Platelet hyperreactivity and a prothrombotic phenotype in mice with a gain-of-function mutation in phospholipase C gamma 2

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ORIGINAL ARTICLE

Platelet hyperreactivity and a prothrombotic phenotype in mice with a gain-of-function mutation in phospholipase Cγ2

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Summary. *Background:* Agonist-induced platelet activation involves different signaling pathways leading to the activation of phospholipase C (PLC) β or PLCγ2. Activated PLC produces inositol 1,4,5-trisphosphate and diacylglycerol, which trigger Ca^{2+} mobilization and the activation of protein kinase C, respectively. PLCβ is activated downstream of Gq-coupled receptors for soluble agonists with only short interaction times in flowing blood. In contrast, PLCγ2 becomes activated downstream of receptors that interact with immobilized ligands such as the collagen receptor glycoprotein (GP) VI or activated integrins. *Objective and methods:* We speculated that PLCγ2 activity might be optimized for sustained but submaximal signaling to control relatively slow platelet responses. To test this hypothesis, we analyzed platelets from mice heterozygous for a gain-of-function mutation in the *Plcg2* gene (*Plcg2*$^{Ali5/+}$). *Results: Plcg2*$^{Ali5/+}$ platelets showed enhanced Ca^{2+} mobilization, integrin activation, granule secretion and phosphatidylserine exposure upon GPVI or C-type lectin-like receptor-2 stimulation. Furthermore, integrin $\alpha_{IIb}\beta_3$ outside-in signaling was markedly enhanced in the mutant platelets, as shown by accelerated spreading on different matrices and faster clot retraction. These defects translated into virtually unlimited thrombus formation on collagen under flow *in vitro* and a prothrombotic phenotype *in vivo*. *Conclusions:* These results demonstrate that the enzymatic activity of PLCγ2 is tightly regulated to ensure efficient but limited platelet activation at sites of vascular injury.

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Introduction

Platelets are small anuclear cells that play a fundamental role in hemostasis. They adhere to the injured vessel wall and recruit other platelets to form a hemostatic plug that is critical in limiting blood loss and initiating vascular repair. On the other hand, platelet–platelet interactions can lead to inordinate thrombus growth, which is a major pathomechanism in the development of acute ischemic disorders, including stroke and myocardial infarction [1].

At sites of injury, platelets are recruited to the exposed extracellular matrix by the interaction of glycoprotein (GP)Ib–V–IX with immobilized von Willebrand factor (VWF). This interaction is characterized by a high dissociation rate, resulting in platelet translocation. For stable adhesion to occur, intracellular signals are required that lead to conformational changes within integrin adhesion receptors, most notably integrin $\alpha_{IIb}\beta_3$, to allow efficient ligand binding. A recent study has suggested that platelet aggregate formation is driven mainly by shear forces, most likely through a GPIb-triggered process [2]. Under flow, GPIb–VWF interactions can elicit intracellular signals and weak $\alpha_{IIb}\beta_3$ activation [3,4]. Full platelet activation, however, requires the concerted action of strong platelet agonists, such as collagens, thromboxane A_2 (TxA_2) and ADP released from activated platelets as well as locally produced thrombin [5]. These stimuli trigger different pathways leading to the activation of phospholipase C (PLC), which comprises a family of enzymes known to be important for platelet activation and integrin regulation. Activated PLC produces the intracellular messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC), and IP_3 triggers Ca^{2+} release from the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR), followed by sustained Ca^{2+} influx through the plasma membrane. This so-called store-operated calcium entry is regulated by the ER/SR-resident Ca^{2+} sensor stromal interaction molecule 1

(Stim1) [6], and occurs through the four transmembrane channel protein Orai1 [6,7]. Ca^{2+} mobilization and PKC activation trigger vital platelet responses such as integrin activation and degranulation [8].

Two major PLC isoforms are expressed in platelets, and they become activated by different signaling pathways. PLCβ becomes activated downstream of G-protein (Gq)-coupled receptors, which are mainly triggered by soluble agonists such as ADP and TxA_2 or locally produced thrombin with only short interaction times in flowing blood [9,10]. In contrast, PLCγ2 is activated by signaling pathways involving tyrosine phosphorylation cascades downstream of receptors that predominantly interact with immobilized ligands and may trigger sustained signaling events. The best characterized PLCγ2-activating receptors in platelets are the immunoreceptor tyrosine-based activation motif (ITAM)-coupled collagen receptor GPVI, FcγRIIa, ligand-occupied integrins, and possibly also GPIb. In addition, the recently identified C-type lectin-like receptor-2 (CLEC-2) also strongly activates PLCγ2 in platelets, and mediates powerful cellular activation. CLEC-2 was identified as the receptor for the snake venom toxin rhodocytin [11], and appears to be involved in the stabilization of newly formed thrombi under flow [12]. The physiologic ligand that stimulates CLEC-2 under these conditions has not been identified, but it is hypothesized to be expressed or immobilized on the surfaces of activated platelets [12]. These observations raise the interesting hypothesis that PLCβ is optimized for fast and maximal activity, whereas PLCγ2 may be designed to mediate sustained, adhesion-dependent signaling events that require prolonged rather than transient PLC activity. To characterize the function of PLCγ2 activity in platelets, a genetically dominant gain-of-function murine model, designated Ali5 [13], was employed. *Plcg2*$^{Ali5/+}$ mice develop severe autoimmune and autoinflammatory symptoms reminiscent of human systemic lupus erythematosus. Biochemical analysis suggests that the mutation D993G in PLCγ2 affects a conserved residue in the catalytic domain of the enzyme, which could compromise autoinhibition in the inactive PLC, facilitating the activation process. In addition, the removal of the negative charge may enhance membrane interaction in the activated state without compromising substrate binding and hydrolysis [14].

In the current study, we show that platelets from *Plcg2*$^{Ali5/+}$ mice display enhanced outside-in signaling of $\alpha_{IIb}\beta_3$ and markedly enhanced thrombus formation and stability upon GPVI/CLEC-2 stimulation *in vitro*, which translates into a prothrombotic phenotype *in vivo*.

Materials and methods

Animals

N-ethyl-*N*-nitrosourea mutagenesis of C3HeB/FeJ male mice was performed as previously described [13]. C3H Ali5-affected female mice were mated with C3H wild-type males, and the heterozygous offspring were used for analysis. Wild-type C3HeB/FeJ mice were used as controls.

Reagents

ADP and high molecular weight heparin were purchased from Sigma (Deisenhofen, Germany), α-thrombin was purchased from Roche Diagnostics (Mannheim, Germany), and apyrase type III was purchased from Sigma-Aldrich (Taufkirchen, Germany). Indomethacin was purchased via a local pharmacy. Collagen-related peptide (CRP) was generated as described previously [15]. The antibody against the activated form of $\alpha_{IIb}\beta_3$ (JON/A–phycoerythrin) was from Emfret Analytics (Würzburg, Germany). All other antibodies were generated and modified in our laboratory as previously described [16]. Rhodocytin was isolated as previously described [17].

