ARTICLE - Coagulation & its Disorders

Mechanisms sensitive to different drugs mediate a pro-adhesive and pro-thrombotic platelet phenotype in anti-phospholipid syndrome

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ONLINE SUPPLEMENTARY DATA

Mechanisms sensitive to different drugs mediate the platelet pro-adhesive/-thrombotic phenotype in anti-phospholipid syndrome.

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Supplementary Methods

Flow cytometry

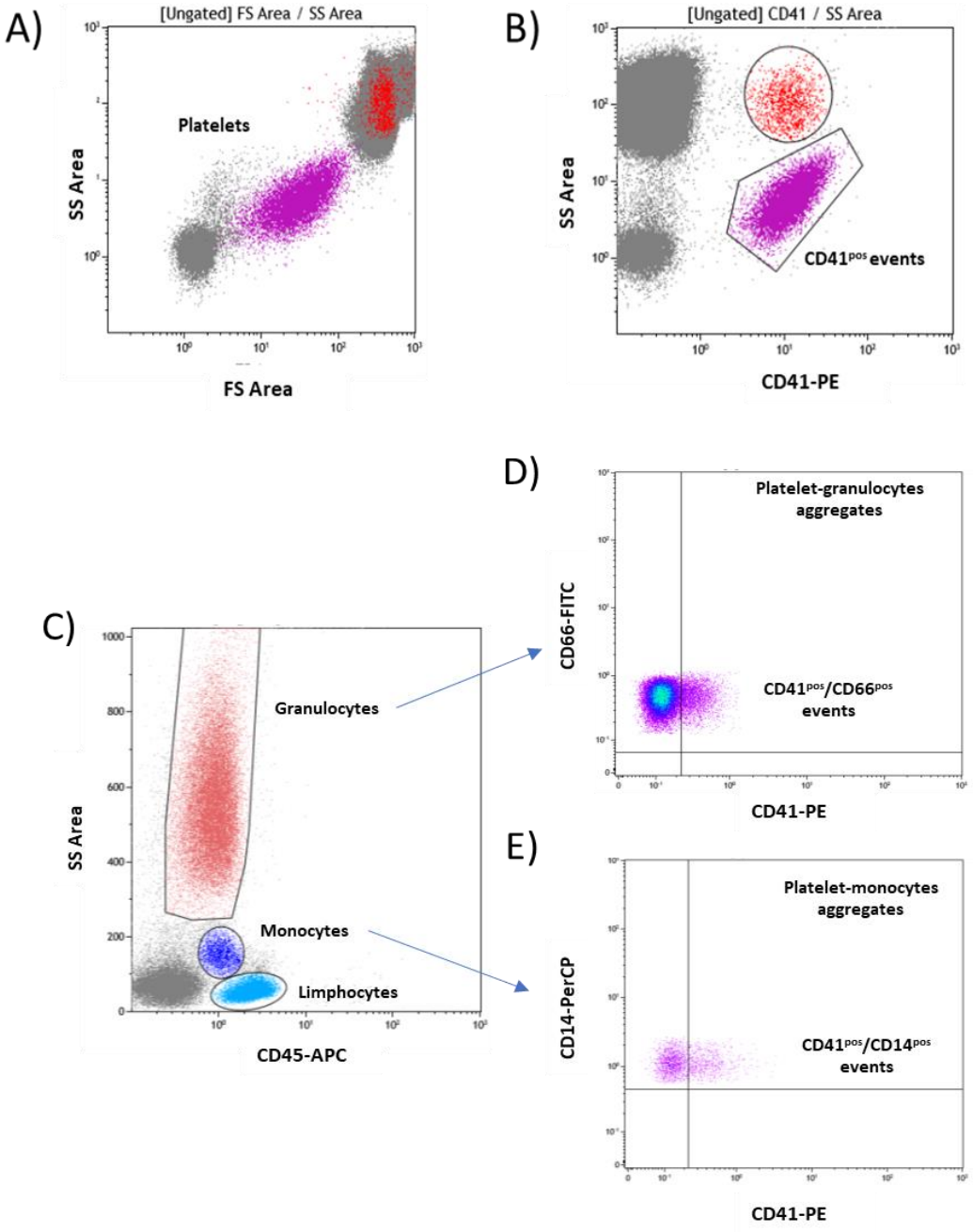
The phenotypic characterization of PAPS platelets were performed on 27 out of 34 enrolled PAPS patients. The assessment of aGPIIbIIIa, intracellular TF, TFPI, ApoER2 and β2GPI was performed as proof-of-concept on 10 patients.

