Nonredundant Roles of Platelet Glycoprotein VI and Integrin αIIbβ3 in Fibrin-Mediated Microthrombus Formation

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Nonredundant Roles of Platelet Glycoprotein VI and Integrin αIIbβ3 in Fibrin-Mediated Microthrombus Formation

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OBJECTIVE: Fibrin is considered to strengthen thrombus formation via integrin αIIbβ3, but recent findings indicate that fibrin can also act as ligand for platelet glycoprotein VI.

APPROACH AND RESULTS: To investigate the thrombus-forming potential of fibrin and the roles of platelet receptors herein, we generated a range of immobilized fibrin surfaces, some of which were cross-linked with factor XIIIa and contained VWF-BP (von Willebrand factor-binding peptide). Multicolor microfluidics assays with whole-blood flowed at high shear rate (1000 s^{-1}) indicated that the fibrin surfaces, regardless of the presence of factor XIIIa or VWF-BP, supported platelet adhesion and activation (P-selectin expression), but only microthrombi were formed consisting of bilayers of platelets. Fibrinogen surfaces produced similar microthrombi. Markedly, tiggering of coagulation with tissue factor or blocking of thrombin no more than moderately affected the fibrin-induced microthrombus formation. Absence of αIIbβ3 in Glanzmann thrombasthenia annulled platelet adhesion. Blocking of glycoprotein VI with Fab 9O12 substantially, but incompletely reduced platelet secretion, Ca^{2+} signaling and aggregation, while inhibition of Syk further reduced these responses. In platelet suspension, glycoprotein VI blockage or Syk inhibition prevented fibrin-induced platelet aggregation. Microthrombi on fibrin surfaces triggered only minimal thrombin generation, in spite of thrombin binding to the fibrin fibers.

CONCLUSIONS: Together, these results indicate that fibrin fibers, regardless of their way of formation, act as a consolidating surface in microthrombus formation via nonredundant roles of platelet glycoprotein VI and integrin αIIbβ3 through signaling via Syk and low-level Ca^{2+} rises.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: blood platelet ■ fibrin ■ microfluidics ■ platelet aggregation ■ thrombin

GP (glycoprotein) VI is a platelet immunoglobulin (Ig) receptor, expressed at 3000 to 4000 copies per platelet, and known to be involved in the onset of thrombus formation.[1,2] Common concept is that GPVI mediates the initial activation of platelets in contact with exposed collagen in the vasculature, assisted by platelet integrins and by GPIb-V–IX which interacts with collagen-bound VWF (von Willebrand factor).[3–5] In the platelet membrane, GPVI is constitutively associated with the Fc receptor γ-chain, containing an intracellular immunoreceptor tyrosine-based activation motif. Ligand binding induces clustering of GPVI and ensuing phosphorylation of the immunoreceptor tyrosine-based activation motif via Src-family kinases.[6] This leads to activation of the tyrosine kinase Syk through its tandem SH2 (Src homology2) domain, culminating in activated phospholipase Cγ2 and ensuing Ca^{2+} mobilization.[2,6]

Since 2015, it has been recognized that GPVI can also act as a receptor for fibrin and fibrinogen.[7–9] Relevance of this finding comes from the earlier observation

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Nonstandard Abbreviations and Acronyms

FXIIIa	factor XIIIa
GPVI	glycoprotein VI
PPACK	D-Phe-Pro-Arg chloromethyl ketone
PRP	platelet-rich plasma
VWF	von Willebrand factor
VWF-BP	von Willebrand factor-binding peptide

Highlights

- Platelet adhesion to a fibrin layer under flow elicits only moderate glycoprotein VI activation.
- The interaction with glycoprotein GPVI (glycoprotein VI) interaction relies on αIIbβ3 and forms small, bilayered thrombi.
- This consolidated thrombus formation is restricted by thrombin binding to fibrin.

that fibrin formation can be both an initial and propagating process in vaso-occlusive thrombus formation upon vascular damage.[10–12] In this setting, the role of GPVI as a functional receptor for fibrin implies a crucial contribution of this receptor interaction in thrombus growth and in the propagation of coagulation. This idea is supported by the observations that (1) GPVI binding to fibrin can trigger platelet procoagulant activity and ensuing thrombin generation[8] and (2) fibrin binds to procoagulant platelets via the cross-linking transglutaminase FXIIIa (factor XIIIa).[13] However, some authors have questioned the role of GPVI as a fibrin receptor in blood.[14]

Integrin αIIbβ3 is known as the conventional platelet receptor for fibrinogen and fibrin, being expressed at 50 000 to 80 000 copies per platelet.[15–17] Similarly to GPVI, integrin αIIbβ3 promotes platelet adhesion and activation via outside-in signaling through Src-family and Syk protein tyrosine kinases.[6,18] Questions then arising are (1) what are the roles of GPVI and αIIbβ3 in fibrin-dependent platelet activation; (2) how do these receptor interactions contribute to thrombus formation; (3) how can they prevent endless growth of the platelet-fibrin thrombus; and (4) which is the role of blood flow and shear in this process.

In the present article, we aimed to answer these questions and resolving the dispute on the role of GPVI. We studied the relative contribution of GPVI and integrin αIIbβ3 in fibrin-dependent platelet activation and thrombus formation under defined flow conditions. Since fibrin is known to bind VWF,[19] we also explored the contribution herein of the VWF receptor, GPIb-V–IX. Our results show that GPVI provides a weakly activating signal that relies on αIIbβ3-dependent platelet adhesion and Syk activation to form small-sized thrombi. Markedly, our data also indicate that the platelet-activating role of thrombin is dampened on fibrin surfaces.

MATERIALS AND METHODS

The authors declare that materials and data are available upon reasonable request from the authors. An extended version of Materials and Methods is available in the Data Supplement.

Major Resources

Please see the Major Resources Table in the Data Supplement.

Blood Withdrawal and Platelet Preparation

Blood was obtained by venepuncture from healthy volunteers (male and female), who had not received antiplatelet or anticoagulant medication for at least 2 weeks. Informed consent was obtained according in compliance with the ethical principles of the Declaration of Helsinki, and studies were approved by the local Medical Ethics Committee (METC 10-30-023, Maastricht University). Blood samples were collected into 3.2% trisodium citrate (Vacuette tubes, Greiner Bio-One, Alphen a/d Rijn, the Netherlands). Blood samples were also obtained from 2 patients with diagnosed Glanzmann thrombasthenia, that is, one homozygous patient lacking expression of integrin αIIbβ3 on platelets, and one heterozygous patient with 50% of normal αIIbβ3 expression.

PRP (platelet-rich plasma) was obtained from the citrated blood by centrifugation at 870*g* for 10 minutes.[20] After addition of 1:10 vol/vol acid-citrate-dextrose (80 mmol/L trisodium citrate, 183 mmol/L glucose, 52 mmol/L citric acid), the PRP was centrifuged at 2360*g* for 2 minutes. Platelet pellets were resuspended into Hepes buffer pH 6.6 (10 mmol/L Hepes, 136 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L $MgCl_2$, 5.5 mmol/L glucose, and 0.1% BSA). After addition of apyrase (1 U/mL) and 1:15 vol/vol acid-citrate-dextrose, another centrifugation step was performed to obtain washed platelets. The platelet pellet was resuspended into Hepes buffer pH 7.45 (10 mmol/L Hepes, 136 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L $MgCl_2$, 5.5 mmol/L glucose, and 0.1% BSA).[20]

Microfluidic Flow Experiments

Glass coverslips were coated for 1 hour with two 1.5 mm diameter spots, each 3 mm apart, which contained the indicated type of fibrin or fibrinogen (upstream) and, when indicated, collagen type III (50 µg/mL) as a reference spot (downstream), and collagen type I (50 µg/mL). This microspot coating procedure eliminates cross-talk of thrombus formation between the adjacent surfaces.[21] For fibrin spots, fibrinogen (1 mg/mL, 0.5 µL) was applied for 30 minutes, after which α-thrombin (20 nmol/L, 1 µL) was supplemented for additional 30 minutes. Where indicated, mixtures (1 µL) of α-thrombin (20 nmol/L), FXIIIa (0.7 µg/mL), and $CaCl_2$ (10 mmol/L) were applied on top of the fibrinogen. Residual FXIIIa activity of the fibrinogen preparation used was determined as 6% in comparison to plasma. Indicated spots were postcoated with the peptide VWF-BP (von Willebrand factor-binding peptide; 100 µg/mL, 1 µL). After the completion of coating, coverslips were blocked with 1% BSA in Hepes buffer pH 7.45 for 30 minutes.

