GPVI signaling is compromised in newly formed young platelets after acute thrombocytopenia in mice

Shuchi Gupta[1,2], Deya Cherpokova[1,3], Markus Spindler[1], Martina Morowski[1], Markus Bender[1] and Bernhard Nieswandt[1]

[1]Institute of Experimental Biomedicine I, University Hospital and Rudolf Virchow Center, University of Würzburg, Würzburg, Germany;

[2]Department of Medicine and Pharmacology, University of Pennsylvania, Philadelphia, PA, USA

[3]Program in Cellular and Molecular Medicine, Boston Children's Hospital, and Department of Pediatrics, Harvard Medical School, Boston, MA; USA

Supplemental Material

Anesthetic drugs: medetomidine (Pfizer, Karlsruhe, Germany), midazolam (Roche Pharma AG, Grenzach-Wyhlen, Germany), fentanyl (Janssen-Cilag GmbH, Neuss, Germany) and antagonists; atipamezol (Pfizer), flumazenil and naloxon (both from Delta Select GmbH, Dreieich, Germany) were used according to the regulation of the local authorities. Thrombin (Roche Diagnostics, Mannheim, Germany), ADP, high-molecular-weight heparin, thiazole orange and human fibrinogen (Sigma-Aldrich, Deisenhofen, Germany), U46619 (Alexis Biochemicals, San Diego, CA, USA), collagen (Kollagenreagens Horm; Nycomed, Munich, Germany), apyrase Type III (Sigma-Aldrich, Diesenhofen, Germany) were purchased. Monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or DyLight-488 and the antibody against the activated form of integrin αIIbβ3 (JON/A-PE), X488 were from Emfret Analytics (Würzburg, Germany). Collagen-related peptide (CRP) was from Collagen Tool kit (UK). Anti-PLCγ2 was from Santa Cruz Biotechnology anti-β-actin, anti-phospho-PLCγ2, anti-LAT, anti-phospho-LAT (Y191), anti-Syk and anti-phospho-Syk were all from Cell Signaling Technology.

Supplemental Methods

Platelet preparation and aggregometry

Mice were bled under isoflurane anaesthesia. Blood was collected in heparin (20 U/ mL, Ratiopharm) and centrifuged twice for 6 min at 300 *g* to obtain platelet-rich plasma (PRP). Platelets were pelleted from PRP by centrifugation for 5 min at 800 *g*, washed twice with Tyrodes–HEPES buffer (N-2-hydroxyethyl-piperazine-N′2-ethanesulphonic acid; 134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 5 mM HEPES, 5 mM glucose, 0.35% BSA, pH 7.4) containing 0.02 U/ mL apyrase and PGI_2 0.1 µg/ mL PGI_2. Briefly, to determine platelet aggregation, changes in light transmission were measured using washed platelets (200 µL with 0.5 x 10^6 platelets/µL) on a Fibrintimer 4 channel aggregometer (APACT Laborgeräte und Analysensysteme, Hamburg, Germany). Aggregation of washed platelets was induced by addition of thrombin, collagen-related peptide (CRP), collagen, convulxin or rhodocytin at indicated concentration.

Electron microscopy of platelets

For transmission electron microscopy, platelets were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and embedded in epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a CM120 transmission electron microscope (Zeiss, Oberkochen, Germany).

Transmission electron microscopy (TEM) of BM-MKs.

For TEM of MKs, the proximal and distal end of femora from vehicle treated control mice or mice 5 days post platelet depletion with anti-GPIbα antibody were cut and the bone marrow was fixed overnight at 4°C using Karnovsky fixative (2% PFA, 2.5% glutaraldehyde in 0.1 M cacodylate buffer). Subsequently, fatty components of the samples were fixed with 2% osmium tetroxide in 50 mM sodium cacodylate (pH 7.2), stained with 0.5% aqueous uranyl acetate, dehydrated with a graded ethanol series

and embedded in epon 812. Ultra-thin sections were stained with 2% uranyl acetate (in 100% ethanol) followed by lead citrate. Images were taken on a JEOL JEM-2100.