Flow cytometry analysis was performed as previously described[1,2] and modified as follows. Briefly, $1x10^6$ platelets/sample, resting or activated with ADP (10μM, 15 min, RT; Sigma Aldrich), were labelled in a direct staining with saturating concentrations of mouse anti-P-selectin (APC; Becton Dickinson), anti-human aGPIIbIIIa (PAC-1; FITC; Becton Dickinson), anti-human Tissue Factor Pathway Inhibitor (TFPI; CF488; Biorbit), anti-ApoER2 (FITC; LS Bio) antibodies. Cell surface-associated TF and β2GPI expression was analysed with an indirect staining as follows. Whole blood, resting or activated with ADP, was incubated with saturating concentration of mouse anti-human Tissue Factor (HTF-1; Thermo Fisher) or anti- β2GPI (100μg/ml), for 15 min at room temperature, fixed for 1 hour with 1% paraformaldehyde at room temperature and labelled with AlexaFluor®633 IgG (1:200; Thermo Fisher) or with FITC anti-human IgG (1:100; Cappel Research Reagents), respectively. Fluorochrome-conjugated isotype (Becton Dickinson), AlexaFluor®633 IgG (1:200; Thermo Fisher), FITC anti-human IgG (1:100; Cappel Research Reagents) controls were used in all the experiments to quantify the background labelling. Anti-CD41 (PE; Beckman Coulter) and anti-CD14 (PerCP; Becton Dickinson) or anti-CD66 (FITC; Becton Dickinson) monoclonal antibodies were used to identify platelets, monocytes, and granulocytes, respectively. For TF intracellular staining, whole blood was fixed for 2 hours with 1% paraformaldehyde, permeabilized for 10 min with a 0.1% Triton X-100 PBS solution, and labelled for 15 min at room temperature in the dark, with saturating concentrations of anti-HTF-1 (PE; Becton Dickinson) and anti-CD61 (PerCP; Becton Dickinson) monoclonal antibodies. Before use, all antibodies were centrifuged at 17,000g, 5 min, at 4°C to remove any aggregate[2]. The gating strategy for flow cytometry analysis is reported in Supplementary Figure S1. Platelets were identified as SSC/FSC and $CD41^{pos}$/low SS scatter events (A and B) in order to avoid inclusion of platelet-leukocyte aggregates. Whole blood leukocytes were identified in a SS-A/αCD45-APC dot plot and heteroaggregates were double-positive events for platelet and leukocyte population markers (CD41pos/CD66pos or CD41pos/CD14pos for platelet-granulocyte and platelet-monocyte aggregates, respectively) and reported as a percentage of positive events within the monocyte or granulocyte population (panel D and E). A total of 10,000 $CD41^{pos}$, 3,000 $CD14^{pos}$ and 20,000 $CD66^{pos}$ events per sample were acquired with Gallios flow

cytometer (Beckman Coulter) equipped with four solid-state lasers at 488nm, 638nm, 405nm, and 561nm. Flow-check Pro Fluorospheres (Beckman Coulter) were used daily according to the manufacturer's instructions to monitor cytometer performance. All data were analyzed with Kaluza analysis software v1.5 (Beckman Coulter) and reported as positive cells' percentage ± standard deviation (SD). Imaging flow cytometry was performed as previously described[3] and modified as follows. Briefly, whole blood samples were labelled as described above. Monocyte and granulocyte were discriminated according to physical parameter and CD45 expression. Platelets were identified based on CD61 expression. Nuclei were stained with Hoechst dye. Images were acquired at 40X magnification with ImageStreamX Mk II (Amnis, Merck), and data were analysed using IDEAS™ software.

