ARTICLE - Hemostasis

Platelet phosphatidylserine is the critical mediator of thrombosis in heparin-induced thrombocytopenia

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Supplemental Material

Supplemental Methods

Patients and sera

Experiments were performed using serum material from HIT patients who were referred to our laboratory between March 2019 and April 2022. The diagnosis of HIT was confirmed by reviewing medical records by two experienced physicians according to current guidelines (e.g. 4Ts-score >3) as well as based on laboratory findings in enzyme-immune assay (EIA) and heparin-induced platelet activation assay (HIPA).[1] In addition, serum samples were collected from healthy blood donors (HCs) at the Blood Donation Centre Tübingen, after a written informed consent has been obtained. Serum samples were stored at -20°C and thawed prior to experiments. To exclude unspecific effects of serum components other than Abs, all sera were heat-inactivated at 56°C for 30 min, which was followed by a centrifugation at 5000g for 5 min. The supernatants were used in this study.

Testing for anti-PF4/heparin antibodies

A commercially available IgG-EIA was used in accordance to manufacturer's instructions (Hyphen Biomed, Neuville-sur-Oise, France). A sample was considered reactive if the optical density (OD) was higher than 0.500. The ability of sera to activate PLTs was tested using the HIPA as previously described.[2] In brief, serum was tested with washed platelets (wPLTs) from four different healthy donors in the absence (buffer alone) or in the presence of heparin (0.2 IU/mL and 100 IU/mL). Reactions were placed in microtiter wells containing spherical stir bars and stirred at approximately 500 revolutions per minute (rpm). Wells were examined optically at five-minute (min) intervals for loss of turbidity. A serum was considered reactive (positive) if a shift from

turbidity to transparency occurred within 30 min in at least two PLT suspensions. Observation time was 45 min. Each test included a diluted serum from a patient with HIT as a weak positive control, collagen (5µg/mL) as strong positive control and a serum from a healthy donor as a negative control.

Preparation of washed platelets

wPLTs were prepared from venous blood samples as previously described.[2,3] Briefly, fresh whole blood (WB) from healthy donors that showed a good PLT response upon activation with HIT sera in the HIPA assay (time to PLT aggregation less than 20 minutes) was withdrawn by cubital venipuncture into acidic-citrate-dextrose (ACD) containing vacutainers (Becton-Dickinson, Plymouth, United-Kingdom) and allowed to rest for 45 min at 37°C. Blood was withdrawn by cubital venipuncture into acidic-citrate-dextrose (ACD) containing vacutainers (Becton-Dickinson, Plymouth, United-Kingdom) and allowed to rest for 45 min at 37°C. After a centrifugation step (120g, 20 min, room temperature [RT], no brake), PLT-rich-plasma (PRP) was gently separated and supplemented with apyrase (5 µL/mL; Sigma-Aldrich, St. Louis, USA) and pre-warmed ACD (333 µL/mL; Sigma-Aldrich, St. Louis, USA). After an additional centrifugation step (650g, 7 min, RT, no brake), the PLT pellet was resuspended in 5 mL of wash-solution (modified Tyrode buffer: 5 mL bicarbonate buffer, 20 percent [%] bovine serum albumin, 10% glucose solution [Braun, Melsungen, Germany], 2.5 U/mL apyrase, 1 U/µL hirudin [Pentapharm, Basel, Swiss], pH 6.3) and allowed to rest for 15 min at 37°C. Following final centrifugation (650g, 7 min, RT, no brake) wPLTs were resuspended in 2 mL of resuspension-buffer (50 mL of modified Tyrode buffer, 0.5 mL of 1 mM MgCl2, 1 mL of 2 mM CaCl2, pH 7.2) and adjusted to $300x10^3$ PLTs/µL after

the measurement at a hematological analyzer (CELL-DYN Ruby, Abott, Wiesbaden, Germany) was performed.