Flow cytometry to determine platelet activation

50 µL of heparinized whole blood was diluted 1:20 in Tyrode-HEPES buffer, incubated with appropriate fluorophore-conjugated monoclonal antibodies for 15 min at room temperature and analyzed on a FACSCalibur instrument (Becton Dickinson, Heidelberg, Germany) to determine glycoprotein expression. For platelet activation studies, washed blood was incubated with different agonists, stained with JON/A-PE and anti P-selectin-FITC antibody in the presence of 2 mM $CaCl_2$ for 15 min at 37 °C and analyzed using platelet FSC/SSC parameters. To study activation profile of aged platelets in circulation, platelets in circulation were labeled with X488 from Emfret Analytics and platelet integrin activation in response to indicated agonists were measured on day 5 post labeling on FACSCanto II (BD Biosciences). Activation status of platelets labeled with X488 was compared to the rest of the platelets in circulation.

Whole cell tyrosine phosphorylation

Washed platelets from vehicle- and antibody-treated mice were stimulated in suspension with 0.1 µg/mL CRP under stirring conditions at 37°C for indicated time points. The samples were run on 4-12% gradient polyacrylamide gels and the proteins were transferred. Membranes were blocked for 2 h in 5% BSA in PBS, followed by incubation with the appropriate primary antibodies overnight at 4°C. The membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at RT and developed using an enhanced chemiluminescence detection system.

Adhesion under flow conditions

24 x 60 mm coverslips were coated overnight with 0.2 mg/mL fibrillar type I collagen at 37°C and blocked on the following day for 1 h with 1% BSA in PBS. Blood

(700 µL) was collected into 300 µL heparin (20 U/mL in TBS, pH 7.3). Platelets were labeled with a DyLight-488 conjugated α-GPIX-Ig derivative (0.2 µg/mL) for 5 min at 37°C. Whole blood was diluted 2:1 in Tyrode's buffer containing Ca^{2+} and filled into a 1 mL syringe. Transparent flow chambers with a slit depth of 50 µm, equipped with the coated coverslips, were connected to the syringe filled with diluted whole blood. Perfusion was performed using a pulse-free pump under high shear stress equivalent to a wall shear rate of 1000 s^{-1} (for 4 min). Thereafter, coverslips were washed for 1 min by perfusion with Tyrode's buffer at the same shear stress and phase-contrast and fluorescent images were recorded from at least five different microscopic fields (40x objective). To avoid discrepancies in aggregate formation due to the reduced platelet counts, blood from antibody-treated mice was reconstituted with isolated blood cells from other antibody-treated mice. Image analysis was performed off-line using MetaVue® software. Thrombus formation was expressed as the mean percentage of total area covered by thrombi and as the mean integrated fluorescence intensity per mm^2.

Mechanical injury of abdominal aorta

Mice were anesthetized by intraperitoneal injection of a combination of midazolam/medetomidine/fentanyl (5/0.5/0.05 mg/kg body weight). The abdominal cavity of anesthetized mice was opened to expose the abdominal aorta. An ultrasonic flowprobe (0.5PSB699; Transonic Systems, USA) was placed around the abdominal aorta and thrombus formation was induced by a single firm compression with a forceps upstream of the flowprobe. Blood flow was monitored until complete occlusion of vessel, or experiments were stopped after an observation period of 30 min.

Proplatelet formation from BM explants.

BM was flushed out from anti-GPIbα antibody-treated and control mice 5 days after treatment in Tyrode-HEPES buffer supplemented with 5% mouse serum and cut in

0.2-0.5 mm pieces. After incubation for 24 h at 37°C and 5% CO_2, Alexa488 conjugated anti-GPIX antibodies were added (0.25 µg; p0p6; Emfret Analytics) to visualize MKs. Percentage of round MKs vs. MKs forming proplatelets was analyzed on a Nikon Eclipse TS100. Fixed samples were analyzed on a Leica TCS SP8 confocal microscope (Leica Microsystems).

Determination of MK ploidy.

