Platelets are essential players in thrombosis and hemostasis, as they "survey" the integrity of the vascular system. Upon damage of the vessel wall, they rapidly adhere to the exposed extracellular matrix (ECM) at the injury site and form a plug that seals the wound. However, under pathological conditions, uncontrolled thrombus formation may cause irreversible occlusion of the vessel and result in acute ischemic disease states, such as myocardial infarction or stroke (57, 88), which are the leading causes of disability and mortality worldwide (71). Therefore, targeting platelet signaling pathways and receptors has become an important therapeutic strategy for prevention and treatment of such acute ischemic events (47, 107). Particularly in ischemic stroke the therapeutic options are limited, as antithrombotic therapy may induce intracranial hemorrhage (1, 64). Thus, there is a strong demand for powerful, yet safe antithrombotic therapy.

Platelets are produced by their bone marrow resident precursors, the megakaryocytes (MKs), in a complex and unique process (72). Thereby, MKs at the sinusoidal blood vessels extend long cytoplasmic protrusions, designated proplatelets, from which platelets or platelet intermediates...
(113) are sequentially released into the blood stream, a process that has only recently been visualized in situ by two-photon microscopy of the mouse bone marrow (10, 58, 119). Changes in the organization of cytoskeletal components are critical for platelet production (6, 10, 56), however, the exact underlying mechanisms remain poorly understood. MKs express a similar repertoire of receptors as platelets, including those that mediate rapid platelet activation on ECM components (see below). How MKs remain relatively refractory in the ECM-rich environment to avoid signaling and consequently premature release of platelets into the bone marrow compartment, is a question of particular interest, with a recent report pointing towards a role of the inhibitory receptor G6b-B (78).

Platelets circulate for about 10 days in humans and about 5 days in mice and most of them never undergo adhesion before they are removed by the reticuloendothelial system. At sites of vascular injury, however, vessel wall components trigger rapid platelet adhesion, activation and subsequent thrombus formation through a complex and tightly regulated process involving multiple interactions of platelet receptors with immobilized ligands and soluble agonists (105). The initial adhesion of platelets to the components of the newly exposed ECM is mediated by the interaction of platelet receptor complex glycoprotein (GP) Ib/V/IX with von Willebrand factor (VWF), which becomes immobilized on collagen (96). This induces a rapid deceleration of the platelets, thereby enabling the interaction of collagen with the immunoglobulin-like receptor GPVI, the major platelet collagen receptor (86). Because of its low affinity for collagen, GPVI does not mediate stable adhesion by itself. However, through an immunoreceptor tyrosine-based activation motif (ITAM) in the associated Fc receptor (FcR) γ-chain, GPVI induces intracellular signaling processes, such as protein kinase C (PKC) activation and calcium (Ca^{2+}) mobilization (21). This leads to cellular activation and the release of secondary platelet agonists, most notably adenosine diphosphate (ADP), and thromboxane A₂ (TXA₂). In parallel, exposed tissue factor (TF) triggers local thrombin generation, which is further supported by the exposure of negatively charged phosphatidylserine on the platelet surface, providing a platform for the prothrombinase complex (50) and the release of inorganic polyphosphates which trigger the intrinsic pathway of coagulation through the activation of coagulation factor XII (FXII) (83). ADP, thrombin and TXA₂ further reinforce and sustain cellular activation by initiating different signaling pathways via G protein-coupled receptors (GPCRs), and recruit additional platelets from the blood stream into the growing thrombus. In addition, platelet activation can also be triggered through the hemITAM-receptor C-type lectin-like receptor 2 (CLEC-2) (77, 116). All these signaling pathways converge into the “final common pathway” of platelet activation, which is the conformational change of adhesion receptors of the integrin class, most notably integrin αIIbβ3 (GPⅡb/Ⅲa), from a low to a high affinity state, allowing firm adhesion, aggregation and stable thrombus formation (88). The scaffold proteins talin-1 and kindlin-3, which both bind to the cytoplasmic tail of β-integrins, as well as the small GTPase Rap1, play essential roles in this process, and their ablation in genetic mouse models resulted in severe hematopoietic defects (26, 81, 87, 92). Also for Rap1-GTP-Interacting Adaptor Molecule (RIAM) a critical role in integrin activation has been suggested – recent studies in knockout-mice, however, showed that RIAM is dispensable for this process in platelets (108).

