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ORIGINAL ARTICLE

Respective roles of Glycoprotein VI and FcγRIIA in the regulation of αIIbβ3-mediated platelet activation to fibrinogen, thrombus buildup, and stability

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Abstract

Background: The interplay between platelets and fibrinogen is the cornerstone of thrombus formation. Integrin αIIbβ3 is the main platelet adhesion receptor for fibrinogen and mediates an outside-in signal upon ligand binding that reinforces platelet activation. In addition, FcγRIIA and glycoprotein VI (GPVI) contribute to platelet activation on fibrinogen, thereby participating in thrombus growth and stability. To date, the relative importance of these two immunoreceptor tyrosine-based activation motif-bearing receptors in these processes remains unknown.

Objective: The aim of this study was to evaluate the relative contributions of FcγRIIA and GPVI to platelet activation on fibrinogen and subsequent thrombus growth and stability.

Methods: We evaluated human and mouse platelet adhesion to fibrinogen in static assays and a flow-based approach to evaluate the contribution of FcγRIIA and GPVI to thrombus growth and stability.

Results: We first confirmed that integrin αIIbβ3 is the key receptor supporting platelet adhesion and spreading on fibrinogen. Using human platelets treated with pharmacological blocking agents and transgenic mouse platelets expressing human receptors, data indicate that GPVI, but not FcγRIIA, plays a prominent role in platelet activation on fibrinogen. Moreover, using a flow-based assay, we observed that blockade of GPVI with 1G5, but not FcγRIIA with IV.3, prevents thrombus growth. Finally, we observed that 1G5, but not IV.3, promotes the disaggregation of thrombi formed on collagen in vitro.

Muhammad Usman Ahmed and Nicolas Receveur contributed equally.

Conclusion: This study provides evidence that GPVI, but not FcγRIIA, induces platelet activation and spreading on fibrinogen, and promotes thrombus buildup and stability.

Essentials

- Glycoprotein VI, but not FcγRIIA:
  - Promotes platelet activation on fibrinogen.
  - Is key in thrombus buildup.
  - Ensures thrombus stability.

1 | INTRODUCTION

Blood platelets play a key role in the arrest of bleeding through their ability to accumulate at site of lesion to form a hemostatic plug. Upon vessel wall injury, the first step of primary hemostasis consists of platelet attachment to a variety of adhesive subendothelial proteins exposed to flowing blood including von Willebrand factor, collagen, laminins, and fibronectin.[1,2] This stage of stable adhesion facilitates the interaction of glycoprotein VI (GPVI) with ligands, notably collagen, initiating platelet activation.[3] Activated platelets then recruit circulating platelets through the formation of bonds between integrin αIIbβ3 and dimeric plasma fibrinogen. This αIIbβ3-fibrinogen interplay is critical, as it regulates thrombus buildup and maintains the stability of the hemostatic plug. Following fibrinogen binding to αIIbβ3, an "outside-in" signal is induced that on one hand upregulates the affinity of resting αIIbβ3 integrins on the same platelet, and on the other hand maintains the molecules already activated in an elevated affinity state for fibrinogen binding. This outside-in signaling cascade has been thoroughly dissected in static adhesion assays where platelets are allowed to adhere to immobilized fibrinogen.[4,5] However, it has since been reported that platelet activation upon adhesion to fibrinogen does not only rely on αIIbβ3 outside-in signaling but is reinforced by the signaling of immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors, notably FcγRIIA.[6,7] FcγRIIA is a low-affinity receptor for IgG found on human but not mouse platelets, which has been shown to regulate thrombus growth.[6-8] More recently we provided evidence that GPVI is also a major regulator of platelet activation on fibrinogen and provided evidence that this regulates both thrombus buildup and stability.[9,10] To date, the relative contributions of FcγRIIA and GPVI to platelet activation on fibrinogen remains unknown. In addition, the importance of these two ITAM-bearing receptors for thrombus development and stability, where fibrinogen is abundant but no other GPVI and FcγRIIA ligand is found, has also never been compared. The aim of this study was to assess the respective abilities of FcγRIIA and GPVI to support platelet activation on fibrinogen and compare their respective contributions to growth and stability of human thrombi using an in vitro flow-based approach. Identification of key receptors that play prominent roles in the regulation of platelet activation following integrin αIIbβ3 engagement with fibrinogen within a thrombus is particularly important from a pharmacological standpoint to therapeutically regulate thrombosis.

