Appropriation of GPIbα from platelet-derived extracellular vesicles supports monocyte recruitment in systemic inflammation

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 doi:10.3324/haematol.2018.215145

Received: December 21, 2018.

Accepted: August 23, 2019.

Pre-published: August 29, 2019.

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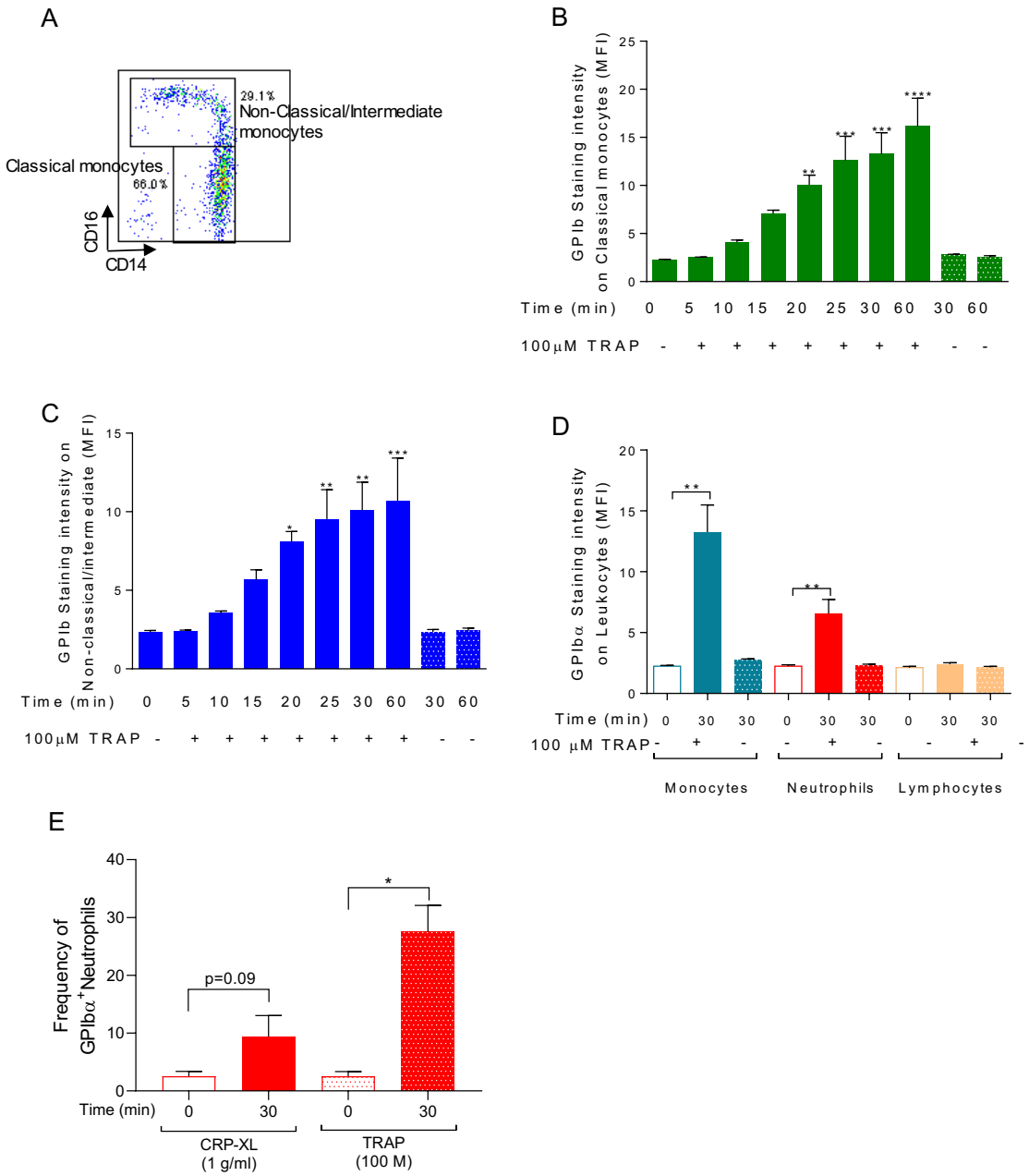
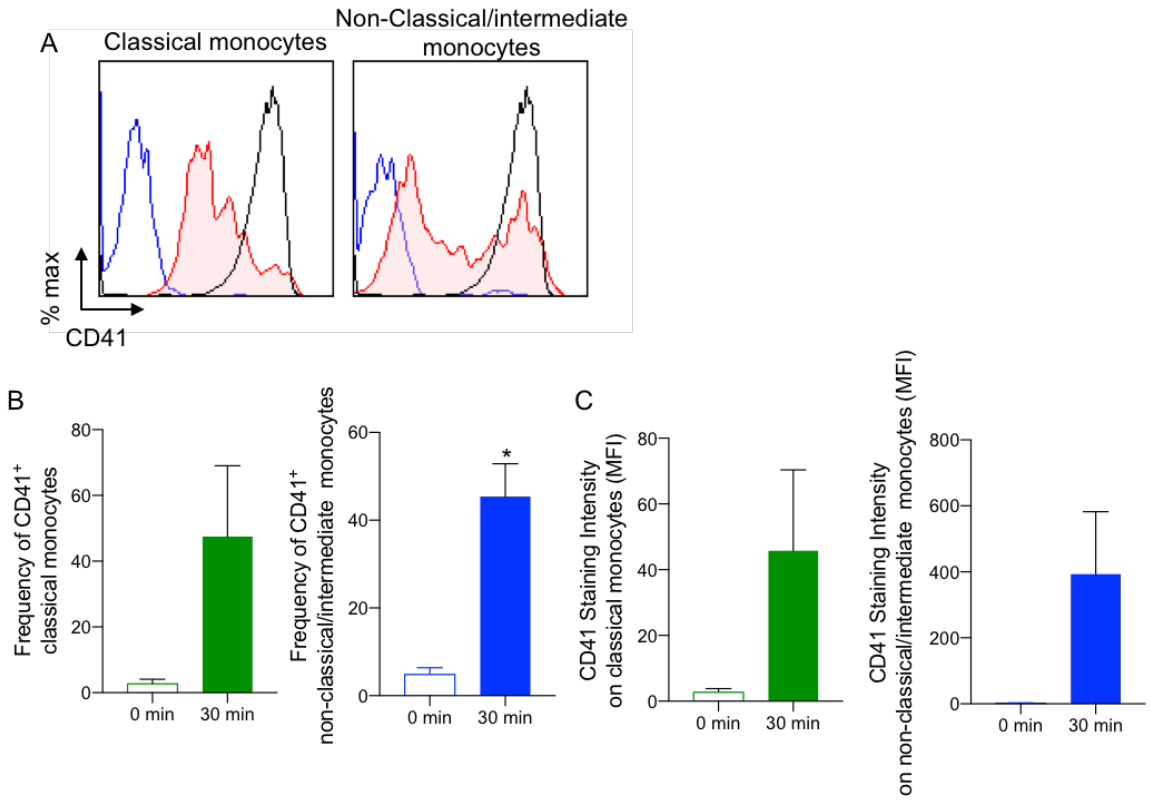
Supporting information:

Supplementary Figure 1



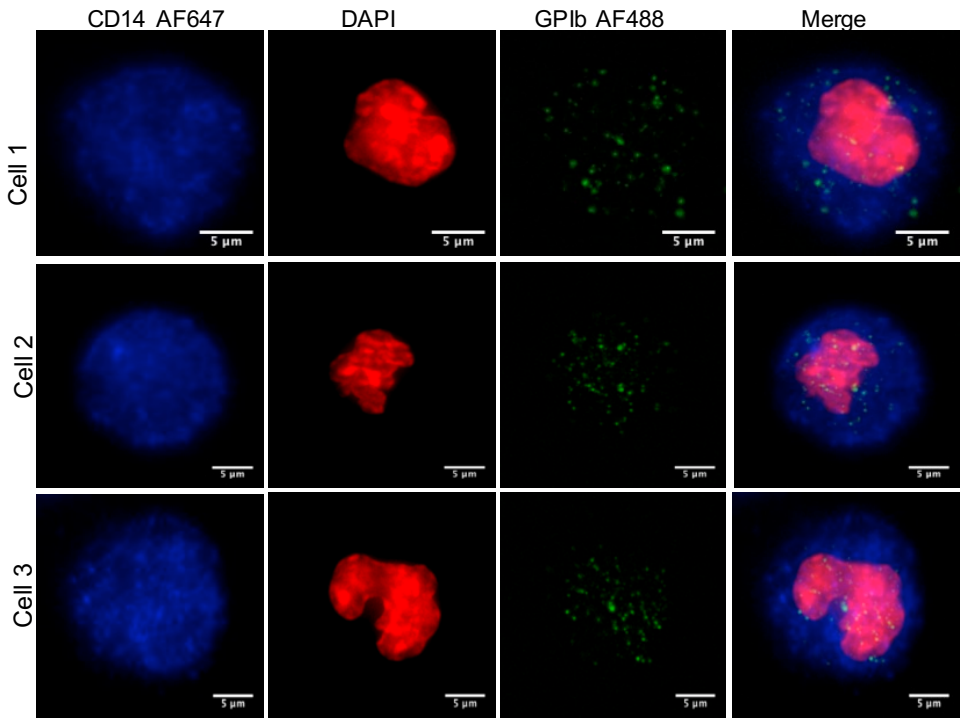
Supplementary Figure 1. Accumulation of GPIbα on leukocyte in stimulated whole blood. (A) Gating strategy to identify classical ($CD14^+CD16^-$) and non-classical/intermediate ($CD14^+CD16^+$) monocytes. (B) Median Fluorescence intensity (MFI) of $GPIb\alpha^+$ staining on Classical monocytes in whole blood stimulated with 100 µM TRAP at 37°C under shear determined by flow cytometry, n=3. (C) MFI of $GPIb\alpha^+$ staining non-classical/intermediate monocytes in whole blood stimulated with 100 µM TRAP at 37°C under shear determined by flow cytometry, n=3. (D) MFI of $GPIb\alpha^+$ staining on monocytes (all subsets), lymphocytes and neutrophils in whole blood stimulated with 100 µM TRAP at 37°C under rolling conditions determined by flow cytometry, n=3. (E) Percentage of $GPIb\alpha^+$ neutrophils in whole blood stimulated with 1µg/ml CRP-XL or 100 µM TRAP at 37°C under shear for 0 and 30 minutes, n=3-5. Data are mean ± s.e.m. ** P ≤ 0.01, *** P ≤ 0.001, **** P≤ 0.0001 compared to the 0 min control (B, C) or to monocytes (D) by analysis of variance (ANOVA) and Dunnett post-test.