Platelet preparation

Mice were bled from the retro-orbital plexus under ether anesthesia. Blood was collected in a tube containing 20 U mL^{-1} heparin, and platelet-rich plasma (PRP) was obtained by centrifugation at $300 \times g$ for 10 min at room temperature. For the preparation of washed platelets, PRP was washed twice at $1000 \times g$ for 8 min at room temperature, and the pellet was resuspended in modified Tyrode's buffer [134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 5 mM HEPES, 5 mM glucose, 0.35% bovine serum albumin (BSA), pH 7.4] in the presence of prostacyclin (0.1 μg mL^{-1}) and apyrase (0.02 U mL^{-1}). Platelets were finally resuspended in the same buffer without prostacyclin (pH 7.4, 0.02 U mL^{-1} apyrase) and incubated at 37 °C for 30 min before use.

Aggregometry

To determine platelet aggregation, light transmission was measured using PRP or washed platelets. Transmission was recorded on a Fibrintimer four-channel aggregometer (APACT Laborgeräte und Analysensysteme, Hamburg, Germany) over 10 min, and was expressed as arbitrary units, with 100% transmission adjusted with plasma. Platelet aggregation was induced by addition of CRP, collagen, U46619 or ADP at the indicated concentrations. Thrombin-induced aggregation was performed with washed platelets [200 μL with 0.5×10^6 platelets μL^{-1} in Tyrode's buffer (pH 7.4) + 2 mM $CaCl_2$].

Flow cytometry

Fifty microliters of blood was collected, washed twice in phosphate-buffered saline (PBS), and diluted 1 : 20 in Tyrode's buffer (pH 7.4). Samples were activated with agonists at the indicated concentrations, and stained with fluorophore-conjugated monoclonal antibodies at saturating concentrations for 10 min at 37 °C. Samples were analyzed directly on a

FACSCalibur (BD Biosciences, Heidelberg, Germany). Platelets were identified by forward scatter/side scatter characteristics.

Adhesion under flow conditions

Rectangular coverslips (24 × 60 mm) were coated with 0.2 mg mL^{-1} fibrillar type I collagen (Nycomed, Singen, Germany) for 1 h at 37 °C and blocked with 1% BSA. Heparinized whole blood was labeled with a DyLight 488-conjugated anti-GPIX Ig derivative at 0.2 μg mL^{-1}, and perfusion was performed as previously described [18]. Image analysis was performed off-line using METAVUE software (Visitron, Puchheim, Germany). Thrombus formation was expressed as the mean percentage of total area covered by thrombi, and as the mean integrated fluorescence intensity per square millimeter.

Intracellular Ca^{2+} measurements

Platelet intracellular free Ca^{2+} measurements were performed as previously described [19]. Briefly, platelets isolated from blood were washed, suspended in Tyrode's buffer without Ca^{2+}, and loaded with Fura-2/AM (5 μM) in the presence of Pluronic F-127 (0.2 μg mL^{-1}) (Molecular Probes, Karlsruhe, Germany) for 30 min at 37 °C. After labeling, platelets were washed once and resuspended in Tyrode's buffer containing either no Ca^{2+} or 1 mM $CaCl_2$. Stirred platelets were activated with agonists, and fluorescence was measured with a PerkinElmer LS 55 fluorimeter. Excitation was alternated between 340 nm and 380 nm, and emission was measured at 509 nm. Each measurement was calibrated using Triton X-100 and EGTA.

Measurement of inositol 1-phosphate (IP_1)

The IP-One enzyme-linked immunosorbent assay (ELISA) kit was from Cisbio (Bagnols-sur-Cèze, France). Washed murine platelets were adjusted to a concentration of 0.7×10^6 μL^{-1} in modified phosphate-free Tyrode's buffer containing 2 mM $CaCl_2$ and 50 mM LiCl. Apyrase, indomethacin and EDTA were added to final concentrations of 2 U mL^{-1}, 10 μM, and 5 mM, respectively. Platelets were activated with the indicated agonists for 5 min at 37 °C (350 × *g*). After stimulation, platelets were lysed in the buffer supplied by the kit. Fifty microliters of lysed platelets was used for the IP_1 ELISA assay, according to the manufacturer's protocol.

Measurement of ATP release

Washed platelets were adjusted to a concentration of 0.4×10^6 μL^{-1}. Platelets were activated with the indicated agonists for 2 min at 37 °C with stirring (1000 × *g*). Following activation, EDTA (3 mM final concentration) and formaldehyde (0.1% final concentration) were added, and platelets were fixed for 2 h. The platelets were then centrifuged for 1 min at 16 000 × *g*, and 100 μL of supernatant was added to 100 μL of absolute ethanol. Samples were stored at − 20 °C until measurement. The levels of ATP in 12.5-μL samples were quantified using a bioluminescence assay kit (Roche Diagnostics, Mannheim, Germany) and a Fluostar Optima luminometer (BMG Lab Technologies, Offenburg, Germany).

Clot retraction assay of murine platelets

Clot retraction studies were performed at 37 °C in an aggregometer tube containing diluted PRP (500 μL, 3×10^8 mL^{-1} platelets), thrombin (5 U mL^{-1}), and $CaCl_2$ (20 mM). Clot retraction was recorded with a digital camera and by measurement of the removable volume of clear fluid at the end of the experiment.

Spreading experiments

Coverslips were coated overnight with 1 mg mL^{-1} human fibrinogen, 200 μg mL^{-1} soluble type I collagen, or 50 μg mL^{-1} laminin, respectively, and then blocked for 1 h with 1% BSA in PBS. Washed platelets of mutant or wild-type mice were resuspended at a concentration of 0.5×10^6 platelets μL^{-1}, and then further diluted 1 : 10 in Tyrode's buffer. Platelets were seeded on the respective coverslips and incubated at room temperature for the indicated times. Platelets allowed to spread on fibrinogen were activated with 0.008 U mL^{-1} thrombin shortly before seeding. Unbound platelets were removed by rinsing with Tyrode's buffer. Adherent platelets were fixed with 4% paraformaldehyde and analyzed by differential interference contrast microscopy. At least 10 images of each sample were taken and analyzed using METAVUE software (Visitron).

Pulmonary thromboembolism model

Mice were anesthetized by intraperitoneal injection of tribromoethanol (Avertin), at 0.15 mL per 10 g body weight, from a 2.5% solution. Anesthetized mice received a mixture of collagen (0.15 mg kg^{-1}) and epinephrine (60 μg kg^{-1}) injected in the jugular vein. The surviving mice were allowed to recover.

