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

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Abstract

Heparin-induced thrombocytopenia (HIT) is a severe immune-mediated prothrombotic disorder caused by antibodies (Ab) reactive to complexes of platelet factor 4 and heparin. Platelets (PLT) and their interaction with different immune cells contribute to prothrombotic conditions in HIT. However, the exact mechanisms and the role of different PLT subpopulations in this prothrombotic environment remain poorly understood. In this study, we observed that HIT patient Ab induce a new PLT population that is characterized by increased P-selectin expression and phosphatidylserine (PS) externalization. Formation of this procoagulant PLT subpopulation was dependent on engagement of PLT Fc-γ-RIIA by HIT Ab and resulted in a significant increase of thrombin generation on the PLT surface. Using an *ex vivo* thrombosis model and multi-parameter assessment of thrombus formation, we observed that HIT Ab-induced procoagulant PLT propagated formation of large PLT aggregates, leukocyte recruitment and most importantly, fibrin network generation. These prothrombotic conditions were prevented via the upregulation of PLT intracellular cAMP with Iloprost, a clinically approved prostacyclin analogue. Additionally, the functional relevance of P-selectin and PS was dissected. While inhibition of P-selectin did not affect thrombus formation, the specific blockade of PS prevented HIT Ab-mediated thrombin generation and most importantly procoagulant PLT-mediated thrombus formation *ex vivo*. Taken together, our findings indicate that procoagulant PLT are critical mediators of prothrombotic conditions in HIT. Specific PS targeting could be a promising therapeutic approach to prevent thromboembolic events in HIT patients.

Introduction

Heparin-induced thrombocytopenia (HIT) is an immune-mediated prothrombotic disorder that is characterized by low platelet (PLT) counts and high risk of thromboembolic events.[1-3] HIT is caused by antibodies (Ab) of the immunoglobulin G (IgG) subclass that recognize complexes of the endogenous protein platelet factor 4 (PF4) and heparin.[4,5] These immune complexes evolve 4-14 days after heparin exposure and harbor the potential to elicit prothrombotic conditions due to their interaction with PLT, neutrophils and monocytes via immune receptor Fc-γ-RIIA.[6-8]

Despite low PLT counts, HIT is associated with an increased risk of thrombosis.[9,10] In fact, Fc-γ-RIIA-mediated PLT activation by HIT immune complexes is associated with intense thrombin generation and prothrombotic potential.[7,11-13] Additionally, binding of HIT Ab to PF4 and glycosaminoglycans on the surface of monocytes has been reported to result in increased tissue factor and microparticle release which promotes thrombin generation and PLT activation.[6,14,15] Furthermore, HIT immune complexes can activate neutrophils via Fc-γ-RIIA and lead to the formation of PLT-neutrophil aggregates as well as the release of prothrombotic neutrophil extracellular traps (NET).[16] Although this evidence directs towards a prominent role of PLT in the pathogenesis of HIT, a better understanding of HIT Ab-mediated PLT changes and the potential relevance of different PLT subpopulations in prothrombotic conditions in HIT patients is needed.

Procoagulant PLT, a distinct subpopulation of activated PLT, are characterized by an increased externalization of the negatively charged phospholipid phosphatidylserine (PS).[17,18] High levels of PLT PS are efficient to promote as-

sembly of coagulation complexes and subsequent thrombin burst.[19,20] Recently, we showed that procoagulant PLT might contribute to the prothrombotic state in patients with COVID-19 and vaccine-induced immune thrombotic thrombocytopenia (VITT).[21,22]

In the current study, we hypothesized that anti-PF4/heparin HIT IgG (HIT Ab) induce a procoagulant PLT response that contributes to the hypercoagulable state in HIT. Using an *ex vivo* model for thrombosis we analyzed the molecular mechanisms of HIT Ab-induced thrombosis. Our data indicate that anti-PF4/heparin IgG Ab can generate procoagulant PLT via the crosslinking of Fc-γ-RIIA leading to increased intracellular calcium and subsequent P-selectin (CD62p) expression as well as PS externalization. Most importantly, thrombus formation by HIT Ab was mediated by PLT PS rather than CD62p expression.

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. Where indicated, IgG fractions were isolated from HIT as well as from control sera using a commercially available IgG purification kit. 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. Additional details are available in the *Online Supplementary Appendix*.

Testing for anti-PF4/heparin antibodies

A commercially available IgG enzyme-immune assay was used in accordance to manufacturer's instructions (Hyphen Biomed, Neuville-sur-Oise, France). The ability of sera to activate PLT was tested using HIPA as previously described.[23] Additional details are available in the *Online Supplementary Appendix*.

Preparation of washed platelets

Washed PLT (wPLT) were prepared from venous blood samples as previously described.[23,24] Additional details are available in the *Online Supplementary Appendix*.

Treatment of platelets with sera/immunoglobulins

A total of 37.5 μL of wPLT or platelet-rich plasma (PRP) were supplemented with 5 μL serum/IgG from HIT patients or controls and incubated for 1 hour under rotating conditions at room temperature. Changes in the expression of CD62p and PS were assessed via flow-cytometry. Additional details are available in the *Online Supplementary Appendix*.

Analysis of heparin-induced thrombocytopenia antibody-induced mechanomolecular signaling mechanisms

In order to investigate the underlying mechanisms that result in HIT Ab-induced procoagulant PLT effects, PLT were pretreated with the Fc-γ-RIIA blocking monoclonal Ab IV.3 or a monoclonal isotype control, prior to HIT serum/IgG treatment. In order to investigate the role of upregulated intracellular levels of cAMP, PLT were pretreated with the prostacyclin analogue Iloprost or vehicle prior to incubation with IgG from HIT patients or healthy controls (HC). Additional details are available in the *Online Supplementary Appendix*.

Specific blockade of platelet surface P-selectin and phosphatidylserine

P-selectin (CD62p) was blocked via the pretreatment of PLT with 5 μg/mL anti-SELP humanized Ab (anti-CD62p, 15 minutes [min] at room temperature; ProteoGenix, Schiltigheim, France). Specific inhibition of externalized PS on the surface of procoagulant PLT was performed via the incubation of PLT with Lactadherin, a calcium-independent ligand of PS.[25,26]

Thrombin generation assay

HIT Ab-induced thrombin generation on PLT was detected using CAT (Stago, Maastricht, the Netherlands) according to the manufacturer's instructions. Additional details are available in the *Online Supplementary Appendix*.

Investigation of antibody-induced thrombus formation

In order 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.[27] Images were processed identically using adjusted threshold settings and exclusion of image artefacts by using Fiji image processing software.[28] Additional details are available in the *Online Supplementary Appendix*.

Statistical analysis

Statistical analysis was performed using GraphPad Prism, Version 8.0 (GraphPad, La Jolla, USA). Comparison between groups was performed by Mann-Whitney U test for unpaired data sets and Wilcoxon matched-pairs signed-rank or paired sample *t* test for paired data sets. *P* values <0.05 were considered statistically significant.

Results

Patient characteristics

In this study, we used serum samples from 37 patients with suspected HIT (Table 1). Twenty-three of 37 (62%) pa-

tients were male and the mean age was 65 years (range, 37-87 years). Twenty-eight of 37 (76%) patients tested positive for anti-PF4/heparin IgG Ab in enzyme-immune assay (mean optical density [OD] 2.12; range, 0.51-3.61). The diagnosis of HIT was confirmed in 20 of 37 (54%) using heparin-induced PLT activation assay (HIPA, median time to platelet aggregation in the presence of 0.2 IU/mL heparin: 5 min; range, 5-25 min]). In the HIT-positive cohort, thrombocytopenia (PLT count <150x10^6/mL) occured in 19 of 20 (95%) and clinical overt thrombosis was observed in nine of 20 (45%) patients, including six pulmonary embolisms, two occlusions of arterial vessels and one disseminated intravascular coagulation (for more details see the *Online Supplementary Table S1*).

Table 1. Patient characteristics.