In addition to integrins, a wide range of other receptors on the platelet surface have been reported or implicated to further stabilize the thrombus, such as ephrins/Eph kinases, junctional adhesion molecules (JAMs), SEM4D, CD150 or CD84 (20). A role for the latter one could, however, not be confirmed by studies on CD84-deficient mice (54).

Critical functions of platelets beyond their role in primary hemostasis and thrombosis have been increasingly recognized by demonstrating that platelets are essential to maintain the vascular integrity in inflamed tissue in a (hem)ITAM-dependent manner, a process that is critical to prevent spontaneous bleeding in multiple inflammatory disease settings (17, 42). In addition, platelets are indispensable for proper development and maintenance of the lymphatic system and CLEC-2 is the essential platelet receptor in this process (18).

In this review, we will summarize recent progress in the investigation of three central platelet receptors which are increasingly recognized as potential targets for the development of novel antithrombotic agents: GPⅠb, GPⅥI and CLEC-2. We will discuss the therapeutic potential of targeting these receptors in distinct thrombotic and thrombo-inflammatory diseases and will also emphasize the risks and potential pitfalls that have to be considered.

**Targeting GPIb in arterial thrombosis and ischemic stroke**

GPⅠb is part of the GPIb/Ⅴ/Ⅸ complex and besides interacting with VWF to initiate thrombus formation, it also binds other ligands, including P-selectin, macrophage antigen 1 (Mac-1), and the coagulation factors XI, XII and thrombin. In humans, the lack or dysfunction of GPIb/Ⅴ/Ⅸ is associated with a rare congenital bleeding disorder, the Bernard-Soulier syndrome, which is characterized by a bleeding phenotype, thrombocytopenia and giant platelets (23).

The essential role of GPIb in arterial thrombus formation was revealed by in vivo studies in mice. GPIb mutant mice in which the extracellular domain was replaced by the human interleukin-4 receptor showed severely prolonged tail bleeding (59) and were profoundly protected from vessel occlusion following chemical injury of mesenteric arterioles (12). This effect was more profound than that seen in VWF⁻/⁻ mice suggesting that also other ligands of GPⅠb may be involved in thrombus formation (12). Similarly, mice treated with a Fab fragment (p0p/B) that blocks the VWF binding site of the murine GPIb receptor exhibited abolished platelet adhesion to the injured carotid artery and were protected from occlusive thrombus formation in vivo (73) (Fig. 1). In addi-
tion, blocking the VWF binding site of GPIb markedly reduced the procoagulant activity of platelets and subsequent thrombin generation in vitro (62). Also in baboons, the antithrombotic potential of targeting the VWF-GPIb axis has been demonstrated: Treatment of baboons with an anti-VWF antibody targeting the VWF-collagen interaction (117), or the application of Fab fragments of a blocking anti-GPIb antibody (24) exerted strong anti-thrombotic effects, whereas bleeding times were not or only moderately prolonged. Due to these findings, different classes of drugs targeting the VWF-GPIb interaction, including humanized antibodies, nanobodies, aptamers and recombinant proteins, have been generated and are under (pre)clinical development (for review see (29, 43)).

In addition, the GPIb–VWF interaction has also emerged as a suitable pharmacological target for prevention and treatment of ischemic stroke. Stroke is the second leading cause of death and disability worldwide (71). In most cases, stroke is caused by a cerebral ischemia due to arterial occlusion of a major or multiple smaller intracerebral arteries. At present, immediate thrombolysis (within 4.5 h after onset) by intravenous application of tissue plasminogen activator (tPA) and more recently, mechanical thrombectomy are the only approved and effective acute stroke treatments (97). However, despite successful reperfusion of a previously occluded cerebral artery, ischemic brain lesions still further expand in significant number of patients, a process referred to as reperfusion injury (28).

