APPENDIX

METHODS

Reagents and antibodies

Abciximab (ReoPro®) was obtained from Janssen Biologics B.V. (Leiden, The Netherlands). Acetyl salicylic acid (ASA, Multiplate® ASA Reagent) was obtained from Roche Diagnostics (Rotkreuz Switzerland). DiOC6, Alexa Fluor® 405 goat anti-rabbit IgG, Zenon® Alexa Fluor® 594 human IgG labeling kit, Zenon® Alexa Fluor® 405 and Zenon® Alexa Fluor® 594 rabbit IgG labeling kits and ProLong®Diamond Antifade Mountant were from Life Technologies (Eugene, Oregon, USA). Alexa Fluor® 488 conjugated goat anti-human-Fc Fab2 and PE-conjugated goat anti-human-Fc IgG came from Invitrogen/life technologies (Frederick, MD, USA). Anti-human IgG-Fc (gamma)-specific PE ab conjugate was obtained from eBioscience (San Diego, CA, USA). Anti- collagen type I and III antibodies (rabbit) purified by immunoaffinity chromatography was obtained from Rockland (Limerick, PA, USA). Human serum albumin and paraformaldehyde came from Sigma-Aldrich (Steinheim, Germany). Collagen (Horm) was obtained from Takeda (Linz, Austria). Anti- human CD41 ab FITC conjugate was purchased from Invitrogen (Frederick, MD, USA). EDTA came from Carl Roth GmbH (Karlsruhe, Germany). Fluorogel came from Electron Microscopy Sciences (Hatfield, PA, USA). Recombinant lepirudin (Refludan®) was obtained from Celgene (Windsor, UK). Revacept® (RP-15-260805, a dimeric GPVI-Fc fusion protein), and Fc protein of human IgG1, were from advanceCOR GmbH (Martinsried, Germany). Anti-mouse ab conjugated with DyLight® -488 was purchased from abcam (Cambridge, UK). Alexa Fluor®-488 conjugated goat anti-human-Fc IgG came from Jackson ImmunoResearch (West Grove, PA, USA), goat anti-human-Fc IgG and goat anti-human-Fc Fab2 came from Thermo Scientific (Rockford, IL, USA). (Rockford, IL, USA). High precision microscope cover glasses (24x60mm, No.1.5H) were obtained from Paul Marienfeld GmbH (Lauda-Königshofen, Germany).

Isolation of human carotid atherosclerotic plaque material

Patient consent was obtained as approved by the Ethics Committee of the Faculty of Medicine of the University of Munich and in accordance with the ethical principles for medical research involving human subjects as set out in the Declaration of Helsinki. Carotid atherosclerotic plaque tissue was donated by patients undergoing endarterectomy for high-grade carotid stenosis, processed, and preserved as described [1, 2]. Plaque homogenates from 5 patients were pooled and stored in aliquots at -80°C (100 mg wet weight/ml). Several plaque pools (n=4) were applied. Aliquots were used for platelet aggregation studies [3, 4] or coated onto glass cover slips for flow studies [2, 5].

Blood collection

Blood was obtained from healthy volunteers who denied taking any medication affecting platelet function for at least 2 weeks as approved by the Ethics Committee of the Faculty of Medicine of the University of Munich and in accordance with the ethical principles for medical research involving human subjects as set out in the Declaration of Helsinki. Blood was collected by venipuncture using a 20-gauge needle in a plastic syringe containing 1/10 volume recombinant lepirudin (dissolved in 0.9% NaCl, final concentration in blood ~200 U/ml; 13 μg/ml). The first 2 ml of blood were discarded. Aggregation measurements and flow experiments were performed between 20 min and 4 h after venipuncture.

