

Supplementary material to:

Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation: regulation by transient integrin activation

Running title: formation of procoagulant microdomains in thrombi

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Methods

Materials. Convulxin was purified to homogeneity from the venom of *Crotalus durissus terrificus* (Latoxan, France).¹ Annexin A5 labeled with Oregon Green (OG)-488, Alexa Fluor-568 (AF568) and AF647 were from Molecular Probes (Leiden, The Netherlands), as were OG488-conjugated human fibrinogen, Syto-44, carboxyfluorescein succinimidyl ester (CFSE) and AF532-labeled streptavidin. Fluorescein isothiocyanate (FITC)-labeled monoclonal antibody against human P-selectin (α -CD62 mAb) was from WAK Chemie (Steinbach, Germany); FITC-labeled anti-CD61 mAb, FITC-labeled PAC1 antibody and control antibody from BD Biosciences (San Jose, CA); (FITC-labeled) anti-phosphotyrosine mAb 4G10 from Upstate Biotechnology (Dundee, UK); anti-actin mAb AC-40 from Sigma (St. Louis, MO). Lotrafiban was a gift from GlaxoSmithKline (Middlesex, UK). Biotin-pentylamine-succinylated bovine serum albumin (BPA-sBSA), prepared by reacting succinylated BSA with biotin-pentylamine, was kindly provided by dr. G. Dale (Dept. of Medicine, Health Science Center, Oklahoma). BPA-sBSA recognizes serotonin binding sites on coated platelets and is less sensitive to oxidation than the parent compound, biotin-BSA-(5HT)_n.² Biotin was detected with AF488- or AF532-labeled streptavidin. Other materials were from sources described earlier.³

Fluorescent coagulation factors. Active site OG-labeled, human factor Xa was prepared by inactivation of native factor Xa with N^{α} -[(acetylthio)acetyl]-(D-Phe)-Pro-Arg-CH₂Cl, and covalent modification with OG488-iodoacetamide following mild NH₂OH treatment, as described.⁴ OG-prothrombin was labeled at the active site by a similar method, following formation of the catalytic site on the prothrombin zymogen by the use of a

staphylocoagulase fragment.⁵ Stoichiometries of OG incorporation into factor Xa and prothrombin were 0.8 and 0.9 mol probe per mol protein, respectively. OG-prothrombin had 0.1% residual native prothrombin, determined as described.⁵ Bovine factor V, purified as described,⁶ was suspended in phosphate-buffered saline and labeled with AF488 protein labeling kit using a PD-10 column (Molecular Probes). The AF-488 labeled factor V contained 15 mol probe per mol protein. After activation with thrombin, the labeled factor Va had retained its cofactor function in enhancing factor Xa activity. SDS-gel electrophoretic analysis under reducing conditions showed that both the heavy and light chains of factor Va were labeled. Concentrations of labeled coagulation factors were confirmed by protein assay.

Collection of human and mouse blood. Blood was obtained from healthy volunteers with full informed consent. For experiments in the absence of coagulation, blood was collected into 40 μ M *D*-Phe-Pro-Arg-chloromethyl ketone (PPACK) in 10% saline, and supplemented hourly with additional 20 μ M PPACK. For experiments involving coagulation, blood was collected in 10% 0.129 M trisodium citrate.

Wildtype 12-week-old C57Bl/6 mice of either sex (20-25 g) were obtained from Charles River (Maastricht, The Netherlands). Blood was collected under anesthesia with ketamine and xylazine (Eurovet, Bladel, The Netherlands) by orbital puncture. For experiments in the absence of coagulation, blood was collected in PPACK/heparin;⁷ for experiments involving coagulation, blood was collected into 0.129 M trisodium citrate. Experiments were approved by the local animal care and use committees.