Supplementary Figure 2



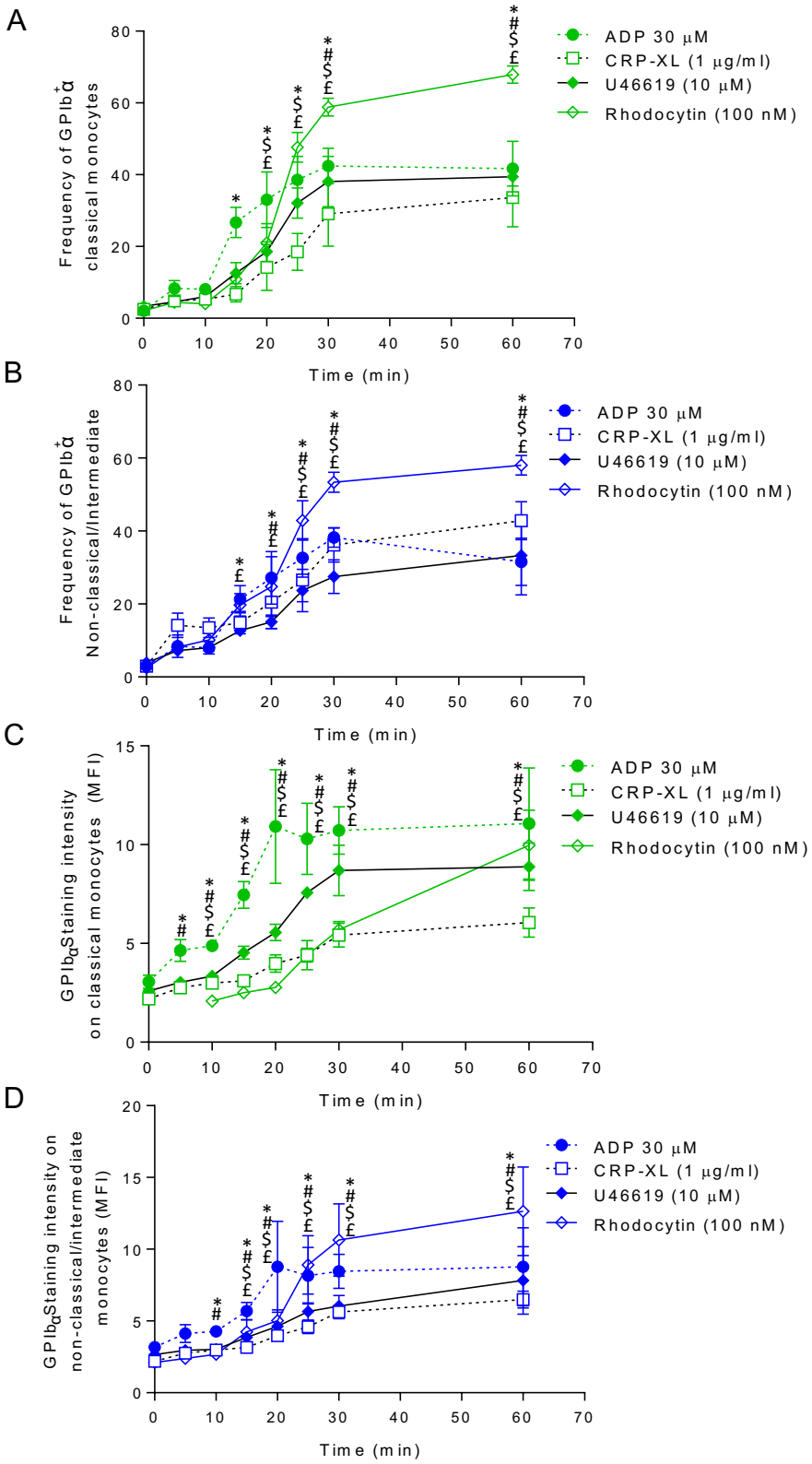
Supplementary Figure 2. Accumulation of CD41 on monocytes in stimulated whole blood. (A, B) Representative plots of CD41 labelling on classical monocytes (A) or non-classical/intermediate monocytes (B) in unstimulated or TRAP (100 µM)-stimulated whole blood under shear for 30 minutes measured by flow cytometry. (C, D) Percentage of $CD41^+$ classical monocytes (C), non-classical/intermediate monocytes (D) in whole blood stimulated with 100 µM TRAP at 37°C under shear at 0 and 30 minutes, n=3. Data are mean ± s.e.m. ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ compared to the 0 min control (C, D) or to monocytes by paired Student's t-test.

Supplementary Figure 3



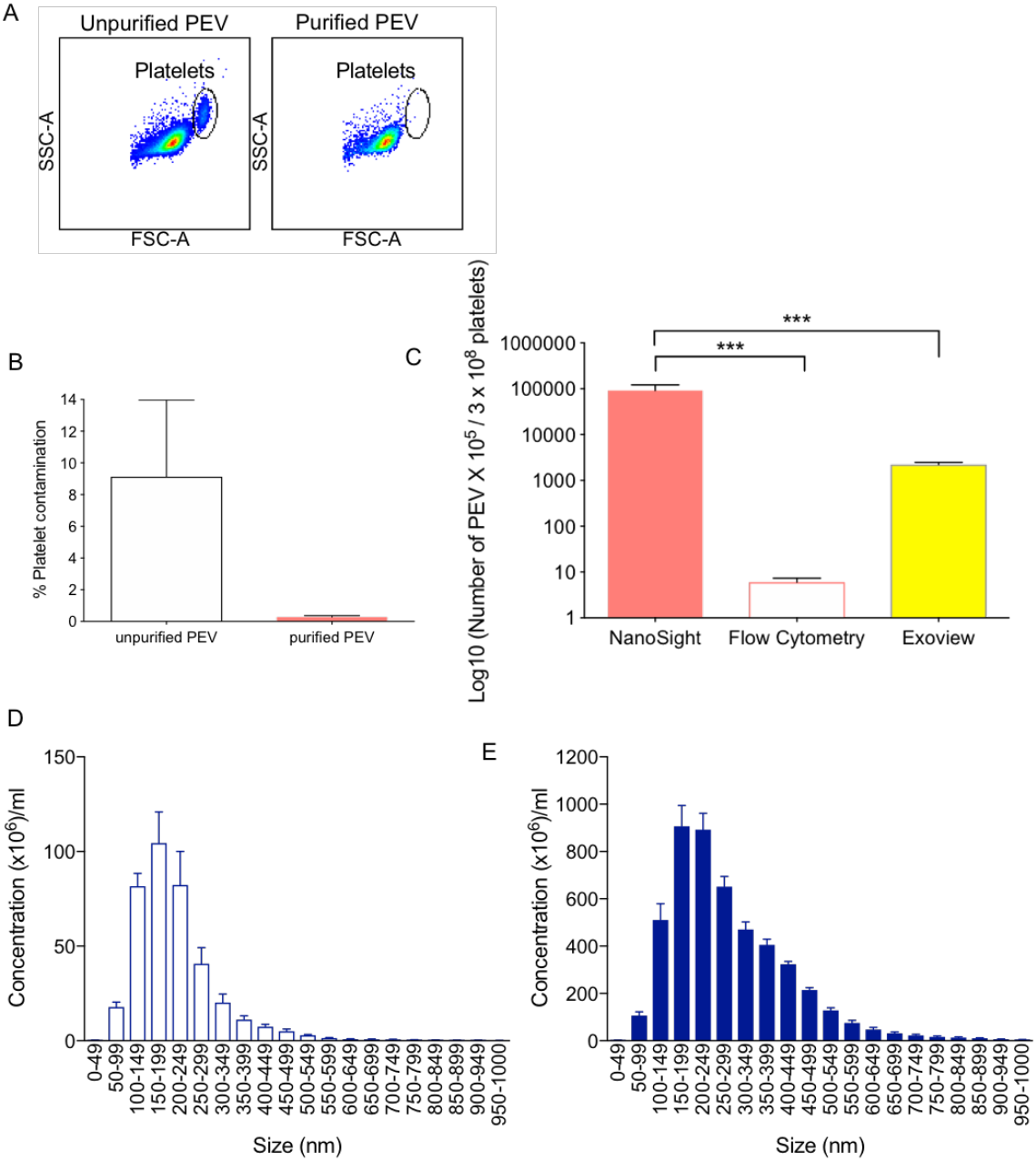
Supplementary Figure 3. Surface and intracellular accumulation of GPIbα on monocytes. Representative images of monocytes labelled with CD14 (blue), GPIbα (green) for PEV and DAPI in nuclei (red) as determined by confocal microscopy.