To determine BM MK ploidy, both femora from control and antibody treatment mice were isolated, BM was flushed and homogenized. Unspecific binding sites were blocked by incubation of the cell suspension with 0.02 µg/ml anti-FcγR antibody (2.4G2, BD Pharmingen). Afterwards, MKs were stained using a fluorescein isothiocyanate-conjugated anti-integrin αIIbβ3 antibody (10 µg/ml, MWReg30, Emfret Analytics) or fluorescein isothiocyanate-conjugated anti-GPVI antibody (10 µg/ml, Emfret Analytics). Finally, the cells were fixed, permeabilized and the DNA was stained using propidium iodide (50 µg/ml; Invitrogen) staining solution with RNaseA (100 µg/ml; Fermentas) in PBS. Analysis was performed by flow cytometry and FlowJo software (Tree Star Inc., Ashland, USA).

Thiazole Orange staining.

5 µL of whole blood was incubated with a combination of thiazole orange (1 µg/mL final concentration) and anti-integrin αIIbβ3 ($F(ab')_2$ fragment, clone MWReg30) for 20 min at room temperature in dark. The blood was diluted to 500 µL with PBS and analyzed on flow cytometry. A virtual gate denoting platelet population was placed on the SSC/$CD41^+$ cells. The platelet population was further differentiated based on the thiazole orange positivity. Platelets positive for thiazole staining were termed reticulated platelets and their mean forward scatter characteristics were compared to non-reticulated platelets in IgG-treated versus NFYPs on day 5 post depletion.

Supplemental data

Tables

Table 1

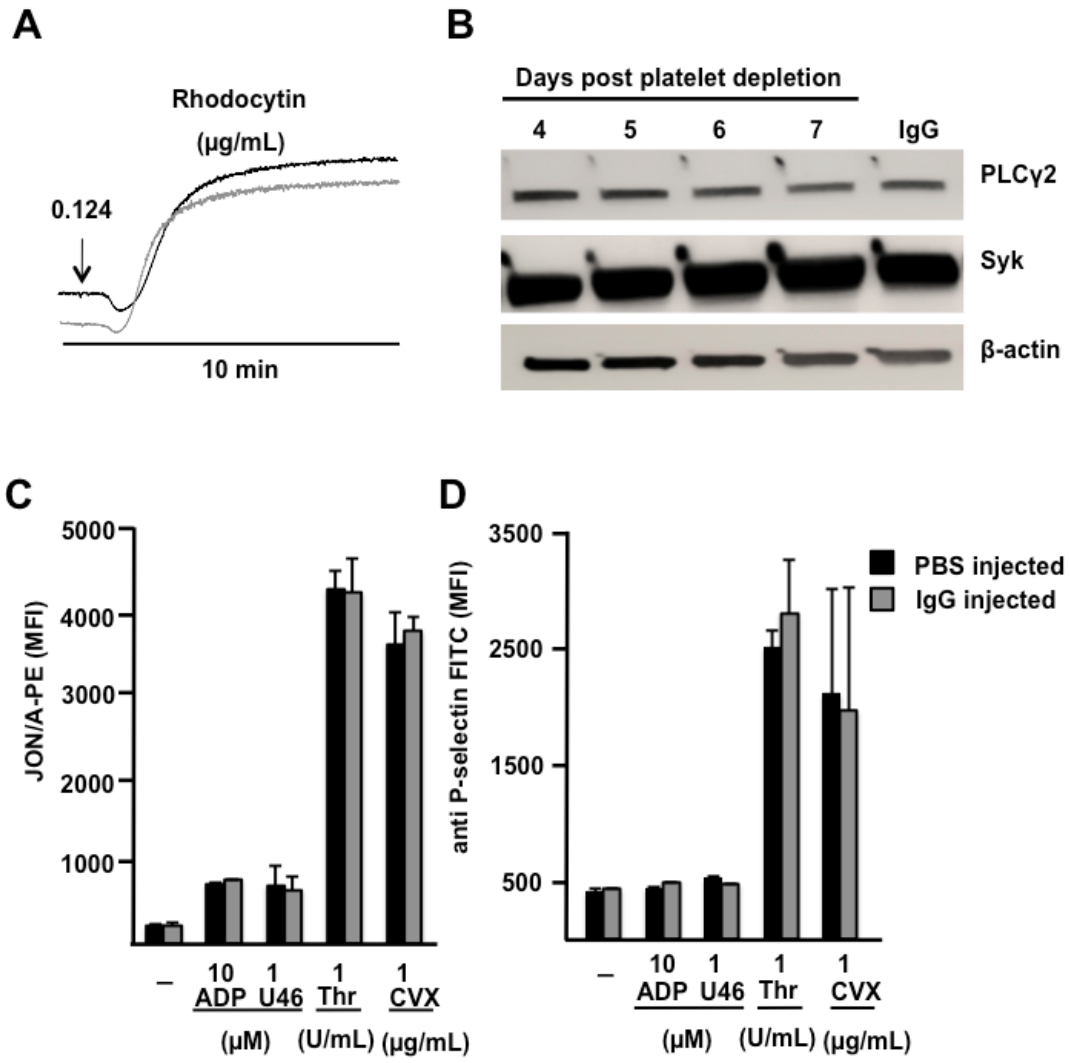
	Vehicle treated	*NFYPs*
GPIb	307 ± 9.0	303 ± 12.2
GPV	283 ± 13.8	297 ± 9.9
GPIX	437 ± 6.7	441 ± 10.0
GPVI	30 ± 2.18	33 ± 5.18
GPIIbIIIa	451 ± 18,8	595 ± 26.5***
CLEC2	124 ± 8.57	149 ± 8.7*

