Immobilized fibrinogen activates human platelets through glycoprotein VI

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ABSTRACT

Glycoprotein VI, a major platelet activation receptor for collagen and fibrin, is considered a particularly promising, safe antithrombotic target. In this study, we show that human glycoprotein VI signals upon platelet adhesion to fibrinogen. Full spreading of human platelets on fibrinogen was abolished in platelets from glycoprotein VI-deficient patients suggesting that fibrinogen activates platelets through glycoprotein VI. While mouse platelets failed to spread on fibrinogen, human-glycoprotein VI-transgenic mouse platelets showed full spreading and increased Ca^{2+} signaling through the tyrosine kinase Syk. Direct binding of fibrinogen to human glycoprotein VI was shown by surface plasmon resonance and by increased adhesion to fibrinogen of human glycoprotein VI-transfected RBL-2H3 cells relative to mock-transfected cells. Blockade of human glycoprotein VI with the Fab of the monoclonal antibody 9O12 impaired platelet aggregation on preformed platelet aggregates in flowing blood independent of collagen and fibrin exposure. These results demonstrate that human glycoprotein VI binds to immobilized fibrinogen and show that this contributes to platelet spreading and platelet aggregation under flow.

Introduction

The immunoglobulin receptor glycoprotein (GP) VI is expressed on megakaryocytes and platelets. GPVI associates with the Fc receptor (FcR) γ-chain in the membrane, and with the Src family kinases (SFK) Lyn and Fyn through its cytosolic tail.[1] Ligand binding clusters GPVI at the platelet surface promoting phosphorylation of the immunoreceptor tyrosine-based motif (ITAM) of the FcR γ-chain by SFK.[2–4] This results in the recruitment of Syk and formation of a LAT-based signalosome that activates PLCγ2 leading to an increase in Ca^{2+}, activation of integrin and secretion of granules.[5]

GPVI is widely known as a platelet activation receptor for fibrillar collagen.[5] However, in recent years, GPVI has been shown to bind to additional ligands, including subendothelial and plasma adhesive proteins such as laminins and fibrin,[6–8] the hormone adiponectin and the transmembrane protein emmprin.[9,10] Several of these interactions are relatively weak and of unclear significance. For example, GPVI supports adhesion and efficient activation of platelets to collagen

and fibrin but is only involved in post-adhesive events on laminin.[6,11] Determining the importance of the interaction of GPVI with each ligand in mediating platelet activation *in vivo* will require development of selective inhibitors.

GPVI is involved in arterial thrombosis and in several of the more recently discovered roles of platelets, including maintenance of vascular integrity at sites of inflammatory challenge.[12] We and others have reported that the absence of GPVI reduces experimental thrombosis in mouse models of atherosclerotic plaque rupture[13,14] and abolishes occlusive thrombus formation following $FeCl_3$ injury.[15] In contrast, the absence or blockade of GPVI has a relatively minor impact on hemostasis in mice[16] and patients deficient in GPVI have a relatively mild bleeding diathesis.[17–19] These results highlight that GPVI is a promising antithrombotic target with inhibitors predicted to have a minor effect on hemostasis.[20]

Following ligand binding, GPVI stimulates signals that convert integrin αIIbβ3 from a low to a high affinity state for fibrinogen and other physiological ligands.[21] Ligand engagement of integrin αIIbβ3 has been reported to generate outside-in signals that are similar to those of GPVI, including activation of Src and Syk kinases, PLCγ2 and Ca^{2+} mobilization.[22–24] Paradoxically, however, human but not mouse platelets generate extensive lamellipodial sheets and stress fibers on fibrinogen whereas on collagen, which stimulates similar signals, full spreading of platelets is seen in both species.[25] One explanation for this difference is the presence of the low affinity immune receptor, FcγRIIA, in the human but not the rodent genome, as FcγRIIA-transfected transgenic mouse platelets exhibit increased spreading and Syk activation upon adhesion to fibrinogen, although the increase in spreading is only partial.[26–28] Outside-in signaling by αIIbβ3 is also mediated by two conserved tyrosines present in a NxxY motif in the integrin β3 intracytoplasmic domain independent of Src and Syk activation. Mutation of these two tyrosine residues to phenylalanine leads to a re-bleeding diathesis that has been attributed to a defect in clot retraction.[29] This shows that engagement of integrin αIIbβ3 leads to activation of multiple signaling pathways.

In the present study, we showed that full spreading of human platelets on fibrinogen is abolished in patients deficient in GPVI and that transgenic mouse platelets expressing human GPVI, in contrast to wild-type platelets, spread fully on fibrinogen. Direct binding of fibrinogen to human GPVI was demonstrated using human GPVI-transfected cell lines and recombinant GPVI. Inhibiting the binding of fibrinogen to GPVI limited platelet aggregation under conditions that excluded involvement of collagen and fibrin.

Methods

Patients

Family 1 and family 2 are two families who are heterozygous or homozygous for an adenine insertion in exon 6 of *GP6* that generates a premature stop codon in position 242 of the protein.[17] They have been described previously.[30] Patient 3 is a 10-year old boy suffering from an autoimmune disease with anti-GPVI antibodies. The platelets of this patient do not aggregate to collagen and GPVI is not detected at the platelet surface using flow cytometry and western blot (*data not shown*).

Mice

Wild-type mice were generated from breeding of heterozygotes or purchased from Harlan Laboratories (Hillcrest, UK) or Charles River (Lyon, France). $GPVI^{-/-}$ mice were provided by Dr Jerry Ware.[31] Mouse platelets expressing human GPVI have been described and characterized previously.[32] Syk chimera mice have been described previously.[27] Ethical approval for animal experimentation was obtained from the French Ministry of Research and UK Home Office in accordance with the European Union guidelines, the Guide for the Care and Use of Laboratory Animals.

Reagents

PRT-060318 was obtained from Caltag Medsystems (Buckingham, UK), REOPRO from E. Lilly (Indianapolis, IN, USA). Recombinant GPVI was made as described elsewhere.[33] Fibrinogen was from Kabi (Bad Homburg, Germany) or from ERL (South Bend, IN, USA). RAM.1 (anti-GPIbβ) was generated in U949.[34] The blocking Fab fragment of monoclonal antibody directed against human GPVI, 9O12.2, and its humanized version are referred to as 9O12 in this manuscript.[35,36] All other reagents were from previously described sources.[11,37] The anti-fibrin antibody 59D8 was obtained from CT Esmon (Oklahoma Medical Research Foundation, OK, USA).[38]

Generation and characterization of RBL-2H3 cells

The cDNA of WT human GPVI[33] was inserted in pSRαNeo between the 5'-XhoI and 3'-BamHI restriction sites. Rat basophilic leukemia cells, RBL-2H3, were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum albumin and stably transfected with 1 μg of DNA corresponding to the empty vector or WThGPVI vector mixed with FuGENE6 (Roche, Boulogne-Billancourt, France) and selected in growth medium containing G418 0.7%; 1 mg/mL geneticin (GibcoBRL, Invitrogen, Cergy Pontoise). Cell surface expression of recombinant GPVI and constitutively expressed integrin αIIbβ3 was confirmed by flow cytometry and immunoblot (*data not shown*).