Blood platelet aggregation under static conditions

Platelet aggregation in blood was determined by multiple electrode aggregometry (MEA) using the Multiplate® device as described previously[3] according to a recently modified protocol [4]. GPVI-Fc (final concentrations in the test cuvette from 6.6nM up to 333nM) was pre-incubated without or with anti-human-Fc goat IgG or anti-human-Fc goat Fab2 in equimolar concentrations for 10min at RT and added to blood samples equilibrated at 37°C for 3 min in the absence of stirring [4]. Antibodies alone, and human Fc after incubation with equimolar concentrations of anti-human-Fc IgG or anti-human-Fc Fab2 served as controls.

Subsequently plaque homogenate (333 µg/ml) or collagen (0.1-0.3µg/ml; as tested to induce the same aggregation values as plaque homogenate) was added, stirring was started and the increase in electrical impedance was recorded continuously for 10 min. The mean value of two independent determinations is expressed in arbitrary "aggregation units" over the time period (AU*min) (cumulative aggregation values).

Quantification of platelet adhesion and thrombus formation in flowing blood

For flow experiments, glass cover slips (Menzel, 24x60mm, # 1.5) coated with pooled plaque homogenates (1:20 dilution in PBS) or Horm® collagen (20 or 100µg/ml) were mounted into parallel plate flow chambers using sticky slides (0.1 Luer sticky-Slides, ibidi®, Martinsried, Germany) which had been blocked with human serum albumin (HSA, 4% in PBS). The flow chamber was then mounted on the stage of a fluorescence microscope (TE2000-E, Nikon) equipped with an incubation chamber (37°C). The flow chambers were perfused with PBS followed by 4% HSA in PBS to block the glass coverslips. Platelets were labeled by incubation of blood with DiOC6 (1µM, 10min at 37°C) before the flow experiment.

GPVI-Fc was incubated without or with equimolar concentrations of anti-human-Fc IgG or anti-human-Fc Fab2 for 10min at RT, and added to blood at a final concentration of 50µg GPVI-Fc/ml (333nM) before perfusion. Human Fc (333nM) incubated with equimolar concentrations of anti-human-Fc IgG or anti-human-Fc Fab2 for 10min at RT was used as control.

If only platelet adhesion without subsequent aggregation was studied, blood was pre-incubated with abciximab (20µg/ml) for 10min at 37°C to block the integrin αIIbβ3.

Blood was perfused through the flow chamber at a shear rate of 600/s via a withdrawal syringe pump (Harvard Apparatus, Holliston, Massachusetts, USA). Fluorescence microscopy (Lambda DG4, Sutter Instruments; excitation: 485/25nm, emission: 528/38 nm) was performed for real time measurement of platelet adhesion and aggregate formation using a

10x air objective (NA 0.4) and a CoolSNAP HQ2 CCD camera (Photometrics). Fluorescence images were continuously recorded (1frame/5sec) and analyzed by quantifying the binary fluorescent area fraction (1.0=total area) using the NIS-element 3.2 (Nikon) software package. The area visualized in this setting was 669µm x 896µm. Values are the mean ± SD (measured every 5 sec) of n experiments with blood from different donors.

In experiments comparing GPVI-Fc*Fab with GPVI-Fc*Fab2-XL, GPVI-Fc (0.6µM) was pre-incubated with Zenon labeling reagent (anti-human-Fc Fab-Alexa Fluor 594) (1.8µM) (GPVI-Fc*Fab), and GPVI-Fc (0.6µM) was pre-incubated with equimolar amounts of anti-human-Fc Fab2 antibodies for 5min at room temperature (GPVI-Fc*Fab2-XL) and added to plaque homogenate- or collagen- coated cover-slips for 5min at 37°C before perfusion with blood (shear 600/s). If samples were analysed by structured illumination microscopy (SIM), equimolar amounts of PE-labeled anti-human-Fc Fab2 was used for GPVI-Fc crosslinking.