The molecular processes involved in this secondary infarct growth have not been fully elucidated yet, but considerable progress in understanding underlying pathomechanisms has been made using animal models of experimental stroke, especially by transient middle cerebral artery occlusion (tMCAO) to induce ischemic stroke in rodents. In this model, a filament is advanced through the carotid artery into the middle cerebral artery to occlude the vessel and to induce cerebral ischemia. Although removal of the filament (typically after 1 h) allows reperfusion of the occluded vessel, progressive stroke develops which resembles the pathology observed in human patients. Meanwhile it is well-established, that besides thrombotic, also inflammatory processes are involved in the acute phase of ischemic stroke involving immune cells, most notably T cells, monocytes/macrophages and neutrophils (32). For instance, studies in mice lacking lymphocytes (Rag1−/−) demonstrated that T cells critically contribute to infarct progression, and that their detrimental effect is not related to adaptive immunity (66, 118). Hence, ischemic stroke is increasingly recognized as a thrombo-inflammatory disease (89).

Different approaches have been used to target platelet function and the coagulation cascade in acute ischemic stroke. These studies identified FXII as a potential target for treatment of this disease, since genetic deletion or its pharmacological inhibition markedly reduced infarct volumes and improved neurological function without affecting hemostasis in mice (48, 63). In contrast, the pharmacologic blockade of GPIIb/IIIa, which has proven to be beneficial in patients undergoing percutaneous coronary intervention, is not effective in the treatment of acute ischemic stroke in humans and mice, but rather worsens the outcome by increasing the risk of intracranial hemorrhage in both species (1, 61, 64).

There is compelling evidence that GPIb might represent a valuable pharmacological target to prevent or treat acute ischemic stroke (89). In the tMCAO model, both the prophylactic (1 h before tMCAO) and therapeutic (1 h after tMCAO) blockade of the VWF binding site on GPIb via administration of anti-GPIbα Fab fragments (pfp/B) dramatically reduced stroke progression and consequently resulted in significantly better neurological scores (64). Importantly, and in contrast to blockade of GPIIb/IIIa, no increased incidence of intracranial bleeding was observed in these mice, although they displayed markedly prolonged tail bleeding times (64). Ultra-high field (17T) magnetic resonance imaging (MRI) studies revealed that the blockade of GPIbα 1 h after tMCAO maintained the blood flow during the reperfusion phase. In contrast, the blood flow continuously decreased in control animals leading to large infarcts after 24 h (93). Similar to anti-GPIbα Fab-treated mice, VWF-deficient mice exhibit significantly reduced infarct volumes and a better neurological outcome following tMCAO as compared to wild-type controls, demonstrating a central role for the GPIb-VWF interaction in ischemic stroke (65, 120). This is further supported by hydrodynamic gene transfer experiments, where mutant forms of VWF were expressed in VWF-deficient animals and revealed that the GPIb-binding site of VWF, but not its binding site for GPIIb/IIIa, is crucial for stroke progression (30). The therapeutic potential of a pharmacological blockade of the GPIb-VWF interaction has been recently further emphasized in studies by Momi et al. (80). The authors showed that ALX-0081, a divalent humanized nanobody directed against the GPIb-binding site on VWF, prevents thrombo-inflammatory damage and infarct progression without increasing intracerebral hemorrhage in an experimental stroke model in guinea pigs. Strikingly, ALX-0081 also dissolved newly formed intracranial thrombi when given at early times after vessel occlusion, indicating that the interaction between GPIb and VWF is not only essential for platelet adhesion, but also for initial thrombus stabilization (80). Another therapeutic approach targeting this interaction could be the application of A disintegrin and metalloprotease with thrombospondin type 1 repeats-13 (ADAMTS13), a protease in the plasma that rapidly cleaves ultra-large VWF. Intravenous application of ADAMTS13 to naive mice provided partial protection from cerebral ischemia after tMCAO (120), while conversely, AdamiTS13−/− mice developed significantly larger infarcts than wild-type controls (39, 120).

GPIb-VWF interaction induces intracellular signaling events that eventually lead to weak integrin activation (23). The underlying mechanisms are only incompletely understood, however, phospholipase D1 (PLD1) seems to play a central function in this process (37). In line with the findings as summarized above, PLD1-deficient animals were markedly protected from stroke progression following tMCAO, again without displaying increased intracranial bleeding (37). Inhibition of PLD isoforms with the small molecule PLD inhibitor, 5-fluoro-2-indolyl-
des-chlorohalopemide (FIPI), reproduced this protection in wild-type mice, thus establishing PLD1 as a potential target for the treatment of ischemic stroke (106).