As a standard, whole blood was flowed over the coated spots using a microfluidics chamber under conditions allowing

coagulation.[12] In brief, 1.0 mL samples of citrated blood were co-perfused with recalcification medium using 2 pulse-free micro-pumps (Model 11 Plus, 70-2212, Harvard Apparatus), and a y-shaped mixing tubing. The recalcification medium (in a second 1 mL syringe) consisted of 32 mmol/L $MgCl_2$ and 63 mmol/L $CaCl_2$ in Hepes buffer pH 7.45. Complete mixing was achieved at a volume ratio of 10 (blood) to 1 (recalcification medium).[22] Flow rates were adjusted to give a total wall-shear rate of 1000 or 100 s^{-1}.

Fluorescent labels added per blood sample were $DiOC_6$ (platelet staining), AF568-annexin A5 (phosphatidylserine, exposure), and Alexa Fluor 647-anti-CD62P mAb (P-selectin expression), as described.[23] When appropriate, samples were preincubated for 10 minutes with vehicle, inhibitor PRT-060318 (10 µmol/L, in 0.4 µg/mL pluronic plus 0.5% DMSO) and Fab 9012 (50 µg/mL, in saline). Inhibition of GPVI was achieved with Fab 9O12 (50 µg/mL), which has previously been shown to interfere in the interaction of GPVI and fibrin.[8] Brightfield and multicolor fluorescence images were recorded per spot over time.[24] Per donor, all control and intervention conditions were repeated at least in duplicates. Collected time series of brightfield and fluorescence microscopic images were analyzed by using predefined scripts,[25] formatted in the open source package Fiji.[26]

Scanning Electron Microscopy

For electron microscopy, fibrin-coated spots were prepared as for flow studies and coated on a Sefar matrix (sieve mesh, pores: 170 µm; Sefar Pharma Ltd) using 96-wells plates.[27] Samples were fixed with 4% paraformaldehyde for 1 hour. After wash with PBS, the samples were dehydrated by a 5-step gradient of ethanol (30%–100%), and then dried by 10-minutes treatment with hexamethyl disilizane/ethanol (1:1) and 1 hour exposure to air. Dried samples were mounted onto aluminium pin studs with 12 mm carbon conductive tabs (Ted Pella, Redding, CA), were sputter coated with gold (Quorum Technologies, Ashford, United Kingdom; vacuum pump: Edwards, Crawley, United Kingdom) on carbon tabs, and imaged. Table-top electron microscopy was performed, as before.[27]

Fibrin Suspension Preparation

Fibrinogen (1 mg/mL) was mixed with $CaCl_2$ (10 mmol/L), FXIIIa (0.7 µg/mL), and thrombin (1 U/mL) and was left to polymerize for 1 hour. Subsequently, D-Phe-Pro-Arg chloromethyl ketone (PPACK; 20 µmol/L) was added to inactivate the thrombin, the clot mixture was agitated until it turned liquid, and it was left for 15 minutes. The gel solution was then ultrasonicated at 20 kHz and amplitude of 80 to 100 µm until clear; this was followed by a centrifugation step at 1000 g for 5 minutes. The obtained pellet was homogeneously resuspended into Hepes buffer pH 7.45.

Platelet Aggregometry

Platelet aggregation was monitored by light transmission aggregometry using an automated Chronolog aggregometer (Havertown PA) at 37 °C with stirring at 1200 rpm. Platelet suspensions (2×10^8/mL) were incubated at 37 °C for 2 minutes, antagonists were added for 10 minutes, followed by agonists sonicated fibrin, collagen-I (5 µg/mL), or α-thrombin (0.1 U/mL).

Cytosolic Ca^{2+} Measurements

Washed human platelets (2×10^8/mL) were loaded with Fluo-4 acetoxy methyl ester (8 µmol/L) and pluronic (0.4 mg/mL) by a 40 minutes incubation in the presence of apyrase (1 U/mL).[28] After centrifugation step in the presence of acid-citrate-dextrose, the Fluo-4-loaded platelets were resuspended into Hepes buffer pH 7.45. Blood samples were supplemented with 10% of autologous Fluo-4-loaded platelets; inhibitors were added after 5 minutes. Changes in cytosolic $[Ca^{2+}]_i$ during flow-dependent adhesion of labeled platelets were recorded for 5 minutes, using a Zeiss LSM 510 confocal microscope, essentially as described before.[29] Time series of fluorescence images were analyzed for changes in fluorescence intensity and for platelet adhesion, using Fiji/image J software.

Data Handling and Statistics

Data are represented as means±SD. Statistical analysis was performed using GraphPad Prism v8 software (San Diego, CA). Significance was determined using a 2-way ANOVA (Dunnett and Sidak multiple comparison test) or a 1-way ANOVA (Dunnett multiple comparison test); differences with $P<0.05$ were considered as significant.

Heatmaps were generated with the program R. For the heatmap representation, all parameters were univariate scaled to 0 to 10.[21] According to earlier procedures,[30] thrombus values of duplicate or triplicate flow runs from one blood donor were averaged to obtain one parameter set per spot. Mean values of control and inhibitor runs were then compared per blood sample. For subtraction heatmaps, a conventional filter of $P<0.05$ (1-way ANOVA) was applied to determine relevant effects, as described before.[25,30]

RESULTS

Fibrin Microstructure of Coated Spots

To assess the suitability of fibrin-coatings for flow chamber studies, we prepared a series of spotted fibrinogen surfaces which were treated with a thrombin mixture in the presence or absence of the cross-linking transglutaminase, FXIIIa, and a peptide (VWF-BP) capable to capture free VWF from blood.[31] Ultrastructural observation of the different preparations by scanning electron microscopy showed that in all conditions multiple layers of fibrin were formed, which presented as microstructures with both thicker and thinner fibers (Figure 1, arrows). The addition of VWF-BP did not alter the overall fiber structure. However, addition of FXIIIa resulted in fibrin fibers that appeared to be less densely packed with an overall thicker size. The latter observation may be due to the local high transglutaminase concentration upon the fibrin formation. For comparison, also fibrinogen-only spots were examined with or without VWF-BP. Electron microscopy did not reveal any fibrous structures in this case (data not shown).

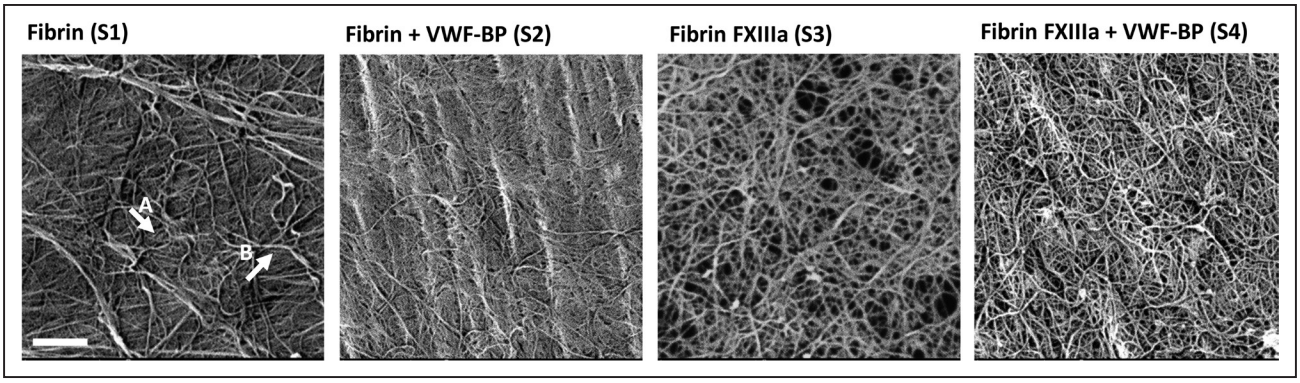

Figure 1. Fibrillar microstructure of immobilized fibrin surfaces.
Representative scanning electron microscopy (SEM) images of immobilized fibrin spots, produced from fibrinogen coatings with or without FXIIIa (factor XIIIa) and VWF-BP (von Willebrand factor-binding peptide). Mixtures were allowed to generate fibrin fibers on a SEFAR (Sefar Pharma Ltd) filter for 30 min. Representative images are shown; scale bar 2 µm. Arrow A points to a thin fiber, arrow B indicates a thick fiber.

