SUPPLEMENTAL INFORMATION

Platelet-RBC interaction mediated by FasL-FasR induces pro-coagulant activity important for thrombosis

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SUPPLEMENTAL METHODS

Chemicals and Antibodies

Accutase® (L11-007) was from PAA Laboratories GmbH. Apyrase (Grade III, from potato). PE Anti-human CD178 (Fas-L) (#306406) was from BioLegend. Cy™5 Annexin V (#559933), PE Mouse Anti-human CD42a (#558819) as well as FITC Mouse Anti-human CD62P (#555523) were from BD Biosciences. CD235a-FITC (#IM2212U) was from Beckman Coulter. FITC-Conjugated monoclonal antibody against human Tissue Factor (#4508CJ) was from Sekisui Diagnostics. Fas-Receptor antibody CD95 (#GTX13549) was from GeneTex. HRP-conjugated Anti-mouse IgG (#NA931) was from GE Healthcare. Recombinant human DcR3 (TNFRSF6B Fc Chimera) (#095407) was from R&D Systems. Fas-ligand-inhibitor (#AF-016) was from Kamiya Biomedical Company. All other reagents were of analytical grade.

Mouse models with altered hematocrit

Anemia mouse model: On two consecutive days 200 µl blood from retro-orbital plexus was collected to reduce the hematocrit up to 30%. The platelet count should be in a range, which does not lead to any bleeding complications. Control mice received RBC (total amount: $1.6x10^9$) and platelet transfusions (up to $45x10^4$/ µL) until a normal hematocrit level (35-45%)

was reached. Anemic mice only received platelet transfusions (up to platelet counts of $45x10^4$/µL). HCT + mouse model: Mice received one injection of RBCs each from donor mice with the same genetic background to induce an increased hematocrit (>50%) in these mice. To this end, $20x10^6$ RBCs/µL were injected intravenously into the recipient mouse and the hematocrit was determined using the hematology analyzer (Sysmex) before the experiment was started. For further details see supplementary figure 3.

Human platelet preparation

Fresh citrate-anticoagulated blood (BD-Vacutainer®; Becton, Dickinson and Company) was obtained from healthy volunteers aged between 18 and 60 years. Blood parameters were analyzed by a hematology analyzer (Sysmex, Norderstedt, Germany). The blood was centrifuged at 200 g for 12 min. The PRP was separated and added to PBS [pH 6.5, 2.5 U/ml apyrase (Sigma), 1 µM PGI_2] in 1:1 volumetric ratio and centrifuged at 1000 g for 6 minutes. The platelet pellet was resuspended in Tyrode buffer [137 mM NaCl, 2.8 mM KCl, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 5.5 mM Glucose, 0.1% HIBSA, pH 7.4]. The platelet count was adjusted as required for the applied functional assay.

Isolation of human white blood cells (WBCs)

1 ml whole blood was mixed with 15 ml RBC lysis buffer (154 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4) and incubated for 5 min. The cells were pelletized by centrifugation at 400 g for 5 min and washed until the pellet was free of RBCs, at least 3 times, and finally resuspended with 1 ml PBS (2% BSA).

Isolation of human red blood cells (RBCs).

For platelet preparation the blood was centrifuged at 200 g for 12 minutes. The platelet rich plasma was separated. The remaining blood is centrifuged at 800 g for 15 minutes in a closed syringe. The plasma was removed and used to prepare cell-free plasma. To separate RBCs from leukocytes the syringe was opened and the red blood cells were collected in a new

container. The leukocytes building a white cell layer on top of the red blood cells were left in the syringe. Red blood cells were washed with the fivefold volume of saline solution (154 mM) three times by centrifugation at 300 g for 10 min. RBCs were stored at 4°C. Where indicated, old RBCs were used because these cells are highly positive for AnnexinV. Old RBCs were isolated from RBC concentrates from the blood bank (from the university clinic of Düsseldorf) that were stored at 4°C for more than 40 days after blood withdrawal. The Ethics Committee of the Heinrich-Heine-University approved the use of RBC concentrates on the patients' consent.

Human erythrocyte ghosts

Isolated RBCs were processed to produce erythrocyte ghost cells. The protocol was modified according to P. Nandy (1). The RBCs were hemolysed by adding 50 times the volume of saline (10 mM NaCl, 0°C) for 15 min. All following steps were performed at 4°C. Not lysed cells were removed by centrifugation at 800 g for 20 min. The pellet was discarded and the supernatant centrifuged at 3500 g for 30 min. The pellet was washed 3 times in saline (10 mM). Ghosts were resealed by mixing gently one part of unsealed ghosts with three parts of cell free plasma (RT). The mixture was stirred for 5 min, followed by centrifugation at 3500 g for 30 min (RT). Part of the erythrocyte ghost is smaller than the primordial RBCs; therefore the number of ghosts used in the experiments was determined through the membrane surface. Concentration of ghosts was calculated for each experiment leading to a membrane surface equal to RBCs in the respective control experiment.

Flow cytometry, static experiments

Cell suspensions, blood components according to the respective experiment, were diluted with PBS to a concentration of 100.000 cells/µl. 50 µl thereof were incubated with 5 µl labeled antibodies for 20 minutes at RT. Staining was stopped by addition of 400 µl PBS and analyzed directly on a FACSCalibur (BD Bioscience). For AnnexinV-Cy5 staining Binding Buffer (10mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) was used instead of PBS and only 4 µl AnnexinV-

Cy5 were necessary. CD42 was used as platelet specific marker. Where indicated, cells were pre-treated with FasR (CD95, 10 µg/ml) antibody for 15 minutes at room temperature. This antibody has been demonstrated to block the apoptotic activity of FasR (2). In other experiments, platelets have been pre-treated with Decoy receptor 3 (DcR3, 10 µg/ml) to inhibit FasL on the surface of platelets. DcR3 competes for binding of FasL with similar affinity as Fas (3). For analysis platelets were gated using their forward- and side-scatter profiles. Externalization of Fas ligand (FasL), CD62 and tissue factor on activated and non-activated platelets was determined by flow cytometry in the presence or absence of RBCs.

Determination of phosphatidylserine (PS) exposure on RBCs and platelets by flow cytometry

PS exposure on the membrane of RBCs and platelets was measured by flow cytometry using Cy™5 AnnexinV (BD Biosciences). AnnexinV binds in presence of calcium to negatively charged phospholipid surfaces, especially PS. For optimal staining it is important to work with defined calcium and salt concentrations. For this reason all preparations for analysis of PS exposure are diluted in Binding Buffer (10x Binding Buffer: 0.1 M Hepes, 1.4 M NaCl, 25 mM $CaCl_2$, pH 7.4). Cells were incubated at room temperature in the dark for 20 minutes with 4 µl Cy™5 Annexin V and 5 µl FITC-conjugated CD235a antibody (Beckman Coulter). CD235a, also known as Glycophorin A, is expressed on the surface of erythroblastic precursor cells, reticulocytes and mature red blood cells. The staining with CD235a antibody allows distinguishing between RBCs and other blood cells upon analysis. The stained cells were resuspended in 400 µl Binding Buffer before analysing by flow cytometry. For this analysis red blood cells were gated using CD235a antibody staining.

HPLC

The release of adenosine triphosphate from platelets, RBCs and ghosts was determined by HPLC. The cells were isolated as described above, diluted with HBSS buffer to a concentration of $2x10^6$ RBC/µl or $2x10^5$ platelets/µl and incubated with L-Arginine (200 µM) at 37°C for 30

minutes. Supernatant was prepared by centrifugation and then analyzed by HPLC. To determine the release of ATP during a flow chamber experiment samples of reconstituted blood were collected before and after the experiment. The cells were discarded and the supernatant was processed. Proteins were precipitated by combining 500 µl supernatant with 500 µl ice-cold 1 M perchloric acid. The supernatant was neutralized with 87 mg dipotassium phosphate. 200 µl of the resulting supernatant were injected onto the HPLC column (Hypersil™ BDS C18 Column Particle Size 3 µm, Thermo Scientific) for measurement of ATP.

Cell lysis and immunoblotting of FasR

Different cell concentrations of RBCs ($1x10^5$/µl, $5x10^5$/µl and $1x10^6$/µl) were lysed over night at 4°C in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1 mM NaF, 1 mM Na_3VO_4, 0.4% ß-Mercaptoethanol, protease inhibitor cocktail (PI)). The lysates were centrifuged at 4°C and 8.400 g for 10 minutes. The supernatants of the RBC lysates were prepared with reducing sample buffer (Laemmli buffer) and denatured at 95°C for 5 minutes, separated on SDS-polyacrylamide gel and transferred onto nitrocellulose blotting membrane (GE Healthcare Life Science). Accordingly, the membrane was blocked with 5% non-fat milk in TBST (20 mM Tris/HCl, 137 mM NaCl, pH 7.3, 0.1% Tween20) at room temperature for one hour. The membrane was incubated with CD95-antibody (IgG, 1:500) at 4°C overnight. After washing with TBST the membrane was incubated for one hour with peroxidase-conjugated secondary antibody (sheep anti-mouse-IgG, 1:1000). Antibody binding was detected by the use of ECL detection reagent (BioRad).

