Platelet Immunoreceptor Tyrosine-Based Activation Motif (ITAM) Signaling and Vascular Integrity

Yacine Boulafia, Paul R. Hess, Mark L. Kahn, Wolfgang Bergmeier

Abstract: Platelets are well-known for their critical role in hemostasis, that is, the prevention of blood loss at sites of mechanical vessel injury. Inappropriate platelet activation and adhesion, however, can lead to thrombotic complications, such as myocardial infarction and stroke. To fulfill its role in hemostasis, the platelet is equipped with various G protein–coupled receptors that mediate the response to soluble agonists such as thrombin, ADP, and thromboxane A2. In addition to G protein–coupled receptors, platelets express 3 glycoproteins that belong to the family of immunoreceptor tyrosine-based activation motif receptors: Fc receptor γ chain, which is noncovalently associated with the glycoprotein VI collagen receptor, C-type lectin 2, the receptor for podoplanin, and Fc receptor γII A, a low-affinity receptor for immune complexes. Although both genetic and chemical approaches have documented a critical role for platelet G protein–coupled receptors in hemostasis, the contribution of immunoreceptor tyrosine-based activation motif receptors to this process is less defined. Studies performed during the past decade, however, have identified new roles for platelet immunoreceptor tyrosine-based activation motif signaling in vascular integrity in utero and at sites of inflammation. The purpose of this review is to summarize recent findings on how platelet immunoreceptor tyrosine-based activation motif signaling controls vascular integrity, both in the presence and absence of mechanical injury. (Circ Res. 2014;114:1174-1184.)

Key Words: blood and lymphatic vessels ■ blood platelets ■ hemostasis ■ inflammation

Platelet Immunoreceptor Tyrosine-Based Activation Motif Signaling in Hemostasis and Thrombosis

A monolayer of endothelial cells (ECs) separates the lumen of blood vessels from the extracellular matrix (ECM). On mechanical disruption of this monolayer, blood gets into contact with the various components of the ECM. Proteins soluble in blood, such as von Willebrand factor and clotting factors, are rapidly deposited in the ECM. As a consequence, platelets are recruited to the site of injury, followed by their activation and firm adhesion. Transient adhesion of platelets (tethering) depends largely on von Willebrand factor and its receptor, the glycoprotein Ib (GPIb)-V-IX complex. Firm adhesion requires the inside-out activation of integrin receptors such as αIIbβ3, α2β1, and α6β1, followed by interaction with their respective ligands in the ECM (see below). Critical to the activation process is signaling provided by G protein–coupled receptors (GPCRs), agonist receptors triggered by soluble mediators such as thrombin (a main protease of the coagulation cascade) or thromboxane A2 (TxA2) and ADP (agonists released by...
activated platelets and red blood cells). The molecular mechanisms underlying the various stages of thrombus formation at sites of vascular injury have been reviewed extensively and will not be discussed in detail. Instead, we will summarize important findings on the contribution of immunoreceptor tyrosine-based activation motif (ITAM) signaling to platelet adhesion at sites of vascular injury.

**Glycoprotein VI**

Collagen has long been known to be a potent activator of platelets as well as a strong adhesive ligand by which activated platelets may adhere to an injured vessel wall in the face of arterial shear forces. These 2 platelet responses to collagen are mediated by structurally and functionally distinct receptors, GPVI and integrin α2β1. GPVI is an Ig domain-containing transmembrane protein that is structurally homologous to immune-type receptors. Its short cytoplasmic tail does not have signaling activity but facilitates the noncovalent association with the ITAM-containing Fc receptor γ (FcRγ) chain. Collagen is an abundant protein in the body that is secreted into the ECM predominantly by fibroblasts. The basement membrane underneath the EC layer contains mostly type IV collagen. Unlike the fibrillar collagens type I, III, and V, which are found in deeper layers of the ECM, type IV collagen forms 2-dimensional networks. Compared with fibrillar collagens, type IV collagen shows lower platelet activating activity. However, collagen type IV is important for GPVI-dependent platelet activation at sites of superficial vascular injury. Interestingly, GPVI is less critical for thrombus formation at sites of more severe injury as documented in various (mouse) models of thrombotic disease. Consistent with this finding, the bleeding times in humans and mice with defective GPVI function are only minimally prolonged. The likely explanation for this unexpected result is that thrombin, produced via the tissue factor–dependent coagulation pathway, provides a strong enough stimulus for platelet activation in the absence of GPVI engagement. It is important to note, however, that the reverse is not true. Deficiency in protease activated receptor 4, the main GPCR expressed on mouse platelets, cannot be compensated for by signaling via GPVI. The hemITAM receptor C-type lectin 2 (CLEC2) may also contribute to platelet activation and thrombus formation in the deeper layers of the ECM (see below). In addition to collagen, GPVI also binds laminins, heterotrimeric GPs consisting of α, β, and γ chains. Laminins, in particular α4 and α5 laminins, are predominantly found in the basement membrane. GPVI has an ≈10-fold lower affinity for laminin when compared with collagen, a fact that may explain why binding of platelets to laminin via integrin α6β1 is a prerequisite for the GPVI–laminin interaction. Elegant studies by Schaff et al recently confirmed the critical role of α6β1 for platelet adhesion at sites of vascular injury. Using α6-deficient mice, the authors demonstrated that α6β1 is critical for platelet adhesion to α4 and α5 laminins in vitro and for normal thrombus formation in vivo. In contrast, platelets deficient in GPVI were normal in their ability to adhere to laminin in vitro, whereas they showed a minor activation defect specifically to α5 laminins. However, binding of GPVI to laminin may play a bigger role in vivo, where laminin is found together with type IV collagen in the basement membrane.