Formation of Only Small-Sized Microthrombi on Fibrin Surfaces Under Flow

We examined how the different types of fibrin(ogen) spots formed with or without FXIIIa and VWF-BP (coded as S1-S6, see Table) were able to support thrombus formation under flow in microfluidics chambers. Therefore, citrated whole blood (labeled with $DiOC_6$, AF568-annexin A5, and Alexa Fluor 647-anti-CD62P mAb) was flowed over sets of 2 spots at defined conditions, in coagulating condition (absence of PPACK).[22] After 10 minutes of flow at arterial shear rate (1000 s^{-1}), on all fibrin(ogen) surfaces, platelets adhered and formed small aggregates, not extending 2 or 3 cell layers, which we characterized as bilayer aggregates or microthrombi (Figure 2A; representative images of all 6 spots in Figure I in the Data Supplement). In contrast, simultaneous flow experiments using collagen-I spots produced larger thrombi composed of multi-layered platelet aggregates (see below), such as reported before.[32] No platelets adhered to coverslip areas in between the coated spots.[21]

Capturing of multicolor fluorescence microscopic images at time points of 2, 4, 6, 8, and 10 minutes allowed to assess the kinetics of the process, in terms of 6 parameters. These were $DiOC_6$ platelet adhesion (P1), thrombus morphological score (P2), thrombus contraction score (P3), bi- or multi-layer score (P4), bi- or multilayer size (P5), P-selectin expression (P6), and phosphatidylserine exposure (P7).[25] End stage bright-field and triple-coloured microscopic images from fibrin-FXIIIa spots (S3) are shown in Figure 2A. To compare the time-dependent parameter increases per type of fibrin spots (S1–S4) and fibrinogen spots (S5–S6), we scaled all values per parameter across surfaces (scale 0–10) and represented the results in a practical heat-map format (Figure 2B).

Overall, the heatmap analysis pointed to similar parameter increases over time for all types of fibrin spots (S1–S4), although rates of platelet adhesion (P1) were slightly higher in spots containing VWF-BP (S2, S4). The latter observation pointed to a moderate enhancement of platelet adhesion but not to bilayer microthrombus formation, by plasma-bound VWF. On all fibrin spots, the adhered platelets gradually increased in P-selectin expression (P6), but remained low in phosphatidylserine exposure (P7), thus indicating an only moderate platelet activation state.[2] Addition of FXIIIa during fibrin formation (S3, S4) slightly increased the formation of platelet aggregates, when compared with no added FXIIIa (S1, S2). Furthermore, the 2 spots with fibrinogen (S5, S6) were even less active in supporting platelet adhesion,

Table. Coding of Spots (*S*) and Parameters (*P*) in Whole-Blood Thrombus Formation

S	Microspot coating	Adhesive receptors	
S1	Fibrin	GPVI, αIIbβ3	
S2	Fibrin+VWF-BP	GPIb, VI, αIIbβ3	
S3	Fibrin-FXIIIa	GPVI, αIIbβ3	
S4	Fibrin-FXIIIa+VWF	GPIb, VI, αIIbβ3	
S5	Fibrinogen	GPVI, αIIbβ3	
S6	Fibrinogen+VWF-BP	GPIb, VI, αIIbβ3	
S7	Collagen-III	GPIb, VI, α2β1	
P	Image type	Description	Unit or scaling
Platelet parameters			
P1	$DiOC_6$	Platelet adhesion	%SAC
P6	AF647 α-P-selectin	Platelet activation	%SAC
P7	AF568 annexin A5	Platelet PS exposure	%SAC
Thrombus parameters			
P2	Brightfield	Thrombus morphology score	1–5
P3	Brightfield	Thrombus aggregation score	1–3
P4	Brightfield	Thrombus contraction score	1–3
P5	Brightfield	Thrombus multilayer coverage	%SAC

Coding of variables in the order of appearance in heatmaps. Assumed relevant platelet-adhesive receptors are indicated per spot. Parameters were evaluated from microscopic images over time periods as indicated in figures. FXIIIa indicates factor XIIIa; GPVI, glycoprotein VI; PS, phosphatidylserine; SAC, surface-area-coverage; and VWF-BP, von Willebrand factor-binding peptide.

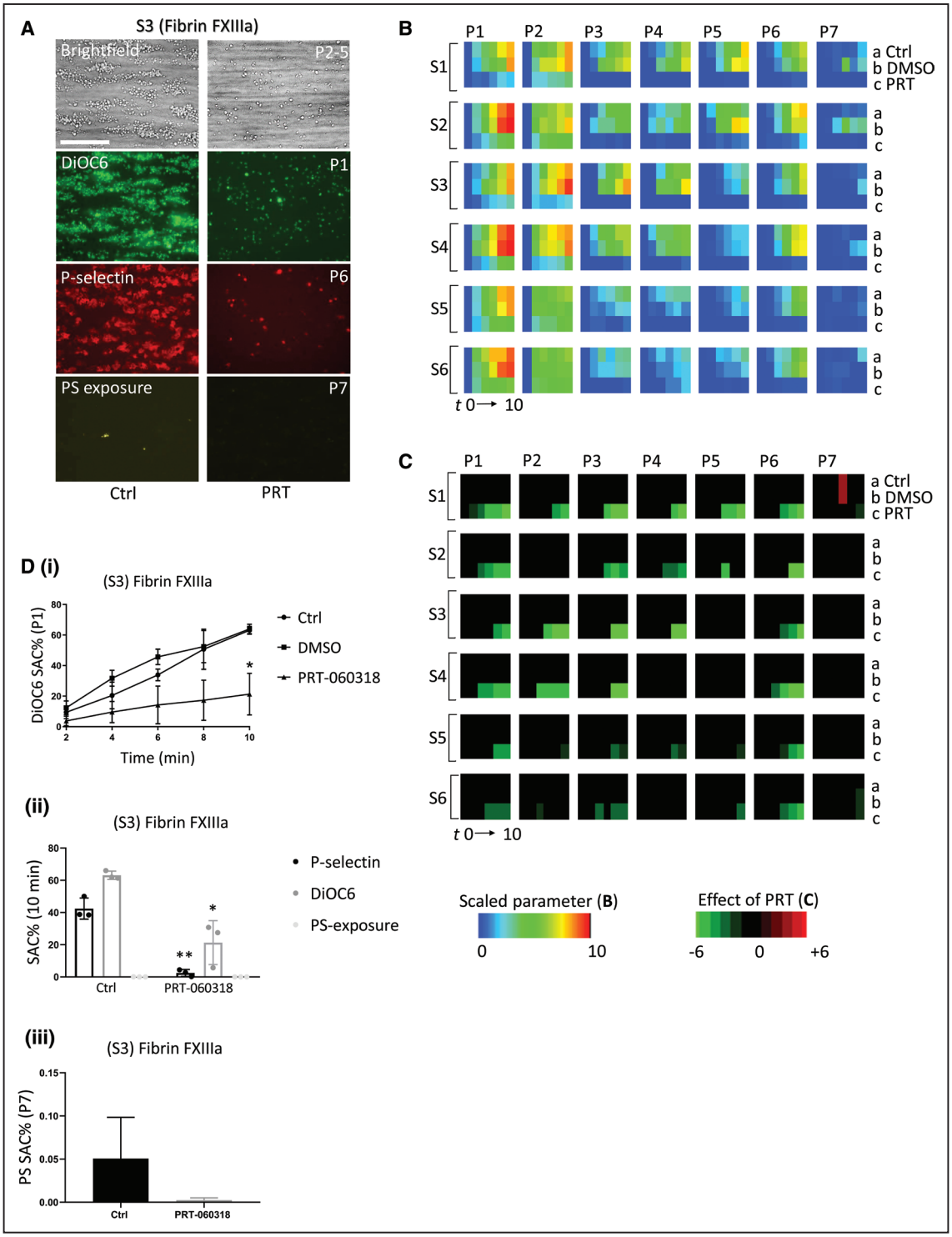

Figure 2. Major role of Syk kinase in microthrombus formation on fibrin or fibrinogen surfaces.
Blood samples preincubated with vehicle (Ctrl) or Syk inhibitor PRT-060318 (PRT, 10 µmol/L), labeled and flowed at 1000 s^{-1} over spots S1–S6 (Table). Surfaces were imaged (brightfield and fluorescence) at time points *t*=0, 2, 4, 6, 8, 10 min to obtain parameters P1 ($DiOC_6$ platelet adhesion), P2 (thrombus morphological score), P3 (contraction score), P4 (bi- or multi-layer score), P5 (bi- or multi-layer size), P6 (P-selectin expression), and P7 (phosphatidylserine [PS] exposure). Effects of PRT were assessed per blood sample, surface, and parameter. **A**, Representative brightfield and fluorescence images of microthrombi on fibrin-FXIIIa (factor XIIIa) spots (S3) after 10 min. Scale bar, 50 µm. **B**, Mean parameter values from 3–4 blood samples over time, univariate scaled to 0–10 per parameter. Heatmap of the scaled parameters, demonstrating effects of PRT. Rainbow colour code indicates scaled values from 0 (blue) to 10 (red). **C**, Subtraction heatmap representing effects of PRT, filtered for relevant differences (n=3–4, $P<0.05$, 2-way ANOVA). Color code showing relevant decrease (green) or increase (red) in comparison to control runs. **D**, **i**, Time-dependent increase in platelet adhesion (P1) in the presence or absence of PRT (**D**, **i**). Mean effects of PRT on platelet adhesion (P1) and platelet activation (P6) after 10 min (**D**, **ii**); mean effects of PRT on platelet PS exposure (P7) after 10 min (**D**, **iii**). SAC indicates surface-area-coverage. Data are means±SD (n=3–4), $*P<0.01$, $**P<0.001$ (2-way ANOVA).

bilayer aggregate formation and platelet activation (P-selectin expression) in comparison to the fibrin surfaces (Figure 2A and 2B).