Supplementary Figure 4



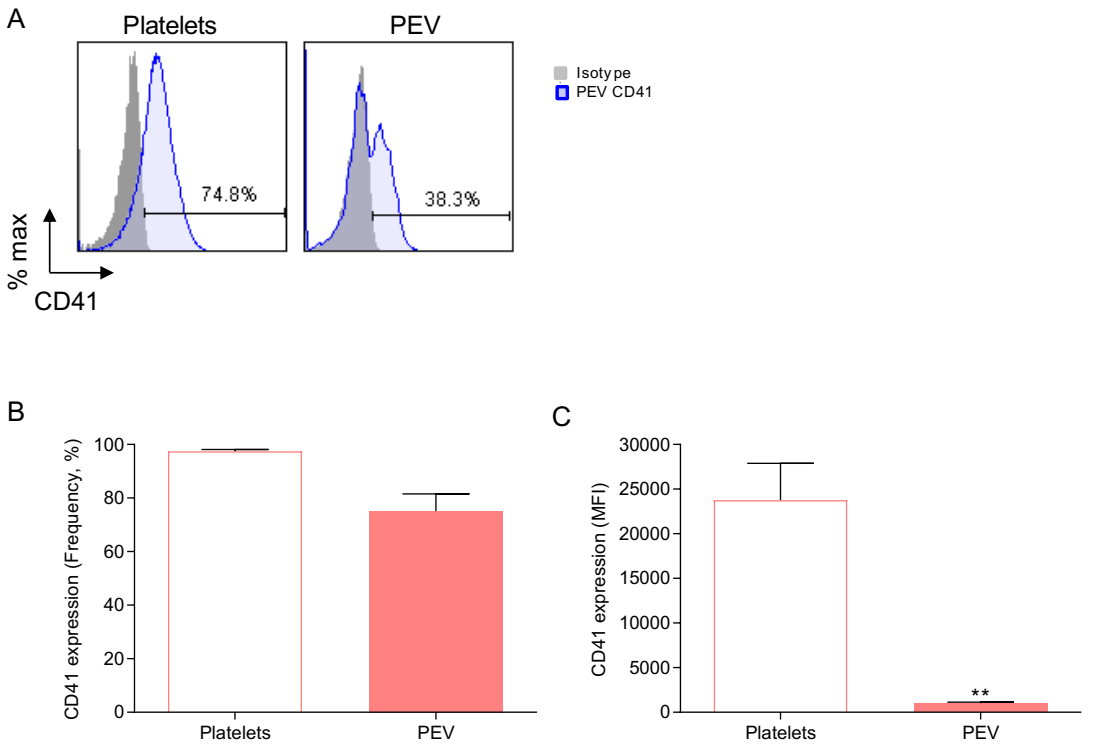
Supplementary Figure 4. Accumulation overt time of GPIbα on monocyte subsets in whole blood under shear and stimulated with different agonists. (A) n=4-7 for ADP, n=4 for CRP-XL, n=3 for U46619 and n=3 for Rhodocytin. Data are shown as mean ± s.e.m. *,#, $, £ P ≤ 0.05, compared to the time point 0 minute control for each agonist * ADP, # CRP-XL, $ U46619 and £ Rhodocytin by analysis of variance (ANOVA) and Dunnett post-test.

Supplementary Figure 5



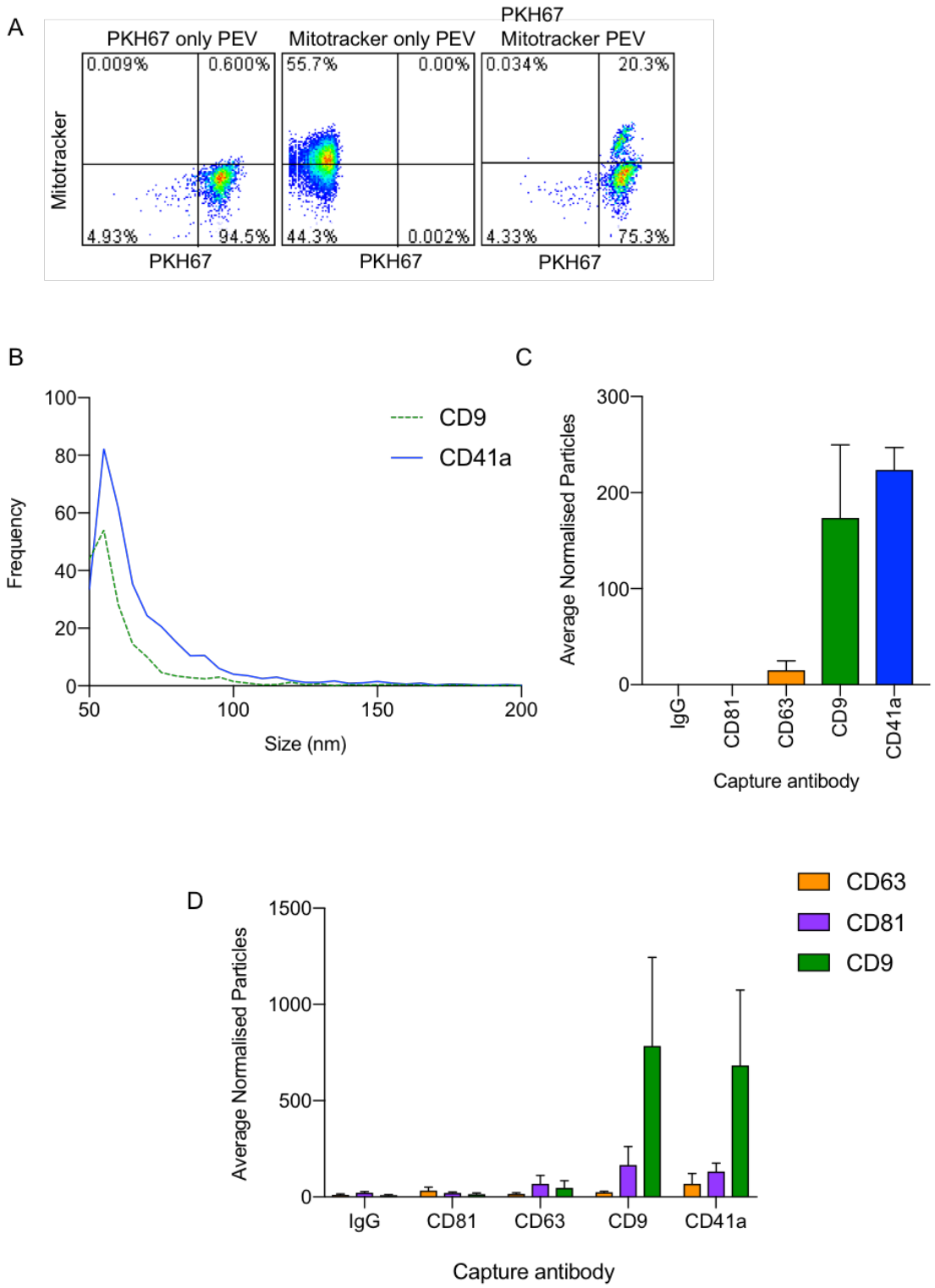
Supplementary Figure 5: Characterisation of PEV. (A) Representative flow cytometry dot-plots showing the number of events in a platelet gate before and after purification of PEV by centrifugation. (B) Contamination of PEV with platelets before vs after purification of PEV by centrifugation, n=5-8. (C) Number of PEV produced from $3x10^8$ platelets stimulated with CRP-XL, quantified by flow cytometry or nanoparticle tracking analysis and enriched in CD41a expression using the ExoView R100 (number of PEV captured on a CD41a spot), n=3-10. (D, E) Unstimulated washed isolated platelets (D) ($3x10^8$/ml) or CRP-XL (1 µg/ml) stimulated (E) were incubated for 30 minutes at 37°C. PEV were isolated in supernatants following centrifugation for 20 minutes at 2000g and 2 minutes at 13000g. Data were acquired using nanoparticle tracking analysis. Data are mean ± SEM. *** P<0.001 by one-way ANOVA and Bonferroni post-test (C).