**C-Type Lectin 2**

CLEC2 was identified in platelets as a receptor for the snake venom toxin rhodocytin and for the transmembrane GP podoplanin (also known as T1α, gp38, and Aggrus). CLEC2 is highly expressed on platelets, but it is also found at a lower level on immune cells (dendritic cells, monocyte/macrophages, neutrophils). The intracellular domain of CLEC2 contains a single YxxL motif (known as a hemITAM motif). Clustering of CLEC2 by podoplanin triggers a powerful platelet signaling response similar to that established for GPVI and FcγRIIA. Podoplanin is a heavily glycosylated type I transmembrane protein expressed in a variety of tissues: kidney podocytes, type-1 lung alveolar cells, lymphatic ECs (LECs), the nervous system, and metastatic tumor cells. The importance of CLEC2 in hemostasis remains controversial. Various studies using mice deficient in CLEC2 showed normal hemostasis in these mice. One study in mice depleted of CLEC2 by infusion of a monoclonal antibody to the receptor observed a significant prolongation of the tail bleeding time, whereas a similar treatment did not affect hemostasis in a different study. The study by Bender et al further investigated the effect of combined deficiency in CLEC2 and GPVI on hemostasis. Although antibody-induced depletion of both receptors from circulating platelets led to a marked prolongation of the tail bleeding time in mice, the defect in hemostasis was much more subtle in animals with a genetic deficiency in both receptors. It is important to remember that both approaches to eliminate CLEC2 from platelets have limitations. Antibody-induced depletion of CLEC2 may affect platelet activation in ways that are independent of CLEC2. Such off-target effects were shown for antibodies to GPVI, which when injected into mice transiently affect thrombin activation of platelets. On the contrary, genetic deletion of CLEC2 affects the integrity of blood vessels and allows blood to enter the lymphatic circulation (see below), alterations that could certainly affect the bleeding time in mice. Assuming there is a role for CLEC2 in hemostasis, the obvious question is how platelet CLEC2 signaling is initiated at the site of vascular injury. Expression of podoplanin, the only known endogenous ligand of CLEC2, has not been documented in cells of the vascular wall. Blood cells, including platelets, also lack podoplanin.

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLEC2</td>
<td>C-type lectin 2</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>FcRγI</td>
<td>Fc receptor γI</td>
</tr>
<tr>
<td>GPib</td>
<td>glycoprotein Ib</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein–coupled receptor</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>LEC</td>
<td>lymphatic endothelial cell</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>SLP-76</td>
<td>SH2 containing leukocyte protein of 76 kDa</td>
</tr>
<tr>
<td>TxA2</td>
<td>thromboxane A2</td>
</tr>
</tbody>
</table>

Boulaftali et al Platelet Signaling and Vascular Integrity 1175
Thus, a critical role for CLEC2 in hemostasis would suggest the existence of a CLEC2 ligand other than podoplanin, or a homotypic interaction between CLEC2 receptors as recently suggested. Additional studies are needed to clarify the underlying mechanism.

**Fc Receptor γRIIA**

Another ITAM receptor expressed on human platelets is a low-affinity member of the Fcγ (IgG binding) family of FcRs, FcγRIIA (CD32A). Mice lack the genetic equivalent of human FcγRIIA, but transgenic mice expressing the human receptor have been used to study its biology in vivo. FcγRIIA allows platelets to play a role in innate immunity by binding to and becoming activated by pathogens opsonized with antibody, thus speeding their clearance. Platelet activation through the platelet Fcγ receptor, FcγRIIA, also plays a critical role in the pathogenesis of various immune-mediated thrombocytopenia and thrombosis syndromes, including heparin-induced thrombocytopenia and thrombosis, bacterial sepsis–associated bacterial sepsis–associated thrombocytopenia and thrombosis, and disseminated intravascular coagulation. Platelet activation through the Gi-coupled receptor for ADP, P2Y12. Platelet ITAM signaling strongly depends on the Ca²⁺/CalDAG-GEFI/Rap1 pathway as deficiency in CalDAG-GEFI protects mice from collagen- and immune complex–induced thrombosis. It is important to remember, however, that signaling molecules downstream of PLCγ2 such as CalDAG-GEFI and protein kinase C (PKC) are also critical for PLCβ-dependent GPCR signaling in platelets. Thus, we focused on literature that evaluated hemostasis and thrombosis but does not affect hemostasis in mice. In contrast, mice deficient in PLCγ2 are protected from experimental thrombosis but also exhibit a marked defect in hemostasis. A comprehensive analysis of the hemostasis and thrombosis phenotypes in mice with defects in signaling molecules upstream of PLCγ2 is shown in the Table. This unexpected discrepancy in results may be explained by different experimental approaches used in the respective laboratories or it may reflect the well-documented role of PLCγ2 signaling downstream of ligand binding to GPIbα. In summary, these studies document that ITAM signaling plays a minor role for platelet adhesion at sites of vascular injury when compared with signaling via platelet GPCRs.