2 | MATERIAL AND METHODS

2.1 | Materials

Fatty acid–free human serum albumin and tetramethylrhodamine (TRITC) phalloidin were provided by Sigma-Aldrich (St. Louis, MO, USA). Fibrinogen was from Fresenius Kabi (Lake Zurich, IL, USA). Horm fibrillar type-I collagen from equine Achilles tendon, used to coat flow chambers (200 µg/ml) was purchased from Takeda (Tokyo, Japan). ReoPro was from Eli Lilly (Indianapolis, IN, USA). The anti-GPVI blocking antibody, 1G5, was developed by Elizabeth Gardiner (ANU, Australia). IV.3 was from Stemcell Technologies (Vancouver, BC, Canada). The mouse IgG2b use as a negative control (clone MPC-11) was from Merck-Millipore (Molsheim, France). RAM-1 is an anti-GPIbβ antibody developed at U1255 (François Lanza). Recombinant hirudin, used as an anticoagulant to directly block thrombin (100 U/mL), was from Transgene (Illkirch-Graffenstaden, France). 3,3'-dihexyloxacarbocyanine iodide ($DIOC_6$) was from Molecular Probes (Paisley, UK). Paraformaldehyde was from VWR (Strasbourg, France).

2.2 | Mice

Mice expressing human GPVI or human FcγRIIA were previously described.[11,12] Ethical approval for the animal experiments was obtained from the French Ministry of Research, in accordance with the guidelines of the Regional Committee for Ethics in Animal Experimentation of Strasbourg (CREMEAS, CEEA-35).

2.3 | Static adhesion experiments

Human and mouse platelets were isolated and static adhesion on immobilized fibrinogen was performed as previously reported.[10]

2.4 | In vitro perfusion experiments

Microfluidic flow chambers were prepared as previously described.[13] Briefly, the chambers were coated with a solution of fibrillary Horm collagen (200 µg/mL) overnight at 4°C and blocked with phosphate