Cell adhesion to fibrinogen

LAB TEK 4 wells were coated with 400 μL/well of collagen (50 μg/mL) or fibrinogen (100 μg/mL) overnight at 37°C. Wells were saturated with human serum albumin (10 mg/mL) for 1 h at 37°C. Trypsinised RBL cells ($3x10^5$ cells/mL) were incubated with phosphate-buffered saline or 9O12 (50 μg/mL) and/or REOPRO (40 μg/mL) for 15 min at 37°C. Subsequently, 100,000 cells were added to the wells (300 μL) for 1 h at 37°C. After three washing steps, cells were fixed with 400 μL paraformaldehyde 4% for 20 min. Pictures were taken with an EVOS optic microscope (x10). Actin was stained with Alexa-488-phalloidin and the nucleus with DAPI.

Washed platelets

Human blood was taken from patients or from healthy donors using 3.8% (v/v) sodium citrate (1:9) as the anticoagulant. Human and mouse washed platelets were obtained by centrifugation using prostacyclin (2.8 μmol/L) and resuspended in modified Tyrode-HEPES buffer as described elsewhere.[37,39]

Platelet spreading

Platelet adhesion to immobilized fibrinogen was achieved as described previously.[37] Images of the platelets were obtained with a Zeiss Axiovert 200 mol/L microscope or with a Leica DMI400 microscope. Platelet surface area was analyzed using ImageJ software (NIH, Bethesda, USA).

Western blotting

For stimulation on fibrinogen, washed platelets were pre-treated with 10 μmol/L indomethacin and 2 U/mL apyrase. Platelets (1.5 mL containing $5x10^8$/mL) were allowed to adhere to 10 cm dishes coated with 100 μg/mL fibrinogen or heat-inactivated bovine serum albumin for 45 min at 37°C. Non-adherent platelets were removed and lysed by addition of 2X lysis buffer (150 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EGTA, 1 mmol/L EDTA, 1% NP-40; pH 7.4, plus 1.25 mmol/L Na_3VO_4, 50 μg/mL AEBSF, 2.5 μg/mL leupeptin, 2.5 μg/mL aprotinin and 0.25 μg/mL pepstatin). Adherent platelets were washed twice with Tyrode buffer then lysed with 1X lysis buffer on ice for 15 min before scraping. Proteins were immunoprecipitated with α-Syk antibody and protein A-sepharose beads for 2 h. The beads were washed, proteins eluted in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), electro-transferred, and western blotted with the stated antibodies. For whole platelet lysates, washed platelets ($5x10^8$/mL) were lysed directly with an equal volume of 2xSDS sample buffer, separated by SDS-PAGE, electro-transferred, and western blotted with the stated antibodies.

Ca^{2+} assay and *in vitro* perfusion assay

Intraplatelet Ca^{2+} concentrations following platelet adhesion to fibrinogen were measured using a dual-dye ratiometric method and hirudinated blood perfusion was performed as previously described.[40] Three-dimensionsal reconstructed images were obtained using the 3D module of Leica LAS X software.

Solid-based binding assay

Binding studies were performed with the recombinant proteins, GPVI-Fc fusion (dimer) and GPVI-His tagged (monomer). Cover slips were coated with collagen or fibrinogen overnight at 4°C. The plates were blocked with 3% bovine serum albumin – phosphate-buffered saline for 1 h and washed prior to addition of monomeric or dimeric GPVI at a concentration of 100 nmol/L for 1 h. After washing, 4 μg/mL of secondary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-human IgG Fc or HRP-conjugated anti-His Tag, were added for 1 h. GPVI binding was detected using 3,3′,5,5′-tetramethylbenzidine. The reaction was stopped with H_2SO_4 (2 mol/L) and absorbance was measured at 450 nm with a spectrofluorometer.

Surface plasmon resonance

Surface plasmon resonance was performed on a Pioneer platform from PALL® FortéBio® (Portsmouth, UK). IF-1 purified fibrinogen was diluted to 100 μg/mL using 10 mmol/L NaAc pH 5.0. IF-1 fibrinogen was adsorbed to the chip surface via amine coupling to a level of 3825 resonance units (RU) at flow-cell 1 and 3423 RU on flow-cell 3. Flow-cell 2 was activated using amine coupling and blocked using 1 mol/L ethanolamine and was the designated reference channel. GPVI analytes were dialysed and diluted to 1 μmol/L using the same batch of running buffer as used for the blanks. Analytes were injected using the OneStep® titration function at a flow rate of 30 μL/min with a 100% loop-inject and 400 s dissociation. The chip surface was regenerated by flushing with 1 mol/L NaCl at 60 μL/min for 10 s, followed by a further 400 s dissociation. Qdat data analysis software (PALL® FortéBio®, UK) was used to analyze the data. Binding data were fitted using a one site K_A/K_D model and analyte aggregation parameters adjusted per binding curve according to goodness of fit and curve type.

Statistics

The statistical analyses were performed using the GraphPad Prism program, version 5.0 (Prism, GraphPad, LaJolla, CA, USA). The values are indicated as mean ± standard error of the mean (SEM). The statistical analysis is described in the Figure legends.

Results

Abolition of spreading on fibrinogen in glycoprotein VI-deficient human platelets

Human platelets undergo robust spreading on immobilized fibrinogen, generating lamellipodial sheets and stress fibers.[41] This is illustrated in Figure 1A with over 90% of platelets from a control donor undergoing full spreading on fibrinogen over 30 min; the small number of partially-spread platelets most likely represent newly adhered cells. In 2013, Matus *et al.* described four unrelated families with index cases who are homozygous for an adenine insertion in exon 6 of human *GP6*, which leads to a premature 'stop codon' in position 242 prior to the transmembrane domain.[17] All four homozygous patients lack expression of GPVI on their platelets and heterozygous relatives express approximately 50% of the receptor. Since then, two further unrelated families with the same mutation have been identified by the same group and also been shown to lack surface expression of GPVI with absent platelet aggregation to collagen.[30] Unexpectedly, in studying platelets from two unrelated index cases in these families, we observed reduced adhesion on immobilized fibrinogen and a failure to form lamellipodial sheets and stress fibers (Figure 1A). The absence of GPVI was confirmed by flow cytometry and by abolition of aggregation to collagen but not to other agonists in both cases[30] (*data not shown*). In contrast, spreading and adhesion of platelets from heterozygote carriers from each family and platelets from a control were similar (Figure 1A). The same result was also seen in a patient with an auto-immune thrombocytopenia associated with the absence of GPVI expression (Figure 1Bi). Adhesion of platelets was blocked by the αIIbβ3 receptor antagonist, REOPRO (Figure 1B), as previously shown in controls. These results demonstrate that adhesion of human platelets on fibrinogen is critically dependent on integrin αIIbβ3 with a minor contribution from GPVI, but that full spreading requires GPVI.

