

*Platelet endothelial cell adhesion molecule-1 inhibits platelet response to thrombin and von Willebrand factor by regulating the internalization of glycoprotein Ib via AKT/glycogen synthase kinase-3/dynamin and integrin  $\alpha$ IIb $\beta$ 3*

Article

Supplemental Material

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## Supplementary Material

### Materials and methods

#### *Reagents*

CRP-XL (monomeric sequence GCI[GPO]<sub>10</sub>GCOG) was prepared as described previously,<sup>1</sup> thrombin was from Sigma Chemical Co. Ltd. (Poole, Dorset, UK). FITC-labelled thrombin was labelled in house. Thrombin receptor activation peptides (TRAP, PAR-1 – SFLLRN, PAR-4 – GYPGQV) were from Bachem (St. Helens, UK). Gly-pro-arg-pro peptide (GPRP) (Sigma) was added where thrombin was present to prevent clotting. PECAM-1 monoclonal antibody (mAb) AB468 and appropriate isotype control antibodies (Millipore, Watford, UK) were dialysed to remove azide, as was PECAM-1 antibody (WM59) (Serotec, UK). PE/Cy5-anti-CD62P, FITC-anti-GPIb $\alpha$  and FITC-anti-GPIb $\beta$  were from BD Biosciences (Oxford, UK), and FITC-anti-fibrinogen was from Dako Ltd (Ely, UK). GPVI (HY101, M.L. Kahn, University of Pennsylvania), PAR1 (Life technologies, Paisley, UK) and PAR4 antibodies (Abcam, Cambridge, UK), respectively, were labelled using a Zenon Alexa Fluor 647 labelling kit (Life Technologies) prior to use. Antibodies against the C- or N-terminal of GPIb $\alpha$  came from Santa Cruz (Dallas, USA). Antibodies recognising mouse GPIb $\alpha$ , GPVI, and integrin  $\alpha$ IIb $\beta$ 3 came from Emfret analytics (Eibelstadt, Germany). VWF was from Haematologic Technologies Inc (Vermont, USA). The purified cobra metalloproteinase, Nk, was a gift from Prof. Rob Andrews, Monash University, Australia.<sup>2</sup> Cytochalasin D, Latrunculin and CHIR99021 all came from Millipore and Dynasore came from Sigma. GSK-3 $\alpha$ / $\beta$  and pSer<sup>21/9</sup> GSK-3 $\alpha$ / $\beta$  antibodies were from Cell Signalling Technologies (New England Biolabs, Hitchin, UK). Hepes buffered saline (HBS; 0.14M NaCl, 5mM KCl, 1mM MgSO<sub>4</sub>, 10mM HEPES (sodium salt), pH7.4) was used for all dilutions. PECAM-1 knockout mice were provided by Prof. T. Mak (University of Toronto, Ontario, Canada) and the DiYF mice were generated in the laboratory of Dr. David R. Phillips (Portola Pharmaceuticals Inc. San Francisco, USA). All protocols involving the use of animals were approved by the University of Reading Local Ethical Review Panel and authorized by a Home Office license.

#### *Phlebotomy*

Fresh blood was taken via standardised phlebotomy into vacuette tubes (Greiner bio-one, Stonehouse, UK) containing 3.2% sodium citrate from drug free donors. Informed consent from human subjects was obtained and procedures approved by the University of Reading Research Ethics Committee.

#### *Mouse platelet preparation*

Blood from PECAM-1 Knockout or DiYF knockin or sibling matched wild-type or heterozygous mice was taken immediately after sacrifice by cardiac puncture into 4% citrate. All experiments were performed blind and genotyping was performed subsequently as previously described.<sup>3, 4</sup>

#### *PECAM-1 cross-linking*

Where applicable, PECAM-1 cross-linking was performed prior to platelet stimulation as follows. Human platelets, in whole blood or platelet rich plasma (PRP), were incubated with anti-PECAM-1 (AB468) or isotype control antibodies (10 $\mu$ g/ml) for 10 minutes followed by an excess of cross-linking antibody, goat-anti-mouse IgG antibody (20 $\mu$ g/ml, Sigma) for 10 minutes. The same procedure was performed for mouse platelets using anti-PECAM-1 antibody (M-185) or isotype-matched control IgG (both Santa Cruz, Dallas, USA) and goat-anti-rabbit cross-linking antibody (Sigma).