Targeting GPVI

The activating platelet collagen receptor, GPVI, is a MK-/platelet-specific transmembrane type I receptor that non-covalently associates with the FcR γ-chain which contains an ITAM. Upon ligand-induced GPVI clustering, the ITAM becomes tyrosine phosphorylated and initiates a series of phosphorylation events finally resulting in cellular activation (34) and the exposure of negatively charged phosphatidylsierines on the platelet surface (68). GPVI has been estimated to be expressed at 4000–6000 copies per platelet partially in a monomeric and dimeric form (13), with one GPVI molecule associating with one FcRγ-chain dimer. Various studies have shown that only the dimeric form of the receptor binds collagen with high affinity (34, 79). Constitutive signaling through GPVI is thought to be prevented by immunoreceptor tyrosine-based inhibition motif (ITIM) containing receptors, such as PECAM-1, CEACAM1/2, or G6b-B and the action of protein-tyrosine phosphatases (116). Furthermore, intracellular proteins, such as SLAP/SLAP2 (25) or CLP-36 (46), have been shown to act as negative regulators of GPVI/FcRγ-chain-mediated signaling in platelets.

A few patients with GPVI-related defects caused by autoantibody-induced receptor loss (5), compound heterozygous (33, 51) or homozygous mutations (76) have been reported so far. These patients suffer merely from a mild bleeding tendency, but their platelets are unresponsive to collagen. Interestingly, this phenotype could be reproduced in different mouse models of GPVI deficiency (8, 60, 70, 84, 85) or with blocking anti-GPVI antibody Fab fragments (73), revealing that GPVI is largely dispensable for normal hemostasis and has a predominant role in the formation of (experimental) arterial thrombi in vivo. Although some controversies exist concerning the functional role of GPVI in certain thrombosis models (8, 36), the vast majority of analyses emphasized a prominent role of the receptor in thrombus formation in injured arteries (88) or on atherosclerotic lesions (27, 49, 67, 91, 95).

In line with this, targeting of GPVI has been suggested to be beneficial in the setting of focal cerebral ischemia due to the observation that interfering with platelet adhesion and activation limits infarct progression without increasing the risk of intracranial bleeding. GPVI-depleted mice developed significantly reduced brain infarct volumes and this was not accompanied by an increase in bleeding complications (64). Although the reduction in brain infarct sizes was less pronounced than after GPIb blockade, GPVI might nevertheless be a suitable target for the treatment of acute stroke. Importantly, in patients, elevated GPVI expression levels in platelets have been shown to be associated with an increased risk of stroke development (15), and indirect evidence for increased GPVI activation in acute ischemic stroke has been provided by the observation that levels of soluble GPVI, which are generated upon platelet activation, are elevated in plasma of stroke patients (3). More direct evidence for a central role of GPVI-dependent signaling in stroke progression was recently provided by studies in mice deficient for the adapter proteins SLAP and SLAP2 whose platelets display a marked hyperreactivity in the GPVI-signaling pathway (25). SLAP/SLAP2 deficiency in platelets resulted in a dramatic increase in infarct volumes following tMCAO (Fig. 2) establishing GPVI as a critical modulator of stroke severity. The pro-inflammatory potential of platelets and the importance of GPVI in thrombo-inflammatory processes were also demonstrated in models of experimental rheumatoid arthritis (16), glomerulonephritis (31) and atherosclerosis (27, 49, 67, 91, 95).

Over the last years several experimental strategies have demonstrated efficacy in inhibiting GPVI function and also anti-thrombotic and anti-inflammatory potential (Fig. 1): One promising strategy turned out to be the direct blockade of the ligand binding site on platelet GPVI by a monovalent agent, such as an antibody Fab fragment, without altering its expression. This approach was shown to efficiently inhibit collagen-induced thrombus formation ex vivo in rats and cynomolgus monkeys (69, 75, 90) and occlusive thrombus formation in injured arteries in vivo in mice and rats (69, 73) without causing a significant bleeding defect. Another targeting strategy may be based on the observation that GPVI can be specifically and irreversibly removed from circulating platelets in vivo by antibody treatment. Monoclonal antibody (JAQ1, 2, or 3) injection in mice resulted in an acquired GPVI deficiency (85, 100) similar to that seen in patients (5) and lasted for several days. This was accompanied by a short transient thrombocytopenia and reduced protease activated receptor (PAR4) activity at early but not later time points (101). These mice displayed only moderately increased bleeding times, but a long-term antithrombotic protection in different experimental arterial thrombosis models. Interestingly, antibody-induced removal of GPVI could also be reproduced in human platelets circulating in NOD/SCID mice, demonstrating that this mechanism could be a powerful antithrombotic strategy in humans (19).