Microchip-based flow chamber analysis. (T-Tas®)

For the comparison of thrombus formation in whole blood and PRP the Total Thrombus-formation Analysis System (T-TAS; Fujimori Kogyo Co., Ltd.,Tokyo, Japan) was used, which is a microchip-based flow chamber system. It is equipped with a pneumatic pump, a flow pressure sensor, and a video microscope. Blood was taken as described and from each

volunteer a sample of whole blood and a sample of PRP were analysed. The platelet counts in whole blood and PRP are adjusted by dilution of the PRP with saline solution (200.000 platelets/µL). The samples were perfused over a microchip coated with collagen and tissue factor (AR-Chip; Fujimori Kogyo Co., Ltd.,Tokyo, Japan) at a flow rate of 600 s^{-1} and a temperature of 37°C. While blood was flowing through the capillary the process has been visually inspected by the built-in light microscope. In addition T-TAS performs a flow pressure analysis. The occlusion time is defined as the lag time for the flow pressure to increase by 80 kPa from baseline. When flow pressure increases to 80 kPa, blood perfusion and flow pressure monitoring stops. At this time, the capillary is presumed to be almost completely occluded by thrombi. The occlusion time value reflects the onset, growth and stability of thrombi inside the capillaries. The thrombus formation was analysed using image analysis software (Zia; Fujimori Kogyo Co., Ltd., Tokyo, Japan). Experiments with murine blood from WT, FasL and FasR knock-out mice were performed accordingly with some exceptions: PRP from WT (WT, *FasR*$^{-/-}$) or *FasL*$^{-/-}$ mice was perfused through the chamber supplemented with RBCs from either WT (WT, *FasL*$^{-/-}$) or *FasR*$^{-/-}$ mice, a flow rate of 1.600 s-1 was used for all murine experiments.

Thrombus formation under flow using the flow chamber system

A parallel plate flow chamber was briefly rinsed with plasma before whole- or recomposed-blood was perfused through the chamber (50 µm x 5 mm). Platelet counts in PRP were adjusted to 200.000/µL and number of RBCs were taken for each run as indicated. The cover slide (24 x 60 mm) was coated with 200 µg/ml type I collagen (Takeda Pharmaceutical) and blocked with 1% BSA. After 5 min (150 s^{-1}, 1000 s^{-1}) or 10 min (1700 s^{-1}) of flow, five pictures from different areas were taken (400x total magnification; Axio Observer.D1, Carl Zeiss). The surface coverage was analyzed using the Zen 2012 software (Carl Zeiss). Where indicated, fixation of RBCs was done with 0.5% glutaraldehyde. RBCs were washed twice with physiologic salt solution and number of RBCs was adjusted to $4x10^6$.

Treatment with DcR3: DcR3 was used at final concentration of 10 µg/ml. DcR3 was added to whole blood and perfused through the flow chamber. Binding of DcR3 to its target structure is

only possible upon platelet activation by collagen in the chamber, therefore a pre-incubation of platelets with DcR3 before perfusion was not successful. A flow chamber run of whole blood supplemented with IgG-Fc served as control. Pre-treatment with FasR antibody: FasR antibody was used in the flow chamber assay at a final concentration of 10 µg/ml. RBCs were pre-incubated with FasR antibody for 15 minutes at room temperature and supplemented with PRP before perfusing through the chamber. Flow chamber experiments using a control IgG antibody served as control. Where indicated cells from thrombi were isolated by Accutase (120 µl/coverslip, solution with protease and collagenolytic activity) treatment to detach and solve adherent and aggregated cells and analyzed by flow cytometry.

Treatment with pan-Caspase inhibitor: For flow cytometry analysis and flow chamber experiments we used isolated human RBCs and platelets and whole blood. Samples were treated with 10 µM pan-Caspase inhibitor (InSolution™ Q-VD-OPh, Non-O-methylated, 10 µM, Calbiochem/Merck, Darmstadt, Germany).

Detection of RBCs and platelets by immunofluorescence: For Immunofluorescence staining of thrombi which were generated under flow conditions (1700 s^{-1}) using WT, FasR and FasL deficient mice we used the unconjugated rat anti-mouse TER-119 antibody (3 µg/ml) against mouse RBCs (#557909, BD Pharmingen™, Becton, Dickinson and Company) and the unconjugated polyclonal rabbit anti-mouse/human GP9 antibody (10 µg/ml) (orb167288, Biorbyt Ltd., Cambridge, England) against platelets as primary antibodies. Secondary antibodies were used as follows: Goat anti rabbit Alexa Fluor 488 (20µg/ml) and goat anti rat Alexa Fluor 568 (20 µg/ml) (Thermo Fischer, previously Lifetechnologies). Samples were analyzed using confocal microcopy (Leica TCS SP8 STED 3x, 63x objective, Wetzlar, Germany).

Thrombin generation in PRP in the absence and presence of RBCs

The calibrated automated thrombography was used to assess thrombin generation in PRP with and without RBCs. Thrombin generation in PRP was determined as described previously (4). Briefly, platelets in PRP were adjusted to 150x10^3/µl with platelet-free plasma. Blocking

anti-FasR-antibody and control IgG was preincubated with platelets for 10 minutes at RT prior to addition of α-thrombin as trigger (final concentration 0.1 U/ml). Thrombin generation was started by adding FluCa-solution containing $CaCl_2$ (final concentration 17 mM) in HEPES-BSA-buffer, pH 7.35. Thrombin-mediated cleavage of the fluorogenic substrate Z-Gly-Gly-Arg-AMC was monitored for 60 minutes at 37°C using a Fluoroskan Ascent fluorescence reader (excitation 390 nm, emission 460 nm wavelengths, Thermo Labsystems, Franklin, MA). Quantification of thrombin formation in PRP plus RBCs was performed according to Ninivaggi M et al. with some modifications (5). Briefly, rhodamine-based thrombin substrate P_2Rho (final concentration 300 µM) and α-thrombin (final concentration 0.1 U/ml)) were added to 30 µl of PRP plus RBCs. Thrombin generation was started by adding $CaCl_2$ (final concentration 17 mM) in HEPES-buffer containing 0.5% BSA. Filter paper discs (5 mm diameter and 180 µm thickness) were wetted with 5 µl of the blood mixture and covered with 40 µl mineral oil. Thrombin-mediated cleavage of the rhodamine-based substrate P2Rho was monitored for 60 minutes at 37°C with a Fluoroskan Ascent fluorescence reader (excitation 485 nm, emission 538 nm wavelengths). The ThrombinoscopeTM, Synapse BV software program was used for calculation of thrombin generation parameters.

Analysis of human thrombi and blood

Arterial thrombi of 29 patients (both genders) were analyzed. All patients suffered from arterial thrombosis from different reasons and underwent thrombectomy. The Ethics Committee of the Heinrich-Heine-University approved the collection and analysis of the tissue based on the patients' consent.

Histology of human thrombi

One part of the thrombus was fixed in formaldehyde. Clarification, dehydration and inclusion in paraffin were carried out. The compounded paraffin-blocks were cut in sections with a thickness of 5 µm by a microtome (Microm HM400). Sections of hydrated and deparaffinised tissues were stained with hematoxylin and eosin (HE). From each thrombus sections of the

middle of the thrombus were analysed. After the staining procedures, images (magnification 25x, 100x, 400x) were obtained with a Carl Zeiss microscope used for this purpose and a AxioCam 105 Colour camera with the software Zen 2012 (blue edition, Carl Zeiss) was used for image capturing.

Electron microscopy of human thrombi

One part of a fresh thrombus was fixed with warmed Karnovsky's fixative (3% formaldehyde, 2.5% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4) for 10 min at 37°C and stored at 4°C. Post-fixation was based on 1.0% osmium tetroxide containing 1.5% K-ferrocyanide in 0.1 M cacodylate buffer for 2 h. After following standard methods, blocks were embedded in glycide ether and cut using an ultra-microtome (Ultracut, Reichert, Vienna, Austria). Ultra-thin sections (30 nm) were mounted on copper grids and analyzed using a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) operating at 120 kV (6).

Flow cytometry of human thrombi

One part of a thrombus was delivered in saline solution. To analyse the cells in the thrombi by flow cytometry they need to be separated. Therefore Accutase was used to detach adherent cells. A small part from the middle of every thrombus was dissolved in 500 µl of Accutase for 30 minutes at 37°C. After filtration (40 µm filter, BD Bioscience) and centrifugation in two containers at 525 x g for 5 minutes the supernatant consisting of Accutase can be removed. One cell pellet was resolved in 500 µl saline solution (Fresenius Kabi Deutschland GmbH). The other cell pellet was resolved in 200 µl Binding Buffer (10x Binding Buffer: 0.1 M Hepes, 1.4 M NaCl, 25 mM $CaCl_2$, pH 7.4). Analogous to the staining procedure described above, flow cytometry was performed.

To differentiate platelets from other cells double-staining with 5 µl FITC- or PE-conjugated CD42 antibody (platelets) was performed simultaneously. To identify the different cell populations in the thrombus staining with 5 µl PE-conjugated CD45 antibody (leukocytes) and 5 µl FITC-conjugated CD235a (RBCs) antibodies of 50 µl resolved cells in saline solution for

20 minutes in the dark was done. To analyse PS exposure on RBCs, the resolved cells in Binding Buffer were incubated at room temperature in the dark for 20 minutes with 4 µl Cy™5 AnnexinV and 5 µl FITC-conjugated CD235a antibody. The stained cells were then resuspended in 400 µl Binding Buffer and analysed in flow cytometry. For this analysis, RBCs were gated using CD235a antibody staining.

$FeCl_3$ injured carotid artery

The right A. carotis communis was prepared and a flow probe (Transonic Systems, 0.5 PSB, AD Instruments) was placed around the carotis. Before starting the measurement, the artery and the surrounding area were moisturized with 0.9% NaCl and blood flow was measured in mL/min. Then a small pad was placed under the artery below the measuring head and the environment of the artery was dried. A 0.5 x 1 mm filter paper (Whatmann No.1) saturated with 15% $FeCl_3$ (Sigma-Aldrich) was placed at the area of the carotid artery below the measuring head for 3 min. After removing the filter paper, the environment of the artery was moisturized with 0.9% NaCl again. Measurement runs till the blood flow stops (full occlusion) (6, 7).

Intravital microscopy of thrombus formation in mesenteric arterioles injured with $FeCl_3$

Intravital microscopy was performed as described previously (6, 8). Mice (4-5 week of age) were anesthetized with Ketamin (Ketavet®, Pfizer, 100 mg/kg) and Xylazin (2% Bernburg, medistar, 5 mg/kg) by intraperitoneal (i.p.) injection. After a median abdominal incision, the mesentery was exteriorized and a filter paper saturated with 20% $FeCl_3$ was used to injure arterioles by topical application for 20 seconds. Thrombus formation was made visible by i.v. injection of DCF (50 µL/mouse, 4.48 M solution in PBS) and observed with a fluorescence microscope (40 ×, Zeiss, Axio Observer, Germany). Time until full occlusion of the vessel (when blood flow had stopped for >1 minute) was measured. Experiments were stopped after 40 minutes.

