Platelet G_i protein $G\alpha_{i2}$ is an essential mediator of thrombo-inflammatory organ damage in mice

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Platelets are crucial for hemostasis and thrombosis and exacerbate tissue injury following ischemia and reperfusion. Important regulators of platelet function are G proteins controlled by seven transmembrane receptors. The G_i protein $G\alpha_{i2}$ mediates platelet activation in vitro, but its in vivo role in hemostasis, arterial thrombosis, and postischemic infarct progression remains to be determined. Here we show that mice lacking $G\alpha_{i2}$ exhibit prolonged tail-bleeding times and markedly impaired thrombus formation and stability in different models of arterial thrombosis. We thus generated mice selectively lacking $G\alpha_{i2}$ in megakaryocytes and platelets (*Gnai2*$^{fl/fl}$*/PF4-Cre* mice) and found bleeding defects comparable to those in global $G\alpha_{i2}$-deficient mice. To examine the impact of platelet $G\alpha_{i2}$ in postischemic thrombo-inflammatory infarct progression, *Gnai2*$^{fl/fl}$*/PF4*-Cre mice were subjected to experimental models of cerebral and myocardial ischemia/reperfusion injury. In the model of transient middle cerebral artery occlusion stroke *Gnai2*$^{fl/fl}$*/PF4*-Cre mice developed significantly smaller brain infarcts and fewer neurological deficits than littermate controls. Following myocardial ischemia, *Gnai2*$^{fl/fl}$*/PF4*-Cre mice showed dramatically reduced reperfusion injury which correlated with diminished formation of the ADP-dependent platelet neutrophil complex. In conclusion, our data provide definitive evidence that platelet $G\alpha_{i2}$ not only controls hemostatic and thrombotic responses but also is critical for the development of ischemia/reperfusion injury in vivo.

G proteins | platelets | ischemia reperfusion injury | $P2Y_{12}$ receptor | thrombosis

Platelet activation at sites of vascular injury is essential for normal hemostasis but also is a major pathomechanism underlying acute ischemic disease states such as stroke or myocardial infarction, which represent leading causes of death and severe disability worldwide (1–3).

Upon vascular injury, exposed extracellular matrix constituents of the damaged vessel wall allow initial adhesion of platelets, initiating intracellular signaling cascades that result in platelets' firm adhesion and aggregation (2, 3). Activated platelets deliver diffusible local mediators, such as ADP or thromboxane A_2, to recruit and activate additional platelets into the growing thrombus. Hence, ADP potentiates the aggregatory effects of other stimuli such as thrombin and collagen and thereby contributes to stable thrombus formation. These mediators orchestrate platelet signaling by activating G protein-coupled receptors (GPCRs) (4). In particular, platelet activation by ADP is mediated by two GPCRs, $P2Y_1$, which couples to the heterotrimeric G protein G_q, and $P2Y_{12}$, which couples to G_i proteins (5). Deficiency of either $P2Y_1$ or $P2Y_{12}$ receptors leads to a reduced aggregation response following ADP stimulation, suggesting a complementary function of the two types of G proteins, G_q and G_i, in the induction of platelet activation (6–9).

Only $P2Y_{12}$ receptors are therapeutically targeted by antagonists, which inhibit platelet aggregation in patients (10, 11). Correspondingly, mice lacking the $P2Y_{12}$ receptor exhibit a profound defect in platelet activation (7, 8) with prolonged bleeding times which correlate with impaired formation and stability of thrombi. However, the impact of this pathway on the progression of thrombo-inflammatory infarcts in the postischemic brain and heart is unknown. The $P2Y_{12}$ receptor is reported to signal selectively through the G protein G_{i2}, although biochemical reconstitution experiments suggest that it interacts with other G_i isoforms such as G_{i3} (11–13). Platelet G_{i2}, however, may interact not only with $P2Y_{12}$ but also with additional GPCRs present in platelets. Regardless of these considerations, and unlike murine platelets deficient in $P2Y_{12}$ receptors, $G\alpha_{i2}$-deficient platelets show only a moderate inhibition of platelet aggregation in vitro, and the translation of this defect into the in vivo situation has not been reported thus far (14, 15).

A different approach to study how $G\alpha_{i2}$ affects in vivo thrombotic activity of platelets came from a knockin mouse line in which regulator of G-protein signaling (RGS)-insensitive $G\alpha_{i2}$ (G184S) was expressed (16). However, the complexity and severity of the phenotype is likely to limit further studies on the progression of platelet-dependent thrombo-inflammatory infarcts (16).

Here, we show that $G\alpha_{i3}$ only partially compensates for the loss of $G\alpha_{i2}$, revealing that platelet $G\alpha_{i2}$ plays a dual role in both thrombus formation in injured vessels and in progression of tissue damage after focal cerebral ischemia or myocardial infarction.