Supplementary Figure 6



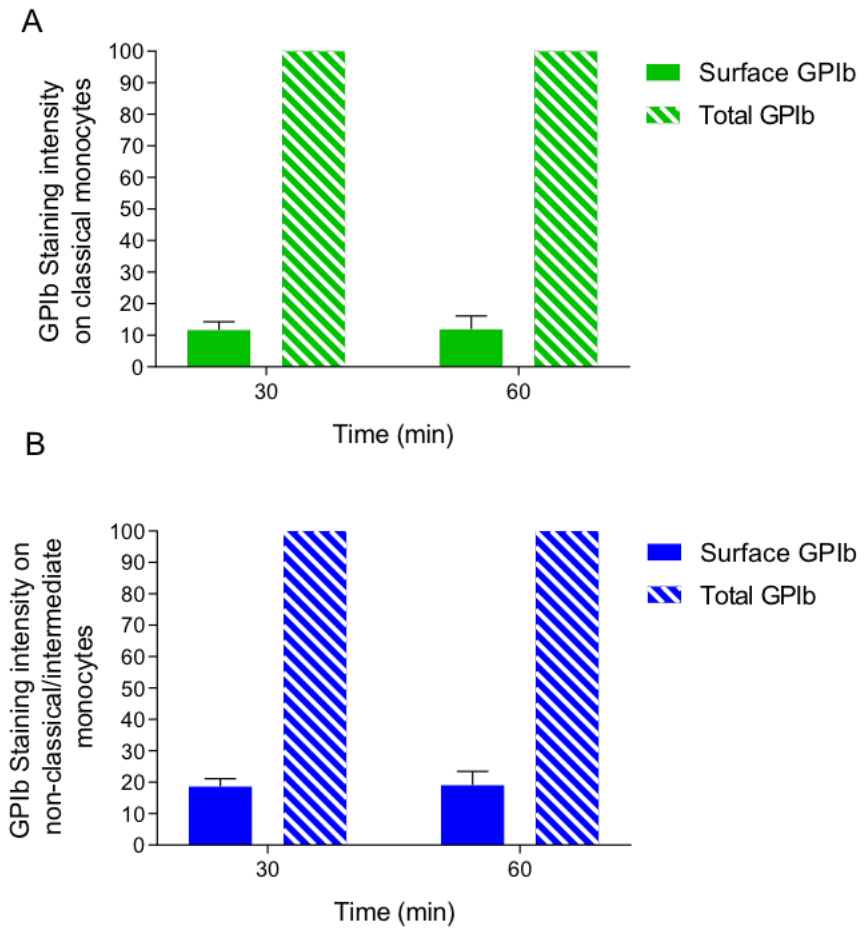
Supplementary Figure 6: Measurement of CD41$^+$ PEV. (A) Representative plot of CD41 intensity on platelets (left histogram) and PEV generated by stimulation of platelets with 1 µg/ml CRP-XL for 30 minutes analysed by flow cytometry (right histogram). (B) Quantification of CD41$^+$ frequency (C) and MFI (D) in platelets and PEV, n=3. Data are mean ± SEM. *P<0.05, ** P<0.01 by unpaired t-test.

Supplementary Figure 7



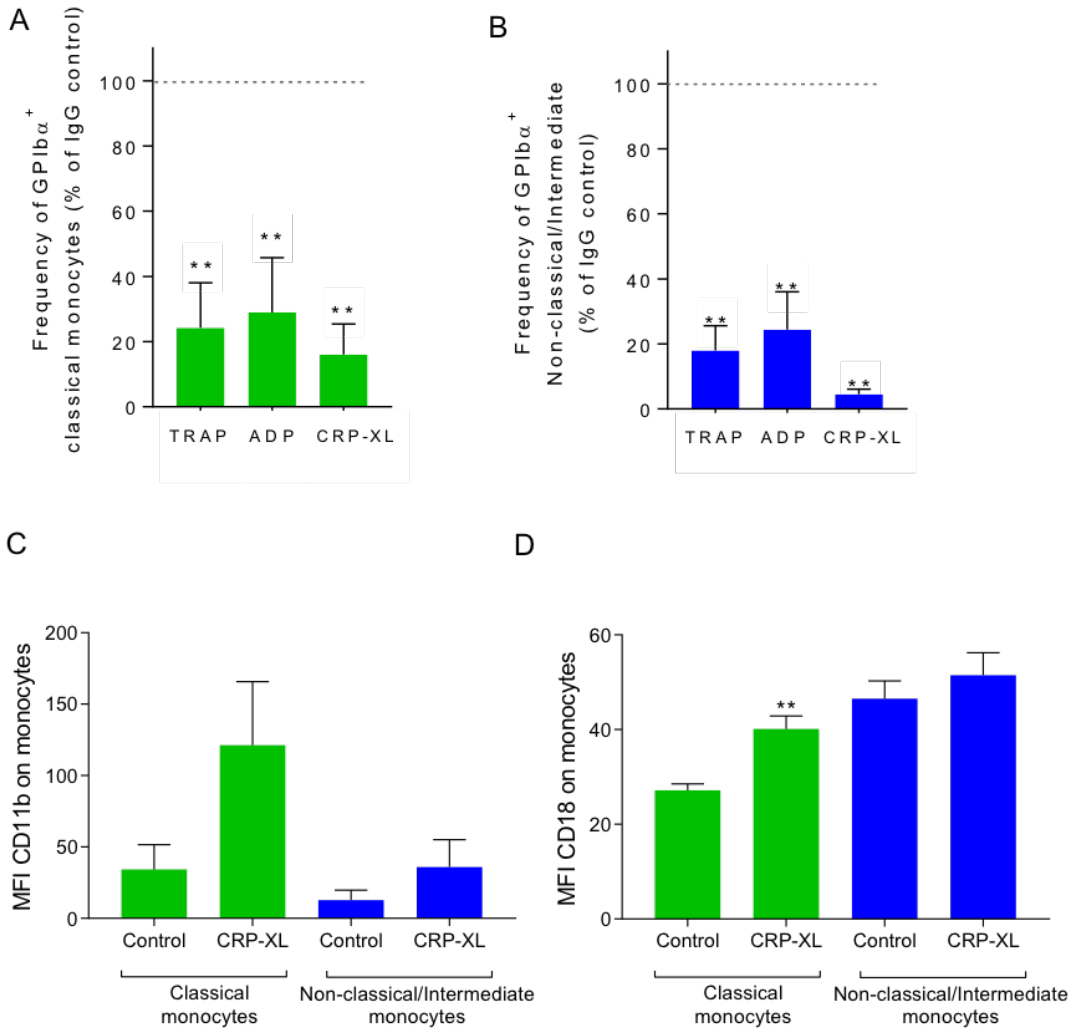
Supplementary Figure 7: Characterisation of PEV. (A) Representative plot of Mitotracker and PKH67 labelled PEV generated by stimulation of platelets with 1 µg/ml CRP-XL for 30 minutes analysed by flow cytometry. (B) Size proportion of $CD41a^+$ and $CD9^+$ PEV using ExoView R-100. (C) Normalised total number of PEV captured on a control antibody (IgG), exosomal markers CD81 and CD63 and platelet markers CD9 and CD41a using the ExoView R-100, n=4. (D) CD81, CD63 and CD9 fluorescence intensities of PEV captured on control IgG, CD81, CD63, CD9 and CD41a using ExoView R-100, n=4. Data are mean ± SEM.

Supplementary Figure 8



Supplementary Figure 8. Surface and intracellular accumulation of GPIbα on monocytes. (A, B) Staining intensity (MFI) of GPIbα on (A) classical monocytes and (B) Non-classical/intermediate monocytes, after incubation with PEV for 30 minutes at 37°C under shear, before (surface) and after (Total) permeabilisation, n=3. Surface expression was normalised to total GPIbα expression. Data are from at least three independent experiments using three donors and are shown as mean ± s.e.m.

Supplementary Figure 9



Supplementary Figure 9. P-selectin blockade and expression of integrins on monocytes in stimulated whole blood. (A, B) Frequency of GPIbα$^+$ classical (A) and non-classical/intermediate (B) monocytes with blockade of P-selectin (G1 clone, 20 µg/ml) in ADP (30 µM), TRAP (100 µM) or CRP-XL (1µg/ml)-stimulated whole blood for 30 minutes under shear, n=3. (C, D) MFI of CD11b ($\alpha_{M)}$ (A) and CD18 ($\beta_{2)}$ (B) on classical monocytes (green bars) and non-classical/intermediates (blue bars) in whole blood stimulated with CRP-XL (1µg/ml) for 30 minutes under shear determined by flow cytometry, n=3. Data are mean ± s.e.m. ** P ≤ 0.01, *** P ≤ 0.001, **** P≤ 0.0001 compared to the normalised IgG control (A, B) by analysis of variance (ANOVA) and Dunnett post-test and using repeated measured two-way ANOVA and Bonferroni post-test (C, D).