Immunoglobulin G preparation

IgG fractions were isolated from HIT as well as from control sera using a commercially available IgG-purification-kit (Melon^{TM}-Gel IgG Spin Purification Kit, Thermo Fisher Scientific, Waltham, USA) as recommended by the manufacturer. In brief, heat inactivated serum was diluted 1:10 in purification buffer and incubated with the kit specific gel IgG Purification Support for 10 min. Subsequently centrifugation through a 10µm pore size filter tube was performed for 1 min at 5800g. The flow-through was collected into 100 kDa pore sized centrifugal filters (Amicon Ultra-4, Merck Millipore, Cork, Ireland) with subsequent concentration to the initial volume of the used serum sample via centrifugation (10-15 min, 2000g, 4°C, with brake). IgG concentrations were measured using NanoDrop One Spectrophotometer at an excitation wavelength of 340 nm (Thermo Fisher Scientific, Waltham, USA).

Treatment of PLTs with sera/IgGs

37.5 µL of wPLTs or PRP were supplemented with 5 µL serum/IgG from HIT patients or controls and incubated for 1 hour under rotating conditions at RT. Afterwards, 5µL of the PLT suspension containing ~1x10^6 PLTs were transferred into a final volume of 100 µL of Hank´s balanced salt solution (HBSS) containing 137 mM NaCl, 1.25 mM $CaCl_2$, 5.5 mM glucose (Carl-Roth, Karlsruhe, Germany). Cell suspension was then incubated with 1 µL anti-CD62p-APC (BD, San Jose, USA), 1 µL Annexin-V-FITC (Immunotools, Friesoythe, Germany) and 2 µL anti-CD42a-PerCP (BD, San Jose, USA) for 30 min at RT in the dark. PLTs that were treated with thrombin receptor activating peptide-6 (TRAP-6; 5 µM, 30 min at RT) and ionomycin ([5 µM, 15 min at

RT], both from Sigma-Aldrich, St. Louis, USA) served as positive controls. Afterwards, PLTs were resuspended with HBSS to a final volume of 500 µL and immediately assessed via flow cytometry (FC; Navios, Beckman-Coulter, Brea, USA).

Analysis of HIT antibody-induced mechanomolecular signaling mechanisms

To investigate the underlying mechanisms that result in HIT Ab-induced procoagulant PLT effects, 75 µL of wPLTs were pretreated with the Fc-gamma-RIIA blocking monoclonal antibody (moAb) anti-CD32 (moAb IV.3; Stemcell™ technologies, Vancouver, Canada) or a monoclonal isotype control (clone SC-2025; Santa Cruz Biotechnology, Dallas, USA) for 30 min at RT prior to HIT serum/IgG treatment. To investigate the role of upregulated intracellular levels of cAMP, PLTs were pretreated with the prostacyclin analogue Iloprost (20 nM; Sigma-Aldrich, St. Louis, USA) or vehicle for 5 min at RT prior to incubation with IgG from HIT patients or HCs.

Specific blockade of platelet surface P-Selectin and phosphatidylserine

P-selectin (CD62p) was blocked via the pretreatment of PLTs with 5 µg/mL anti-SELP humanized Ab (anti-CD62p, 15 min, RT; ProteoGenix, Schiltigheim, France). Using a modified FC approach that detects PLT-leukocyte aggregates, 5 µg/mL of anti-CD62p were found to efficiently block the interaction of TRAP-6 (10µM, 30 min, RT) activated wPLTs with neutrophil granulocytes that were purified via density gradient centrifugation (data not shown).[4-6]

Specific inhibition of externalized PS on the surface of procoagulant PLTs was performed via the incubation of PLTs with Lactadherin, a calcium independent ligand of PS (200nM, 15 min, RT; Haematologic Technologies, Essex Junction, USA).[7,8] Sufficient blocking concentrations of Lactadherin were assessed via calibrated automated thrombogram (CAT; data not shown).