Mouse platelets expressing human glycoprotein VI undergo full spreading on fibrinogen

Mouse platelets adhere and undergo limited spreading on human or mouse fibrinogen, forming filopodia and limited lamellipodia but not stress fibers (Figure 2A). A similar response is seen in platelets deficient in GPVI (Figure 2A), whereas adhesion is abolished in the absence of the integrin β3-subunit (Figure 2B). A similar level of adhesion is seen in human GPVI transgenic mouse platelets but is associated with the formation of lamellipodial sheets and stress fibers (Figure 2C). These results demonstrate that full spreading but not adhesion of mouse platelets is dependent on human GPVI and not mouse GPVI. One potential explanation for these results is that human but not mouse GPVI is able to bind to fibrinogen and mediate platelet activation.

Fibrinogen binds to monomeric human glycoprotein VI

To test whether fibrinogen is able to bind to GPVI, increasing concentrations of recombinant soluble GPVI extracellular domain, expressed either as a monomer (GPVI-ex) or dimer (GPVI-Fc), was flowed over immobi-

lized fibrinogen and binding monitored by surface plasmon resonance. As shown in Figure 3A, clear binding of monomeric GPVI (k_a = 1.17 ± 0.01 x 10^4 $M^{-1}s^{-1}$) was observed with a k_d of 3.94 ± 0.01 x 10^{-3} s^{-1}. Binding was fitted to a single site with an equilibrium dissociation constant (K_D) of 336 ± 1 nmol/L. In contrast, binding of dimeric GPVI to fibrinogen was not detected at concentrations up to 1 μmol/L (Figure 3Ai). In a second approach, fibrinogen was immobilized on a plastic surface and a solid phase binding assay was performed. There was increased binding of monomeric GPVI, but not dimeric GPVI, which was inhibited by D-dimer (Figure 3Aii) where the binding motif in fibrin resides.[30] To further investigate the ability of GPVI to bind to fibrinogen, we transfected rat RBL-2H3 basophilic cells, which constitutively express integrin αIIbβ3 at low levels, with human GPVI and studied adhesion to immobilized fibrinogen. We observed a 3-fold increase in adhesion of GPVI-transfected cells to fibrinogen and to collagen relative to the adhesion of mock-transfected control cells (Figure 3Bi, ii). RBL-2H3 cells expressing human GPVI also formed stress fibers upon adhesion to fibrinogen. The human GPVI-blocking monoclonal antibody 9012 Fab blocked the increase in adhesion. Blocking the integrin αIIbβ3 with REOPRO reduced cell adhesion to immobilized fibrinogen to the same level as 9O12 Fab, with no further inhibition in the presence of both inhibitors (*data not shown*), indicating the presence of additional binding proteins for fibrinogen in the adherent cell line although binding to these was not sufficient to induce spreading (Figure 3Biii and not shown). These results demonstrate that GPVI binds to immobilized fibrinogen and is able to contribute to cell adhesion.

Spreading of human platelets but not mouse platelets is dependent on Syk

The formation of lamellipodial sheets and stress fibers in human platelets on fibrinogen and collagen is blocked by the inhibitors of Src and Syk tyrosine kinases, PP2 and PRT060318, respectively (Figure 4Ai & ii). Adhesion of human platelets to fibrinogen induces phosphorylation of Syk which co-precipitates with the phosphorylated FcR γ-chain (Figure 4Aiii). These results provide further evidence of GPVI activation in human platelets by immobilized fibrinogen. In contrast, the morphological modifications of

