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Glycoprotein VI in securing vascular integrity in inflamed vessels

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Abstract

Glycoprotein VI (GPVI), the main platelet receptor for collagen, has been shown to play a central role in various models of thrombosis, and to be a minor actor of hemostasis at sites of trauma. These observations have made of GPVI a novel target for antithrombotic therapy, as its inhibition would ideally combine efficacy with safety. Nevertheless, recent studies have indicated that GPVI could play an important role in preventing bleeding caused by neutrophils in the inflamed skin and lungs. Remarkably, there is evidence that the GPVI-dependent hemostatic function of platelets at the acute phase of inflammation in these organs does not involve aggregation. From a therapeutic perspective, the vasculoprotective action of GPVI in inflammation suggests that blocking of GPVI might bear some risks of bleeding at sites of neutrophil infiltration. In this review, we summarize recent findings on GPVI functions in inflammation and discuss their possible clinical implications and applications.

KEYWORDS
bleeding, glycoprotein VI, inflammation, platelets, vascular integrity

Essentials

- Platelets can stop bleeding independently of integrin-mediated aggregation in inflamed organs.
- GPVI contributes to aggregation-independent hemostasis in the inflamed skin.
- GPVI supports sealing of neutrophil-induced vascular breaches by nonaggregated platelets.
- Evaluation of anti-GPVI drugs should take into account the risk of inflammatory bleeding.

1 | GPVI IN PRIMARY HEMOSTASIS AND THROMBOSIS

Glycoprotein VI (GPVI) is a type I transmembrane protein of 58-60 kDa that belongs to the immunoglobulin superfamily and whose expression is restricted to the megakaryocyte lineage.[1] It was initially identified as a collagen receptor in the 1980s based on clinical evidence that platelets from patients presenting with mild bleeding disorders were specifically unresponsive to fibrillar collagen, a defect that was associated with a deficiency in GPVI.[2,3] The cloning of GPVI few years later.[1,4,5] allowed the development of various experimental tools including genetically-modified mice, recombinant forms of GPVI, and anti-GPVI antibodies, which have all been crucial in dissecting GPVI structural organization and functions. Those tools helped define GPVI as the primary receptor for platelet/collagen interactions and subsequent platelet activation.[6-9] They have also made possible the demonstration that GPVI forms highly competent dimers at the platelet surface whose clustering upon adhesion to collagen further increases its avidity and downstream signaling.[10-14] The signaling activity of GPVI depends on its physical association

with the homodimeric γ chain common to Fc receptors (FcRγ), whose cytoplasmic domains each bears a signaling motif known as immunoreceptor tyrosine-based activation motif (ITAM).[15,16] Upon ligand binding, the Src family kinases Lyn and Fyn mediate phosphorylation of the ITAM tyrosine residues that transmit activation signals.[17]

Although GPVI in its dimeric form binds collagen with a relatively high affinity,[10,18] it is considered as a signaling rather than an adhesion receptor, as its interaction with collagen is a prerequisite for the activation of platelet integrins, including that of the collagen receptor integrin α2β1.[6–8,19,20] GPVI-dependent signaling indeed ensures firm adhesion and aggregation through activation of platelets. In addition, it has been shown that GPVI interaction with immobilized collagen related peptide (CRP), a synthetic peptide that mimics the triple-helical structure of collagen and specifically engages GPVI,[21,22] is transient and not sufficient for firm platelet adhesion under flow, thus arguing against a role for GPVI in triggering platelet adhesion to collagen.[20,23] Instead, it has been proposed that the interaction between GPIbα and collagen-bound von Willebrand factor (VWF) would play a prominent role in initiating platelet adhesion to collagen, especially at higher shear, with GPVI-dependent signaling taking over integrin activation subsequently.[20,23] Nonetheless, it should be noted that several studies have shown that GPVI permits platelet adhesion to fibrillar collagen irrespective of integrin activation in both static and flow conditions.[6,7,23–25] Moreover, absence or inhibition of GPVI was shown to cause a drastic reduction in platelet adhesion to fibrillar collagen, despite normal levels of GPIbα.[6,7,26,27] Therefore, although GPIbα primes adhesion to collagen and integrin activation reinforces it and allows aggregation, there is evidence that GPVI can support platelet adhesion to fibrillar collagen. In recent years, laminins,[28,29] fibrin,[30,31] and fibronectin[32,33] have also been identified as adhesive and/or activating ligands for GPVI. The fact that GPVI has multiple ligands suggests that GPVI interactions with the injured or diseased vessel wall could be more complex than what can be deduced from its interactions with CRP or purified collagen alone.