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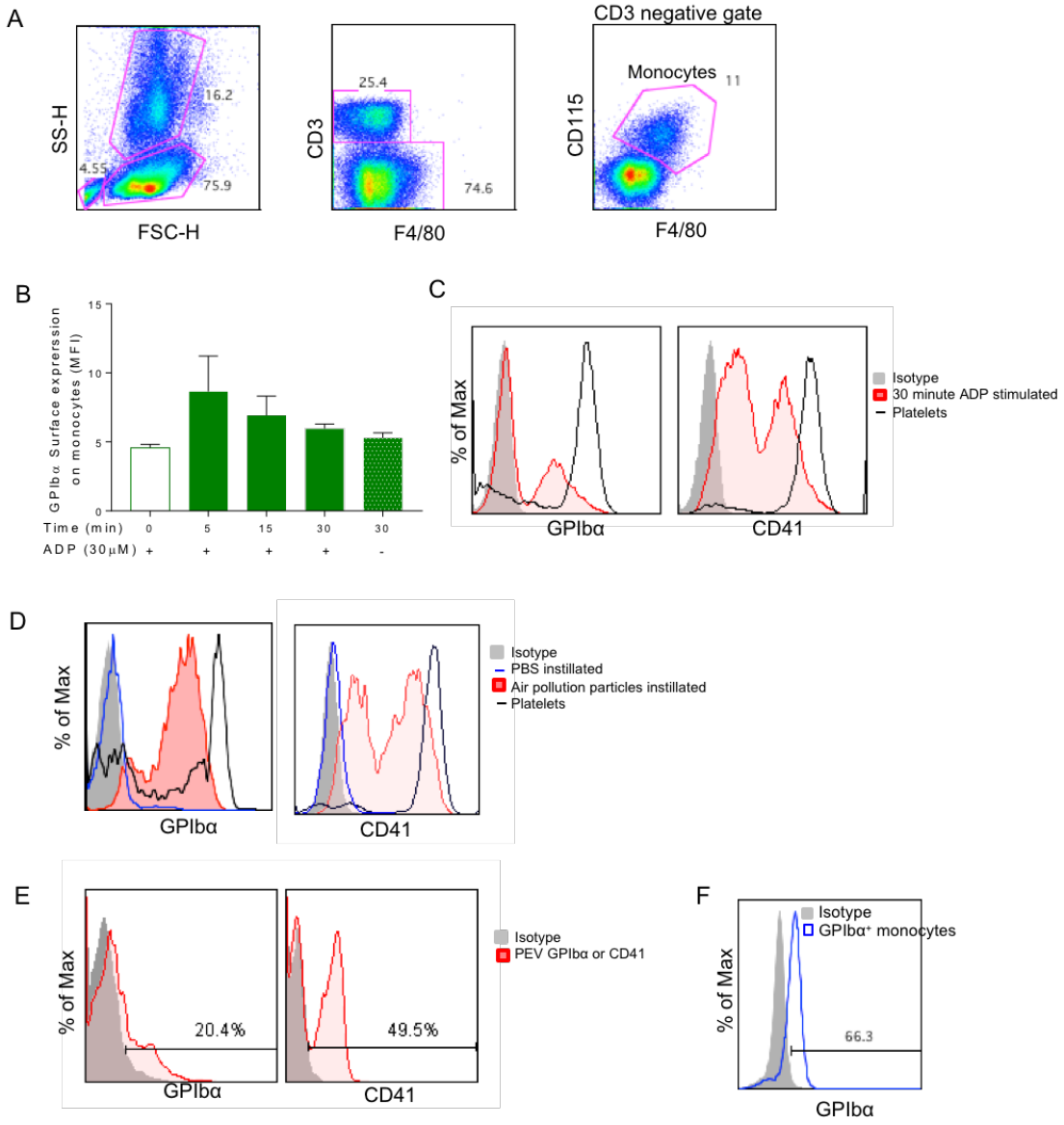
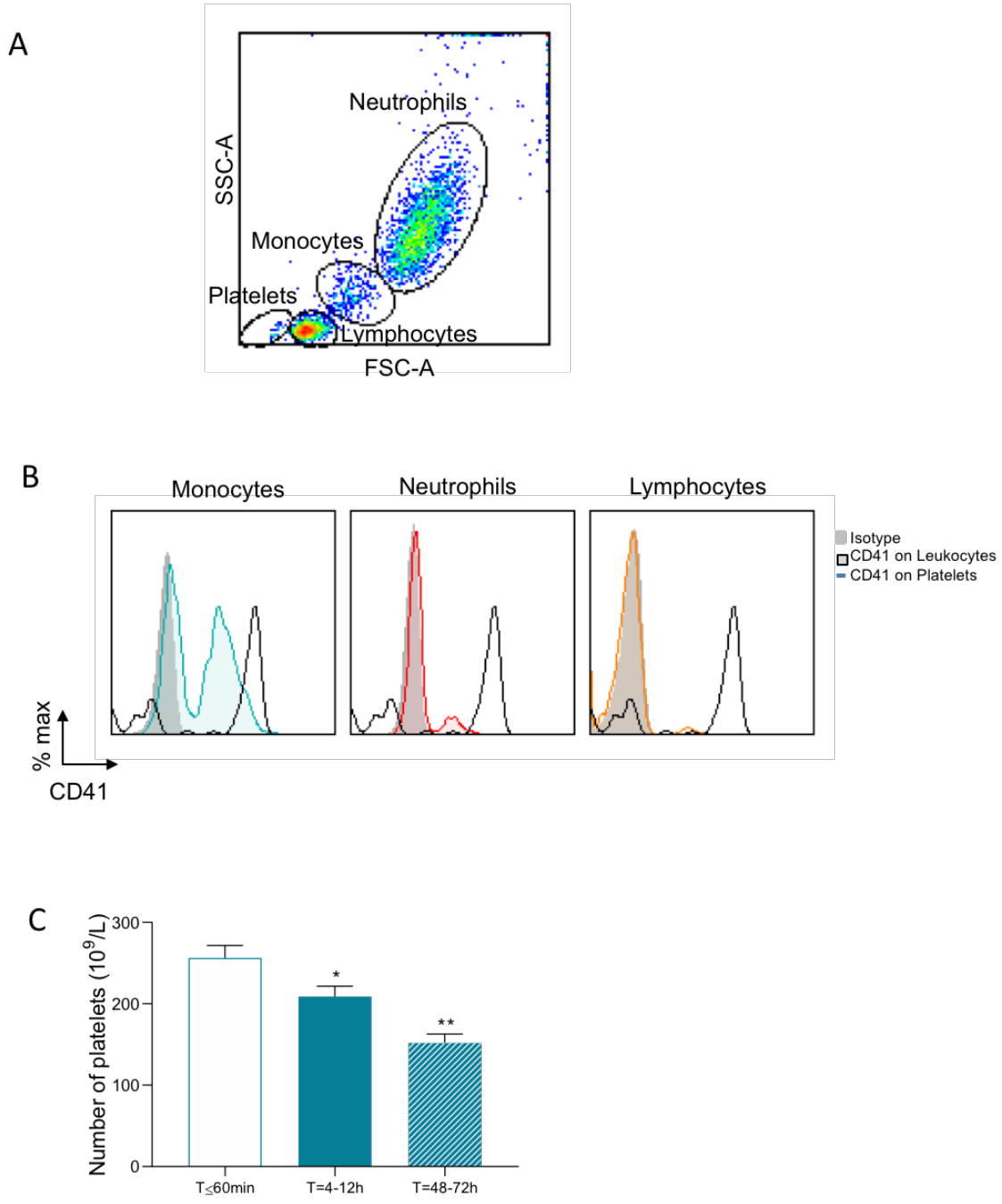
Supplementary Figure 10



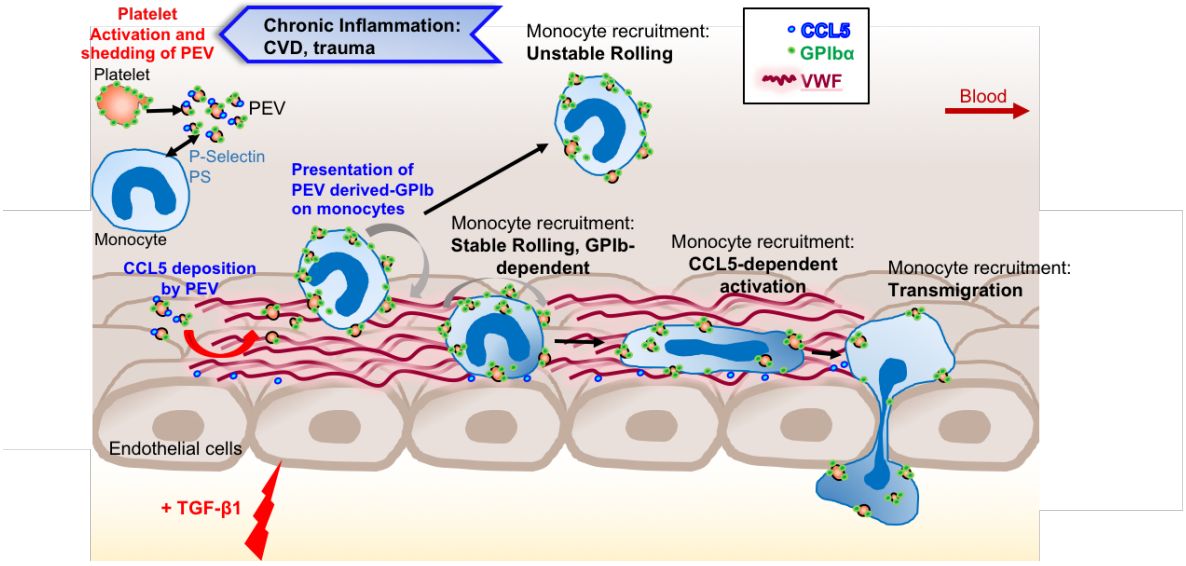
Supplementary Figure 10. Accumulation of mouse GPIbα or CD41 on monocytes. (A) Gating strategy to identify mouse monocytes by flow cytometry. (B) MFI of GPIbα^+ monocytes in whole blood stimulated with 30 μM ADP at 37°C under shear determined by flow cytometry, n=4. (C) Representative histogram of mouse monocytes bound to GPIbα$^+$ (left histogram) or CD41$^+$ (right histogram) PEV in murine whole blood stimulated with ADP (30μM) for 30 minutes under shear stress. (D) Representative histogram of mouse monocytes bound to GPIbα$^+$ (left histogram) or CD41$^+$ (right histogram) PEV in control mice without instillation, PBS and air pollution particles instillated animals. (E) Representative histograms of GPIbα$^+$ (left histogram) or CD41$^+$ (right histogram) isolated murine PEV generated upon platelet stimulation with ADP (30μM) for 30minutes measured by flow cytometry (F) Representative histogram of human monocytes bearing GPIbα from murine PEV used to inject in the platelet depleted IL4R/GPIbα-Tg mice for intravital microscopy of the TGFβ1-stimulated cremaster muscle. Data are from at least three independent experiments using three mice and are shown as mean ± s.e.m.