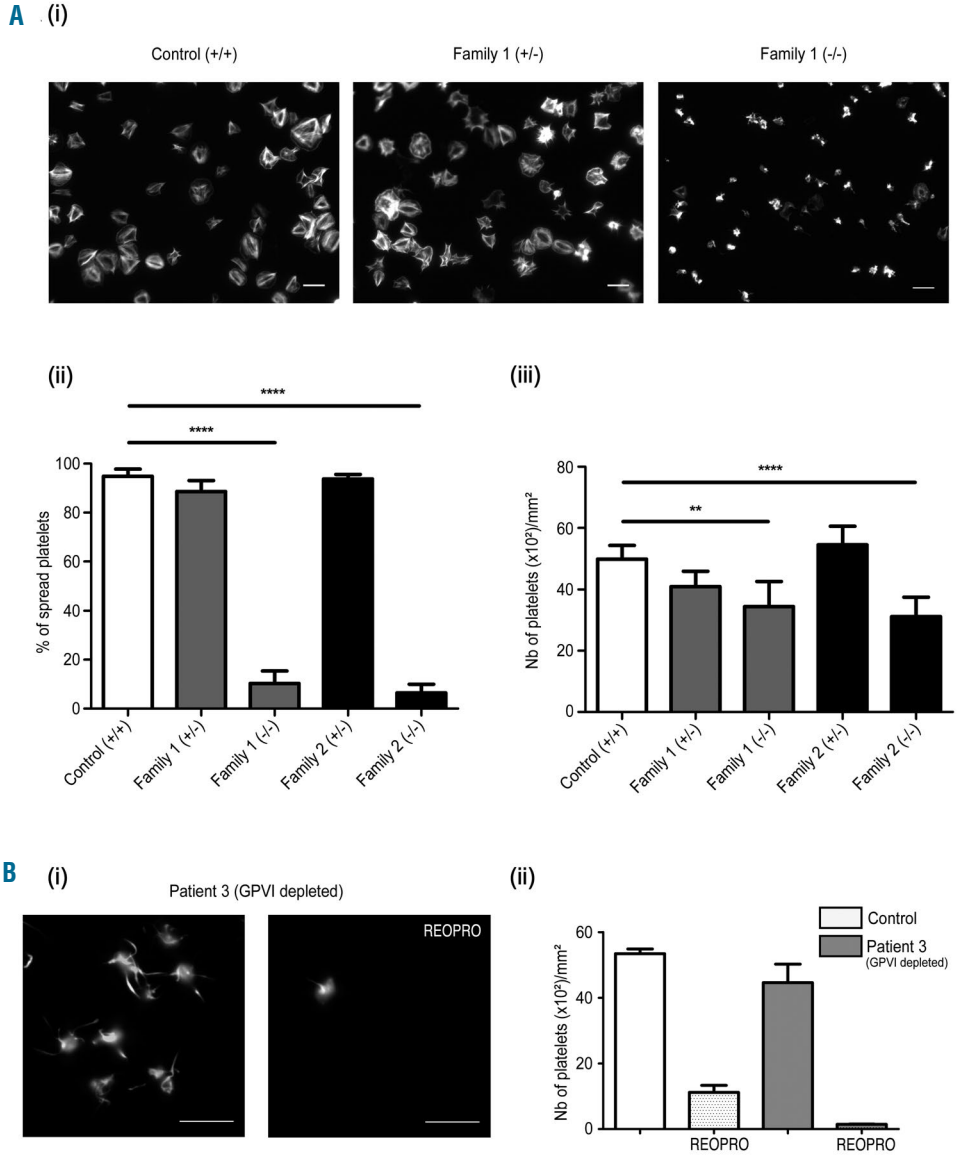

Figure 1. Glycoprotein VI supports platelet adhesion and spreading on immobilized fibrinogen. (A, B) Washed human platelets were allowed to adhere to immobilized fibrinogen (10 μg/mL) for 30 min. (A)(i). Representative epifluorescence images of fibrinogen-adherent platelets from healthy donors (Control) or members of a family with a mutation in the *GP6* gene (Heterozygotes: Family 1 +/-; homozygotes: Family 1 -/-). Scale bars represent 10 μm. (A)(ii). Bar graph representing the percentage of platelets spreading on fibrinogen. Spreading is expressed as the mean±SEM in five random fields, in two separate experiments (one-way ANOVA, Kruskal-Wallis post-hoc test, ***P<0.0002; ****P<0.0001). (A)(iii). Bar graph representing the number of platelets adhering to immobilized fibrinogen per mm² of a control (Control) and two families with a mutation in the *GP6* gene. Adhesion is expressed as mean±SEM in five random fields, in two separate experiments (one-way ANOVA, Kruskal-Wallis post-hoc test, **P<0.002; ****P<0.0001). (B). Washed platelets from a control or a patient with an immune thrombocytopenic purpura presenting with undetectable levels of GPVI on platelets (Patient 3) were allowed to adhere to fibrinogen (100 μg/mL). (B)(i). Representative epifluorescence images of washed platelets from patient 3 adhering to immobilized fibrinogen for 30 min, in the presence or absence of REOPRO (40 μg/mL). Scale bars represent 10 μm. (B)(ii). Bar graph represents the number of platelets adhering to fibrinogen per mm². Adhesion is expressed as mean±SEM in eight random fields, in two separate experiments.

mouse platelets on fibrinogen is blocked by the Src kinase inhibitor PP2 but not by the Syk kinase inhibitor PRT060318 (Figure 4B). Morphological changes of mouse platelets on fibrinogen are also not altered in platelets from irradiated mice transplanted with Syk-deficient fetal liver (Figure 4B) or from PF4.Cre-Syk$^{fl/fl}$ mice (*Online Supplementary Figure S1*). Western blotting for Syk confirmed lack of expression of the tyrosine kinase in the two transgenic models (Figure 4B and not shown). Thrombin stimulated full spreading of wild-type and Syk-deficient platelets (Figure 4B). Formation of lamellipodia and stress fibers in human GPVI transgenic mouse platelets was blocked by PRT060318 (Figure 4C). The ability of Src and Syk inhibitors to block spreading of human platelets and human GPVI transgenic mouse platelets on fibrinogen is consistent with platelet activation by GPVI. This is supported by demonstration of phosphorylation of the FcR γ-chain. The limited spreading of mouse platelets on fibrinogen is mediated through a Src-dependent but Syk-independent pathway. Together, these results support a model in which immobilized fibrinogen activates human but not mouse platelets through GPVI.