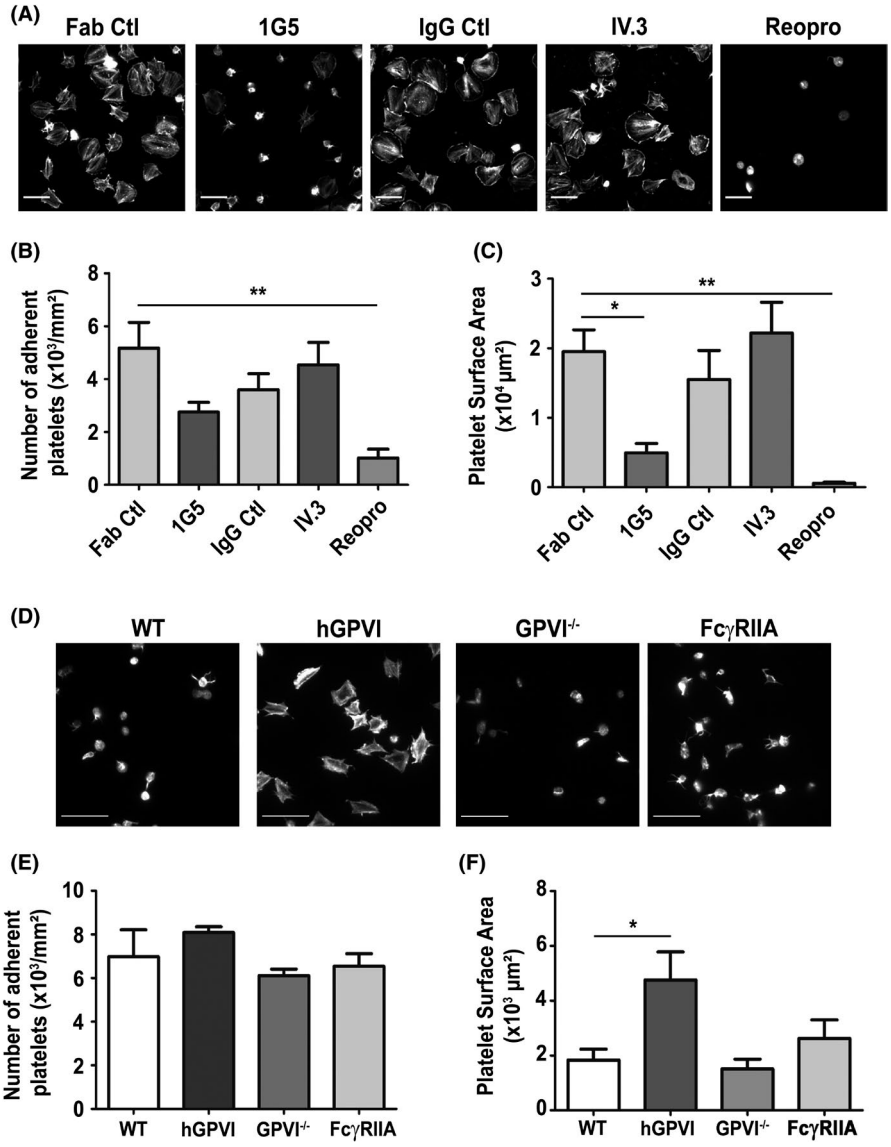

FIGURE 1 Glycoprotein VI (GPVI) but not FcγRIIA promotes platelet activation after adhesion to immobilized fibrinogen. A-C, Washed platelets from healthy human donors treated with antibody reagents against αIIbβ3 (ReoPro: 40 µg/ml) FcγRIIA (IV.3: 10 µg/mL) or GPVI (1G5: 10 µg/ml) were allowed to adhere to human fibrinogen for 40 minutes, and fixed with paraformaldehyde (PFA) and stained with phalloidin-tetramethylrhodamine (TRITC) (2 µg/mL). A, Representative epifluorescence images of washed platelets adhering to fibrinogen. Scale bars represent 10 µm. B, Bar graph representing the number of platelets adhering to immobilized fibrinogen per mm². Adhesion is expressed as mean±SEM in eight random fields, in six separate experiments (One-way analysis of variance [ANOVA], Bonferroni post hoc test: $^{*}P < .05$, $^{**}P < .001$; n = 6). C, Bar graph representing the surface area of platelets spreading over immobilized fibrinogen per 10^3 mm². Spreading is expressed as mean ± standard error of the mean (SEM) in eight random fields, in six separate experiments (One-way ANOVA, Bonferroni post hoc test: $^{**}P < .001$, $^{***}P < .001$; n = 6). D-F, Washed platelets from wild-type (WT) mice or mice expressing human FcγRIIA (hFcγRIIA mice) or GPVI-deficient mice (GPVI$^{-/-}$ mice) or mice expressing human GPVI (hGPVI mice) were allowed to adhere to human fibrinogen for 40 minutes, and fixed with PFA and stained with phalloidin-TRITC (2 µg/mL). D, Representative epifluorescence images of washed platelets adhering to fibrinogen. Scale bars represent 10 µm. E, Bar graph representing the surface area of platelets spreading over immobilized fibrinogen per 10^3 mm². Adhesion is expressed as mean ± SEM in eight random fields, in four separate experiments (one-way ANOVA, Bonferroni post hoc test: $P > .05$). F, Bar graph representing the number of platelets spreading on immobilized fibrinogen per mm². Spreading is expressed as the mean ± SEM in eight random fields, in four separate experiments. Significance was attained using a one-way ANOVA, Bonferroni post hoc test: $^{***}P < .001$

buffered saline (PBS) 10 mg/mL human serum albumin for 30 minutes at room temperature. Hirudinated (100 U/mL) whole blood from healthy human volunteers was perfused through the coated capillaries with a syringe pump (Harvard Apparatus, Holliston, MA, USA) at 37°C and various flow rates. Thrombus stability was studied in real time by differential interference contrast microscopy (Leica DMI4000B; Leica Microsystem, Mannheim, Germany) using a 40×, 1.25 numerical aperture oil objective and a Hamamatsu CMOS

ORCA FLASH-4 LT camera (Hamamatsu Photonics, Hamamatsu, Japan). For thrombus formation, whole blood was incubated with $DIOC_6$ (1 μM) to label platelets. Fluorescence emission was measured in the range of 490 to 595 nm after excitation with a 488-nm argon-ion laser using a confocal Leica SP8 inverted microscope with a resonant scanner and a 40× oil objective. Series of optical sections in xyz were taken from the base to the peak of the thrombi (Leica LAS X software). Images were then stacked and the volume of the thrombi was determined with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.5 | Statistical analysis

Statistical analyses were performed using Prism, version 5.0 (GraphPad Software, La Jolla, CA, USA).