Two-photon laser scanning microscopy (TPLSM) and confocal microscopy. TPLSM was performed with a BioRad 2100 multiphoton system. Excitation was with a Spectra Physics Tsunami Ti:Sapphire laser, tuned and mode-locked at 800 nm, producing pulses of 100 fs wide (repetition rate 82 MHz), which was connected to an upright Nikon E600FN fluorescence microscope.⁸ Two photomultipliers with separate pinholes detected fluorescence, selected by optical filters, at 508-523 nm and 570-620 nm. A third photomultiplier detected fluorescence above 660 nm from a parallel-placed red diode laser, exciting at 647 nm. This system was used for multicolor, confocal scanning of thrombi in flow chambers during or after perfusion, and for deep scanning of intact carotid arteries, mounted in a home-build perfusion chamber. Thrombi were double or triple labeled to detect fluorescence from the probes OG488 or FITC (color-coded green), AF568 or Syto-44 (color-coded blue), and AF647 (color-coded red). Recording was at the following conditions: iris was open; photomultiplier was maximal (except when recording in confocal); no background was subtracted; threshold was automatically set by out-of-focus recording. Two recording variables were used: the laser power 3-25% (set at a minimum to get high quality images at a scanning rate of 166 lines/second and 2-3 Kalman averaging); the optical zoom which varied between 1-4 times. When combining confocal (one-photon) and two-photon recording (e.g. when measuring AF647 fluorescence together with OG488 or AF568 fluorescence), confocal images were scanned before the two-photon images, to avoid bleaching. Optical sections were scanned in Kalman filtering mode without further image processing. Note that background fluorescence was low, as red or infrared light was used for excitation of 1-photon or 2-photon probes, respectively.

Single-photon, two-color confocal laser scanning microscopy, using a Leica confocal microscope (DM, IRE2; Leica, Milton Keynes, UK), was performed as described.⁹ Analysis of all confocal images (gray level bit maps) and 3D reconstruction of images were with ImagePro/LaserPix software (Media Cybernetics, Silver Spring, MD). Degree of co-localization of two fluorophores was evaluated using the Pearson's correlation coefficient (R_r), which describes the overlap between two colored patterns, and is independent upon pixel intensity values. The overlap coefficient (R) and the sub-coefficients k_1 and k_2 , which vary with differences in color intensities, were also calculated.

Thrombus formation on collagen under flow. Human or murine blood was perfused at shear rate of 1000 s^{-1} over a collagen surface, as described.¹⁰ Briefly, glass coverslips were coated with fibrillar Horm type-I collagen, blocked with Hepes buffer pH 7.45 (5 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4 and 1% BSA), and then placed in a parallel plate flow chamber (slit depth of 50 μm). Blood pre-incubated for 15 minutes with inhibitors and/or probes, was perfused for up to 4 minutes over the collagen. Thereafter, the flow chamber was post-perfused for a further 4 minutes with Hepes buffer, pH 7.45 containing 2 mM CaCl_2 and 1 U/mL heparin. For coagulation experiments, citrate-anticoagulated blood was co-perfused with 10 vol% of Hepes buffer, pH 7.45 containing 20 pM tissue factor and 200 mM CaCl_2 upon entry into the flow chamber.¹⁰

Bright-field phase-contrast and fluorescence images of adherent platelets were also recorded using a non-confocal two camera system.¹¹ Surface coverage with platelets

was analyzed using ImagePro software. Data from >10 different fields of view were averaged per experiment without image processing.

Thrombus formation *in vivo*. Twelve-week-old mice were anesthetized by subcutaneous injection of ketamine and xylazine, followed by continuous infusion of ketamine. Fluorescently labeled compounds were administered intravenously through a PE-10 catheter in the tail vein.¹² Carotid arteries were dissected free and ligated vigorously for 5 minutes to induce vascular injury. In other animals, the adventitial surface of the carotid artery was damaged by local application of a filter paper (0.5×1.0 mm) with 1 M FeCl₃ for 5 minutes. With either method, thrombus formation was allowed to proceed for 10 minutes, after which fluorescence in the still intact vessel was monitored by TPLSM. For *ex vivo* evaluation, 5 mm of the artery was carefully removed, mounted between two micropipettes in a perfusion chamber, and subjected to 1.0 atmosphere pressure.⁸ Mounted arteries were post-labeled for 30 minutes with Syto-44 (2 μM in phosphate-buffered saline), to stain for nuclei.