Determination of the kinetics of cross-linked and not cross-linked GPVI-Fc binding to collagen fibers in flowing blood

GPVI-Fc was incubated with PE-labeled anti-human-Fc Fab2 antibodies in a 20:1 molar ratio for GPVI-Fc labeling (GPVI-Fc*Fab2), or with unlabeled Fab2 antibodies containing 10% PE-conjugated anti-human-Fc Fab2 in a 1:1 molar ratio for GPVI-Fc crosslinking (GPVI-Fc*Fab2-XL). The mixtures were added to blood (333nM GPVI-Fc f.c) containing abciximab before perfusion over collagen at a shear rate of 600/sec. Fluorescence images (excitation: 560/25nm, emission: 605/35 nm) were continuously recorded (1frame/sec) using a 10x air objective (NA 0.4). The binary fluorescent area fraction was quantified after appropriate thresholding using the NIS-element 3.2 (Nikon) software.

Advanced imaging of binding of cross-linked and not cross-linked GPVI-Fc and platelet adhesion to collagen

Three different microscopes were used for live imaging of binding of GPVI-Fc complexes and platelet adhesion to collagen under flow (a, Nikon microscope), for samples fixed after flow (b, SIM), and for imaging of binding of GPVI-complexes to collagen in vitro (b, SIM and c, STED):

a) A Nikon TE2000-E microscope was used for simultaneous differential interference contrast (DIC) and fluorescence microscopy high magnification in flow experiments. Collagen and platelets were visualised by DIC, and PE-labeled GPVI-Fc (GPVI-Fc: anti-human-Fc-PE ab ratio 200:1) was detected by fluorescence microscopy (excitation: 560nm, emission: 605nm) using a 100x oil objective (TE2000-E, Nikon) in real time (frame/5sec)

b) For higher optical resolution (≈ 100 nm), an ELYRA PS.1 (Carl Zeiss Microimaging) microscope was used for structured illumination microscopy (SIM) of fixed samples after addition of PE-labeled GPVI-Fc (cross-linked or not cross-linked) to blood as well as after incubation of collagen with AlexaFluor488-labeled GPVI-Fc (cross-linked or not cross-linked) without subsequent perfusion of blood. In some experiments, collagen-coated glass cover-slips were stained with anti-collagen type I and type III 1st abs (dilution 1:100, 15min at RT), and after washing (0.5% HSA in PBS) with AlexaFluor405-conjugated 2nd ab (dilution 1:100, 10min at RT) before they were mounted onto sticky slides and into parallel plate flow chambers as before (see above). GPVI-Fc was incubated with PE-conjugated anti-human-Fc IgG or Fab2 ab in molar GPVI-Fc: ab ratios of 200:1 or 20:1 (for GPVI-Fc labeling), or 1:1 (for GPVI-Fc crosslinking). In the experiments with molar GPVI-Fc: ab ratios of 20:1 and 1:1, the PE-conjugated antibodies were diluted with 90% of unlabeled antibodies. PE-labeled GPVI-Fc samples (not cross-linked or cross-linked) were then added to blood before perfusion. Blood contained abciximab to only observe platelet adhesion without aggregation.

At 3 to 5 min after blood flow, samples were fixed by perfusion with 4% PFA in PBS containing 5mM EDTA for 5 min. Platelets were then stained with an anti-CD41 ab (dilution 1:200 in washing buffer; 2min) and subsequently with DyLight® 488-conjugated 2nd Ab (dilution 1:100; 2min). After fixation and between the staining steps, the samples were perfused for 1 min with washing buffer (0.5% HSA in PBS) containing 6.5µg/ml Refludan. The flow chambers were filled with FluorGel or Prolong®Diamond Antifade Mountant, and samples were analysed by SIM [6]. Thin (150nm) z-sections (total z was 2-2.5µm) of images were collected in three or five rotations for each channel. Images were reconstructed using ZEN software (Carl Zeiss MicroImaging) based on the structured illumination algorithm developed by Heintzmann and Cremer [7]. For 3D rendering ImagePro Premier 3D v9.1 (Media Cybernetics Inc., Rockville, Maryland) was applied. Line intensity profiles were generated in ZEN software by drawing a curved line tracing along a collagen fiber at the central focal plane. Percentage of intensity was calculated based on the maximal intensity level measured in the quantified region.