3 | RESULTS AND DISCUSSION

To investigate the respective roles of integrin αIIbβ3, FcγRIIA, and GPVI in platelet adhesion to fibrinogen, human washed platelets were deposited on immobilized fibrinogen (100 μg/mL) in the presence of antibodies blocking αIIbβ3, FcγRIIA or GPVI (ReoPro, IV.3, or 1G5 respectively). Epifluorescence images showed that ReoPro (40 μg/mL) dramatically reduced platelet adhesion to fibrinogen (68%), while IV.3 had no effect and 1G5 presented only a nonsignificant tendency of reduced platelet adhesion (Figure 1A, B). This result confirmed that αIIbβ3 is the key platelet adhesion receptor for fibrinogen. We next evaluated the contribution of FcγRIIA and GPVI to platelet activation in this assay, by quantifying platelet spreading. We observed that 1G5, but not IV.3, markedly inhibited platelet spreading on fibrinogen (IgG control: 1.55 ± 0.42 × 10^4 μm²; IV.3: 2.22 ± 0.44 × 10^4 μm²; 1G5: 0.49 ± 0.13 × 10^4 μm²; Fab control: 1.95 ± 0.31 × 10^4 μm²; *P < .05; **P < .001) (Figure 1C). In parallel, and as expected, we confirmed that ReoPro had a dramatic impact on platelet spreading on fibrinogen (Fab control: 1.95 ± 0.31 × 10^4 μm²; ReoPro: 0.06 ± 0.02 × 10^4 μm²; **P < .001) (Figure 1C). We observed that the lack of effect of IV.3 was also obtained with a range of concentration varying from 5 μg/mL up to 20 μg/mL (Figure S1). These results were in agreement with experiments performed with washed mouse platelets. Indeed, while wild-type (WT) platelets did not spread on fibrinogen, we observed that those expressing human GPVI but not human FcγRIIA were able to spread (WT: 1.83 ± 0.39 × 10^3 μm², hGPVI: 4.75 ± 1.02 × 10^3 μm², $GPVI^{-/-}$: 1.51 ± 0.36 × 10^3 μm²; FcγRIIA: 2.62 ± 0.68 × 10^3 μm²; **P < .05) (Figure 1D-F). Together, these results confirm the key role played by αIIbβ3 in platelet adhesion to fibrinogen and indicate that human GPVI but not human FcγRIIA plays an important role in platelet activation on this surface resulting in spreading.

We next investigated the role of platelet FcγRIIA and GPVI in thrombus buildup by using an in vitro flow-based assay. We first preformed aggregates by perfusing hirudinated whole blood from healthy donors over immobilized type I fibrillar collagen for 90 seconds at an arterial wall shear rate of 750 s^{-1} and stained adherent platelets with $DIOC_6$ (green). We then perfused autologous blood treated with the anti-GPIbβ antibody RAM.1-A647 to label platelets (red) in the presence of IV.3 or 1G5 for 6 minutes at 750 s^{-1} to visualize real-time thrombus progression and compare the growth to a control IgG or Fab fragments. Three-dimensional reconstructed confocal microscopy images indicated that the second (red) population of platelets formed thrombi over the top of the first population of green aggregates with no difference between blood treated with either IV.3 or a control IgG (Figure 2A). In contrast, blood treated with 1G5 resulted in the formation of smaller thrombi (Figure 2A). This result was confirmed by measuring thrombus volume over time (Figure 2B) or at final time points (Figure 2C), which indicated that the aggregate volumes were similar in the presence of IV.3 and control, and markedly reduced when 1G5 was compared with a Fab control (IgG control: 1.1 ± 0.57 × 10^5 μm^3; IV.3: 1.1 ± 0.9 × 10^5 μm^3, 1G5: 0.6 ± 0.3 × 10^5 μm^3, Fab control: 1.6 ± 0.6 × 10^5 μm^3; P < .05), (Figure 2B, C). In addition, we confirmed under venous blood flow conditions (200 s^{-1}) that IV.3 was also not reducing thrombus growth (Figure 2D-F). Of note, both antibodies were used at concentrations where they efficiently inhibited platelet aggregation in response to an anti-CD9 antibody (activating via Fc engagement of FcγRIIA) for IV.3 or to collagen for 1G5 (Figure S2). These results indicate that human GPVI, but not FcγRIIA, plays an important role in thrombus growth under arterial blood flow conditions.