Considering the central role of GPVI in mediating collagen-induced platelet activation, as well as its ability to potentiate other platelet activation pathways,[34–36] one could expect from GPVI to be a major player in primary hemostasis. However, although their platelets are highly refractory to collagen-induced activation, patients with genetic or acquired GPVI deficiency only have a mild bleeding disorder.[2,3,37–41] Those patients present a bleeding phenotype comparable to that of patients with moderate thrombocytopenia, which includes easy bruising, a prolonged bleeding time, and so-called spontaneous bleeding events like petechiae and epistaxis. Autoantibodies to GPVI can be associated with idiopathic thrombocytopenic purpura,[2,37] which can be considered as a confounding factor in the setting of abnormal bleeding. Hence, it is worth noting that the bleeding phenotype of the first patient described with anti-GPVI antibodies persisted despite correction of thrombocytopenia by steroid therapy.[2] This indicated that the patient's bleeding tendency was a consequence of platelet dysfunction rather than of reduced platelet counts. Consistent with observations made in GPVI-deficient patients, inhibition, immunodepletion, or genetic deletion of GPVI in animals only had minor consequences on primary hemostasis.[42,43] In contrast to its limited impact on primary hemostasis, GPVI deficiency conferred remarkable protection against thrombosis in a variety of in vitro and in vivo experimental models, including flow chamber-based assays using human atherosclerotic plaque material.[27,33,42–58] For these reasons, and despite some controversies, GPVI has been proposed as a promising target for antithrombotic therapy with reduced bleeding risk as compared to current antiplatelet therapies based on administration of aspirin and/or P2Y12 inhibitors.[42,43]

The mild bleeding manifestations in patients with GPVI deficiency suggest that either GPVI plays a modest role in hemostasis or, more likely, that its absence is overcome by compensatory and/or redundant mechanisms. Among the many pathways of platelet activation, the protease-activated receptors (PARs) for thrombin and the relatively newly discovered hem-ITAM-containing platelet activation receptor for podoplanin, C-type lectin-like receptor 2 (CLEC-2), have both shown functional redundancy with GPVI in mouse models of hemostasis and thrombosis.[59,60] It has also been shown that the requirement for GPVI-dependent signaling through Syk in platelet spreading on various matrix proteins can be bypassed by thrombin.[35] Taken together, these data suggest that GPVI deficiency is compensated for in situations where vascular injury leads to significant thrombin generation and/or podoplanin exposure. Those situations likely encompass most traumatic vascular injuries, which might explain why GPVI patients do not present severe bleeding. Indeed, besides lymphatic endothelial cells, several types of podoplanin-expressing cells (inflammatory macrophages,[61–66] fibroblasts[65,66]) have been described in the vicinity of blood vessels and could therefore engage platelet CLEC-2 through vascular breaches.

Around 10 years back, inflammation was identified as a cause of nontraumatic bleeding in thrombocytopenia.[67] The fact that inflammation can cause bleeding in thrombocytopenia has hinted that spontaneous bleeding in patients with thrombocytopenia or platelet dysfunction may not always be "spontaneous" but rather evoked by unsuspected underlying inflammatory reactions. In addition, it has revealed a so far unnoticed and uncharacterized protective function of platelets in inflammation where they continuously prevent bleeding from inflamed vessels. Experiments in mouse models of inflammation have indicated that, surprisingly, the hemostatic function of platelets in inflammation can operate independently of platelet aggregation, the main and best known mechanism of action of platelets in hemostasis.[68] As presented in further detail below, there is experimental evidence to suggest that there are situations where GPVI could play a predominant role in preventing inflammatory bleeding, notably in the skin. In the light of these findings, it is worth noting that inflammation-induced bleeding in humans and mice can take the form of cutaneous petechiae,[67,69,70] a manifestation shared by both thrombocytopenic and GPVI-deficient patients.

2 | PLATELETS AND GPVI IN INFLAMMATION

Platelets have long been considered primarily through the lens of hemostatic platelet plug formation and thrombosis, who both rely on integrin-mediated platelet aggregation. They are, however, increasingly recognized as major actors of inflammatory reactions and immune responses.[71-77] Platelets intervene at various stages of inflammatory reactions. Whereas platelets enhance the barrier function of the quiescent endothelium and stop bleeding in case of vessel injury,[68] in the context of inflammation, they contribute to opening of endothelial junctions and thereby to edema formation.[70,78-82] In addition, platelets promote leukocyte recruitment and infiltration in many tissues and organs under a range of inflammatory conditions.[26,31,70,78-92] Platelets also regulate several effector functions of innate immune cells including degranulation,[93,94] oxidative activity,[95,96] phagocytosis,[95] or the formation of neutrophil extracellular traps.[83,97] Besides these proinflammatory effects, platelets also act to limit inflammation-associated collateral damage to the host by preventing bleeding in inflamed organs.[68,98] With respect to the role of GPVI in inflammation, GPVI has been involved in the recruitment of platelets and leukocytes to the inflamed vessel wall,[26,33,80,84,94,99] the regulation of vascular permeability[80] and

leukocyte activation,[94,100,101] and in the prevention of inflammatory bleeding[94,102] (Table 1).