Tail bleeding time

Mice were anesthetized with Ketamin (100 mg/kg) and Xylazin (5 mg/ml) i.p. and the tail tip was cut with a scalpel at a position where the diameter of the tail is 2.25 – 2.5 mm using a gauge. The tail was immersed in PBS (37°C). The time from the incision to the cessation of bleeding was recorded (no blood flow for 1 minute) (6).

Mouse model of the inferior vena cava (IVC)

1. IVC ligation to analyze platelet-RBC interaction at the injured vessel: Mice were anesthetized by intraperitoneal injection of fentanyl, midazolam, and medetomidine. After a median laparotomy the IVC was exposed by atraumatic surgery ligated below the left renal vein. Side branches were not ligated or manipulated. All mice with bleedings or any injury of the IVC during surgery were excluded from further analysis.

2. Mouse model of flow restriction in the IVC (9): The animals were matched in age, sex and weight. Fas-Antibody (20 µg, GeneTex) and the isotype control antibody (IgG1, 20 µg, Cell Signaling) were administered into the tail vein of C57BL/6 before surgery as controls for the *FasL*$^{-/-}$ animals served C57BL/6. After median laparotomy and exposition of the IVC a wire was positioned on the vessel as a spaceholder and the vessel was ligated in order to achieve a flow reduction in the IVC. For avoiding a complete occlusion the spaceholder was removed. The sidebranches of the IVC have not been manipulated or ligated during this procedure. In order to measure the thrombus weight after 48 hours the mice were sacrificed, blood was asserved and the IVC was excised just below the renal veins and proximal to the confluence of the common iliac veins.

Preparation of platelets and erythrocytes for intravital microscopy

Murine platelets were isolated from whole blood of C57BL6 (WT) or *FasL*$^{-/-}$ mice and labeled with RhodaminB as reported and injected into C57BL6 mice (10). The RhodaminB-labeled platelet suspension was adjusted to a final concentration of 150.000 platelets/µl and 50 µl were injected i.v. via tail vein catheter. Erythrocytes were isolated from citrated whole blood of

C57BL6 (WT) or *FasR*$^{-/-}$ mice, labeled with DCF, and 3x10^7 were injected i.v. into C57BL6 mice.

Intravital epifluorescence microscopy

Imaging was performed with a high-speed wide field Olympus BX51WI fluorescence microscope using a long-distance condenser and a 20x (NA 0.95) water immersion objective with an Olympus MT 20 monochromator and an ORCA-ER CCD Camera (Hamamatsu). For image acquisition and analysis Cell^R software (Olympus) was used. Cell interactions were quantified in four fields of view (50x100 µm) per animal 30, 60, and 90 minutes after ligation. Interactions were defined as co-localization for at least three consecutive images (204 ms).

Statistical analysis

Data are demonstrated as mean ± standard error of the mean (s.e.m.). Statistical significance was analyzed by Student´s paired t-test (2 tailed) and by One-way Anova multiple comparison and Tukeys multiple comparison, two-way Anova Sidaks multiple comparison (Graph pad prism) as indicated in the figure legends. P values < 0.05 were considered to be statistically significant.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Effects of RBCs and WBCs on thrombus formation under flow. (A-B) Whole blood or PRP supplemented with different concentrations of RBCs or WBCs were perfused over a collagen coated surface at a shear rate of 1.000 s^{-1}. PRP contains 200.000 platelets/µl. **(A)** Representative phase contrast images at the end of the perfusion period. Scale bar 100 µm. **(B)** Quantification of surface coverage with blood components as indicated (n=3-5). **(C)** No hemoglobin release upon thrombus formation under flow. Flow chamber experiments on collagen were performed at a shear rate of 1000 s^{-1} using either whole blood, 2x10^5 platelets/µl

and $4x10^6$ RBCs/µl, $4x10^6$ RBCs/µl alone or plasma. RBC lysates served as positive control. The difference in optical density at 412 nm from samples taken before and after a flow chamber experiment was determined. **(D)** Thrombus formation using PRP and increasing concentrations of RBCs. Representative images are shown. Scale bar 50 µm. **(E)** Quantification of thrombus formation depending on the amount of RBCs. Comparison of physiological ($4x10^6$ RBCs/µl) and increased ($8x10^6$ RBCs/µl) concentration of RBCs. **(F and G)** Thrombus formation using PRP supplemented with fresh and old RBCs ($4x10^6$ RBCs/µl), respectively. **(F)** Representative images are shown. Scale bar 50 µm. **(G)** Mean surface coverage per visual field. Data represents the arithmetic mean ± s.e.m (n=3). ** $P < 0.01$, *** $P < 0.001$. One-way Anova Dunnets multiple comparison test (B-C, E) and Student's t-test (G). PRP=platelet-rich plasma; RBC=red blood cell; WBC=white blood cell.

Figure S2. **No significant alterations in plasma ATP content before and after flow through. (A)** ATP concentration in plasma was measured by HPLC before (black column) and after (grey column) flow chamber experiments using either $2x10^5$ platelets/µl and $4x10^6$ RBCs/µl or ghosts and $2x10^5$ platelets/µl. Data represents the arithmetic mean ± s.e.m. (n=3). Student's t-test.

Figure S3. **Analysis of mice with altered hematocrit**. **(A)** Size/volume of fixed RBCs compared to fresh RBCs. **(B)** Scheme of anemia induction in wildtype mice. **(C)** Determination of the hematocrit in control and anemic mice (n = 17-22). **(D)** Hematocrit was measured in mice that receive injection of RBCs (HCT+). Each symbol represents one animal. Data represents the arithmetic mean ± s.e.m. (n=5), *** $P < 0.001$, Student's t-test (A).

Figure S4. Characterization of different RBC populations. Determination of **(A)** CD235, **(B)** FasR and **(C)** FasL expression by flow cytometry. **(D)** Volume / size of different RBCs were measured by an automatic cell counter (Sysmex). **(E)** Morphology of different RBCs was analyzed by Pappenheim's staining (scale bar 20 µm) and **(F)** by Differential interference

contrast (DIC) microscopy (scale bar 50 µm). **(G-H)** RBC deformability was measured by ektacytometry using a Laser-assisted optical rotational cell analyzer (LORCA) (n=4-10). Data are mean ± s.e.m. ** P < 0.01, *** P < 0.001. One-way Anova Dunnets multiple comparison test.

Figure S5. Accutase treatment of thrombi is suitable for separation of cells and does not lead to PS exposure of RBCs per se. **(A)** Representative images of the separation of cells from a thrombus grown on collagen matrix under flow using Accutase. Upper panel left image shows the collagen matrix used in flow chamber experiments before thrombus formation. The black arrowhead indicates a collagen fiber. Representative phase contrast image of thrombus formation on collagen with whole blood at the end of the perfusion period (upper panel right). The black arrowhead indicates a collagen fiber while the black arrow indicates a thrombus. Collagen matrix after treatment with Accutase where most of the cells were detached and washed away (lower panel, left image). While the black arrowhead indicates a collagen fiber, the white arrowhead shows a remaining cell. Cells detached from the collagen matrix by Accutase treatment (lower panel, right image). Separated cells are indicated by the white arrowhead, white arrow shows two cells detached from the collagen surface but still attached to each other. Scale bar 50 µm. **(B)** Comparison of AnnexinV binding to RBCs before (left column) and after (right column) Accutase treatment. Data represents the arithmetic mean ± s.e.m. (n=3). Student's t-test.

Figure S6. CD36, CXCL16 and caspases do not effect thrombus formation or PS exposure of RBCs. (A) Effects of aspirin and clopidogrel on PS exposure of RBCs. RBCs and platelets were preincubated with CRP (5 µg/ml), aspirin (100 µM) and clopidogrel (100 µM) and stained with AnnexinV to determine PS exposure of RBCs by flow cytometry (n=6). **(B-C)** Whole blood obtained from *Cd36*$^{+/+}$ and *Cd36*$^{-/-}$ was perfused over a collagen coated surface at a shear rate of 1000 sec^{-1} (n=5). **(B)** Representative phase contrast images at the end of the perfusion period. Scale bar 50 µm and **(C)** mean surface coverage per visual field.

(D) Thrombus formation on collagen using human PRP ($2x10^5$ platelets/µl) and RBCs ($4x10^6$ RBCs/µl) with a shear rate of 1000 sec^{-1}. Control (+IgG) is shown on the left (black column) and PRP ($2x10^5$ platelets/µl) and RBCs ($4x10^6$ RBCs/µl) pre-treated with anti-CD36 and anti-CXCL16 is shown on the right (grey column) (n=3). (**E**) Representative Western blots of human red blood cell lysates using FasR (CD95) antibody (n=3). **(F)** FasR expression in platelets (plts), RBCs and RBCs supplemented with resting and ADP activated platelets was determined by Western blot. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control, n=3. (**G**) Thrombus formation in the presence of a pan-caspase inhibitor was analyzed using whole blood. Platelets were labeled with mepacrine and surface coverage (left panel) and relative platelet deposition as measured by integrated fluorescence intensity (thrombus volume,IFI) (right panel) were determined (n=6). (**H**) AnnexinV binding of RBCs in the presence and absence of a pan-caspase inhibitor was measured by flow cytometry (n=4). One-way Anova Dunnets multiple comparison test (A, H) and Student's t-test (C-D, G).

Figure S7. Platelet activation by RBCs and WBCs. Platelet activation was determined by P-selectin exposure (degranulation marker) and integrin activation (PAC-1 binding) using flow cytometry. **(A)** P-selectin exposure and **(B)** PAC-1 binding in the presence and absence of RBCs and an inhibiting FasR antibody (n=7). **(C)** P-selectin exposure and **(D)** integrin activation (PAC-1) in the presence and absence of WBCs (n=6). **(E)** FasL exposure of platelets in the presence and absence of WBCs (n=7). Data are mean ± s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, One-way Anova Dunnets multiple comparison test (A-E).