We next compared the role of FcγRIIA and GPVI in thrombus stability in an in vitro perfusion assay. Therefore, we preformed aggregates by flowing human hirudinated blood over collagen for 3 minutes at 750 s^{-1}, before perfusing PBS for 12 minutes at 750 s^{-1} over the thrombi in the presence of IV.3 or 1G5. We observed that IV.3 had no effect on human platelet aggregate stability when compared to a control IgG (Figure 3A). This observation was confirmed by a quantification showing that the number of disaggregating thrombi was indistinguishable between aggregates treated with a control IgG and those treated with IV.3 (IgG control: 2.3 ± 0.6 × 10^{-4} disaggregating thrombi/μm^2; IV.3: 2.3 ± 1.1 × 10^{-4} disaggregating thrombi/μm^2; ns, P > .5), (Figure 3B). Moreover, the time of initiation of disaggregation was also similar between both conditions (IgG control: 9.0 ± 0.6 min; IV.3: 9.5 ± 1 min; ns, P > .5), (Figure 3C). In sharp contrast, we observed under similar experimental conditions that perfusion of PBS-containing 1G5 resulted in a marked disaggregation when compared to IV.3 and control Fabs (Figure 3A). Quantification confirmed that the number of disaggregating thrombi was significantly elevated with 1G5, and the time taken to disaggregate was shortened when compared to IV.3 and control (Fab control: 2.3 ± 0.6 × 10^{-4} disaggregating thrombi/μm^2 and 10.1 ± 0.2 minutes, 1G5: 10.9 ± 0.7 × 10^{-4} disaggregating thrombi/μm^2 and 4.7 ± 0.1 minutes; IgG control: 2.3 ± 0.6 × 10^{-4} disaggregating thrombi/μm^2 and 9.0 ± 0.6 minutes; IV.3: 2.3 ± 1.1 × 10^{-4} disaggregating thrombi/μm^2 and 9.5±1 minutes; ***P < .001; **P < .01) (Figure 3B, C). These results indicate that blockade of GPVI, but not of FcγRIIA, promoted efficient and rapid disaggregation of human platelet thrombi.