Flow cytometry. Diluted PRP or washed platelets (1×10^7 platelets/mL) were activated with 50 ng/mL convulxin and 4 nM thrombin in the presence of 2 mM CaCl₂ for 5-30 minutes (no stirring). Samples were incubated with fluorescent labels and/or antibodies, as described in the text. For double labeling experiments, probes exciting at 488 and 647 nm were added simultaneously at saturating concentrations. Detection of fluorescence was with a FACScan flow cytometer, equipped with an argon and a red diode laser (Becton-Dickinson, Franklin Lakes, NJ). For analysis, platelets were gated based on their

forward scatter/side scatter (FSC/SSC) characteristics, rigorously excluding small events (e.g., microparticles). A minimum of 10,000 events was counted per assay. Control measurements were performed with unlabeled/stimulated and labeled/unstimulated platelets. List-mode data were analyzed using WinMDI 2.8 software (<http://facs.scripps.edu>).

Protein tyrosine phosphorylation in adherent platelets. After flow experiments, adherent platelets on collagen-coated coverslips were stained with AF647-annexin A5, and then fixed for staining with FITC-4G10 anti-phosphotyrosine mAb. Alternatively, platelets on coverslips were carefully lysed with ice-cold NP-40-based lysis buffer pH 7.4 (300 mM NaCl, 20 mM Tris-HCl, 2 mM EGTA, 2 mM EDTA, 2 mM Na₃VO₄, 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin, 2% NP-40). Protein was quantified in the lysates with a BioRad DC protein kit. Lysates with calculated equal protein amounts were resolved on 10% SDS-PAGE gels, then transferred to blotting membranes by semi-dry transfer. Membranes were immunoblotted using an ECL system (Amersham Biosciences, Bucks, UK) with 4G10 mAb followed by stripping of the membranes and re-probing with AC-40 anti-actin mAb to verify equivalent loading.¹³

Statistics. Differences were tested on significance with the Mann-Whitney U test using the statistical package for social sciences (SPSS 11.0, Chicago, IL). Results are given as mean values ± SD, unless otherwise indicated.

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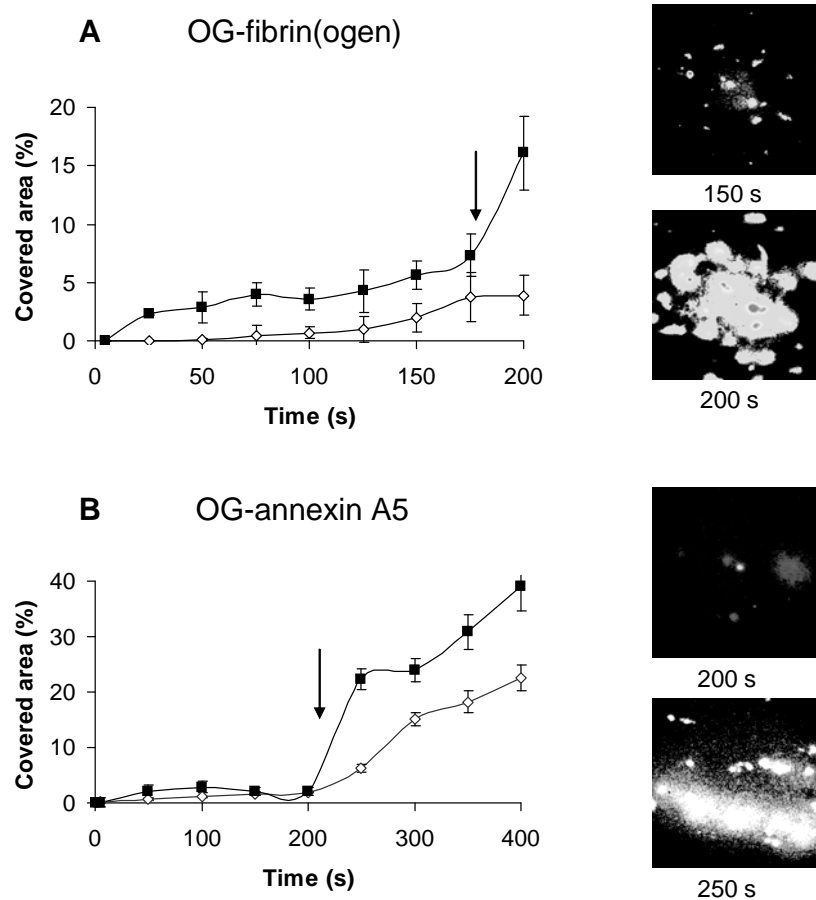


