Supplemental material

Detailed Methods

Reagents

4-Amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate and Prolong Gold antifade reagent were obtained from Invitrogen, (Mississauga, ON, Canada). L-Arginine, N-nitro-L-arginine methyl ester hydrochloride (L-NAME), N-([3-(Aminomethyl)phenyl]methyl)-ethanimidamide dihydrochloride (1400W), TXB_2, prostacyclin, sildenafil, superoxide dismutase (SOD), poly-lysine solution, bovine serum albumin, gelatin, anti-sialic acid acetylesterase (HPA038053) and monoclonal anti-β-actin (clone AC15) antibody were obtained from Sigma-Aldrich. 1-Oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine (TEMPOL) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies recognizing human eNOS at the C- (clone M221) and N- (clone 6H2) terminals, sGC alpha 1 subunit (clone GC10), PKG (PRKGR1B) (ab9050200), VASP (ab26650), activated caspase-3 (ab13847), COX-1 (clone EPR5866), MMP-2 (clone EPR1184), and a mouse IgG1 isotope control (ICIGG1) were obtained from Abcam (Cambridge, MA, USA). Unlabeled PAC-1, anti-human CD42b-PE (clone HIP1), anti-human CD41a-PE-Cy7 (clone HIP8), mouse IgG1-PE (clone MOPC-21), and anti-mouse IgM-PE-Cy7 antibody (clone R6-60.2) were obtained from BD Pharmingen (Mississauga, ON, Canada). DyLight488-conjugated donkey anti-mouse IgG, Alexa488-conjugated goat $(F(ab')_2$ anti-mouse IgG, R-phycoerythrin-conjugated goat $(F(ab')_2$ anti-mouse IgG, PerCP-conjugated anti-rabbit IgG, R-phycoerythrin-conjugated anti-rabbit IgG, and rabbit and goat IgG whole molecule (ChromPure) were obtained from Jackson ImmunoResearch, Inc. (West Grove, PA, USA). Goat anti-human caveolin-1 antibody (AF5736) was purchased from R&D Systems (Minneapolis, MN, USA). ABT-737 was obtained from Selleck Chemicals. 2-{4,5-Bis[(6-(2-ethoxy-2-oxoethoxy-2-methylquinolin-8-ylamino)methyl]-6hydoxy-3-oxo-3H-xanthen-9-yl}benzoic acid FL2E nitric oxide sensor (CuFL2E) was obtained from STREM (Newburyport, MA, USA). 1 μm fluorescent yellow-green and 0.5 μm fluorescent orange polystyrene latex beads were obtained from Sigma. Size standard 5 μm and 3.8 μm Spherotech beads were obtained from Beckman Coulter. FITC anti-human CD41b (clone HIP2), PE anti-human CD45 (clone

HI30), FITC IgG3 (clone J606) were obtained from BD Pharmingen. Cardiac and lung-derived human microvascular endothelial cells (HMVEC) were obtained from Lonza (Walkersville, MD, USA). HUVEC-C and Meg-01 were obtained from the ATCC.

An eNOS inhibiting peptide, as described by Sessa and colleagues,[1] containing caveolin-1 amino acids 82-95 fused to the C terminus of the *Antennapedia* internalization sequence was synthesized by the Alberta Proteomics and Mass Spectrometry Facility. The peptide sequence (NH_2-RQIKIWFQNRRMKWKKDGIWKASFTTFTVT-COOH) was confirmed by mass spectrometry. The peptide was dissolved in H_2O:acetic acid (7:3 – condition used for purification) to 2 mM and subsequently diluted to a stock 100 μM solution with Tyrode's buffer containing 10 mM HEPES. The corresponding vehicle for the peptide was prepared in the same manner.

Platelet isolation and aggregometry

The study was approved by the University of Alberta Human Research Ethics Board. Following informed consent blood was obtained from healthy volunteers (22-35 years of age) who had not taken any drugs known to affect platelet function for 2 weeks prior to the study. Prostacyclin-washed platelet suspensions ($2.5x10^8$/mL) were prepared as described previously.[2] Washed platelets were pre-incubated (2 minutes at 37°C) in a lumi-aggregometer (Chronolog, Havertown, PA, USA) with either vehicle or L-arg, L-NAME, SOD, TEMPOL, 1400W, and/or AP-Cav-AB. Platelet aggregation was then initiated with collagen (1 - 10 μg/mL) or thrombin (0.01 – 0.03 U/mL) (Chronolog) and monitored by Aggro-Link software for further 3 - 4 minutes.

Determination of DAF-FM specificity for nitric oxide

In order to assess the specificity of DAF-FM for NO, DAF-FM-stained platelets were pre-incubated with L-arg (100 μM) and L-NAME (100 μM), SOD (100U/mL), TEMPOL (100 μM), or 1400W (30 μM) for 2 min at 37°C in a lumi-aggregometer (Chronolog, Havertown, PA). Platelet aggregation was initiated by collagen (5μg/mL) and monitored by Aggro-Link software for a further 4 min as described above. Following aggregation, DAF-FM-stained platelet samples were incubated with anti-CD42b-PE antibody

(1:100) for 10 minutes in the dark and then diluted to a final volume of 1 mL with saline before analysis with a Beckman Coulter Quanta SC flow cytometer with Cell Lab Quanta analysis software. 10,000 platelet-specific events were analyzed and compensation was performed to account for fluorophore spectral overlap.

Additional experiments were carried out with platelets stained with the fluorescent NO sensor 2-{4,5-bis[(6-(2-ethoxy-2-oxoethoxy-2-methylquinolin-8-ylamino)methyl]-6hydoxy-3-oxo-3H-xanthen-9-yl}benzoic acid FL2E nitric oxide sensor (CuFL2E). CuFL2E staining of platelets was carried out as described above for DAF-FM-stained platelets. CuFL2E-stained platelets were then pre-incubated with L-arg (100 μM) and L-NAME (100 μM) or SOD (100U/mL) for 2 min at 37°C in the lumi-aggregometer. Platelet aggregation was initiated by collagen (5μg/mL) and monitored by Aggro-Link software for a further 4 min as described above. Following aggregation, CuFL2E-stained platelet samples (10 μl) were diluted to a final volume of 1 mL with saline before analysis on a Becton Dickinson Aria III BSL-2 FACS or Beckman Coulter Quanta SC flow cytometer. 10,000 events were analyzed and the mean fluorescence on FL1 determined. The results were expressed as CuFL2E mean fluorescence following subtraction of the mean autofluorescence of unstained platelet samples.

To confirm the integrity of platelet membranes resting platelets were incubated at room temperature for 30 minutes in the presence or absence of 50% ethanol. Subsequently platelet samples were incubated with anti-CD41a-FITC antibody (1:100) and propidium iodide (5 μg/ml) for 10 minutes in the dark and then diluted to a final volume of 1 mL with saline before analysis with a Beckman Coulter Quanta SC flow cytometer with Cell Lab Quanta analysis software. 10,000 events were analyzed.

To determine if DAF-FM may detect changes in NO production by maximally stimulated endothelial cells HMVEC-C were cultured under standard conditions in EGM-2 MV as described previously.[3] HMVEC-C were washed 2x with 2 ml of PBS and then incubated in PBS for 30 minutes at 37^{o}C, after which DAF-FM diacetate (5 μM) was added to the HMVEC-C and incubated for a further 20 minutes. The DAF-FM was washed out 2x with 2 ml PBS, and 100 μM L-arginine in Tyrodes's buffer added to the HMVEC-C. Subsequently, the HMVEC-C were incubated for 10 minutes at 37^{o}C in the presence or absence of recombinant $VEGF_{165}$ (R&D Systems). The HMVEC-C were then trypsinized,

washed 1x with cold PBS, and the DAF-FM fluorescence analyzed by flow cytometry. 10,000 events were analyzed.

HMVEC-C eNOS confocal microscopy

HMVEC-C cells (seeding density: 5,000 cells/cm²) were cultured in an incubator (37°C, 5% CO_2, 90% humidity) for 3 days on sterile glass coverslips in 6-well tissue culture plates. Subsequently, cells were fixed in 4% formaldehyde in Tyrode's Buffer for 20 min and washed three times for 5min in PBS with gentle agitation. Cells were permabilized with 0.1% Triton X-100 for 25 minutes and washed three times for 5 minutes in PBS with gentle agitation. Next, cells were blocked in blocking buffer (5% goat serum in PBS and 0.02% Tween 20) for 2 hours. Mouse monoclonal eNOS M221 (1.25 μg/ml, Abcam), eNOS 6H2 (26 μg/ml, Abcam) primary antibodies and IgG1 isotype control (1.25 μg/ml and 26 μg/ml, Abcam) were added to blocking buffer. Coverslips were incubated on parafilm with primary antibodies for 1 hour and washed three times for 5min. with PBS and 0.02% Tween 20 with gentle agitation. Goat anti-mouse IgG (H+L), F(ab')2 fragment specific Alexa Fluor 488 (15 μg/ml, Jackson Immuno Research) secondary antibodies were added to blocking buffer and incubated on parafilm with coverslips for 1 hour. Next, coverslips were washed three times for 5min. with PBS and 0.02% Tween 20 with gentle agitation. 5 μl of Alexa Fluor 568 Phalloidin (Invitrogen) was diluted in 200 μl PBS and incubated on parafilm with coverslips for 1 hour. Coverslips were washed 3 times with PBS and mounted onto microscope slides along with 5 μl of ProLong Gold Antifade solution. Slides were left for 24 hours in 4°C, next sealed with nail polish and imaged when dry. An Olympus IX-81 (Olympus America Inc., Melville, NY, U.S.A.) motorised microscope base with Yokagawa CSU10 (Yokogawa Electric Corporation, Tokyo, Japan) spinning disk confocal scan-head equipped with digital camera Hamamatsu EMCCD (C9100-13) (Hamamatsu, Photonics K.K., Hamamatsu City, Japan) was used to image HMVEC-C cells. Magnification was set to 60X/1.42 Oil and lasers used were for Alexa 488 - 50mW 491nm laser and for Alexa 568 - 50mW 561nm laser. Images were acquired using Perkin Elmer's Volocity software (Perkin Elmer Inc., Waltham, MA, USA).

Activated caspase-3 flow cytometry

In order to investigate the circulatory age of eNOS^{neg} to eNOS^{pos} platelet subpopulations intracellular flow cytometry for activated caspase-3 was performed. Human platelet samples were fixed and prepared for intracellular eNOS flow cytometry as described in main methods. Next, the samples were incubated overnight at 4^{o}C with anti-activated caspase-3 antibody (4 μg/mL) (Abcam) or as control concentration matched rabbit IgG (Jackson ImmunoResearch). The platelet samples were washed three times with 1mL of PBS and then incubated with PerCP-conjugated secondary antibody (1:100) for 2 hrs in the dark with rocking. Following another round of washing, platelets were incubated with anti-CD41-PE-Cy7 antibody for 15 minutes and then diluted to a final volume of 1 mL with PBS before analysis with a Quanta SC MPL flow cytometer as described in main methods. 10,000 events were analyzed.

As a positive control for activated caspase-3, prior to processing for flow cytometry, platelets were incubated with 10 nM ABT 737 (Selleck) for 2 h at 37^{o}C. In addition, immunoblot analysis for activated caspase-3 of control and ABT 737 samples was performed to confirm the specificity of the antibody for activated caspase-3.

Determination of eNOS^{pos} and eNOS^{neg} platelet volume

The electronic volume of non-fluorescent 5 μm standard size beads, 3.8 μm Spherotech beads, 1 μm fluorescent yellow-green and 0.5 μm fluorescent orange beads was measured using a Quanta SC MPL flow cytometer with the protocol used to detect eNOS-based platelet subpopulations. The volume of 0.5 – 5 μm beads in μm^3 was calculated by the formula $V = 4/3pr^3$ and plotted against their mean electronic volume (in arbitrary units). Linear regression analysis was performed (GraphPad Prism 3.0) to determine the slope and the Y-intercept, and mean electronic volumes (EV) of eNOS^{pos} and eNOS^{neg} platelet subpopulations were converted into μm^3 based on the formula μm^3 = (mean EV – 11.16)/2.432.