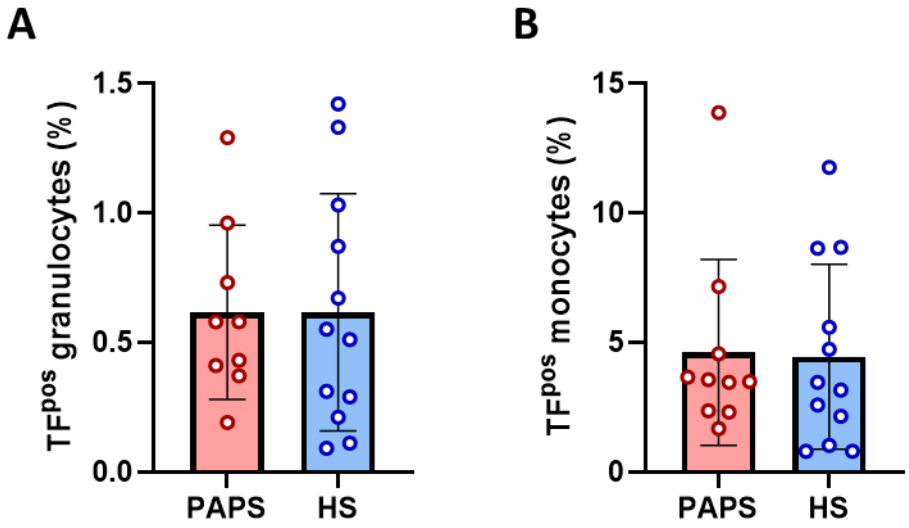
Supplementary Figures

Supplementary Figure S1



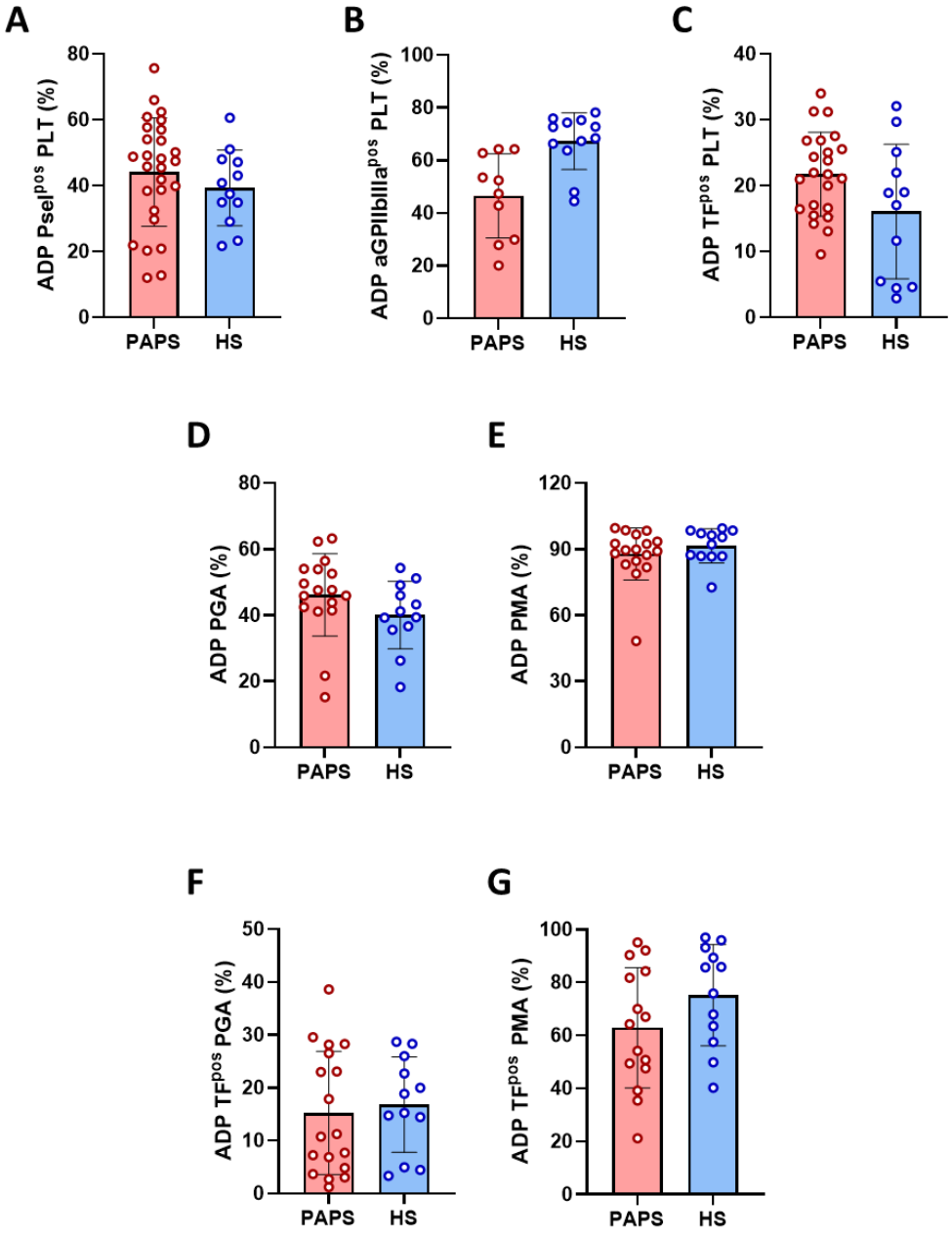
Supplementary Figure S1. Flow cytometry gating strategy for the identification of platelets and platelet-leukocyte aggregates. Platelet population (purple) in whole blood is displayed in FS/SS-A dot plot (A) and identified by αCD41^{pos}/low SS scatter events (B). Whole blood leukocytes are identified in SS-A /αCD45-APC dot plot (C) and platelet-granulocyte (D) and platelet-monocyte (E) aggregates were identified as CD41^{pos}/CD66^{pos} and CD41^{pos} /CD14 ^{pos} events, respectively.