Figure S8. Characterization of wild-type, FasL and FasR knock-out mice. **(A)** The number of platelets and RBCs and the hematocrit was measured in an automatic cell counter (Sysmex). (n=5-8). **(B-C)** AnnexinV binding of freshly isolated (**B**) and RBCs in whole blood (**C**) using wild-type, FasL and FasR knock-out mice. (n=5). Data are mean ± s.e.m. One-way Anova Dunnets multiple comparison test (B-C).

Figure S9. Flow cytometric analysis of platelet activation and AnnexinV binding of RBCs. Whole blood was obtained from patients upon thrombectomy. Blood samples from healthy volunteers served as controls. **(A)** Exposure of P-selectin and FasL was determined by flow cytometry using the respective PE labelled antibodies (n=3-5). **(B)** Flow cytometric analysis of PS exposure of platelets and RBCs using AnnexinV (n=4-6). Data represents the arithmetic mean ± s.e.m. * $P < 0.05$, Student's t-test (A-B).

SUPPLEMENTAL VIDEOS

Movie S1. Movie of *ex vivo* thrombus formation of whole blood from healthy volunteers. Blood was perfused over a collagen-coated coverslip at shear rate 1000 sec^{-1} using a flow chamber system. Representative time lapse movie of platelet adhesion and thrombus formation is shown.

Movie S2. Movie of *ex vivo* thrombus formation of PRP from healthy volunteers. Blood was perfused over a collagen-coated coverslip at shear rate 1000 sec^{-1} using a flow chamber system. Representative time lapse movie of platelet adhesion and thrombus formation is shown.

Movie S3. Movie of *ex vivo* thrombus formation using PRP and fresh RBCs ($4x10^6$/µl) isolated from whole blood of healthy volunteers. Blood was perfused over a collagen-coated coverslip at shear rate 1000 sec^{-1} using a flow chamber system. Representative time lapse movie of platelet adhesion and thrombus formation is shown.

Movie S4. Movie of *ex vivo* thrombus formation using PRP supplemented with freshly prepared ghosts isolated from whole blood of healthy volunteers. Blood was perfused over a collagen-coated coverslip at shear rate 1000 sec^{-1} using a flow chamber system. Representative time lapse movie of platelet adhesion and thrombus formation is shown.

Movie S5/6. *In vivo* platelet adhesion and thrombus formation after injury of mesenteric arterioles in FasR antibody treated WT mice. WT mice were injected with IgG antibody (20 µg/mouse, control, Movie S5) or FasR antibody (anti-FasR, 20 µg/mouse, Movie S6) and mesenteric arterioles were injured by topical application of 20% $FeCl_3$. Platelets were fluorescently labelled by intravenous injection of DCF and thrombus formation was observed till full occlusion of the vessel using a fluorescent microscope (Zeiss Axio-Observer, objective: 10 x)

Movie S7/8/9. *In vivo* platelet adhesion and thrombus formation in FasR knock-out and FasL knock-out mice after injury of mesenteric arterioles. WT mice (Movie S7), FasR knock-out mice (Movie S8) and FasL knock-out mice (Movie S9) received DCF (i.v.). Mesenteric arterioles of mice were exteriorized and vessel injury was done by topical application of $FeCl_3$. Platelet adhesion and aggregate formation was monitored by fluorescence microscopy (Zeiss Axio-Observer, objective: 10x).

Movie S10/11/12. Cell-cell interactions of platelets and RBCs after ligation of the IVC *in vivo*. WT Mice receiving platelets and RBCs from C57BL/6 mice (Movie S10), mice receiving RBCs from FasR knock-out mice (Movie S11) and mice with platelet transfusions from FasL knock-out mice (Movie S12) received DCF to label RCBs and RhodaminB to label platelets (i.v.). After median laparotomy the IVC was exposed and ligated below the left renal vein. Platelet and RBC interactions 60 min. after IVC ligation were monitored by intravital epifluorescence microscopy (Olympus BX51WI fluorescence microscope, objective: 20x).

SUPPLEMENTAL TABLE LEGENDS

Table S1. Characteristics of patients who underwent surgical thrombectomy.

SUPPLEMENTAL REFERENCES

1. Nandy P, Dutta Choudhury M, and Chakrabarti P. Effect of sealing on the incorporation of pyrene in goat erythrocyte ghosts — A fluorescence study. *J Biosci.* 1983;5(2):163-6.
2. Dabrowska A, Kim N, and Aldovini A. Tat-induced FOXO3a is a key mediator of apoptosis in HIV-1-infected human CD4+ T lymphocytes. *Journal of immunology (Baltimore, Md : 1950).* 2008;181(12):8460-77.
3. Sheikh MS, and Fornace AJ, Jr. Death and decoy receptors and p53-mediated apoptosis. *Leukemia.* 2000;14(8):1509-13.
4. Jurk K, Lahav J, H VANA, Brodde MF, Nofer JR, and Kehrel BE. Extracellular protein disulfide isomerase regulates feedback activation of platelet thrombin generation via modulation of coagulation factor binding. *Journal of thrombosis and haemostasis : JTH.* 2011;9(11):2278-90.
5. Ninivaggi M, Apitz-Castro R, Dargaud Y, de Laat B, Hemker HC, and Lindhout T. Whole-blood thrombin generation monitored with a calibrated automated thrombogram-based assay. *Clinical chemistry.* 2012;58(8):1252-9.
6. Fotinos A, Klier M, Gowert NS, Munzer P, Klatt C, Beck S, et al. Loss of oligophrenin1 leads to uncontrolled Rho activation and increased thrombus formation in mice. *Journal of thrombosis and haemostasis : JTH.* 2015;13(4):619-30.
7. Jarre A, Gowert NS, Donner L, Munzer P, Klier M, Borst O, et al. Pre-activated blood platelets and a pro-thrombotic phenotype in APP23 mice modeling Alzheimer's disease. *Cellular signalling.* 2014;26(9):2040-50.
8. Elvers M, Herrmann A, Seizer P, Munzer P, Beck S, Schonberger T, et al. Intracellular cyclophilin A is an important Ca(2+) regulator in platelets and critically involved in arterial thrombus formation. *Blood.* 2012;120(6):1317-26.
9. von Bruhl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *The Journal of experimental medicine.* 2012;209(4):819-35.

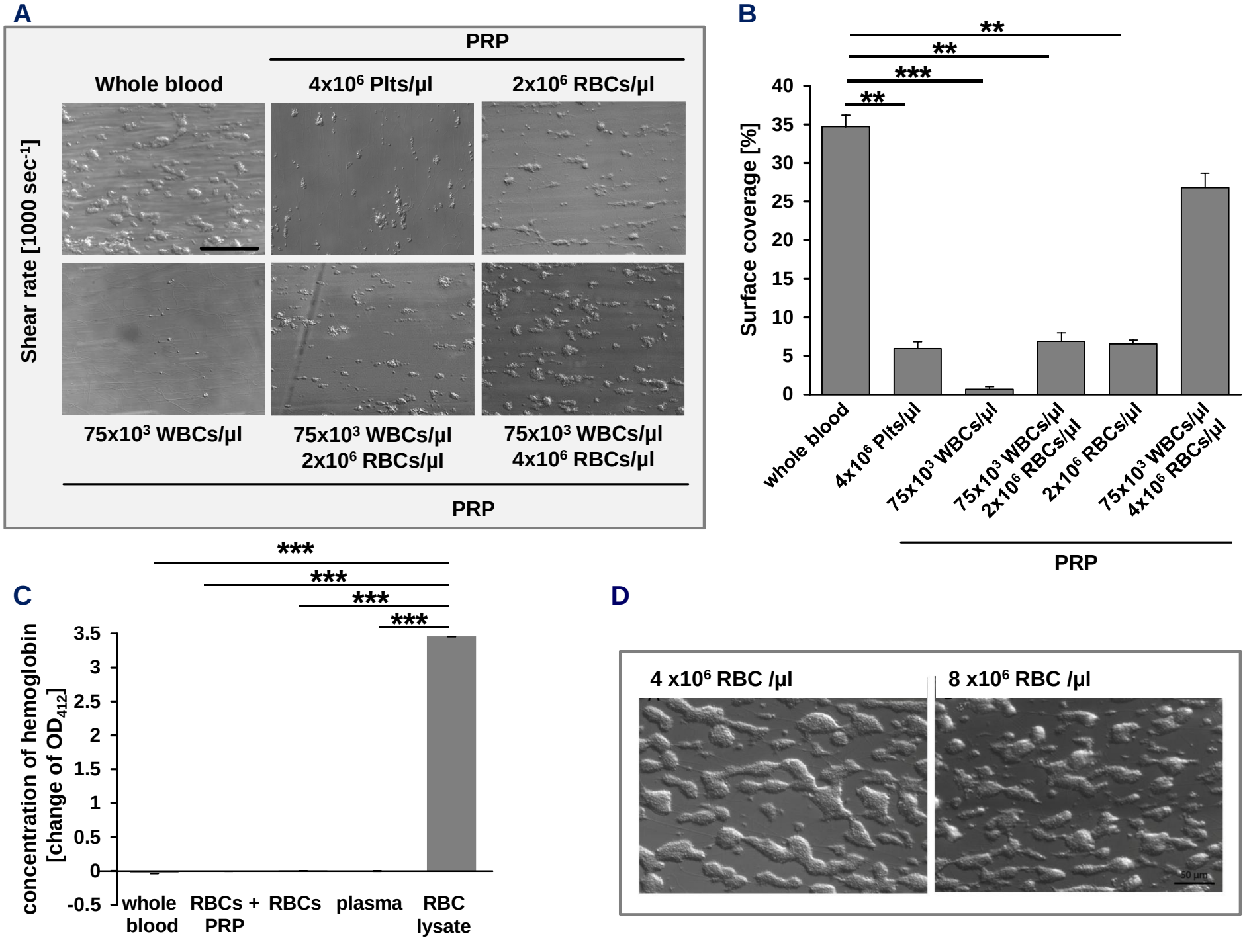
10. Massberg S, Brand K, Gruner S, Page S, Muller E, Muller I, et al. A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. *The Journal of experimental medicine.* 2002;196(7):887-96.