Activated integrin α_{IIb}/β_3 measurement

In order to investigate the integrin α_{IIb}/β_3 activation by eNOS^{pos} and eNOS^{neg} platelet subpopulations, platelets were stimulated with collagen (3 μg/mL) and aggregation was allowed to proceed to 50% light transmittance. At this point platelet samples were fixed, permeabilized, and incubated with eNOS (clone M221) and secondary antibody as described above. Subsequently, samples were incubated with PAC-1 antibody (2.5 μg/mL) for 15 minutes and washed three times with PBS and incubated with PE-Cy7 conjugated anti-mouse IgM antibody (2 μg/mL) (clone R6-60.2) for 15 minutes in the dark. The platelet samples were once again washed 3x with PBS and then diluted to a final volume of 1 mL before analysis by a Quanta SC MPL flow cytometer. 100,000 platelet-specific events (1-4 μm diameter) were analyzed.

Platelet fluorescence activated cell sorting (FACS)

In order to separate NO-producing from non-NO-producing platelets fluorescence activated cell sorting was performed on DAF-FM-stained platelets re-suspended at 1.5×10^7/mL in Tyrode's buffer + L-arginine (100 μM). Fluorescence activated cell sorting was performed on a Becton Dickinson Aria III BSL-2 sorter. DAF-FM fluorescence was excited with the 488 nm laser and detected on FL1. To discriminate between NO-producing and non-NO-producing platelets, sorting regions were established to encompass the top and bottom 20% fluorescence of DAF-FM-stained platelets, respectively. Platelet sorting was carried out at a rate of 15,000-20,000 events/second for 3 – 3.5 h typically resulting in yields of approximately 3.0×10^7 of each NO-producing and non-NO-producing platelets in 70 – 100 ml of PBS.

For further experimentation, platelets were isolated by the addition of PBS + 3% BSA at a ratio of 1:9 of sorted platelets in PBS. Subsequently, PGI_2 (0.067 μg/ml) was added to the sorted platelets, and the platelets were centrifuged at 900 g for 20 minutes at room temperature. Following centrifugation the buffer was removed and the pelleted platelets were resuspended in 1 ml of fresh PBS + 0.3% BSA and centrifuged for a further 10 minutes at 900 g. The resulting platelets were resuspended in 30 μl of Tyrode's buffer, counted, and further diluted to a concentration of 2.5×10^8/ml

typically yielding 40 – 50 μl of both NO-producing and non-NO-producing platelets at this concentration. Platelets were subsequently either fixed with an equal volume of 8% formaldehyde in Tyrode's buffer for intracellular flow cytometry or in other experiments allowed to rest for a further 30 minutes before platelet aggregometry.

Aggregometry of fluorescence activated cell sorted platelets

DAF-FM-negative and -positive FACS sorted platelets (30 μL of 2.5 × 10^8/mL) were incubated at 37°C with stirring (900 RPM) for 2 minutes in a lumi-aggregometer. Collagen (1 μg/mL) was added and aggregation allowed to proceed for a further 4 minutes. Subsequently, aggregated platelets were fixed with an equal volume of 8% formaldehyde in Tyrode's buffer and incubated for 5 minutes. 10 μL of samples were mounted on microscope slide and imaged using an Olympus CKX41 phase-contrast microscope (Olympus America Inc., Melville, NY) equipped with an *Infinity 1* digital camera. Photomicrographs were captured at the top, bottom, right, left, and center of each slide. The platelet aggregate areas in each field of view were measured using ImageJ software, and the results were expressed as the mean platelet aggregate area.

Video confocal microscopy

In order to assess adhesion by NO-producing and low/non-NO-producing platelets confocal microscopy was performed. DAF-FM loaded platelets (1.5 x 10^8/mL) pre-incubated with 100 μM L-arginine were added to collagen-coated (10 μg/mL) coverslips in a Chamlide TC-A Live Cell Chamber mounted on a Wave FX Olympus IX-81 microscope. The chamber was incubated at 37°C and fluorescent yellow-green beads (1:1,000,000) were added to the cover slips to aid in focusing prior to addition of platelets. Images were captured every 10 seconds with a Hamamatsu EMCCD (C9100-13) camera for approximately 15 minutes. Platelets with peak fluorescence above 1000 units were classified as NO-producers, while platelets with peak fluorescence below 1000 units were classified as low/none-NO-producers.

In addition, platelets were allowed to aggregate for a further 45 minutes, post adhesion experiment coverslips containing collagen adhered and aggregated platelets were fixed for 20 minutes in 4% formaldehyde in Tyrode's buffer to examine the 3-dimensional aggregate architecture. Coverslips were then permeablized with 0.1% Triton X-100, washed, blocked and incubated with eNOS antibody as described above. All steps up to this point were carried out in room light to bleach residual DAF-FM fluorescence. Subsequently, coverslips were incubated in the dark with anti-mouse Alexa488 $(F(ab')_2$ as described above along with Alexa568 Phalloidin to image F-actin. Z-stack images were captured every 0.3 μm on a Leica TCS SP5 microscope. Fluorescence was excited with 488-nm and 543 laser lines.

Flow chamber experiments

In order to assess the functional role of $eNOS^{neg}$ and $eNOS^{pos}$ platelets under flow conditions experiments were carried out with the Spinning Disc Confocal Microscope WaveFX (Olympus IX-81 motorized microscope base) equipped with a BIOPTECHS FCS2 flow chamber assembly and pump. Flow chamber coverslips were incubated 10 μg/mL Type I fibrillar collagen and 10 μl of diluted 1 μm fluorescent yellow-green beads (1:1,000,000) was added to aid in focusing. The experiment was performed at 37°C and DAF-FM-loaded human platelets 1.5 x 10^8/mL in Tyrodes were pumped through the chamber at a flow rate of 50 μl/min for 10 min. Images were captured every 10 seconds with a Hamamatsu EMCCD (C9100-13) camera and non-adhered platelets which passed through the flow chamber were collected for further analysis of $eNOS^{neg/pos}$ subtype by intracellular flow cytometery.

Flow cytometry to establish platelet purity

Human platelets were isolated as described in main methods. Human polymorphonuclear (PMN) and mononuclear (MNC) were isolated using Lympholyte-poly (Cedarlane, Hornby, ON, Canada) and platelet-leukocyte flow cytometry was performed on FACScan and Quanta SC flow cytometers as described previously.[4] Platelets were identified based on CD41b-FITC surface immunostaining and

leukocytes were identified based on CD45-PE immunostaining. Compensation was performed based on individually stained samples and 100,000 events were collected.

RT-PCR

Human leukocyte and platelet RNA was isolated using the PARIS™protein and RNA isolation system (Applied Biosystems, Carlsbad, CA, USA). The RNA was reverse transcribed using Sensiscript reverse transcriptase (Qiagen, Mississauga, ON, Canada). Thereafter, PCR was performed using an Eppendorf Mastercycler eprgradient thermocycler. 1 ng of transcribed DNA was used for each PCR reaction in 20 μl using 1 μM of CD45 (sc-29251-PR) or β-actin (sc-108069-PR) primer pairs obtained from Santa Cruz Biotechnology. Reaction products were separated on a 1% agarose gel and visualized following ethidium bromide staining using an Alpha Innotech Multiimage Light Cabinet.

HUVEC-C and Meg-01 eNOS RT-PCR was performed using a Bio-Rad S1000 thermal cycler with human eNOS primer pairs (Forward-GTC CTG CAG ACC GTG CAG C and Reverse-GGC TGT TGG TGT CTG AGC CG) or GAPDH primer pairs (Forward-GAG AAG GCT GGG GCT CAT TT and Reverse-AGT GAT GGC ATG GAC TGT GG) as endogenous control. Reaction products were separated on a 2% agarose gel and visualized following ethidium bromide staining using a Bio-Rad VersaDoc MP5000 molecular imager. To further confirm that the cDNA sequence matched to that of eNOS visualized bands were excised and submitted for DNA sequencing by the University of Alberta Faculty of Science Molecular Biology Facility.

Intracellular platelet esterase flow cytometry

To investigate platelet esterase levels in $eNOS^{pos}$ and $eNOS^{neg}$ platelet subpopulations, three-color intracellular flow cytometry was performed to measure sialic acid acetylesterase. Intracellular flow cytometry for platelet eNOS was carried out with the M221 clone as described in main methods. Subsequently, anti-sialic acid acetylesterase antibody (2 μg/mL) or corresponding concentration-matched rabbit IgG were incubated overnight at 4°C. Next, the platelets were washed three times with 1mL of PBS. Following, Alexa488-conjugated secondary antibody (1:100) (eNOS detection) was added

to platelet samples and incubated along with R-phycoerythrin-conjugated secondary antibodies (1:100) for the detection of sialic acid acetylesterase, respectively. Samples with secondary antibodies were incubated for 2 h with rocking in the dark. Finally, once again the platelets were washed with PBS and incubated with anti-CD41-PE-Cy7 for 15 minutes and then diluted to a final volume of 1 mL with PBS before analysis by flow cytometry.

Flow cytometry was carried out with a Quanta SC MPL flow cytometer. Fluorescence was induced with a 488 nm argon laser and was detected on FL1 (525 nm BP filter) and FL2 (568 nm BP filter) and/or FL3 (620 nm LP filter). FSD (fluorescence surface density) and FC (fluorescence concentration) measurements were made and 10,000 platelet-specific events were analyzed for each experiment. Compensation was performed using Cell Lab Quanta analysis software to account for fluorophore spectral overlap.

Platelet COX-1 and MMP-2 flow cytometry

To investigate MMP-2 and COX-1 in eNOS^{pos} and eNOS^{neg} platelet subpopulations three-color flow cytometry was performed. Intracellular staining for platelet eNOS was carried out as described in main methods. Subsequently, for MMP-2 or COX-1 determination, eNOS-stained platelets were then incubated with primary antibodies (anti-MMP-2 10 μg/ml; anti-COX-1 1.5 μg/ml) or corresponding concentration-matched isotype/IgG controls overnight at 4°C. Next, the platelets were washed with PBS (3x). Following, samples were incubated in the dark with PE-conjugated secondary antibodies (1:100) for the detection of MMP-2 and COX-1. Platelets were then washed with PBS and incubated with anti-CD41a-PE-Cy7 antibodies for 15 minutes and then diluted to a final volume of 1 mL with PBS before analysis by flow cytometry.

Flow cytometry was carried out with a Quanta SC flow cytometer. FSD and FC measurements were made and 10,000 platelet-specific events were analyzed for each experiment. Compensation was performed using Cell Lab Quanta analysis software to account for spectral overlap.

eNOS-GFP Transgenic Mice

Approval for the current study was obtained from the University of Alberta Animal Care and Use Committee. Breeding pairs of eNOS-GFP transgenic mice (C57BL/6) were provided by Dr. Robert Krams (Imperial College, London, UK) and have been described by van Harpen et al.[5] The transgenic mice express human eNOS fused to GFP protein driven by the human eNOS promoter. The mice were regenerated by initially mating homozygous male eNOS-GFP transgenics with wild type female C57BL/6 mice (JAX). WT mice were used as controls and were obtained from nontransgenic littermates. Mice were maintained on a 12 hr light/dark cycle with food and water ad libitum. Mice used for flow cytometry experiments were of both genders aged 14 to 16 weeks. Mice were anaesthetized using isoflurane (1.5-3%) and 37°C body temperature was maintained using circulating water heating pad. Surgical plane anesthesia was assessed by checking for lack of pedal reflex on all four paws. Blood samples were obtained via cardiac puncture and were anticoagulated with 3.15% sodium citrate (1/9 ratio). Prostacyclin (1 μg/ml) was added to the blood and platelet rich plasma (PRP) was obtained after centrifugation at 300g for 5 minutes.

Flow cytometry was performed using Quanta SC flow cytometer (Beckman Coulter, Mississauga, ON, Canada). Fresh platelet rich plasma (20 μl) was incubated in dark for 15min at 22°C with 2.5 μg/ml of PerCP/Cy5.5 labeled rat anti-mouse CD41 antibody or 2.5 μg/ml PerCP/Cy5.5 rat IgG_1 isotype control (BioLegend, San Diego, CA, USA). After incubation samples were diluted to 400 μl with PBS. Twenty thousand events per sample were collected and mouse platelets were identified based on positive PerCP/Cy5.5 CD41 antibody staining in FL3 set to log scale. CD41 positive events were further subgated and GFP fluorescence detected in FL1 on a linear scale. A dot plot of FL3 vs. FL1 was created and percentage of GFP positive events among CD41 positive events was calculated. Further, the mean FL1 auto-fluorescence of WT platelets was subtracted from the mean fluorescence of eNOS-GFP hemizygous and homozygous mouse platelets.