Determination of phosphatidylserine (PS)-exposing platelets after perfusion

Adhesion experiments under flow conditions (1000 s^{-1}) were performed with heparanized whole blood containing PPACK (30 μM) [18]. Rectangular coverslips were coated with type I collagen (Nycomed), rinsed with saline, and blocked with 1% BSA. To prevent coagulation, the chamber and tubing were prewashed with Tyrode's buffer supplemented with 1 U mL^{-1} heparin. The blood was perfused through the flow chamber by using a 1-mL syringe and a pulse-free pump at a shear rate of 1000 s^{-1} for 4 min. The flow chamber was rinsed with Tyrode's buffer supplemented with 1 U mL^{-1} heparin and 2 mM $CaCl_2$ at the same shear rate for another 4 min. Exposure of PS was

detected with OG488-labeled annexin A5 (1 μg mL^{-1}). Phase-contrast and fluorescent images were obtained from at least seven different collagen-containing microscopic fields, which were randomly chosen.

Data analysis

Results are shown as means ± standard deviations from at least three individual experiments per group. Differences between wild-type and *Plcg2*$^{Ali5/+}$ mice were assessed by the Mann–Whitney *U*-test. *P*-values < 0.05 were considered to be statistically significant.

Results

The significance of PLCγ2 for platelet activation was analyzed by comparing wild-type mice with mice heterozygous for a point mutation in the *Plcg2* gene that leads to a single amino acid substitution in the catalytic domain of the enzyme that results in a gain-of-function mutation (*Plcg2*$^{Ali5/+}$). This point mutation did not lead to increased tyrosine phosphorylation of the enzyme in response to GPVI stimulation, as shown by whole cell tyrosine phosphorylation experiments. Interestingly, a tendency for slightly faster dephosphorylation of bands comigrating with PLCγ2 and FcR γ-chain at 150 kDa and 14 kDa, respectively, was observed in mutant platelets at the latest time point (120 s; Fig. S1A). However, immunoprecipitation experiments confirmed comparable increases in tyrosine phosphorylation of PLCγ2 in control and mutant platelets up to 90 s after stimulation (Fig. S1B). C3H-inbred *Plcg2*$^{Ali5/+}$ mice develop normally, but after birth gradually show spontaneous inflammation and autoimmunity, the severity of which depends on age and gender [13]. For the current study, only mice that did not yet show any obvious signs of inflammation were used. Blood platelet counts, platelet size, and the expression of prominent platelet surface receptors, including $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, GPIb, GPV, GPIX, CLEC-2, and GPVI, were normal, demonstrating that the mutation had no effect on platelet production (Table 1).

Table 1 Platelet count, size and glycoprotein (GP) expression in control and *Plcg2*$^{Ali5/+}$ mice

	Wild type	*Plcg2*$^{Ali5/+}$
Platelets (nL^{-1})	1076 ± 23.1	1170 ± 22.7
MPV (fl)	5.3 ± 0.2	5.5 ± 0.06
GPVI	66 ± 7.4	64 ± 4.4
CLEC-2	96 ± 7.0	103 ± 3.0
$\alpha_{IIb}\beta_3$	553 ± 34	485 ± 54.9
GPIb	391 ± 22.6	408 ± 22.4
GPV	273 ± 12.6	282 ± 16
GPIX	452 ± 29.8	466 ± 43.7
α_2	103 ± 6.0	107 ± 8.0
β_1	132 ± 11.0	138 ± 8.6
CD9	1258 ± 86.6	1345 ± 145.2

Platelet counts per nanoliter and platelet size in femtoliters, shown as mean platelet volume (MPV), were analyzed with an automated blood analyzer (Sysmex; $n = 6$ mice per group). To determine platelet GP expression in *Plcg2*$^{Ali5/+}$ mice, diluted whole blood was stained with fluorophore-labeled antibodies at saturating concentrations for 15 min at room temperature, and fluorescence from gated events was measured by flow cytometry using a FACSCalibur (Becton Dickinson, Heidelberg, Germany). Results are given as the mean fluorescence intensity ± standard deviation for at least six mice per group.

Increased ITAM-triggered IP_3 production and Ca^{2+} mobilization in Plcg2$^{Ali5/+}$ platelets

To test the significance of the Ali5 mutation for PLC activity in platelets, we measured IP_3 production in response to the PLCβ-activating agonist thrombin and the GPVI/PLCγ2-stimulating agonist convulxin (CVX). For this, we quantified the stable IP_3 metabolite IP_1 by ELISA [20]. Whereas the increases in IP_1 level in response to thrombin were comparable between wild-type and *Plcg2*$^{Ali5/+}$ platelets, the response to CVX was markedly increased in the mutant cells as compared with controls, indicating enhanced activity of the mutant PLCγ2 in platelets (Fig. 1A). Similar results were obtained when GPVI was stimulated with CRP (data not shown).

The increased ITAM-triggered IP_3 production resulted in markedly enhanced Ca^{2+} mobilization from intracellular stores in *Plcg2*$^{Ali5/+}$ platelets upon CVX or CRP stimulation as assessed by measuring changes in intracellular Ca^{2+} concentration in the absence of extracellular Ca^{2+}. In contrast, thrombin-induced Ca^{2+} mobilization was indistinguishable between wild-type and mutant platelets (Fig. 1B,C). The increased GPVI-induced Ca^{2+} mobilization in *Plcg2*$^{Ali5/+}$ platelets also translated into markedly increased (store-operated) Ca^{2+} entry (Fig. 1B,C) in these cells, whereas overall Ca^{2+} responses to thrombin were similar in wild-type and mutant platelets. Thus, upon GPVI stimulation, *Plcg2*$^{Ali5/+}$ platelets showed Ca^{2+} responses of similar amplitude to those seen upon thrombin stimulation, whereas in wild-type platelets, PLCγ2 activation evoked much lower Ca^{2+} amplitudes.

Plcg2$^{Ali5/+}$ platelets show enhanced responses upon GPVI and CLEC-2 stimulation

To study the functional consequences of altered PLCγ2 activity in *Plcg2*$^{Ali5/+}$ platelets, we performed standard aggregometry with PRP or washed platelets, using different agonists. In response to thrombin, ADP, or the stable TxA_2 analog U46619, *Plcg2*$^{Ali5/+}$ platelets aggregated normally at all tested concentrations (Fig. 2A), although a slightly faster reversion of ADP-induced aggregation was consistently observed with mutant platelets as compared with wild-type controls. In contrast, when the cells were stimulated with collagen, CRP (Fig. 2B), or CVX (not shown), a clearly enhanced response was seen in mutant platelets that was most evident at low agonist concentrations. At threshold concentrations of collagen or CRP that did not induce aggregation of wild-type platelets, *Plcg2*$^{Ali5/+}$ platelets responded with irreversible aggregation. At high collagen or CRP concentrations, aggregation of wild-type and *Plcg2*$^{Ali5/+}$ platelets was not significantly different. Additionally, similarly increased reactivity was seen when