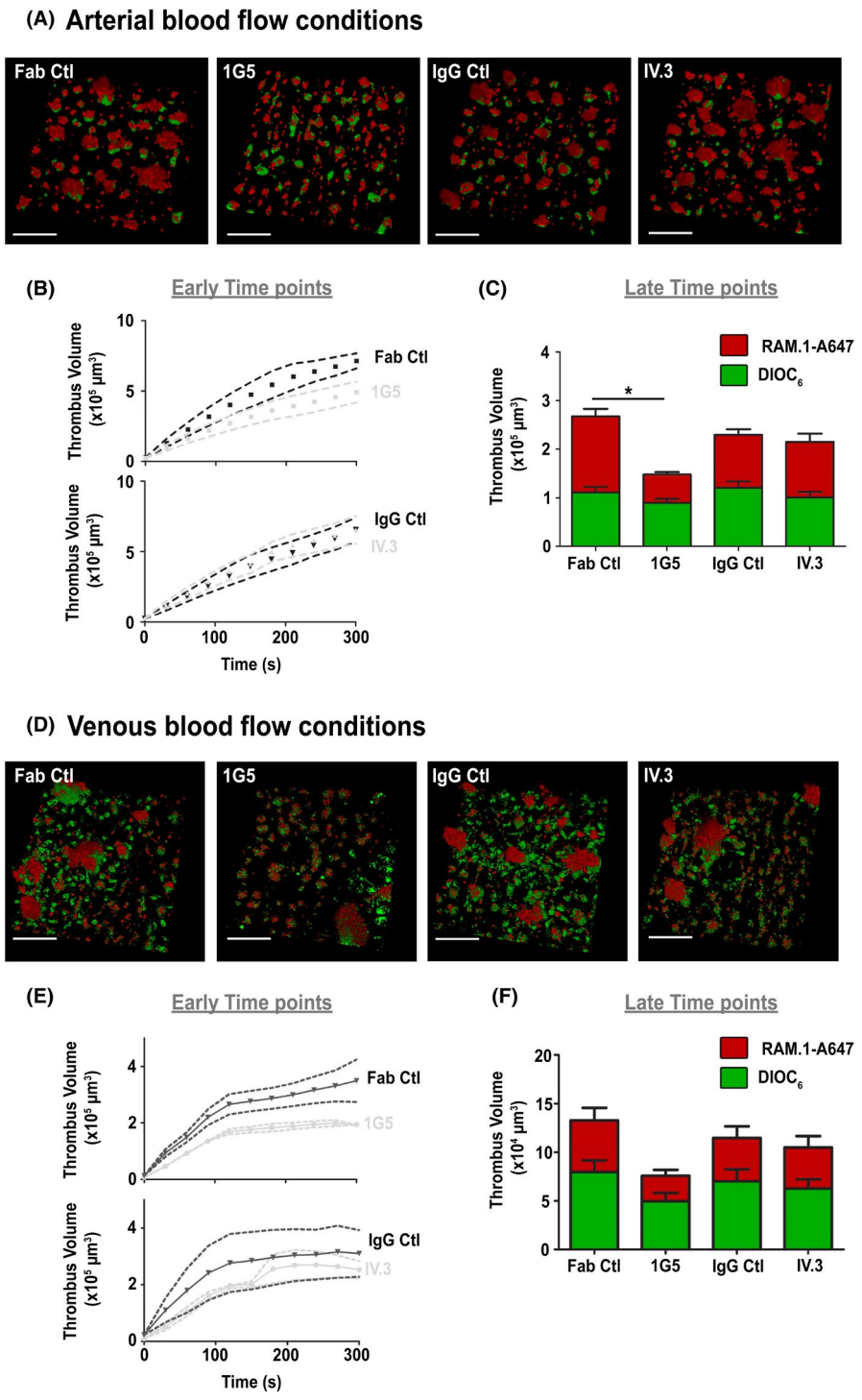

FIGURE 2 Glycoprotein VI (GPVI) but not FcγRIIA promotes thrombus growth in an in vitro flow-based assay. A, Representative three-dimensional reconstructed confocal images of human thrombi treated with anti-GPVI or anti-FcγRIIA agents obtained after perfusing hirudinated whole blood stained with 2 µg/mL anti-GPIbβ antibody RAM.1-A647 (in red) over human aggregates during 6 minutes at 750 s^{-1}. The preformed thrombi were obtained by perfusing human hirudinated whole blood stained with 1 µmol/L 3,3'-dihexyloxacarbocyanine iodide ($DIOC_6$; in green) over fibrillar collagen during 1 minute 30 seconds at 750 s^{-1}. Scale bar represents 50 µm. B, Kinetic of human thrombus growth in the presence of a Fab control, an anti-GPVI Fab (1G5), an IgG control or an anti-FcγRIIA (IV.3), (10 µg/mL) (n = 6). C, Quantification of human (n = 6) thrombus volumes after treatment with anti-GPVI or anti-FcγRIIA agent by confocal microscopy. Kruskal-Wallis test and post hoc Dunn multiple comparison test, *$P < .05$, n = 5. D, Representative three-dimensional reconstructed confocal images of human thrombi treated with anti-GPVI or anti-FcγRIIA agents obtained after perfusing hirudinated whole blood stained with 2 µg/mL anti-GPIbβ antibody RAM.1-A647 (in red) over human aggregates at 200 s^{-1}. The preformed thrombi were obtained by perfusing human hirudinated whole blood stained with 1 µmol/L $DIOC_6$ (in green) over fibrillar collagen during 1 minute 30 seconds at 750 s^{-1}. Scale bar represents 50 µm. E, Kinetic of human thrombus growth in the presence of a Fab control, an anti-GPVI Fab (1G5), an IgG control or an anti-FcγRIIA (IV.3), (n = 6). F, Quantification of human (n=6) thrombus volumes after treatment with 1G5 or IV.3 agent by confocal microscopy