Table 2

	Vehicle treated	*MWReg30 antibody treatment*
GPIb	408 ± 73.0	362 ± 52.5
GPV	260 ± 51.0	306 ± 41.0
GPIX	339 ± 7.4	371 ± 53.0
GPVI	50 ± 0.5	48 ± 6.9
GPIIbIIIa	491 ± 21.0	558 ± 10.9 **
CLEC2	84 ± 8.3	92 ± 19.0

Surface glycoprotein expression of newly formed young platelets on day 5 was determined by flow cytometry. Diluted whole blood from the vehicle-treated and polyclonal rat anti-mouse GPIbα antibody (Table 1) or MWReg30 (Table 2) treated mice was incubated with FITC-labeled antibodies detecting extracellular domains of indicated surface glycoproteins at saturating concentrations for 15 min at RT and analyzed directly by flow cytometry. Results are expressed as mean fluorescence intensity ± SD (n=5) and are representative of 4 individual experiments.*P < 0.05, **P < 0.01, ***P < 0.001.

Suppl. Figure 1

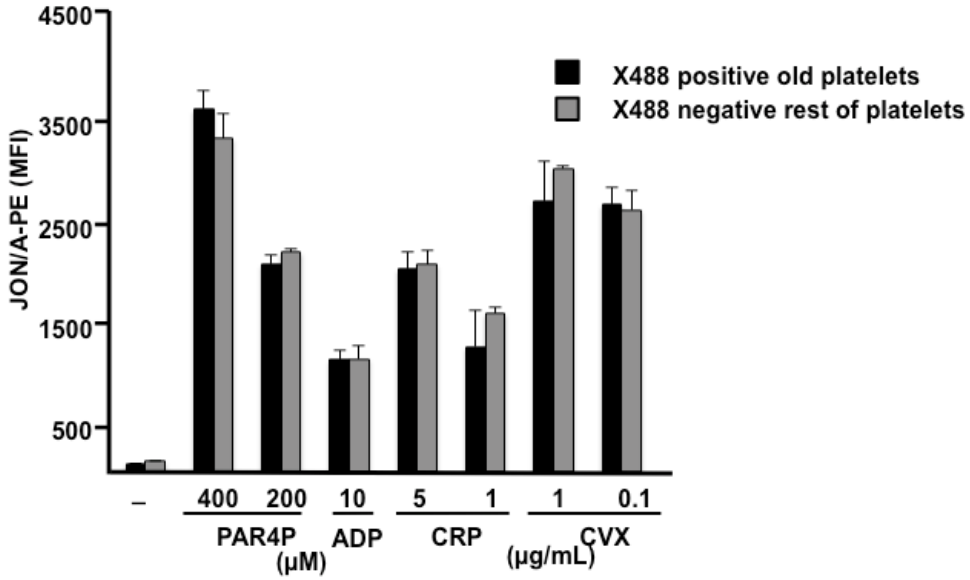


Supplemental Figure 1: (A) Washed control platelets (black line) or NFYPs (gray line) were stimulated with rhodocytin, and light transmission was recorded on a Fibrintimer 4-channel aggregometer. (B) Total protein expression of Syk and PLCγ2 was detected using Western blot analysis. Platelets were harvested from mice from day 4 until day 7 post injection of anti-GPIbα antibodies and were blotted. Syk and PLCγ2 total protein expression was detected using specific antibodies. Proteins were visualized by enhanced chemiluminesence (ECL). Total β-actin was used as loading controls. The blot images were further processed using Image J software (National

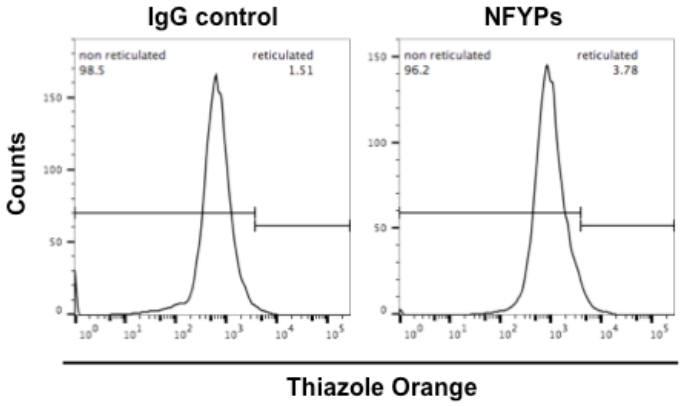
Institute of Health, USA). Mice were injected with PBS (black bar) or with 4 µg/g polyclonal non-immune rat immunoglobulin (gray bar) and (C) integrin activation by JON/A and (D) P- selectin exposure as a measure of α-granule release in response to indicated agonists on day 5 post injection.