Immunoblotting under reducing/denaturing conditions

Platelet pellets were sonicated in homogenizing buffer containing 1 μM β-cyclodextrin. Immunoblot analysis was performed under reducing/denatureing conditions as described previously[6, 7]. Briefly,

platelet pellets were lysed in homogenizing buffer containing 2% SDS. Lysed platelet or endothelial cells samples (10 – 100 μg protein per lane) were subjected to 7% (eNOS), 12% (sGC, PKG, VASP, COX-1) or 15% (activated caspase-3) SDS-PAGE. After electrophoresis and transfer of gel onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA), the membranes were blocked overnight in blotting buffer with 5% non-fat milk. Subsequently, membranes were incubated with eNOS (clone M221) antibody (1 μg/mL) for 2 hrs at room temperature. For sGC, PKG, and VASP immunoblots membranes were incubated overnight in blotting buffer with 5% non-fat milk and containing either sGC (1 μg/mL), PKG (1 μg/mL), VASP (2 μg/mL), COX-1 (1.5 μg/ml), or activated caspase-3 (2 μg/mL). Subsequently, anti-mouse (1:5,000 for eNOS) or anti-rabbit (1:3,000) horseradish peroxidase–labeled antibodies were used as the secondary antibody (Sigma-Aldrich). The immunoreactive bands were visualized with an ECL Plus kit (Amersham Biosciences, San Francisco, CA). In some instances, membranes were stripped and probed with a β-actin-HRP antibody (1:35,000) (Sigma-Aldrich), which was used as a loading control. Chemiluminescence was detected using a VersaDoc MP5000 molecular imager with Quantity One software (Bio-Rad).

TXB_2 measurement by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)

FACS-sorted DAF-FM-positive and negative platelets (100 μl) were incubated at 37^{o}C under stirring conditions (900 rpm) in the presence of L-arginine (100 μM) and stimulated with collagen (10 μg/ml) for 4 minutes. Subsequently, platelets were separated from their releasates by centrifugation and the releasates were stored at -80^{o}C before further LC-ESI-MS analysis. The stable metabolite of TXA_2, TXB_2, was analyzed in 50 μL of platelet releasate using LC-ESI-MS (Waters Micromass ZQ 4000 spectrometer). An equivolume (50 μL) of absolute ethanol containing 0.5 μg/mL of TXB_2-D4, as the internal standard, and 50 μg/mL of butylated hydroxytoluene was added to each sample; thereafter, the samples were evaporated to dryness and reconstituted with 25 μL acetonitrile. The mass spectrometer

was run in negative ionization mode with single ion monitoring: m/z= 369 for TXB_2 and m/z = 373 for TXB_2-D4. The nebulizer gas was acquired from an in house high purity nitrogen source. The temperature of the source was set at 150°C, and the capillary and cone voltage were 3.51 kV and 25 V, respectively. An isocratic separation was performed on a reverse phase C18 column (Alltima HP, 150 × 2.1 mm) at 35°C using acetonitrile/water/acetic acid (40/60/0.01, v/v/v) as mobile phase. The concentrations were calculated based on peak area, and the linear ranges of the calibration curve was from 0.001 μg/mL to 10 μg/mL. The results were subsequently normalized to platelet count and express as ng of $TXB_2/10^8$ platelets.

Gelatin zymography and β-actin loading-controlled zymography

Gelatin zymography, which is 1000x more sensitive than immunoblot,[8] was used to measure platelet-secreted pro-MMP-2. Gelatin zymography was performed on platelet releasates obtained during aggregometry of collagen-(3 μg/ml)-stimulated platelets. Platelet releasates were obtained at 1 minute prior to the addition of collagen, at shape change, at 50% and maximal light transmittance as measured by a lumi-aggregometer with Aggro-Link software. Platelets were separated from releasates by centrifugation (1000 g for 10 minutes) and the releasates stored at -80^{o}C until analysis. Subsequently, gelatin zymography was performed as described previously.[9, 10] Briefly, Zymography was performed using 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with co-polymerized gelatin (2 mg/mL). After electrophoresis, gels were washed 3· for 20 min in 2% Triton X-100. Next, gels were washed 2· for 20 min in developing buffer (50 mM Tris– HCl pH 7.6, 150 mM NaCl, 5 mM CaCl2, and 0.05% NaN3) and then incubated in developing buffer at 37 ^{o}C overnight. Gels were stained with 0.05% Coomassie Brilliant Blue and then destained in a 4% ethanol and 8% acetic acid solution. Gelatinolytic activity was detected as transparent bands against a blue background of Coomassie-stained gelatin. Seventy-two kilodaltons pro-MMP-2 activity was identified by comparison with standards from conditioned medium of HT-1080 human fibrosarcoma cells. A novel β-actin loading-controlled zymography was used to measure pro-MMP-2 levels within the lysates of DAF-FM-sorted platelets as recently described by us in detail.[11]

cGMP ELISA

A cGMP ELISA kit was obtained R&D Systems. FACS-sorted DAF-FM-positive and negative platelets (100 μl) were incubated at 37°C under stirring conditions (900 rpm) in the presence of L-arginine (100 μM) and sildenafil (10 μM) and stimulated with collagen (10 μg/ml) for 10 minutes. Subsequently, platelets were separated from their releasates by centrifugation and the releasates were stored at -80°C before further analysis. Subsequently, platelets were lysed in 100 μl of homogenizing buffer and the cGMP ELISA was carried out according to manufacturers instructions. cGMP levels within FACS-sorted DAF-FM-negative platelets were expressed as a percent of FACS-sorted DAF-FM-positive platelet levels.

Mass Spectrometry Analysis

Human PGI_2-washed platelets were isolated following staining with DAF-FM diacetate as described in main methods and subsequently incubated with L-arg (100 μM) at 37°C for 6 minutes. Platelet samples (200 μl aliquots) were transferred to 2 ml Eppendorf tubes and 15 μl of a 0.1 M acetic acid solution was added, stirred for 3 seconds, and then 1000 μl of ethyl acetate added. The samples were stirred for 15 seconds and then frozen by keeping the mixture in a dry ice bath for 5 minutes. The samples were then centrifuged (14,000 rpm) at 4°C for 10 minutes to separate the organic layer from which an 800 μl aliquot of the solvent was taken and transferred to glass vial to evaporate the solvent under vacuum (rotavapor) and a nitrogen atmosphere. The residues were reconstituted by adding 200 μl of acetonitrile. Aliquots of the solution were injected into the mass spectrometer for analysis by electrospray ionization. The spectrometric analyses were carried out using a Waters Micro Mass ZQ-4000 single quadruple mass spectrometer (ESI-MS), recording the corresponding fragmentation patterns in both positive and negative ionization modes. The DAF-FM signal ($C_{21}H_{14}F_2N_2O_5$; *m/z* = 413.1) was detected in positive ionization mode [ES+], and the final metabolic product benzotriazole derivate ($C_{21}H_{10}F_2N_3O_5$) was detected as the corresponding triple anion of form (*m/z*= 421.1**)** in negative ionization mode [ES-].

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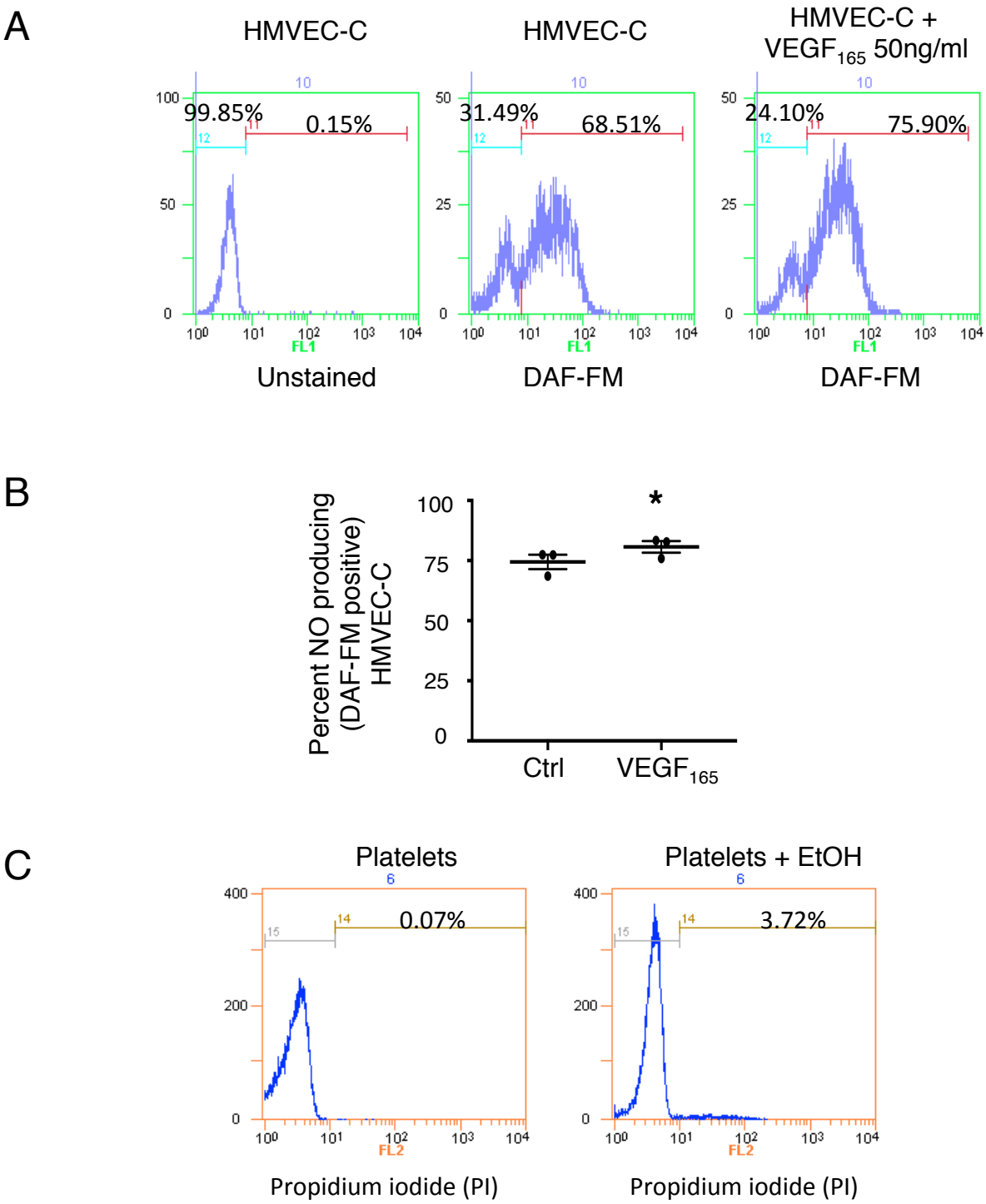
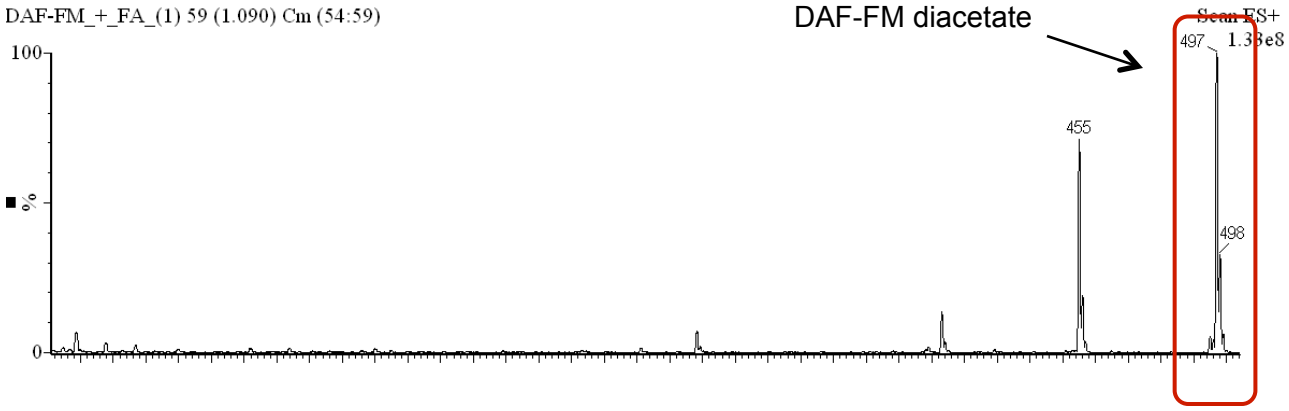


Figure S1. (**A**) Representative flow cytometry histograms and (**B**) summary data demonstrating DAF-FM is able to detect changes in NO production of maximally stimulated (50 ng/ml $VEGF_{165}$) cardiac-derived human microvascular endothelial cells (HMVEC-C). N = 3. *, $P < 0.05$. **(C)** Representative flow cytometry histograms demonstrating that isolated platelets have intact cells membranes as they are impermeable to propidium iodide (PI) and therefore would not accumulate DAF-FM that may have contacted NO generated by leukocytes. Brief treatment with 50% ethanol (EtOH) causes a small proportion of platelets to become permeable to PI. Representative histograms are one of three independent experiments.