Supplementary Figure 11



Supplementary Figure 11. Monocytes accumulate PEV derived marker CD41 in trauma patients. (A) Gating strategy on platelets, monocytes, lymphocytes and neutrophils in the Golden Hour patient samples. (B) Representative histogram of CD41 expression on monocytes, lymphocytes and neutrophils at 60 minutes after trauma. (C) Number of platelets in the circulation of patients after trauma (<60min, 4-12h and 48-72h), n=33. Data are mean ± s.e.m. * P ≤ 0.05, ** P ≤ 0.01 by ANOVA and Dunnett post-test to T=<60min.

Supplementary Figure 12



Supplementary Figure 12. Alternative thrombo-inflammatory mechanism of monocyte recruitment mediated by PEV. Platelet activation results in release of PEV which have preferential affinity for monocytes in the circulation. P-selectin- and PS-dependent accumulation of PEV on monocytes leads to transfer of platelet-derived molecules such as GPIbα. Monocytes utilise PEV-derived GPIbα to adhere to VWF exposed at the EC surface triggered by TGF-β1 stimulation. CCL5 transferred on the EC surface by PEV will then allow monocyte activation and transmigration. This mode of monocyte recruitment offers an alternative pathway for monocytes and to migrate into the atherosclerotic plaque and contribute to inflammation.

Supplementary Material and Methods

Blood donors and the Golden Hour study

We obtained blood samples from healthy adult volunteers after written informed consent and with the approval of the University of Birmingham Local Ethical Review Committee (ERN_07-058). Blood obtained from the Golden Hour study cohort was ethically approved by a national research ethics committee (reference 13/WA/0399). In the Golden Hour study, blood was collected within an hour of major traumatic injury (ISS>8), 4 to 12 hours after and 48 to 72 hours after injury. All blood samples were stored at room temperature and used within six hours of the venepuncture.

Leukocyte-PEV aggregate formation in whole blood in response to treatment with platelet agonists

To determine platelet-leukocyte aggregate formation, blood was collected from healthy volunteers into a tube containing 10% citrate phosphate dextrose solution (CPDA, Sigma-Aldrich, Poole, UK). Blood was incubated at 37°C following treatment with 1µg/ml CRP-XL (provided by Prof. Farndale Group, Department of Biochemistry, University of Cambridge) or 30µM ADP (Sigma) (P2Y1 P2Y12 agonist) or 10 µM U46619 (Cayman Chemical, Michigna, USA) (Thromboxane A2 receptor agonist) or 100nM Rodocytin (gift from Johannes Eble, University of Munster) (CLEC-2 agonist) or 100 µM TRAP (Alta Bioscience, Birmingham, UK) (PAR1 agonist) for up 2 hours on a roller mixer to apply shear to the samples. For controls, blood was either incubated, untreated for 30 or 60 minutes, or fixed immediately after phlebotomy. Following incubation, 300µl of blood was fixed with 300µl of 1% (w/v) formaldehyde (Sigma-Aldrich) for 10 minutes followed by two wash steps at 500g for 5 minutes in 3ml PBS (Sigma-Aldrich). Samples were incubated with the following antibodies; Phycoerythrin (PE) labelled anti-CD14 (MEM-18) (ImmunoTools), FITC labelled anti-CD16 (YYF701101) (Alere, Waltham, US) and APC labelled CD42b (HIP1) (BD-Pharmingen, Oxford, UK) or relevant isotype controls (Ebioscience, Altrincham, UK). In some experiments, monocytes were labelled using: FITC-labelled anti-CD49d (44H6, Abcam), anti-CD11b (ICRF44, Ebioscience), anti-CD18 (212701, R/D Systems), or anti-PSGL1 (FLEG,

Ebioscience), or PE-labelled anti CD29 (P5D2, R/D Systems) and. Red cells were lysed using ACK lysis buffer (Gibco, Life technologies, Paisley, UK) according to manufacturer's instructions. To remove any residual ACK an additional wash step was added using 10ml PBS. Samples were suspended in 300µL PBS. Data were acquired using a Dako Cyan flow cytometer. A total of $2x10^4$ events were collected in the monocyte gate (CD14 positive cells).

Summit version 4.3 was used to analyse acquired flow cytometry data. Monocyte population was defined by size and granularity, as well as by CD14 and CD16 expression, to ensure minimal contamination by other blood cells. Monocytes fell into two populations, $CD14^+CD16^-$ (Classical monocytes) (~90% population) and $CD14^{+/dim}CD16^+$ (Non- classical/Intermediate monocytes) (~10% population) (Supplementary Figure 1A). GPIbα and CD14 dual positive staining indicated Classical monocyte-PEV formation and GPIbα and CD16 dual positive indicated Non- classical/Intermediate monocytes-PEV. The neutrophil and lymphocyte populations were defined by size and granularity and PEV identified using GPIbα. The zero minute time point was used for gating on GPIbα positive events for each of the leukocyte subsets. The gates were not changed between samples and leukocyte platelet-aggregates were recorded for each time point. To determine the MFI of the platelet/PEV-leukocyte aggregates, a gate was set on the platelet/PEV-leukocyte aggregates, which would include the equivalent of 95% of the total population of resting platelets expressing GPIbα. This was defined as the 'platelet' gate. A gate was set to include the remaining events, with a low GPIbα MFI. This was defined as the 'PEV' gate. This gating strategy allowed us to quantify the MFI for a specific accumulation of PEV on monocytes without including rare platelet-leukocyte aggregates.

In some experiments, 20µg/ml of mouse anti-human P-selectin blocking antibody (G1) or unlabelled mouse IgG1 (clone P3.6.2.8.1) (Ebiosience), or 10µg/ml anti-human CD31 blocking antibody (clone L133.1, BD Bioscience), or 10µg/ml anti-human ICAM-2 (clone AF244, Biotechne), or 20µg/ml anti-human CD18 (clone IB4, Merk Millipore), or 30µg/ml anti-human

PS (clone 05-719, Merk Millipore) were added to 300µL of whole blood (collected into 10% v/v CPDA from healthy volunteers) before the aggregation assay.

PEV generation

Whole blood was collected in 10% CPDA solution with adenine (Sigma-Aldrich) and centrifuged at 250 x g for 15 minutes after addition of 10% v/v theophylline solution (60mM theophylline 4.8mM D-glucose in phosphate buffered saline without Ca^{2+}/Mg^{2+}; 0.2µm pore-filtered) (Sigma-Aldrich). Platelet-rich plasma was collected and washed in theophylline-albumin solution (theophylline solution with0.15% bovine albumin fraction V solution) by centrifugation at 800g for 15 minutes. The number of platelets was counted using a Beckman Coulter Z2 cell & particle counter according to manufacturer's instructions. Platelets were resuspended in 100µl per 3 x 10^8 platelets and stained with APC anti-CD42b antibody (HIP1) (BioLegend) (20 µl per 100µl containing 3 x 10^8 platelets) or APC isotype (BioLegend) (5 µl per 100µl containing 3 x 10^8 platelets) for 30 minutes at 4˚C. Platelets were alternatively labeled with PKH67 dye using the PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich) as manufacturer's instructions. Briefly, platelet pellets containing 3 x 10^8 platelets each, were resuspended in 750 µl Diluent C. In a fresh tube 15 µl of dye was diluted in 750 µl of Diluent C. The dye solution was added to the cell solution and left to incubate at room temperature for 5 minutes with periodic mixing. An equal volume of 1.5% Bovine Albumin Fraction V solution (7.5%, Life Technologies) in diluted theophylline solution was added to the stained cells and left to incubate for 1 minute to stop the reaction. Cells were then washed by centrifugation at 700g for 10 minutes at 20-25 ˚C. The pellet was rewashed twice in 10 ml of the same solution. Stained platelet pellets were resuspended in 1ml PBS-Albumin solution (0.15% bovine albumin fraction V solution, phosphate buffered saline with Ca2+/Mg2+) (0.2µm pore-filtered) and stimulated with 1µg/ml CRP-XL at 37 ˚C for 30 minutes to generate PEV. Samples were centrifuged at 2000 x g for 20 minutes to pellet platelets. The supernatants containing PEV were transferred in fresh tubes leaving behind ~100 µl in order to prevent contamination with the platelet pellet and were further centrifuged at 13 000 x g for 2 minutes to pellet any platelet

remnants. The supernatants containing pure PEV were then transferred in fresh tubes leaving behind ~100 µl in order to prevent contamination with pellet. Samples were analysed using an Accuri C6 Flow Cytometer (BD Bioscience, Oxford, UK). PEV gating was determined by running microspheres of <1µm in size. PEV gated on size with this method were on average 80-90% positive for GPIbα (Figure 3A) and were used in our recruitment assays to bind to monocytes.