In other experiments collagen was incubated directly with fluorescent -labeled GPVI-Fc (cross-linked or not cross-linked). Collagen- coated glass slides were incubated for 20 min at RT with GPVI-Fc (666nM) which had been either cross-linked (molar ratio of GPVI-Fc: ab 1:1) or not (molar ratio of GPVI-Fc: ab 10:1) by incubation with AlexaFluor488 conjugated anti-human-Fc IgG or anti-human-Fc Fab2 for 10min.

c) For highest optical resolution (<100 nm), stimulated emission depletion (STED) nanoscopic imaging was performed using a Leica SP8 STED 3X (Leica, Germany) to visualize the binding of cross-linked and not cross-linked GPVI-Fc to collagen. Collagen coated coverslips were stained simultaneously with anti-collagen type I and type III antibodies (0.5µg/10µl per sample = 333nM, directly labeled with Zenon® AlexaFluor594 Fab) and with GPVI-Fc (0.5µg/10µl= 333nM) which had been either cross-linked (molar ratio

of GPVI-Fc: ab 1:1) or not (molar ratio of GPVI-Fc: ab 10:1) by incubation with Alexa Fluor 488 conjugated anti-human-Fc IgG or anti-human-Fc Fab2. The slides were incubated for 20 min at RT, fixed with 4% PFA (after washing), and embedded with Prolong®Diamond Antifade Mountant. 3D STED imaging was performed using a 100x oil objective (NA 1.4). A tunable white light laser source was used to optimally excite the applied fluorophores. Depletion was performed at 592nm and 775nm for AlexaFluor488 and AlexaFluor594 respectively. Images were collected in a sequential scanning mode using hybrid diode detectors to maximize signal collection while reducing background noise and the cross-talk between the channels. Image reconstructions were performed using the LAS X software package (Leica, Germany) and deconvolution was applied with the Huygens Professional software package (Scientific Volume, the Netherlands).

Analytical ultracentrifugation (AUC) sedimentation velocity experiments

Analytical ultracentrifugation was carried out with a ProteomLab XL-A (Beckman, Krefeld, Germany) supplied with fluorescence optics (Aviv Biomedical Inc, USA). The samples (0. 5ml) were loaded into assembled cells with quartz windows and 12 mm path length charcoal-filled Epon double sector centerpieces, and were centrifuged at 28000 rpm in an eight-hole Beckman–Coulter AN50-Ti rotor. Fluorescence was excited at a wavelength of 488 nm and had an emission range of 505-565 nm. Sedimentation was monitored by taking one scan every 90 seconds. Data analysis was carried out with the program Sedfit using a non-model based continuous Svedberg distribution method (c(S)), with time (TI) and radial (RI) invariant noise on [8]. The partial specific volume for the ab was taken from the literature [9].

The following samples (0.5 ml) were prepared in 0.1 M PBS: in order to saturate GPVI-Fc with anti-Fc ab a GPVI-Fc: ab ratio of 10:1 was chosen: GPVI-Fc (2.5µM; 375 µg/ml) was incubated with anti-human-Fc Fab2-Alexa Fluor 488 (0.25µM) or anti-human-Fc IgG-Alexa Fluor 488 (0.25µM) (GPVI-Fc*Fab2, GPVI-Fc*IgG). For GPVI-Fc crosslinking, GPVI-Fc (0.4µM; 60 µg/ml) was mixed with anti-human-Fc Fab2-Alexa Fluor 488 (0.4µM) or anti-

human-Fc IgG-Alexa Fluor 488 (0.4µM) in a ratio of 1:1 (GPVI-Fc*Fab2-XL, GPVI-Fc*IgG-XL). HSA was added to reach a final concentration of 0.05%.