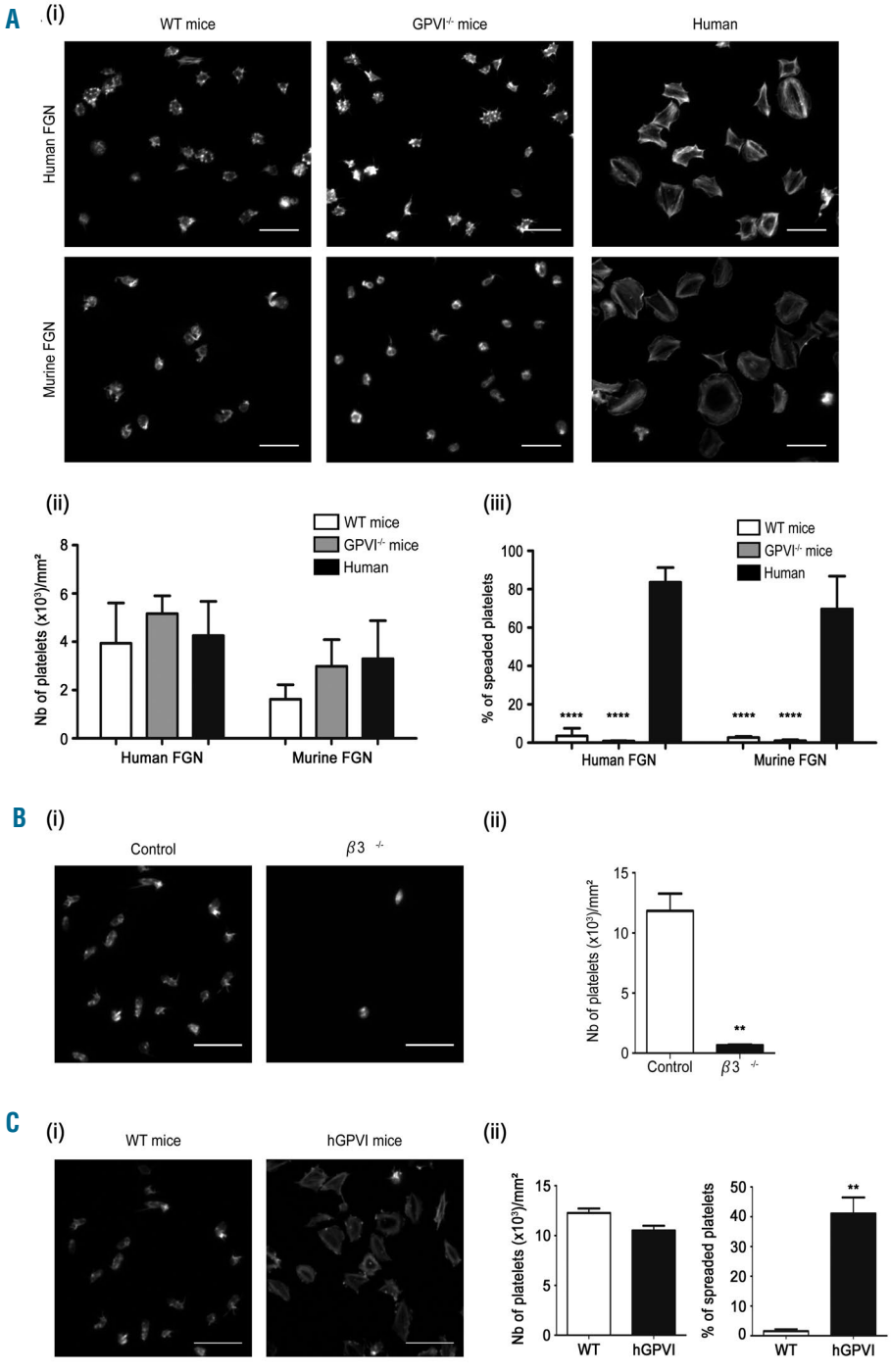

Figure 2. Human but not mouse glycoprotein VI supports platelet adhesion and spreading on immobilized fibrinogen. (A). Washed platelets from wild-type mice (WT mice), GPVI-deficient mice (GPVI$^{-/-}$ mice) or from healthy donors (Human) were allowed to adhere to human or mouse fibrinogen (FGN) for 30 or 45 min, respectively, and fixed with PFA and stained with Alex-488-phalloidin (4 μg/mL). (A)(i). Representative epifluorescence images of washed platelets adhering to fibrinogen. Scale bars represent 10 μm. (A)(ii). Bar graph representing the number of platelets adhering to immobilized fibrinogen per mm². Adhesion is expressed as mean±SEM in five random fields, in three separate experiments (two-way ANOVA, Bonferroni post-hoc test: P>0.05). (A)(iii). Bar graph representing the percentage of platelets spreading on immobilized fibrinogen. Spreading is expressed as the mean±SEM in five random fields, in six separate experiments. Significance was attained using a two-way ANOVA, Bonferroni post-hoc test: ****P<0.001. (B). Washed control (WT) or β3-deficient (β3$^{-/-}$) platelets were allowed to adhere to fibrinogen for 60 min, fixed with PFA and stained with TRITC-phalloidin (2 μg/mL). (B)(i). Representative epifluorescence images of washed mouse platelets adhering to fibrinogen. Scale bars represent 10 μm. (B)(ii). Bar graph representing the number of platelets adhering to immobilized fibrinogen per mm². Adhesion is expressed as the mean±SEM in eight random fields, in four separate experiments (Mann-Whitney test, **P<0.001). (C). Washed platelets from wild-type mice (WT mice) or mice expressing human GPVI (hGPVI mice) were allowed to adhere to fibrinogen for 60 min, fixed with PFA and stained with TRITC-phalloidin (2 μg/mL). (C)(i). Representative epifluorescence images of washed platelets adhering to fibrinogen. Scale bars represent 10 μm. (C)(ii). Bar graph (left) representing the number of platelets adhering to immobilized fibrinogen per mm². Adhesion is expressed as the mean±SEM in eight random fields, in four separate experiments (Mann-Whitney test, P>0.05). Bar graph (right) representing the percentage of platelets spreading on immobilized fibrinogen. Spreading is expressed as the mean±SEM in eight random fields, in four separate experiments (Mann-Whitney test, **P<0.001).

Fibrinogen stimulates an increase of Ca^{2+} in human glycoprotein VI-transgenic mouse platelets

The observation that platelets expressing human but not mouse GPVI undergo full spreading suggests that signals from human GPVI are of significance. To investigate this, a dual-dye Ca^{2+} assay was used to monitor cytoplasmic Ca^{2+} levels as a marker of PLCγ2 activation. Analysis of single platelet Ca^{2+} profiles by confocal microscopy highlighted that signals generated on fibrinogen are composed of Ca^{2+} spikes (Figure 5A). The number of Ca^{2+} spikes in mouse platelets expressing human GPVI was significantly increased relative to the number in wild-type platelets and was blocked in the presence of PRT-060318 (Figure 5Ai-ii) highlighting the critical role of Syk in Ca^{2+} mobilization. These results demonstrate that human GPVI stimulates Ca^{2+} signaling in fibrinogen-adherent mouse platelets.

Fab 9012 blocks aggregate growth of humanized glycoprotein VI mouse platelets

Fibrinogen plays a critical role in hemostasis and arterial thrombosis through crosslinking of platelets in the growing thrombus. In addition, we now show that fibrinogen induces platlet activation by GPVI. To establish whether