Given the presence of 6% residual FXIIIa in the fibrinogen preparation used for the coating, we checked if transglutaminase contributed to platelet-activating effects by generating spots of fibrin (S1) and fibrin-FXIIIa (S3) in the presence of the FXIIIa inhibitor T101. However, after 10 minutes of whole-blood flow, both end stage images (Figure IIA in the Data Supplement) and scaled time-dependent parameters P1,5-7 did not show significant effects of this inhibitor (Figure IIB and IIC in the Data Supplement). Together, these results pointed to a no more than modest role of FXIIIa-induced cross-linking in fibrin-dependent microthrombus formation.

Major Role of Syk Kinase in Microthrombus Formation on Fibrin Independently of Coagulant Strength

To assess tyrosine kinase-dependent signaling, blood samples were preincubated with the selective Syk kinase inhibitor PRT-060318, known to completely abolish the thrombus formation on collagen-like surfaces.[30] This inhibitor lowered platelet adhesion and abolished the bilayer aggregate formation and P-selectin expression on all prepared fibrin and fibrinogen spots (Figure 2A and 2B). Subtraction heatmaps of the scaled treatment effects over time showed for essentially all parameters a relevant reduction with PRT-060318, but not with DMSO vehicle (Figure 2C). For instance, on fibrin-FXIIIa spots, PRT-060318 gradually suppressed platelet adhesion parameters P1 to P2 (Figure 2D, i and 2D, ii) and even tended to suppress the low phosphatidylserine exposure (Figure 2D, iii).

Subsequent flow experiments were performed with fibrin-FXIIIa (S3) spots, which were used as a standard. We first examined how the formation of microthrombi relied on the extent of coagulation, that is, thrombin generation. Therefore, blood samples were incubated either with the thrombin inactivator PPACK, or with 5 or 10 pM tissue factor to trigger the extrinsic coagulation pathway. During 10 minutes of flow at 1000 s^{-1}, microscopic images again were captured and analyzed for $DiOC_6$ platelet adhesion (P1), multilayer score (P5), P-selectin expression (P6), and phosphatidylserine exposure (P7). Markedly, the presence of either PPACK and tissue factor did not significantly change platelet adhesion, size of microthrombi or the P-selectin expression at fibrin-FXIIIa spots, when compared with control runs (Figure IIIA in the Data Supplement). In addition, Syk inhibition with PRT-060318 had a similar lowering effect on all parameters, regardless of the presence of PPACK or tissue factor (Figure IIIB in the Data Supplement).

A marked finding was that, for all variable coagulation conditions, the extent of phosphatidylserine exposure remained low for fibrin-FXIIIa spots (0.1–0.4 SAC%). On the contrary, parallel flow experiments on collagen-I spots caused formation of large-sized platelet thrombi, with high staining for $DiOC_6$ and high phosphatidylserine exposure, with or without tissue factor (Figure IV in the Data Supplement). Hence, the low level of phosphatidylserine exposure observed on fibrin-FXIIIa spots was not due to limitations of the flow set-up, in agreement with earlier findings.[22]

To further assess the apparently limited role of thrombin in the microthrombus formation on fibrin spots, we compared the effects of PPACK with the thrombin receptor antagonist atopaxar and the thrombin inhibitor refludan. When added to the blood, neither end stage images nor (subtraction) heatmaps of scaled parameters P1,5-7 indicated any effect of these interventions (Figure VA and VB in the Data Supplement). In addition, we measured the ability of thrombi formed on fibrin-FXIIIa and collagen-I spots to support (phosphatidylserine-dependent) thrombin generation, using an earlier described procedure based on the thrombin-induced cleavage of substrate Z-GGR-ACM (Z-Gly-Gly-Arg aminomethyl coumarine).[33] The observed no more than minimal thrombin generation on fibrin-FXIIIa spots supported the conclusion that the procoagulant activity of the fibrin surface is low in comparison to the collagen-I surface (Figure VC and VD in the Data Supplement). Together, these results indicated that the low thrombogenic effect of fibrin surfaces relies on Syk kinase signaling, which is relatively independent of coagulation triggering.

Shear-Dependent Contribution of Integrin αIIbβ3 in Microthrombus Formation on Fibrin

Considering that also integrin αIIbβ3 interaction with fibrin(ogen) can trigger Syk activation,[6,34] we went on to determine the role of this integrin in the microthrombus formation. Therefore, blood samples from 2 patients with Glanzmann thrombasthenia were obtained and flowed over fibrin-FXIIIa spots (S3) at arterial (1000 s^{-1}) or venous (100 s^{-1}) shear rate. Markedly, with blood from the homozygous patient, completely lacking platelet surface expression of αIIbβ3, the platelets failed to adhere to fibrin, regardless of the shear rate (Figure 3A and 3B). Control experiments indicated that VWF was present on the S3 (fibrin-FXIIIa) surfaces (see below). With blood from the heterozygous patient, presenting with reduced platelet αIIbβ3 expression, the platelets again did not adhere at the high shear rate. However, there was substantial platelet adhesion (P1) at the low shear rate. In the latter case, the normal P-selectin expression (P6) pointed to residual platelet activation. Taken together, these results pointed to a crucial, shear-dependent role of the αIIbβ3 integrin in the flow-dependent platelet interaction with fibrin.