Significance

Platelet activation is crucial for hemostasis and thrombosis but also contributes to inflammation and progression of tissue damage following ischemia/reperfusion injury. Here we demonstrate that platelet activation through the G_i protein $G\alpha_{i2}$ not only controls hemostatic responses but also thrombo-inflammatory tissue damage following cerebral and cardiac ischemia. Our report on a dual role of G_i proteins in platelet function opens new options for pharmaco-therapeutic strategies fighting ischemic diseases such as heart attack and stroke.

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Materials and Methods

$G\alpha_i$-Deficient Mouse Strains. The generation and basal phenotypic characterization of $G\alpha_{i2}$-deficient and $G\alpha_{i3}$-deficient mice have been described elsewhere (17–20). All mice were maintained in isolated ventilated cages or under specific pathogen-free conditions according to national guidelines for animal care at the animal facilities of the Universities of Düsseldorf, Tübingen, and Würzburg. Animal experiments used 10- to 14-wk-old mice of either sex and were conducted in accordance with current laws in combination with the regulations of the local authorities Regierungspräsidium of Dusseldorf, Tubingen, or Wurzburg.

Immunoblot Analysis of $G\alpha_i$ Proteins. Preparation of cell membranes and immunoblot analysis has been described previously (21, 22).

Platelet Preparation and Aggregation. Platelet preparation and aggregation was measured as described previously (23).

Tail-Vein Bleeding Assay. Mice were anesthetized, and tail bleeding was assessed using either the filter paper method or the PBS method as described elsewhere (23, 24).

Aorta Occlusion Model. As described previously, thrombus formation was induced by a single firm compression of the vessel with a forceps downstream of the flow probe (25).

Intravital Microscopy of Thrombus Formation in $FeCl_3$-Injured Mesenteric Arterioles. This thrombus model was carried out as detailed elsewhere (26).

Transient Middle Cerebral Artery Occlusion. Focal cerebral ischemia was induced in mice by 1 h of transient middle cerebral artery occlusion (tMCAO) as described in ref. 25.

Assessment of Functional Outcome and Determination of Infarct Size. Neurological function was assessed by three independent investigators 24 h after tMCAO in a blinded manner. A modified Bederson score was used to determine global neurological function. Motor function and coordination were graded using the grip test (25, 27). The animals were killed immediately thereafter, and brains were sliced and stained with 2,3,5-triphenyltetrazolium chloride (TTC) (25).

Murine Model of Myocardial Ischemia. Murine model of myocardial ischemia/reperfusion injury was performed as described previously (28).

Determination of Platelet–Neutrophil Complex Formation. Platelet–neutrophil complex (PNC) formation was measured by flow cytometry as described previously (28).

Statistics. Statistical evaluation was performed as indicated in figure legends. A value of $P \leq 0.05$ was considered to be statistically significant.

Additional detailed information on materials and methods is provided in *SI Materials and Methods*.

Results

$G\alpha_{i2}$ Plays a Critical Role in Hemostasis. Earlier in vitro studies with platelets from $G\alpha_i$ KO mice had indicated a selective role for $G\alpha_{i2}$ in ADP-induced platelet aggregation (14, 15). Here, we observed that $G\alpha_{i2}$-deficient platelets exhibited a moderately reduced aggregation response not only to ADP but also to other stimuli such as collagen or collagen-related peptide (CRP) (Fig. S1*A*). Mechanistically, a defect in integrin αIIbβ3 activation (Fig. S1*C*) and degranulation-dependent P-selectin exposure (Fig. S1*B*) following various stimuli was evident, but the stimulated rise of intracellular calcium was unaffected (Fig. S2).

To assess the role of $G\alpha_i$ in platelet function further, we examined the ability of platelets from $G\alpha_i$-deficient mice to form aggregates on collagen under flow (Fig. S3). WT and $G\alpha_{i3}$-deficient platelets adhered to collagen fibers and formed aggregates that consistently grew into large thrombi. In contrast, $G\alpha_{i2}$-deficient platelets initially adhered to collagen and were able to form smaller aggregates, but these aggregates subsequently failed to propagate into stable thrombi (Fig. S3 *B* and *C*). These results show that $G\alpha_{i2}$ has a significant role in forming stable aggregates under high-shear flow conditions. To test whether this defect translated into a bleeding diathesis, we measured tail-bleeding times in $G\alpha_{i2}$-deficient mice using the filter paper method (Fig. 1). We found markedly prolonged bleeding times in $G\alpha_{i2}$-deficient mice compared with $G\alpha_{i3}$-deficient and WT mice, and bleeding had to be stopped manually in 50% of the $G\alpha_{i2}$-deficient animals to avoid excess blood loss (Fig. 1*A*). In addition, $G\alpha_{i2}$-deficient mice showed considerable variations in tail-bleeding intensity (Fig. 1*B*). Together, these data show that $G\alpha_{i2}$ deficiency results in a moderate defect in platelet aggregation in vitro which translates into a severe hemostatic defect in vivo.