Table 1. Characteristics of patients who underwent surgical thrombectomy

ID	**Patient (year of birth)**	**Date of Sampling**	**Place of removal**	**Weight [kg]**	**body height [cm]**	**smoker**
1	Adult female (1941)	May 25th 2014	forearm arteries	90	163	ex
2	Adult female (1939)	May 30th 2014	femoro-distal bypass	51	162	ex
3	Adult male (1945)	June 16th 2014	common femoral artery	40	174	yes
4	Adult male (1954)	July 09th 2014	femoro-popliteal bypass	120	181	ex
5	Adult male (1952)	July 11th 2014	femoro-popliteal bypass	70	172	ex
6	Adult female (1929)	July 18th 2014	femoro-popliteal bypass	74	158	no
7	Adult male (1954)	July 21st 2014	forearm arteries	72	173	yes
8	Adult male (1961)	July 23rd 2014	femoral and popliteal artery	75	180	yes

9	Adult female (1939)	August 13rd 2014	femoro-popliteal bypass	67	168	yes
10	Adult female (1935)	August 29th 2014	A. rad., A. uln. sin.	61	171	no
11	Adult male (1946)	October 04th 2014	femoro-popliteal bypass	92	183	yes
12	Adult male (1960)	October 11st 2014	common femoral artery	68	167	yes
13	Adult female (1934)	October 22nd 2014	forearm arteries	79	170	no
14	Adult male (1936)	November 01st 2014	A. poplitea dex.	76	169	no
15	Adult male (1946)	November 01st 2014	femoro-popliteal bypass	92	183	yes
16	Adult male (1953)	January 04th 2015	Iliac arteries	72	180	no
17	Adult male (1951)	January 04th 2015	Iliac arteries	73	176	no
18	Adult female (1943)	January 21st 2015	fem-fem crossover bypass	66	176	no
19	Adult female (1939)	January 28th 2015	forearm arteries	53	158	no
20	Adult female (1959)	February 08th 2015	femoro-popliteal bypass	37	161	no
21	Adult male (1952)	April 26th 2015	femoro-popliteal bypass	69	172	yes

22	Adult female (1930)	June 06th 2015	femoral artery	NA	NA	no
23	Adult female (1940)	June 08th 2015	radial artery	74	164	no
24	Adult male (1948)	June 10th 2015	femoro-popliteal bypass	77	182	yes
25	Adult male (1939)	June 19th 2015	femoro-popliteal bypass	76	178	no
26	Adult male (1951)	July 14th 2015	brachial artery	110	180	yes
27	Adult male (1944)	July 29th 2015	femoral artery	87	190	yes
28	Adult female (1944)	September 29th 2015	femoral artery	90	176	No
29	Adult female (1958)	October 07th 2015	femoral artery	50	163	yes


A
PRP
Whole blood
4x10⁶ Plts/µl
2x10⁶ RBCs/µl
Shear rate [1000 sec⁻¹]
75x10³ WBCs/µl
75x10³ WBCs/µl
2x10⁶ RBCs/µl
75x10³ WBCs/µl
4x10⁶ RBCs/µl
PRP
B
**
**
***
**
Surface coverage [%]
40
35
30
25
20
15
10
5
0
whole blood
4x10⁶ Plts/µl
75x10³ WBCs/µl
75x10³ WBCs/µl
2x10⁶ RBCs/µl
2x10⁶ RBCs/µl
75x10³ WBCs/µl
4x10⁶ RBCs/µl
PRP
C
***
***
***
***
concentration of hemoglobin
[change of OD412]
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
whole blood
RBCs + PRP
RBCs
plasma
RBC lysate
D
4 x10⁶ RBC /µl
8 x10⁶ RBC /µl
50 µm

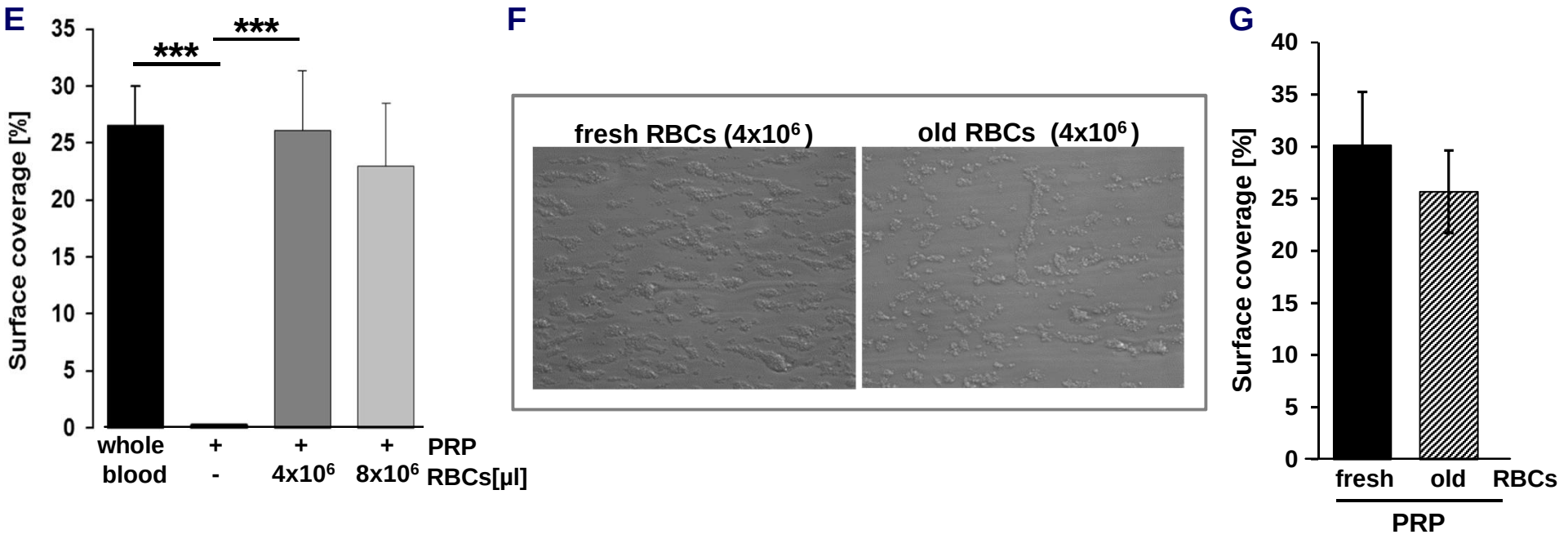

E
F
G
***
***
Surface coverage [%]
0
5
10
15
20
25
30
35
whole blood
+
-
+
4x10^6
+
8x10^6
PRP
RBCs[µl]
fresh RBCs (4x10^6)
old RBCs (4x10^6)
Surface coverage [%]
0
5
10
15
20
25
30
35
40
fresh
old
RBCs
PRP

Figure S1

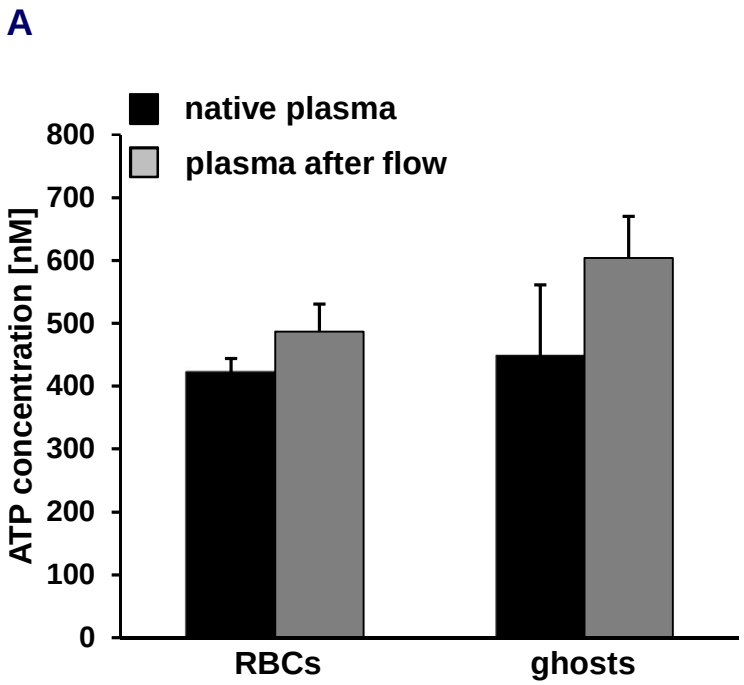

A
native plasma
plasma after flow
ATP concentration [nM]
800
700
600
500
400
300
200
100
0
RBCs
ghosts