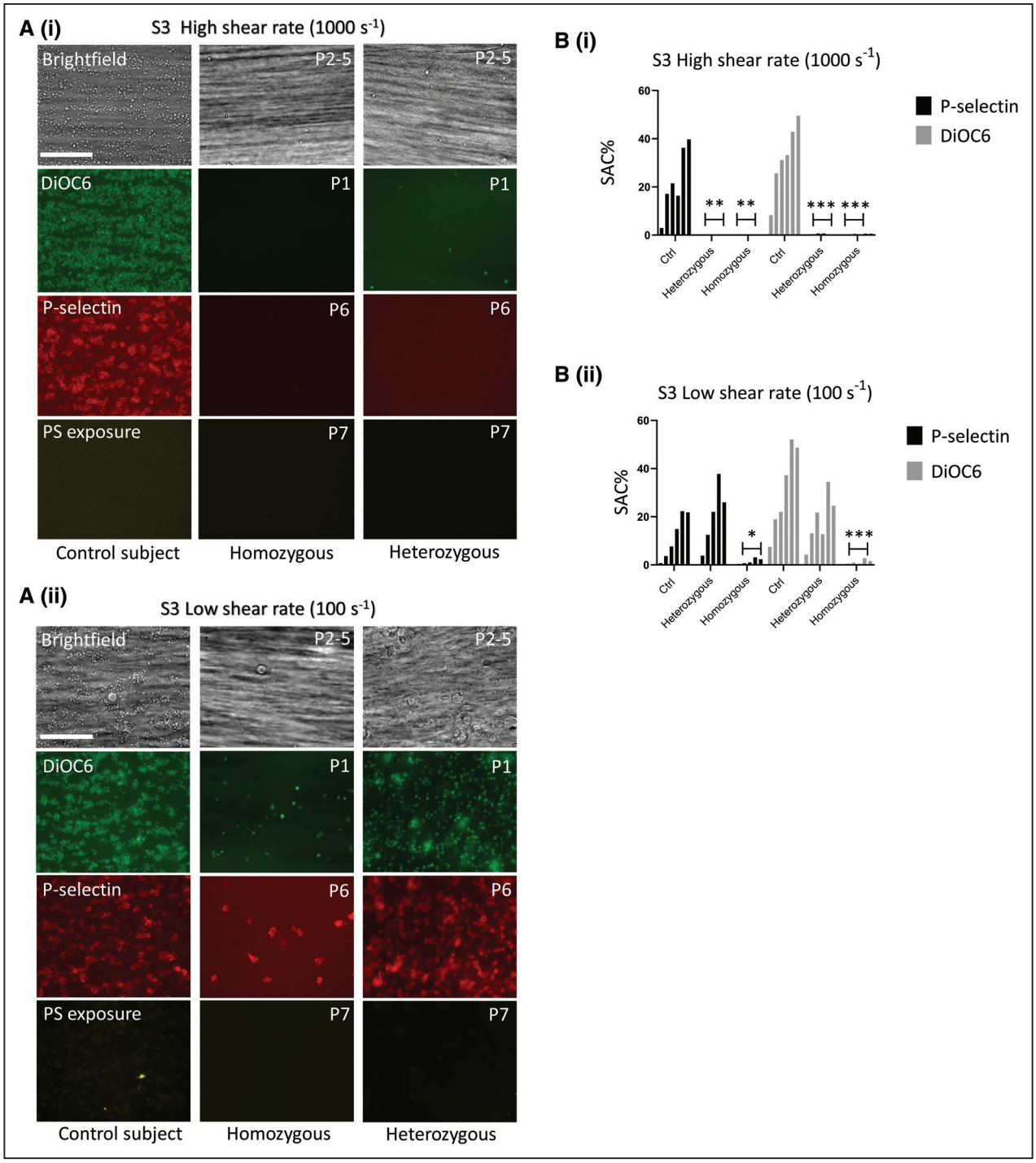

Figure 3. Abolished microthrombus formation on fibrin in Glanzmann thrombasthenia.
A, Representative brightfield and fluorescence images from microthrombi after flow of blood from control subjects or 2 Glanzmann patients over spots of fibrin-FXIIIa (factor XIIIa; 10 min). Flow perfusion was at arterial shear rate of 1000 s^{-1} (**A**, **i**) or at venous shear rate of 100 s^{-1} (**A**, **ii**). Scale bars 50 µm. **B**, Time-dependent increases in platelet activation (P-selectin expression, P6) and platelet adhesion ($DiOC_6$, P1) for controls (Ctrl, n=4), heterozygous and homozygous patients at time points t=0, 2, 4, 6, 8, and 10 min. Graphs at shear rates of 1000 s^{-1} (**B**, **i**) and 100 s^{-1} (**B**, **ii**). SAC indicates surface-area-coverage. Means±SD (n=4 for controls), $^{**}P<0.001$, $^{***}P<0.0001$ (2-way ANOVA).

Complementary Roles of GPVI, αIIbβ3, and GPIb-V–IX in Microthrombus Formation on Fibrin

Subsequent flow experiments were performed with spots of fibrin-FXIIIa (S3) in combination with downstream collagen-III (S7), which was considered as a reference platelet-activating surface. The comparative analysis of fibrin-FXIII and collagen-III surfaces showed that the microthrombi on fibrin were less contracted and activated than those on the collagen-III (Figure 4A and 4B).

Considering that both GPVI and integrin αIIbβ3 can activate Syk kinase, in this setting we established the role of GPVI in the thrombus-forming process at both surfaces. Whole-blood samples were treated with anti-GPVI Fab 9O12 and flowed over the spots for 10 minutes at high shear rate of 1000 s^{-1}. Microscopic images, captured from these surfaces over time, were analyzed for the same parameters as before (P1,5-7). The results showed that, for fibrin-FXIIIa (S3), GPVI inhibition caused significant decreases of platelet adhesion, bilayered aggregation, and P-selectin expression (Figure 4A and 4B). The effects of Fab 9O12 on platelet adhesion and activation were similar for fibrin-FXIIIa and collagen-III spots. However, these were lower in comparison to Syk inhibition (compare Figures 2C and 4C). This notion agrees with a complementary activation pathway of Syk kinase, involving integrin αIIbβ3.

Fibrin has previously been shown to bind to VWF.[19] For the fibrin-FXIIIa spots exposed to flowing blood, we could confirm the binding of plasma-derived VWF by staining with an fluorescein isothiocyanate-labeled anti-VWF antibody (Figure 5A). The presence of VWF was yet lower than on the reference spot, collagen-III. To determine contribution of the VWF-GPIb-V–IX axis to the microthrombus, we used an established blocking anti-GPIbα antibody, RAG35.[35] For both spots S3 (fibrin-FXIIIa) and S7 (collagen-III), the addition of RAG[35] antibody resulted in a marked reduction in platelet adhesion, bilayer formation, and P-selectin expression, already observable from the first minutes of flow (Figure 5B and 5C). Subtraction heatmaps, however, pointed to larger effect for collagen-III than for fibrin-FXIIIa spots (Figure 5D).

Role of Syk and GPVI in Fibrin-Induced Platelet Aggregation

To further confirm the moderate signaling via Syk kinase and GPVI in fibrin-induced platelet activation, we examined the aggregation response of platelets upon stimulation with a sonicated, homogeneous fibrin suspension. This suspension was treated with PPACK to remove thrombin traces. Similar to the results of microthrombus formation, pretreatment of platelets with Syk inhibitor PRT-060318 abrogated the fibrin-induced aggregation (Figure 6A and 6B). Similarly, the GPVI blocking Fab 9O12 suppressed fibrin-induced aggregation, but a residual shape change and aggregation remained. In comparison, both inhibitors also antagonized the platelet aggregation induced by collagen-I, but not by thrombin (Figure 6). As expected, treatment with the integrin antagonist tirofiban blocked the fibrin-induced aggregation response by >80% (n=3, data not shown).

Fibrin-Induced Platelet Ca^{2+} Signaling Under Flow

In collagen-induced platelet activation, GPVI adhesion under flow is known to induce a prolonged and high Ca^{2+} signal, leading to massive P-selectin expression and phosphatidylserine exposure.[21,36] To investigate the Ca^{2+} signal of platelets flowed over fibrin-FXIIIa, blood samples were supplemented with autologous Fluo-4-loaded platelets, and fluorescent $[Ca^{2+}]_i$ rises were measured in real time by confocal microscopy. The results show a consistent, moderate increase in Fluo-4 fluorescence in fibrin-adhered platelets, which was suppressed by Fab 9O12 (Figure 7A). A near complete major reduction in fluorescence increase was observed upon Syk inhibition with PRT-060318. The effects on Fluo-4 fluorescence increases paralleled effects on platelet adhesion (Figure 7B). However, detailed analysis of traces from single adhered platelets confirmed suppression of transient, spiking Ca^{2+} signal generation in the presence of Fab 9O12 or PRT-060318 (Figure VIA through VIC in the Data Supplement). Of note, no platelet adhesion could be observed in the presence of integrin inhibitor, tirofiban. Taken together, these results confirm that fibrin interaction activates platelets via Syk kinase and GPVI.

Fibrin in Consolidating Microthrombus Formation

We explored why the fibrin-adhered platelet were insensitive to thrombin, for instance in flow with tissue factor. As a first approach, we adapted an earlier protocol, where single immobilized platelets in a flow chamber were triggered to generate star-like fibrin fibers.[37] These fibrin-forming platelets were postperfused for up to 10 minutes with blood samples, again containing labels for platelets ($DiOC_6$), and P-selectin and phosphatidylserine exposure. Recording of brightfield and tri-colour fluorescence images from the same microscopic fields after blood flow showed considerable overlap between the staining (Figure VIIA in the Data Supplement). Detailed analysis showed that the stainings concentrated around the immobilized platelets, rather than at the extending fibrin fibers (Figure VIIB in the Data Supplement). Thus, the $DiOC_6$-labeled microaggregates showed high overlap with the P-selectin exposing prior immobilized platelets, which was confirmed by measuring the overlap coefficients R (Figure VIIC in the Data Supplement). This overlap was further increased by blood flow in the presence of integrin αIIbβ3 antagonist tirofiban.