$G\alpha_{i2}$ Deficiency in Mice Alters the in Vivo Thrombotic Profile. To assess the effect of $G\alpha_{i2}$ deficiency on occlusive thrombus formation in vivo, we used two well-established models of arterial thrombosis. In the first model, vascular injury was induced in the abdominal aorta mechanically by a single firm compression with a forceps, and blood flow was monitored with an ultrasonic flow probe (25). In WT and $G\alpha_{i3}$-deficient mice, injury-provoked thrombosis resulted in complete and irreversible occlusion of the vessels within 10 min (Fig. 2 *A* and *B*). In contrast, platelets from $G\alpha_{i2}$-deficient mice did not form occlusive thrombi within the observation time of 30 min (Fig. 2 *A* and *B*). In a second model, we monitored thrombus formation in $FeCl_3$-injured mesenteric arterioles using intravital microscopy (26). In all WT mice examined, the formation of small platelet aggregates started within 10 min after injury (Fig. 2 *C* and *D*), resulting in complete vessel occlusion within 20 min (Fig. 1*E* and Movie S1). In contrast, although the initial adhesion and formation of small aggregates was similar in arterioles from WT and $G\alpha_{i2}$-deficient mice (Fig. 2 *C* and *D*), the formation of stable and occlusive thrombi did not occur in the majority of arterioles during the observation period of 40 min (Fig. 2*E* and Movie S2), revealing a pivotal role for $G\alpha_{i2}$ in occlusive thrombus formation in vivo.

Up-Regulated $G\alpha_{i3}$ Partially Compensates for the Missing $G\alpha_{i2}$. Deletion of one $G\alpha_i$ isoform in mice can provoke an up-regulation of the expression levels of the remaining isoforms, which may substitute for some but not all functions (17, 19, 29–33). Because we observed a clear-cut phenotype in the $G\alpha_{i2}$ KO mice, we analyzed the expression of $G\alpha_i$ isoforms in their platelets. As in human platelets, we detected two $G\alpha_i$ proteins, i.e., $G\alpha_{i2}$ and $G\alpha_{i3}$, by immunoblotting (Fig. S4 *A* and *B*). Importantly, a significant up-regulation of platelet $G\alpha_{i3}$ in the absence of $G\alpha_{i2}$ was

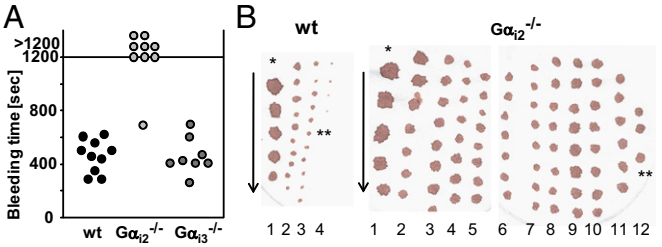

Fig. 1. Prolonged bleeding times in global $G\alpha_{i2}$-deficient mice. (*A*) Tail-bleeding times of WT ($n = 10$; 7.9 ± 2.0 min), $G\alpha_{i2}$-deficient ($G\alpha_{i2}^{-/-}$; $n = 9$), and $G\alpha_{i3}$-deficient ($G\alpha_{i3}^{-/-}$; $n = 8$; 7.9 ± 2.3 min) mice. Each symbol represents one individual mouse. (*B*) Filter papers showing 4 and 12 rows of consecutive bleeding spots after tail-tip amputation from a WT and a $G\alpha_{i2}$-deficient mouse, respectively. Each spot represents one time point of 20-s intervals. The single asterisk indicates the first bleeding spot, and the double asterisks indicate the last bleeding spot after tail-tip amputation. Note that initially the size of the bleeding spots from the $G\alpha_{i2}$-deficient mouse decreased continuously (rows 1–7) but then increased (rows 8–10). Eventually, the bleeding spots decreased again but had not stopped at the end of the test (rows 11–12).