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

ID: identification; PLT: platelet; M: male; F: female; OD: optical density; EIA: enzyme-immune assay; HIPA: heparin-induced platelet activation assay; Pos.: positive; Neg: negative. †Reference range PLT count (150-450x10^9/L); #1-17: patients with suspected heparin-induced thrombocytopenia (HIT); #18-37: patients with confirmed HIT.

Membrane phospholipid symmetry of platelets is rearranged by sera from heparin-induced thrombocytopenia patients

In order to investigate the ability of HIT Ab to induce new PLT populations, wPLT from healthy individuals were incubated with sera from 37 patients with suspected HIT in the presence of buffer, low- (0.2 IU/mL) or high-dose (100 IU/mL) heparin. As shown in Figure 1A, marked changes in CD62p expression and PS externalization were induced by sera from HIT patients (HIPA-positive) in the presence of low-dose (0.2 IU/mL) heparin but not in the presence of high-dose (100 IU/mL) heparin (mean percent of CD62p and PS double-positive PLT [mean %] ± standard error of the mean [SEM]: 38.65±5.79% *vs.* 1.14±0.26%; *P*<0.0001; Figure 1A). In contrast, no significant alteration in the expression levels of PLT CD62p and PS was observed by testing sera from patients with suspected but not confirmed HIT (HIPA-negative) compared to sera from HC in the presence of low-dose (0.2 IU/mL) heparin (mean % ± SEM: 0.63±0.12% *vs.* 1±0%; *P*=0.0565; Figure 1A). In order

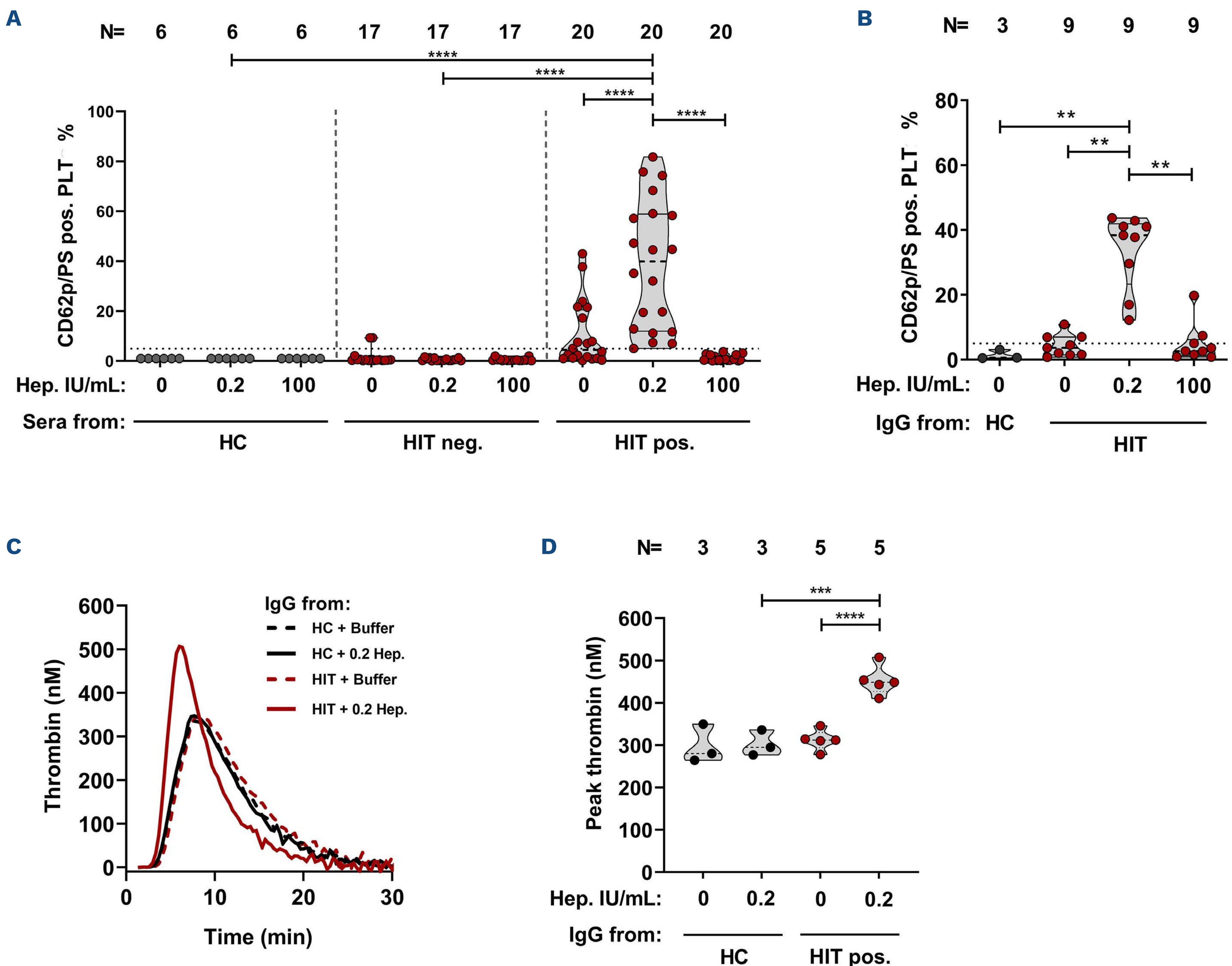

Figure 1. Heparin-induced thrombocytopenia antibodies induce procoagulant platelets and thrombin generation in a heparin-dependent manner. (A) Platelets (PLT) were incubated with sera from patients with suspected heparin (Hep)-induced thrombocytopenia (HIT, N=37), control sera (healthy controls [HC], N=6) or (B) with corresponding immunoglobulin G (IgG) isolates and tested for changes in the expression levels of P-selectin (CD62p) and phosphatidylserine (PS) via double staining in flow cytometry. Where indicated, PLT were co-incubated with low- (0.2 IU/mL) or high-dose (100 IU/mL) heparin. (C) Representative thrombin generation curve induced on PLT after incubation with IgG from HC (black lines) or HIT patients (red lines) in the presence of buffer or heparin (0.2 IU/mL). Each curve represents the amounts of generated thrombin over time in the presence of buffer (dashed lines) or heparin (0.2 IU/mL, [solid lines]). (D) Data were quantified as peak thrombin generated (nM) using Thrombinoscope software and Graphpad prism. The number of patients/IgG tested is reported in each graph. Violin plots showing the distribution of the values were generated using Graphpad Prism 8. **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*< 0.0001. ns: non-significant; CAT: calibrated automated thrombogram; pos.: positive; neg.: negative.

to investigate whether our findings were solely due to IgG-PLT interactions and not induced by unspecific serum-mediated effects, we next investigated IgG fractions from corresponding HIT patients (*Online Supplementary Figure S1*). Compared to sera, HIT IgG isolates not only showed similar binding properties to PF4/heparin complexes (*Online Supplementary Figure S2*) but also induced a significant increase in procoagulant PLT formation in a heparin-dependent manner (mean % ± SEM: 33.71±3.88% *vs.* 4.30±1.13%; *P*=0.0039; and *vs.* 1.40±0.84%; *P*=0.0091, respectively; Figure 1B).

Heparin-induced thrombocytopenia antibodies mediate increased thrombin generation and thrombus formation

When IgG from HC were incubated with test PLT in the presence of low-dose (0.2 IU/mL) heparin, no significant increase in thrombin generation was observed compared to buffer (mean peak thrombin [nM] ± SEM: 302.70±17.47 *vs.* 297.90±26.20; *P*=0.3258; Figure 1C, D). In contrast, IgG from confirmed HIT cases were able to induce higher levels of thrombin on the PLT surface (mean peak thrombin [nM] ± SEM: 452.70±15.67 *vs.* 312.00±10.76; *P*=<0.0001; Figure 1C, D).