In an earlier study, we observed high binding of Oregon Green 488-labeled thrombin to fibrin-containing thrombi.[11] This could be confirmed using the present coagulant flow conditions for fibrin-FXIIIa and collagen-I spots (Figure VIIIA and VIIIB in the Data Supplement). As a control, blocking of the coagulation process with PPACK suppressed cleavage of the Oregon Green 488-labeled prothrombin probe, with as a consequence binding to phosphatidylserine-exposing platelets only

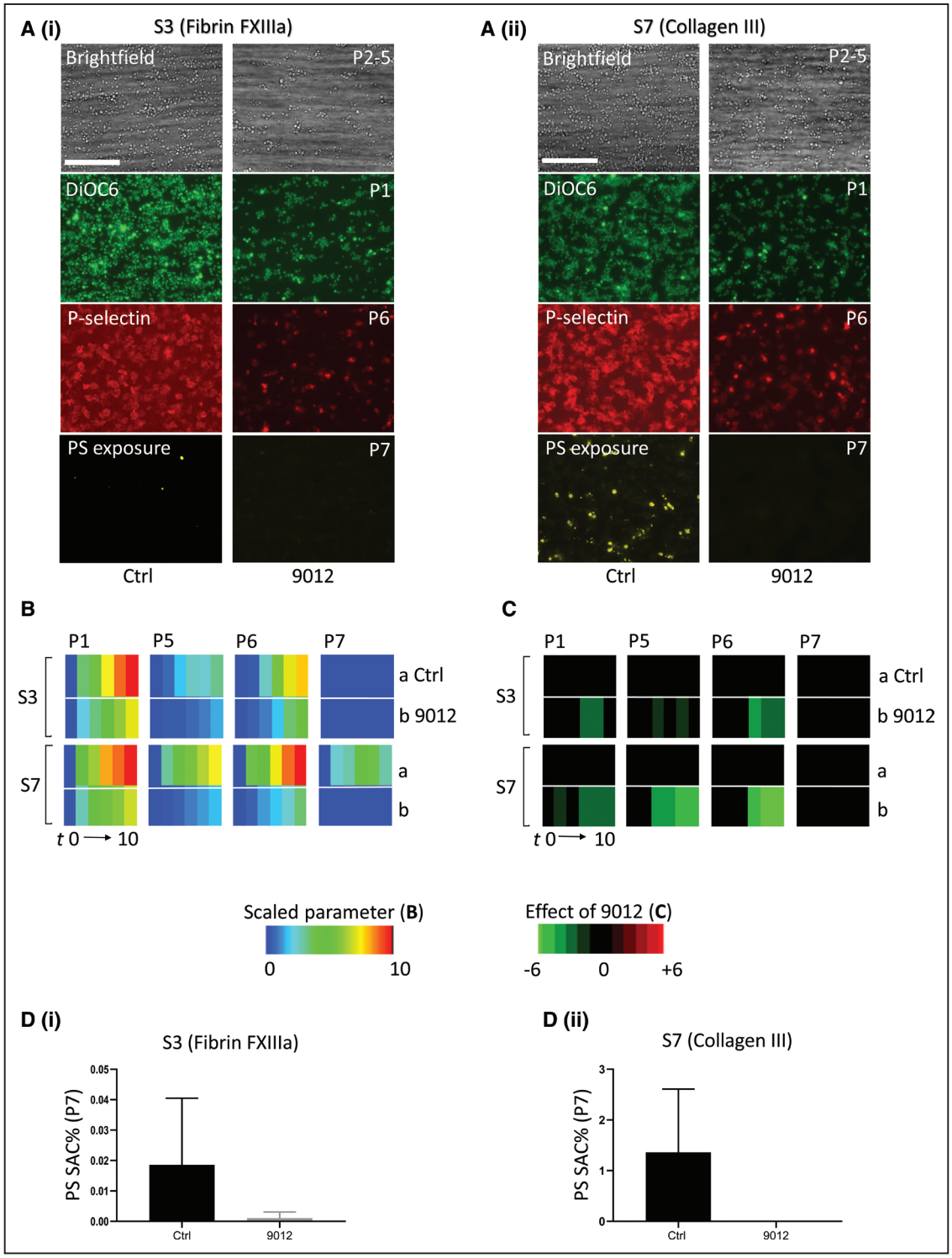

Figure 4. Effect of GPVI (glycoprotein VI) inhibition on parameters of microthrombus formation on immobilized fibrin-FXIIIa (factor XIIIa) or collagen-III.

Blood samples preincubated with vehicle (Ctrl) or GPVI blocking agent (9012 Fab, 50 µg/mL) were flowed over spots S3 (fibrin-FXIIIa, upstream) and S7 (collagen-III, downstream) for 10 min at 1000 s^{-1}. Microthrombi formed were imaged to obtain parameters P1 ($DiOC_6$ platelet adhesion), P5 (bi- or multilayer size), P6 (P-selectin expression), and P7 (PS exposure). **A**, Representative brightfield and fluorescence images from fibrin-FXIIIa (**A**, **i**) and collagen-III spots (**A**, **ii**) at 10 min. **B**, Heatmap of scaled parameters, demonstrating mean effects of GPVI inhibition on thrombus formation per spot. Effects of GPVI inhibition were assessed per blood sample, surface, and parameter. Mean values from individual blood samples were univariate scaled to 0–10 per parameter. Rainbow color code indicates scaled values between 0 (blue) and 10 (red). **C**, Subtraction heatmaps representing scaled effects of GPVI inhibition, filtered for relevant changes ($P<0.05$, 2-way ANOVA per surface and parameter). Colour code represents decrease (green) or increase (red) in comparison to control runs. Scale bar 50 µm. **D**, Graphs representing PS exposure (P7) for fibrin-FXIIIa (**D**, **i**) and collagen-III (**D**, **ii**) at 10 min. SAC indicates surface-area-coverage. Data are means±SD (n=7), *$P<0.05$ (2-way ANOVA).

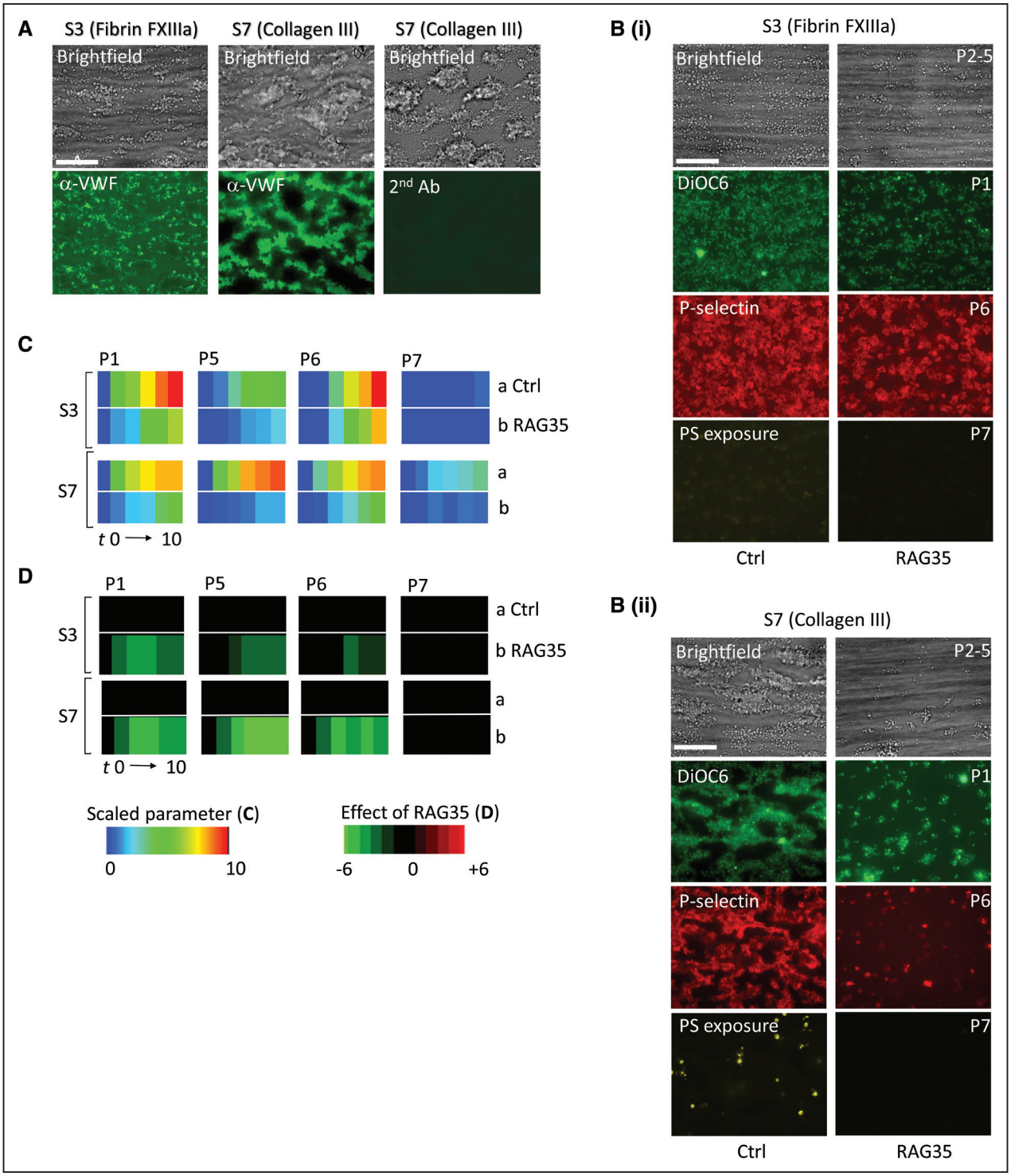

Figure 5. Roles of VWF (von Willebrand factor) and GPIb-V–IX (glycoprotein Ib-V–IX) in microthrombus formation on immobilized fibrin-FXIIIa or on collagen-III.