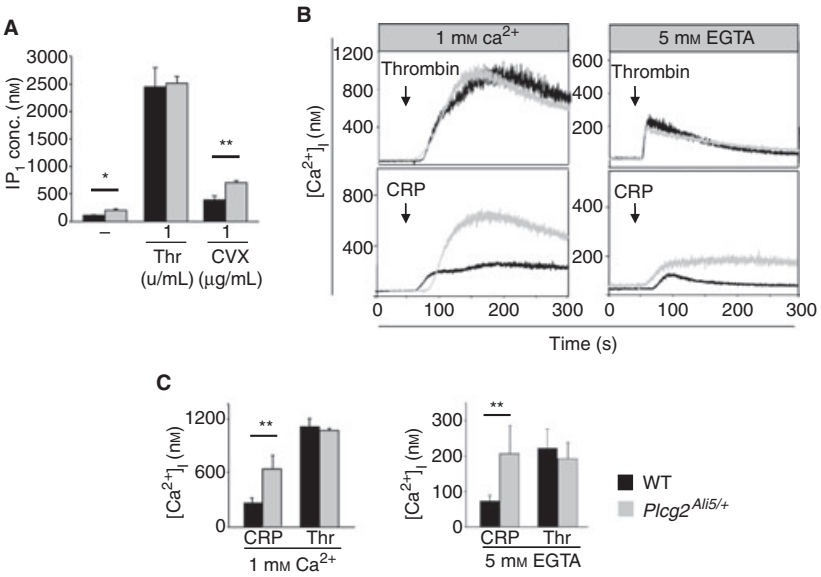

Fig. 1. Increased phospholipase C (PLC) γ2 activation and Ca^{2+} mobilization in *Plcg2*$^{Ali5/+}$ platelets upon immunoreceptor tyrosine-based activation motif (ITAM) stimulation. (A) Quantification of inositol 1-phosphate (IP_1) produced upon platelet activation. Washed platelets were stimulated with the indicated agonists for 5 min at 37 °C. Platelets were lysed in the presence of LiCl, and IP_1, a specific metabolite of inositol 1,4,5-trisphosphate, was quantified using an enzyme-linked immunosorbent assay. Results are given as the mean IP_1 concentration (nM) ± standard deviation (SD) (n = 3 per group). (B) Time course of intracellular Ca^{2+} mobilization in wild-type (WT) platelets (black) and *Plcg2*$^{Ali5/+}$ platelets (gray curve) in response to thrombin (Thr) and collagen-related peptide (CRP). The experiments were performed in the absence and presence of extracellular Ca^{2+}. The results shown are representative of six individual experiments. (C) Maximal increase in cytosolic Ca^{2+} concentrations of wild-type platelets (black bars) and *Plcg2*$^{Ali5/+}$ platelets (gray bars) after activation with the indicated agonists (thrombin, 0.1 U mL^{-1}; CRP, 5 μg mL^{-1}). Results are given as mean $[Ca^{2+}]_i$ (nM) ± SD ($n \geq 6$ mice each); $^{**}P < 0.01$. CVX, convulxin.

Plcg2$^{Ali5/+}$ platelets were stimulated with the CLEC-2-activating snake venom toxin rhodocytin (Fig. 2C).

Flow cytometric analysis of degranulation-dependent P-selectin exposure and $\alpha_{IIb}\beta_3$ activation confirmed enhanced activation of *Plcg2*$^{Ali5/+}$ platelets in response to GPVI and CLEC-2 stimulation that was most evident at low and intermediate agonist concentrations, whereas G-protein-coupled agonists induced comparable activation of control and mutant platelets (Fig. 2D,E). The enhanced degranulation of *Plcg2*$^{Ali5/+}$ platelets was also confirmed by an increase in agonist-induced release of ATP, which is stored in dense granules (Fig. 2F). Together, these results demonstrate enhanced activation and aggregation of *Plcg2*$^{Ali5/+}$ platelets upon GPVI and CLEC-2 stimulation, but unaltered responses to G-protein-coupled agonists.

Enhanced integrin outside-in signaling in Plcg2$^{Ali5/+}$ *platelets*

PLCγ2 has been shown to be involved in outside-in signal transduction of ligand-occupied $\alpha_{IIb}\beta_3$ in platelets [21,22], but it is not clear whether its activity is a limiting factor in this process. To test this directly, washed control and *Plcg2*$^{Ali5/+}$ platelets were allowed to spread on immobilized fibrinogen, a process that is dependent on $\alpha_{IIb}\beta_3$ outside-in signaling [22]. It is known that mouse platelets, in contrast to human platelets, do not fully spread on fibrinogen without exogenous stimulation [23]. Therefore, in order to increase the efficiency of adhesion and spreading, the cells were stimulated with 0.008 U mL^{-1} thrombin. Under these conditions, platelets of both groups were able to spread fully, but a marked difference in kinetics between control and *Plcg2*$^{Ali5/+}$ platelets became evident (Fig. 3A, Videos VS1 and VS2). After 1 min, approximately 40% ± 2.3% of the mutant platelets were fully spread, whereas the wild-type platelets only adhered to the fibrinogen matrix or started to develop filopodia. After 5 min, the first fully spread wild-type platelets were observed (30% ± 9%) whereas 70% ± 11% of the mutant platelets were already fully spread. These data demonstrate that PLCγ2 activity determines, to a great extent, the efficacy of $\alpha_{IIb}\beta_3$-dependent platelet spreading.

Next, we tested the ability of the mutant platelets to spread on a fibrinogen matrix in the absence of exogenous agonists. Under these conditions, wild-type platelets attached to the matrix but were unable to fully spread, whereas 67% ± 3% of the *Plcg2*$^{Ali5/+}$ platelets fully spread within 60 min (Fig. 3B, Videos VS3 and VS4). Accelerated spreading of *Plcg2*$^{Ali5/+}$ platelets was also observed when collagen (Fig. 3C) or laminin (Fig. S2) matrices were used.