**Platelet ITAM Signaling and Blood–Lymphatic Separation**

**Lymphatics in the Cardiovascular System**

Cardiovascular function requires distinct blood and lymphatic vascular networks to circulate blood and drain interstitial fluid from the periphery. The partitioning of these 2 vascular compartments represents a fundamental adaptation underlying the physiology of mammals and related vertebrates; however, our understanding of the biological processes that give rise to 2 distinct networks remains incomplete. The lymphatic system performs additional functions that include dietary fat absorption, where it transports chylomicrons from the small intestine to the blood, and adaptive immune responses, where it transports antigens and antigen presenting cells to lymph nodes, connects lymph nodes together, and returns lymphocytes to the blood. Lymphatics form an extensive vascular network that originates during embryonic development from a subset of venous ECs in both the cardinal vein and intersomitic vessels that acquire lymphatic identity. Starting at E9.75 in mice, induction of the transcription factor prospero homeobox protein 1 in these venous ECs activates lymphatic identity, and these newly specified LECs bud out of the cardinal
Table. Comprehensive Analysis of the Hemostasis and Thrombosis Phenotypes in Mice With Defects in Signaling Molecules Upstream of PLCγ2

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Approach</th>
<th>Mobilization</th>
<th>Secretion</th>
<th>Aggregation</th>
<th>Thrombosis</th>
<th>Bleeding</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fyn/Lyn</td>
<td>Fyn−/−</td>
<td>↓</td>
<td>↓ α-Granules</td>
<td>(Only to low-dose agonist)</td>
<td>Normal thrombus formation on collagen in vitro</td>
<td>Normal</td>
<td>62–64</td>
</tr>
<tr>
<td></td>
<td>Lyn−/−</td>
<td>↑ (Delay)</td>
<td>† α-Granules</td>
<td>† (Delay)</td>
<td>† Thrombus formation on collagen in vitro</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lyn−/−</td>
<td>↓</td>
<td>† α-Granules</td>
<td>† (Delay)</td>
<td>† Thrombus formation on collagen in vitro</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fyn−/−</td>
<td>↓</td>
<td>† α-Granules</td>
<td>† (Delay)</td>
<td>† Thrombus formation on collagen in vitro</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Syk−/−</td>
<td>↓↓↓ Dense granules</td>
<td>↓↓↓</td>
<td>↓↓↓</td>
<td>↓↓↓ Thrombus formation on collagen in vitro</td>
<td>Normal</td>
<td>16,65–67</td>
</tr>
<tr>
<td></td>
<td>Inhibitor</td>
<td>↓↓↓ Dense granules</td>
<td>↓↓↓</td>
<td>↓↓↓</td>
<td>↓↓↓ Thrombus formation on collagen in vitro</td>
<td>Normal</td>
<td>68–70</td>
</tr>
<tr>
<td>LAT</td>
<td>Lat−/−</td>
<td>↓↓ Dense granules</td>
<td>↓↓ (Only to low-dose agonist)</td>
<td>↓↓ (Only to low-dose agonist)</td>
<td>↓↓ Thrombus formation on collagen in vitro</td>
<td>Normal</td>
<td>16,71–74</td>
</tr>
<tr>
<td>Gad</td>
<td>Gad−/−</td>
<td>↓↓ Dense granules</td>
<td>↓↓ (Only to low-dose agonist)</td>
<td>↓↓ (Only to low-dose agonist)</td>
<td>↓↓ Thrombus formation on collagen in vitro</td>
<td>Normal</td>
<td>72,73</td>
</tr>
<tr>
<td>Vav</td>
<td>Vav1−/−</td>
<td>↓↓</td>
<td>↓↓ α-Granules</td>
<td>↓↓ α-Granules</td>
<td>↓↓ (Overcome at high agonist concentrations)</td>
<td>↓↓ Thrombus formation on collagen in vitro</td>
<td>45,76</td>
</tr>
<tr>
<td></td>
<td>Vav2−/−</td>
<td>↓↓</td>
<td>↓↓ α-Granules</td>
<td>↓↓ α-Granules</td>
<td>↓↓ (Overcome at high agonist concentrations)</td>
<td>↓↓ Thrombus formation on collagen in vitro</td>
<td>45,76</td>
</tr>
<tr>
<td></td>