A DAF-FM diacetate



B Resting Platelets + DAF-FM diacetate + L-arg

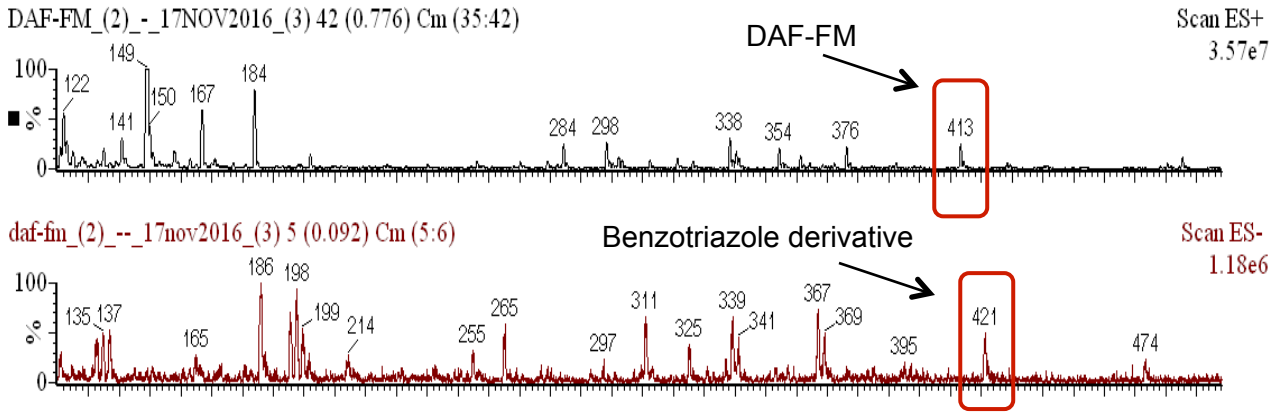


Figure S2. (A) Representative mass spectrometry analyses of DAF-FM diacetate and **(B)** DAF-FM and its benzotriazole derivative (NO-bound form) within resting platelets incubated with L-arg (100 μM). Platelet esterases convert DAF-FM diacetate into DAF-FM trapping it within the platelelet. The DAF-FM signal ($C_{21}H_{14}F_2N_2O_5$; *m/z* = 413.1) was detected in positive ionization mode [ES+], and the final metabolic product benzotriazole derivate ($C_{21}H_{10}F_2N_3O_5$) was detected as the corresponding triple anion form (*m/z*= 421.1**)** in negative ionization mode [ES-]. Representative spectra are from one of three independent experiments.

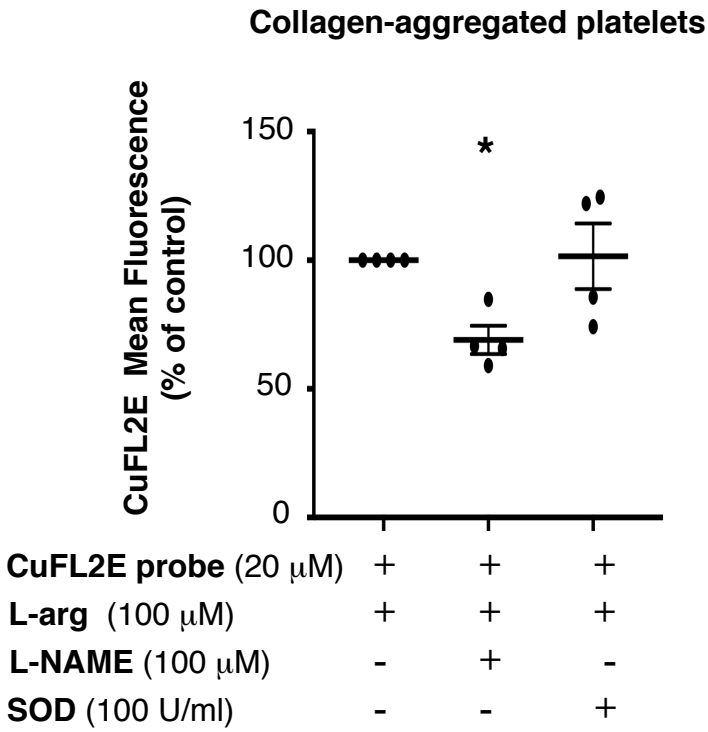


Figure S3. A single flow cytometry experiment demonstrating platelet NO-production as measured by the FL2E nitric oxide sensor (CuFL2E). L-NAME reduces CuFL2E fluorescence but not SOD. Platelet NO-production was stimulated by collagen (5 μg/ml). N = 4 independent experiments. *, $P < 0.05$ vs. L-arg.

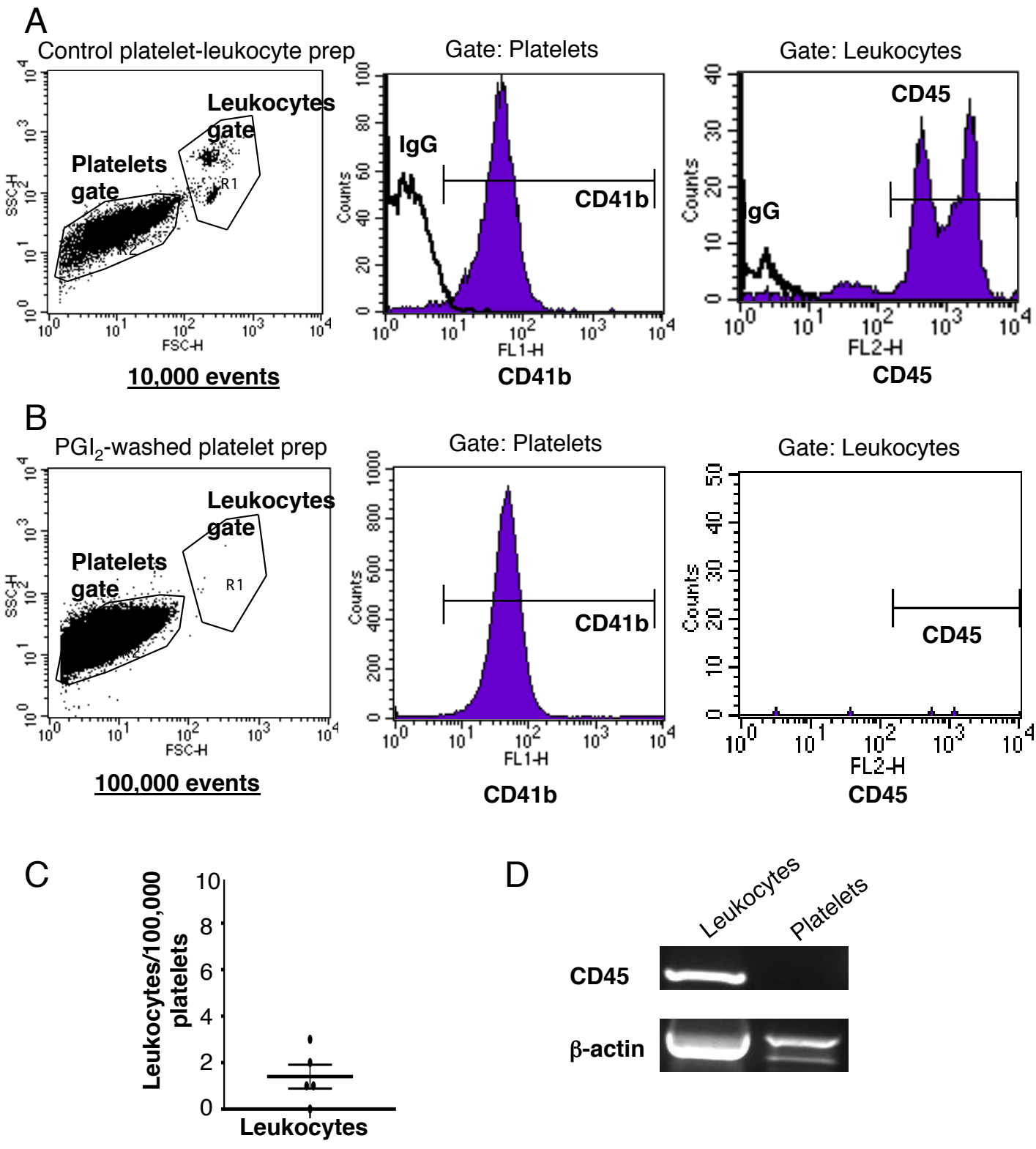
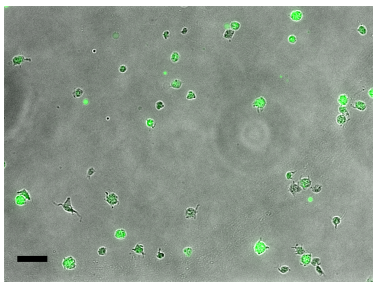
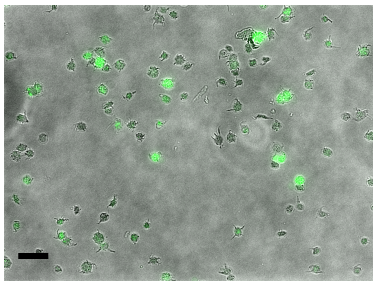


Figure S4. (A) Control experiment in which Lympholyte™-isolated leukocytes were mixed with isolated human platelets to establish a platelet-leukocyte flow cytometry protocol. IgG indicates isotype controls. CD41b-positive events indicate platelets and CD45-positive events indicate leukocytes. (**B**) Representative PGI_2-washed platelet prep demonstrating 2 leukocytes per 100,000 isolated platelets. (**C**) Summary data demonstrating the high purity of PGI_2-washed platelet preps. N = 5. **(D)** RT-PCR demonstrating absence of detectable CD45 mRNA in platelet isolations. Representative of N = 3 experiments.



L-Arg



L-Arg
+
L-NAME

Figure S5. Platelet-derived NO Limits Aggregate Formation. DAF-FM fluorescence microscopy of resting platelets (RP) in the presence of L-arg without and with L-NAME (both 100 μmol/L). Scale bars = 10 μm. Representative images are from N = 3 independent experiments**.**

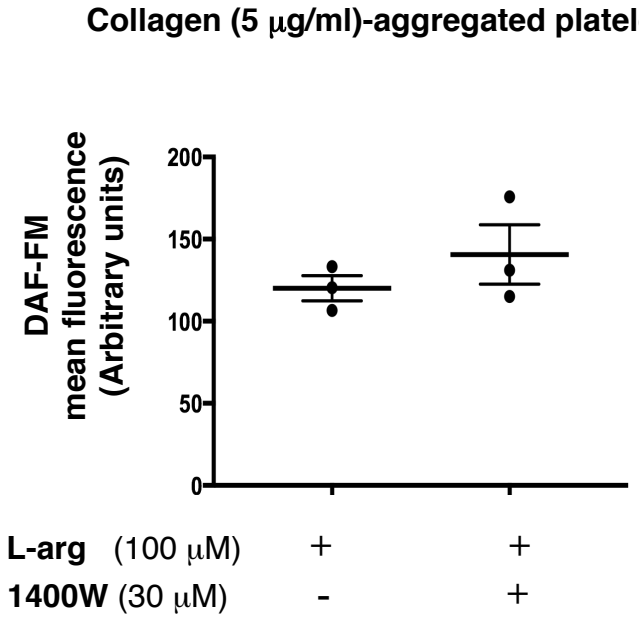


Figure S6. Summary flow cytometry data demonstrating that the iNOS-selective antagonist 1400W does not reduce platelet DAF-FM staining. N = 3. *, P > 0.05.