The role of GPVI in platelet recruitment to the inflamed vasculature has been assessed and demonstrated in various models of inflammation including atherosclerosis in hypercholesterolemic animals,[26,33] rheumatoid arthritis,[80,84] myocardial ischemia-reperfusion,[103-105] and immune complex (IC)-induced glomerulonephritis[99] and dermatitis[94] (Table 1).

In hypercholesterolemic mice and rabbits, GPVI was shown to mediate transient but not firm adhesion of platelets to atherosclerotic endothelium. Chronic inhibition of those GPVI-dependent interactions with the diseased vessel wall by anti-GPVI antibodies or GPVI competitive antagonists reduced lesion formation and corrected endothelial dysfunction in atherosclerotic arteries.[26,33] These results exemplify how, even in the absence of aggregation, GPVI engagement can contribute to inflammation and thereby to atheroprogression in experimental atherosclerosis.

Rheumatoid arthritis is another situation where GPVI promotes inflammation independently of aggregation. In a mouse model of autoimmune rheumatoid arthritis, platelets recruited to the inflamed joints promoted inflammation by increasing the permeability of the synovial microvasculature and the recruitment of neutrophils.[80,84] These proinflammatory effects of platelets involved the release of

TABLE 1 GPVI functions in inflammation. A non-exhaustive list of the various effects of GPVI deficiency or blockade in models of inflammation. anti-GBM: anti–glomerular basement membrane antibody; K/BxN serum: serum from K/BxN a mouse strain known to develop severe autoimmune inflammatory arthritis

Inflammation model	Effect of GPVI deficiency or blockade	References
Acute dermatitis (IgG immune-complex mediated)	• skin bleeding at the reaction site • reduced platelet recruitment to the inflamed skin • reduced reaction-induced serotonin secretion	[94, 102]
Peritonitis (IgG immune-complex mediated)	• decreased MMP-9 release in the peritoneal cavity • no bleeding	[94]
Rheumatoid arthritis (K/BxN serum)	• decreased vascular permeability in the inflamed joint • decreased production of proinflammatory platelet microparticles	[80, 84]
Glomerulonephritis (anti-GBM antibody)	• reduced platelet recruitment to the inflamed glomerulus	[99]
Myocardial Ischemia-Reperfusion (30 min long ligation of the left coronary artery)	• reduced neutrophil recruitment • reduced expression of inflammatory cytokines in the myocardium • reduced microthrombosis • improved microperfusion • reduced infarct size • intramyocardial bleeding	[103–105]
Cerebral Ischemia-Reperfusion (60 min long occlusion of middle cerebral artery)	• reduced infarct size • reduced thrombosis in ischemic cortices • improved microperfusion • no bleeding	[113, 116, 136]
Lung infection (*Klebsiella pneumoniae*)	• increased bacterial growth in infected lungs • increased MMP-9 release in the bronchoalveolar space • increased inflammatory cytokines in the bronchoalveolar space • reduced platelet recruitment and activation in the lung • reduced platelet-leukocyte complex formation in the lung • minor bleeding	[148, 149]
Acute lung injury (LPS inhalation)	• lung bleeding	[102]
Atherosclerosis (ApoE$^{-/-}$ mice)	• reduced platelet adhesion to atherosclerotic endothelium • reduced atherosclerotic lesion formation	[26, 33]

serotonin and microparticles in synovial fluid. Platelet serotonin and microparticles, respectively, induced endothelial gap formation in the synovial vasculature and the secretion of the neutrophil chemoattractant interleukin-8 by synovial fibroblasts, thus amplifying arthritis. The contribution of various platelet activation pathways to the inflammatory functions of platelets in this model was investigated using genetically deficient mice and pharmacologic blockade. The G protein-coupled receptors for thromboxane A2 (TP) and ADP (P2Y12), GPIbα, and integrin αIIbβ3 were all found to be dispensable for microparticles generation and development of joint inflammation, which instead relied on GPVI.[84]