Nanoparticle tracking analysis (NTA) to determine the size and number of PEV.

Total PEV concentration and size distribution in PEV samples were determined using a Nanosight LM10 (Malvern Instruments, UK) equipped with NTA software 2.2 as described previously.[1] Maximum possible detection was achieved by optimizing the concentration of PEV to get 10^8-10^9/ml, by diluting with filtered phosphate buffered saline (PBS; Sigma). Approximately 300µL of the diluted sample was introduced into the Nanosight chamber mounted on the light microscope platform, and a laser was illuminated by laser at an angle to the optical axis of the microscope. The scattered light illuminates the particles and the video images were captured through digital camera attached to the microscope at a rate of 30/s for 60 seconds. NTA software 2.2 analyses the sample by tracking the movement of individual particles to determine the particle size and total particle count. Pre and post-acquisition parameters for measuring PEV samples were optimized and maintained constant between samples. The camera gain set to 1 and camera shutter set to 250 and the detection threshold set at 8. Each video was analysed to give the mean, mode, and median vesicle size and an estimate of the concentration of PEV/ml. On average, this method generated $1.6x10^8$ ± $2.7x10^7$PEV/ml.

PEV Characterisation by ExoView R-100

PEV were detected by the ExoView R100 reader (NanoView Biosciences, Boston, MA, USA). This technique utilises chip-based antibody capture and Single Particle Interferometric Reflectance Imaging Sensor (SP-IRIS) technology to capture and resolve particle size down between 50-200 nm. Captured PEV can then be labelled with secondary fluorescent

antibodies, thus allowing for identification of different populations of EVs and for identification of vesicles <50 nm in diameter. Tetraspanin Plasma chips (NanoView Biosciences, Boston, MA, USA) were used for all samples. These silicon chips were arrayed with capture antibodies against human CD63, CD81, CD9, CD41a and Mouse IgG.

PEV were thawed via a 10-minute incubation at 37ºC in a water bath. PEV were then transferred to 1.5ml Eppendorf(s) and centrifuged at 3000 x g for 20 minutes to pellet any remnant cellular material. Supernatants were collected and diluted 1:100 in PBS with 0.05% tween (PBST). Chips were carefully placed in the centre of separate wells in a 24 well plate. 35 µl of diluted sample was applied to each chip. 1ml of distilled, 0.22 filtered water was added to the void space between wells to form a humidity chamber; the plate was sealed and allowed to incubate for 16 hours at room temperature in the dark. Wells containing chips were washed three times in PBST. Following the final wash, a fluorescent antibody cocktail of CD9-Alexa 488, CD81-Alexa-555 and CD63-Alexa-647 was applied with a final immunostaining ratio of 1:6000. The plate was incubated for one hour at room temperature in the dark. Wells containing chips were then washed with PBST once and washed a further three times with PBS.A final wash was performed with distilled, 0.22 filtered water. After each wash the plate was shaken at 500 rpm (LSE Digital Microplate Shaker, Corning) for three minutes. The chips were then dried and imaged with the ExoView R100 reader using the nScan2 Version 2.76 acquisition software. Data were analysed using NanoViewer Version 2.82 with fluorescence gating based on Mouse IgG capture background.

Monocyte-PEV aggregate generation

Some assays required isolated monocyte-PEV aggregates. PBMC were collected from EDTA-anticoagulated whole blood using histopaque density gradient centrifugation as described previously.[2] Monocytes were isolated using $CD14^+$ beads (Miltenyi Biotec, Surrey, UK) according to manufacturer's instructions, to yield isolated monocytes with >95% purity. Unlabelled PEV were generated (as described above) and stored at -20˚C. $1x10^6$ monocytes were suspended in 0.15% w/v PBSA (control) or PEV (generated from $3x10^8$ platelets), and

samples were incubated at 37°C for 30 minutes on a roller mixer to apply shear to the samples. Approximately 2-3 x 10^8 platelets and 1 x 10^6 monocytes can be found in 1 ml of blood. We therefore generated PEV from the number of platelets found in 1 ml of blood to add to the number of monocytes found in 1 ml of blood in order to standardise our assays as close to physiological conditions as possible. Monocyte-PEV aggregates were either labelled for flow cytometry with PE labelled anti-CD14 (MEM-18) (Immuno Tools), FITC labelled anti-CD16 (YYF701101) (Alere) and APC labelled anti-CD42b (clone HIP1) (BD-Pharmingen) or relevant isotype controls (Ebioscience, Altrincham, UK) as described above or used for flow-based adhesion assays on VWF and EC (see below).

Microscopy

Unstained isolated monocytes (1 x 10^6) were incubated with purified unstained PEV (generated from 3 x 10^8 platelets) on a roller for 30 minutes at 37°C. The cell suspension was then centrifuged at 400g for 6 minutes and the pellet containing monocyte-PEV aggregates was resuspended in medium 199 without phenol red (Thermo Fisher Scientific). 1.5 x 10^5 monocytes were plated on glass microwell plates (MatTek Corporation, Massassushets, US) that had been coated in advance with poly-l-lysine solution (Sigma) for 5 minutes at RT, washed with tissue culture grade water and left to dry for 2-3 hours before introducing cells and medium. Aternatively, cells were plated in ibiTreat slides (Thistle Scientific). Plated cells were incubated at 37°C for 30 minutes in a CO_2 incubator. The cells were then fixed in 2 % formaldehyde solution (Sigma) for 10 minutes. Following fixation, the surface was blocked with BSA (Thermo Fisher Scientific) for 10 minutes.and washed with Dulbeco's phosphate buffered saline (Sigma). Cells were incubated with anti-CD14-Alexa Fluor 647 (clone EPR3653, 1:200, abcam), DAPI (5 ng/ml), FCR blocking reagent (1:100, Miltenyi Biotec) and either GPIbα alpha / IgG1 isotype-Alexa Fluor 488 (Clone HIP1, 1:200, Novus Biologicals) for 60-90 minutes at 4°C and washed in PBS. Plates were saved at 4°C until imaging. Fluorescent images produced by an N-STORM microscope (Nikon, Tokyo, Japan) or a Zeiss LSM 780 microscope were processed using the ImageJ software. Z-stacks produced by fluorescence microscopy

were used to create composite images to show total PEV-derived GPIbα on monocytes. PKH67 labelling on monocytes/PEV aggregates were imaged by z-stacking (0.2um step) with a confocal microscope (Zeiss LSM780). Z-stacks were used to produce composite images showing total PEV signal on the monocytes using FIJI software (maximum projection for PKH67 and Dapi, average projection for CD14).

Flow based adhesion assay on purified VWF

Capillary tubes of rectangular cross section (0.1 × 1.0 mm, 50 mm long, Camlab, Cambridge, UK) were coated with 0.1 mg/mL VWF (HTI, Vermont, USA -HCVWF-0190) overnight at 4°C. The capillaries were washed and blocked with PBS containing 2% bovine serum albumin (BSA) for 2 hours at room temperature. Capillaries were then rinsed with 0.15% PBSA (PBS containing 0.15% BSA), and connected to a flow system containing reservoirs filled with either monocytes ± PEV, or PBSA (wash buffer). Monocytes ± PEV were i prepared as described above and were perfused through the VWF-coated microcapillary at a wall shear rate of $1000s^{-1}$ for 1 minute. Shear rate was then reduced to $100s^{-1}$, for a further 5 minutes. Monocyte ± PEV adhesion was monitored at $100s^{-1}$. At least 6 different microscope fields (20 × objective) were captured for 10s. Image analysis was performed off-line using ImagePro plus software (DataCell Limited, Berkshire, United Kingdom). The numbers of adherent cells were counted in each field, averaged and converted to cells per mm^2 per 10^6 cells perfused. In some experiments, GPIbα was blocked using an anti-GPIbα antibody (clone 6B4, courtesy of Prof. H. Deckmyn, University of KU Leuven Kulak) at 20 µg/ml during incubation of monocytes with PEV for 30 minutes under shear.