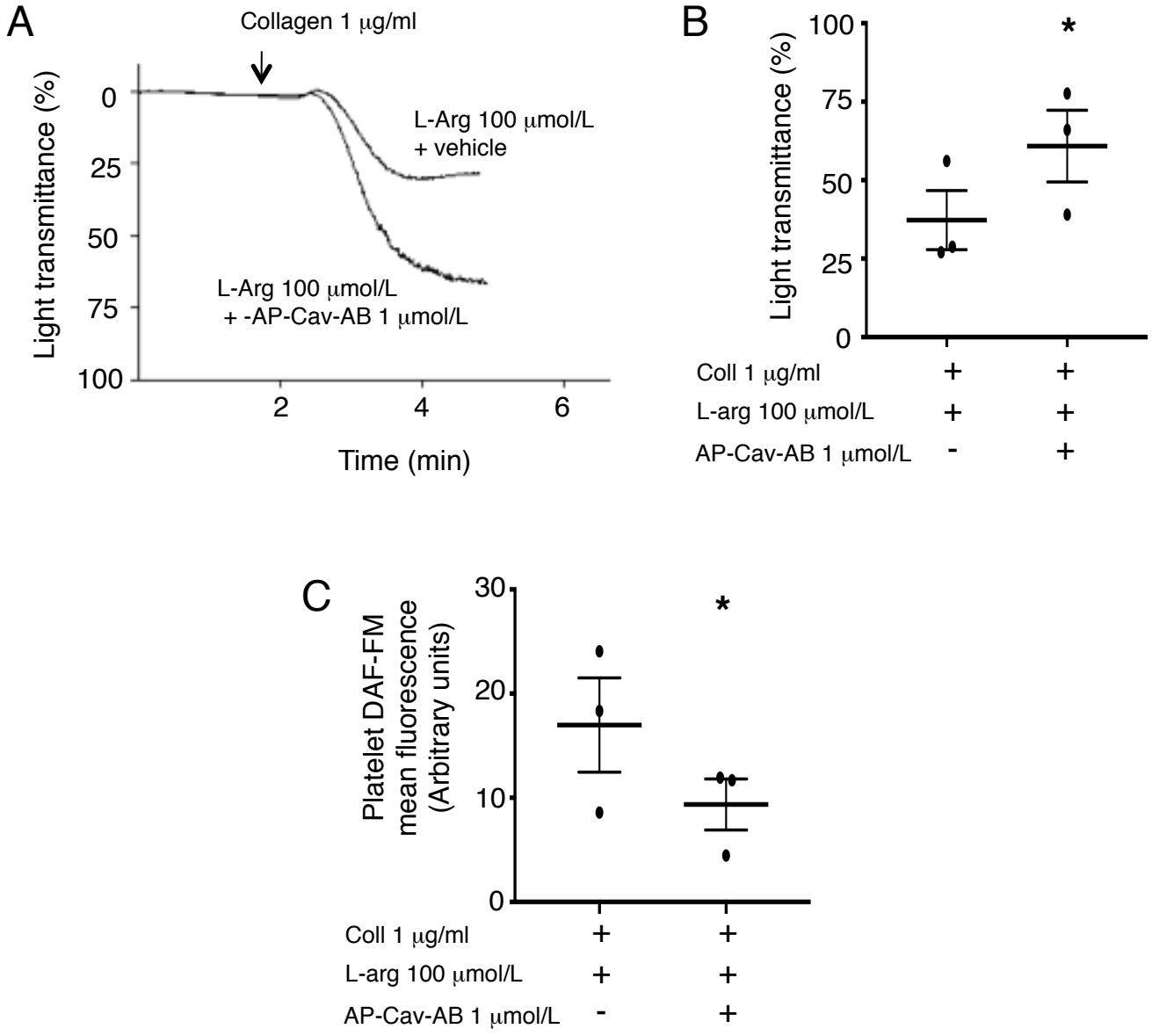


Figure S7. (A and B) Representative platelet aggregation trace and summary data demonstrating that the eNOS-inhibiting peptide AP-Cav-AB enhances platelet aggregation. **(C)** Summary flow cytometry data demonstrating AP-Cav-AB reduces platelet DAF-FM fluorescence. N = 3. *, $P < 0.05$.

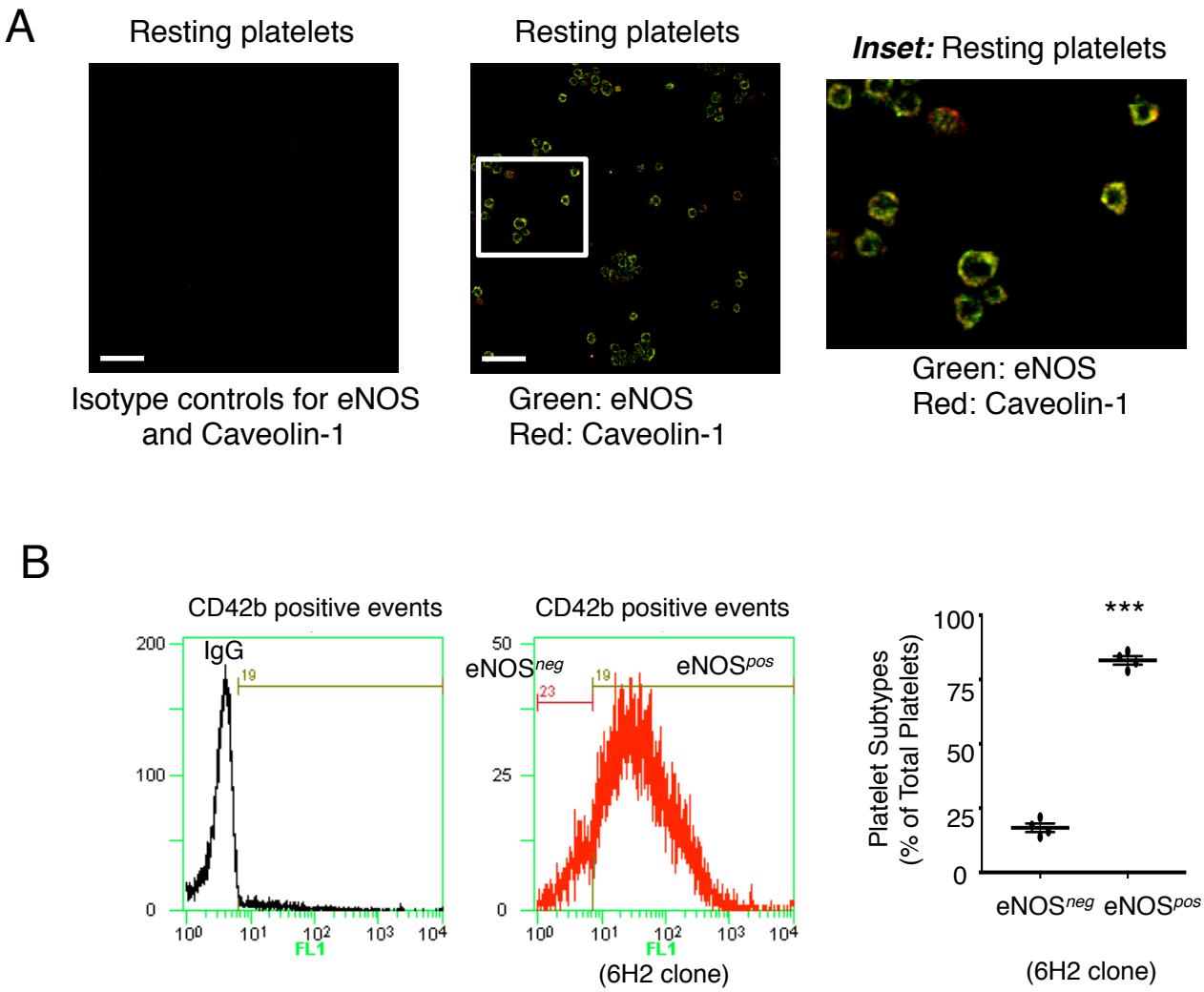
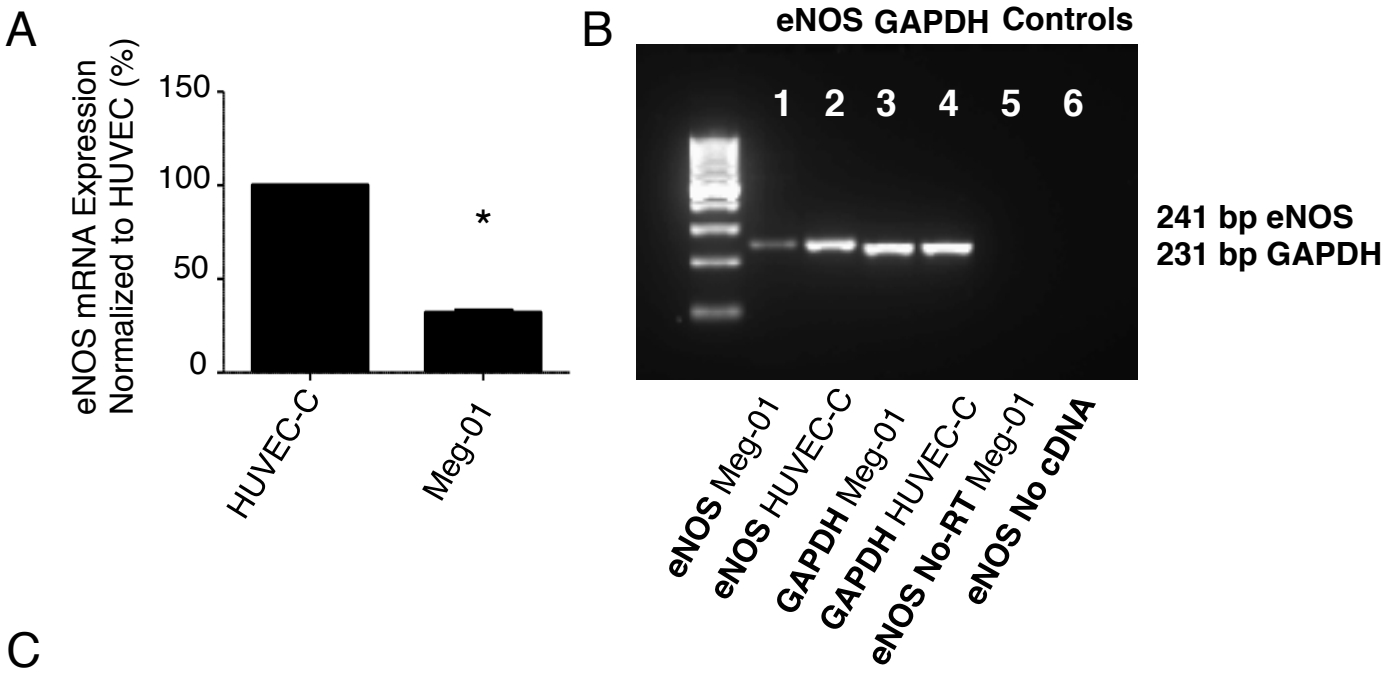


Figure S8. (A) Immunofluorescence confocal microscopy of isotype controls and platelet eNOS (green) and caveolin-1 (red). Yellow indicates co-localization of eNOS and caveolin-1. Scale bar = 10 μm. All representative images from at least N = 3 independent experiments. **(B)** Representative flow cytometry histogram of IgG isotype control and eNOS^{neg} and eNOS^{pos} platelet subpopulations identified by an antibody (6H2 clone) recognizing the N-terminal of eNOS. Summary data comparing percentage of eNOS^{neg} to eNOS^{pos} platelets using the eNOS 6H2 antibody clone. N = 4 independent experiments. ***, P = 0.003. **(C)** Control histograms demonstrating HMVEC lysates block staining of eNOS^{pos} platelets.



C

Homo sapien nitric oxide synthase 3 (NOS3)
Refseq mRNA

3733-3804
3729-3892

Negative strand:

AAAAGCTCTGGGTGCGTATGCGGCTTGTCACCTCCTGGGT
GCGCAGCGTGAGCCCGAAAATGTCTTCGTGGTAGCGTTGC
TGATCCCGCAGCACGCCGATGACGTCGCCGGCCTCGTCCA
GCTCCATGTCGCCCTCCGTCGCCAGGATGCGCTGCACGGT
CTGCA

eNOS matching 165 nucleotide sequence
3667-3831

GAAAAGCTCTGGGTGCGTATGCGGCTTGTCACCTCCTGGG
TGCGCAGCGTGAGCCCGAAAATGTCTTCGTGGTAGCGTTG
CTGATCCCGCAGCACGCCGATGACGTCGCCGGCCTCGTCC
AGCTCCATGTCGCCCTCCGTCGCCAGGATG

eNOS matching 150 nucleotide sequence
3683-3832

Positive strand:

GTCATCGGCGTGCTGCGGGATCAGCAACGCTACCACGAAG
ACATTTTCGGGCTCACGCTGCGCACCCAGGAGGTGACAAG
CCGCATACGCACCCAGAGCTTTTCCTTGCAGGAGCGTCAGT
TGCGGGGCGCAGTGCCCTGGGCGTTCGACCCTCCCGGCT
CAGA

eNOS matching 164 nucleotide sequence
3729-3892

TCGGCGTGCTGCGGGATCAGCAACGCTACCACGAAGACAT
TTTCGGGCTCACGCTGCGCACCCAGGAGGTGA

eNOS matching 72 nucleotide sequence
3733-3804

Figure S9. (A) RT-PCR summary data comparing levels of eNOS expression between HUVEC cells and Meg-01 cells. N = 3, P < 0.05. **(B)** Representative 2% agarose gel showing eNOS 241 bp cDNA band in Meg-01 cells (lane 1) and HUVEC-C cells (lane 2). **(C)** DNA sequencing of Meg-01 samples following electrophoretic separation on 2% agarose gel confirmed DNA fragments correspond to eNOS sequence.