The clinical observations of increased circulating platelet-derived factors (e.g, serotonin, platelet factor 4, β-thromboglobulin), their presence in urine, and the localization of platelets in glomerular capillaries in various forms of glomerulonephritis have suggested that platelets are also involved in the pathogenesis of human renal diseases.[106–108] Like in rheumatoid arthritis, platelets are suspected of increasing vascular permeability and leukocyte infiltration in the inflamed glomerulus. They would also favor local cellular and matrix remodeling, thus further promoting glomerular injury and dysfunction. Although platelet aggregates have been described in biopsies from patients with glomerulonephritis, they are not always present. Several hypotheses have been proposed to explain the paucity of morphologically intact platelets in human glomerular lesions. These hypotheses include the possibility of a hit-and-run model where platelets interacting transiently with the vessel wall exit the glomerular circulation after releasing their granular content.[107–109] An alternative explanation is that, having released their granules, platelets might become difficult to identify in transmission electron microscopy.[107–109] Renal intravital microscopy in a mouse model of IC-mediated glomerulonephritis has brought some insights on the interactions between platelets and the inflamed glomerulus vasculature.[99] Platelet recruitment to the glomerulus started within minutes of inflammation initiation and increased progressively during the course of the reaction. Primary adhesion of platelets was unaffected by inhibition of GPIbα but was reduced by GPVI deficiency, thus indicating that early platelet recruitment was independent of GPIbα and mediated by GPVI. More precisely, GPVI initiated platelet adhesion by permitting platelet tethering but was not sufficient for subsequent stable adhesion, which required integrin αIIbβ3 activation. Platelets recruited to the inflamed glomerulus then stimulated neutrophil recruitment in an ADP and P-selectin-dependent way.[99,110] Interestingly, whereas platelets enhanced neutrophil recruitment in this model, the opposite was also true. In fact, neutrophil depletion prior to glomerulonephritis induction markedly reduced GPVI-dependent platelet adhesion and accumulation within the glomerulus. This result was a first indication that there are situations where neutrophils unmask and/or provide binding sites for GPVI in the inflamed vasculature. Electron microscopy observations that alterations of the endothelial morphology occur very rapidly after glomerulonephritis initiation argue in favor of an increased exposure of subendothelial GPVI ligands to circulating platelets in the inflamed glomerulus.[99]

More recent data have suggested that besides regulation of endothelial permeability and leukocyte recruitment, prevention of inflammatory bleeding is another function that can depend on GPVI-mediated platelet recruitment to the inflamed vasculature[94,102,103] (Table 1). Studies based on the combination of mouse models of inflammation and severe thrombocytopenia (< less than 3% of normal mouse platelet count) have helped to demonstrate that platelets continuously secure the inflamed vasculature by preventing bleeding from the very onset of inflammation.[67,68] This protective action of platelets was reported in many different models of acute inflammation including various models of dermatitis,[67,70,94,102,111–113] IC-mediated glomerulonephritis,[114] endotoxin- and bacteria-induced acute lung injury,[61,67,102,111,113,115] and myocardial and cerebral ischemia-reperfusion injury,[67,103,113,116] as well as in models of viral infection[117,118] and solid tumors.[119–121] Strikingly, in several of these models, prevention of inflammatory bleeding was maintained in mice compromised for platelet aggregation at sites of traumatic vessel injury.[67,102,113] In particular, in contrast to thrombocytopenic mice, mice lacking GPIbα or integrin αIIbβ3 showed no bleeding in IC-mediated dermatitis or solid tumors.[67,113,119] Moreover, platelets lacking the thrombin receptor PAR4 and treated with inhibitors of the P2Y12 and TXA2 activation pathways were fully capable of ensuring hemostasis during IC-mediated dermatitis and LPS-induced lung inflammation when transfused to thrombocytopenic mice.[102] Conversely, transfusion of platelets lacking GPVI, CLEC-2, or their common downstream transducer SLP-76, failed to restore hemostasis in the inflamed skin and lungs of thrombocytopenic mice.[102] Taken together, these results have indicated that GPVI and CLEC-2 could play a predominant role in aggregation-independent inflammation-associated hemostasis.[122] Nevertheless, because the role of these receptors was suggested on the basis of transfusion experiments in which platelet counts were only partially restored, it is conceivable that relative thrombocytopenia might have sensitized recipients to inflammatory bleeding, thus magnifying the impact of GPVI and CLEC-2 deficiency. Using GPVI$^{-/-}$ mice, which have normal platelet count, the role of GPVI in limiting bleeding in IC-induced dermatitis has since been confirmed and investigated further.[94]