#### *Flow cytometry*

Citrated whole blood or PRP was diluted 1:10 (or 1:10,000 when using Dynasore, to reduce the inhibition of Dynasore by plasma proteins) in HBS. Where indicated platelets were incubated for 30 min and 37°C, following which PECAM-1 or control antibody cross-linking

was performed as appropriate. Samples were then incubated for 20 minutes at room temperature either with CRP-XL, thrombin, or TRAP, together with FITC-anti-fibrinogen and PE/Cy5-anti-CD62P antibodies, or surface receptor specific antibodies as indicated. Reactions were stopped by 100 fold dilution in 0.2% formyl saline.

To measure total GPIIb/IIIa (internal stores as well as that expressed on the surface) citrated PRP was diluted 1:10 in HBS and PECAM-1 or isotype control antibody cross-linking was performed. Samples were fixed with 2% formyl saline, permeabilised using BD Phosflow perm buffer III (BD Bioscience, Oxford, UK) washed, resuspended in HBS, and incubated with FITC-anti-GPIIb/IIIa antibodies for 20 minutes at room temperature.

Data were acquired on either a FACSCalibur or an Accuri C6 flow cytometer (BD) and were recorded as percentage of cells positive or median fluorescence intensity (MFI).

#### *Platelet adhesion to VWF under flow.*

DIOC<sub>6</sub> (Sigma Aldrich, UK) labelled human citrated blood, with or without PECAM-1 cross-linking, was perfused over VWF (100µg/ml) coated Vena8 BioChip (Cellix Ltd, Ireland) at a shear rate of 20 dynes/cm<sup>2</sup> for 10 minutes at 37°C. To prevent platelet-platelet binding, Integrelin (2µg/ml) was included in all samples. The channels were then washed with Tyrodes-HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM Na<sub>2</sub>HPO<sub>4</sub>, 12mM NaHCO<sub>3</sub>, 20mM HEPES, 5mM glucose and 1mM MgCl<sub>2</sub>, pH 7.3) for 60 seconds and platelet adhesion was measured in six randomly selected fields of view per channel, using a Nikon eclipse (TE2000-U) microscope (Nikon Instruments, UK). The number of adherent platelets, the area covered and sum fluorescence intensity were calculated using Slidebook 5 software (Intelligent Imaging Innovations, USA).

#### *Platelet preparation and stimulation for Western blot analysis*

Washed platelets were prepared by differential centrifugation.<sup>5</sup> Platelets were re-suspended to a density of 8x10<sup>8</sup> per mL, in Tyrodes-HEPES buffer and rested for 30 minutes at 30°C before stimulation. Washed, rested platelets were either not activated or stimulated with CRP-XL following PECAM-1 cross-linking. The reaction was terminated by the addition of an equivalent volume of ice-cold lysis buffer (20mM Tris, 300mM NaCl, 10mM EDTA, 2% (v/v) Nonidet P40, 1mmol/L phenylmethylsulfonyl fluoride, 1µg/mL pepstatin A, 10µg/mL aprotinin, 10µg/mL leupeptin, and 2mmol/L sodium orthovanadate, pH 7.3). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting onto polyvinylidene difluoride (PVDF) membrane were performed using standard techniques.<sup>6</sup> Membranes blocked using 5% (w/v) bovine serum albumin in Tris-buffered saline-Tween (BSA/TBS-T) (20mM Tris, 0.14M NaCl, 0.01% Tween, pH 7.6). Primary antibodies were diluted in 2% (w/v) BSA/TBS-T and incubated over night at 4°C. Species-specific fluorescently-labelled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were added for 1 hour at room temperature. Blots were visualised using a Typhoon Trio fluorescence scanner (GE healthcare, Buckinghamshire, UK) and analysed using Image Quant TL (GE).