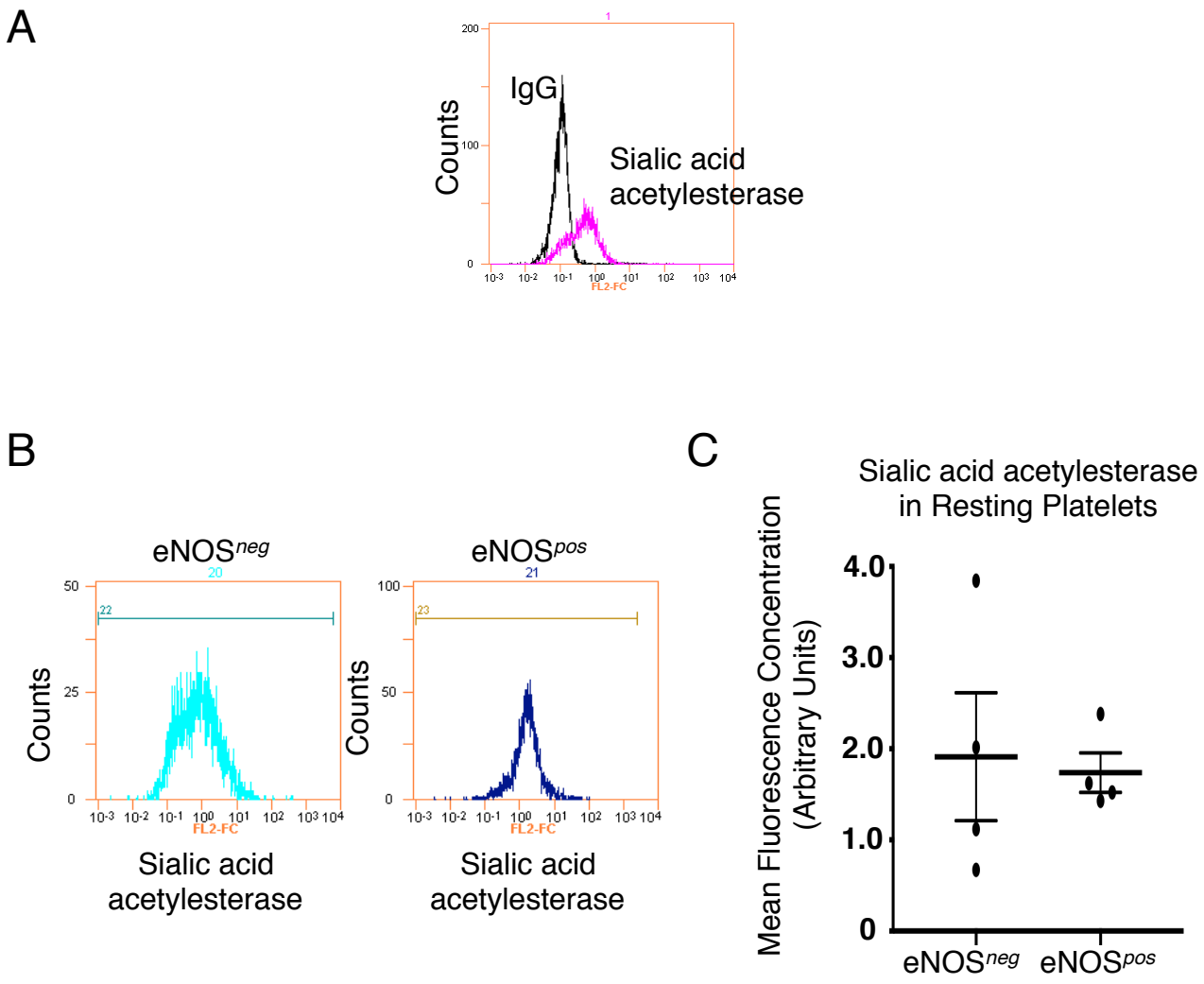


Figure S10. (**A**) Representative platelet intracellular flow cytometry histogram demonstrating detection of sialic acid acetylesterase. (**B**) Representative fluorescence concentration (FC) histograms of sialic acid acetylesterase within eNOS^{neg} and eNOS^{pos} platelet subpopulations. (**C**) Summary data comparing sialic acid acetylesterase in eNOS^{neg} and eNOS^{pos} platelets. N = 4. P > 0.05.

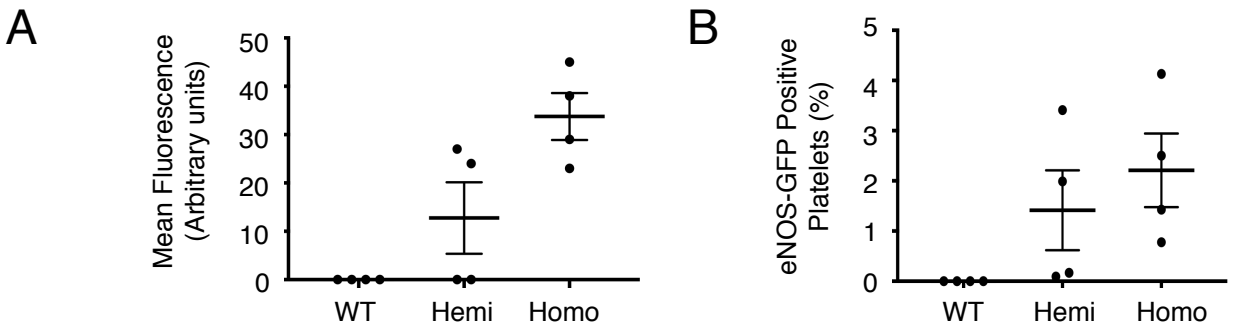


Figure S11. (**A**) Mean fluorescence intensity of wild type (WT) and hemizygous (Hemi) and homozygous (Homo) eNOS-GFP mouse platelets and **(B)** percent eNOS-GFP positive platelets. N = 4 mice in each group.

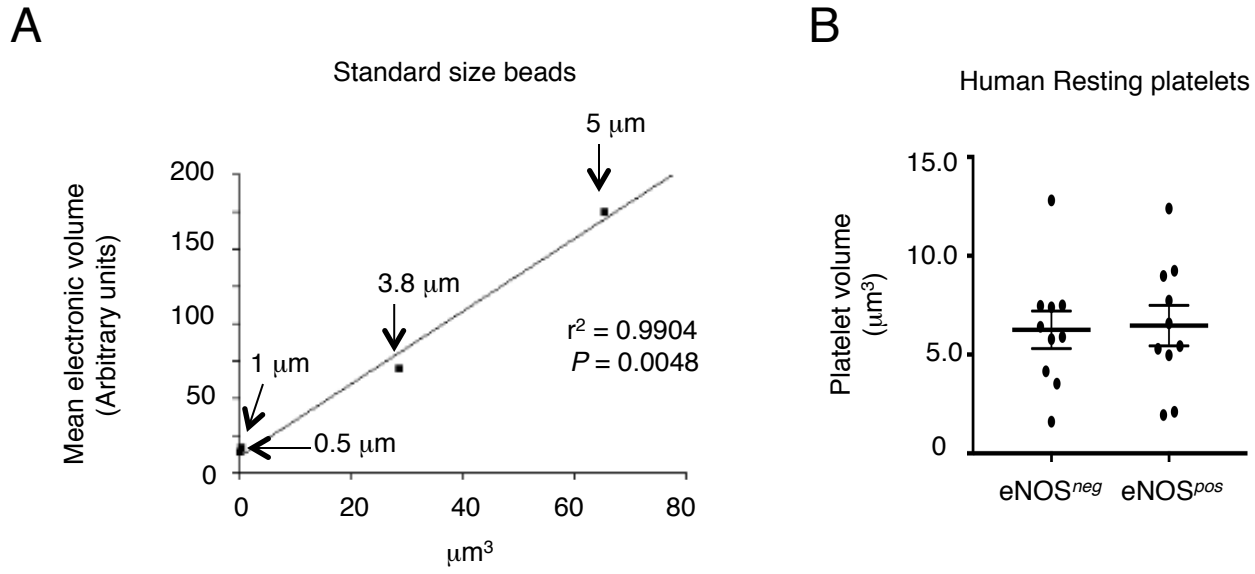


Figure S12. eNOS-based platelet subpopulations are of equivalent size. (A) Linear regression volume analysis of beads of known diameter and **(B)** summary data of eNOS^{neg} and eNOS^{pos} platelet volumes. N = 10.

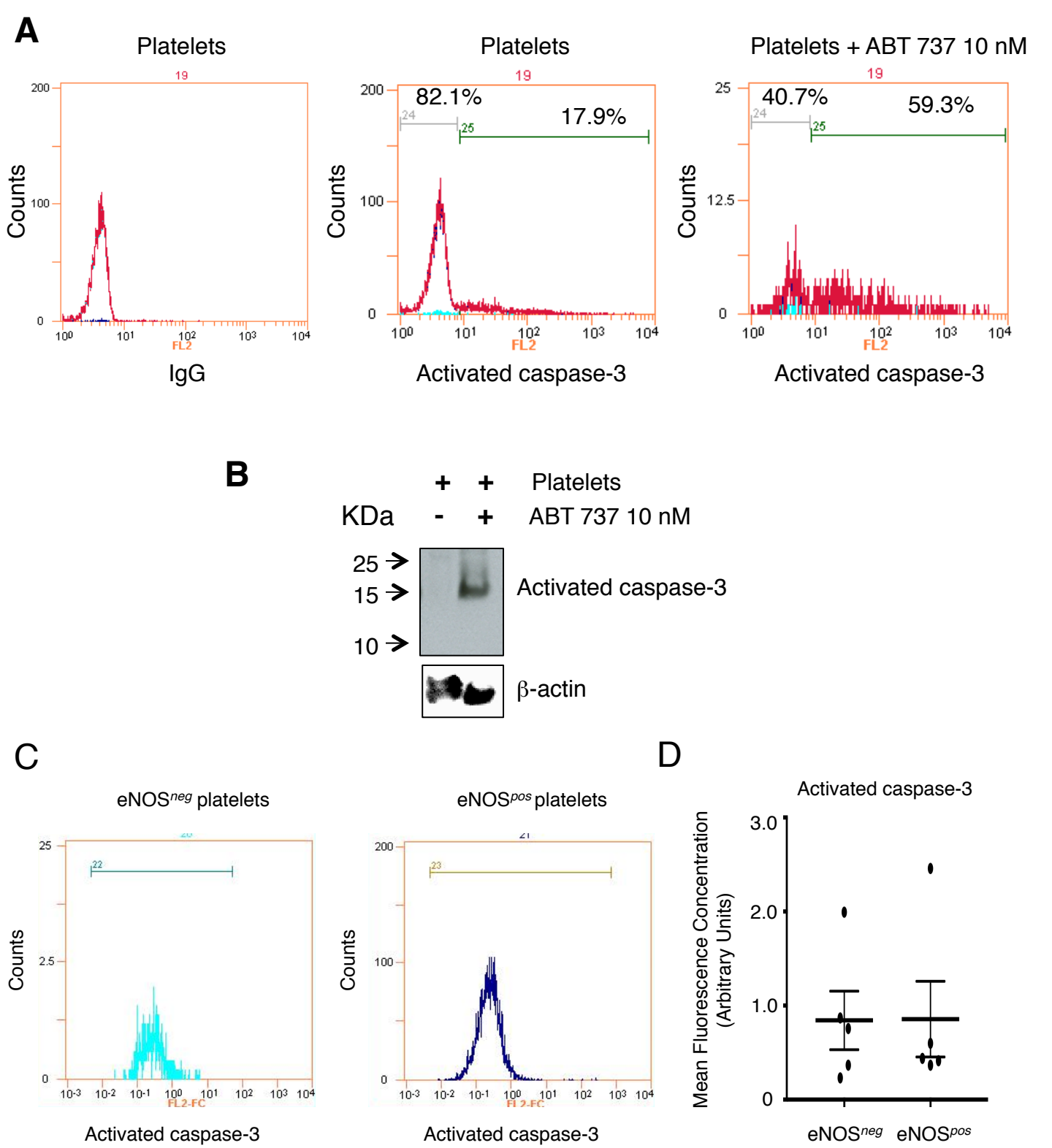


Figure S13. (**A**) Control intracellular flow cytometry histograms of platelet activated caspase-3 in the absence and presence of the platelet apoptosis-inducing compound ABT 737. (**B**) A control immunoblot demonstrating the specificity of the antibody used in flow cytometry for activated caspase-3 within control and ABT 737 treated platelets. **(C)** Representative intracellular flow cytometer fluorescence concentration histograms and **(D)** summary data of activated caspase-3 in eNOS^{neg} and eNOS^{pos} platelets. N = 4. P > 0.05.