Figure S2

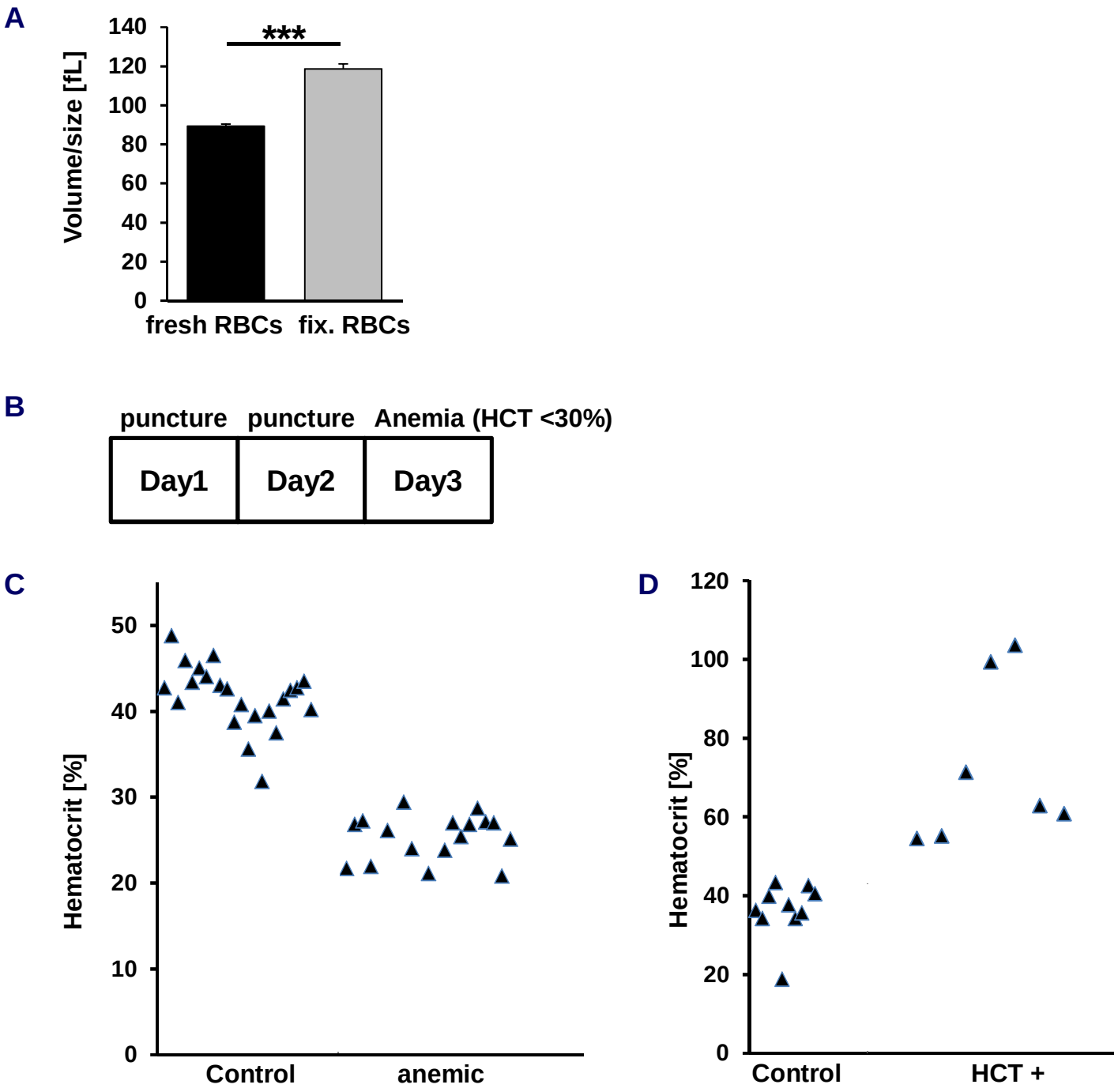
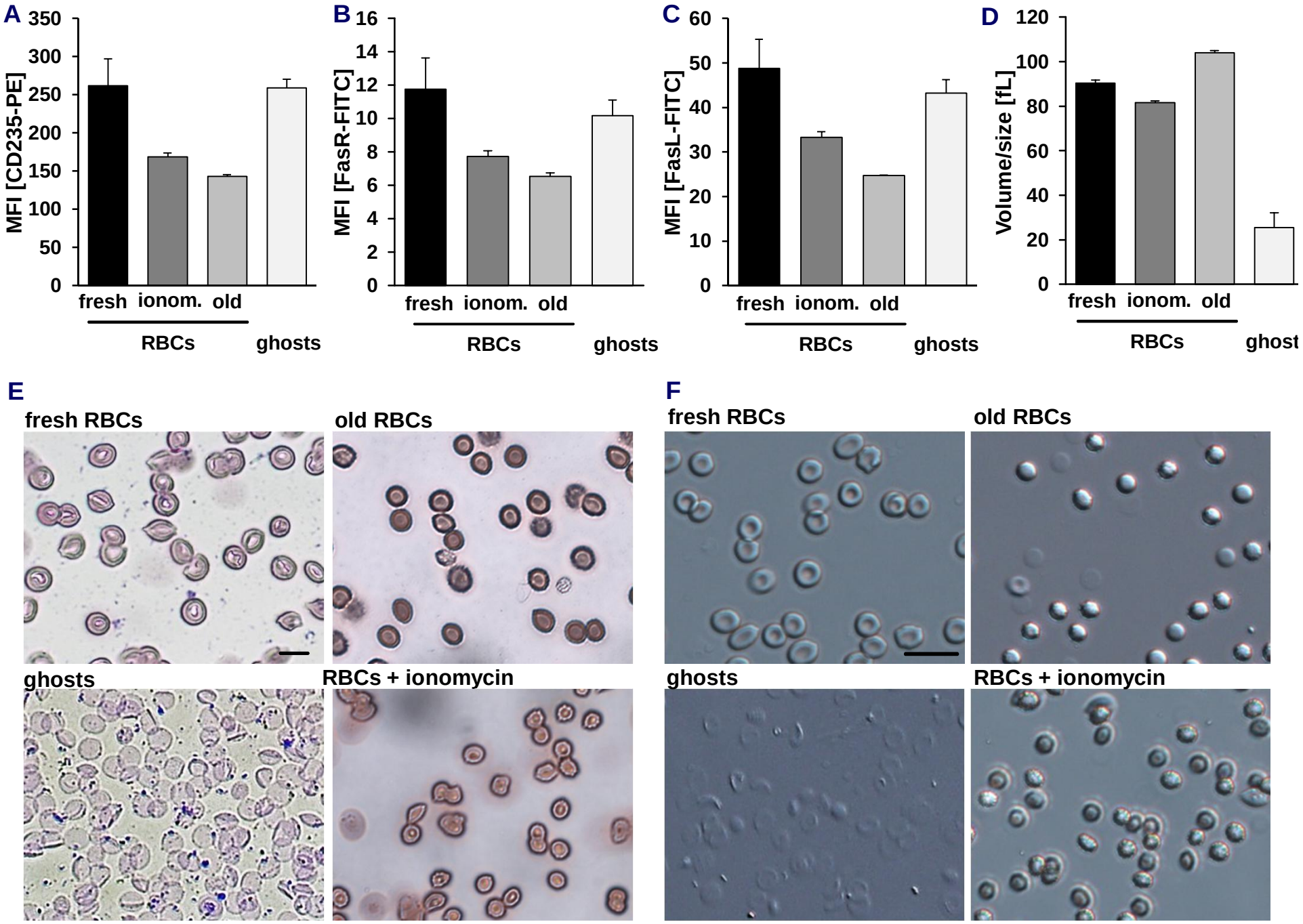

A
***
140
120
100
80
60
40
20
0
Volume/size [fL]
fresh RBCs
fix. RBCs
B
puncture
puncture
Anemia (HCT <30%)
Day1
Day2
Day3
C
50
40
30
20
10
0
Hematocrit [%]
Control
anemic
D
120
100
80
60
40
20
0
Hematocrit [%]
Control
HCT +

Figure S3


A
MFI [CD235-PE]
350
300
250
200
150
100
50
0
fresh
ionom.
old
RBCs
ghosts
B
MFI [FasR-FITC]
16
14
12
10
8
6
4
2
0
fresh
ionom.
old
RBCs
ghosts
C
MFI [FasL-FITC]
60
50
40
30
20
10
0
fresh
ionom.
old
RBCs
ghosts
D
Volume/size [fL]
120
100
80
60
40
20
0
fresh
ionom.
old
RBCs
ghost
E
fresh RBCs
old RBCs
ghosts
RBCs + ionomycin
F
fresh RBCs
old RBCs
ghosts
RBCs + ionomycin