<td>Vav3−/−</td>
<td>↓↓</td>
<td>↓↓ α-Granules</td>
<td>↓↓ α-Granules</td>
<td>↓↓ (Overcome at high agonist concentrations)</td>
<td>↓↓ Thrombus formation on collagen in vitro</td>
<td>45,76</td>
</tr>
<tr>
<td></td>
<td>Vav1−/−/Vav2−/−</td>
<td>↓↓</td>
<td>↓↓ α-Granules</td>
<td>↓↓ α-Granules</td>
<td>↓↓ (Overcome at high agonist concentrations)</td>
<td>↓↓ Thrombus formation on collagen in vitro</td>
<td>45,76</td>
</tr>
<tr>
<td></td>
<td>Vav1−/−/Vav3−/−</td>
<td>↓↓</td>
<td>↓↓ α-Granules</td>
<td>↓↓ α-Granules</td>
<td>↓↓ (Overcome at high agonist concentrations)</td>
<td>↓↓ Thrombus formation on collagen in vitro</td>
<td>45,76</td>
</tr>
<tr>
<td></td>
<td>Vav1/2/3−/−</td>
<td>↓↓</td>
<td>↓↓ α-Granules</td>
<td>↓↓ α-Granules</td>
<td>↓↓ (Overcome at high agonist concentrations)</td>
<td>↓↓ Thrombus formation on collagen in vitro</td>
<td>45,76</td>
</tr>
<tr>
<td>Slp76</td>
<td>Slp76−/−</td>
<td>↓↓↓ α-Granules</td>
<td>↓↓↓</td>
<td>↓↓↓</td>
<td>↓↓↓ Thrombus formation on collagen in vitro</td>
<td>Normal</td>
<td>45,76</td>
</tr>
<tr>
<td>PLCγ2</td>
<td>PLCγ2−/−</td>
<td>↓↓</td>
<td>↓↓ α-Granules</td>
<td>↓↓ α-Granules</td>
<td>↓↓ Thrombosis in a superficial laser injury model</td>
<td>↑↑</td>
<td>77–80</td>
</tr>
<tr>
<td>Rac1</td>
<td>Rac1−/−</td>
<td>↓↓</td>
<td>↓↓ α-Granules</td>
<td>↓↓ α-Granules</td>
<td>↓↓ Thrombosis in a superficial laser injury model</td>
<td>↑↑</td>
<td>16,81–83</td>
</tr>
<tr>
<td></td>
<td>Inhibitor</td>
<td>↓↓</td>
<td>↓↓ α-Granules</td>
<td>↓↓ α-Granules</td>
<td>↓↓ Thrombosis in a superficial laser injury model</td>
<td>↑↑</td>
<td>16,81–83</td>
</tr>
<tr>
<td>STAT3</td>
<td>Inhibitor</td>
<td>↓↓</td>
<td>↓↓ α-Granules</td>
<td>↓↓ α-Granules</td>
<td>↓↓ Thrombosis in a superficial laser injury model</td>
<td>↑↑</td>
<td>16,81–83</td>
</tr>
</tbody>
</table>

Gad indicates growth factor receptor–bound protein-2 adaptor downstream of Shc; HIT, heparin-induced thrombocytopenia and thrombosis; LAT, linker for activation of T cells; PLCγ2, phospholipase Cγ2; STAT3, signal transducer and activator of transcription 3; ↓, mild; ↓↓, moderate; and ↓↓↓, severe defect in platelet response.

vein and interosseous vessels with the help of vascular endothelial growth factor-C to form the primary lymph sacs, early structures that give rise to the entire lymphatic network. The structure and function of the lymphatic system differ significantly from that of the blood vascular system. The blood vascular system propels blood at high pressures generated by mechanical pumping of the heart to circulate blood in a closed loop through pulmonary and systemic vessels. In contrast, lymph fluid is acquired from tissues through a permeable vascular network in which lymph flow is maintained by the contraction of collecting vessel walls, external compression, and valves. Blind-ended lymphatic capillaries with loose endothelial junctions absorb interstitial fluid and coalesce into larger collecting vessels that then drain lymph into lymphatic ducts where protein-rich lymph fluid and immune cells are returned to venous blood. A series of intraluminal valves divide lymphatic vessels into functional units called lymphangions that promote forward lymph flow. In addition, a bicuspid lymphovenous valve is present at the site of connection where lymph drains into blood. The lymphovenous valves are thought to prevent blood from entering the low pressure lymphatic system, and in humans, these valves are found at the right lymphatic duct–subclavian vein junction and the thoracic duct–subclavian vein junction.