Next, we established an *ex vivo* HIT thrombosis model that utilizes tetra staining to visualize the contribution of procoagulant PLT (Annexin V-positive, [red]), non-procoagulant PLT ($DiOC_6$-positive, [green]), leukocytes (Hoechst 33342-positive, [blue]) and Fibrin -(ogen) (magenta) to HIT Ab-induced thrombus.[29-31] In order to resemble venous shear conditions, experiments in the *ex vivo* thrombosis model were performed at venous shear rates as venous thrombosis is common in HIT patients.[3,32,33] In a first proof of concept experiment, we tested whether our thrombosis model resembles the pathophysiology of HIT, namely heparin-dependent multi-cellular activation and subsequent vessel occlusion. As shown in Figure 2, the presence of heparin (0.2 IU/mL) resulted in a significant increase of thrombus formation while such changes were not detectable under buffer conditions (mean cumulative area of thrombus total surface area coverage [%SAC]:

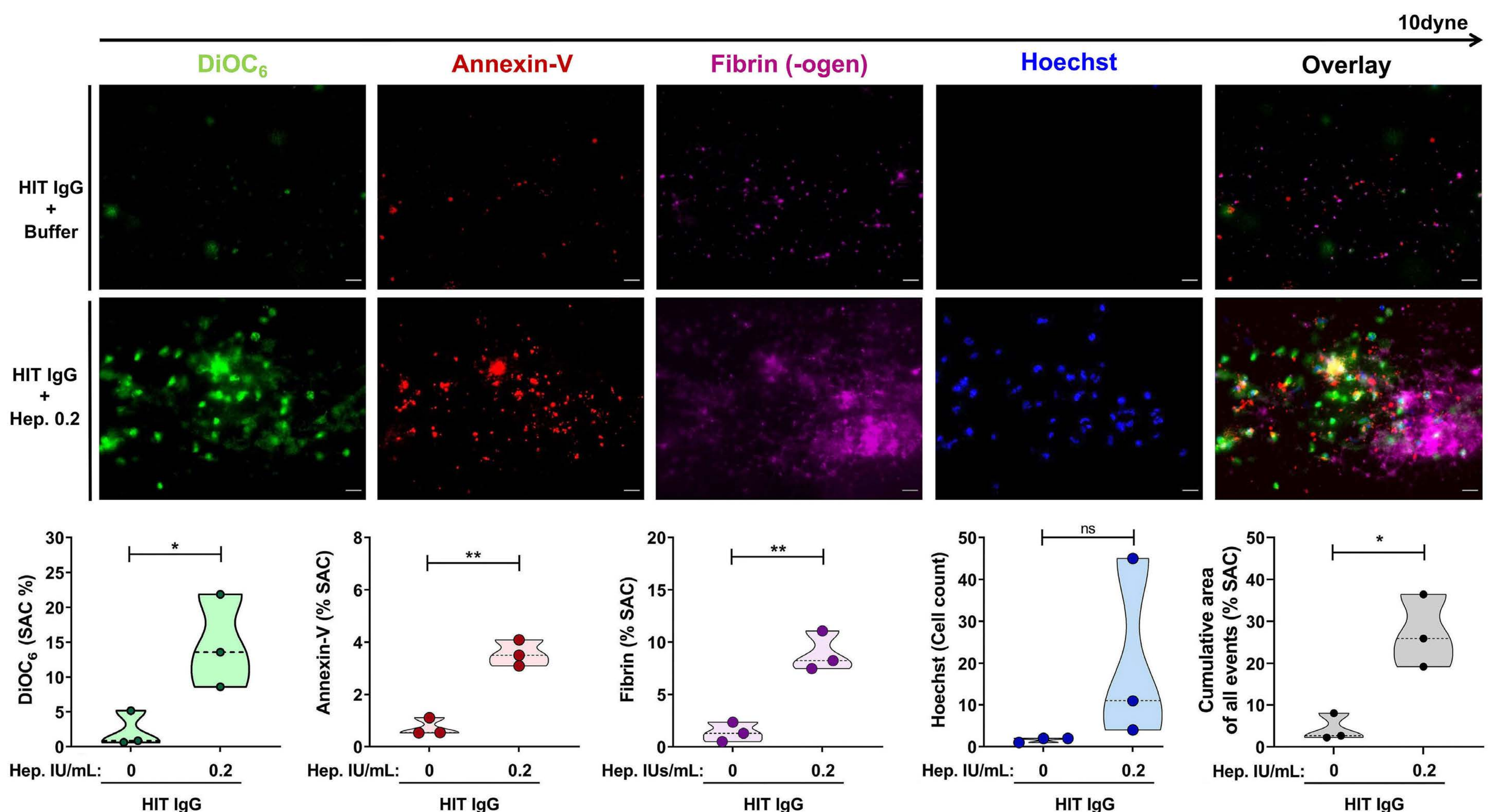

Figure 2. Heparin dependency of the *ex vivo* heparin-induced thrombocytopenia thrombosis model. Platelet-rich plasma from healthy individuals was incubated with Immunoglobulin G (IgG) from heparin (Hep)-induced thrombocytopenia (HIT) patients in the presence of buffer (upper panel) or heparin (lower panel) prior to labeling of platelets (PLT) with $DiOC_6$ (green), procoagulant PLT with AF647 Annexin V (red), AF546 Fibrinogen (magenta) and leukocytes with Hoechst 33342 (blue). After labeling, samples were reconstituted into autologous whole blood. Samples were then recalcified and perfused through microfluidic channels at a venous shear rate of $250s^{-1}$ (10 dyne) for 10 minutes. Images were acquired at x40 magnification in different fluorescence channels using a Zeiss Axio Observer 7 microscope. Scale bar 20μm. Images were processed identically using adjusted threshold settings and exclusion of image artefacts using Fiji image processing software. Violin plots showing the percentage of total surface area coverage (% SAC) by $DiOC_6$, phosphatidylserine (PS), Fibrin (-ogen), number of Hoechst-positive labeled cells and cumulative area with $DiOC_6$, PS and Fibrin (-ogen) labeled thrombus captured in the microfluidic channel. *P<0.05, **P<0.01 and ***P<0.001. ns: non-significant.

27.20±5.02% *vs.* 4.31±1.87%; *P*=0.0110; Figure 2). Interestingly, procoagulant PLT were observed to circulate and attach to the collagen surface of the microfluidic system from the beginning in our experiments (mean %SAC Annexin V ± SEM: 3.57±0.29% vs. 0.72±0.19%; *P*=0.0012; Figure 2; *Online Supplementary Movie S1*).
Furthermore, circulating procoagulant PLT incorporated later into the growing thrombus (*Online Supplementary Movie S2*). Additionally, heparin-dependent deposition of non-procoagulant PLT was observed in the presence of HIT Ab (mean % SAC $DiOC_6$ ± SEM: 14.69±3.86% vs. 2.21±1.48%; *P*=0.020; Figure 2; *Online Supplementary Movie S3*). Moreover, the multicellular composition of HIT Ab-mediated thrombosis in our microfluidic system was confirmed by an increased heparin-dependent recruitment of leukocytes to the thrombi (mean number of Hoechst-positive cells ± SEM: 20±13 *vs.* 2±0; *P*=0.141; Figure 2). Finally, HIT Ab were able to activate the plasmatic coagulation system in our assays as indicated by increased Fibrin (-ogen) network generation (mean % SAC Fibrin (-ogen) ± SEM: 8.93±1.09% *vs.* 1.38±0.54%; *P*=0.0047; Figure 2; *Online Supplementary Movie S4*). No increased thrombus formation was induced by IgG from HC in the presence of low-dose (0.2 IU/mL) heparin (*Online Supplementary Figure S3*).