To further investigate the functional consequences of the PLCγ2 mutation for $\alpha_{IIb}\beta_3$-dependent processes, we studied clot retraction [24]. PRP of control and *Plcg2*$^{Ali5/+}$ mice was stimulated with 5 U mL^{-1} thrombin, and allowed to clot, and subsequent clot retraction was monitored for 5 h. Consistent with the results of the spreading assay, clot retraction was significantly accelerated in *Plcg2*$^{Ali5/+}$ platelets, with partial and almost complete retraction after 60 min and 120 min,

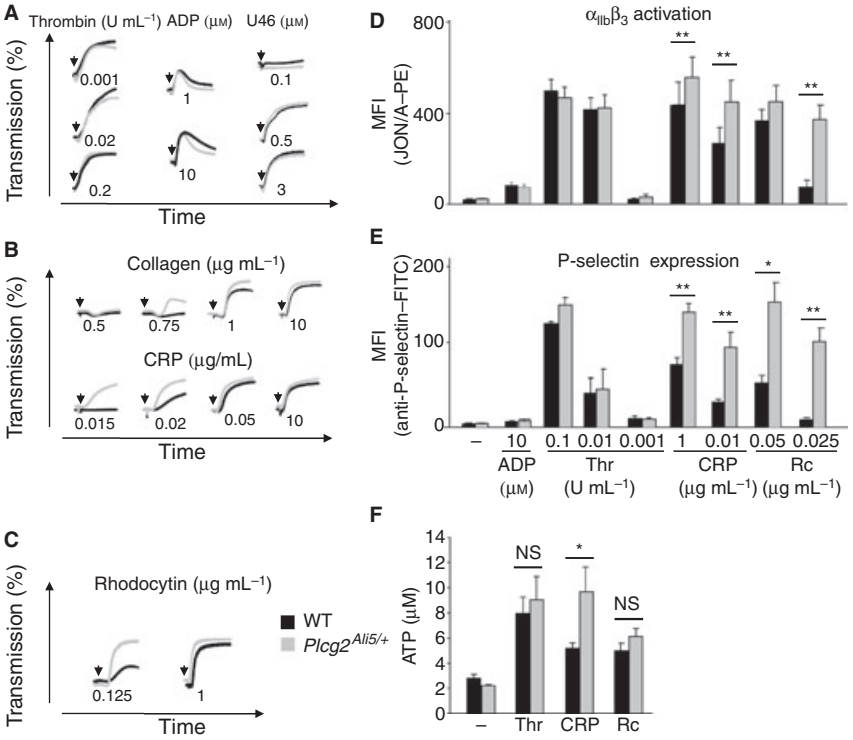

Fig. 2. Increased platelet responses upon glycoprotein (GP) VI and CLEC-2 stimulation. (A–C) Platelets from wild-type (WT) mice (black line) and *Plcg2*$^{Ali5/+}$ mice (gray line) were activated with G-protein-coupled agonists (A), immunoreceptor tyrosine-based activation motif-coupled agonists (B), or rhodocytin (Rc) (C). Light transmission was recorded on a Fibrintimer 4 channel aggregometer over 10 min, and is expressed as arbitrary units, with 100% transmission adjusted with plasma. The results shown are representative of four individual experiments. (D, E) Washed blood from wild-type and *Plcg2*$^{Ali5/+}$ mice was incubated for 10 min with the indicated agonists in the presence of JON/A–phycoerythrin (PE) antibody directed against the activated form of mouse integrin $\alpha_{IIb}\beta_3$ (D) or a fluorescein isothiocyanate (FITC)-conjugated anti-mouse P-selectin antibody (E). The cells were gated by forward scatter/side scatter characteristics. Data shown are mean fluorescence intensity (MFI) ± standard deviation (SD) ($n = 6$ per group). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (F) Measurement of released ATP. Washed platelets were incubated for 2 min at 37 °C with the indicated agonists and fixed. Using a luminometric assay ATP present in the supernatant was determined. Results are given as mean ATP concentration (μM) ± SD ($n = 6$ per group). CRP, collagen-related peptide; NS, not significant; Thr, thombin.

respectively, whereas the process was completed only after 240–300 min in wild-type platelets (Fig. 3C, left). At the end of the observation period of 300 min, both wild-type and *Plcg2*$^{Ali5/+}$ platelets showed complete clot retraction, and the amount of remaining fluid determined at that time point was indistinguishable between the two groups (Fig. 3D). Together, these results demonstrate that PLCγ2 activity is a major determinant of integrin outside-in signaling in platelets.

Virtually unlimited thrombus growth of Plcg2$^{Ali5/+}$ *platelets on collagen under flow*

To investigate the functional consequences of increased PLCγ2 activity under more physiologic conditions, we analyzed platelet adhesion and thrombus formation on collagen under flow in a whole blood perfusion system [25]. Platelets were fluorescently labeled, and the blood was perfused over a collagen-coated surface at different wall shear rates (150, 1000 and 1700 s^{-1}). Wild-type and *Plcg2*$^{Ali5/+}$ platelets adhered to collagen fibers, and the formation of platelet aggregates was initiated within 2 min. However, whereas aggregates grew slowly in wild-type blood, fast and virtually unlimited thrombus growth was seen in *Plcg2*$^{Ali5/+}$ blood at all tested shear rates. As a result, aggregates of *Plcg2*$^{Ali5/+}$ platelets displayed an altered shape and a dramatically increased total thrombus volume as compared with the control at the end of the perfusion period (Fig. 4A,B, Videos VS5 and VS6). In contrast, the surface area covered by platelets was similar between the two groups (Fig. 4C). These results demonstrate that PLCγ2 activity determines, to a great extent, thrombus size under flow conditions independently of the prevailing shear forces.

In collagen-adherent platelets, GPVI, $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ trigger sustained Ca^{2+} responses leading to surface exposure of procoagulant P-Selectin (PS), which is essential for local thrombin production [25,26]. To test whether limited PLCγ2 activity is also a regulator of this process, blood from wild-type and *Plcg2*$^{Ali5/+}$ mice was anticoagulated with PPACK and heparin, and perfused over fibrillar collagen at a wall shear rate of 1000 s^{-1}. PS exposure on collagen-adherent platelets was determined by annexin A5 staining. Whereas the surface area covered by platelets at the end of the perfusion period was similar between the two groups, the number of PS-exposing platelets was markedly increased in *Plcg2*$^{Ali/5+}$ blood as compared with the control (Fig. 4D–E). Thus, increased