Thrombin generation assay

HIT Ab-induced thrombin generation (TG) on PRP was detected using Calibrated Automated Thrombogram (CAT; Stago, Maastricht, Netherlands) according to the manufacturer's instructions. In brief, venous blood from healthy individuals was withdrawn into vacutainers containing sodium citrate 0.105 M (3.2%; BD, Plymouth, UK) and allowed to rest for 20 min at RT. PRP was prepared by centrifugation (20 min, 120g, no brake) and adjusted with autologous platelet poor plasma (PPP; 10 min, 2000g, RT) to a PLT count of 150x106/mL. Afterwards, PRP was treated with IgGs from HC or HIT patients in the presence of buffer or low- (0.2 IU/mL) dose heparin and incubated for 60 min at RT under rotating conditions. Following incubation, samples were washed (10 min, 700g, no brake) and the remaining PLT pellet gently resuspended with fresh PPP. 80 µl of the cell suspension were dispensed into the well of round-bottom 96 well-microtitre plates (Fluoroskan Ascent, ThermoLabsystems, Helsinki, Finland) and supplemented with 20 µL of PRP-reagent containing recombinant tissue factor (Thrombinoscope BV, Maastricht, The Netherlands). 20 µL of fluorogenic substrate and calcium (FluCa-Kit reagent, Thrombinoscope BV, Maastricht, The Netherlands) were dispensed automatically by the device in each well. Fluorescence was acquired for 60 min with a 390-nm excitation/460-nm emission filter set, and parameters automatically calculated by dedicated software (Thrombinoscope BV, Maastricht, The Netherlands). When indicated, PLTs were preincubated with moAb IV.3 or isotype control (20 µg/mL) for 30 min at RT. To investigate the effect of cAMP elevation on PLTs thrombin generation potential, PRP was preincubated with Iloprost (20nM) or vehicle for 5 min at RT prior to the incubation of HIT IgGs. For specific inhibition of CD62p and PS,

PLTs were incubated with indicated concentrations of anti-CD62p and Lactadherin (both 15 min at RT) after HIT IgG incubation and further handled as described above.

Investigation of antibody-induced thrombus formation

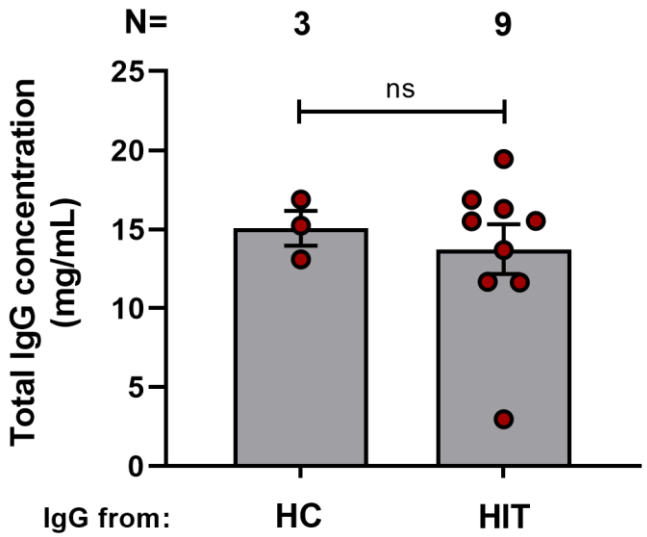
To investigate the effect of Ab-induced PLT alterations on the coagulation cascade and subsequent thrombus formation**,** an ex vivo model for thrombus formation (BioFlux 200, Fluxion Biosciences, Alameda, USA) was used according to the recommendations of the ISTH Standardization Committee for Biorheology.[9] Briefly, microfluidic channels were coated with collagen (100 µg/mL; Collagen Horm, Takeda, Linz, Austria) overnight at 4°C and blocked with 1% of human serum albumin (Kedrion, Barga, Italy) 1 h at RT before perfusion. WB of healthy individuals with blood group O was collected into 0.105 M sodium citrate containing monovettes (Sarstedt, Nuembrecht, Germany) that were supplemented with Corn trypsin inhibitor (5 µg/mL; Santa Cruz Biotechnology, Dallas, USA) and allowed to rest for 30 min at RT. After splitting the WB into aliquots of 200 µL, PRP was isolated via centrifugation (15 min, 120g, RT, no break). Afterwards, 37.5 µL of the supernatant PRP were gently separated and incubated with 5 µL control or HIT IgG under rotating conditions (60 min, RT). After the incubation period, samples were labelled with 3,3′-Dihexyl-oxacarbocyaniniodid ($DiOC_6$, 2.5 µM; Sigma Aldrich, Saint Louis, USA), Alexa Fluor (AF) 647-Annexin A5 (1:200), AF 546 human fibrinogen (8.5 µg/mL) and Hoechst 33342 (3 µg/mL; Thermo Scientifc, Carlsbad, USA) and added to WB during reconstitution. When indicated, the separated PRP was pretreated with moAb IV.3 or isotype control at a concentration of 20 µg/mL for 30 min at RT. For cAMP elevation in PLTs, PRP was pretreated with Iloprost (20 nM) or vehicle control (5 min, RT) prior to IgG incubation. For specific inhibition of CD62p and PS, PLTs were incubated with

anti-CD62p Ab (5 µg/mL) and Lactadherin (200nM) for 15 min at RT. To avoid excess amounts of Lactadherin that could interfere with different blood coagulation factors, PLTs were washed (700g, RT, no brake) and resuspended with fresh PPP after HIT IgG incubation was performed. Finally, reconstituted WB samples were recalcified and run at a shear rate of $250s^{-1}$ (10 dyne) for 10 min as previously described.[3,10]