**Blood–Lymphatic Separation During Embryonic Development**

Beginning almost 20 years ago, genetic studies in mice have revealed an unexpected role for ITAM signaling during embryonic vascular development. Mice lacking the essential ITAM signaling effectors Syk, SLP-76, or PLCγ2 display blood-filled lymphatic vessels during embryonic stages and die postnatally because of impaired lymphatic function. Tissue-specific deletion in the megakaryocyte lineage...
revealed that ITAM signaling in the platelet mediates blood–lymphatic separation, with upstream components identified through deletion of the platelet CLEC2 receptor and its ligand podoplanin expressed on LECs. Mice deficient in CLEC2 or podoplanin exhibit blood-filled lymphatics during fetal life and die shortly after birth like mice lacking Syk or SLP-76. Although these phenotypes all arise because of loss of platelet activation by LECs, it has been unclear precisely how activated platelets affect lymphatic development in embryos. Some studies have used in vitro approaches to suggest that activated platelets may release granule contents that regulate LEC growth and that this is therefore an angiogenic role for platelets. However, we have recently demonstrated that the entry of blood into the developing lymphatic network in late gestation embryos is through the lymphovenous junction and that the role of platelets is to mediate an unexpected form of intervascular hemostasis that supports the lymphovenous valve and prevents blood from entering the thoracic duct. An important outstanding question is whether platelet function at the lymphovenous junction or valve can explain the early blood–lymphatic mixing observed in animals lacking platelets or podoplanin -CLEC2 signaling to activate them. Deficient embryos exhibit blood-filled lymph sacs as early as E11.5, a time point before when the lymphovenous junction has been demonstrated to arise during lymphatic development. Thus, it is possible that platelet CLEC2 signaling mediates intervascular hemostasis in a distinct manner at early time points in lymphatic growth. Although additional studies are needed to address this developmental role, it is clear that the role of platelets in this context is to perform a unique form of hemostasis and not to regulate lymphangiogenesis.

**Blood–Lymphatic Separation in the Adult**

Lethally irradiated mature mice transplanted with bone marrow deficient in CLEC2, Syk, or SLP-76 develop blood-filled lymphatic vessels and die because of lymphatic dysfunction, revealing a lifelong requirement for platelet ITAM signaling to maintain blood–lymphatic separation. This role is also supported by inducible postnatal deletion of the O-glycan synthase enzyme required for podoplanin expression on LECs, which also produces the blood-filled lymphatic phenotype. Platelets and LECs would not be predicted to interact; however, the genetic experiments described above identify a critical interaction that begins in the embryo and continues throughout life where platelets in circulating blood come into contact with LECs to initiate platelet ITAM signaling and regulate blood–lymphatic separation. The site where platelet CLEC2 signaling mediates blood–lymphatic separation was recently determined by examining induced CLEC2 deficiency states in both neonatal and mature animals. These studies reveal that mice lacking this signaling pathway develop blood-filled lymphatic vessels through retrograde filling of the lymphatic network with blood despite the presence of lymphovenous and lymphatic valves and also identify the terminal thoracic duct as the location where blood first enters the lymphatic vascular system after loss of CLEC2. The lymphovenous valve has been thought to function alone in preventing the backflow of blood into the lymphovenous junction; however, these data suggest that an additional platelet-dependent mechanism has escaped notice. To search for platelets at the terminal thoracic duct, the lymphovenous junction was examined and fibrin-containing platelet thrombi were observed within the lymphatic vascular environment of this site (≈25%) in wild-type but not CLEC2-deficient mice. To study the relationship between valves and platelets at the lymphovenous junction, mice heterozygous for prospero homeobox protein 1 or mice lacking the integrin α9 subunit were analyzed. Recent studies demonstrated that Prox1+/- embryos exhibit lymphovenous valve defects, whereas Itgb3+/- embryos are characterized by severe lymphatic valve developmental defects. These mice were found to have an augmented frequency of lymphovenous thrombi (≈100%) and clots that extend deeper within the thoracic duct than those observed in wild-type controls, suggesting that platelets can compensate for impaired valve function. To stress the lymphovenous junction by disabling platelet-mediated hemostasis, Itgβ3−/- mice lacking integrin-mediated platelet aggregation were examined. These animals still form clots within the lymphatic vascular environment yet have marked filling of the thoracic duct with blood, suggesting that integrin-mediated platelet aggregation through αIIbβ3 is not needed for thrombus formation in the lymphatic system yet is essential in preventing lymphovenous backflow. Together, these data support a hemostatic mechanism of platelet function at the lymphovenous junction that maintains blood–lymphatic separation throughout life. Unlike canonical hemostasis which limits hemorrhage from damaged vessels, lymphovenous hemostasis operates within an uninjured intravascular environment under low flow, low shear conditions; therefore, the contribution of coagulation and platelet degranulation may differ from arterial or venous thrombosis. Preliminary studies of lymphovenous hemostasis have identified a divergent role for integrin-mediated platelet aggregation compared with arterial hemostasis where αIIbβ3 is not required for thrombus growth but does contribute to thrombus stability in the prevention of lymphovenous backflow. These studies highlight an unexpected platelet-dependent hemostatic response that functions alongside the lymphovenous valve to maintain the lymphatic system. The activation of platelet CLEC2 receptors by lymphatic endothelial podoplanin is first observed during lymphatic development where it prevents blood from entering the immature system at a time when valves are not yet formed. However, genetic and pharmacological studies demonstrate that the requirement for this hemostatic pathway extends throughout life, including in mature animals in which the lymphovenous valves are fully functional. The basis for this requirement is not yet established, but it is likely that this hemostatic mechanism is necessary to prevent pressure gradients from driving venous blood into lymphatic vessels. Compared with central venous pressure (5–10 mm Hg), the lymphatic pressure is low (1–2 mm Hg). Changes in body position, fluid status, or disease states such as congestive heart failure can further increase this pressure gradient and thus lead to backflow of blood into lymphatic vessels. Importantly, lymphovenous valve insufficiency and reflux of blood into the thoracic duct was recently described for patients with congestive heart failure. The identification of this platelet-dependent safety mechanism may...
have clinical implications. First, application of antiplatelet therapies to patients with congestive heart failure to reduce the risk of myocardial infarction and stroke may have detrimental effects on lymphatic function. Because these patients have chronically elevated pulmonary venous pressures, it is likely that lymphatic drainage in the lung plays an important role in preventing pulmonary edema. Thus, antiplatelet therapies may protect against arterial thrombosis at the expense of lymphovenous hemostasis and worsen congestive heart failure symptoms. Second, new drugs targeting the Syk kinase that are intended to treat chronic inflammatory conditions, such as rheumatoid arthritis, may impair lymphatic function. The fact that these patients are expected to take anti-Syk agents for extended periods of time raises this risk. Our ability to predict the impact of antiplatelet and anti-Syk agents is limited at this time because this pathway has been explored almost exclusively in mouse models. Extending these studies to patients and clinically relevant scenarios will provide needed insight into whether and how impairment of this hemostatic pathway affects human health and disease.