Intravital microscopy analysis of the skin vasculature during IC-induced dermatitis showed that, like in models of IC-induced glomerulonephritis,[99,114] platelet recruitment to the inflammation site started within minutes of reaction induction. Early platelet recruitment occurred without signs of thrombosis and in the form of transient or firm adhesion of individual platelets to the vessel wall and adherent neutrophils in post-capillary venules. Remarkably, platelet recruitment was prevented by neutrophil depletion and reduced, but not abolished, in GPVI$^{-/-}$ mice compared to their wild-type littermates.[94] These results indicated that GPVI contributed to prevention of inflammatory bleeding by mediating, in part, the recruitment of platelets at sites of neutrophil infiltration. They also implied that infiltrating neutrophils

unmasked binding sites for GPVI on the vessel wall, which was confirmed by the presence of endothelial gaps and the adhesion of microspheres coated with a chimeric form of GPVI in post-capillary venules where neutrophils accumulated and extravasated. The fact that GPVI deficiency did not completely abrogate platelet recruitment to inflamed skin vessels supported the idea that, as previously hinted by transfusion experiments,[102] redundant and/or compensatory mechanisms made up in part for the loss of GPVI. Consistent with these results, cutaneous inflammatory bleeding was less severe in GPVI^{−/−} mice than in mice immunodepleted for platelets.
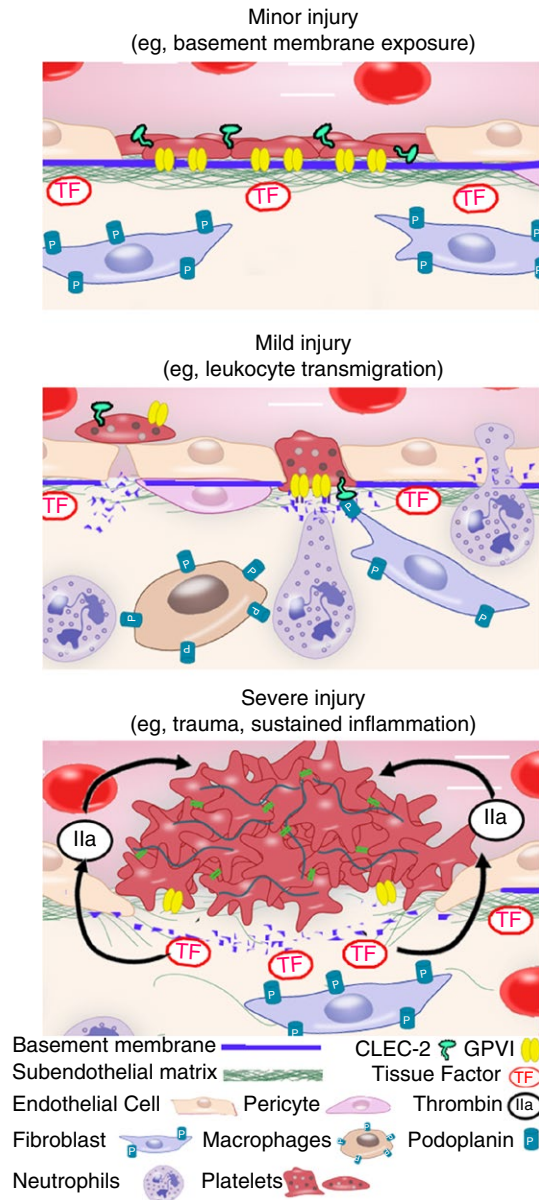
How platelets recruited through GPVI at sites of neutrophil infiltration prevent bleeding independently of aggregation? Dampening of neutrophil histotoxic activities represents a mechanism of action by which platelets could limit injury and bleeding. This possibility has however been dismissed on the grounds that markers of neutrophil activation during IC-mediated inflammation were reduced in thrombocytopenic and GPVI^{−/−} mice as compared to wild-type mice.[94] These data argued that platelets stimulated rather than prevent neutrophil activation during IC-mediated inflammation, and that GPVI contributed to this effect. They also added to growing indications that GPVI can regulate innate immune cell activation and function,[100,123] an effect that might involve the interaction of GPVI with one of its newly identified ligand, EMMPRIN, a surface receptor widely expressed in human tumors but also platelets, monocytes, and activated lymphocytes.[101,124]

Considering that GPVI binds various components of the subendothelial matrix, it is plausible that single platelets or sheets of platelets stop bleeding by covering small areas where the basement membrane gets exposed and disrupted by neutrophils. The role of GPVI in this inflammation-associated hemostatic process might not be restricted to platelet adhesion as it was shown that defective ITAM signaling, either due to a genetic deficiency in SLP76 or to combined pharmacological inhibition of the Src-family kinases Syk and Bruton's tyrosine kinase (Btk) partially impaired the ability of platelets to prevent inflammatory bleeding.[94,102] Thus, GPVI-mediated platelet responses likely also contribute to sealing of vascular breaches caused by neutrophils. In that regard, it was shown that even when not causing aggregation, collagen binding to GPVI can induce platelet shape change[125] and release of soluble platelet factors such as angiopoietin-1, serotonin, and ATP, all of which are regulators of endothelial cell barrier function or survival.[126] How platelet secretion and aggregation can be mechanistically uncoupled remains to be determined but a first glimpse was provided recently by studies showing the existence of specific forms and pathways of platelet secretion in response to low-dose agonists.[127,128] A recent study by Deppermann et al., showed that mice lacking platelet alpha- and/or dense-granule secretion showed no signs of inflammatory bleeding in IC-induced dermatitis or LPS-induced lung inflammation.[113] These findings suggest that platelet shape change and spreading might be more important than granule secretion for GPVI-dependent inflammation-associated hemostasis.