Flow based adhesion assay on EC monolayers

Human dermal blood endothelial cells (HDBEC) (Promocell, Heidelberg, Germany) were passaged four times and frozen. HDBEC were defrosted in $25cm^2$ flasks and seeded into Ibidi 6 channels µ-slides (Ibidi, Martinsried, Germany). After 24 hours, HDBEC were stimulated with TGF-beta (10 ng/ml, R/D Systems) for 24 hours in order to up-regulate VWF at the cell surface. Monocyte ± PEV were prepared as described above and were perfused on HDBEC

monolayers in the μ-slides for 4 minutes at a wall shear stress of 0.1Pa. Monocyte ± PEV adhesion and migration was assessed using a phase-contrast videomicroscope as described previously.[3] Manipulations and microscopy were carried out at 37°C and at least 6 different microscope fields (20 × objective) were analysed. Recordings were digitized and analysed offline using Image-Pro Plus software (DataCell, Finchampstead, U.K.). The numbers of adherent cells were counted in each field, averaged and converted to cells per mm^2 and multiplied by the known surface area of the EC monolayer to calculate the total number adherent. This number was divided by the known total number of monocytes perfused to obtain the percentage of the monocytes that had adhered. In some experiments, GPIbα was blocked using an anti-GPIbα antibody at 20 μg/ml (clone 6B4) during incubation of monocytes with PEV for 30 minutes under shear.

FITC-Phalloidin staining of monocyte bound to platelets

For the FITC-phalloidin stainings, blood was collected from healthy volunteers by venepuncture of the ante-cubital vein, into vacutainers containing dipotassium ethylenediamine tetra acetic acid (K_2 EDTA**)** (Becton & Dickinson UK. Ltd, Oxford). FITC-phalloidin (Molecular Probes, 300U) stock (5.6μM) was prepared by dissolving 0.1mg (300 units) in 1.5ml of methanol and stored at -20ºC until used. A Cell Permeabilisation Kit (Invitrogen, Molecular Probes) was used according to the manufacturer's instructions. Cells then stain with FITC-phalloidin and were visualised in an Olympus microscope (Olympus Optical Co. LTD, Japan). The cells were viewed on the x100 oil immersion objective.

Animal experiments

All experiments were performed in accordance with Home Office Guidelines and with approval of a local ethics committee. In each experiment C57BL/6 *IL4R/GPIbα*−Tg or wild-type (WT) animals with the same background were allocated at random to different experimental groups. Importantly, mice from the same litter were randomly distributed amongst the experimental groups and where possible, were equally distributed amongst experimental groups.

Leukocyte-PEV aggregate formation in murine whole blood in response to treatment with platelet ADP

Platelet-leukocyte aggregate formation was assayed in blood collected from WT mice into a tube containing 10% citrate phosphate dextrose solution (CPDA, Sigma-Aldrich, Poole, UK) following cardiac puncture. Blood was incubated at 37°C on a roller mixer to apply shear to the samples, following treatment with 30μM ADP (Sigma) for up 30 minutes and samples for analysis were retrieved at 5, 15 and 30 minutes. For control samples, blood was either fixed shortly after collection or incubated on a roller mixer for 30 minutes without ADP. Samples for analysis were fixed with 1% (w/v) formaldehyde (Sigma-Aldrich) for 10 minutes, washed twice (500g for 5 minutes). Red cells were lysed using ACK lysis buffer (Gibco, Life technologies, Paisley, UK) according to manufacturer's instructions. Residual ACK was removed by washing in a large volume of PBS. Samples were incubated with the following antibodies for 30 minutes at 4°C, Phycoerythrin (PE) labelled anti-CD115 (clone AFS98), APC labelled anti-F4/80 (clone BM8), PerCp Cy5.5 labelled anti-CD3 (clone 145-2C11) (all from Ebioscience, Altrincham, UK), FITC labelled anti-GPIbα (XiaG5, Emfret, UK) and anti-CD41 (MWReg30, BD Pharmingen) or relevant isotype controls (Emfret, UK). Samples were suspended in 300μL PBS. Data were acquired using a Dako Cyan flow cytometer. Flowjo (Treestar) was used to analyse acquired flow cytometry data. Monocyte population was defined by size and granularity, T-cells where gated out using CD3 and monocytes gated using F4/80 and CD115 (Supplementary Figure 9A). Percentage of GPIbα positive monocytes was determined using gating on the isotype control for each animal.

Intratracheal instillations of air pollution particles

Male 9–12-week-old wild type (WT) C57BL/6 mice, with an average body weight of 25 g were obtained from Harlan UK Limited, Oxford, UK and maintained in the Biomedical Services Unit, Birmingham University, UK. Intratracheal (IT) instillations were performed as previously described.[4] Briefly, mice were anaesthetised using intraperitoneal injections of metetomidine (60 mg/kg) and ketamine (10 mg/kg) and a fine polyethylene catheter (external diameter 0.61 mm and internal diameter 0.28 mm) passed into the trachea via the mouth under direct

visualisation of the vocal cords. 400 µg of air pollution particles (Urban particulate matter, Standard Reference Material 2786; National Institute for Standards and Technology, Gaithersburg, MD 20899, USA) in 50 µL sterile PBS or PBS alone were instilled. Mice were given 0.1 mL atipamezole to reverse the metetomidine and hydrated with two 0.5 mL saline subcutaneous injections, one immediately post IT and another 6 h later.

To determine monocyte-platelet aggregate formation, blood was collected from treated mice 48 hours after instillation, by cardiac puncture into a tube containing 10% citrate phosphate dextrose solution (CPDA, Sigma-Aldrich, Poole, UK). Blood was processed and assayed for flow cytometry as described above.

Intravital imaging of the cremaster muscle and the carotid artery

Mouse PEV were generated by stimulating isolated platelets with 10 µg/ml CRP-XL [5, 6] and purified as described in the section on PEV generation from human platelets. Human monocytes were isolated as described above, and incubated with mouse PEV from 30 minutes under shear. CFSE (ThermoFisher) was added at 10µM during the last 10 minutes of incubation to label monocytes. Monocyte/PEV were washed twice in PBS and resuspended in PBS-BSA for adoptive transfer. When desired, GPIbα function was blocked using an anti-GPIbα antibody at 50 µg/ml (Xia.B2, Emfret) during incubation of monocytes with PEV for 30 minutes under shear.

IL4R/GPIbα−Tg mice were genotyped by measuring expression of the IL4R (anti-human CD124-Phycoerythrin, BD Biosciences) using flow cytometry. For intravital microscopy, anti-human IL4 receptor antibody (2.5 µg/g, R/D Systems) was administrated *to IL4R/GPIbα*−Tg mice intravenously 24 hours before microscopy.[7] The resultant thrombocytopenic *IL4R/GPIbα*−Tg animals were injected intrascrotally with 80 µg/kg TGF-β1. After 4 hours, anesthetized animals (100 mg/kg ketamine hydrochloride, Zoetis UK, UK; 10 mg/kg xylazine hydrochloride, Chanelle Animal Health, UK; intraperitoneally) underwent tracheotomy and carotid cannulation to facilitate infusion of labelled cells. The cremaster

muscle was then exposed and CFSE labelled monocytes/PEV (1.5million) ± anti-GPIbα antibody were injected via the carotid artery. Post-capillary venules with a diameter of 20–40 μm were selected for study and at least three vessels in each mouse were video-recorded for monocyte/PEV adhesion. Data are expressed as adhesive interactions of monocytes per mm of vessel segment/minute.

ApoE$^{-/-}$ ^{mice} were genotyped using the ISOLATE Genomic kit according to manufacturer's instructions. At 10 weeks, ApoE$^{-/-}$ mice were placed on a Western diet (21.4% cocoa butter [w/w] and 0.2% cholesterol [w/w]; Special Diet Services, UK) for 6 weeks. Anesthetized animals (100 mg/kg ketamine hydrochloride, Zoetis UK, UK; 10 mg/kg xylazine hydrochloride, Chanelle Animal Health, UK; intraperitoneally) underwent tracheotomy and left carotid cannulation to facilitate infusion of labelled cells. The right carotid was then exposed and CFSE labelled monocytes/PEV (1.5million) ± anti-GPIbα antibody injected via the left carotid artery. Recruitment to the carotid using a 10x objective and recorded for 4 minutes near the carotid bifurcation. Data are expressed as adhesive interactions of monocytes per mm of vessel segment per minute.

Exposed carotid and cremaster muscle were visualized using an inverted intravital microscope (Olympus IX-81, Olympus, Essex, UK). Data were stored digitally and analysed off-line (Slidebook, Intelligent Imaging Innovations, Denver, CO, USA).

Flow cytometry of Golden Hour samples

25 μl aliquots of citrated whole blood were mixed with 74 μl of Hepes buffered saline (0.145 mMol/L NaCL, 5 mMol/L KCL, 1mMol/L $MgSO_4$) and stained with 1 μg/ml FITC-labelled mouse anti-human CD41 (clone 5B12; Dako, Cambridgeshire, UK) or its concentration-matched isotype control for 10 minutes at room temperature (RT). Post incubation, samples were fixed for 10 minutes at RT in 2% formaldehyde, after which red blood cells were lysed. For flow cytometric analysis, which was performed on an Accuri C6 flow cytometer (BD Biosciences, Oxford, UK), monocytes were gated according to their forward and sideward

scatter properties and CD41 expression measured as the percentage of $CD41^+$ monocytes and median fluorescent intensity. Data was analysed using CFlow software (BD Biosciences).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Normality of data was checked using the Kolmogorov–Smirnov test. Differences between individual treatments or groups were analysed by paired or unpaired t-test as appropriate. One or two-way analysis of variance (ANOVA) was used for multiple group comparison followed by a post hoc analysis where appropriate, using Bonferroni test for comparisons between groups or Dunnet tests for comparisons to a control group. P values ≤ 0.05 were considered significant.

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