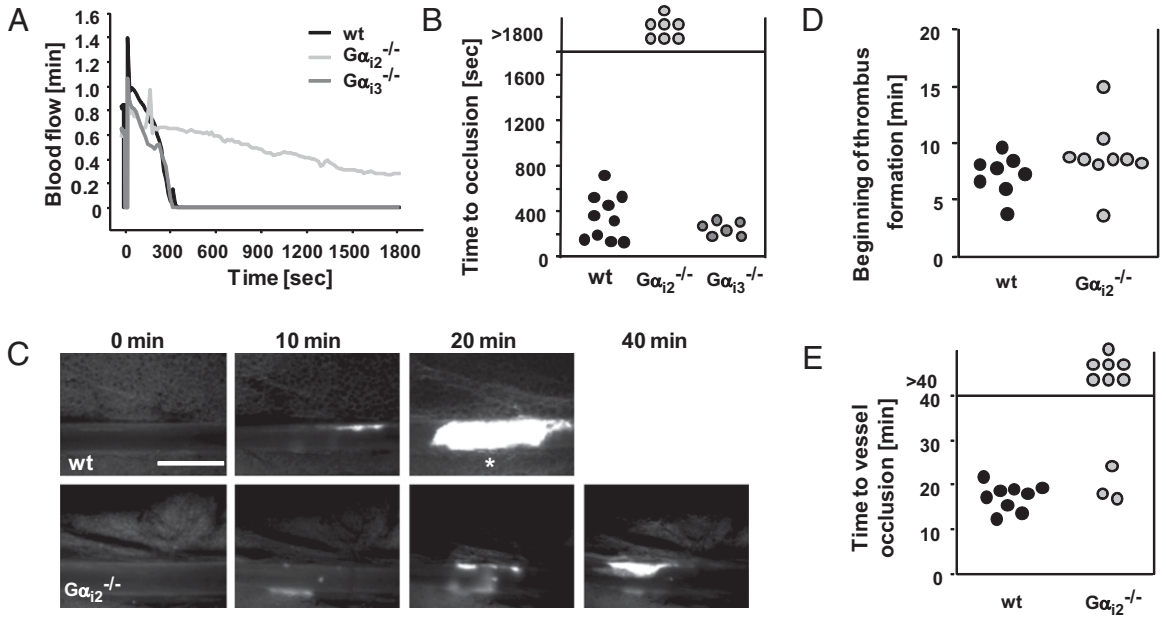

Fig. 2. Defective thrombus formation in global $G\alpha_{i2}$-deficient mice. (*A* and *B*) The abdominal aorta of WT ($n = 10$), $G\alpha_{i2}$-deficient ($n = 7$), and $G\alpha_{i3}$-deficient mice ($n = 6$) was injured by tight compression with a forceps, and blood flow was monitored for 30 min. (*A*) Representative blood-flow recordings. (*B*) Time to stable vessel occlusion. Each symbol represents one mouse. Mean times to occlusion were 6.1 ± 3.4 min for WT mice and 4.5 ± 1.0 min for $G\alpha_{i3}^{-/-}$ mice ($P > 0.05$; Welch test). $G\alpha_{i2}^{-/-}$ mice showed no occlusion within the observation time. (*C*) Thrombus formation after $FeCl_3$-induced injury of mesenteric arterioles was monitored with intravital fluorescence microscopy (see also Movies S1 and S2). Representative images were taken at the indicated time points. (Scale bar: 100 μm.) The asterisk indicates stable occlusion of the vessel. (*D*) Thrombus formation began at 7.2 ± 1.7 min for arterioles from WT mice ($n = 8$) and at 8.9 ± 2.9 min for arterioles from $G\alpha_{i2}^{-/-}$ mice ($n = 9$); $P > 0.05$; Welch test. (*E*) Time to stable occlusion was 17.9 ± 3.0 min for arterioles from WT mice ($n = 9$), but 7 of 10 arterioles from $G\alpha_{i2}^{-/-}$ mice showed no occlusion within the observation time. Each symbol represents one vessel.

evident. A similar picture emerged from analysis of pertussis toxin (PTX)-catalyzed [^{32}P]ADP ribosylation of $G\alpha_i$ proteins (Fig. S4*C*). From these data one may conclude that the up-regulated and functionally intact $G\alpha_{i3}$ cannot rescue defects caused by the missing $G\alpha_{i2}$. Nonetheless, an ADP-induced platelet aggregation still occurred in the absence of $G\alpha_{i2}$, suggesting that $G\alpha_{i3}$ may partially compensate for $G\alpha_{i2}$. To explore this possibility in more detail, we administered PTX, a selective pan-$G\alpha_i$ inhibitor, into WT mice (Fig. S5) and analyzed platelet aggregation and thrombus formation ex vivo and in vivo (Figs. S1, S3, and S6). In summary, the results demonstrated a much stronger PTX-mediated inhibition of platelet activation, aggregation, and thrombus formation than seen with $G\alpha_{i2}$ deficiency, suggesting a partial compensatory role for $G\alpha_{i3}$ in these processes.

Selective $G\alpha_{i2}$ Deficiency in Platelets Is Accompanied by $G\alpha_{i3}$ Up-Regulation. Next, the specific role of $G\alpha_{i2}$ in platelets was studied in a mouse line that was generated by breeding conditional $G\alpha_{i2}$ mice (*Gnai2*$^{fl/fl}$) with mice expressing Cre recombinase under the control of the platelet factor 4 (PF4) promoter (19, 34). The efficient and platelet-specific deletion of $G\alpha_{i2}$ was confirmed, and an increased expression of $G\alpha_{i3}$ became evident by immunoblotting (Fig. S7 *A* and *B*). Like the global $G\alpha_{i2}$-deficient mice, *Gnai2*$^{fl/fl}$/*PF4*-Cre mice displayed unaltered platelet counts and volume as well as expression of prominent platelet surface receptors (Tables S1 and S2). Platelet aggregation measurements (Fig. S7*C*), flow cytometric analysis of integrin αIIbβ3 activation (Fig. S7*D*), and degranulation-dependent P-selectin exposure (Fig. S7*E*) presented defects caused by a selective deficiency of $G\alpha_{i2}$ in platelets in response to various stimuli. Interestingly, in the presence of a specific $P2Y_{12}$ antagonist, ARC69931, ADP-induced aggregation of $G\alpha_{i2}$-deficient platelets was reduced further (Fig. S7*F*), indicating that they still responded to thienopyridines and further supporting our data that $G\alpha_{i3}$ is a potential downstream regulator of $P2Y_{12}$. In addition, we ruled out the possibility that reduced ATP release is responsible for these defects (Fig. S7*G*).