Fc-γ-RIIA signaling induces procoagulant platelets in heparin-induced thrombocytopenia

We next aimed to dissect the underlying molecular mechanisms leading to HIT Ab-induced procoagulant PLT formation and increased prothrombotic potential. Pretreatment of PLT with Fc-γ-RIIA blocking monoclonal Ab IV.3 significantly reduced HIT Ab-induced procoagulant PLT formation (mean % ± SEM: 46.74±4.97% *vs.* 5.48±1.12%, *P*=0.0313; *Online Supplementary Figure S3A*) as well as thrombin generation on test PLT (mean peak thrombin [nM] ± SEM: 423.70±18.92 *vs.* 283.3±24.95; *P*=0.0085; *Online Supplementary Figure S3B, C*). Most importantly, blockade of PLT Fc-γ-RIIA inhibited HIT Ab-induced procoagulant PLT deposition (mean % SAC Annexin V ± SEM: 4.83±0.23% *vs.* 1.12±0.43%; *P*=0.0042; Figure 3). Similarly, a significant reduction in the deposition of non-procoagulant PLT was observed in the presence of monoclonal Ab IV.3 (mean % SAC $DiOC_6$ ± SEM: 20.99±0.97% *vs.* 2.69±1.15%; *P*=0.0062; Figure 3). Fur-

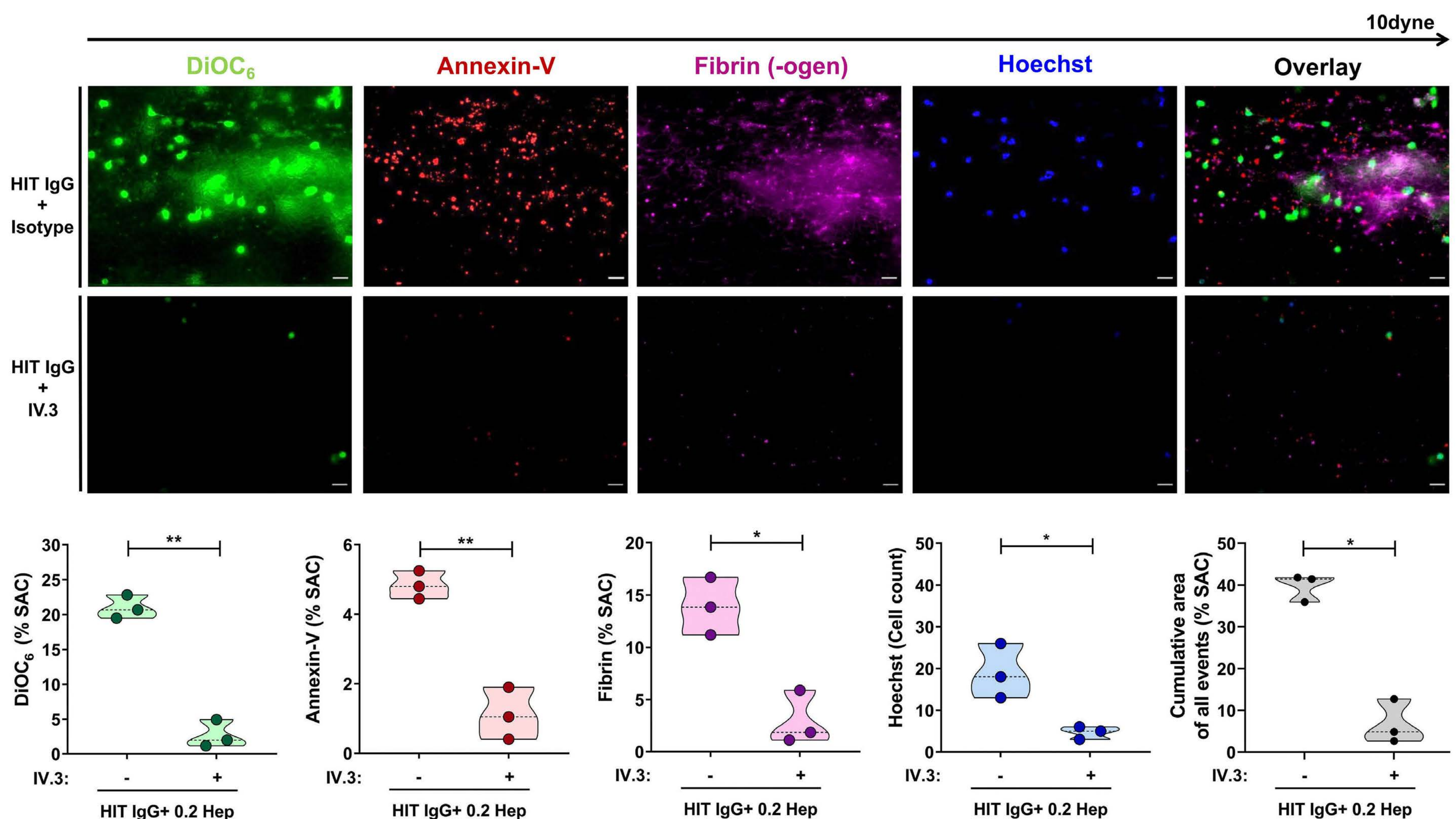

Figure 3. Fc-γ-RIIA inhibition prevents thrombus formation by heparin-induced thrombocytopenia immunoglobulin G. Platelets (PLT) from healthy individuals were incubated with immunoglobulin G (IgG) from heparin (Hep)-induced thrombocytopenia (HIT) patients in the presence of heparin (0.2 IU/mL), and monoclonal antibody IV.3 (lower panel) or isotype control (upper panel) before reconstitution into whole blood and perfusion through microfluidic channels at a venous shear rate of $250s^{-1}$ (10 dyne) for 10 minutes. After perfusion, images were acquired at x40 magnification. Scale bar 20μm. Violin plots showing the percentage of total surface area coverage (% SAC) by $DiOC_6$, phosphatidylserine (PS), Fibrin (-ogen), count of Hoechst-positive labeled cells and cumulative total % SAC with $DiOC_6$, PS and Fibrin(-ogen) labeled thrombus captured in the microfluidic channel. **P*<0.05, ***P*<0.01 and ****P*<0.001.

thermore, blockade of PLT Fc-γ-RIIA resulted in a marked inhibition of fibrin deposition and leukocyte attachment to the thrombus surface compared to isotype control (mean % SAC Fibrin (-ogen) ± SEM: 13.90±1.59% *vs.* 2.95±1.49%; *P*=0.0317; and mean number of Hoechst-positive cells ± SEM: 19±4 vs. 5±1; *P*=0.0257, respectively; Figure 3).

cAMP elevation using Iloprost prevents heparin-induced thrombocytopenia antibody-induced thrombosis *ex vivo*

Sustained high levels of intracellular calcium are a typical feature of procoagulant PLT.[34] Prostacyclins are known to be able to inhibit intracellular calcium release via binding of PLT prostacyclin receptor (IP-R), activation of membrane bound adenylyl cyclase and subsequent elevation of intracellular cAMP.[35] The pretreatment of PLT with the prostacyclin analogue Iloprost clearly inhibited HIT Ab-induced procoagulant PLT formation (mean % ± SEM: 35.99±5.98% *vs.* 4.20±1.08%; *P*=0.0313; *Online Supplementary Figure S4A*). Iloprost pretreatment also reduced procoagulant PLT-induced thrombin generation (mean peak thrombin [nM] ± SEM: 295.80±17.70 *vs.* 125.80±7.08; *P*=0.0026; *Online Supplementary Figure S5B, C*). Most importantly, the deposition of non-procoagulant PLT and procoagulant PLT was significantly reduced when PLT were pretreated with Iloprost prior to HIT IgG incubation (mean % SAC $DiOC_6$ ± SEM: 17.97±2.25% *vs.* 2.19±0.76%; *P*=0.0054; and mean % SAC Annexin V ± SEM: 4.09±0.24% *vs.* 1.42±0.19%; *P*=0.0003, respectively; Figure 4). Additionally, Iloprost pretreatment reduced Fibrin (-ogen) deposition and prevented the recruitment of leukocytes to thrombi (mean % SAC Fibrin (-ogen) ± SEM: 10.40±1.41% *vs.* 3.49±0.90%; *P*=0.0037; and mean number of Hoechst-positive cells ± SEM: 14±5 *vs.* 2±0; *P*=0.0257, respectively; Figure 4).

Phosphatidylserine but not P-selectin is essential to propagate HIT antbody-mediated thrombosis

HIT Ab-induced procoagulant PLT formation and subsequent prothrombotic changes were significantly inhibited via PLT Fc-γ-RIIA blockade as well as intracellular cAMP elevation. However, these data do not allow a discrimination, whether increased CD62p or high PS on the surface of Ab-induced procoagulant PLT is the causing factor for an increased prothrombotic potential.