3 | BACK TO THE PAST: DIRECT EVIDENCE THAT PLATELETS CAN SECURE INJURED VESSELS INDEPENDENTLY OF AGGREGATION

Recent data converge to suggest that non-aggregated platelets adhering, spreading and/or releasing their granular content, in part through GPVI engagement, help to seal neutrophil-induced vascular breaches, thereby providing hemostasis in inflamed tissues (Figure 1).[98] But is there any direct evidence of platelets maintaining vascular integrity in the absence of aggregation? In a series of studies ranging from the 1960s to the 1970s, using electron microscopy, Hans Rudolf Baumgartner showed that platelets rapidly adhered and spread out to form a continuous monolayer that covered the subendothelium exposed following ballooning-induced endothelial denudation in large arteries (Figure 1).[129–131] In contrast to thrombi that disappeared rapidly, this "pseudo-endothelium" made of non-aggregated platelets persisted in time and rendered the vascular surface nonthrombogenic.[129,130] Collagenase treatment of the subendothelial surface prevented the formation of this platelet carpet, which in light of recent findings might be considered as a hint for a role of platelet/collagen interactions, and thus for GPVI, in this function. In a non-traumatic model of endothelial denudation, Baumgartner showed that single platelets filled small endothelial gaps forming following reserpin-induced capillary dilatation.[132] Areas of wider basement membrane exposure were covered by several platelets, however, "platelet aggregates as seen during the formation of platelet thrombi were never observed".[132] Baumgartner concluded that "in certain physiological conditions platelets function as 'bouche-trou' when for any reason the endothelial separate and the underlying basement lamina becomes exposed". One could argue that Baumgartner's observations might not apply to inflamed vessels as endothelial denudation only partially models the vascular modifications and damage accompanying inflammation. In fact, inflammation is not only associated with exposure of the basement membrane through endothelial gap formation but also with leukocyte-mediated damage to it.[98,133,134] Nonetheless, Baumgartner's studies provided evidence that, even under flow conditions, platelet adhesion to the basement membrane does not necessarily cause thrombus formation.

In studies contemporary to Baumgartner, Palade, Majno, Cotran, and other illustrious pathologists published electron microscopy images of the interactions between platelets and microvessels in the context of inflammation.[98] Most of these studies show images of single platelets sealing endothelial gaps in microvessels at sites of increased permeability or neutrophil extravasation (Figure 1).[98] Therefore, visual evidence that, besides fibrin deposits and platelet aggregates, nonaggregated platelets can repair small breaches in inflamed vessels has been available for over 50 years. Yet, it was only until recently that the importance of these interactions for the prevention of inflammatory bleeding, as well as their underlying mechanisms, including the role of GPVI engagement, were unraveled.

Minor injury
(eg, basement membrane exposure)

Mild injury
(eg, leukocyte transmigration)

Severe injury
(eg, trauma, sustained inflammation)

Basement membrane	CLEC-2	GPVI
Subendothelial matrix	Tissue Factor	TF
Endothelial Cell	Pericyte	Thrombin IIa
Fibroblast	Macrophages	Podoplanin P
Neutrophils	Platelets	

FIGURE 1 Platelets can maintain vascular integrity in multiple ways depending on the type and severity of vascular injury. Upper panel: The early work of Hans R. Baumgartner showed that in case of minor injury restricted to the endothelial layer (e.g, endothelial denudation, vasodilatation, and opening of endothelial junctions), platelets adhere and spread over the exposed basement membrane acting as "bouche-trou", either individually or through the formation of a nonthrombogenic "pseudo-endothelium" in wider areas of denuded basement membrane. Notably, the adhesion of platelets to the exposed subendothelial matrix is collagenase-sensitive, indicating a possible role for platelet/collagen interactions, and thus for GPVI, in this function. Middle panel: Recent and early studies indicate that, in case of mild injury where the basement is damaged (e.g, by neutrophil infiltration during acute inflammatory reactions), platelets seal vascular breaches by filling endothelial gaps and/or bridging endothelial junctions over areas of basement membrane disruption. Platelet GPVI contributes to the recruitment and adhesion of platelets to these sites. Engagement of platelets through CLEC-2 by podoplanin-expressing cells in direct vicinity of blood vessels (e.g, fibroblasts and inflammatory macrophages) provides a backup mechanism in the absence of GPVI. Lower panel: In a more classical view of hemostasis, in case of severe injury like in trauma or sustained inflammatory reactions, the exposure of the deeper layers of the vessel wall and/or of tissues surrounding vessels leads to thrombin generation and full platelet activation, thereby triggering platelet aggregation through integrin $\alpha IIb\beta 3$