Determination of the *in vitro* closure time with the platelet function analyzer PFA 200®

The PFA 200® device (Siemens Healthcare, Erlangen, Germany) is a further development of the Thrombostat system which simulates primary haemostasis and has been established to represent bleeding time *in vitro* [10, 11]. The instrument aspirates citrate-anticoagulated blood (0.8ml) under constant vacuum from a reservoir through a capillary and a small hole in a membrane filter which is coated with collagen and ADP or collagen and epinephrine. The time required to obtain full occlusion of the aperture is reported as “in vitro closure time".

References

1. Brandl R, Richter T, Haug K, Wilhelm MG, Maurer PC, Nathrath W. Topographic analysis of proliferative activity in carotid endarterectomy specimens by immunocytochemical detection of the cell cycle-related antigen Ki-67. Circulation 1997;**96**(10):3360-8.

2. Reininger AJ, Bernlochner I, Penz SM, Ravanat C, Smethurst P, Farndale RW, Gachet C, Brandl R, Siess W. A 2-step mechanism of arterial thrombus formation induced by human atherosclerotic plaques. J Am Coll Cardiol 2010;**55**(11):1147-58.

3. Toth O, Calatzis A, Penz S, Losonczy H, Siess W. Multiple electrode aggregometry: a new device to measure platelet aggregation in whole blood. Thromb Haemost 2006;**96**(6):781-8.

4. Bampalis VG, Brantl SA, Siess W. Why and how to eliminate spontaneous platelet aggregation in blood measured by multiple electrode aggregometry. J Thromb Haemost 2012;**10**(8):1710-4.

5. Dwivedi S, Pandey D, Khandoga AL, Brandl R, Siess W. Rac1-mediated signaling plays a central role in secretion-dependent platelet aggregation in human blood stimulated by atherosclerotic plaque. J Transl Med 2010;**8**:128.

6. Elia N, Ott C, Lippincott-Schwartz J. Incisive imaging and computation for cellular mysteries: lessons from abscission. Cell 2013;**155**(6):1220-31.

7. Heintzmann R, Cremer C. Axial tomographic confocal fluorescence microscopy. Journal of microscopy 2002;**206**(Pt 1):7-23.

8. Schuck P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. Biophys Journal 2000;**78**(3):1606-19.

9. Gallagher JS, Voss EW, Jr. Molecular weight of a purified chicken antibody. Immunochemistry 1969;**6**(2):199-206.

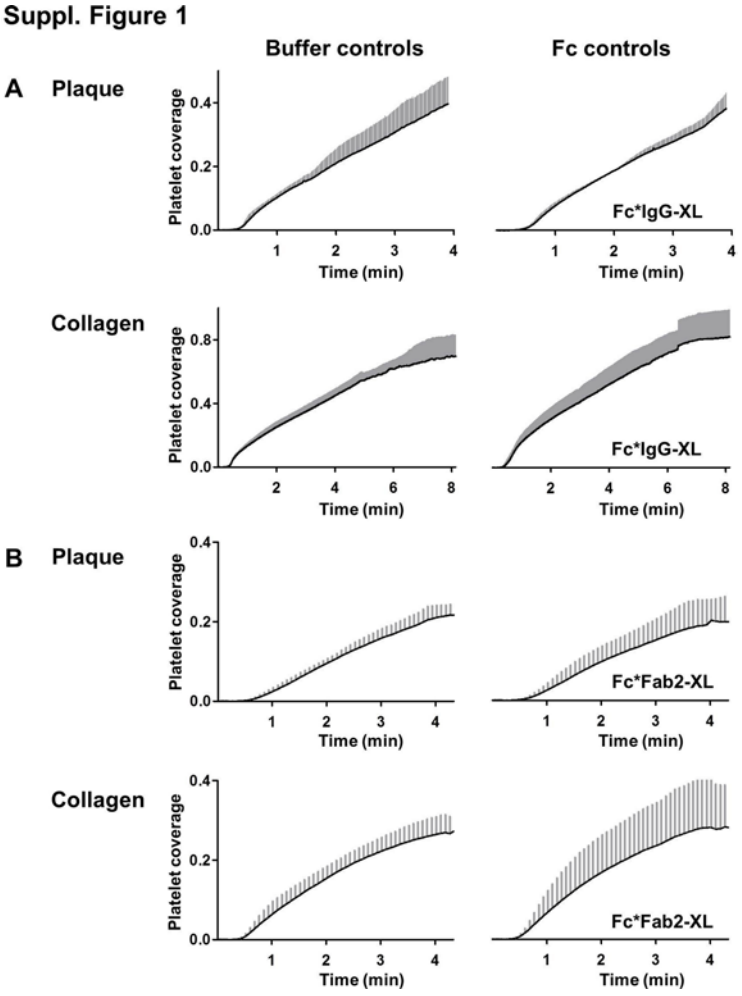
10. Kratzer MA, Negrescu EV, Hirai A, Yeo YK, Franke P, Siess W. The Thrombostat system. A useful method to test antiplatelet drugs and diets. Semin Thromb Hemost 1995;**21 Suppl 2**:25-31.