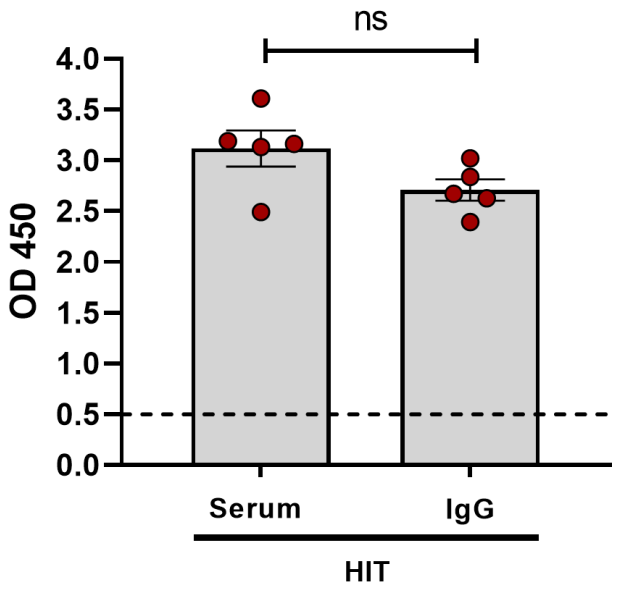
Ethics

Studies involving human material were approved by the Ethics Committee of the Medical Faculty, Eberhard-Karls University, Tübingen, Germany (Application number: 140/2022BO2) and were conducted in accordance with the Declaration of Helsinki.