Suppl. Figure 2

A



B

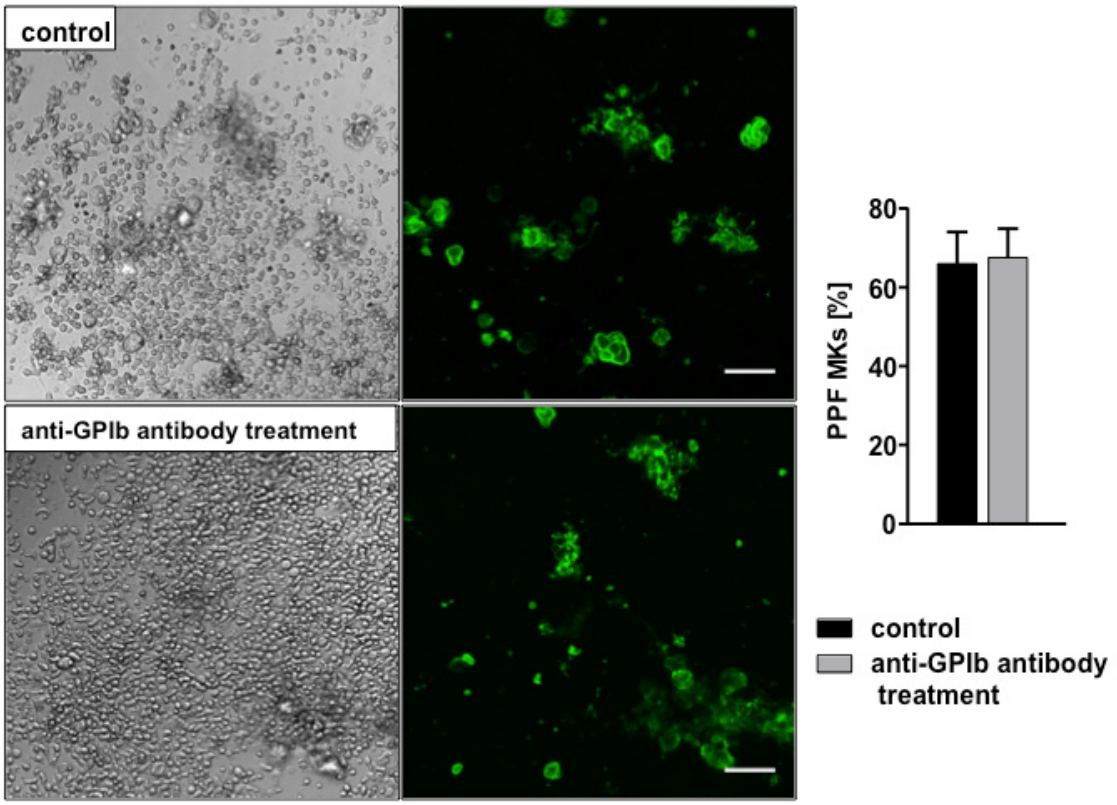


	IgG treated	*NFYPs*
Mean FSC (non-reticulated platelets)	**6850 ± 733.3**	**7957 ± 1330.7**
Mean FSC (reticulated platelets)	**15167 ± 1816.5**	**16672 ± 305.5**

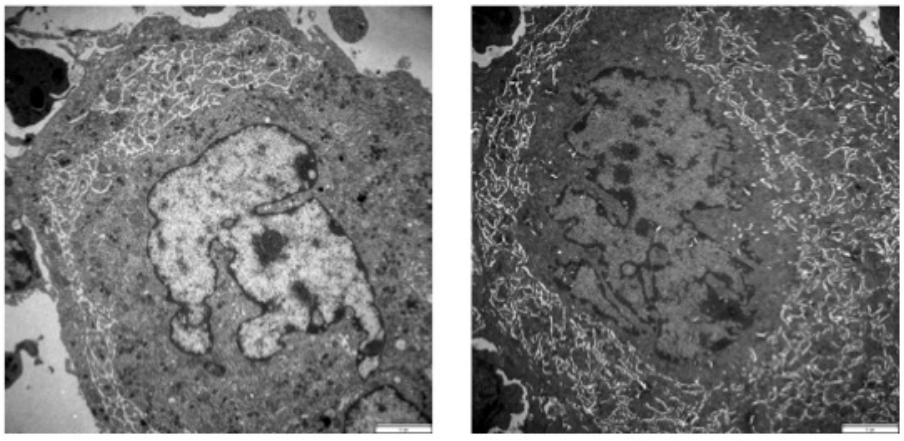
Supplemental Figure 2: (A) Mice were injected with *in vivo* mouse platelet labeling antibodies (X488 from Emfret Analytics) to label all the circulating platelets. Platelets were activated with indicated agonists, and integrin activation as detected by JON/A binding was measured on day 5 post labeling. Integrin activation of old platelets labeled with X488 (black bars) was compared to the rest of platelets in circulation (gray bar). (B) 5 µL of whole blood was incubated with a combination of thiazole orange (1 µg/mL final concentration) and anti-integrin αIIbβ3 (F(ab')$_2$ fragment, clone MWReg30) for 20 min at room temperature in dark. The blood was diluted to 500 µL with PBS and analyzed on flow cytometry. A virtual gate denoting platelet population was placed on the SSC/CD41$^+$ cells. The platelet population was further differentiated based on the thiazole orange positivity. Platelets positive for thiazole staining were termed reticulated platelets and their mean forward scatter characteristics (FSC) were compared to non-reticulated platelets in IgG treated versus NFYPs on day 5-post depletion. Values are mean ± SD.