4 | GPVI SECURING INFLAMED VESSELS: CLINICAL IMPLICATIONS AND APPLICATIONS

In preclinical studies, GPVI antagonists have proven to effectively inhibit thrombosis without significantly affecting primary hemostasis.[42,43] As a consequence, GPVI might appear as a new and ideal target for antithrombotic therapy that would combine efficacy with safety. In this context, the fact that GPVI deficiency or blockade in mice is associated with an increased risk of bleeding in the inflamed skin,[94,102] heart,[103] and possibly lungs[102] (Table 1), suggest that targeting GPVI might not be as safe as initially thought. Nevertheless, besides cutaneous petechiae which, as presented in the present review, can be caused by inflammation, inflammatory bleeding is not a common observation in

GPVI-deficient patients. There are several possible explanations for the lack of clinical reports of inflammation-induced bleeding in GPVI-deficient patients. First, we know from mouse studies that the role of GPVI in inflammation-associated hemostasis can be partly compensated for through engagement of other platelet receptors like CLEC-2.[102,122] Second, it is important to recall that platelets are extremely potent at preventing inflammatory bleeding, as inflammatory bleeding was observed only in mice with severe thrombocytopenia and could be rescued by restoring platelet counts to levels as low as 5-10% of normal mouse platelet count.[67,68,98] In a comparable manner, it was shown that even trace amounts of surface GPVI were sufficient to maintain residual function including adhesion to collagen under static conditions.[9,135] Moreover, consistent with the notion that even a low copy number of functional GPVI is sufficient for hemostasis, the parents of the first GPVI-deficient patient identified as such had platelets who retained normal function despite containing approximately 50% the normal amount of GPVI.[3] Last but not least, the protective role of GPVI in inflammation observed in the inflamed skin and lungs cannot be generalized to all inflammatory situations. In fact, not all organs are prone to inflammatory bleeding[98] (Table 1). No bleeding was observed in mice with severe thrombocytopenia that were subjected to rheumatoid arthritis or to peritonitis.[79,80,84,94] Furthermore, even in organs that are prone to inflammatory bleeding, GPVI is not always required for inflammation-associated hemostasis (Table 1). For example, GPVI was shown to be dispensable for the prevention of hemorrhagic

transformation following cerebral ischemia-reperfusion, which instead more classically relies on integrin αIIbβ3.[113,116,136] Therefore, not all organs are at risk of inflammatory bleeding, and even less with respect to the use of GPVI inhibitors. Nonetheless, the bleeding risk associated with the use of GPVI antagonists might be worth investigating in models of inflammation. Apart from animal models of inflammation, the tourniquet test may provide a simple clinical test for this purpose. Historically, like the bleeding time, the tourniquet test was used to diagnose thrombocytopenia and hemostasis defects.[137] Nowadays, it is part of the World Health Organization–recommended tests for the diagnosis of hemorrhagic fevers like dengue, a condition which incidentally nicely illustrates how the combination of infection and thrombocytopenia can cause bleeding. The tourniquet test is basically a model of mild cutaneous ischemia-reperfusion injury induced by a sphygmomanometer cuff inflated around the upper arm to stop blood flow for several minutes, before being deflated to allow reperfusion in the forearm. The test is considered positive when more than 10-20 petechial bleeding spots per square inch are observed. The formation of petechiae in the tourniquet test has long been interpreted as a consequence of capillary fragility, but recent studies have shown that it involved leukocyte-mediated vascular damage.[138–140] This test might thus turn useful to predict the anti-GPVI drugs-associated risk of inflammatory bleeding.

There is one situation where targeting the ability of platelets to prevent inflammatory bleeding may be beneficial. Experiments in tumor-bearing mice have shown that, like in inflamed organs, platelets continuously prevent leukocyte-induced bleeding in solid tumors independently of aggregation.[119,121,141] Immunodepletion of platelets potentiated the effect of chemotherapy in mice by improving drug accumulation specifically to the tumor site through induction of tumor bleeding.[120] Targeting platelet function was thus proposed as a way to improve chemotherapy.[142] Yet, the identity of the platelet receptor(s) involved in the prevention of tumor bleeding remains unknown. If GPVI were to be involved in this function, this might open the way to new applications for anti-GPVI drugs in the field of cancer.