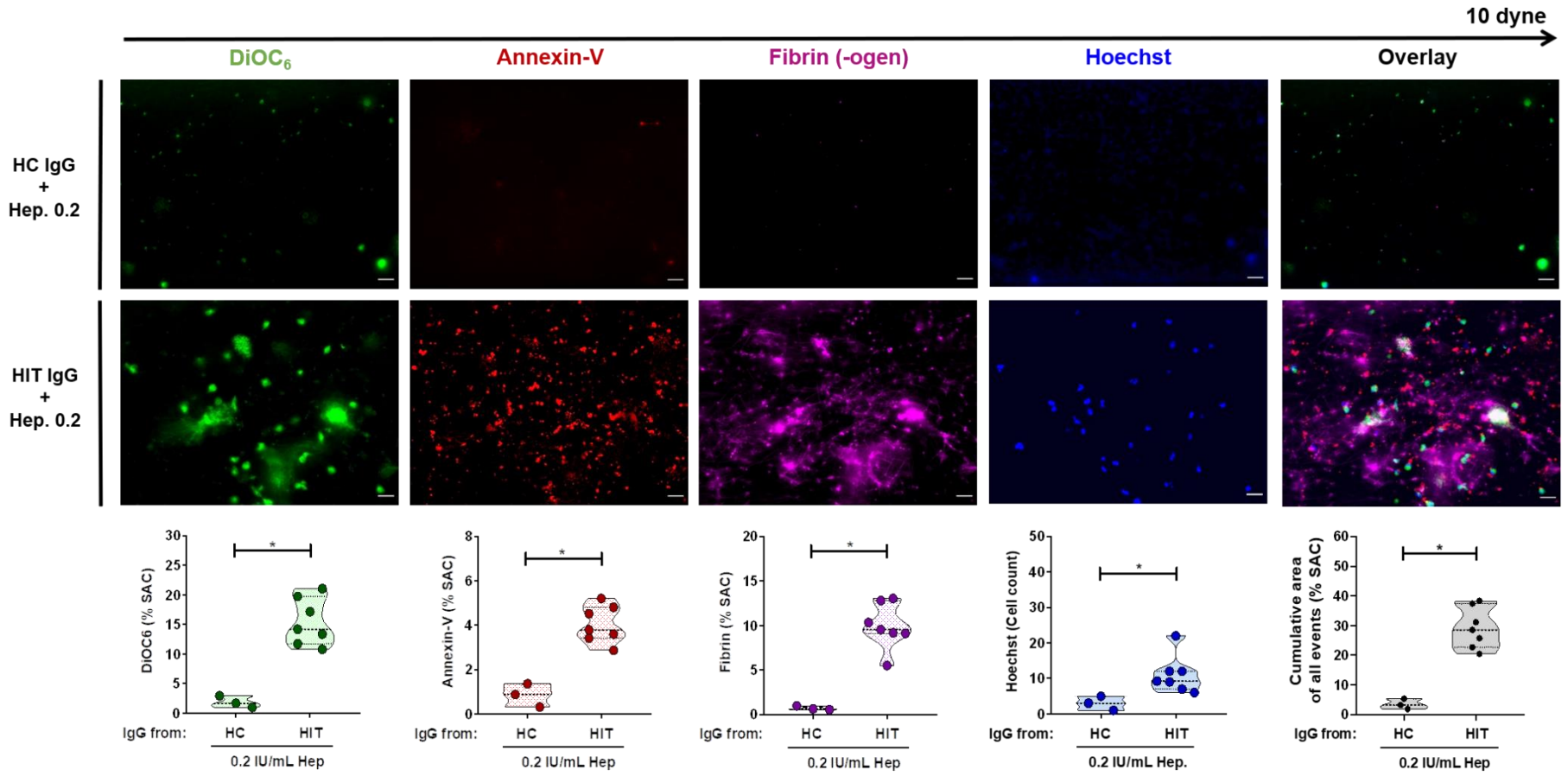
Supplemental Figures and Tables



Supplemental Figure 1. Concentrations of IgG fractions isolated from HC- and HIT patient sera. After isolation from serum, concentrations of HC- or HIT IgG fractions were assessed using a NanoDrop One spectrophotometer. P values were calculated using the Mann-Whitney U test. ns, not significant.