Figure. I. Time-dependent accumulation of labeled fibrin(ogen) and annexin A5 in thrombi formed in the absence or presence of coagulation. Human blood containing 0.2 mg/mL OG-fibrinogen (**A**) or 0.5 $\mu\text{g/mL}$ OG-annexin A5 (**B**) was perfused over collagen at a shear rate of 1000 s^{-1} , and fluorescent images were captured from the collagen surface. PPACK-anticoagulated blood was used (open diamonds) or, alternatively, citrate-anticoagulated blood that was co-perfused with 2 pM tissue factor and 2 mM free CaCl_2 (final concentrations) to reach physiological $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentrations (filled squares). Data show mean surface area coverage with fluorescence \pm SD ($n = 4$); arrows indicate appearance of microscopic clots. Images ($150 \times 150 \mu\text{m}$) shown are examples of experiments in which coagulation occurred (150-250 s).

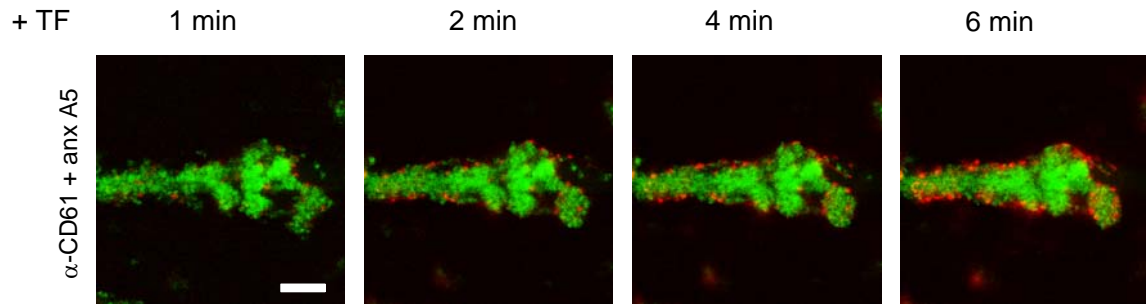


Figure. II. Time dependency of segregation of microdomains of aggregated and procoagulant platelets. Human blood was perfused over collagen in the presence of tissue factor/ CaCl_2 for 1-6 minutes, as described for Figure 1. Blood was pre-labeled with FITC- α -CD61 (*green*) and AF647-annexin A5 (*red*). TPLSM images were recorded from the collagen surface during perfusion. Representative images are shown; bar represents 20 μm .