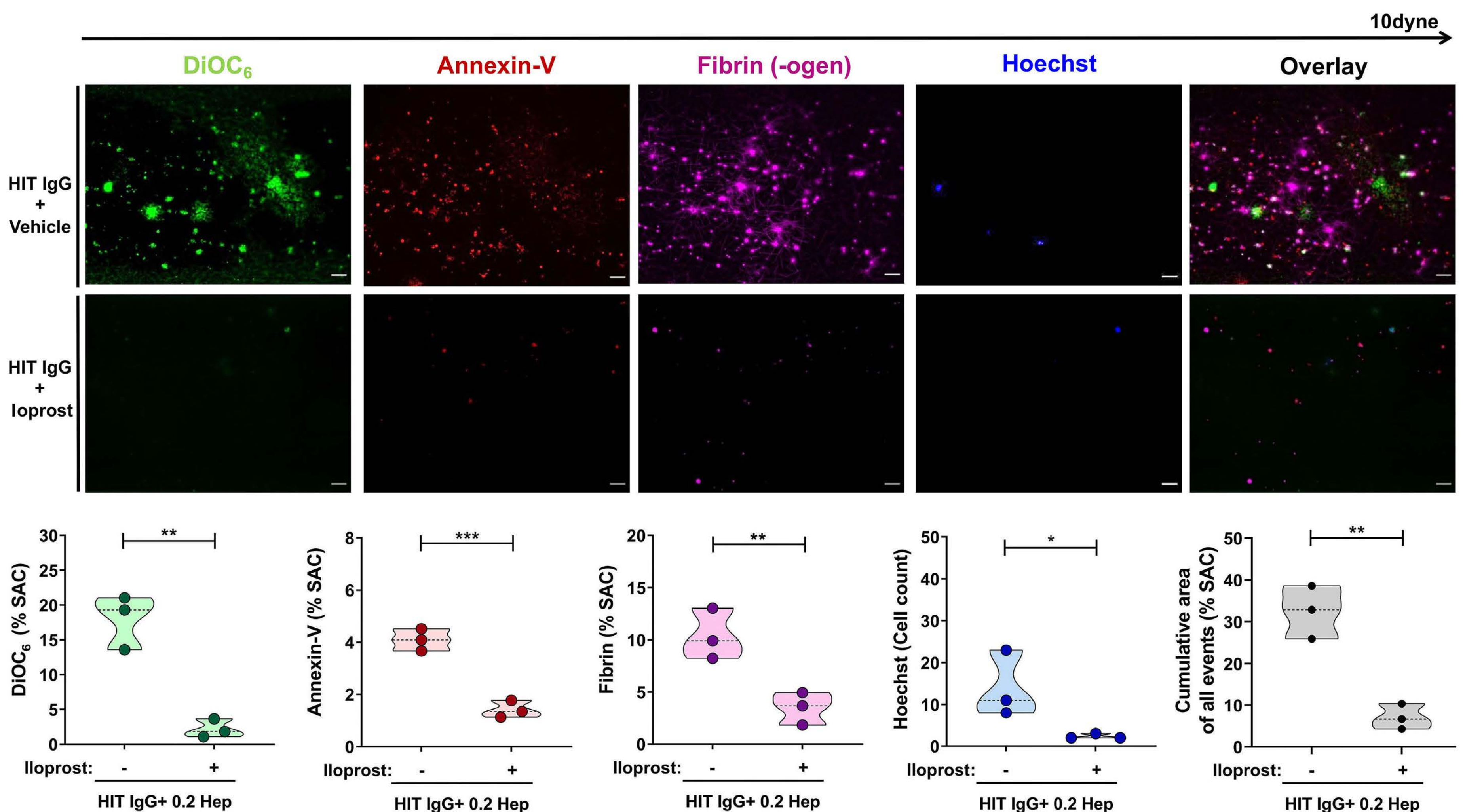

Figure 4. Upregulation of platelet cAMP protects from heparin-induced thrombocytopenia antibody-induced thrombus formation. Platelets (PLT) from healthy individuals were incubated with immunoglobulin G (IgG) from heparin-induced thrombocytopenia (HIT) patients in the presence of vehicle (upper panel) or Iloprost (20 nM, [lower panel]) and heparin (Hep) (0.2 IU/mL). After reconstitution into autologous whole blood and recalcification, samples were perfused through microfluidic channels at a venous shear rate of $250s^{-1}$ (10 dyne) for 10 minutes. After perfusion, images were acquired at x40 magnification. Scale bar 20μm. Violin plots showing the percentage of total surface area coverage (% SAC) by $DiOC_6$, phosphatidylserine (PS), Fibrin (-ogen), count of Hoechst-positive labeled cells and cumulative % SAC with $DiOC_6$, PS and Fibrin (-ogen) labeled thrombus captured in the microfluidic channel. **P*<0.05, ***P*<0.01 and ****P*<0.001.

We observed a slight inhibition of thrombin generation in the presence of the CD62p blocking Ab when PLT were incubated with IgG from HC as well as from HIT patients. However, HIT IgG were still able to induce a significantly higher increase in thrombin generation compared to HC despite the inhibition of CD62p (Figure 5A, B). In our *ex vivo* thrombosis model, anti-CD62p Ab was able to reduce leukocyte deposition on the thrombus surface (mean number of Hoechst-positive cells ± SEM: 16±3 *vs.* 4±1; *P*=0.0434; Figure 6). The reduction of leukocyte recruitment did not affect thrombus formation as no significant alterations in the deposition of procoagulant and non-procoagulant PLT was observed compared to control (mean % SAC Annexin V ± SEM: 4.80±0.67% *vs.* 5.27±0.21%; *P*=0.262; and mean % SAC $DiOC_6$ ± SEM: 17.85±2.91% *vs.* 19.78±1.78%; *P*=0.124, respectively; Figure 6). Moreover, no changes in plasmatic coagulation, namely Fibrin (-ogen) desposition, were observed in the presence of anti-CD62p (mean % SAC Fibrin (-ogen) ± SEM: 9.71±1.42% *vs.* 11.68±2.31%; *P*=0.256; Figure 6).

Finally, we investigated the prothrombotic role of PS on the surface of HIT Ab-induced procoagulant PLT. Notably, blocking PS with Lactadherin resulted in a significant inhibition of HIT Ab-induced procoagulant PLT-mediated thrombin generation compared to vehicle (mean peak