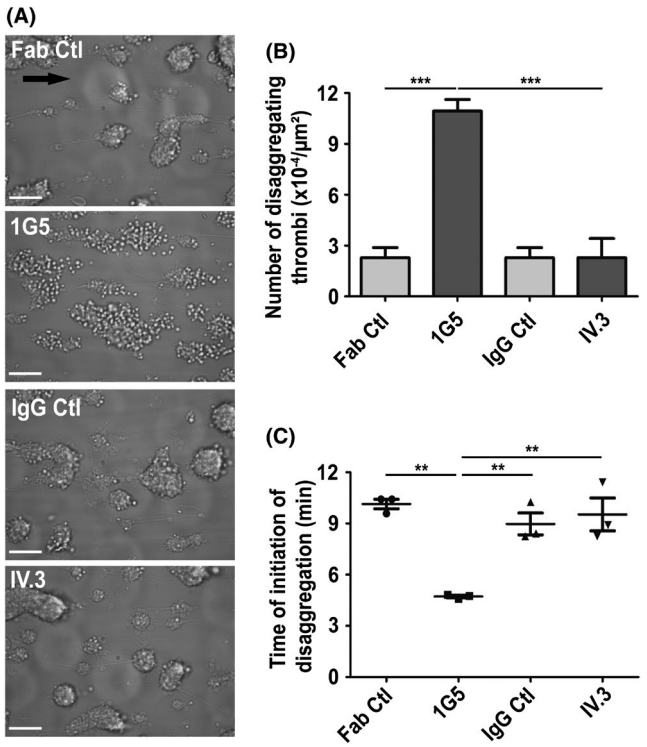

FIGURE 3 Glycoprotein VI (GPVI) but not FcγRIIA contributes to thrombus stability in an in vitro flow based assay. A–C, Hirudinated (100 U/mL) human whole blood was perfused for 3 minutes at 750 s^{-1} through microfluidic flow chambers coated with a solution of type I fibrillar collagen (200 µg/mL) to preform aggregates. A, Representative differential interference contrast microscopy images of thrombi subjected to anti-GPVI or anti-FcγRIIA agent or IgG control(s) diluted in phosphate buffered saline. The black arrow indicates the flow direction. Scale bars represent 20 µm. B, Bar graphs represent the number of disaggregating thrombi $\times 10^{-4}/\mu m^2$; Bonferroni multiple comparison test; ***$P < .001$, n = 3. E, Scatter plots represent the time at which disaggregation was initiated, expressed in minutes; Bonferroni multiple comparison test; **$P < .01$, n = 3

In summary, this study shows that GPVI, but not FcγRIIA, promotes human platelet activation and spreading onto immobilized fibrinogen. As a consequence, GPVI plays a prominent role in supporting both thrombus growth and stability, as we already reported, and acts beyond its role as a collagen receptor. In contrast, we could not confirm previous reports proposing an important role of FcγRIIA in promoting platelet activation on fibrinogen, and subsequent thrombus buildup and stability. The reason why we did not observe an inhibitory effect with IV.3 on platelet spreading as previously reported[6,7] is unclear, but cannot be linked to deployment of suboptimal inhibitor concentrations, as we used twofold greater concentrations of IV.3. In addition, we verified the inhibitory capacity of our IV.3 batch by blocking anti-CD9 antibody-induced aggregation, which is mediated via FcγRIIA. Among experimental differences used between both studies, one can find (i) direct efficient thrombin inhibitors (hirudin vs P-Pack); (ii) different collagen sources and concentrations (200 µg/mL vs 50 µg/mL) both known to promote thrombus growth; and (iii) distinct channel sizes (0.1 mm/1 mm vs 0.1 mm/0.4 mm), but the use of arterial shear rates (750 s^{-1} vs 2888 s^{-1} followed by 880 s^{-1}). Of note, the evidence supporting a role of FcγRIIA in platelet spreading on fibrinogen in the publication by Boylan and colleagues[6] was based on the use of platelets from a patient with low FcγRIIA levels. Interestingly, this patient was also deficient in GPVI, which may explain the observed reduced platelet spreading on fibrinogen, in agreement with our observations here. In addition, our results did not identify a contributory role for FcγRIIA in regulating platelet thrombus formation and stability, which was expected, as IgGs or immune complexes, the natural ligands of this receptor, are not known to be present in a physiological or pathophysiological context of thrombus formation after vessel injury. Whether pathological thromboinflammatory situations where FcγRIIA may contribute to thrombus formation exist, remain to be determined. In conclusion, this work indicates that GPVI, but not FcγRIIA, participates in platelet activation to fibrinogen within a growing thrombus to facilitate thrombus buildup and maintain stability.

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RELATIONSHIP DISCLOSURE

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

MUA, NR, EJB, and CM acquired and analyzed the data and participated in the writing of the manuscript. CG, MJP, and BH contributed to the writing of the manuscript. PHM and EEG conceived and designed the research, interpreted the data, wrote the manuscript, and handled funding and supervision.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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