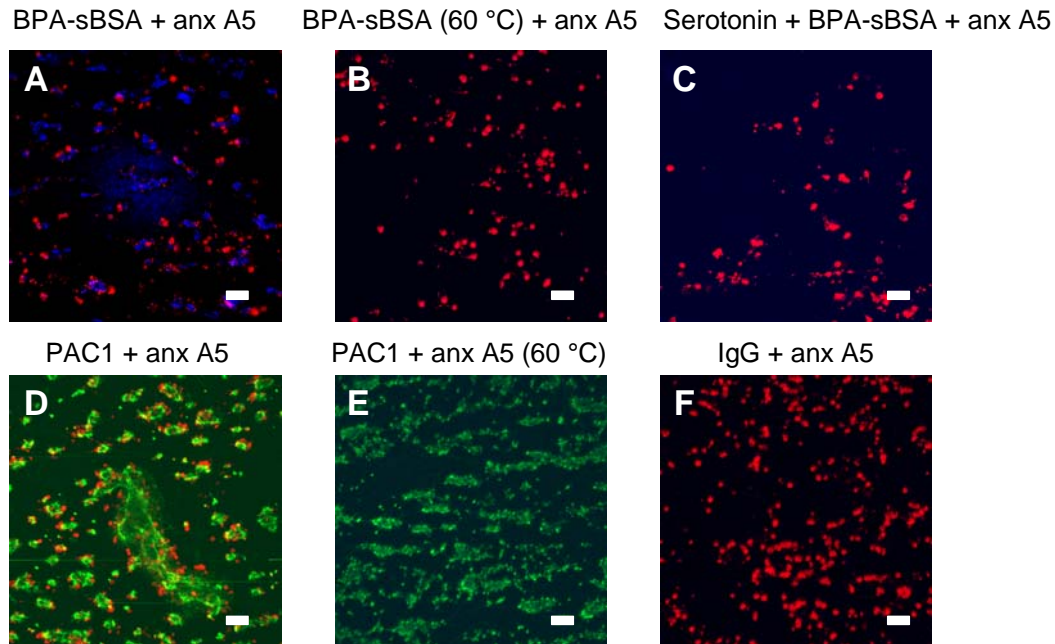


Figure. III. Heterogeneity of platelet activation during thrombus formation under flow. Human, PPACK-anticoagulated blood was perfused over collagen, as described for Figure 1. Blood was pre-incubated with the following labels: **(A)** pre-incubation with 50 µg/mL BPA-sBSA, and thrombi were post-labeled with 1 µg/mL AF532-labeled streptavidin plus AF647-annexin A5; **(B)** pre-incubation with BPA-sBSA plus heat-denatured AF647-annexin A5 (5 minutes 60 °C); **(C)** pre-incubation with 1 mM serotonin, followed by staining with BPA-sBSA and AF647-annexin A5. **(D)** 0.6 µg/mL FITC-PAC1 plus 0.5 µg/mL AF647-annexin A5; **(E)** FITC-PAC1 plus heat-denatured AF647-annexin A5 (5 minutes 60 °C); **(F)** 0.5 µg/mL FITC-IgG with AF647-annexin A5. TPLSM images are shown of FITC-PAC1 (*green*), BPA-sBSA (*blue*) and/or AF647-annexin A5 (*red*) fluorescence. Images are representative of 4-8 experiments; bars indicate 20 µm.

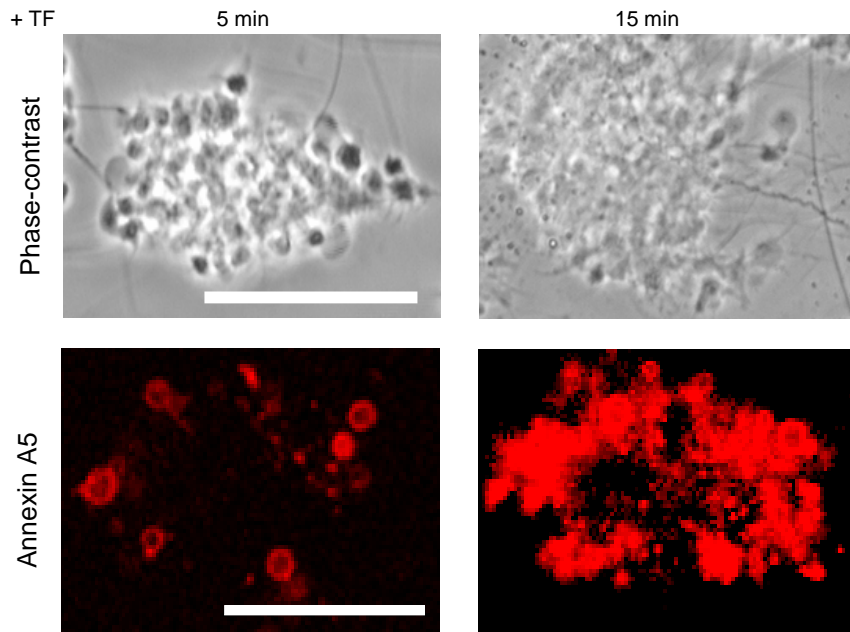


Figure IV. Bleb-shaped annexin A5-binding platelets formed on fibrin-containing aggregates. Human thrombi were formed on collagen during perfusion with tissue factor/ CaCl_2 for 5-15 minutes at shear rate of 1000 s^{-1} . The thrombi were post-labeled with AF647-annexin A5 (*red*); phase contrast and fluorescence images were recorded as for Figure 1. Representative images shown after 5 and 15 minutes of perfusion, as indicated. Bars indicate $20 \mu\text{m}$.