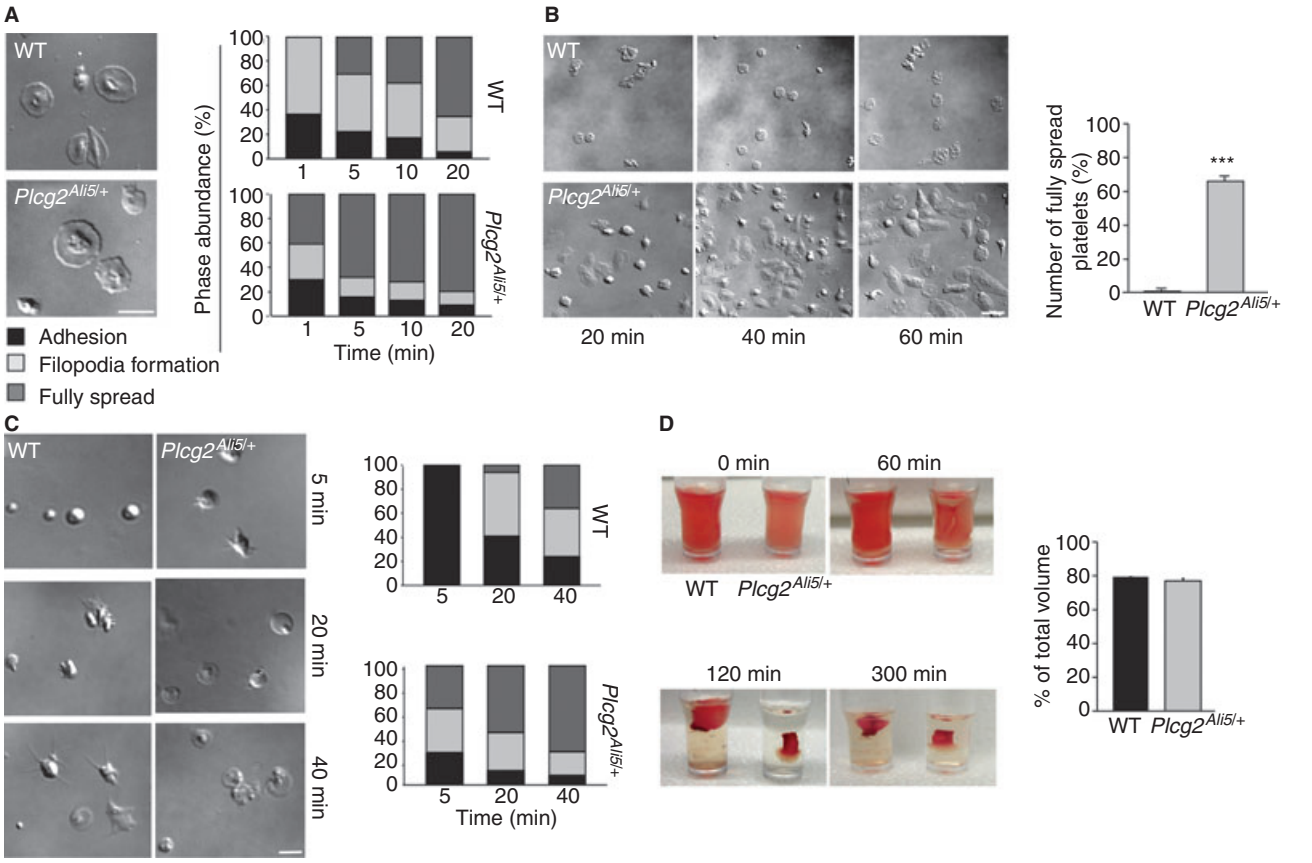

Fig. 3. Enhanced integrin outside-in signaling in $Plcg2^{Ali5/+}$ platelets. (A) Washed platelets from the indicated mice were stimulated with 0.008 U mL^{-1} thrombin and allowed to adhere and spread on immobilized human fibrinogen (1 mg mL^{-1}) for 20 min. Differential interference contrast (DIC) images taken after 20 min, representative of five individual experiments, are shown. The number of adherent platelets, the degree of filopod formation and the number of fully spread platelets were determined at different time points (1, 5, 10 and 20 min). Bar: 5 μm. (B) Platelet spreading and adhesion on immobilized human fibrinogen without exogenous stimulus. Representative DIC images taken after 20, 40 and 60 min, respectively. The bar graph depicts mean values ± standard deviation (SD) ($n \geq 5$ mice per group); ***$P < 0.001$. Bar: 5 μm. (C) Platelet spreading and adhesion on soluble collagen. Representative DIC images taken after 5, 20 and 40 min, respectively, are shown. The bar graph depicts mean values ± SD ($n \geq 5$ mice per group). Bar: 5 μm. (D) Platelet-rich plasma was stimulated with 5 U mL^{-1} thrombin in the presence of 20 mM $CaCl_2$ to induce clot retraction. Representative images show the ongoing process at different time points (0, 60, 120 and 300 min). The remaining fluid was determined at the end of the experiment. The bar graph depicts mean values ± SD ($n = 3$ mice per group). WT, wild type.

PLCγ2 activity results in enhanced coagulant activity of platelets.

Increased mortality of $Plcg2^{Ali5/+}$ mice in a model of pulmonary thromboembolism

To investigate the *in vivo* significance of the platelet hyperreactivity in $Plcg2^{Ali5/+}$ mice, the animals were challenged in a model of pulmonary thromboembolism triggered by intravenous injection of collagen and epinephrine (0.15 mg kg^{-1}; 60 μg kg^{-1} body weight) [27]. At these agonist doses, all $Plcg2^{Ali5/+}$ mice died within 30 min after injection, whereas 90% of the wild-type mice survived and recovered completely (Fig. 5A). In line with this, the numbers of circulating platelets 2 min after the challenge were significantly lower in $Plcg2^{Ali5/+}$ mice than in controls (Fig. 5B), whereas the number of obstructed vessels in the lungs was significantly increased in the mutant animals (Fig. 5C,D). These results demonstrate that increased PLCγ2 activity in platelets leads to largely uncontrolled thrombotic responses *in vivo*.

Discussion

In this study, we have shown that the expression of a hyperactive variant of PLCγ2 in platelets leads to accelerated signaling through the major platelet receptors GPVI, CLEC-2, and $\alpha_{IIb}\beta_3$. This dysregulation translated into virtually unlimited thrombus formation on collagen under flow conditions *in vitro* and a prothrombotic phenotype *in vivo*. Thus, a tight limitation of PLCγ2 activity, even in response to maximal thrombogenic stimuli, appears to be essential for appropriate platelet activation at sites of endothelial damage to ensure sealing of the wound while avoiding vessel obstruction.

Despite the expression of mutant hyperactive PLCγ2, $Plcg2^{Ali5/+}$ mice display normal platelet production and circulating platelets are not in a preactivated state. This was