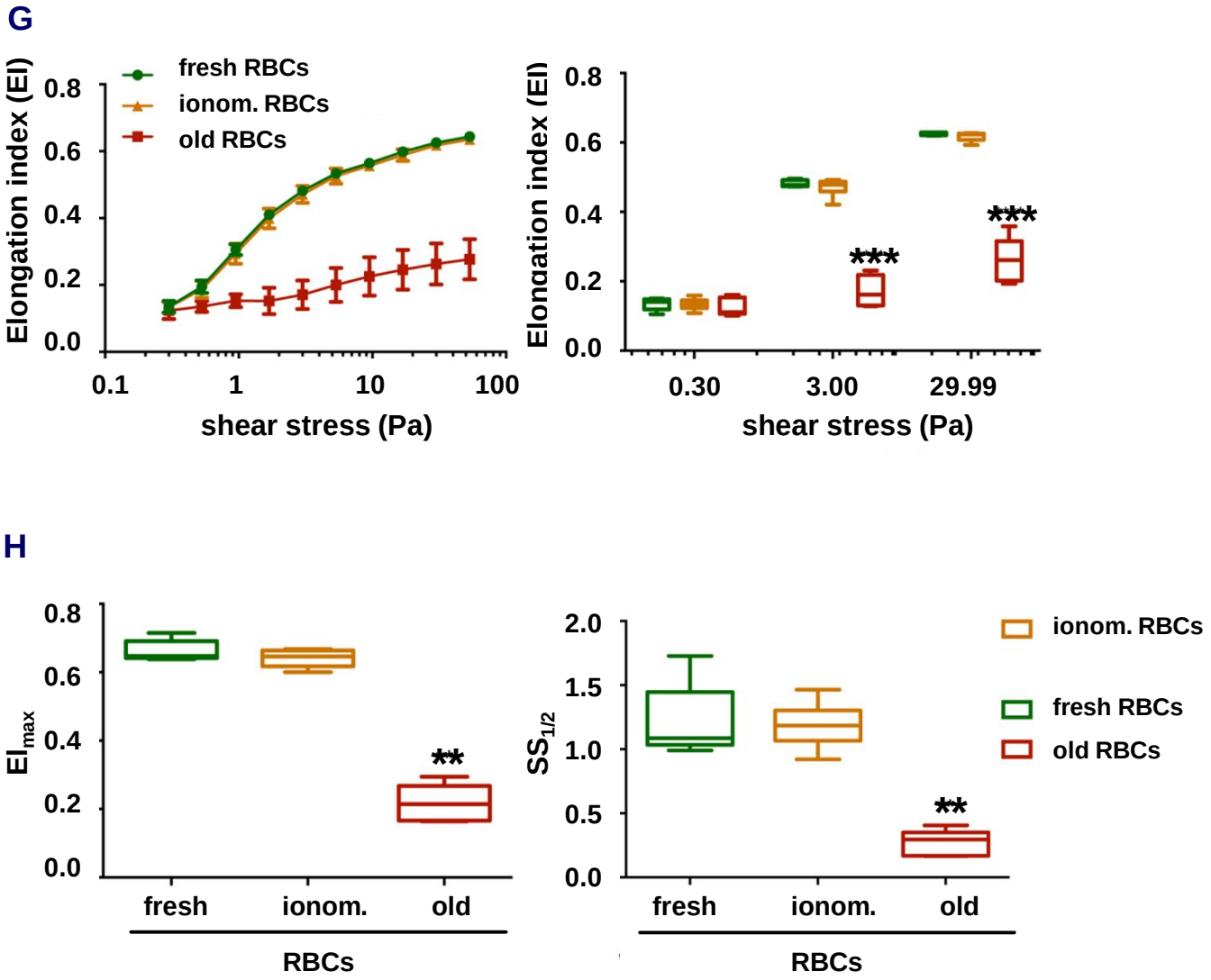
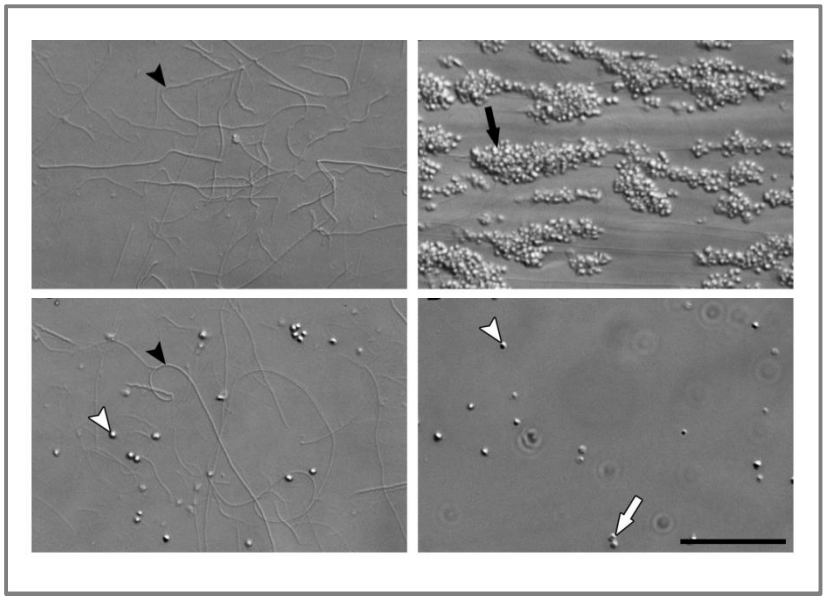

G
fresh RBCs
ionom. RBCs
old RBCs
Elongation index (EI)
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
shear stress (Pa)
Elongation index (EI)
0.30
3.00
29.99
shear stress (Pa)
***
***
H
EImax
**
fresh
ionom.
old
RBCs
SS1/2
**
ionom. RBCs
fresh RBCs
old RBCs

Figure S4

A



B

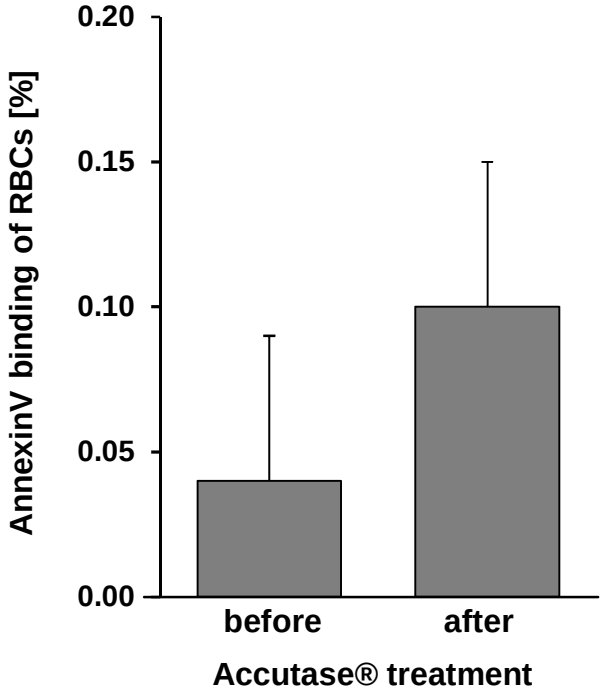

0.20
0.15
0.10
0.05
0.00
AnnexinV binding of RBCs [%]
before
after
Accutase® treatment

Figure S5

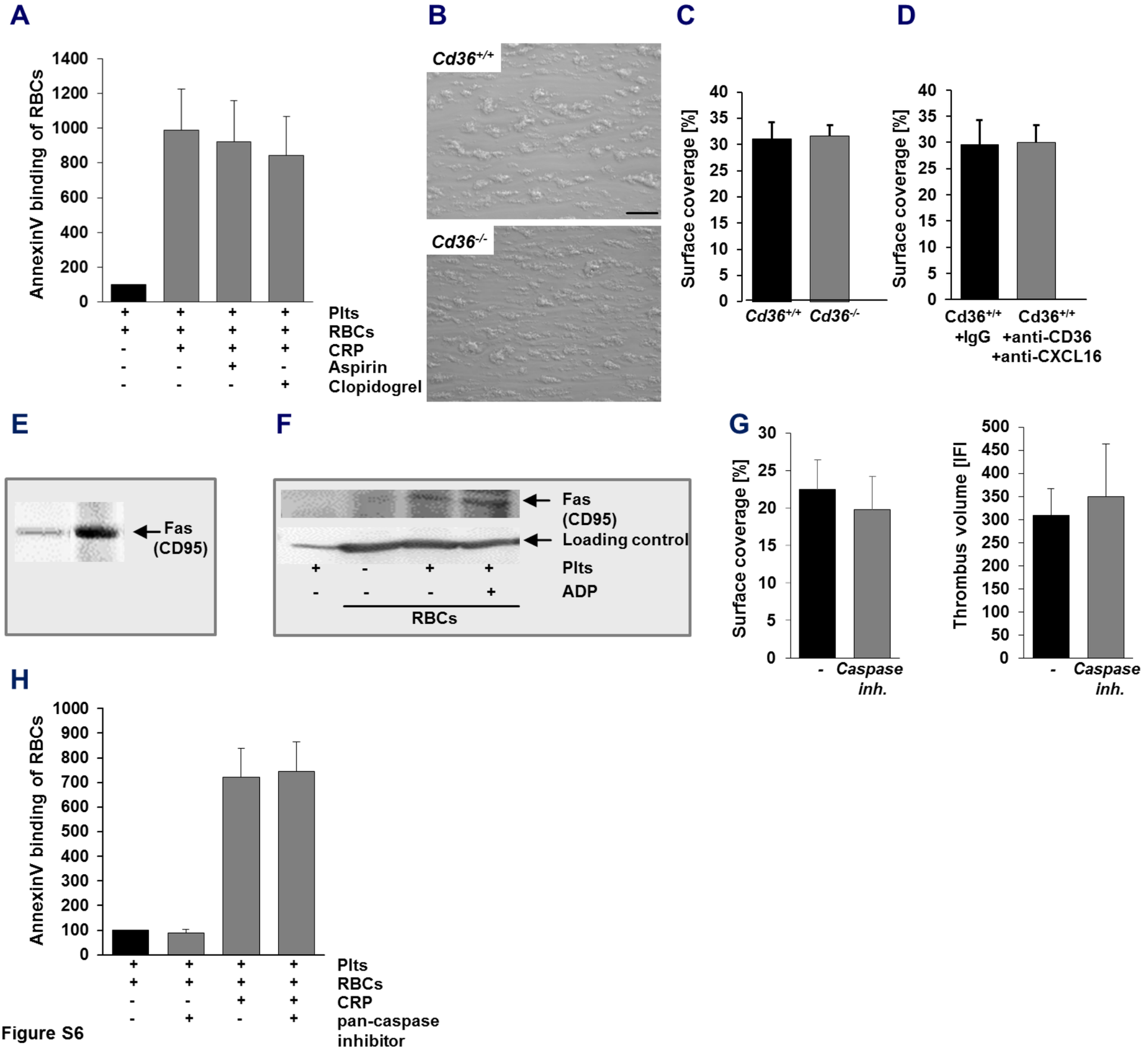

A
AnnexinV binding of RBCs
1400
1200
1000
800
600
400
200
0
+ + + +
+ + + +
- + + +
- - + -
- - - +
Plts
RBCs
CRP
Aspirin
Clopidogrel
B
Cd36+/+
Cd36-/-
C
Surface coverage [%]
40
35
30
25
20
15
10
5
0
Cd36+/+
Cd36-/-
D
Surface coverage [%]
Cd36+/+ +IgG
Cd36+/+ +anti-CD36 +anti-CXCL16
E
Fas (CD95)
F
Fas (CD95)
Loading control
+ - + +
- - - +
Plts
ADP
RBCs
G
Surface coverage [%]
30
25
20
15
10
5
0
-
Caspase inh.
Thrombus volume [IFI
500
450
400
350
300
250
200
150
100
50
0
-
Caspase inh.
H
AnnexinV binding of RBCs
1000
900
800
700
600
500
400
300
200
100
0
+ + + +
+ + + +
- - + +
- + - +
Plts
RBCs
CRP
pan-caspase inhibitor