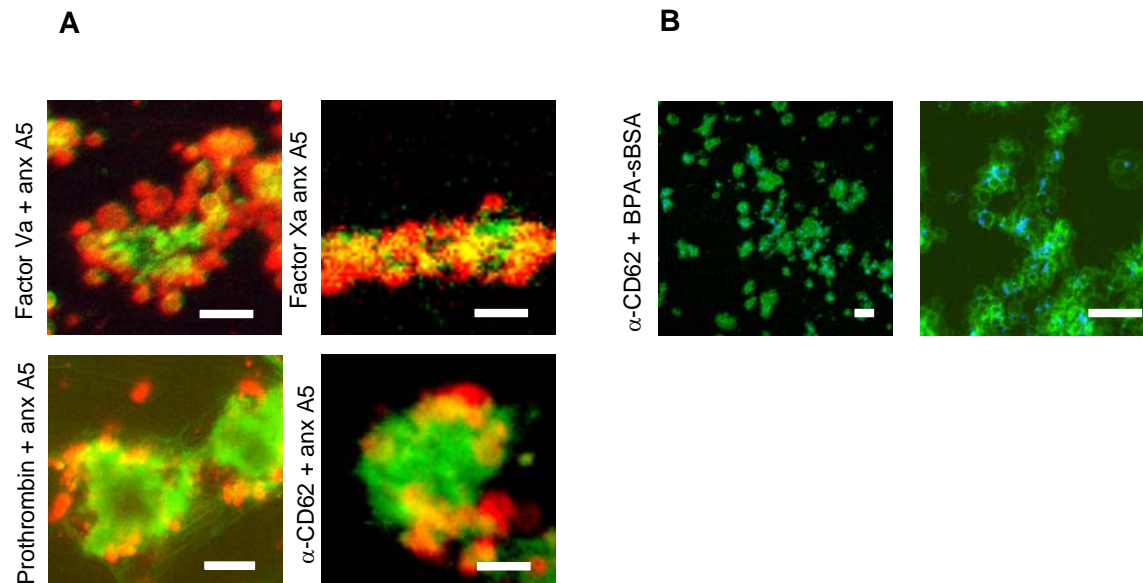


Figure. V. Heterogeneity in platelet binding of coagulation factors, annexin A5 and BPA-sBSA in thrombi. Human thrombi were formed on collagen under flow in the presence of tissue factor/ CaCl_2 , as described for Figure 1. **(A)** Blood was pre-labeled with $0.5 \mu\text{g/mL}$ AF647-annexin A5 (*red*) plus either AF488-factor Va (20 nM), OG-prothrombin (200 nM), OG-factor Xa (100 nM) or FITC- α -CD62 mAb ($1.25 \mu\text{g/mL}$) (*green*). **(B)** Blood was pre-labeled with $1.25 \mu\text{g/mL}$ FITC- α -CD62 mAb (*green*) together with $50 \mu\text{g/mL}$ BPA-sBSA and $1 \mu\text{g/mL}$ AF532-labeled streptavidin (*blue*). Representative TPLSM fluorescence images are shown of AF647-annexin A5 (*red*), coagulation factor or antibody (*green*), and BPA-sBSA (*blue*) fluorescence. Optical zoom 3-4 \times . Yellow color indicates co-localization of green and red staining; bars indicate $20 \mu\text{m}$.