**Platelet ITAM Signaling and Vascular Integrity in Inflammation**

**GPVI, Atherosclerosis, and Enhanced Vascular Permeability**

In addition to hemostasis and thrombosis, platelets are important modulators of inflammatory reactions. Their role in inflammation is partially explained by their ability to interact and communicate with leukocytes and vascular cells. These interactions are mediated by various receptor–ligand pairs, including P-selectin—PSGL-1, GPIbα—Mac1, CD40L—CD40, and αIIbβ3—ICAM1. Platelets also deposit chemokines on activated endothelium, thereby enhancing leukocyte recruitment and promoting the progression and propagation of chronic inflammation. GPVI is also critically involved in platelet adhesion to activated endothelium. Endothelial dysfunction is considered to be a predictive sign of atherosclerosis in patients and correlates with the progression of the disease.

Platelets also deposit chemokines such as P-selectin on activated endothelium, thereby enhancing leukocyte recruitment and promoting the progression and propagation of chronic inflammation. GPVI is also critically involved in platelet adhesion to activated endothelium. Endothelial dysfunction is considered to be a predictive sign of atherosclerosis in patients and correlates with the progression of the disease. GPVI can interact with the activated atherosclerotic endothelium in the absence of plaque rupture. Intravital microscopy studies demonstrated that administration of soluble GPVI-Fc and anti-GPVI (JAQ1) antibody inhibited platelet adhesion to the activated endothelium in apolipoprotein E–deficient mice. As a consequence of this inhibition, endothelial function was improved in atherosclerotic rabbits. The role of GPVI in the absence of plaque rupture in atherosclerosis is unlikely to be attributed to the interaction with collagen/laminin but was suggested to occur through fibronectin which is secreted by activated platelets or ECs at vascular lesions. Similarly, in an experimental animal model of myocardial infarction, recombinant GPVI-Fc molecules bound to activated endothelium via vitronectin and prevented platelet/endothelial interaction, thereby reducing infarct size and preserving cardiac function.