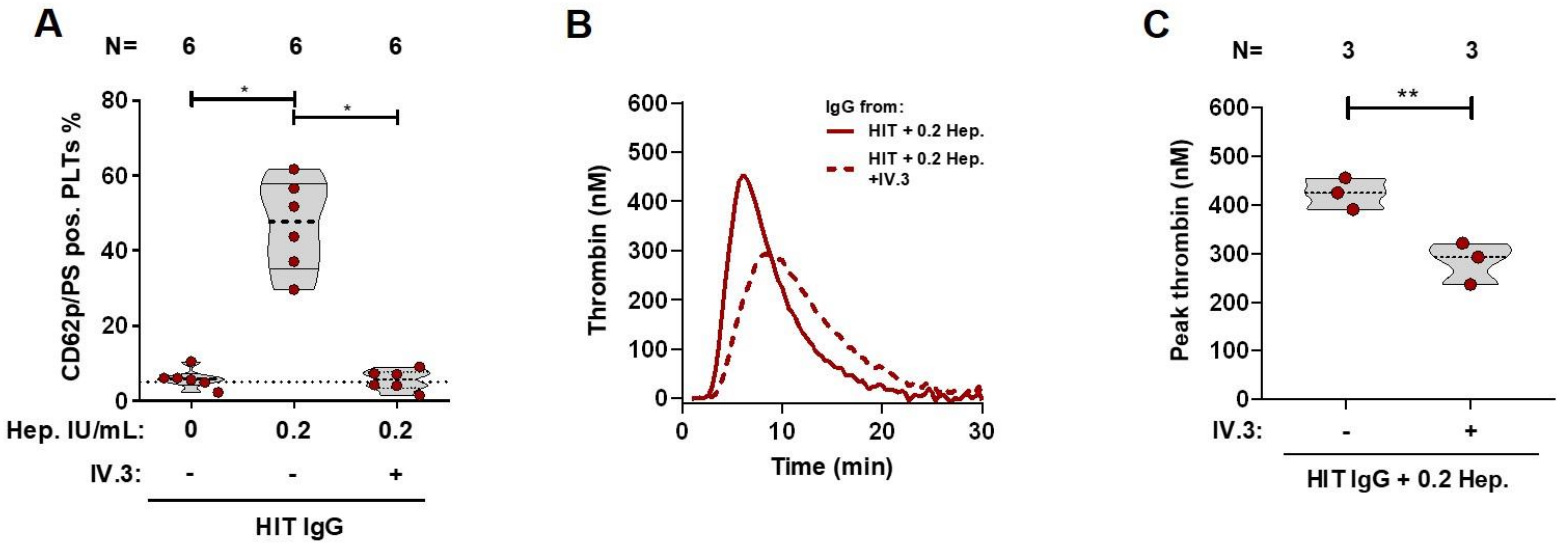


Supplemental Figure 2. Anti-PF4/heparin HIT Ab-titres detected in HIT patient sera and corresponding IgG isolates. Sera or corresponding IgG isolates from HIT patients (n=5) were screened for IgG-antibodies against PF4/heparin complexes using a commercially available ELISA kit (Hyphen Biomed). The cut-off (dashed line) for samples to be considered positive was an optical density (OD) ≥0.5.



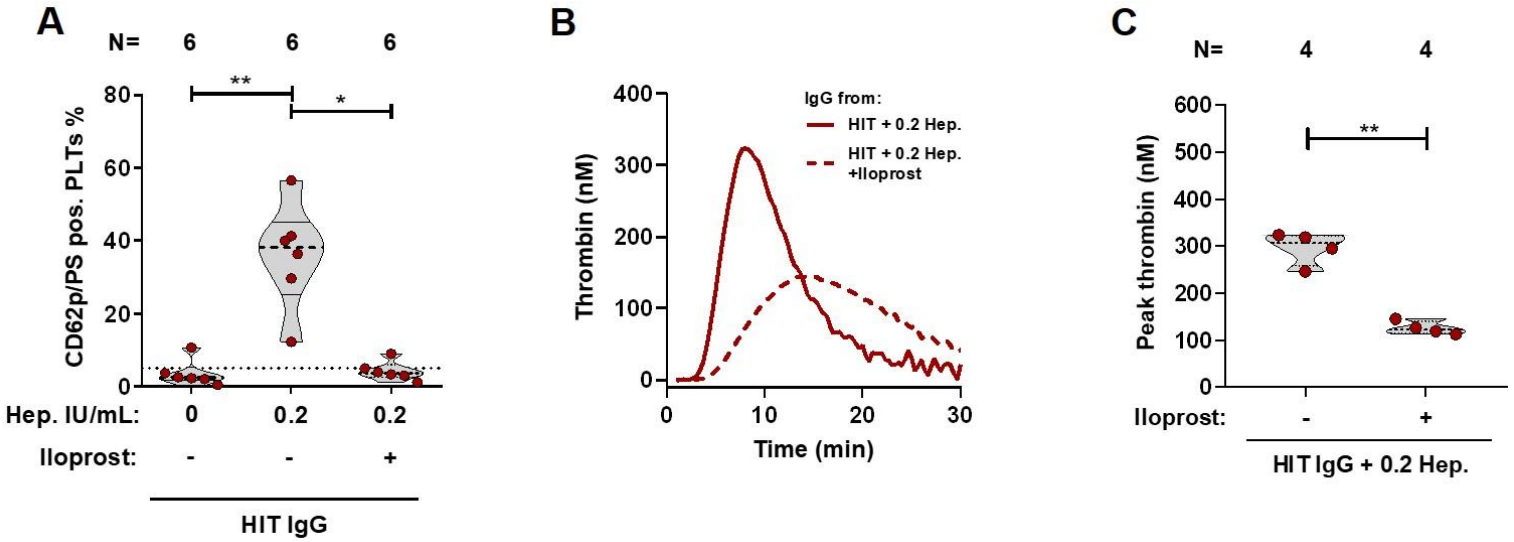
Supplemental Figure 3. Procoagulant platelets contribute to anti-PF4/heparin IgG mediated thrombus formation. PLTs from healthy individuals were incubated with IgGs from controls (HC) or HIT patients in the presence of low-dose (0.2 IU/mL) heparin and labelled with $DiOC_6$, AF647 Annexin-V, AF546 Fibrinogen and Hoechst 33342 prior to reconstitution into autologous whole blood and

perfusion through microfluidic channels. After perfusion, images were acquired at x40 magnification. Scale bar 20µm. Violin plots showing the percentage of total surface area coverage (%SAC) by $DiOC_6$, PS, Fibrin-(ogen), count of Hoechst positive labelled cells and cumulative area of $DiOC_6$, PS and Fibrin-(ogen) labelled thrombus in the microfluidic channel. *p<0.05, **p<0.01 and ***p<0.001. ns, non-significant; HC, healthy control; PLT, platelet; PF4, platelet factor 4; IgG, immunoglobulin G; PS, phosphatidylserine.