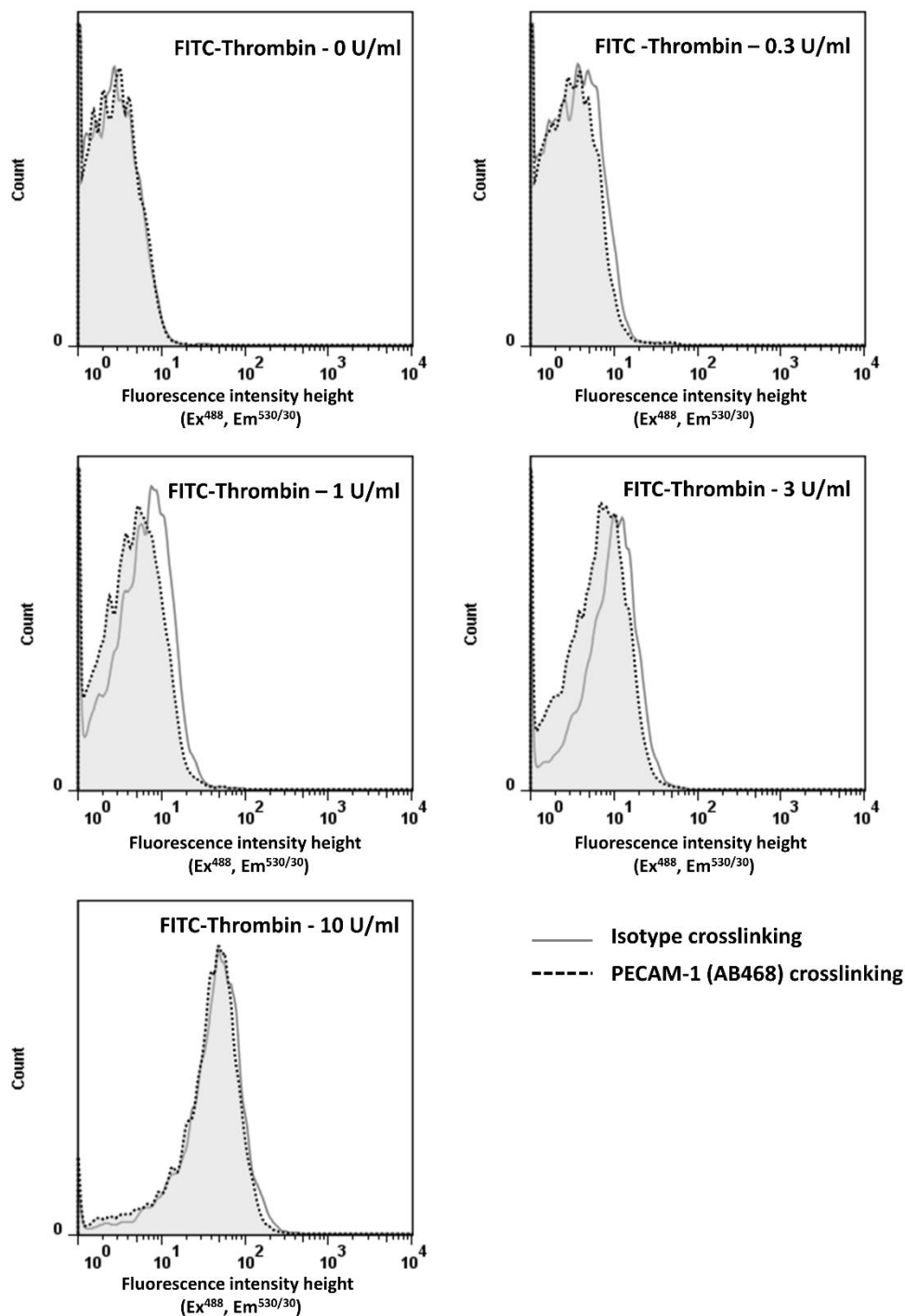
#### *Statistics*

Data are presented as mean±standard deviation of the mean (SD). Statistical analyses were performed using PRISM 5 GRAPHPAD software (GraphPad Software Inc, La Jolla, CA, USA). Data were compared using a Student's T-Test, One-way ANOVA or Two-way ANOVA and Bonferroni post-test analysis as appropriate.

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**Supplementary Figure I** – Representative flow cytometry histograms showing the binding of FITC-labelled thrombin to platelets in the presence (black dotted line) or absence (grey solid line) of PECAM-1 (AB468) crosslinking.



**Supplementary Figure II** – Representative flow cytometry histograms showing the binding of FITC-labelled GPIIb $\alpha$  antibodies to platelets in the presence (black dotted line) or absence (grey solid line) of PECAM-1 (AB468) crosslinking, on the two different flow cytometers used in this study.