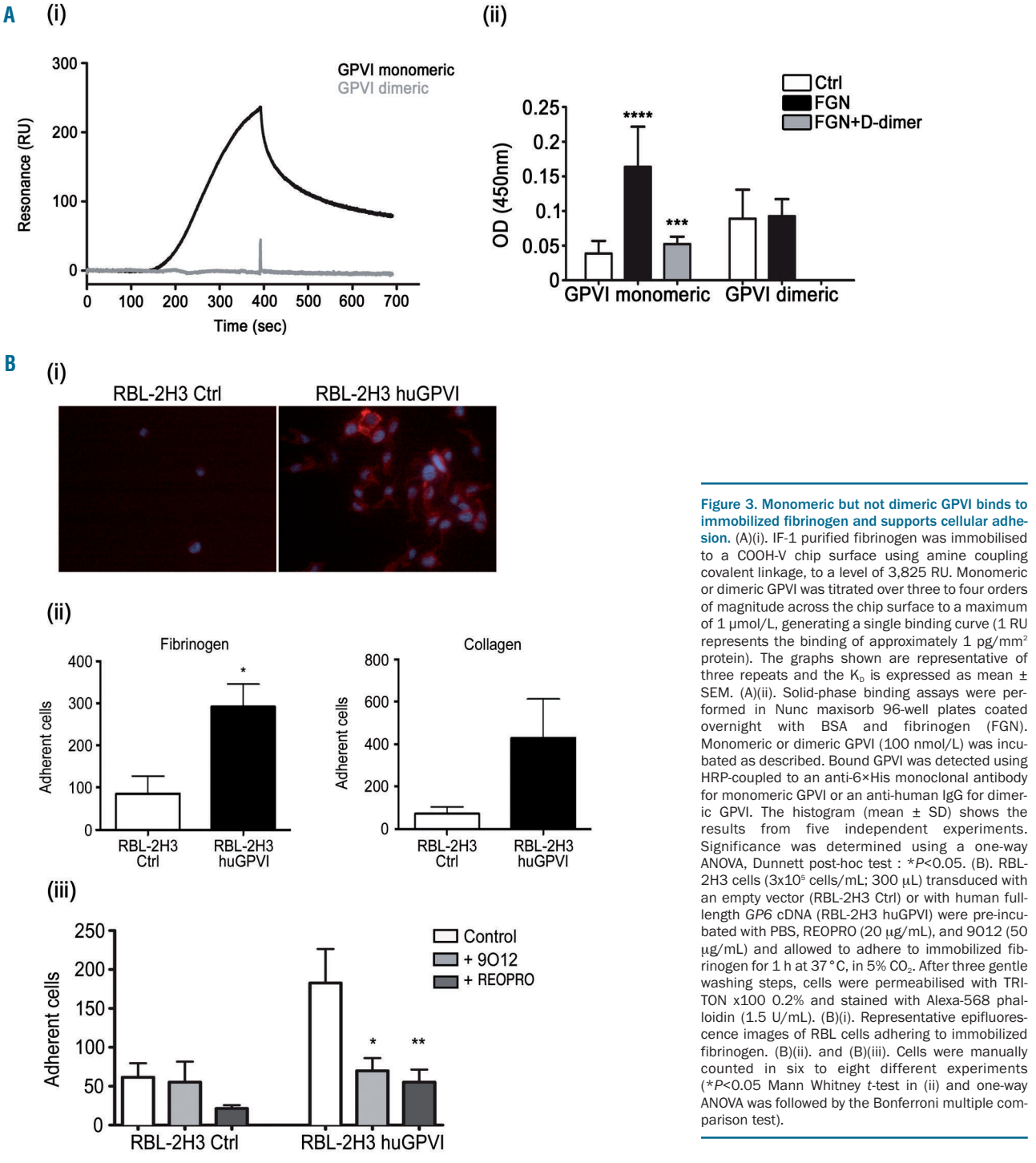

Figure 3. Monomeric but not dimeric GPVI binds to immobilized fibrinogen and supports cellular adhesion. (A)(i). IF-1 purified fibrinogen was immobilised to a COOH-V chip surface using amine coupling covalent linkage, to a level of 3,825 RU. Monomeric or dimeric GPVI was titrated over three to four orders of magnitude across the chip surface to a maximum of 1 µmol/L, generating a single binding curve (1 RU represents the binding of approximately 1 pg/mm^2 protein). The graphs shown are representative of three repeats and the K_D is expressed as mean ± SEM. (A)(ii). Solid-phase binding assays were performed in Nunc maxisorb 96-well plates coated overnight with BSA and fibrinogen (FGN). Monomeric or dimeric GPVI (100 nmol/L) was incubated as described. Bound GPVI was detected using HRP-coupled to an anti-6×His monoclonal antibody for monomeric GPVI or an anti-human IgG for dimeric GPVI. The histogram (mean ± SD) shows the results from five independent experiments. Significance was determined using a one-way ANOVA, Dunnett post-hoc test : *P<0.05. (B). RBL-2H3 cells (3x10^5 cells/mL; 300 µL) transduced with an empty vector (RBL-2H3 Ctrl) or with human full-length *GP6* cDNA (RBL-2H3 huGPVI) were pre-incubated with PBS, REOPRO (20 µg/mL), and 9O12 (50 µg/mL) and allowed to adhere to immobilized fibrinogen for 1 h at 37 °C, in 5% CO_2. After three gentle washing steps, cells were permeabilised with TRITON x100 0.2% and stained with Alexa-568 phalloidin (1.5 U/mL). (B)(i). Representative epifluorescence images of RBL cells adhering to immobilized fibrinogen. (B)(ii). and (B)(iii). Cells were manually counted in six to eight different experiments (*P<0.05 Mann Whitney t-test in (ii) and one-way ANOVA was followed by the Bonferroni multiple comparison test).

activation of GPVI by platelet-bound fibrinogen participates in platelet aggregation we performed an *in vitro* flow adhesion assay under conditions that prevent activation of GPVI by collagen and by fibrin. To achieve this, we generated a platelet aggregate over type I fibrillar collagen using hirudin-treated blood to prevent formation of fibrin. We then perfused additional blood from the same donor over the aggregate at a wall shear rate of 300 s^{-1} in the presence or absence of the Fab fragment of the GPVI blocking monoclonal antibody 9O12. As expected, we were unable to detect the presence of fibrin in the aggregate using a specific antibody (*data not shown*). The aggregate continued to