Platelet ITAM signaling is also critical in inflammatory arthritis. Although it was long recognized that platelets from patients with arthritis are hyper-responsive, it was not clear whether platelets contribute to the progression of the disease. Studies in a mouse model of rheumatoid arthritis, done by Boilard et al., demonstrated such a causal relationship, because mice depleted of all circulating platelets were protected from joint inflammation. Interestingly, mice lacking GPVI were also protected from experimental arthritis, suggesting that platelet ITAM signaling is critical for disease progression. Additional work proposed the following underlying mechanisms: exposure of platelets to collagen and laminin at sites of inflammation leads to the production of interleukin–1–rich, proinflammatory microparticles, platelet fragments that are small enough to diffuse into the synovial fluid. Alternatively, platelet microparticles may be transported into the joint by inflammatory cells. Furthermore, activated platelets may actively weaken the endothelial barrier at sites of inflammation, a process dependent on the release of serotonin from their dense granules. Consistent with these findings, GPVI is important for platelet and leukocyte recruitment to the inflamed capillaries in experimental glomerulonephritis. Taken together, these studies suggest that GPVI is an attractive therapeutic target in inflammatory diseases beyond hemostasis and thrombosis. To date, the contribution of platelet CLEC2 and FcyRIIA to the pathogenesis of these diseases has not been evaluated. Confirmatory studies in mice defective in signaling molecules downstream of GPVI are also missing. Genetic targeting or inhibition in platelets of ITAM signaling molecules such as Syk, however, is difficult because they are also critical for proper immune cell function. Furthermore, even platelet-specific targeting such as achieved in megakaryocyte/platelet-specific conditional knockout mice is complicated by the marked defects in vascular development documented for these animals.

**Platelets and Maintenance of Vascular Integrity in Inflammation**

Platelets have long been recognized to support the integrity of the vasculature. Structural endothelial abnormalities such as thinning, fenestration, and increased permeability have been shown for severely thrombocytopenic humans as well as animals depleted of virtually all circulating platelets. Importantly, however, thrombocytopenia is often not associated with hemorrhage, suggesting that an additional trigger such as inflammation is required for bleeding to occur. This multihit concept was confirmed in elegant studies by Goerge et al., who showed that acute severe thrombocytopenia in mice does not lead to hemorrhage unless these animals are challenged by inflammation. These studies also showed (1) that thrombocytopenia resulted in hemorrhage only at the site of inflammation and (2) that important platelet adhesion receptors such as GPIb-V-IX and αIIbβ3 integrin were not required for this platelet function. Thus, the contribution of platelets to the maintenance of vascular integrity in inflammation does not depend on the platelet’s ability to form a hemostatic plug. At this point, little is known about how platelets protect the inflamed vasculature. Vasoactive factors released from activated platelets may prevent hemorrhage by strengthening EC barrier function or by dampening the inflammatory response. Numerous candidate factors, including ADP released from dense granules, serpins, and metalloproteinase inhibitors released from α-granules, reactive...
oxygen scavengers,\textsuperscript{126} TREM-like transcript-1 proteolytically shed from the platelets surface,\textsuperscript{127,128} and the vasoactive lipid sphingosine-1 phosphate,\textsuperscript{129} have been identified. The extent to which these factors contribute to inflammatory hemostasis, however, is not well defined.

In addition to our lack of understanding with regard to the platelet protective activity, little is known about what triggers platelet activation at sites of inflammation and what signaling response is required. Addressing these questions requires mice with platelet-specific signaling defects, because the pathways regulating cellular activation are similar between platelets, inflammatory, and vascular cells.

This can be achieved by conditional deletion of genes in the megakaryocyte lineage, an approach that depends on the availability of mice with loxP-flanked genes that can be crossed to mice expressing Cre recombinase under the PF4 (platelet factor 4) promoter.\textsuperscript{130} As an alternative approach, we recently described a method for the adoptive transfer of platelets into thrombocytopenic mice. In this approach, thrombocytopenia is induced in transgenic mice with antibodies that recognize human interleukin-4 receptor, a heterologous antigen expressed on circulating platelets in these animals.\textsuperscript{131,132} These mice are then transfused with genetically or chemically inhibited platelets, which are not destroyed by the circulating anti–human interleukin-4 receptor antibodies. The main characteristics of this novel tool are as follows: (1) a fast and reliable method to generate mice with platelet-specific signaling defects, (2) the ability to combine genetic and pharmacological approaches to loss-of-function studies, and (3) an increased sensitivity for platelet defects attributable to the ability to establish a lower peripheral platelet count in experimental animals. It is also important to remember that deletion of genes in megakaryocytes/platelets only can lead to marked vascular changes (see above). This limitation is not relevant for the adoptive transfer system. Using this approach, we identified a critical role for GPVI, CLEC2, and the downstream adapter protein SLP-76 in the maintenance of vascular integrity at sites of immune complex–induced inflammation in the skin as well as lipopolysaccharide-induced inflammation in the lung.\textsuperscript{132} Surprisingly, our studies further demonstrated that platelet GPCR signaling is not required for the maintenance of vascular integrity in inflammation. Unlike platelets lacking functional CLEC2 or GPVI, platelets defective in signaling via the major GPCRs responding to thrombin, ADP and TxA\textsubscript{2} were fully capable of supporting vascular integrity at sites of inflammation.