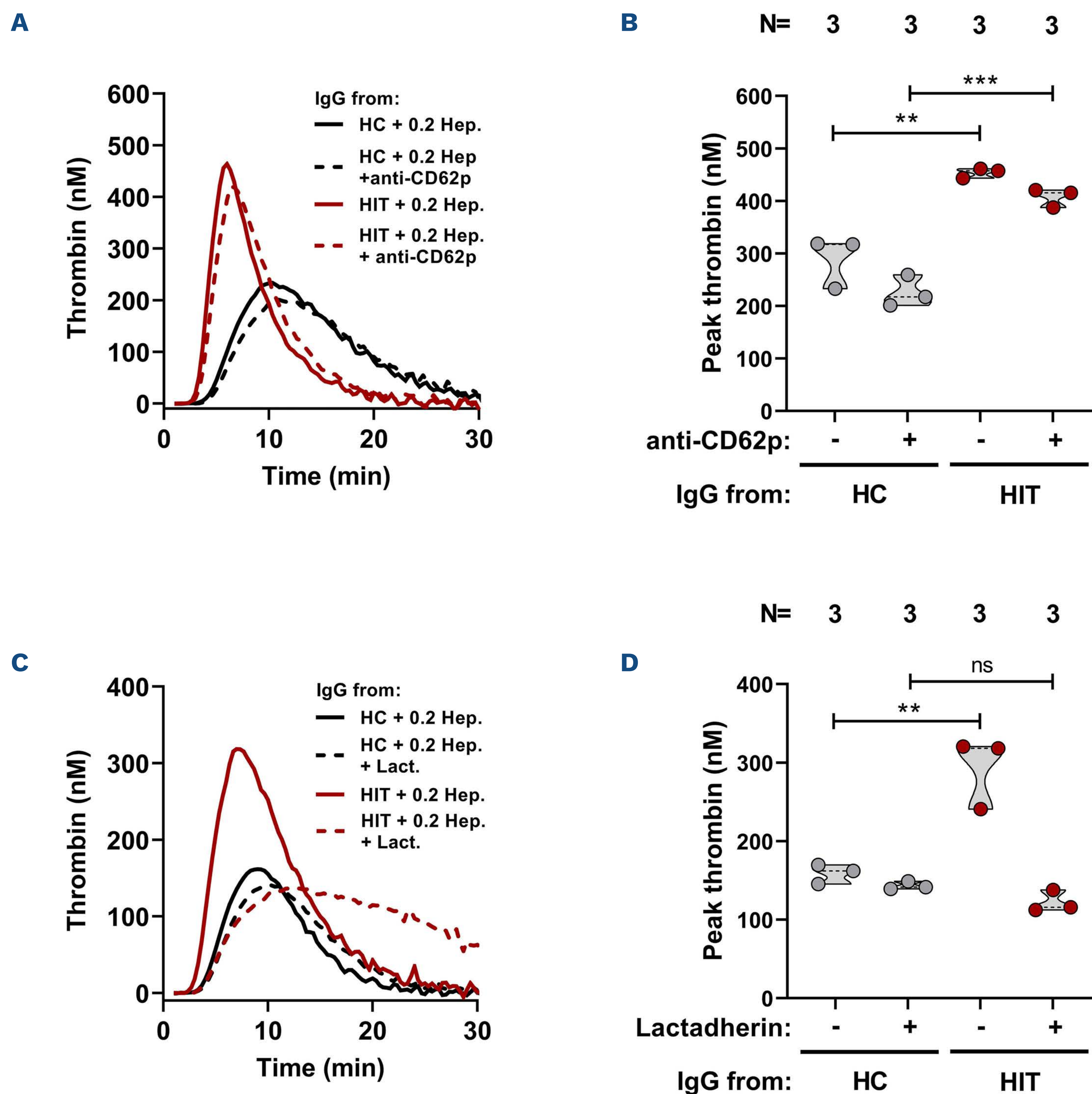

Figure 5. Increased platelet phosphatidylserine and not CD62p causes higher thrombin generation in heparin-induced thrombocytopenia. Panel (A) shows thrombin generation potential on platelets (PLT) after incubation with wit immunoglobulin g (IgG) from heparin-induced thrombocytopenia (HIT) patients (red line) or healthy controls (HC) (black line) in the presence of vehicle or anti-CD62p blocking antibody (Ab). (C) Thrombin generation on PLT that were incubated with different HIT patient IgG (red line) or HC IgG (black line) and treated with Lactadherin (Lact.) or vehicle, before calibrated automated thrombogram (CAT) analysis was performed. Each curve represents the amounts of generated thrombin over time induced by HIT IgG in the presence of vehicle (solid lines) or anti-CD62p blocking Ab or Lactadherin (dashed lines), respectively. (B and D) Data were quantified as peak thrombin generated (nM) using Thrombinoscope software and Graphpad prism. **P*<0.05, ***P*<0.01 and ****P*<0.001. ns: non-significant; CD62p: P-selectin.

thrombin [nM] ± SEM: 305.60±22.24 *vs*. 151.80±30.28; *P*=0.0040; Figure 5C, D; *Online Supplementary Table S2*). Our hypothesis that increased PS could be the critical mediator of increased thrombus formation was further reinforced as PS blockade with Lactadherin resulted in significant reduction of HIT Ab-induced thrombus formation (mean cumulative area of thrombus % SAC: 33.37±1.97% *vs*. 6.00±1.97%; *P*=0.0005; Figure 7). Treatment of PLT with HIT IgG upon Lactadherin incubation resulted in marked reduction of procoagulant PLT deposition (mean % SAC Annexin V ± SEM: 4.29±0.82% *vs*. 0.912±0.20%; *P*=0.0357; Figure 7). Additionally, the presence of Lactadherin prevented non-procoagulant PLT deposition on the collagen surface (mean % SAC $DiOC_6$ ± SEM: 18.25±2.58% *vs*. 2.20±0.58%; *P*=0.0080; Figure 7). Most importantly, PS inhibition interfered with plasmatic coagulation as a nearly complete inhibition of fibrin (-ogen) network deposition was observed (mean % SAC Fibrin (-ogen) ± SEM: 10.83±0.53% *vs*. 2.88±1.30%; *P*=0.0067; Figure 7). Interestingly, also multicellular thrombus composition was affected, as a reduction of leukocytes, although not significant, was observed in the presence of Lactadherin (mean number of Hoechst positive cells ± SEM: 14±6 *vs*. 1±1; *P*=0.0602; Figure 7).

Discussion

Thromboembolic events leading to high morbidity and mortality are frequent and still unpredictable complications in HIT.[3] Of all laboratory findings, thrombocytopenia seems to be the most pronounced change associated with thrombosis in HIT.[9,10] Although recent studies indicate the involvement of multicellular effector mechanisms, the question whether PLT alone are capable of initating a prothrombotic response in HIT, remains elusive. In this study, we found that HIT IgG Ab isolated from serologically and clinically confirmed HIT cases induce a new PLT subpopulation, namely procoagulant PLT. Our data indicate that Fc-γ-RIIA and subsequent intracellular calcium-dependent signaling pathways mediate the generation of procoagulant PLT in HIT. Most importantly, we found that procoagulant PLT-induced prothrombotic conditions are mediated by externalized PS rather than P-selectin. These findings direct towards an essential role of procoagulant PLT in the pathophysiology of thromboembolic complications in HIT. Our findings might have potential therapeutic as well as diagnostic relevance for the management of patients with suspected HIT.