11. Kundu SK, Heilmann EJ, Sio R, Garcia C, Davidson RM, Ostgaard RA. Description of an in vitro platelet function analyzer--PFA-100. Semin Thromb Hemost 1995;**21 Suppl 2**:106-12.

Figure Legends

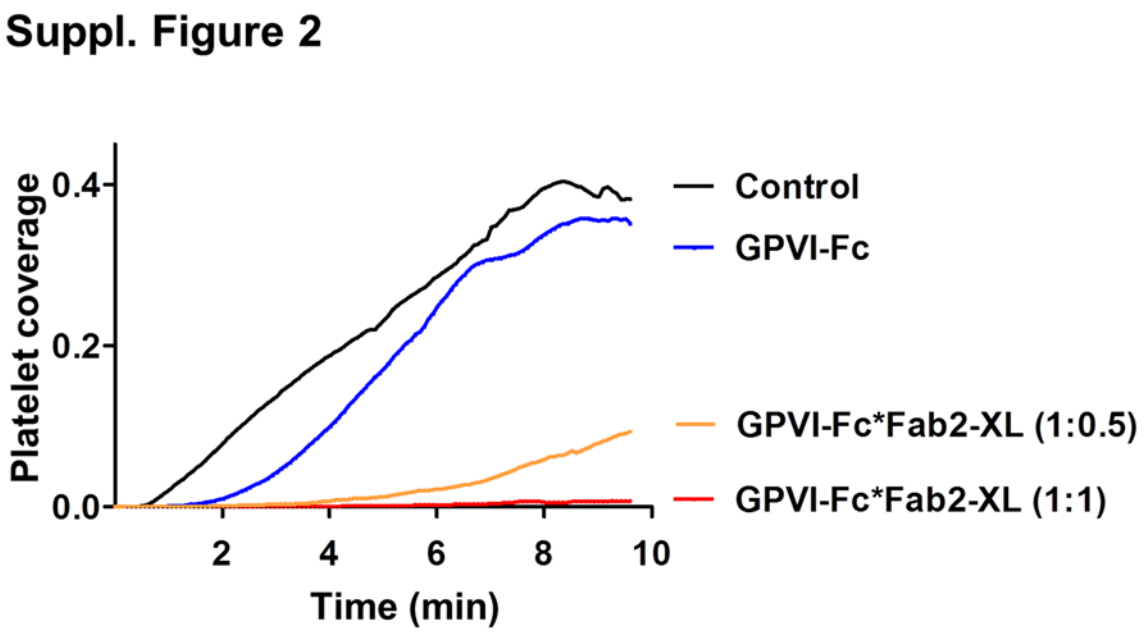
Suppl. Figure 1: Cross-linking of Fc control protein with anti-human-Fc IgG (A) and anti-human-Fc Fab2 (B) does not affect platelet aggregation induced by collagen or plaque under flow conditions as compared to untreated blood

Fc control protein (Fc; 333nM) was incubated for cross-linking with equimolar concentrations of anti-human-Fc IgG or Fab2 antibodies. Buffer (control), Fc + anti-human-Fc IgG (Fc*IgG-XL), or Fc + F(ab)2 (Fc*Fab2-XL) were added to blood containing DiOC6 for platelet visualisation before perfusion over plaque homogenate or collagen at a shear rate of 600/sec. Fluorescence images of platelet deposition were continuously recorded (1frame/5sec) and analyzed as detailed in "methods". Mean ± SD; n=3.