Blood samples preincubated with vehicle (Ctrl) or GPIb blocking antibody RAG35 (20 μg/mL) were flowed at 1000 s^{-1} over spots S3 (fibrin-FXIIIa, upstream) and S7 (collagen-III, downstream). After 10 min, microthrombi formed were imaged to obtain parameters P1 ($DiOC_6$ platelet adhesion), P5 (bi- or multilayer size), P6 (P-selectin expression), P7 (phosphatidylserine [PS] exposure). **A**, Staining of microthrombi formed on S3 and S7 spots after 10 min for VWF using fluorescein isothiocyanate-labeled anti-VWF antibody (green) or irrelevant control antibody. **B–E**, Effects of RAG35 antibody on thrombus parameters were assessed over time per blood sample for S3 and S7 surfaces. Scale bar 50 μm. Shown are representative end stage brightfield and fluorescence images for fibrin-FXIIIa (**B**, **i**) and collagen-III (**B**, **ii**). Furthermore, heatmap of univariate scaled parameters (0–10), indicating increased build-up of microthrombi over time in the absence or presence of RAG35 antibody (**C**). Rainbow colour code shows scaled values between 0 (blue) and 10 (red). In addition, subtraction heatmap representing scaled effects of GPIb blocking (**D**). Filtering was applied for relevant changes (n=6, $P<0.05$, 2-way ANOVA per surface and parameter). Colour code represents decrease (green) or increase (red) in comparison to control runs. Means±SD (n=6), $P<0.05$ (2-way ANOVA).

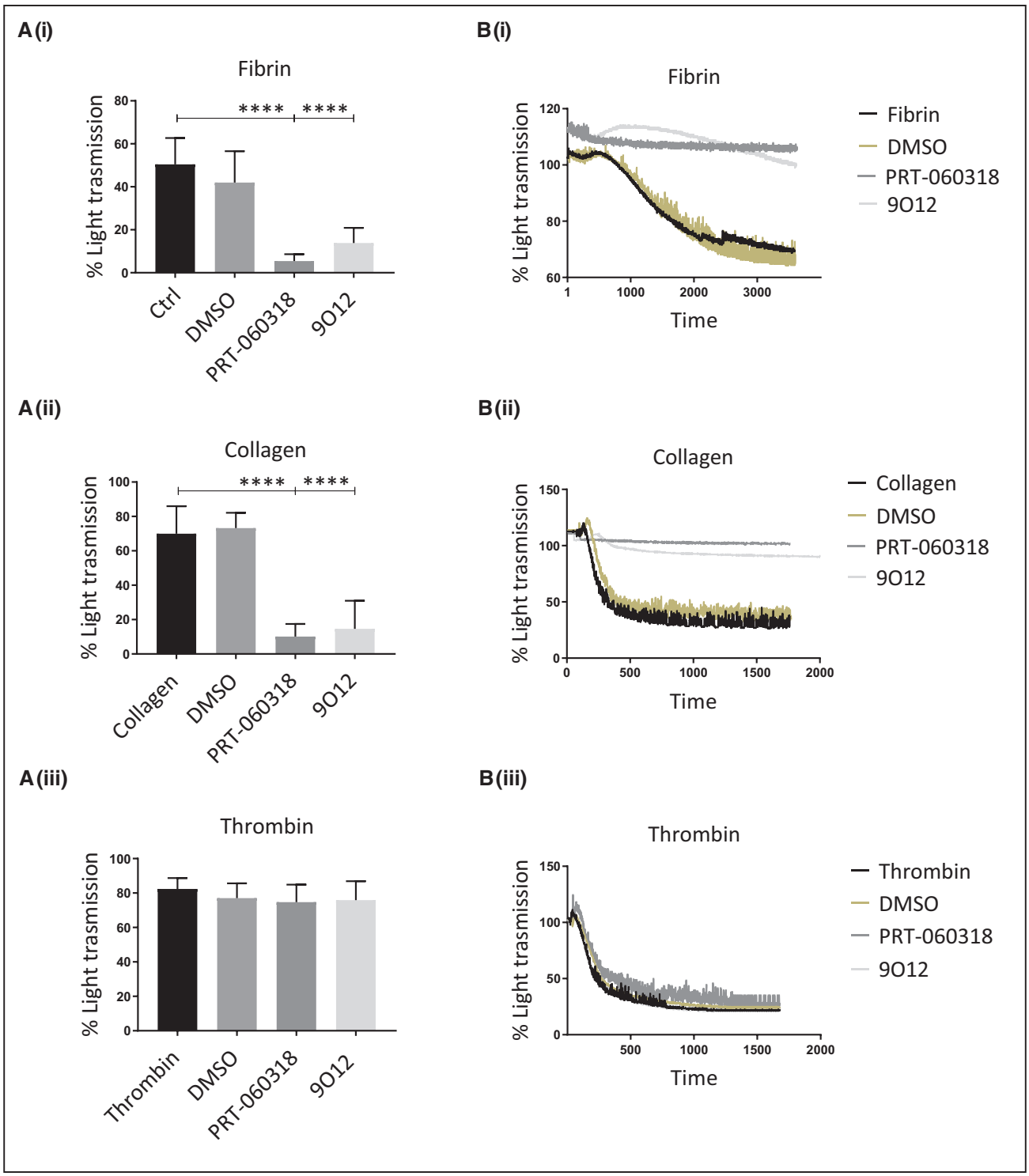

Figure 6. Comparative effects of GPVI (glycoprotein VI) or Syk inhibition on fibrin-mediated platelet aggregation. Platelets in suspension were treated with Syk inhibitor PRT-060318 (10 µg/mL) or GPVI-inhibitor 9O12 (50 µg/mL) for 10 min, before agonist addition. Platelet aggregation was monitored by conventional light transmission aggregometry. **A**, Quantitation of maximal aggregation upon stimulation with fibrin, collagen, or thrombin. **B**, Representative aggregation traces upon stimulation with indicated agonist. Mean±SD (n=5), ****$P<0.0001$ (1-way ANOVA).

(see Figure VIIIA, ii and VIIIB, ii in the Data Supplement and [11]). Taking together, these data suggest that fibrin fibers provide a relatively poor surface for newly adhering platelets, but can trap locally cleaved prothrombin.

DISCUSSION

While GPVI has been identified as a receptor for fibrin and also fibrinogen,[7–9] the relative strength of the