GPVI can principally be down-regulated by internalization/ degradation or ectodomain shedding, both of which require signaling through the FcRγ-chain ITAM (94). In vitro studies revealed that ADAM10 and ADAM17 are the GPVI sheddases depending on the shedding-inducing stimulus, whereby ADAM10 seems to play the major role (2, 4, 7, 40). However, the targeted (therapeutic) down-regulation of GPVI in vivo appears to occur through a more complex mechanism, since the antibody-induced GPVI down-regulation was not prevented in platelets of ADAM10/17 double-deficient mice (4). Although the approach of GPVI immunodepletion maybe a promising antithrombotic and anti-inflammatory strategy based on the results described, it is important to note that an anti-GPVI therapy principally has to be carefully evaluated. Anti-GPVI treatment severely compromised the hemostatic function in mice lacking CLEC-2 (9) or integrin α2β1, or in mice concomitantly treated with aspirin (44). This indicates that other receptors could compensate for defective GPVI function in hemostasis and might explain the observed mild bleeding tendencies in humans and mice lacking GPVI. These findings could be very important with regard to potential anti-GPVI treat-
ment of patients with other inherited or acquired platelet defects. This is also supported by a recent study from the Bergmeier group showing that the ITAM-containing receptors, GPVI and CLEC-2, are critical for maintenance of the vascular integrity at sites of inflammation (17).

Finally, another proposed antithrombotic strategy relies on the competitive inhibition of GPVI binding sites on its substrate collagen by a soluble dimeric GPVI-Fc fusion protein (74, 114, 115). The antithrombotic potential of GPVI-Fc fusion proteins appears, however, to be limited, as they failed to produce a detectable antithrombotic effect in different murine vascular injury models under conditions where direct targeting of GPVI was highly effective in preventing occlusive thrombus formation (45).

Taken together, experimental results point to GPVI as a promising pharmacological target for the treatment of thrombotic and inflammatory diseases because:

- the receptor is only expressed in platelets and MKs,
- its blockade, antibody-induced downregulation, competitive inhibition or genetic deficiency results in markedly reduced pathological thrombus formation without causing major bleeding complications in experimental animal models and
- GPVI is emerging as a key regulator of platelet-dependent inflammatory processes.

However, critical functions of platelets beyond their role in primary hemostasis and thrombosis, e.g., to maintain the vascular integrity in inflamed tissue (17), have to be considered.

**HemITAM-signaling – emerging functions of platelets beyond thrombosis and primary hemostasis**

The second ITAM-containing receptor on mouse platelets is CLEC-2, an ~32 kDa type II transmembrane protein, which signals via phosphorylation of a single conserved YXXL motif (hemITAM) in its cy-
CLEC-2 is expressed as a homodimer on MKs and platelets (~2000 copies per platelet) and at lower levels on some immune cells (41, 82, 102). CLEC-2 has been identified as the receptor for the powerful platelet activating snake venom toxin rhodocytin (109) and the sialoglycoprotein podoplanin (110), the only currently known endogenous ligand of CLEC-2. Since podoplanin is not expressed on platelets and vascular endothelial cells, it has been proposed that a hitherto unidentified CLEC-2 ligand may exist which becomes exposed or released at sites of injury or on growing thrombi and contributes to thrombus stabilization and eventually vessel occlusion (77).