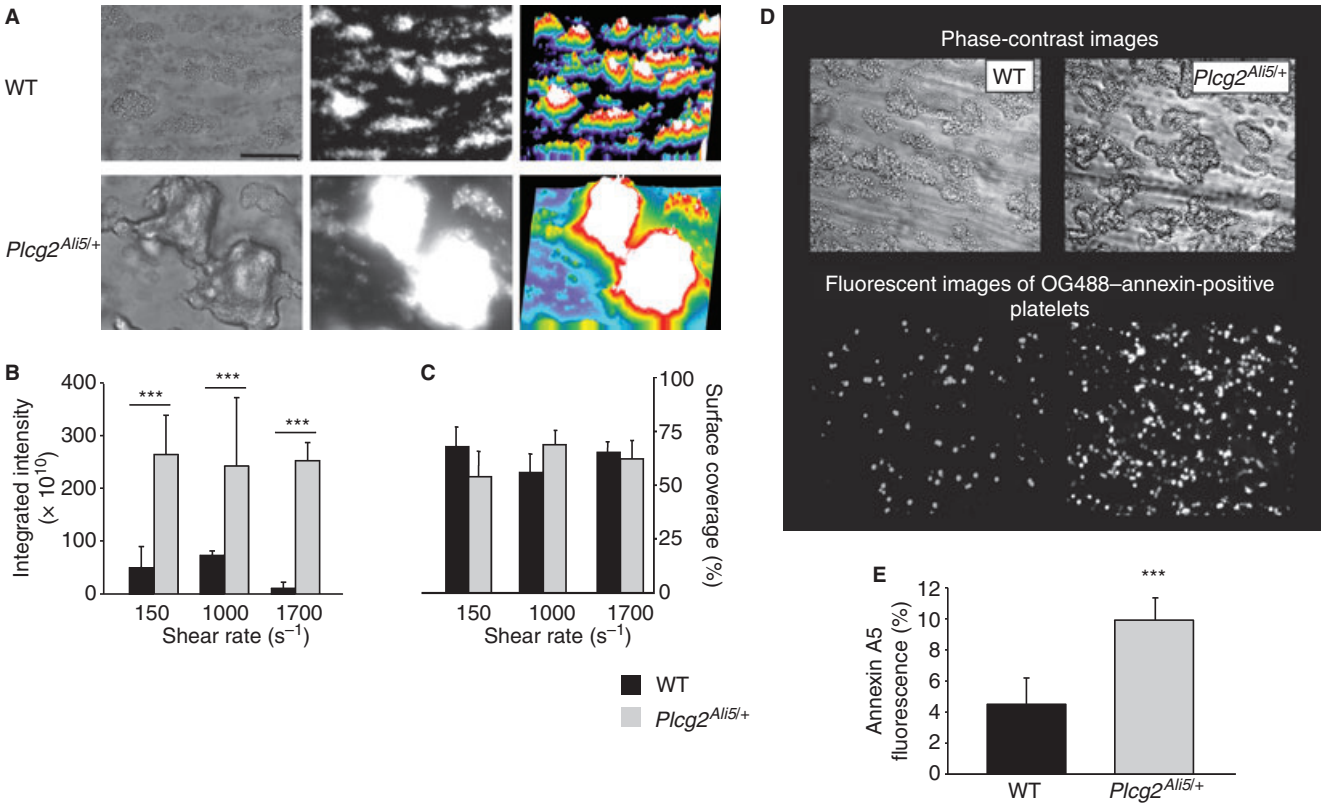

Fig. 4. Gain-of-function mutation of phospholipase C (PLC) γ2 induces elevated thrombus formation on collagen under flow. Whole blood was perfused over a collagen-coated (0.2 mg mL^{-1}) surface at the indicated shear rates, and then washed with Tyrode's buffer for the same perfusion time. (A) Representative phase-contrast and fluorescent images at a shear rate of 1000 s^{-1}. Bar: 100 μm. The profile intensity is shown for wild-type (WT) and $Plcg2^{Ali5/+}$ thrombi. (B) Relative thrombus volume as measured by the integrated fluorescent intensity of fluorescently labeled platelets per visual field. (C) Mean thrombus surface coverage. All bar graphs depict mean values ± standard deviation (SD) ($n \geq 5$ mice per group). ***$P < 0.001$. (D) Determination of phosphatidylserine exposure as a measure of procoagulant activity of platelets by OG488–annexin A5 staining. Fluorescent images and statistical analysis (E) of annexin A5 fluorescence.

confirmed by the observations that the platelet lifespan in the mutant animals is indistinguishable from that in control animals (~ 5 days; data not shown) and that stimulation of the cells with weak agonists such as epinephrine or serotonin did not lead to enhanced activation as compared with the control (not shown). This strongly suggests that hyperactive PLCγ2 has no influence on the basic enzymatic activity of the enzyme. This is also supported by largely unaltered tyrosine phosphorylation of the mutant enzyme as compared with the control before and upon platelet activation, although a tendency for slightly faster dephosphorylation was observed 120 s after stimulation (Fig. S1).

A critical role of PLCγ2 in platelet activation *in vitro* and *in vivo* has been demonstrated in previous studies showing defective GPVI-mediated activation *in vitro* [28,29] and impaired thrombus formation at sites of superficial vascular damage in a model of laser-induced injury in mice lacking the enzyme [30]. In the latter study, Nonne *et al.* demonstrated that thrombus formation partially involves PLCγ2 activation, depending on the severity of the vascular lesion. At sites of deep laser injury, PLCγ2 deficiency can be functionally compensated for by thrombin/Gq/PLCβ-dependent activation, leading to the formation of large, stable thrombi. These results demonstrate that strong Gq/PLCβ stimulation is sufficient to induce powerful thrombotic activity of platelets that can lead to vessel occlusion. Remarkably, however, lack of PLCγ2 leads to impaired primary hemostasis, suggesting that PLCγ2-dependent signaling in platelets is important for sealing of a wound, although this defect can be partially compensated by PLCβ, as revealed by moderately increased bleeding times in $Plcg2^{-/-}$ mice as compared with β_3-deficient or GPIbα-deficient mice [28]. Taken together, these findings indicate that PLCγ2 is an important mediator of thrombus growth and stability in primary hemostasis and arterial thrombosis in mice, but also that PLCβ-dependent signaling pathways can partially compensate for its loss in these processes.

Stimulation of PLCβ or PLCγ2 results in different kinetics and amplitudes of Ca^{2+} mobilization in platelets. Under maximal agonist stimulation, PLCβ induces an intracellular Ca^{2+} increase with a high amplitude but fast reversion, whereas PLCγ2 mediates submaximal but sustained Ca^{2+} mobilization. We found that signaling of the hyperactive PLCγ2 induced a strong increase in intracellular Ca^{2+} concentrations that was comparable to that elicited by thrombin stimulation. As a consequence, $Plcg2^{Ali5/+}$ platelets exhibited virtually unlimited thrombus growth under flow