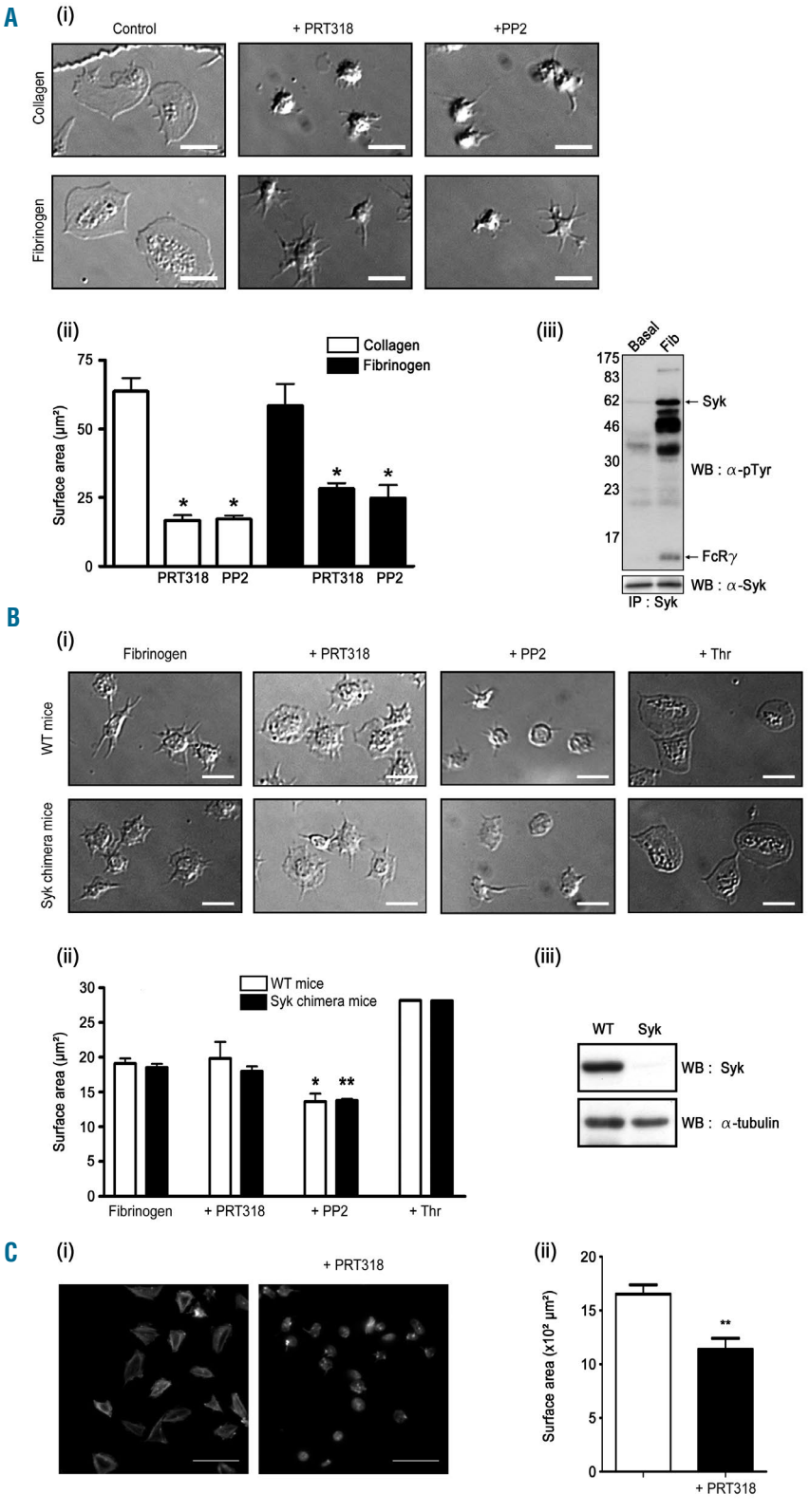

Figure 4. Syk promotes platelet spreading on fibrinogen downstream of glycoprotein VI but not in integrin αIIbβ3 outside-in signaling. Washed human platelets, or washed platelets from wild-type mice (WT mice), Syk chimera mice (Syk chimera mice) and mice expressing human GPVI (hGPVI mice) were allowed to adhere to fibrinogen or to collagen in the presence of either the Src inhibitor PP2 (20 µmol/L), the Syk inhibitor (5 µmol/L), thrombin (0.1 U/mL), or vehicle control, for 30 min (human platelets) or 45 min (mouse platelets) at 37°C followed by fixation with PFA. (A)(i). Representative DIC images of human platelets adhering to fibrinogen or collagen. Scale bars represent 5 µm. (A)(ii). Bar graph representing the surface area of platelets spreading on fibrinogen. Spreading is expressed as the mean±SEM in five or more random fields, in three separate experiments. Significance was determined by one-way ANOVA and Bonferroni multiple comparison test: *P<0.01. (A)(iii) Representative western blot from human platelets following adhesion to fibrinogen. Following immunoprecipitation of Syk, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot for phosphotyrosine. Membranes were then stripped and reprobed for Syk to confirm equal protein loading. Blots are representative of three separate experiments. (B)(i). Representative DIC images of mouse platelets adhering to fibrinogen. Scale bars represent 5 µm. (B)(ii). Bar graph representing the surface area of platelets spreading on fibrinogen. Spreading is expressed as the mean±SEM in five or more random fields, in three separate experiments. Significance was determined using one-way ANOVA, with the Bonferroni post-hoc test: *P<0.05, **P<0.001. (B)(iii). Expression of Syk was measured by western blotting platelet whole-cell lysates with an α-Syk antibody. Membranes were then stripped and reprobed with an anti-α-tubulin antibody to confirm equal protein loading. Blots are representative of three separate experiments. (C)(i). Representative epifluorescence images of mouse platelets adhering to fibrinogen. Scale bars represent 10 µm. (C)(ii). Bar graph representing the surface area of platelets spreading on fibrinogen. Spreading is expressed as the mean±SEM in eight random fields, in five separate experiments (Mann-Whitney test, **P<0.01).

grow in the presence of a Fab control but was dramatically inhibited in the presence of Fab 9O12 (Figure 6A and *Online Supplementary Figure S2*). In contrast, and as previously shown, blockade of GPVI did not impair aggregation measured by light transmission aggregometry in response to ADP, U46619 and thrombin.[35] These results demonstrate a critical role for GPVI in aggregate growth under flow when the roles of collagen and fibrin are negated. In contrast, fibrinogen does not induce activation of platelets in suspension either because the interaction is dependent on activation of integrin αIIbβ3 or because it cannot crosslink GPVI.

Discussion

In this study we show that human GPVI binds to immobilized fibrinogen and that this leads to intracellular signals, which drive the formation of lamellipodial sheets and stress fibers in human platelets and in human GPVI-expressing mouse platelets. This explains the previously paradoxical observation that only human platelets form lamellipodial sheets and stress fibers on a fibrinogen surface, despite mouse platelets being able to form both actin structures in the presence of G protein-coupled receptor agonists such as thrombin. We also show that the interaction of fibrinogen with GPVI is important for aggregate growth providing a new understanding of hemostasis and thrombosis.

We recently identified fibrin as a novel ligand for GPVI[7,8,42] and have shown that binding resides in the D-dimer region.[30] The observation that fibrinogen also activates GPVI should not, therefore, be a surprise. Nevertheless, this was unexpected and came from the observation that human platelets deficient in GPVI adhere to but do not spread on fibrinogen. This raises the question as to why this has been previously overlooked. One reason is that mouse platelets do not spread on fibrinogen and thus there is no defect in the absence of GPVI. A second reason is the low level of phosphorylation of the FcR γ-chain induced by fibrinogen in human platelets relative to that by collagen and other GPVI-agonists. This may reflect the extent to which each ligand is able to cluster GPVI and, in the case, of fibrinogen, the dependency on the interaction with integrin αIIbβ3. A third reason is that fibrinogen binds selectively to monomeric GPVI whereas the original binding studies were performed with dimeric GPVI.[7,8] It is worth noting that we have reported a reduced number of dimers on immobilized fibrinogen relative to collagen.[42]

Adhesion of human platelets to fibrinogen is dependent on integrin αIIbβ3. At present, it is not known whether binding to integrin αIIbβ3 is critical for activation of GPVI or simply to promote adhesion such that activation of GPVI can occur. As a dimer, fibrinogen should be able to bind two GPVI monomers, but alternatively the interaction with integrin αIIbβ3 may be required to support activation of monomeric GPVI. A similar role for an integrin in the activation of an ITAM receptor has been reported in other hematopoietic cells with the postulate that the integrin and the ITAM receptor would be associated via a linker protein.[43]

Fibrinogen is present in whole blood at a concentration of 2 - 4 mg/mL but does not induce platelet activation. This may be explained by an inability of soluble fibrinogen to bind GPVI in suspension due to conformational differences between circulating and immobilized fibrinogen. Alternatively it may be due to the inability of the dimeric fibrinogen to cluster GPVI on the platelet surface in suspension or because of a dependency on binding to integrin αIIbβ3. While the affinity of fibrinogen for GPVI is in the range of that for collagen for GPVI,[44,45] we have shown that fibrinogen (and fibrin) bind selectively to monomeric GPVI and this would not be sufficient to induce activation because of the absence of crosslinking. The reason why human platelets, but not mouse platelets, spread on fibrinogen is unclear. Based on the fact that human and mouse GPVI share 64% homology,[33] one could imagine that only human GPVI binds to fibrinogen or that both bind to this adhesive protein but only human GPVI is able to promote activation.

GPVI is primarily known as the major signaling receptor for collagen. However, in recent years, GPVI has been shown to bind to other ligands including laminin, the transmembrane protein emmprin, adiponectin, histones and fibrin.[6-8,10,47] The physiological significance of many of these interactions is uncertain, in part because of their low affinity or because of whether they acutally occur *in vivo*. The interaction that has received the greatest attention is that with fibrin which lies at the interface of the core and shell of the growing platelet aggregate.[7,8] This interaction