Supplemental Figure 4. Inhibition of Fc-gamma-RIIA signal transduction prevents generation of procoagulant platelets and increased thrombin generation by HIT antibodies. [**A**] HIT IgG-induced changes in PLTs PS externalization and CD62p expression were analyzed via Annexin-V-FITC and CD62p-APC double staining in the presence of isotype (-) or IV.3 moAb. Data are shown as percentage of Annexin-V-FITC and CD62p-APC double positive-labeled PLTs. [**B**] Representative thrombin generation curve induced on PLTs after incubation with IgG from different HIT patients in the presence of isotype (-) or IV.3 moAb. Each curve represents the amounts of generated thrombin over time induced by HIT IgG in the presence of heparin (0.2 IU/mL) and isotype (solid red line) or IV.3 (dashed red line) moAb. [**C**] Data were quantified as Peak thrombin generated (nM) using thrombinoscope software and graphpad prism.

*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. CAT, Calibrated Automated Thrombogram; IgG, immunoglobulin G; moAb, monoclonal Ab, PLT, platelet; PRP, platelet-rich plasma; CD62p, P-selectin; PS, phosphatidylserine. N, number of samples



Suppl. Figure 5. HIT antibody-induced procoagulant platelet and increased thrombin formation can be inhibited by iloprost. [**A**] HIT IgG-induced changes in PLTs PS externalization and CD62p expression were analyzed via Annexin-V-FITC and CD62p-APC double staining in the presence of vehicle (-) or Iloprost (20nM). Data are shown percentage±SEM of Annexin-V-FITC and CD62p-APC double positive-labeled PLTs. [**B**] Representative thrombin generation curve induced on PLTs after incubation with IgG from different HIT patients in the presence of heparin (0.2 IU/mL) and vehicle or Iloprost. Each curve represents the amounts of generated thrombin over time induced by HIT IgG in the presence of vehicle (solid red line) or Iloprost (dashed red line). [**C**] Data were quantified as Peak thrombin generated (nM) using thrombinoscope software and graphpad prism. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. CAT,

Calibrated Automated Thrombogram; IgG, immunoglobulin G; PLT, platelet; PRP, platelet-rich plasma; CD62p, P-selectin; PS, phosphatidylserine; N, number of samples.

Patient ID	gender	age	PLT-count †	Mean OD EIA	HIPA	Time to aggregation in HIPA		4Ts score	Thrombosis
						0.2 IU/mL	100IU/mL		
1	m	54	114	0.109	neg.	x	x	5	yes
2	f	79	120	0.131	neg.	x	x	4	no
3	f	56	79	0.071	neg.	x	x	3	no
4	m	79	283	0.091	neg.	x	x	4	no
5	m	70	41	0.111	neg.	x	x	3	no
6	f	48	133	0.099	neg.	x	x	3	no
7	m	45	27	0.072	neg.	x	x	5	no
8	m	63	32	0.183	neg.	x	x	4	no
9	f	74	95	0.073	neg.	x	x	5	no
10	f	68	83	2.36	neg.	x	x	5	yes
11	m	67	424	1.1895	neg.	x	x	3	no
12	m	40	100	1.776	neg.	x	x	6	yes
13	f	83	114	0.566	neg.	x	x	5	no
14	m	59	80	0.732	neg.	x	x	4	no
15	m	61	211	0.546	neg.	x	x	3	yes
16	f	67	103	0.508	neg.	x	x	4	no
17	m	65	101	0.7285	neg.	x	x	5	no
18	m	76	142	2.729	pos.	20	neg.	5	no
19	m	71	59	2.807	pos.	5	neg.	6	no
20	m	77	184*	2.216	pos.	5	neg.	8	yes
21	m	83	119	1.130	pos.	25	neg.	5	no
22	m	71	114	2.731	pos.	15	neg.	3	no
23	m	57	60	2.490	pos.	10	neg.	4	no
24	f	79	98	1.740	pos.	10	neg.	6	no
25	f	74	35	1.967	pos.	10	neg.	3	no
26	f	41	135	2.205	pos.	10	neg.	6	no
27	m	80	80	3.190	pos.	5	neg.	6	no
28	f	79	22	2.871	pos.	5	neg.	5	no
29	f	58	76	2.755	pos.	15	neg.	7	yes
30	m	45	37	2.742	pos.	5	neg.	7	yes
31	m	77	63	2.520	pos.	5	neg.	6	yes
32	f	87	81	2.342	pos.	5	neg.	7	yes
33	f	46	62	1.864	pos.	5	neg.	7	yes
34	m	49	16	3.130	pos.	5	neg.	5	no
35	m	77	46	3.162	pos.	5	neg.	5	yes
36	m	37	20	2.786	pos.	5	neg.	7	yes
37	m	54	23	3.609	pos.	5	neg.	7	yes