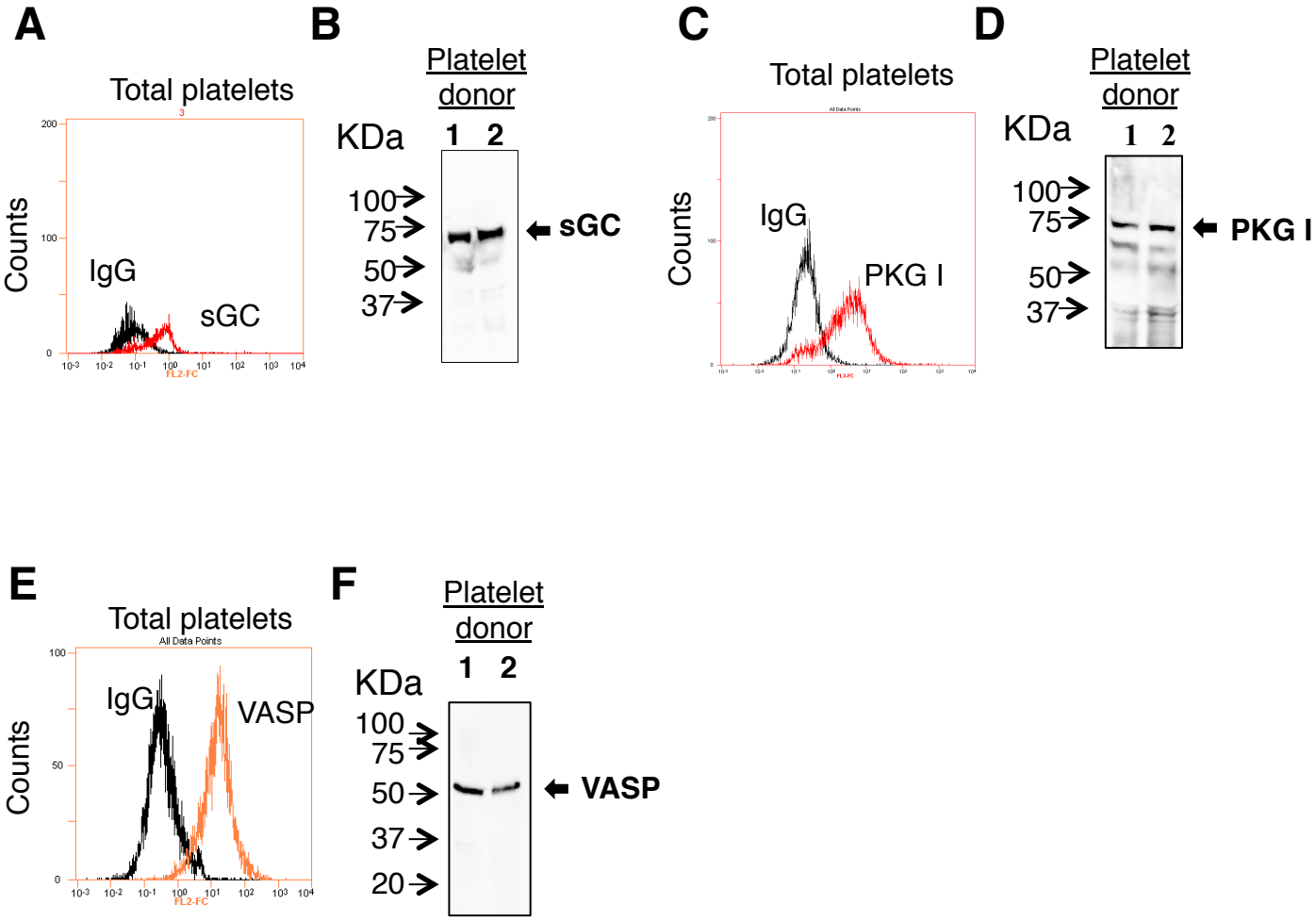


Figure S14. (**A**) Control platelet intracellular flow cytometry histogram demonstrating detection of sGC, and (**B**) platelet immunoblot demonstrating the specificity of the antibody for sGC. (**C**) Control platelet intracellular flow cytometry histogram demonstrating detection of PKG I, and (**D**) platelet immunoblot demonstrating the specificity of the antibody for PKG I. (**E**) Control platelet intracellular flow cytometry histogram demonstrating detection of VASP, and (**F**) platelet immunoblot demonstrating the specificity of the antibody for VASP.

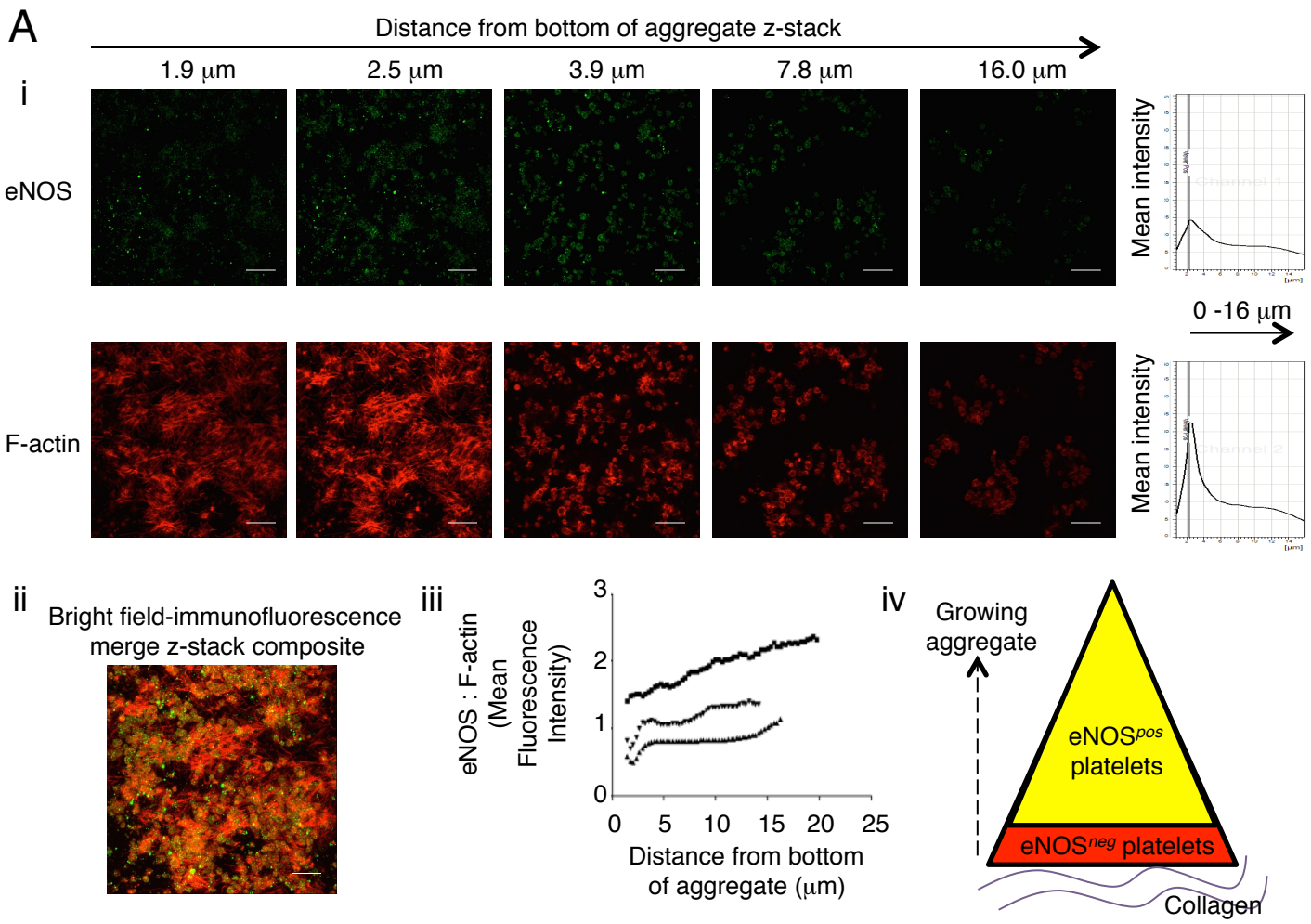


Figure S15. (A) (i) Representative immunofluorescence confocal microscopy z-stacks and mean fluorescence intensity measurements demonstrating platelet eNOS (green) and F-actin (red) within the 3-dimensional structure of an aggregate. Scale bar represents 10 μm. (ii) Z-stack composite of (i). (iii) A graph demonstrating the changes in the ratio of eNOS:F-actin fluorescence within the z-plane of aggregates from 3 independent experiments, and (iv) a cartoon depicting the growth of an aggregate upon a base of eNOS^{neg} platelets. Z-stack images were captured every 0.3 μm on a Leica TCS SP5 microscope equipped with a 100x/1.4 oil NA objective. The ratio of green to red fluorescence determined for each z-stack using Leica LAF AS software.

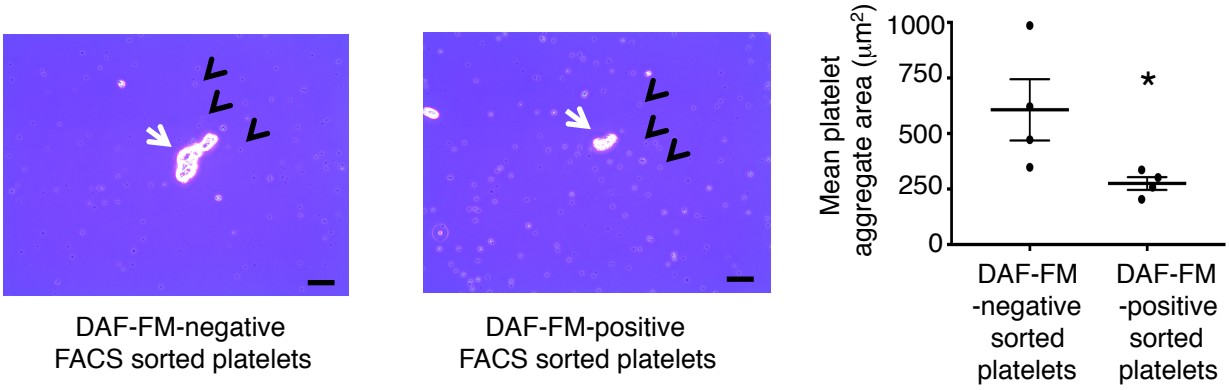


Figure S16. Representative phase-contrast microscopy and summary data of platelet aggregate size following aggregation with collagen (1 μg/mL) of DAF-FM FACS-sorted platelets in the presence of L-arg (100 μM). N = 4. *P < 0.05 vs. DAF-FM-negative sorted platelets. White arrows indicate aggregates. black arrows indicate individual platelets. Scale bars = 25 μm.

