REGULAR ARTICLE

blood advances

Cooperative effect between anti-PF4/H and anti-PF4 antibodies increases cell activation and thrombotic risk in HIT

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Key Points

- Anti-PF4/heparin and anti-PF4 antibodies can cooperate to induce platelet activation, thrombocytopenia, and thrombus formation.
- The synergistic effect between PF4-specific antibodies is FcγRIIA dependent and occurs in the absence of heparin.

Heparin-induced thrombocytopenia (HIT) is a severe complication of heparin therapy, frequently associated with thrombosis. Immunoglobulin G (IgG) antibodies to heparin-platelet factor 4 (PF4/H) complexes play a central role in HIT by activating platelets and leukocytes via Fc gamma Receptor IIa (FcγRIIA). However, some patients also develop IgG against unmodified PF4 (anti-PF4), but their implication in the pathophysiology of HIT is unclear. Therefore, we assessed the impact of the joint presence of anti-PF4/H and anti-PF4 antibodies on cellular activation, platelet count, and thrombus formation, using chimeric monoclonal IgG1 antibodies specific for either PF4/H complexes (5B9) or PF4 alone (1E12). As expected, 5B9 coincubated with washed platelets without heparin did not induce platelet activation, but when a nonactivating concentration of 1E12 was present with 5B9, significant platelet activation was observed. This functional cooperation was Fc dependent and involved FcγRIIA receptors, given that it was no longer detectable with $F(ab')_2$ fragments of 1E12 or 5B9 or with ibrutinib, which inhibits the FcγRIIA pathway. 5B9 at a nonactivating concentration of 1E12 also induced thrombus formation without heparin under flow conditions. Furthermore, when the 2 antibodies were injected together into human FcγRIIA/human PF4 transgenic mice, thrombocytopenia always occurred, with pulmonary thrombi in one-third of the injected mice, similar to that observed after injection of 5B9 and heparin. These results support that functional cooperation may exist between anti-PF4 antibodies of different specificity and promote cell activation, thrombocytopenia, and thrombosis. This process may also increase the risk of thrombosis in HIT even after heparin treatment has been discontinued.

Introduction

Heparin-induced thrombocytopenia (HIT) is a serious complication of heparin therapy owing to an atypical immune response often resulting in thrombocytopenia and thrombotic complications.[1] Classically, patients develop immunoglobulin G (IgG) antibodies (Abs) directed against platelet factor 4

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(PF4) modified by heparin that cross-link IFc gamma Receptor IIa (FcγRIIA) expressed by platelets, neutrophils, and monocytes.[2] Apart from anti–heparin-PF4 (PF4/H) Abs, some patients may also develop Abs against unmodified PF4 that activate platelets in the absence of heparin.[3,4] Importantly, anti-PF4 Abs coexist with anti-PF4/H Abs in ~30% of patients with HIT,[3-5] who may exhibit more severe clinical manifestations, with deeper thrombocytopenia and longer platelet count (PC) recovery after heparin withdrawal.[4] However, the impact of the coexistence of both anti-PF4 and anti-PF4/H Abs on the pathophysiology of HIT is poorly understood. Previously, Nguyen et al showed using single molecule-force spectroscopy that anti-PF4 Abs bind to PF4 and thereby allowed the binding of anti-PF4/H Abs to this chemokine without heparin.[6] However, this cooperative Ab effect has never been confirmed at the cellular level.

Few monoclonal Abs (mAbs) have been developed to study the pathophysiology of HIT. Among them, 5B9, a chimeric IgG1 anti-PF4/H, and 1E12, 1C12, and 2E1, which are specific to PF4 alone, have a human Fc fragment and therefore well mimic the cellular effects induced by human PF4-specific Abs found in affected patients.[7,8]

The use of these tools allowed us to evaluate the effect of the simultaneous presence of anti-PF4 and anti-PF4/H Abs in plasma on cell activation in vitro and on PC and thrombus formation in a humanized mouse model.

Materials and methods

Materials and plasma samples

The chimeric anti-PF4/H monoclonal IgG1 5B9 and the anti-PF4 monoclonal IgG1 1E12, 1C12, and 2E1 have been produced by B Cell Design.[7,8] A chimeric IgG1 anti–endothelial growth factor receptor Ab (cetuximab, Merck) was used as a negative control. To suppress the possibility of Fc-mediated responses with PF4-specific mAbs, we obtained $F(ab')_2$ fragments of 5B9 and 1E12, using the FabRICATOR Fab'2 kit (Genovis). In addition, the deglycosylated forms of 5B9 (DG-5B9), 1E12 (DG-1E12), and cetuximab (DG-control Ab) were obtained after incubation overnight of each Ab (1 mg/mL) with 40 U of N-Glycosidase F (Sigma-Aldrich), followed by removal of the enzyme using a Vivaspin 50 kDa column (Sartorius).[9] Importantly, the absence of undegraded IgG or nonglycosylated forms in each preparation was verified by mass spectroscopy. The monoclonal anti-FcγRIIA Ab IV.3 (STEMCELL Technologies) and the bacterial protease IdeS (IgG-degrading enzyme derived from *Streptococcus pyogenes* from Genovis) that cleaves the hinge region of IgG were used to block FcγRIIA-dependent platelet activation and suppress Ab binding to FcγRIIA receptors.[10] Finally, high concentrations of IV immunoglobulins (LFB) were used to prevent platelet activation induced by anti-PF4/H and anti-PF4 Abs.[11]