Supplementary Figure S2



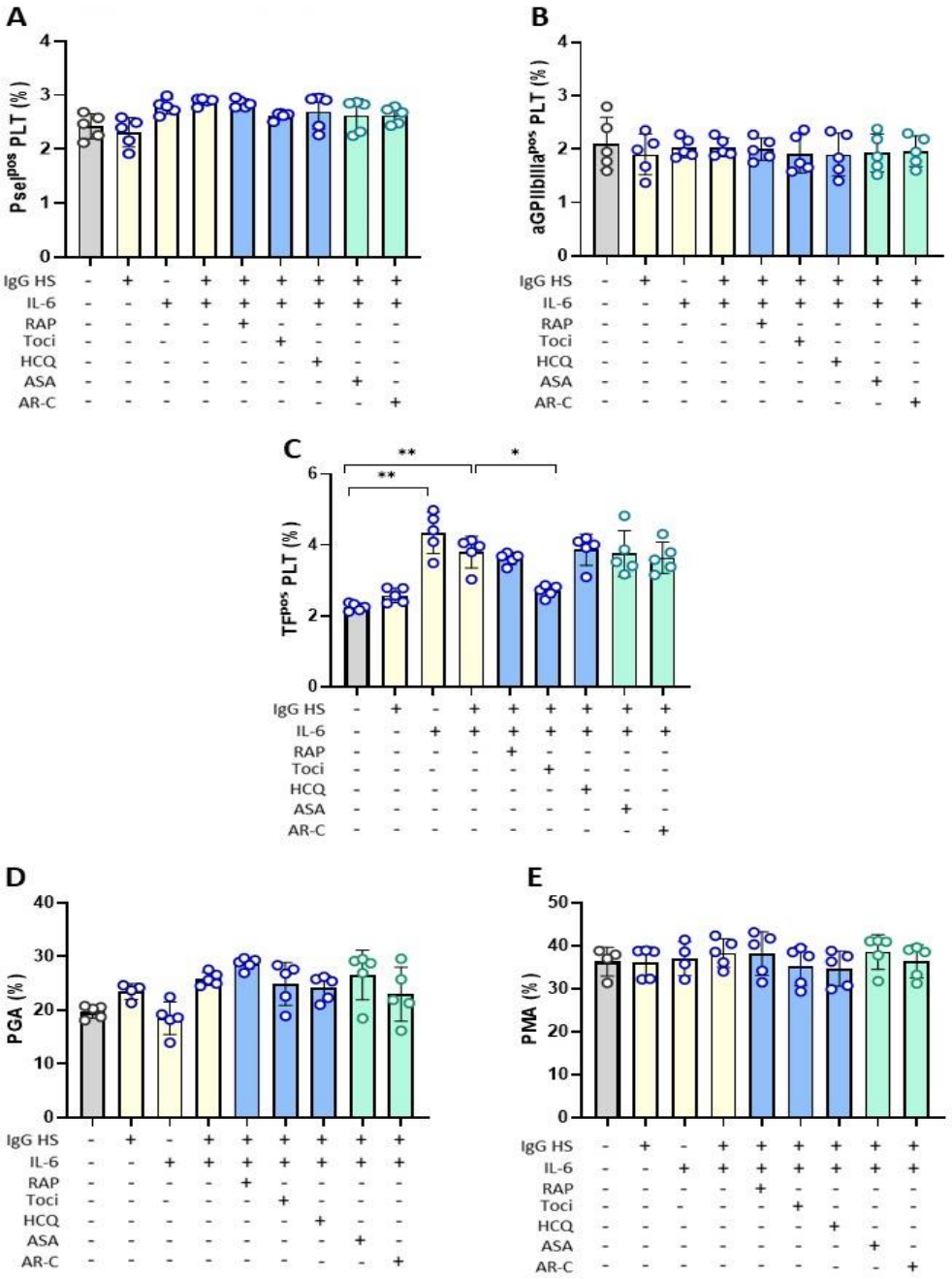
Supplementary Figure S2. TF^{pos}-granulocytes and monocytes in PAPS patients and HS. The percentage of TF^{pos}-granulocytes (A) and -monocytes (B) were evaluated by whole blood flow cytometry in primary APS (PAPS) patients and in healthy subjects (HS). Data are reported as individual values (PAPS n=9 for A; n=10 for B; HS n=12 for A, B) and mean ± SD of antigen-positive cells.