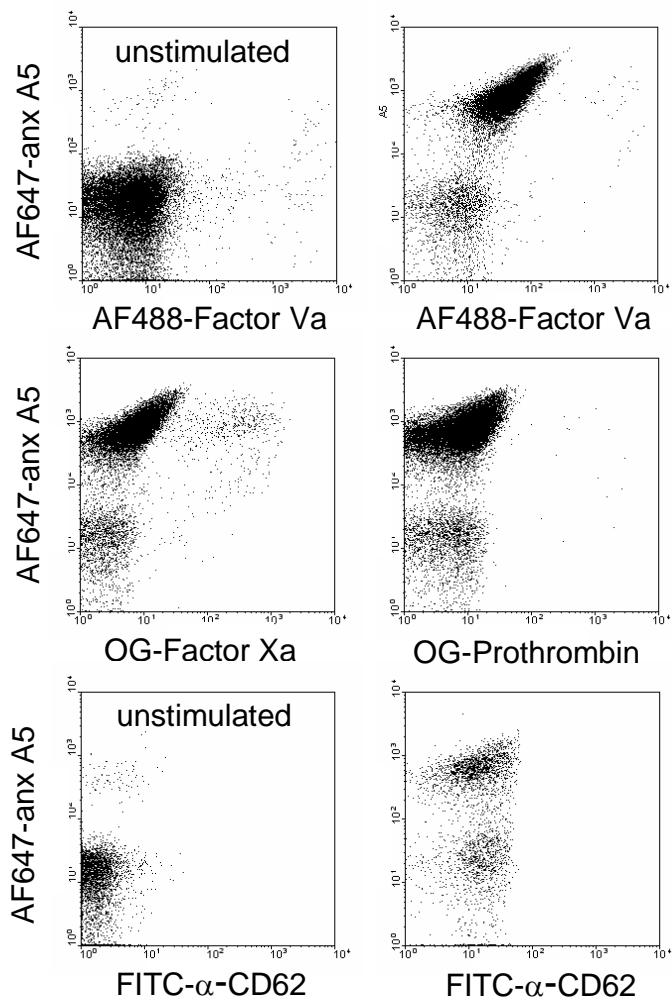


Figure. VI. Heterogeneity in binding properties of PS-exposing platelets. Platelets in suspension were stimulated or not with 50 ng/mL convulxin, 4 nM thrombin and 2 mM CaCl_2 for 10 minutes. Two-color flow cytometry after staining with AF647-annexin A5 (0.5 $\mu\text{g}/\text{mL}$) plus either 20 nM AF488-factor Va (20 nM), OG-factor Xa (100 nM), OG-prothrombin (200 nM) or FITC- α -CD62 mAb (1.25 $\mu\text{g}/\text{mL}$). Shown are dot plots of *FL1* (AF488, OG) versus *FL4* (AF647). Plots are representative of at least 4 experiments.