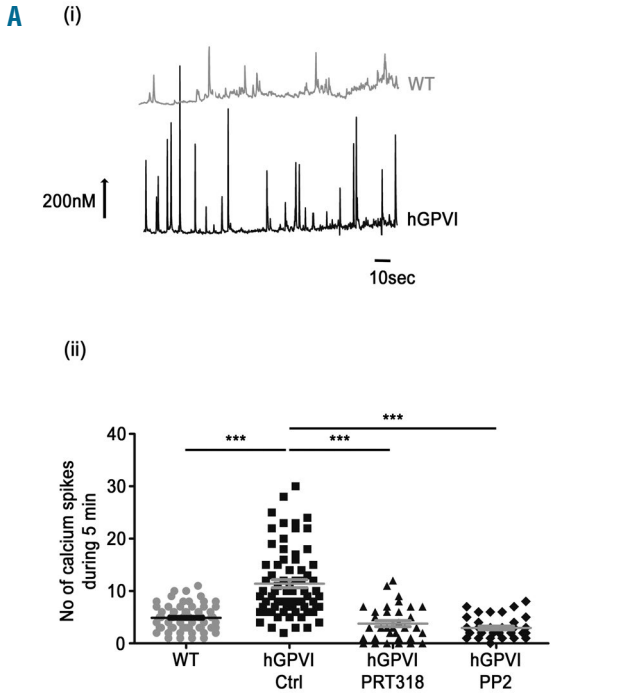

Figure 5. Human glycoprotein VI, but not integrin αIIbβ3 plays a major role in the regulation of the calcium signaling after platelet adhesion to fibrinogen. Washed platelets from wild-type (WT) or mice expressing human GPVI (hGPVI) were loaded with Oregon-green Bapta-1-AM and Calcein red orange and deposited on immobilized fibrinogen (100 μg/mL). Modifications in fluorescence of individual adherent platelets were monitored for 7 min by confocal microscopy and the Ca^{2+} concentrations were determined as detailed in the Methods section. (A)(i). Typical time-course Ca^{2+} profile of one representative platelet adhering to fibrinogen. (A)(ii). Dot plot representing the number of calcium spikes over a period of 5 min. Each point represents an individual platelet. The results are presented as the mean±SEM of five independent experiments (one-way ANOVA, Bonferroni post-hoc test, ***P<0.001).

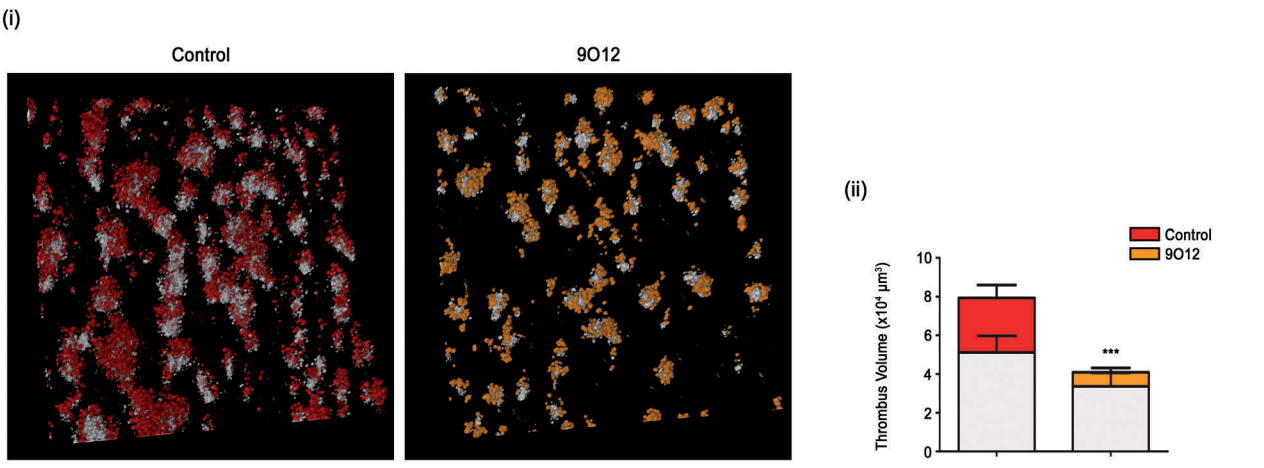

Figure 6. Blockade of human glycoprotein VI limits platelet accumulation to a growing aggregate. (A)(i). Hirudinated human whole blood labeled with $DIOC_6$ (1 µmol/L) was perfused over immobilized collagen (200 µg/mL) to preform aggregates for 2 min 30 s, before perfusing hirudinated blood from the same donor in the presence of the Alexa Fluor 647-conjugated monoclonal antibody against GPIbβ (5 µg/mL) and with a Fab control (Control) or the blocking anti-GPVI antibody 9O12 (50 µg/mL). (A)(i). Representative 3D reconstructions from confocal images of aggregates obtained after 7 min 30 s of blood perfusion at 300 s^{-1}. Preformed aggregates are represented in gray, aggregates formed in the presence of a Fab control are represented in red and aggregates formed in the presence of the Fab 9O12 are depicted in orange. (A)(ii). Bar graph representing the volume of the platelet aggregates (mean±SEM) in eight random fields, in six separate experiments performed with different blood donors (Mann-Whitney test, ***P<0.001). The gray, red and orange colors represent the volume of the preformed aggregates, the aggregates formed in the presence of a Fab control and the aggregates formed in the presence of the Fab 9O12, respectively.

takes place at a critical checkpoint in aggregate consolidation and aggregate growth. Thus, GPVI has the potential to both initiate and propagate thrombus formation through interactions with collagen and fibrin and, it appears now, also with fibrinogen. Moreover, while collagen and fibrin are localized at the base of the thrombus and in the core, respectively, fibrinogen is found throughout the aggregate. This suggests a model in which thrombus growth could be sustained by GPVI/fibrinogen, potentially in association with other adhesive proteins. Indeed, in addition to fibrinogen other adhesive proteins such as von Willebrand factor and fibronectin have been shown to support thrombus growth.[47–50] Whether these proteins participate in GPVI-mediated platelet aggregation is unclear since von Willebrand factor is not known to be a ligand of GPVI and fibronectin does not directly promote platelet adhesion and activation through GPVI.[51] Selective inhibition of the interaction of GPVI with collagen, fibrinogen and fibrin is required to establish their respective contributions to platelet activation in hemostasis and thrombosis.

The discovery that GPVI initiates and propagates platelet aggregation at sites of vessel injury suggests a major role in hemostasis and thrombosis. Paradoxically, however, mice and humans deficient in GPVI only have at most a mild bleeding diathesis, which in the case of humans may be due to additional confounders such as a low platelet count as seen in patients with immune-induced thrombocytopenia caused by antibodies to GPVI. The relatively minor role of GPVI in hemostasis can be explained by redundancy in pathways of platelet activation, with the GPIb-von Willebrand factor axis initiating hemostasis, and ADP, thromboxane and thrombin inducing powerful activation. Additionally, the reactive fibrillar type I and III collagen present in deeper layers of the vessels would limit the role of GPVI in the hemostasic response following superficial injury. On the other hand, the discovery that fibrin and immobilized fibrinogen activate GPVI may be of significance at sites of fibrinogen deposition or fibrin formation in diseased vessels following inflammation or loss of vascular integrity. The ability of fibrin and immobilized fibrinogen to activate GPVI may also reflect yet-to-be-discovered new roles for GPVI.

In conclusion, in the present study, we have identified immobilized fibrinogen as a novel activator of human but not mouse GPVI and have shown that this interaction supports platelet aggregation under flow. This further emphasizes the contribution of GPVI to platelet activation in thrombosis.

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