Supplementary Figure S3



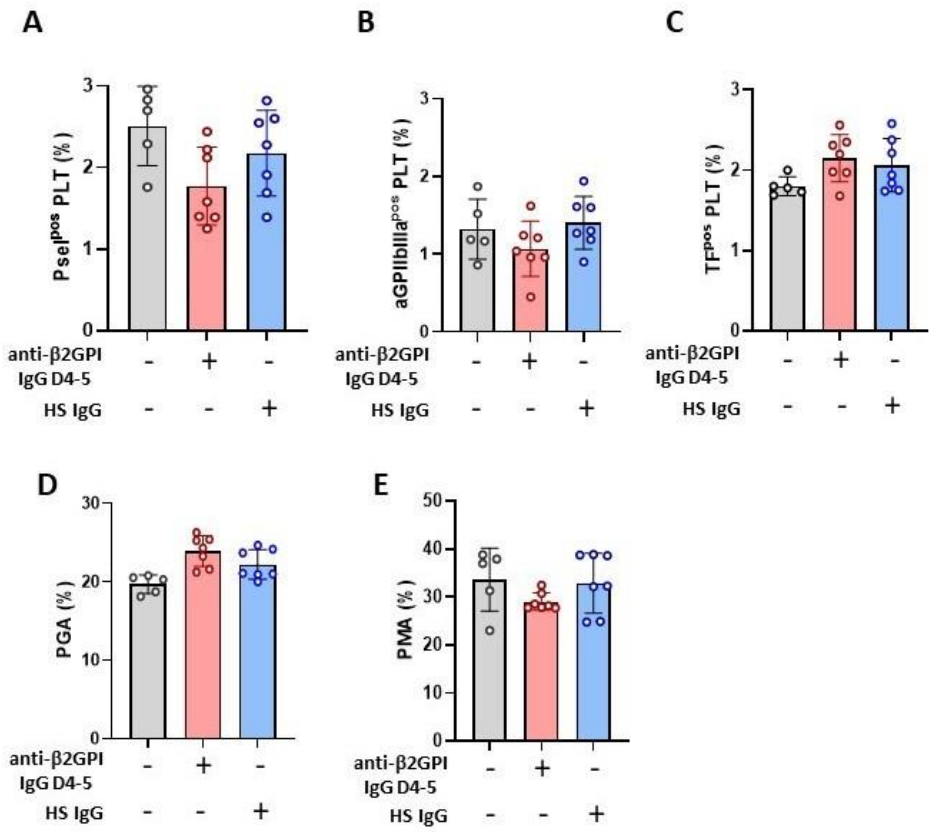
Supplementary Figure S3. Markers of platelet activation in PAPS patients and HS upon ex vivo activation. Platelet-associated P-selectin (A), activated GPIIbIIIa (aGPIIbIIIa) (B), tissue factor (TF) (C) expression, as well as total and TF^{pos}-platelet-granulocyte (PGA) (D, F) and -platelet-monocyte (PMA) (E, G) aggregate formation were evaluated by flow cytometry upon ex vivo stimulation of blood from primary APS (PAPS) patients and healthy subjects (HS) with ADP (10µM, 15min, room temperature). Data are reported as individual values (PAPS n=17-27 for A, C-F; n=10-15 for B, G; HS n=12 for A-G) and mean ± SD of antigen-positive cells.

Supplementary Figure S4



Supplementary Figure S4. Pharmacological inhibition of IgG-mediated P-Selectin and TF platelet expression. Percentages of platelets expressing P-Selectin, activated GPIIbIIIa (aGPIIbIIIa) and tissue factor (TF) (A-C) were analyzed upon healthy subjects (HS) whole blood incubation with HS IgG in the presence or absence of IL-6 (100 pg/ml), ApoER2 (RAP, 13.7 µg/ml) or IL-6R inhibitors [tocilizumab (toci), 300 µg/ml], hydroxychloroquine (HCQ, 100 µg/ml), Aspirin (ASA, 8 µM) and the selective platelet $P2Y_{12}$ antagonist AR-C69931MX (AR-C, 100nM). Comparable experiments measuring platelet-granulocyte (PGA) (D) and -monocyte (PMA) (E) aggregates were performed. Data are reported as individual value and mean ± SD of antigen-positive cells. *p<0.05, **p<0.01.

Supplementary Figure S5



Supplementary Figure S5. In vitro effect of anti-β2GPI-D4,5 IgG on the adhesive and thrombophilic platelet phenotype. Percentages of platelets expressing P-Selectin (Psel), activated GPIIbIIIa (aGPIIbIIIa) and tissue factor (TF) (A-C) were analyzed upon healthy subjects (HS) whole blood incubation with anti-beta2 glycoprotein I (β2GPI)-domain (D)4,5-positive IgG (red bars; n=7) or HS IgG (blue bars; n=7). Comparable experiments measuring platelet-granulocyte (PGA) (D) and -monocyte (PMA) (E) aggregates were performed. The expression of platelet activation markers in whole blood of HS was reported as reference (grey bars; n=5). Data are reported as individual value and mean±SD of antigen-positive cells.

Supplementary References

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