Together, these findings confirm the notion that hemostasis at sites of inflammation requires a platelet response(s) different to that important at sites of vascular injury (Figure). Various questions arise from these unexpected findings. For example, although the main ligands for GPVI, collagen, and laminin are highly expressed in the vessel wall, it is not so clear what triggers the activation of CLEC2 at sites of inflammation. Some organs that are particularly vulnerable to hemorrhage such as the lung, the brain, and the kidney contain cell types that express high levels of podoplanin.\textsuperscript{133} Other tissues, however, such as the skin do not contain podoplanin-positive cells. One possibility is that podoplanin is delivered to the extravascular space by infiltrating podoplanin-positive macrophages.\textsuperscript{133} Alternatively, a hitherto unrecognized ligand other than podoplanin may trigger platelet CLEC2 signaling in these situations. Conditional deletion of podoplanin in various tissues will be required to clarify the underlying mechanism. A second important question to be answered is why this novel form of hemostasis depends so strongly on signaling via the ITAM but not the GPCR pathway. It is difficult to imagine that this pathway selectivity simply reflects the availability of agonists at sites of inflammation. Weakening of EC barrier function leads to plasma leakage into the inflamed tissue, followed by the activation of coagulation and the generation of thrombin.\textsuperscript{134} Furthermore, platelet activation via ITAM receptors and protease activated receptor 4 leads to the release of the second-wave mediators ADP and TxA\textsubscript{2}. Thus, it seems more likely that ITAM receptors trigger a unique platelet response, which is crucial in the setting of inflammation. For example, there is increasing evidence that individual platelet agonist receptors can trigger distinct granule release reactions, although to date these studies have been focused on platelets activated via GPCRs.\textsuperscript{135} Better established is the critical role for ITAM signaling in platelet microparticle release and surface phosphatidylserine exposure\textsuperscript{136,137} as well as shedding of surface receptors.\textsuperscript{138} Both microparticles and soluble GPs could serve as diffusible mediators in a low-flow environment such as the inflamed tissue. The identification of the required platelet response(s) will be a critical first step for a better understanding of how these cells safeguard vascular integrity at sites of inflammation.

In humans, marked thrombocytopenia, such as observed in idiopathic thrombocytopenia purpura, does not necessarily lead to bleeding.\textsuperscript{139} However, hemorrhage is frequent in patients with Wiskott–Aldrich syndrome, a clinical complication characterized by marked thrombocytopenia, recurrent infections, and an elevated risk for development of autoimmune disease.\textsuperscript{140,141} Thus, it seems likely that both thrombocytopenia and an additional trigger such as inflammation are required to compromise the integrity of the vasculature in humans. Moreover, patients deficient in GPVI expression or function often present with ecchymoses, that is, hematomas that are not caused by trauma,\textsuperscript{10,142} suggesting that humans also depend on platelet ITAM signaling for maintenance of vascular integrity. To date, no patients with defects in CLEC2 expression/function have been described.

Conclusions

Platelet-mediated hemostasis has classically been defined in the context of plug formation at sites of vessel injury and high fluid shear forces. Platelet integrins and GPCR activation pathways are essential in this context and form the foundation of modern antiplatelet therapies. In contrast, studies of ITAM-coupled platelet receptors are revealing new aspects of hemostasis that extend far beyond classic arterial injury responses. These include inflammatory and lymphovenous hemostasis, processes that depend on platelet ITAM signaling and platelet responses that have yet to be defined. In addition to our deficit in understanding of the basic mechanisms by which platelets safeguard vascular integrity, we lack information on when these inflammatory and lymphovenous hemostatic responses are most used in normal physiology and under pathophysiologic conditions? Without this knowledge, it will be difficult to predict which patients taking antiplatelet agents are at an increased risk of
inflammatory or lymphovenous hemorrhage. Future studies addressing these questions have the potential to reveal new roles for platelets in common diseases and new effects—both good and bad—of antiplatelet therapies in patients.

**Acknowledgments**

We thank David S. Paul and Nigel Mackman for help with figure design and formatting.

**Sources of Funding**

This work was funded by a fellowship by the American Heart Association (12POST12040088 to Y. Boulaftali) and by the National Heart, Lung, and Blood Institute, National Institutes of Health, grants R01 HL106009 (to W. Bergmeier) and HL072798 (to M.L. Kahn)

**Disclosures**

None.

**References**

Platelet Signaling and Vascular Integrity


Kitchens CS, Pendergrass JF. Human thrombocytopenia is associated with structural abnormalities of the endothelium that are ameliorated by glucocorticosteroid administration. Blood. 1986;67:203–206.


Platelet Immunoreceptor Tyrosine-Based Activation Motif (ITAM) Signaling and Vascular Integrity

Yacine Boulaitali, Paul R. Hess, Mark L. Kahn and Wolfgang Bergmeier

Circ Res. 2014;114:1174-1184
doi: 10.1161/CIRCRESAHA.114.301611

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved. Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/114/7/1174

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/