Selective Deficiency of $G\alpha_{i2}$ in Platelets Impairs Hemostasis. Next, we assessed role of platelet $G\alpha_{i2}$ in hemostasis by performing bleeding-time assays. In WT (*Gnai2*$^{+/+}$) mice and most *Gnai2*$^{fl/fl}$ control mice, tail bleeding stopped within 10 min (Fig. S7*H*). In contrast, the majority of *Gnai2*$^{fl/fl}$/*PF4*-Cre^{+} mice showed greatly prolonged tail bleeding similar to that in global $G\alpha_{i2}$-deficient mice. A second bleeding assay in which the tip-amputated tail was inserted into a tube with PBS confirmed prolonged bleeding (Fig. 3*B*) and fluctuations in bleeding intensity (Movies S3 and S4). In conclusion, selective deletion of $G\alpha_{i2}$ in platelets and megakaryocytes is sufficient to produce a pronounced bleeding defect.

Platelet-Specific $G\alpha_{i2}$ Deficiency Results in Neuroprotection Following Cerebral Ischemia/Reperfusion Injury. Platelets are thought to integrate pathways that orchestrate both thrombotic and inflammatory processes, resulting in a thrombo-inflammatory cascade in acute ischemic disease states in which tissue damage occurs despite successful recanalization of occluded vessels, a process referred to as "reperfusion injury" (3, 35, 36). To evaluate the impact of platelet $G\alpha_{i2}$ on infarct progression, we challenged *Gnai2*$^{fl/fl}$/*PF4*-Cre mice in the tMCAO model in which a filament reversibly occludes the middle cerebral artery and reduces the regional cerebral flow by >90% (25). After 1 h the filament is removed to allow reperfusion for 24 h. Subsequently, mice are tested for their motor and coordination functions. Interestingly, assessment of the global neurologic function (Bederson score) (Fig. 4*A*) revealed that *Gnai2*$^{fl/fl}$/*PF4*-Cre mice developed significantly fewer neurologic deficits and had significantly better (higher) scores in motor function tests (Fig. 4*B*) than littermate controls. These findings were accompanied by a reduction in infarct volumes by >60% in *Gnai2*$^{fl/fl}$/*PF4*-Cre mice

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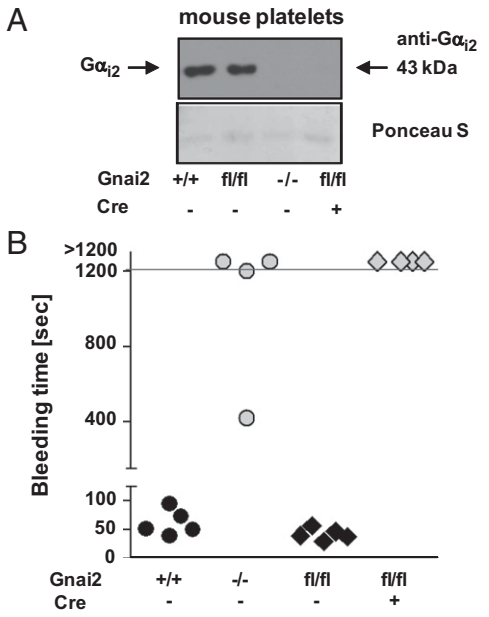

Fig. 3. Prolonged bleeding times in mice selectively lacking $G\alpha_{i2}$ in platelets. (*A*) Cell membranes (20 μg) from platelets isolated from global (*Gnai2*$^{-/-}$) or megakaryocyte/platelet-specific $G\alpha_{i2}$-deficient (*Gnai2*$^{fl/fl}$/*PF4*-Cre^{+}) mice were analyzed by immunoblotting using a $G\alpha_{i2}$-antibody. PF4-Cre–negative littermates (*Gnai2*$^{fl/fl}$/*PF4*-Cre^{-}) and WT animals (*Gnai2*$^{+/+}$) were used as controls. Equal loading was controlled by staining with Ponceau S. (*B*) Tail-bleeding times of WT (*Gnai2*$^{+/+}$) ($n = 5$), $G\alpha_{i2}$-deficient (*Gnai2* $^{-/-}$) ($n = 4$), *Gnai2*$^{fl/fl}$/*PF4*-Cre^{-} ($n = 5$), and *Gnai2*$^{fl/fl}$/*PF4*-Cre^{+} ($n = 4$) mice measured with the PBS method. Mean bleeding time was 42.4 ± 4.5 s for WT mice and 60.2 ± 9.9 s for *Gnai2*$^{fl/fl}$/*PF4*-Cre^{-} mice, but in three of four global $G\alpha_{i2}$-deficient mice and in all *Gnai2*$^{fl/fl}$/*PF4*-Cre^{+} mice the bleeding had to be stopped manually after 20 min to prevent lethal bleeding. Each symbol represents one individual.

compared with controls, as evaluated by 2,3,5-triphenyltetrazolium chloride (TTC) staining of brain sections (Fig. 4 *C* and *D*). In summary, $G\alpha_{i2}$ deficiency in platelets significantly protects mice from postischemic brain damage and results in a better outcome following experimental stroke.