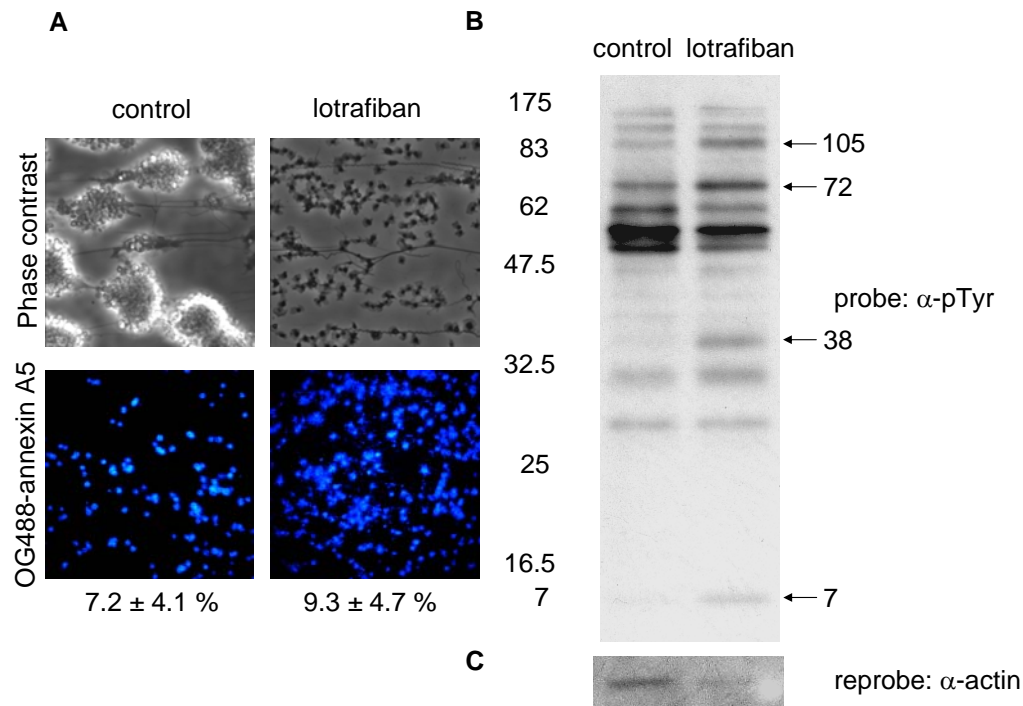


Figure VII. Role of integrin α IIb β 3 in tyrosine phosphorylation of collagen-adherent platelets. Human blood pre-treated with vehicle (control) or 10 μ M lotrafiban was flown over collagen for 4 minutes at a shear rate of 1000 s^{-1} , and stained with OG488-annexin A5. Numbers below images indicate mean surface area coverage \pm SD. (A) Representative phase-contrast (120 \times 120 μ m) and fluorescence (150 \times 150 μ m) images. (B) Flow chambers were perfused with lysis buffer to remove adherent platelets, and lysates (equalized for protein quantity) were run on a SDS-PAGE gel, transferred to PVDF and blotted for phosphotyrosine using mAb 4G10; (C) blots were stripped and re-probed for actin using mAb AC-40. Representative data of 2-4 experiments. Note the low actin staining for the lotrafiban lane, indicating the increased phosphorylation of 7, 38, 72 and 105 kDa proteins is underestimated for this gel.

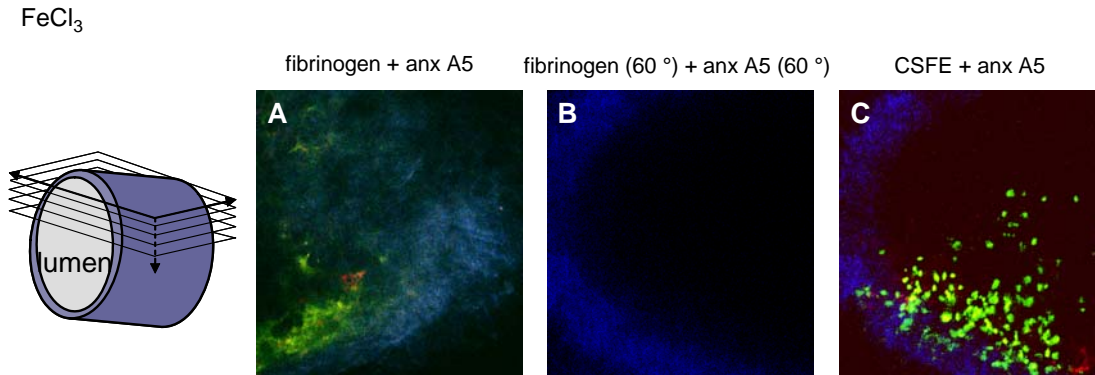


Figure VIII. Heterogeneity in arterial thrombi induced by FeCl₃ application. Mice were infused with the indicated probes (200 µg each): **(A)** OG-fibrinogen plus AF568-annexin A5; **(B)** heat-natured OG-fibrinogen and heat-denatured AF568-annexin A5 (5 minutes 60 °C); or **(C)** autologous CFSE-labeled platelets (5 µg/ml) and AF568-annexin A5. Then, the carotid artery was damaged by local application of saturated FeCl₃ (see Figure 6). Thrombus formation proceeded for 10 minutes, after which fluorescence inside the arteries was recorded by TPLSM (33% laser intensity). **(A)** Distinct patches of fibrin(ogen) (*green*) and annexin A5 (*red*) at the luminal side of the vessel wall (*blue* autofluorescence). **(B)** Control image after injection of heat-denatured fibrinogen and annexin A5. Note absence of green and red staining. **(C)** Accumulation of CFSE-labeled platelets (*green*), partly also binding to annexin A5 (*yellow*) at the luminal side of the vessel wall. Images represent 206×206 µm, and are from two or more mice.

Movie A. Heterogeneity in arterial thrombi induced by carotid ligation. Mice were infused with OG-fibrinogen and AF-annexin A5 and the carotid artery was damaged by tight ligation for 5 minutes. Thrombus formation proceeded for 10 minutes, after which fluorescence inside the artery was recorded by TPLSM. Three-dimensional reconstruction of fibrin(ogen) (*green*) and annexin A5 (*red*) fluorescence in the ligated artery.