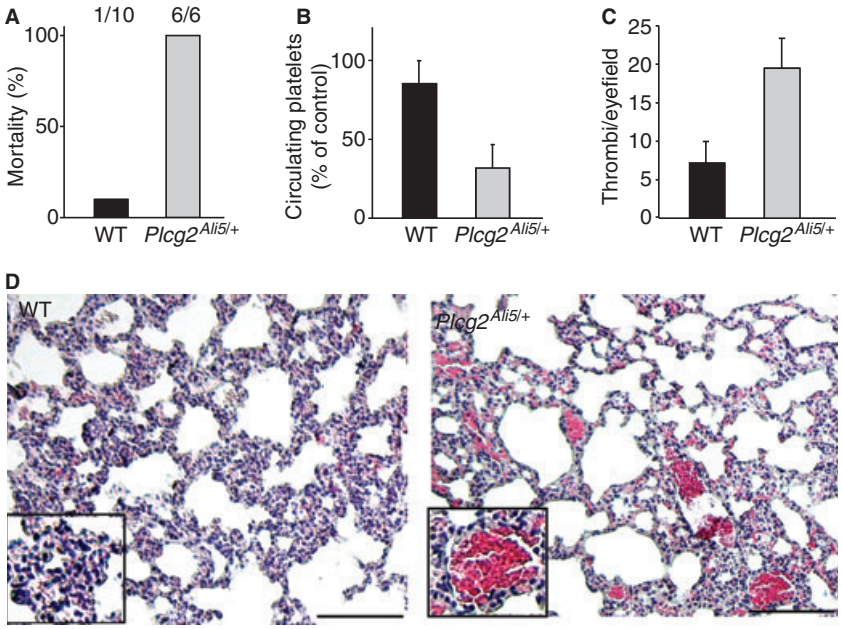

Fig. 5. Collagen-induced pulmonary thromboembolism model. Lethal pulmonary thromboembolism after injection of collagen and epinephrine in anesthetized wild-type (WT) and *Plcg2*$^{Ali5/+}$ mice. (A) Mortality rate upon injection of 150 μg of collagen per kg of body weight. Animals alive 30 min after the challenge were considered to be survivors. (B) Platelet counts in control and *Plcg2*$^{Ali5/+}$ mice 2 min after infusion of collagen/epinephrine. (C, D) Representative pictures of hematoxylin and eosin-stained lung sections; thrombi per visual field were counted at × 20 magnification. Mean ± standard deviation for 50 fields per group is shown. Bar: 200 μm.

in vitro that translated into a prothrombotic phenotype *in vivo*. This was also confirmed by a marked increase in PS exposure upon interaction with collagen that, under physiologic conditions, leads to increased local generation of thrombin at the platelet surface [31]. This indicates that the enzymatic activity of PLCγ2 needs to be precisely regulated to allow efficient but limited platelet activation at sites of vascular injury.

Our data suggest that PLCβ and PLCγ isoforms are differentially regulated to control either fast and reversible or sustained adhesion-dependent processes in platelet activation and aggregation. Such differential regulation of distinct cellular processes is also found in other cell types. PLCγ has been demonstrated to be crucial for pre-BCR and BCR-dependent selection events during B-cell differentiation [32], but appears to be also critical for T-cell and natural killer cell function [33]. In these cell types, PLCγ leads to sustained signals with long ligand exposure times that allow prolonged activation in response to ligand binding. Whereas PLCγ2 deficiency leads to a block in B-cell differentiation as well as a loss of T-cell-independent antibody production and inhibited IgM receptor-induced Ca^{2+} flux [34], hyperactive PLCγ2 specifically increases extracellular Ca^{2+} entry into B cells, which is comparable to the increased Ca^{2+} mobilization in platelets observed in this study (Fig. 1), and leads to expansion of innate inflammatory cells, identifying PLCγ2 as a key regulator in immune responses [13]. In contrast, PLCβ becomes activated downstream of receptors that trigger fast but reversible responses, such as chemokine receptors [35], suggesting that different kinetics of these PLCs might also be responsible for appropriate responses in immune cells.

Thrombus formation is a multiple step process that involves signaling through two major activatory receptors that stimulate PLCγ2, namely GPVI and CLEC-2. GPVI is of central importance for platelet activation on subendothelial collagens [36], whereas it may not have a major direct function in subsequent thrombus growth. In contrast, a very recent study on mice with a transient antibody-induced CLEC-2 deficiency revealed that CLEC-2 is crucial for the activation of newly recruited platelets in a growing thrombus but is not required for adhesion to the damaged vessel wall [12]. As a consequence, CLEC-2-deficient mice displayed severely impaired thrombus formation *in vitro* and protection from arterial thrombosis *in vivo* [12]. Locally produced thrombin was not sufficient to achieve stable thrombus formation in those mice, demonstrating that CLEC-2 signaling is essential for this process to occur *in vivo*. On the basis of these data, we speculate that, as well as GPVI and $\alpha_{IIb}\beta_3$, increased CLEC-2-dependent signaling contributes to the virtually unlimited thrombus growth of *Plcg2*$^{Ali5/+}$ platelets under flow. This suggests that tight regulation of CLEC-2 and PLCγ2 activity is required to avoid excessive thrombus growth and vessel occlusion.

Our studies confirm that PLCγ2 is involved in $\alpha_{IIb}\beta_3$ outside-in signaling [22], and reveal that its activity is a limiting step in this process. Mutant platelets spread significantly faster on a fibrinogen matrix under stimulating conditions. Remarkably, hyperreactive PLCγ2 was sufficient to induce full spreading of murine platelets under non-stimulating conditions. In previous studies, McCarty *et al.* demonstrated that stimulation of murine platelets with either thrombin or ADP strongly enhances the degree of

lamellipodia formation, whereas human platelets bind and fully spread on immobilized fibrinogen in the absence of external agonists [23], like *Plcg2*$^{Ali5/+}$ platelets. This indicates that a difference in the degree of integrin-dependent PLCγ2 activation may exist between human and murine platelets. Furthermore, PLCγ2 is not essential for $\alpha_{IIb}\beta_3$-mediated clot retraction, most likely because this process is regulated by different molecules, for example PLCβ, Rho kinase, and PLCγ1 [21]. However, this process was also enhanced in the mutant mice, demonstrating that PLCγ2 activity is also a rate-limiting factor in this process.

Taken together, our findings show that PLCγ2 activity is a major determinant of appropriate platelet activation to ensure efficient but limited thrombus formation. This indicates that modulation of PLCγ2 activity in platelets might be a promising approach for antithrombotic therapy. Indeed, mice lacking the PLCγ2-coactivating small GTPase Rac1 are protected from arterial thrombosis [20]. Similarly, GPVI deficiency caused by antibody treatment also protects mice from arterial thrombosis [36] and ischemic brain infarction [37], while only moderately affecting hemostasis.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Tyrosine phosphorylation assays of *Plcg*$^{Ali5+/-}$ and wild-type platelet lysates.
Fig. S2. *Plcg*$^{Ali5+/-}$ platelets spread faster on laminin than wild-type platelets.
Video S1. Time lapse video of platelet spreading on fibrinogen (wild-type).
Video S2. Time lapse video of platelet spreading on fibrinogen (*Plcg2*$^{Ali5/+}$).
Video S3. Time lapse video of spreading on fibrinogen without exogenous stimulus (wild-type).
Video S4. Time lapse video of spreading on fibrinogen without exogenous stimulus (*Plcg2*$^{Ali5/+}$).
Video S5. Time lapse video of thrombus formation on collagen under flow in a whole blood perfusion system (wild-type).
Video S6. Time lapse video of thrombus formation on collagen under flow in a whole blood perfusion system (*Plcg2*$^{Ali5/+}$).

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