Platelet-Specific $G\alpha_{i2}$ Deficiency Protects Against Myocardial Ischemia/Reperfusion Injury. To address the specific role of platelet $G\alpha_{i2}$ in a second clinically relevant model of thrombo-inflammatory organ damage, we assessed myocardial ischemia/reperfusion injury in control mice and mutant littermates (Fig. 5). In this acute invasive model, the left coronary artery is occluded for 1 h. We used a hanging-weight system for coronary artery occlusion as described previously (28, 31). Inspection of TTC-stained heart discs immediately visualized differences between *Gnai2*$^{fl/fl}$/*PF4*-Cre mice and controls (Fig. 5*A* and Fig. S8*B*). For statistical analysis, the degree of myocardial destruction was calculated as percentage of infarcted myocardium to area at risk (AAR). Although the AAR did not differ between the two groups (Fig. S8*A*), infarct size was 43.0 ± 3.7% of AAR in control mice but was significantly reduced to 21.8 ± 3.3% of AAR in *Gnai2*$^{fl/fl}$/*PF4*-Cre mice (Fig. 5*B*).

These results identified platelet $G\alpha_{i2}$ as an important intracellular switch that controls not only platelet-dependent thrombosis but also thrombo-inflammatory infarct progression during reperfusion following stroke and cardiac ischemia.

Accumulating evidence suggests that platelets are part of the immune cell arsenal. In particular, cross-talk between platelets and leukocytes is a common feature of immune reactions with formation of PNCs affecting the extent of inflammatory tissue damage (28, 37–39). Therefore we examined the formation of PNCs after myocardial ischemia/reperfusion injury (Fig. 5*C*) and found that PNC levels in *Gnai2*$^{fl/fl}$/*PF4*-Cre mice (45.4 ± 12.7) were less than 10% of the levels in littermate controls (587.8 ± 189.6), resulting in less tissue destruction (Fig. S8*C*). Moreover, in the absence of infarction, platelets and neutrophils from *Gnai2*$^{fl/fl}$/*PF4*-Cre mice showed defective PNC formation upon ADP stimulation (Fig. 5*D*).

In conclusion, $G\alpha_{i2}$ integrates signaling pathways controlling both platelet aggregation and inflammation during ischemia/reperfusion injury, thereby enabling platelets to bridge hemostasis and inflammation.

Discussion

Activated platelets not only are essential for thrombus initiation, formation, and stabilization but also promote inflammation and progression of tissue damage even after reperfusion of previously occluded arteries (40). Activation of platelets involves numerous cell-surface receptors coupled to G proteins, and the $G\alpha_{i2}$ protein has been suggested to be important in this scenario, but its exact in vivo function has long remained undefined. Here, we show that $G\alpha_{i2}$ is crucial for thrombus stabilization in the course of hemostasis and experimental thrombosis. In addition, our study reveals an important function of platelet $G\alpha_{i2}$ in the development