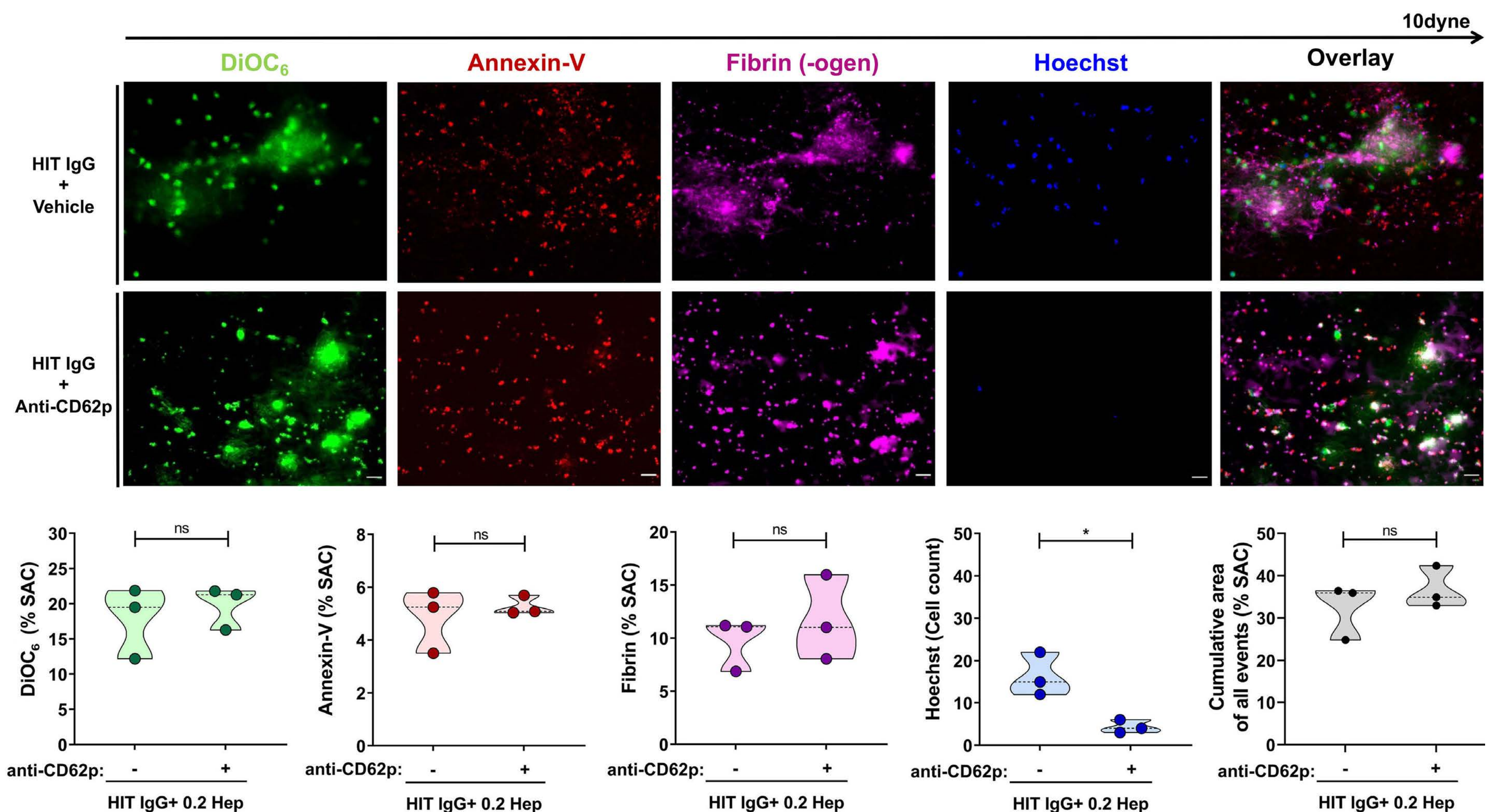

Figure 6. Heparin-induced thrombocytopenia antibodies can induce thrombus independent of CD62p. Platelets (PLT) from healthy individuals were incubated with heparin-induced thrombocytopenia (HIT) patient immunoglobulin G (IgG) and treated with vehicle (upper panel) or anti-CD62p blocking antibody (lower panel). After reconstitution into autologous whole blood and recalcification, samples were perfused through microfluidic channels at a venous shear rate of $250s^{-1}$ (10 dyne) for 10 minutes. After perfusion, images were acquired at x40 magnification. Scale bar 20 μm. Violin plots showing the percentage of total surface area coverage (% SAC) by $DiOC_6$, phosphatidylserine, Fibrin (-ogen), count of Hoechst-positive labeled cells and cumulative area of $DiOC_6$, PS and Fibrin (-ogen) labeled thrombus in the microfluidic channel. **P*<0.05, ***P*<0.01 and ****P*<0.001. ns: non-significant. Hep: heparin.

We observed that the ability to generate procoagulant PLT is restricted to sera from patients with laboratory- and clinically-confirmed HIT. These data confirm recent findings by Lee *et al*. who showed that HIT patient plasma samples have the ability to induce a PLT phenotype that shows increased signal of CD62p (P-Selectin) and the arsenic-based PLT necrosis marker 4-(N-(S-glutathionylacetyl)amino) phenylarsonous acid (GSAO) using whole blood and patient plasma. The most significant difference in our setting was the absence of any additional PLT agonist (SFLLRN, thrombin analogue) that was necessary to induce the HIT patient-plasma induced PLT phenotype observed by Lee *et al.*[36] In fact, in our study IgG fractions from these sera modulate the expression of CD62p and PS on the surface of PLT. Hence, the ability of HIT immune complexes to induce a procoagulant PLT phenotype seems to be independent of costimulatory signals i.e., additional PLT activation by extracellular agonists like thrombin in patient sera.[6,36] The discrepancy could be also due to different experimental settings. We incubated isolated wPLT with HIT Ab in the absence of other cells in our experiments.[37] Preactivation of PLT during preparation of wPLT has also to be considered as a preanalytical factor that could further explain why dual receptor activation for procoagulant PLT formation was not necessary in our studies.[38,39] Additionally, a higher concentration of PF4/heparin-HIT Ab immune complexes in the fluid phase could interact directly with PLT Fc-γ-RIIA in our experimental conditions, which may have amplified Fc-γ-RIIA-mediated signaling and resulted in increased procoagulant PLT formation despite the lack of other costimulatory signals. However, and despite the use of wPLT and IgG isolates, we cannot exclude potential contribution of other cofactors.

In order to evaluate the biological relevance of HIT Ab-induced procoagulant PLT, we analyzed the interaction with the plasmatic coagulation system and other blood cells using a PLT-based thrombin generation assay and an *ex vivo* thrombosis model, respectively. HIT Ab-mediated significant increase in thrombin generation on PLT in a heparin-dependent fashion. Previously, HIT IgG-activated monocytes have been shown to be a main source of tissue factor release which results in the activation of factor VII and subsequent increased thrombin generation.[6] Our finding directs towards the ability of HIT Ab to induce a procoagulant PS containing PLT surface that provides an