5 | CONCLUSION

Recent studies have shown that, in various inflammatory situations, and from the very onset of inflammation, platelets ensure hemostasis independently of aggregation. In some of these situations, exemplified by IC-mediated dermatitis, platelets recruited early to inflammation sites partly through GPVI seal small vascular breaches caused by infiltrating leukocytes, thereby preventing bleeding. These recent data considered together with observations made in past electron microscopy studies indicate that sealing of those breaches involves platelets filling gaps in the endothelial lining and spreading over damaged areas of the exposed basement membrane. Both GPVI adhesion and signaling properties would thus be required for this function. The role of GPVI in this unconventional form of hemostasis shows that platelets can stop bleeding in multiple ways, depending on the type and severity of vascular injury, which likely determine the pathways and extent of platelet activation in a context-dependent manner.

6 | ISTH BERLIN REPORT

The duality of platelets, which exert both potentially damaging and protective actions in inflamed tissues,[143] is reflected in the works presented at the ISTH 2017 in Berlin. The group of Michael Hickey extended its previous observations on the proinflammatory role of platelets in a mouse model of IC-induced acute glomerulonephritis. After showing that GPVI and integrin αIIbβ3 mediate platelet recruitment to the inflamed glomerus, thus allowing subsequent platelet P-selectin-dependent recruitment of neutrophils.[99,110] they now provide evidence that platelet activation also contributes to stimulation of neutrophil intravascular activation. In particular, their new results show that the ADP/P2Y12 and thromboxane/TP receptor pathways stimulate neutrophil oxidative activity in inflamed glomerular capillaries.[144]

The regulatory action of platelets towards immune cell activities is not restricted to neutrophils.[83] In her presentation, Julie Rayes from the group of Steve Watson in Birmingham showed that platelets modulate the proinflammatory phenotype of podoplanin-expressing inflammatory macrophages during caecal ligation and puncture-induced sepsis. In this model, deletion of platelet-CLEC-2 resulted in an increased severity of sepsis that was associated with a dysregulation of inflammatory cytokine production, thus indicating a protective role of platelet CLEC-2.[63] These findings showing a protective role of platelet CLEC-2 in inflammation have since been published[64] and are in line with those of a recent study demonstrating that platelet CLEC-2 protects against LPS-induced lung injury via its interaction with podoplanin on inflammatory alveolar macrophages.[61] While it was previously shown that engagement of platelet CLEC-2 by podoplanin on inflammatory macrophages triggers platelet activation,[145] these new data indicate that this interaction induces reciprocal regulation between platelets and inflammatory macrophages. In a more general way, these results are consistent with a model where platelets and leukocytes regulate their recruitment and activation during inflammatory reactions in a mutual and reciprocal manner. It is worth mentioning that, in general, recent studies on the role of the podoplanin/CLEC-2 axis in inflammation have used PF4 cre mice to generate platelet-specific CLEC-2-deficient mice. Recent studies have shown that the PF4-driven cre recombinase is expressed on a subset of immune cells and activated dentritic cells, thus suggesting the possible implication of cells other than platelets in the observed effects.[146,147]

A series of studies converged to support the concept that platelets can ensure inflammation-associated hemostasis independently

of major platelet activation pathways, but also that the functional hierarchy between platelet receptors for this function varies with the cause and site of inflammation. The group of Tom van der Poll showed that, in contrast to the previously reported role of GPVI and CLEC-2 in the prevention of bleeding in models of IC-induced dermatitis and LPS-induced lung injury, neither GPVI nor CLEC-2 were needed for inflammation-associated hemostasis in a model of sepsis induced by *Klebsiella pneumoniae* lung infection.[148,149] Notably, GPVI-depleted mice showed increased bacterial growth in lungs, thus indicating that GPVI contributes to host defense during pneumonia-derived sepsis. In a model of inflammatory bowel disease induced by oral administration of dextran sodium sulfate, the group of Cécile Oury showed that prevention of intestinal bleeding by platelets involved P2X1 but not P2Y12 or GPVI.[150] In collaboration with the group of Steve Watson, we showed that GPVI played the major role in preventing bleeding in IC-mediated dermatitis, with CLEC-2 being involved only in the absence of GPVI.[62] In addition, we showed that neither receptor plays a prominent part in preventing bleeding in LPS-inflamed lungs, which partly relied on GPIb.

Collectively, these results demonstrate that the contribution of platelet adhesion receptors to vascular integrity varies between vascular beds and inflammatory challenges. Among the parameters that vary with the vascular bed and inflammatory stimulus, the type and expression levels of ligands of platelet activating and inhibitory receptors, the type of leukocytes involved, local hemodynamic factors (e.g, arterial vs. venous and systemic vs. pulmonary pressures and resistances), as well as the severity of the injury, likely contribute to determine how platelets are engaged for inflammation-associated hemostasis.

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