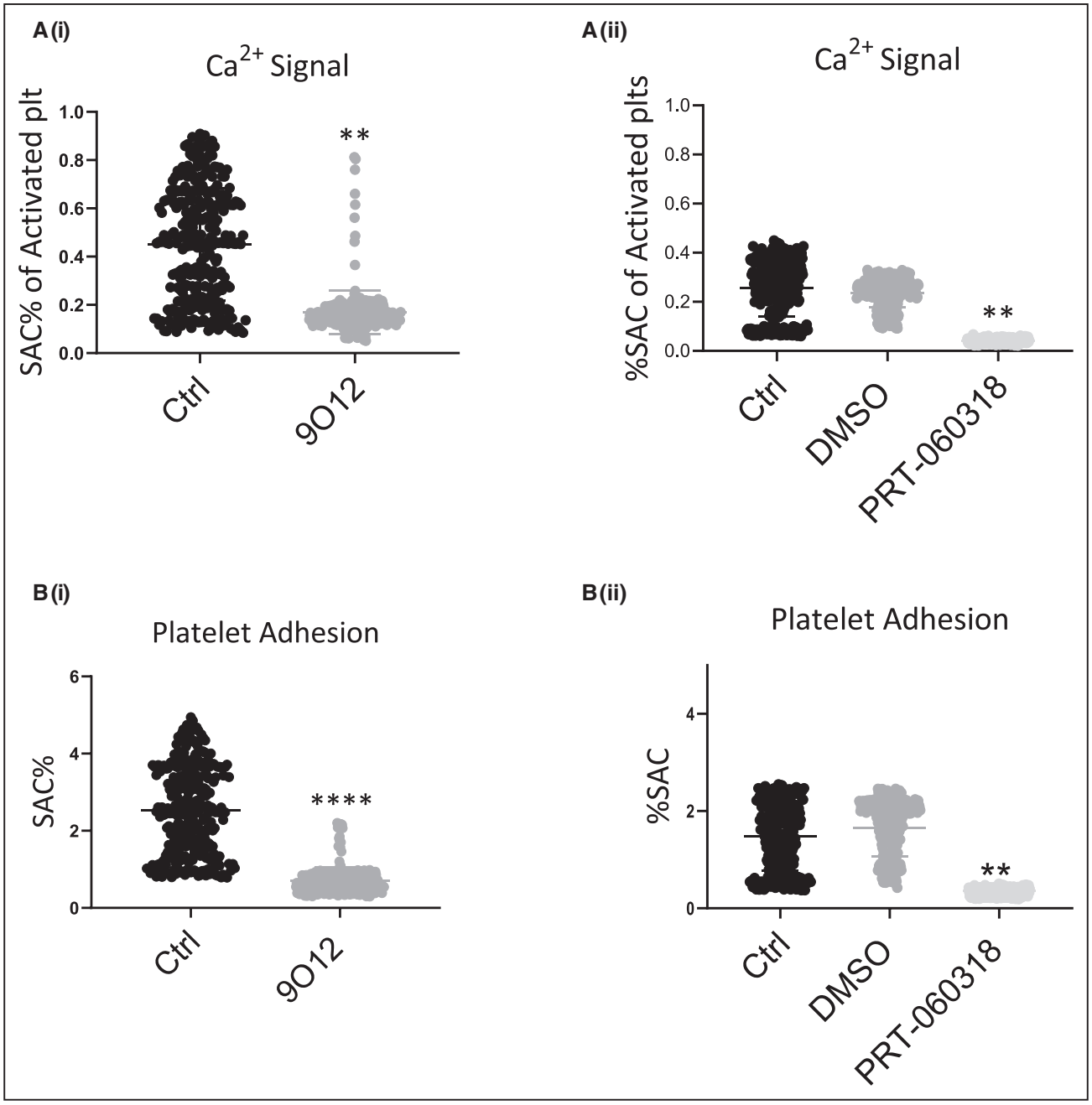

Figure 7. Effects of GPVI (glycoprotein VI) or Syk inhibition on Ca^{2+} signaling in fibrin-adhered platelets under flow. Blood samples containing autologous Fluo-4-loaded platelets were preincubated with vehicle (Ctrl), GPVI blocking agent 9O12 Fab (50 µg/mL) or Syk inhibitor PRT-060318 (10 µmol/L), and flowed over S3 spots (fibrin-FXIIIa), as for Figure 2. Fluorescence changes in cytosolic $[Ca^{2+}]_i$ of adhered platelets were recorded by confocal microscopy for 5 min. Time series of fluorescence images were analyzed for threshold increases in fluorescence intensity representing platelet activation (**A**, **i** and **ii**), and for fluorescence coverage as a measure of platelet adhesion (**B**, **i** and **ii**). Parts present data from parallel flow runs; dots represent values from analyzed images. SAC indicates surface-area-coverage. Bars are means (n=3 experiments), $^{**}P<0.001$, $^{****}P<0.0001$ (1-way ANOVA).

platelet-activating effect of fibrin via GPVI has not been examined in detail. It has been established that blood flow over immobilized collagens or collagen-related peptides via GPVI causes strong platelets activation responses, that is, a prolonged Ca^{2+} signal, high integrin activation, P-selectin expression, phosphatidylserine exposure, and massive thrombus formation.[21,38–40] The present data, using a variety of immobilized fibrin surfaces, point to a weaker GPVI-dependent platelet-activating effect of blood flow over fibrin, in that fewer platelets adhered showing transient Ca^{2+} signals, residual P-selectin expression and limited phosphatidylserine exposure, altogether resulting in the formation of only small-sized microthrombi.

The microthrombus formation under flow appeared to be hardly influenced by producing the fibrin with or without added VWF-BP or FXIIIa. However, this does not rule out a role of VWF or FXIIIa in the thrombus-forming process, because fibrin can capture VWF from the blood plasma,[19] and FXIIIa can also be produced in coagulating plasma and released from activated platelets.[13] Evidence for a relative weakness of fibrin (in comparison to collagen) as platelet-activating surfaces was further corroborated by experiments showing that fibrin fibers extending from immobilized platelets are relatively ineffective in trapping newly perfused platelets, when compared with the immobilized platelets themselves.

Comparative analysis of the microthrombi on fibrin-FXIIIa and collagen-III spots indicated that the platelets on fibrin had a lower activation state than those on collagen. Nevertheless, on both surfaces, inhibition of Syk (PRT-060318) or blockade of GPVI (Fab 9O12) suppressed the flow-dependent platelet adhesion, aggregate formation, and activation (P-selectin expression). In agreement with these findings, also light transmission aggregation studies using stirred platelet suspensions showed that the fibrin-induced aggregation process is abolished by both PRT-060318 and Fab 9O12.

Complete or partial defects in expression of integrin αIIbβ3 (Glanzmann patients) resulted in an annulled platelet adhesion to fibrin under flow, which in case of partial deficiency was limited to the high shear rate condition. In addition, we could establish a role of GPIb-V to IX by using the blocking anti-GPIbα antibody RAG35.[35] This antibody substantially but not completely decreased platelet adhesion to both fibrin-FXIIIa and collagen-III spots, while the remaining adhered platelets still displayed P-selectin expression. Together, these findings point to complementary and nonredundant roles of GPVI, αIIbβ3, and GPIb-V-IX complex in the microthrombus formation on fibrin surfaces. Since the tyrosine kinase Syk is known to be phosphorylated and activated downstream of both GPVI and αIIbβ3,[6,30,41] our results suggest that concomitant activation via both receptors is required for formation of the microthrombi. This idea is supported by a previous study showing that Syk phosphorylation is a continuous process in murine thrombus growth, and that secondary Syk inhibition can annul platelet adhesion even on preformed thrombi under flow.[42] A nonredundant contribution of GPVI and αIIbβ3 can also be derived from the observation that perfusion of blood from patients with GPVI deficiency over fibrin spots resulted in an abolished aggregate formation, although individual platelets still adhered (unpublished data, but see [43]).

Novel related observations were (1) the low phosphatidylserine exposure of platelets on fibrin (although still dependent on low-level GPVI), (2) the relative inability of thrombin to alter fibrin-dependent microthrombus formation, and (3) a low-level thrombin generation of platelets on fibrin in comparison to collagen. An explanation for these observations is the finding, supported by earlier studies,[11] that fibrin captures (fluorescent-labeled) thrombin, apparently without ability to cleave its substrate Z-GGR-ACM. This agrees with the earlier notion of irreversible thrombin binding to a fibrin network.[44] A suggestion then is that under the present microfluidic conditions fibrin-bound thrombin is unable to activate platelets. Clearly, more research needs to be done to better understand this phenomenon. Hence, our present findings lead to the concept that on fibrin a low platelet GPVI activation and an inactivation of thrombin induces only weak support of thrombus formation; or in other words, that platelet interaction with fibrin in particular consolidates the process of thrombus formation. However, we cannot rule out that under certain (patho)physiological static or flow conditions the role of fibrin is enlarged.[45]

The overall observation of fibrin-induced microthrombus formation suggests that fibrin fibers act as consolidating elements of the thrombus shield, such in contrast to vascular collagens which trigger the formation of larger size thrombi. Given that thrombus growth is regulated by secondary mediators, such as ADP and thromboxane A_2, which activate platelets in the thrombus core, it is not evident that the fibrin-GPVI interaction substitutes the high GPVI activation induced by collagens. A local inactivation of thrombin by fibrin may further contribute to this dampening process.

ARTICLE INFORMATION

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G. Perrella designed and performed experiments, analyzed and interpreted data, and wrote the article. J. Huang performed experiments and wrote the article. R.A.S. Ariëns provided the protocol for fibrin generation and edited the article. M. Jandrot-Perrus provided Fab 9O12 and edited the article. S.P. Watson edited the article. J.W.M. Heemskerk designed experiments, supervised research, interpreted data, and wrote the article.

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Disclosures

J.W.M. Heemskerk is founder and co-owner at FlowChamber b.v. The other authors report no conflicts.

Supplemental Material

Online Figures I–VIII
Online Table

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