Suppl. Figure 3

A

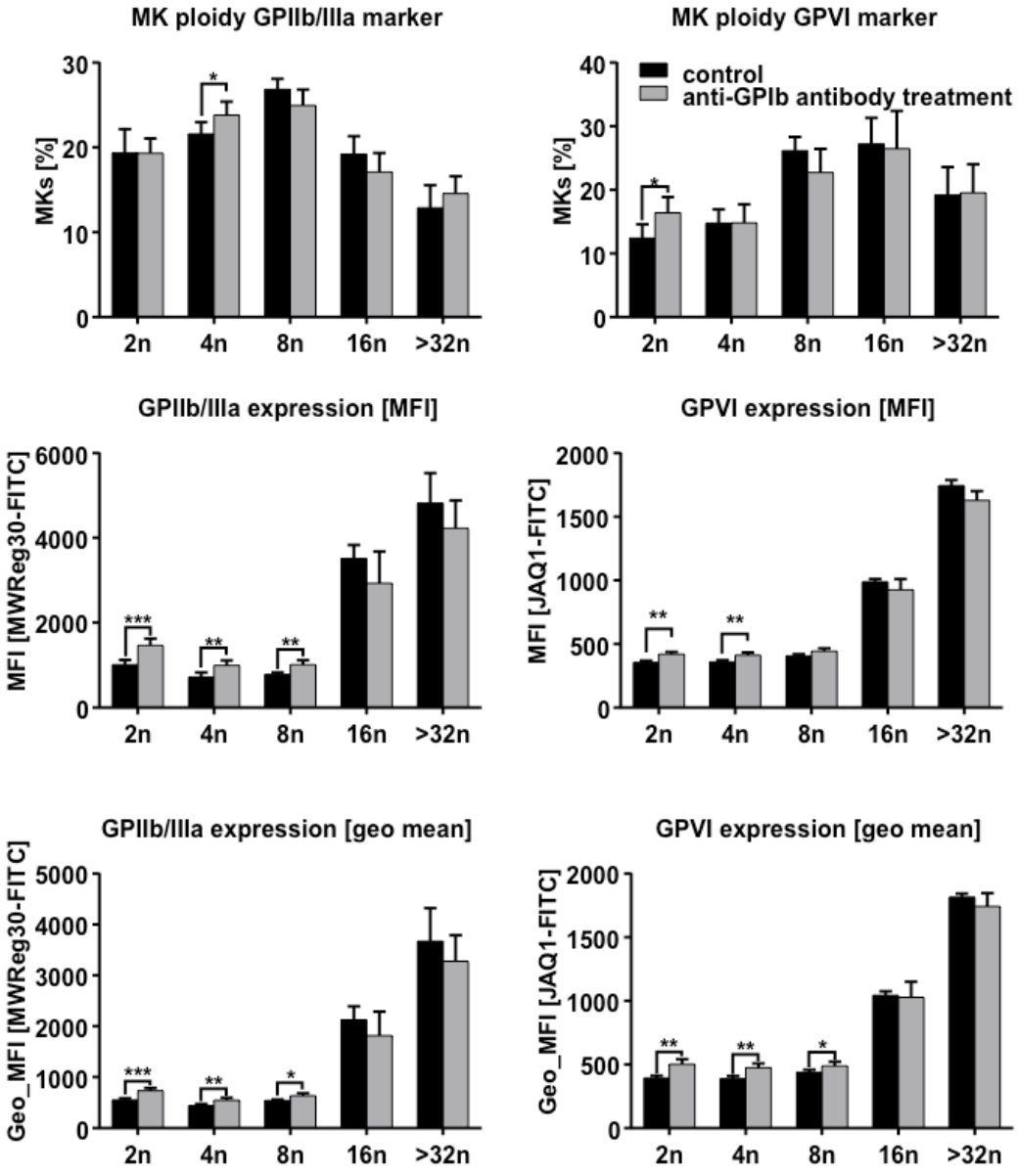


B



C

Suppl. Figure 3



Supplemental Figure 3. (A) Percentage of proplatelet-forming MKs from BM explants of control and platelet-depleted mice was determined. Values are mean ± SD, 12 explant/condition were analyzed. Representative images are shown. MKs were stained with Alexa488 conjugated anti-GPIX antibodies. (B) TEM analysis of control BM-MKs (left) and MK from mice 5 days post antibody treatment (right). Representative images for n=3 are shown. Scale bar: 3 µm. (C) BM-MKs from

control and mice 5 days post antibody treatment were stained with megakaryocyte-specific antibodies (anti-GPIIb/IIIa or anti-GPVI) and DNA with propidium iodide. Ploidy and glycoprotein expression was determined by flow cytometric analysis. Values are mean ± SD; n=5. (*), $P < 0.05$; (**), $P < 0.01$; (***), $P < 0.001$.