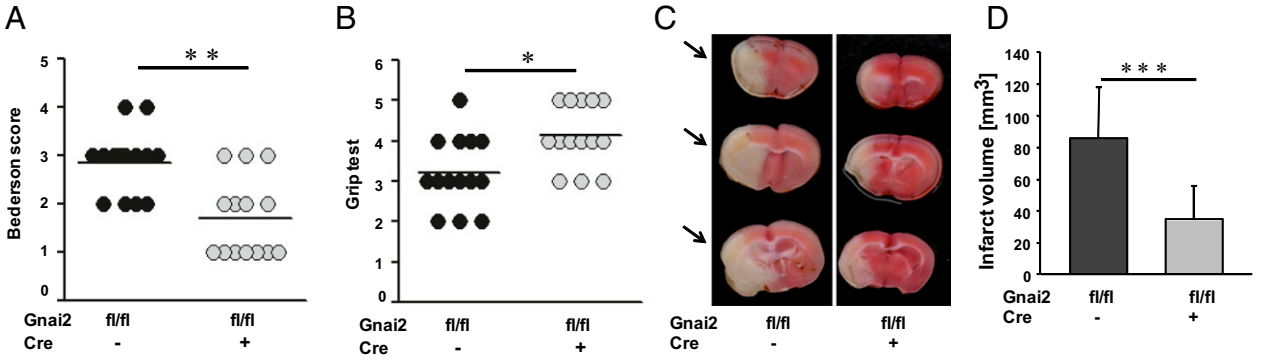

Fig. 4. Platelet-specific deletion of $G\alpha_{i2}$ protects against reperfusion injury following transient cerebral ischemia. Consequential neurologic defects after cerebral brain infarction were investigated in the tMCAO model. (*A* and *B*) Bederson score (*A*) and grip test (*B*) determined 24 h after tMCAO of Gnai2$^{fl/fl}$/PF4-Cre^{+} mice ($n = 14$) and littermate controls ($n = 14$). (*C*) Representative images of three corresponding coronal sections from control and *Gnai2*$^{fl/fl}$/*PF4*-Cre^{+} mice stained with TTC 24 h after tMCAO. Red areas represent vital brain tissue, and white areas indicate cerebral infarctions (arrows). (*D*) Mean infarct volumes were 86.1 ± 34.5 mm^3 for *Gnai2*$^{fl/fl}$/*PF4*-Cre^{-} mice and 31.7 ± 21.4 mm^3 for *Gnai2*$^{fl/fl}$/*PF4*-Cre^{+} mice. Data are presented as mean ± SD and were analyzed with the Mann–Whitney *u* test; $*P < 0.02$, $**P < 0.002$, and $***P = 0.0002$.

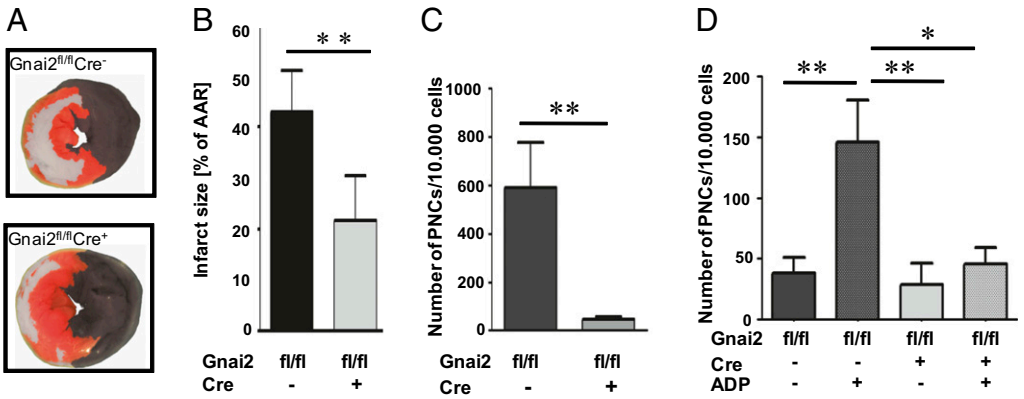

Fig. 5. Platelet-specific deletion of $G\alpha_{i2}$ protects against reperfusion injury following myocardial ischemia. (*A* and *B*) *Gnai2*$^{fl/fl}$/*PF4*-Cre^{+} mice ($n = 7$) and control mice ($n = 5$) were exposed to 1 h of ischemia and 2 h of reperfusion. Hearts were stained with Evans Blue to determine the AAR and with TTC to mark vital tissue (red) and necrotic tissue (white). (*A*) Representative heart slices of a *Gnai2*$^{fl/fl}$/*PF4*-Cre^{+} mouse and a littermate control with infarcted areas of 22% and 43%, respectively. Images were contrasted for better visibility. The original images of these heart slices are shown in Fig. S8*B*. (*B*) Infarct size was calculated as percentage of AAR. Data are shown as mean ± SEM; $^{**}P < 0.01$ (*t* test). (*C*) Number of PNCs after 1 h of ischemia detected in whole blood by flow cytometry in *Gnai2*$^{fl/fl}$/*PF4*-Cre^{+} mice ($n = 10$) and littermates ($n = 9$). (*D*) Number of PNCs per 10,000 cells at basal levels (*Gnai2*$^{fl/fl}$/*PF4-Cre*$^{-}$: 38.3 ± 12.5; *Gnai2*$^{fl/fl}$/*PF4-Cre*$^{+}$: 28.4 ± 17.7) and after ADP stimulation (*Gnai2*$^{fl/fl}$/*PF4-Cre*$^{-}$: 146.1 ± 34.6; *Gnai2*$^{fl/fl}$/*PF4-Cre*$^{+}$: 45.6 ± 13.3). Data are shown as mean ± SEM and were analyzed with ANOVA followed by Dunnett's test; $^{*}P < 0.05$ and $^{**}P < 0.005$ compared with *Gnai2*$^{fl/fl}$/*PF4*-Cre^{-} +ADP.

of cerebral and myocardial ischemia/reperfusion injury. These findings emphasize a thrombo-inflammatory role of platelets and put platelet $G\alpha_{i2}$ in the center of controlling both hemostasis and inflammation.

Previous studies focused on acute responses of isolated platelets from $G\alpha_i$ KO mice to stimuli, leaving the question on the in vivo relevance of G_i signaling unanswered (14, 15, 41). We show that global $G\alpha_{i2}$ but not $G\alpha_{i3}$ deficiency produces a marked increase in bleeding time, although spontaneous hemorrhage was not evident. The same profound bleeding phenotype was seen in *Gnai2*$^{fl/fl}$/*PF4*-Cre mice, demonstrating a central role of platelet $G\alpha_{i2}$ in hemostasis. Of note, a similar primary hemostatic defect is seen in mice lacking the G_i-coupled ADP receptor $P2Y_{12}$ (5, 6, 8).