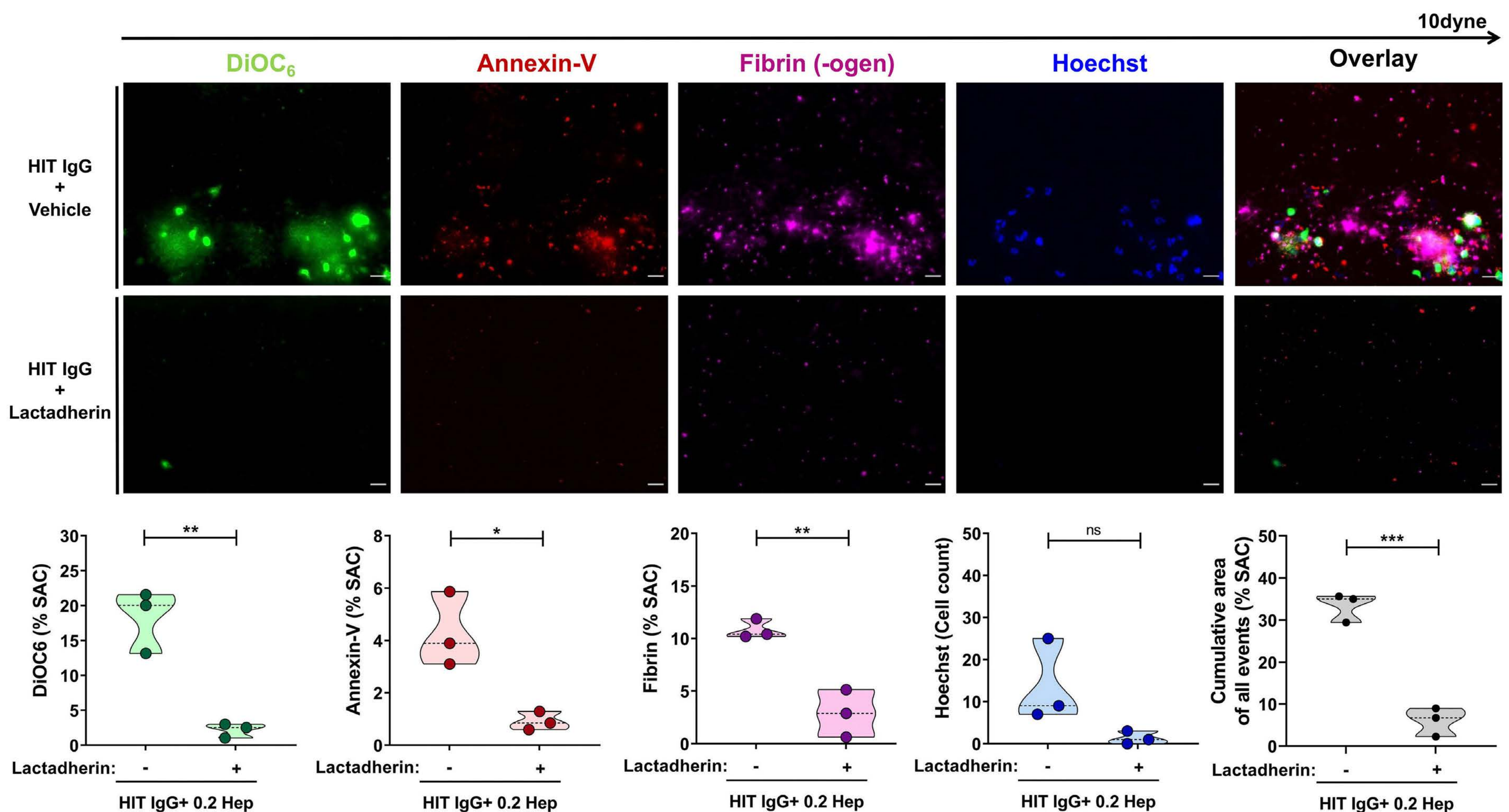

Figure 7. Platelet phosphatidylserine externalization is essential for heparin-induced thrombocytopenia antibody-induced thrombus formation. Platelets (PLT) from healthy individuals were incubated with heparin-induced thrombocytopenia (HIT) patient immunoglobulin G (IgG) and treated with vehicle (upper panel) or Lactadherin (lower panel). After reconstitution into autologous whole blood and recalcification, samples were perfused through microfluidic channels at a venous shear rate of $250s^{-1}$ (10 dyne) for 10 minutes. Images were acquired at x40 magnification. Scale bar 20μm. Violin plots showing the percentage of total surface area coverage (% SAC) by $DiOC_6$, phosphatidylserine (PS), Fibrin (-ogen), count of Hoechst-positive labeled cells and cumulative area with $DiOC_6$, PS and Fibrin (-ogen) positive labeled thrombus in the microfluidic channel. *$P<0.05$, **$P<0.01$ and ***$P<0.001$. ns: non-significant. Hep: heparin.

assembly site for increased thrombin generation. Furthermore, these results indicate a potential capability of HIT Ab-induced procoagulant PLT to cause increased *ex vivo* thrombosis. We optimized our thrombosis model to allow specific analysis of PLT-mediated thrombosis. In this model, PLT that were incubated with IgG from HIT patients but not HC, induced increased thrombus formation in the presence of heparin. Interestingly, PLT aggregates showed two different PLT phenotypes, namely procoagulant PLT (Annexin V-positive/$DiOC_6$-negative) and non-procoagulant PLT (Annexin V-negative/$DiOC_6$-positive).[30,31] Recent evidence highlights that procoagulant PLT primarily evolve on the surface of the growing thrombus where they promote thrombin generation.[40] In our model, circulating HIT Ab-induced procoagulant PLT attached directly to the collagen surface and later incorporated directly into the growing thrombus. Apart from this PS-positive PLT population, non-procoagulant PLT also adhered to collagen in our model. The fact that the recruitment of non-procoagulant PLT and leukocytes was inhibited by PS blockade suggests that procoagulant PLT are not only an important component in the thrombus structure but also propagate the deposition of other PLT subpopulations as well as immune cells into the growing thrombus body.[41]

Of potential therapeutic interest is the observation that the prostacyclin analogue Iloprost significantly reduces HIT Ab-induced procoagulant PLT and thrombus formation. Interestingly, in a recent clinical study patients with acute HIT undergoing cardiac surgery were treated with Iloprost in addition to heparin.[42] HIT Ab levels remained stable and no HIT Ab-induced reactivity on PLT was observed during the procedure, despite the presence of heparin. Most importantly, compared to the non-HIT control group, the incidence of thrombotic and thromboembolic events was similar (5.4% *vs*. 5.1%, respectively). Nevertheless, according to current HIT therapy guidelines, the use of prostacyclins is limited to cardiac surgery patients with acute HIT where surgery delay is not feasible.[43] Our results suggest that Iloprost may have a potential role in the treatment of a broader HIT patient collective. However, further clinical studies are needed to test the effectiveness and safety of Iloprost in patients with HIT.

HIT Ab-induced PLT-neutrophil interplay via P-selectin/P-selectin glycoprotein ligand-1 was reported to result in NET formation and subsequent increased thrombosis in mice.[16,44] In our experiments, blocking P-selectin reduced the recruitment of leukocytes to the thrombus, but did not affect procoagulant PLT-mediated thrombin generation or thrombus formation. This finding indicates that HIT Ab-induced procoagulant PLT can cause thrombus formation in the absence of direct PLT-leukocyte interactions. In contrast, the blockade of PS with Lactadherin significantly impaired the ability of HIT Ab-induced procoagulant PLT to promote thrombin generation and thrombus formation. These data indicate an indispensable role of PS on HIT Ab-induced procoagulant PLT to link cellular with plasmatic components of the coagulation cascade, leading to thrombin burst and subsequent Fibrin(-ogen) network formation. Lactadherin also prevented the deposition of $DiOC_6$-positive labeled PLT in the thrombus structure. This observation enforces our theory that procoagulant PLT might initiate the deposition of other PLT phenotypes such as activated PLT.

The findings of our study might have several important clinical implications. Currently no predictive biomarker exists that distinguish between HIT patients with high risk for thrombosis and those with "only" thrombocytopenia. The detection of procoagulant PLT in HIT patients via FC could help in risk stratification for prophylactic or therapeutic anticoagulation. Without any doubt, targeting PS to inhibit propagation of the prothrombotic condition in HIT is another promising clinical aspect of our results. Although our data regarding the potential use of PS blockade to prevent thrombosis might be promising, it has to be considered that hemostatic defects with increased tail bleeding has been reported when Lactadherin was investigated in an animal model.[26] However, in this study healthy mice were used. The prothrombotic nature of HIT might lead to different findings. Therefore, further investigations that assess the effect of Lactadherin in different animal models (e.g., HIT mouse model) might shed light whether the inhibition of Ab-induced thrombus formation is associated with an increased bleeding risk. As our data provide further insights regarding the molecular mechanisms of thromboembolic complications in HIT, some technical aspects might limit immediate translation. First, our data are based on testing sera from a relatively small retrospective patient cohort. Despite significant formation of procoagulant PLT was induced by all sera from patients with confirmed HIT, thrombosis was only detected in approximately 50% of our patient cohort. In our study, we did not perform deeper examinations to detect asymptomatic thrombosis during the follow-up period (e.g., routine duplexsonography etc.). Another explanation might be a high clinical awareness of treating physicians in our specialized center which may have resulted in prompt discontinuation of heparin and advanced treatment with alternative antiocoagulants. Second, our study does not provide data on the dynamics of thrombus formation. Future research attempts should focus on the sequence of events and the relevance of intercellular interactions to enable better identification of procoagulant PLT role in the initiation and propagation of thrombus formation. Additionally, procoagulant PLT-leukocyte interactions have to be investigated in future research attempts to conclude whether neutrophils and other immune cells are required for procoagulant PLT-mediated thrombus formation. Finally, while our *ex vivo* thrombosis

model might provide information on the interaction between blood cells, further investigations are needed to assess the influence of other cell types (e.g., endothelial cells). Additional *in vivo* approaches that utilize Fc-γ-RIIA transgenic mice or a humanized PLT transfusion mouse model would allow robust confirmation of our results.
In conclusion, our study suggests an indispensable role of procoagulant PLT in the pathophysiology of HIT-associated thrombosis. The inhibition of HIT Ab-induced procoagulant PLT formation with Iloprost or the inhibition of prothrombotic effects with PS targeting specific therapeutics could be a promising approach to prevent the onset of thromboembolic events in HIT patients.

Disclosures
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Contributions
JZ and TB designed the study. KA and TB were responsible for the treatment of the patients. JZ and KA collected and analyzed the clinical data. JZ, AS, KW, HJ, LP, and KA performed the experiments. JZ, AS, KA, GU and TB analyzed the data, interpreted the results and wrote the manuscript. All authors read and approved the manuscript.

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Data-sharing statement
Data generated from this study are available from the corresponding author upon reasonable request.

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