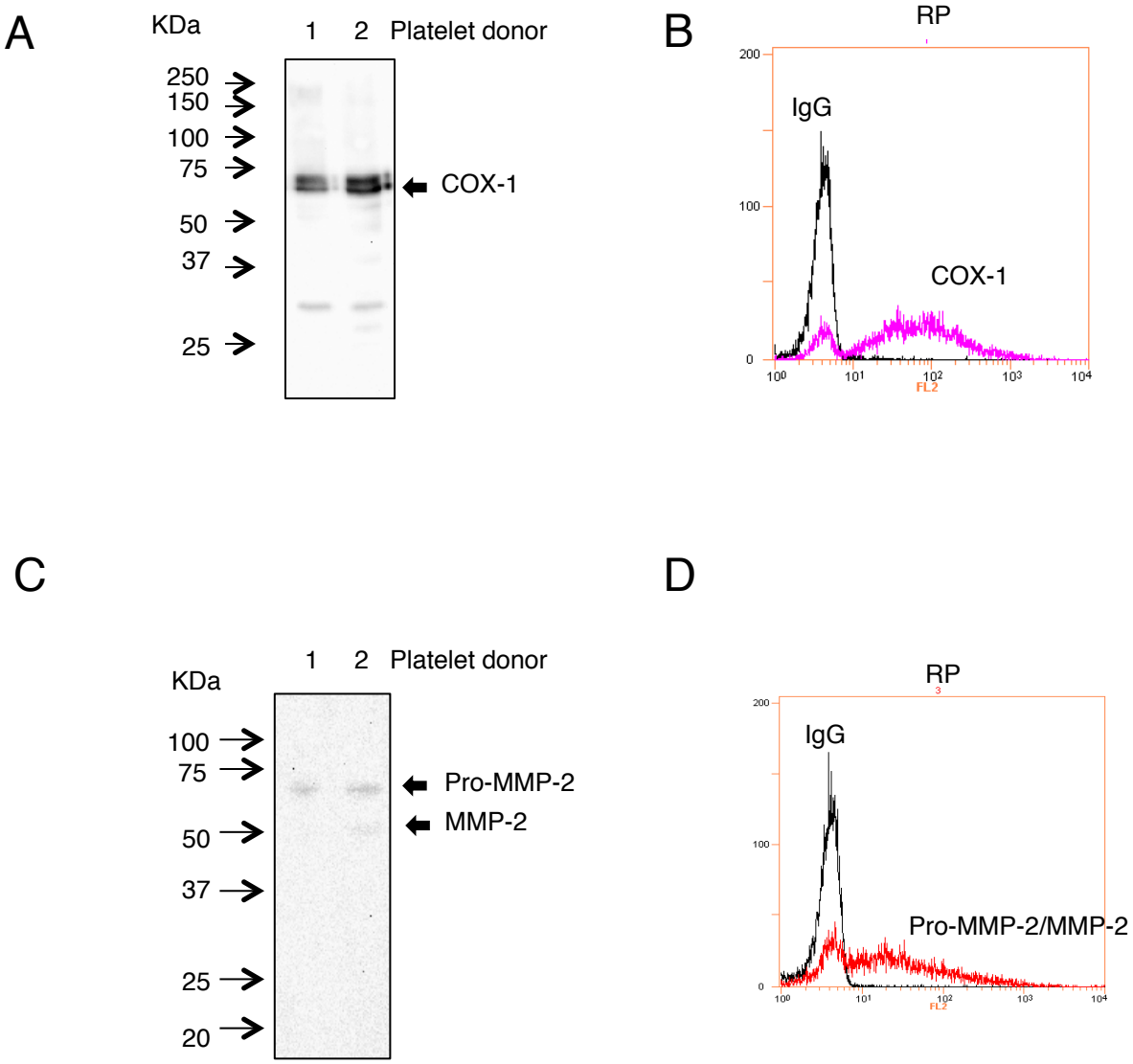


Figure S17. (A and C) Immunoblot demonstrating the specificity of COX-1 and MMP-2 antibodies, respectively. (B and D) Flow cytometry histograms demonstrating the detection of platelet COX-1 and pro-MMP-2/MMP-2, respectively. RP – resting platelets.

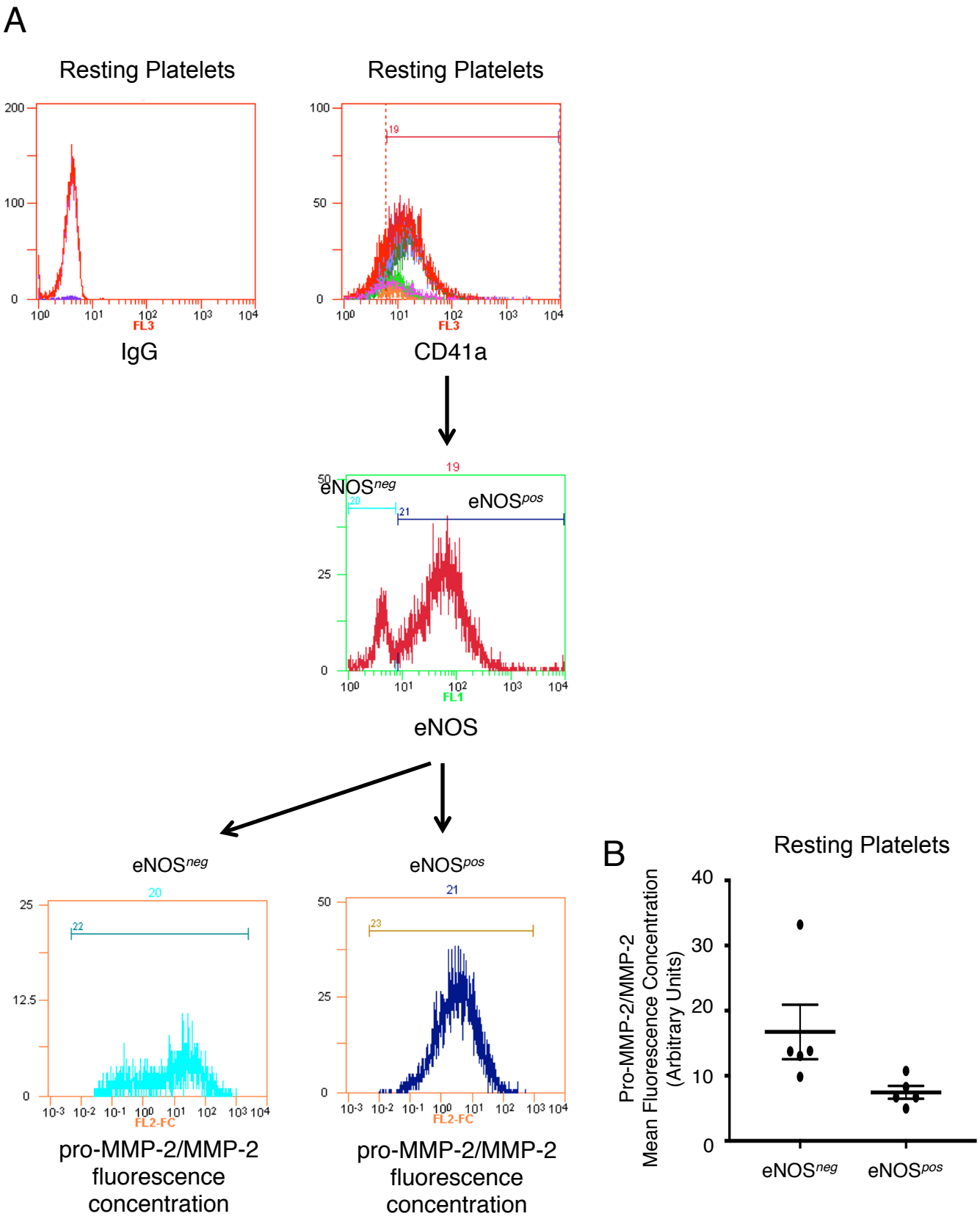


Figure S18. (A) Example of the gating strategy utilized in 3-color platelet flow cytometry experiments. **(B)** Summary data of platelet pro-MMP-2/MMP-2 fluorescence concentration in eNOS^{neg} and eNOS^{pos} platelets. N=5 independent experiments. * P = 0.08.

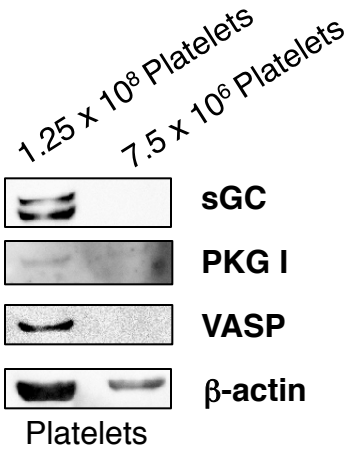


Figure S19. Representative immunoblots demonstrating that immunoblotting is not sensitive enough to detect the proteins in the eNOS-signalling pathway within 7.5 x 10^6 platelets the typical yield following a FACS-sorting isolation.

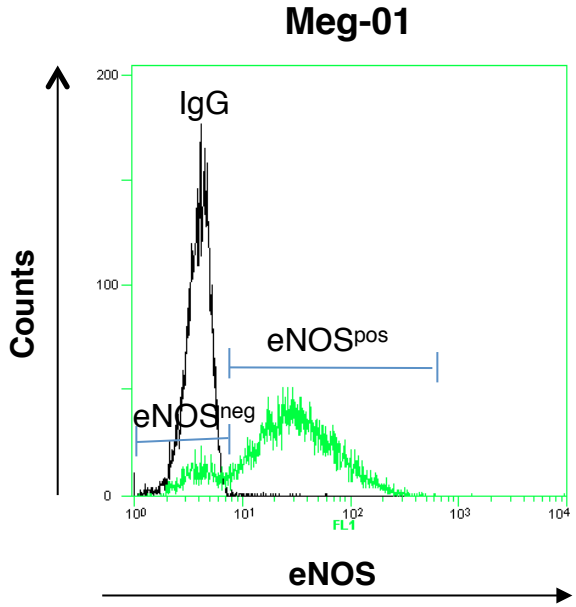


Figure S20. A preliminary flow cytometry histogram overlay demonstrating eNOS-negative and eNOS-positive subpopulations of human Meg-01 (megakaryoblasts).

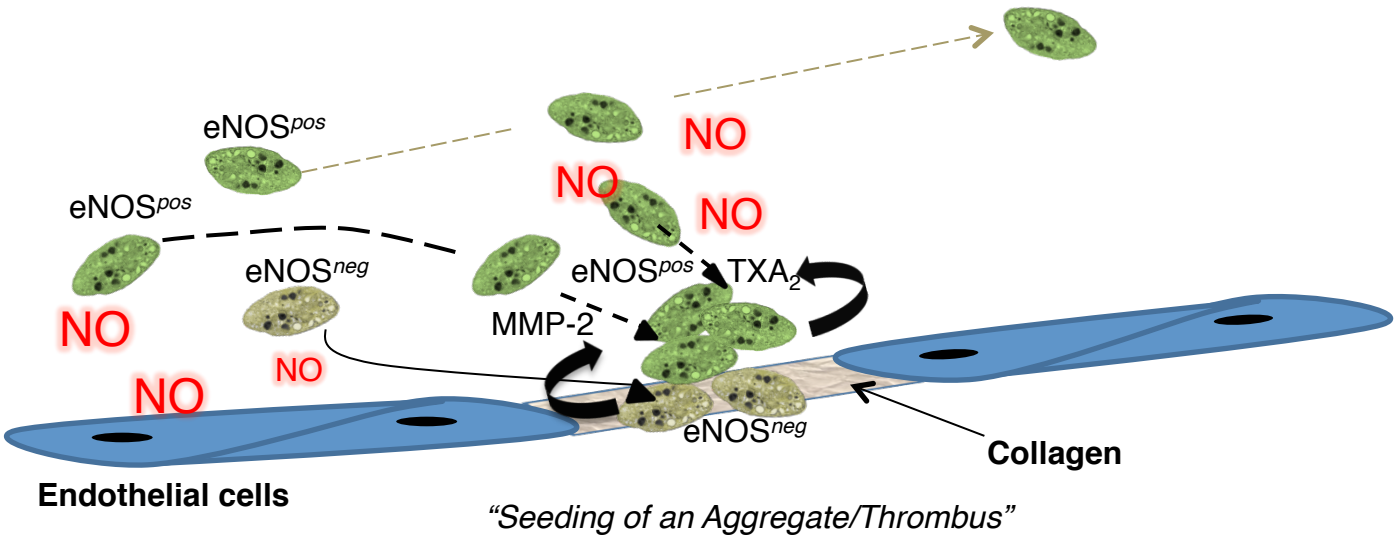


Figure S21. (**A**) A cartoon summarizing the "seed platelet" hypothesis in which NO-refractory eNOS^{neg} platelets preferentially initiate platelet adhesion and aggregation, while eNOS^{pos} platelets form the bulk of an aggregate and limit its size.