Figure S6

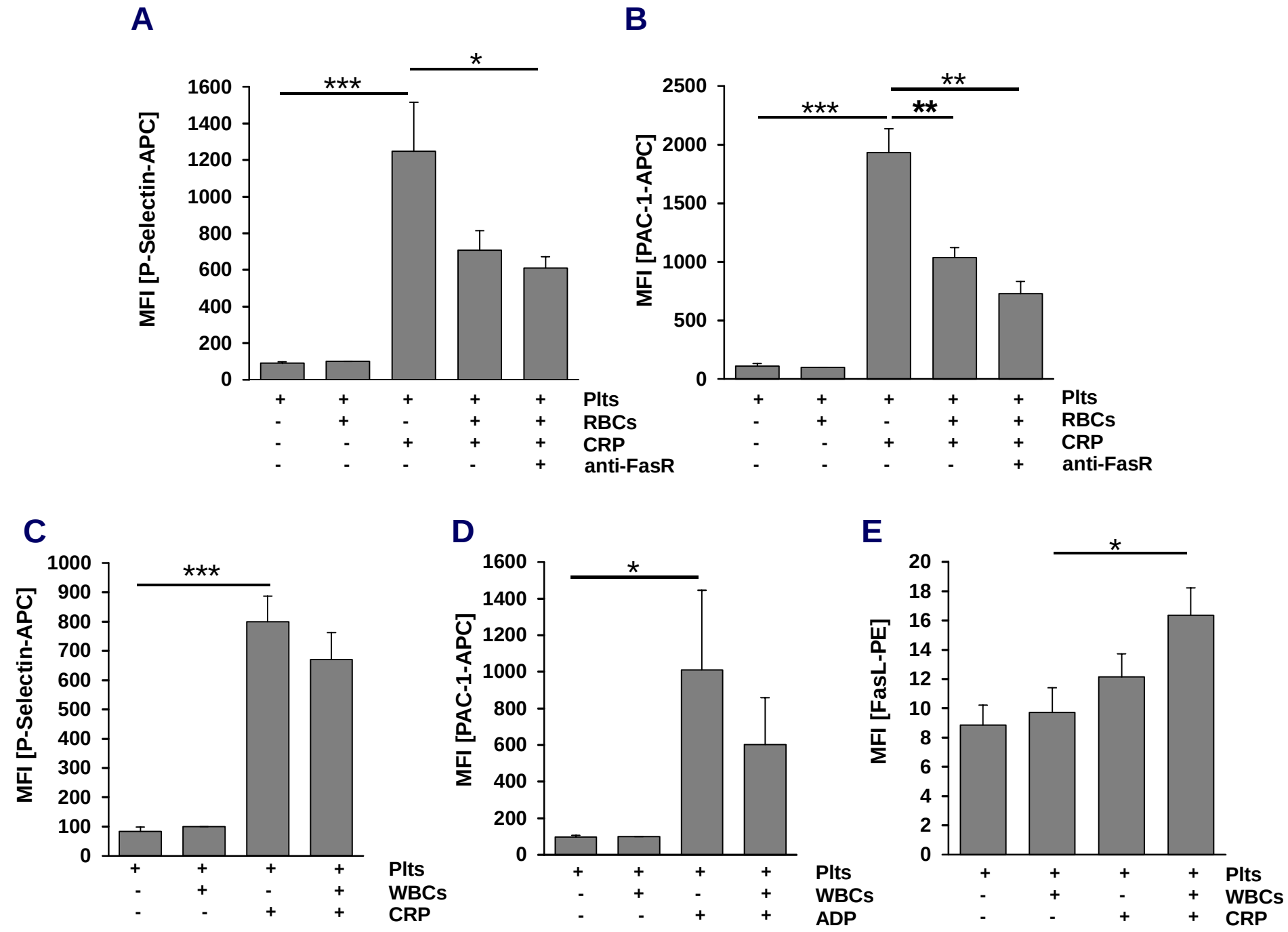
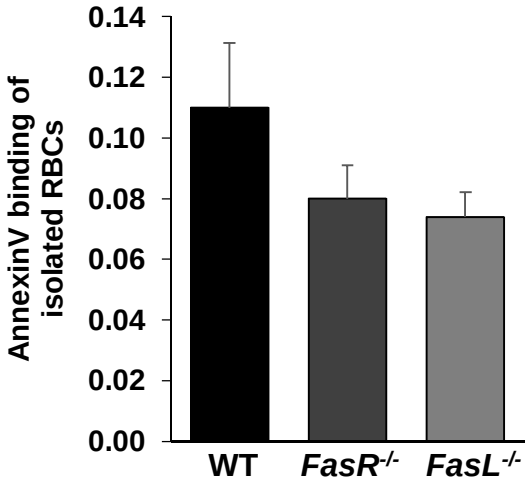

A
B
C
D
E
MFI [P-Selectin-APC]
MFI [PAC-1-APC]
MFI [FasL-PE]
***
*
**
Plts
RBCs
CRP
anti-FasR
WBCs
ADP

Figure S7

A

	Wild-type	*FasR*$^{-/-}$	*FasL*$^{-/-}$
Platelets (x10^3/µl)	1143.00 ± 88.73	923.00 ± 61.34	858.00 ± 104.76
RBCs (x10^6/µl)	9.47 ± 0.8	9.02 ± 0.65	8.61 ± 0.26
Hematocrit (%)	48.40 ± 5.06	49.20 ± 3.97	43.20 ± 1.37

B



C

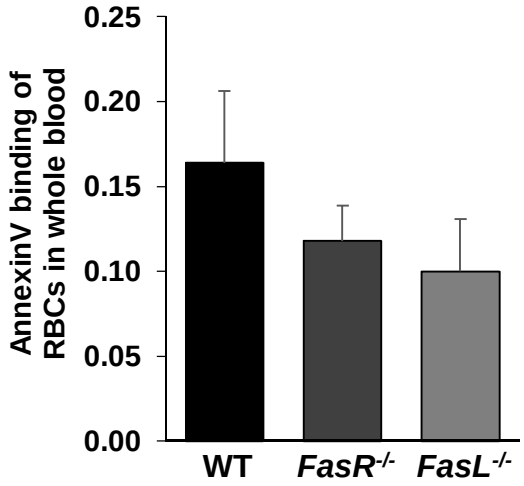


Figure S8

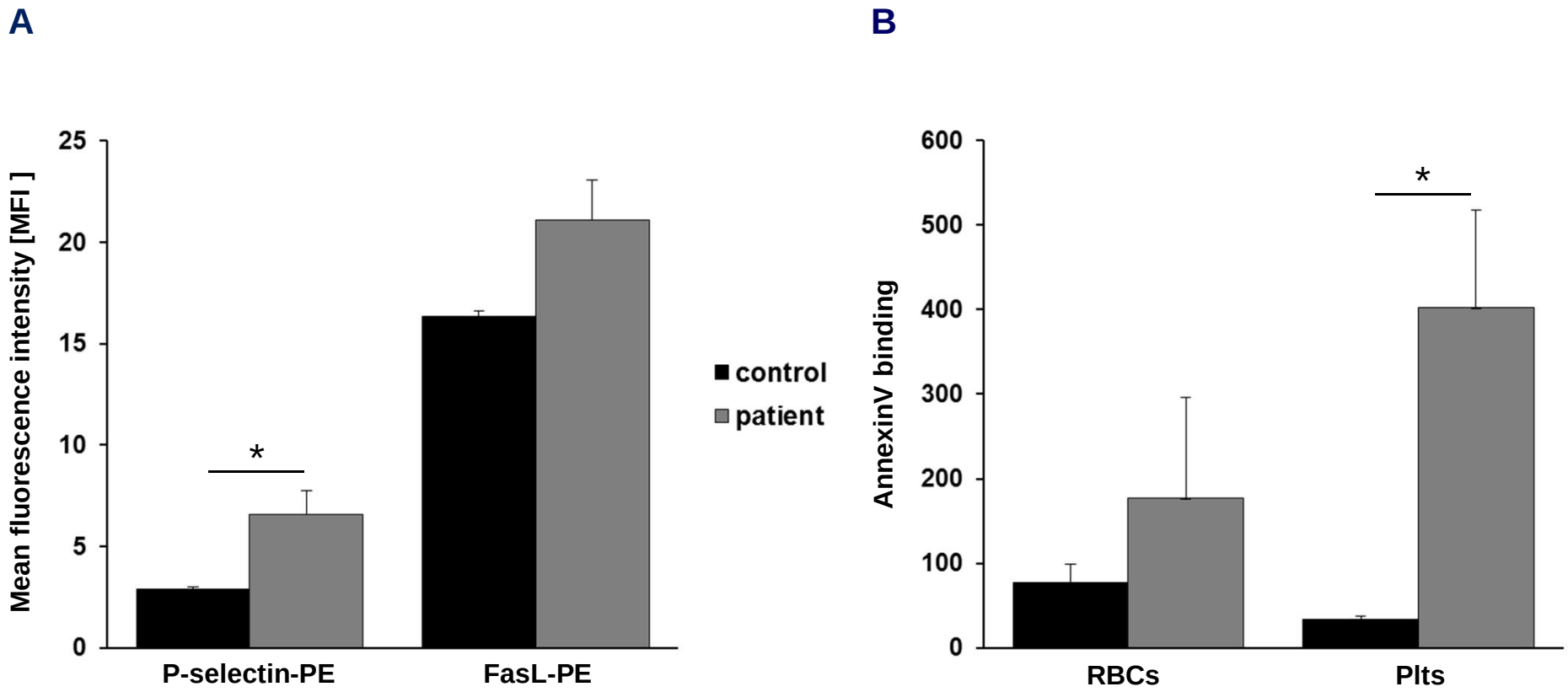

A
Mean fluorescence intensity [MFI]
25
20
15
10
5
0
*
P-selectin-PE
FasL-PE
control
patient
B
AnnexinV binding
600
500
400
300
200
100
0
*
RBCs
Plts

Figure S9