Suppl. Figure 2: Degree of inhibition of plaque-induced platelet aggregation under flow by cross-linked GPVI-Fc depends on the molar ratio of GPVI-Fc: cross-linking antibody.

GPVI-Fc was incubated or not (GPVI-Fc) with anti-human-Fc Fab2 antibodies either in equimolar concentrations (1:1 ratio) or in a molar ratio of 1:0.5 (GPVI-Fc: anti-human-Fc Fab2). Buffer (control), GPVI-Fc (50µg/ml), and GPVI-Fc*Fab2-XL (1:0.5, and 1:1 molar ratios) were added to blood containing DiOC6 for platelet visualisation before perfusion over plaque homogenate at a shear rate of 600/sec. Fluorescence images of platelet deposition were continuously recorded (1frame/sec) and analyzed as detailed in "methods". The experiment is representative of two others.



Suppl. Figure 3: Comparison of inhibition of plaque- induced platelet aggregation under flow of cross-linked GPVI-Fc and the anti-GPVI antibody 5C4.

GPVI-Fc was incubated not (GPVI-Fc) with anti-human-Fc Fab2 or IgG antibodies in equimolar concentrations (1:1 ratio; GPVI-Fc*Fab2-XL, GPVI-Fc*IgG-XL).

5C4 (2.5µg/ml), GPVI-Fc (50µg/ml), GPVI-Fc*Fab2-XL or GPVI-Fc*IgG-XL were added to blood containing DiOC6 for platelet visualisation before perfusion over plaque homogenate at a shear rate of 600/sec. Fluorescence images of platelet deposition were continuously recorded (1frame/5sec) and analyzed as detailed in “methods”.

Suppl. Figure 4: Size distribution pattern of not cross-linked and cross-linked GPVI-Fc complexes as analyzed by analytical ultracentrifugation

GPVI-Fc (2.5µM (10:1 ratio) or 400nM (1:1ratio) was mixed with anti-human-Fc IgG-Alexa Fluor 488 (top) or anti-human-Fc Fab2-Alexa Fluor 488 (bottom) either in a 10:1 molar ratio (dark and light blue: GPVI-Fc*IgG, GPVI-Fc*Fab2) or in a 1:1 molar ratio for GPVI-Fc crosslinking (dark and light green: GPVI-Fc*IgG-XL; red, brown: GPVI-Fc*Fab2-XL). Samples were centrifuged, and sedimentation was monitored by taking one scan every 90 seconds. Details are in "methods". **A** c(S) Sedimentation coefficients of the samples performed as duplicates. Asterisk, high molecular weight complexes. **B** Possible GPVI-Fc - antibody complexes.

Suppl. Figure 5: SIM imaging of stable platelet adhesion to collagen fibers after addition of GPVI-Fc, GPVI-Fc*IgG-XL or GPVI-Fc*Fab2-XL to blood.

Collagen coated onto glass coverslips was stained with anti-collagen type I and type III Ab and AlexaFluor405-conjugated 2nd Ab (blue). GPVI-Fc (50µg/ml), or equimolar concentrations of GPVI-Fc*IgG-XL or GPVI-Fc*Fab2-XL were added to blood before start of perfusion (shear rate 600/s). After 4min of flow, platelets were fixed and stained with anti-CD41 antibody and DyLight® 488-conjugated 2nd Ab (green). The SIM micrographs are maximum intensity projections of 0.15 µm z-sections (total z: 2.5 µm). The image is representative of five others from different experiments.