Initially, we confirmed previous data that $G\alpha_{i3}$ deficiency did not result in a platelet-specific phenotype. However, PTX treatment of mice produced a much stronger reduction of platelet function than $G\alpha_{i2}$ deficiency. Interestingly, and in contrast to previous reports, we noticed an up-regulation of $G\alpha_{i3}$ expression in $G\alpha_{i2}$ KO mice which we identified to be the only PTX substrates in platelets (12–15, 41). Therefore, we assume that $G\alpha_{i3}$ partially compensates for the missing $G\alpha_{i2}$. In fact, $G\alpha_{i3}$ has been shown to interact with $P2Y_{12}$ in a purified reconstituted system (12). Further support for this conclusion comes from the finding that the specific $P2Y_{12}$ receptor antagonist ARC69931 blunts the residual response to ADP in $G\alpha_{i2}$-deficient platelets. Taken together, the platelet $P2Y_{12}$ receptor signals through two $G\alpha_i$ isoforms with $G\alpha_{i2}$ being the pathophysiologically dominant one.

In tail-bleeding assays we observed variability in bleeding intensity in $G\alpha_{i2}$-deficient mice which paralleled defective formation and stabilization of arterial thrombi seen in two arterial thrombosis models. These findings agree with impaired thrombus stability seen in $P2Y_{12}$ receptor-deficient mice (5, 6) and are complementary to reports from a heterozygous RGS-insensitive $G\alpha_{i2}$ mutant (+/G184S) demonstrating increased platelet accumulation and aggregation at vascular injury sites in vivo, although the effects on bleeding times in this mouse model were not reported (42, 43). Thus, $G\alpha_{i2}$ represents the dominant signaling entity downstream of the ADP-binding $P2Y_{12}$ receptor where either its absence or its uncontrolled increased signaling activity has a profound impact on thrombus formation and stability, indicating that balanced $G\alpha_{i2}$ activity assures normal hemostasis and thrombosis.

Platelets also contribute to secondary infarct progression by inflammatory mechanisms that may differ from those involved in thrombus formation (3, 36). Interestingly, *Gnai2*$^{fl/fl}$/*PF4*-Cre mice displayed a significant protection against cerebral ischemia, and this protection was associated with a marked reduction in neurological deficits and better performance in motor functions, highlighting the pathophysiological importance of $G\alpha_{i2}$ in platelets during reperfusion injury. Interestingly, microscopic analysis of brain slices showed no increased incidence of intracranial hemorrhage in *Gnai2*$^{fl/fl}$/*PF4*-Cre mice following tMCAO, indicating that inhibition of $G\alpha_{i2}$ signaling may represent a safe approach to prevent or treat acute stroke. This assumption is corroborated by data from a second model in which *Gnai2*$^{fl/fl}$/*PF4*-Cre mice also were protected from myocardial ischemia/reperfusion injury. This finding supports and extends our view that early steps in platelet activation and amplification mechanisms are critical factors in infarct development (35, 44, 45).

It is noteworthy that the data presented here argue for a concerted action of activated platelets and leukocytes to aggravate infarct progression. Moreover, in both cell types $G\alpha_{i2}$ seems to represent a central switch of regulation. Although the central role of $G\alpha_{i2}$ in leukocyte function is well established, our study also indicates a corresponding role for $G\alpha_{i2}$ in platelets. Previously, pharmacological inhibition of the G_i-coupled ADP $P2Y_{12}$ receptors has been reported to inhibit PNCs and platelet-dependent leukocyte activation (46, 47), whereas other ex vivo or in situ studies suggested that $P2Y_{12}$ receptor blockers decrease cardiac reperfusion injury by mechanisms other than inhibition of intravascular coagulation (48, 49). Our data clearly show that lack of the $P2Y_{12}$ signal-transducing $G\alpha_{i2}$ in platelets results in the reduced formation of ADP-dependent PNCs which correlates with a significant reduction in cardiac reperfusion injury. Because platelets express also other G_iPCRs relevant for putative platelet immune functions, a future aim will be to dissect the pathophysiological role of G_i proteins for these signaling pathways (50). In this context, it is tempting to speculate about roles for other $G\alpha_i$ proteins, such as $G\alpha_{i3}$, which is expressed in platelets, but its function remains to be elucidated.

Taken together, our findings reveal that the thrombotic and inflammatory functions of platelets converge at the level of the signal transducer $G\alpha_{i2}$. As a consequence, the severity of stroke or cardiac infarcts is determined by a platelet-intrinsic

$G\alpha_{i2}$-dependent interrelation of thrombus formation and immune-mediated processes. These findings may have significant implications for the development of novel strategies to prevent or treat acute ischemic diseases.

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