CLEC-2 is expressed as a homodimer on resting platelets and clustering of the receptor upon ligand binding leads to powerful cellular activation (55). Receptor engagement of GPVI or CLEC-2 leads to the initiation of a downstream signaling cascade, characterized by the involvement of the Src family kinases (SFKs) Lyn and Fyn, the tyrosine kinase Syk, several adapter proteins, including LAT, SLP76 and Grb2 (35), which culminates in the activation of effector enzymes, such as phosphoinositide-3-kinase and PLCγ2 (116). Importantly, recent studies suggested a significant difference in the proximal events in GPVI- and CLEC-2-signaling: Phosphorylation of the ITAM on the FcR γ-chain is mediated by SFKs, followed by the recruitment, phosphorylation and activation of Syk (98, 104), whereas Syk is essential for phosphorylation of the hemITAM, with SFKs being predominantly involved in the regulation of downstream signaling events (103).

Initial studies on the role of CLEC-2 in thrombosis and hemostasis suggested that the receptor might become a target for antithrombotic therapy, because immunodepletion of CLEC-2 from circulating platelets (77) or genetic ablation of the receptor (9, 111) led to a significant protection of mice in models of occlusive arteriolar thrombosis, but only a moderate prolongation of bleeding times (Fig. 1). However, recent studies demonstrated a previously unrecognized functional redundancy of GPVI and CLEC-2 in hemostasis and the maintenance of vascular integrity during inflammation (9, 17), which questions the suitability of the hemITAM receptor as an antithrombotic target. Furthermore, besides its requirement for thrombus formation and stability and for preventing hemorrhage at sites of inflammation, CLEC-2 plays a crucial role in a plethora of other (patho)physiological processes, including tumor metastasis (reviewed in (112)) and lymphatic vascular development (18). Genetic ablation of CLEC-2 resulted in embryonic or neonatal lethality associated with blood-filled lymphatics and severe edema formation (14, 111), a phenotype which strongly resembles the one observed during embryonic development in mice deficient in key (hem)ITAM-signaling molecules, including Syk, SLP76 and PLCγ2, and importantly, podoplanin (18). Recent studies utilizing tissue-specific deletion approaches identified that (hem)ITAM-signaling in platelets mediates blood/lymphatic separation and further demonstrated an essential role of the interaction between platelet CLEC-2 and podoplanin on lymphatic endothelial cells in this process (14, 38). It has been controversially discussed how activated platelets enable blood/lymph vessel separation, but recent studies revealed that this may occur through a platelet hematic function that prevents blood from entering the lymphatic systems at the level of the thoracic duct (53). In addition, platelet CLEC-2-podoplanin interactions have been implicated in the maintenance of high endothelial venule integrity (52) and the development of lymph nodes (11), indicating that targeting of CLEC-2 might substantially alter immune responses which may not be desirable in patients at cardiovascular risk.

Taken together, recent studies establish CLEC-2 as a central receptor in arterial thrombosis, but also vascular integrity at inflammatory sites and blood/lymphatic separation, thereby raising awareness about the possibility of severe side-effects of anti-CLEC-2-based antithrombotic therapeutics. Furthermore, a growing body of experimental evidence documents a functional redundancy between CLEC-2 and GPVI in platelet hemostatic and anti-inflammatory functions, thus emphasizing the importance of evaluating the functional status of CLEC-2 prior to and during GPVI targeting. Notably, this requirement for thorough evaluation of the impact on receptor expression and function applies also to currently tested therapeutic agents which block common downstream signal-
ing molecules, such as the tyrosine kinase Syk.

**Conclusion**

GPIb and GPVI have emerged as promising pharmacological targets for powerful and safe prevention and treatment of thrombotic and anti-inflammatory diseases, whereas interference with CLEC-2 may represent an effective, but clearly not specific and safe antithrombotic approach. The MK-/platelet-specific expression of GPIb and GPVI and the possibility to efficiently block or immunodeplete them without causing major defects in platelet hemostatic functions further emphasize the suitability of both receptors as targets in the development of antithrombotic and antithrombo-inflammatory therapeutics. However, further studies are needed to better characterize the mechanisms by which platelet receptor signaling orchestrates the initiation and progression of complex thrombotic and (thromb)o-inflammatory disease states. This knowledge is pivotal for the improvement of existing antithrombotic strategies and may contribute to the development of more effective and safer therapies of thrombotic and thrombo-inflammatory disorders in the future.

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**Conflict of interest**

The authors declare no conflict of interest.

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