Supplemental Table 1. Clinical and serological characteristics of the patient cohort. PLT, platelet; m, male; f, female; OD, optical density; EIA, enzyme-immune assay; HIPA, heparin-induced platelet activation assay; † reference range PLT-count (150-450 x10^9/L); #1-17: patients with suspected HIT; #18-37: patients with confirmed HIT. *patient #20 has a drop of PLT count>50%.

IgG from:	Peak thrombin (nM)	AUC (nM*min)
HC	151.80±30.28	1516.00±26.72
HC+Lact.	159.10±7.15	1597.00±28.24
HIT	305.60±22.24	2883.50±47.35
HIT+Lact.	151.80±30.28	3236.75±236.30

Supplemental Table 2. Antibody-induced changes in thrombin generation in the presence or absence of lactadherin. HIT patient IgG (n=3) induced changes in thrombin generation were detected in PRP in the presence or absence of lactadherin (200nM) by the use of the CAT thrombin generation assay and compared to healthy control (HC) IgG (n=3). Each patient was tested in two independent experiments. Data are presented as mean±SEM. AUC, area under the curve.

Supplemental Movies

Supplemental Movie 1. Attachment of HIT antibody-induced procoagulant platelets to a collagen coated microfluidic channel under venous shear conditions.

PLTs from healthy individuals were incubated with IgGs from HIT patients in the presence of low-dose (0.2 IU/mL) heparin and AF647 Annexin-V (red) prior to reconstitution into autologous whole blood and perfusion through microfluidic channels at 10 dyne. Video shows attachment of circulating procoagulant platelets to the collagen channel surface in the early phase of the perfusion experiment. Scale bar 50µm.

Supplemental Movie 2. Localization of antibody-induced procoagulant platelets during HIT Ab-induced thrombus formation.

PLTs from healthy individuals were incubated with IgGs from HIT patients in the presence of low-dose (0.2 IU/mL) heparin and labelled with $DiOC_6$, AF647 Annexin-V

and AF546 Fibrinogen prior to reconstitution into autologous whole blood and perfusion through microfluidic channels at 10 dyne. The sequence shows the integration of HIT Ab-induced procoagulant (red) PLTs during thrombus formation at x40 magnification. Scale bar 20µm.

Supplemental Movie 3. Accumulation of non-procoagulant platelets in HIT Ab-mediated thrombus. PLTs from healthy individuals were incubated with IgGs from HIT patients in the presence of low-dose (0.2 IU/mL) heparin and labelled with $DiOC_6$, AF647 Annexin-V and AF546 Fibrinogen prior to reconstitution into autologous whole blood and perfusion through microfluidic channels at 10 dyne. The sequence shows the integration of $DiOC_6$ positive (green) labelled cells during thrombus formation at x40 magnification. Scale bar 20µm.

Supplemental Movie 4. Fibrin (-ogen) generation during HIT antibody-induced thrombus formation ex vivo. PLTs from healthy individuals were incubated with IgGs from HIT patients in the presence of low-dose (0.2 IU/mL) heparin and labelled with $DiOC_6$, AF647 Annexin-V and AF546 Fibrinogen prior to reconstitution into autologous whole blood and perfusion through microfluidic channels at 10 dyne. The sequence shows the deposition of AF546 labelled Fibrin (-ogen) (magenta) during thrombus formation at x40 magnification. Scale bar 20µm.

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