Plasma samples from 18 patients with definite HIT were used, and all contained platelet-activating anti-PF4/H Abs (enzyme immunoassay [EIA]; mean optical density at 450 nm [OD_{450nm}], 2.34 ± 0.79; LIFECODES PF4 IgG EIA, Immucor GTI Diagnostics with positive serotonin release assay [SRA]), without any reactivity against unmodified PF4. In addition, plasma samples containing nonactivating Abs to PF4/H (mean OD_{450nm} 1.74 ± 0.37, negative SRA) from 18 other patients were also studied. All blood samples were obtained after informed consent according to the Helsinki Declaration principles, and the local ethics board approved their collection (DC 2008-308).

SRA

SRA was performed as previously described.[12] Briefly, 14C-serotonin–labeled platelets from healthy donors were incubated with 1E12, 5B9, control Ab, $F(ab')_2$ of 5B9, $F(ab')_2$ of 1E12, and/or patients' plasma samples for 1 hour at room temperature. Then, the radioactivity was measured in supernatants, and results were expressed as a percentage release of serotonin (percent).

Impedance aggregometry assay

Whole blood impedance aggregometry assay using the Multiplate analyzer (Roche) was adapted from Morel-Kopp.[12] Whole blood from healthy donors was incubated without or with unfractionated heparin (UFH; 0.1 IU/mL) for 5 minutes before the addition of 5B9 and/or 1E12 and/or control Ab. Changes in impedance were recorded over 15 minutes, and results were expressed as area under the curve values. A cutoff of 74 area under the curve was defined after testing 21 healthy donors (mean + 3 standard deviation).

Flow cytometry analysis

Washed platelets from healthy donors were obtained from whole blood collected on 0.129 M sodium citrate and used at a final count of 300 × 10^9/L. Platelets (50 µL) were incubated with 5B9 or its $F(ab')_2$ (both without UFH), 1E12 or its $F(ab')_2$, or control Ab, for 30 minutes with PE CD62P labeled Ab (Becton Dickinson) to evaluate platelet activation. As positive controls, platelets were incubated with 5B9 (50 µg/mL) and UFH (0.01 IU/mL). The same conditions were used to study platelet binding of Abs, by using deglycosylated forms of 5B9 and 1E12, labeled with Alexa Fluor 488 (DG-5B9-AF488 and DG-1E12-AF488) (Thermo Fisher Scientific). To assess the role of the FcγRIIA pathway, platelets were incubated for 5 minutes with Bruton tyrosine kinase inhibitor, ibrutinib (2 µM), and/or IV.3 (10 µg/mL) before incubation with PF4-specific Abs. For all flow cytometry experiments (CytoFLEX S, Beckman Coulter), platelets were identified with PerCP-Cy5.5 anti-CD41a mAb (clone HIP8, Becton Dickinson), and PE- or PerCP-Cy5.5 isotype IgG were tested as controls. Platelet activation level was evaluated by measuring the median fluorescence intensity (MFI) of CD62P-PE Abs compared with those of resting platelets. The platelet binding of deglycosylated forms of 5B9 and 1E12 was expressed as MFI ratio, considering the binding of each Ab alone as the baseline reference.

Microfluidic whole blood thrombosis model

Whole blood from donors identified as being sensitive to the synergistic effect of 5B9 and 1E12 (ie, good responders) was collected on 0.129 M sodium citrate and incubated for 10 minutes with 5B9 and/or 1E12 and/or control Ab. Then, blood samples were recalcified to 3 mM $CaCl_2$ and perfused at a shear rate of 20 µL/min (500 s^{-1}) in microfluidic channels (Vena8 Fluoro+, Cellix) precoated overnight at 4°C with 120 µg/mL purified human von Willebrand factor (LFB). To assess the controls, whole blood was preincubated for 10 minutes with UFH (1 or 100 IU/mL), 10 µg/mL of IV.3 (STEMCELL Technologies), 20 mg/mL of IV immunoglobulin (Privigen; CSL Behring), or 0.02 U/µg IgG of IdeS (Genovis)

before adding a PF4-specific mAb. Platelets and fibrin deposition were visualized by adding to whole blood DiOC6 (10 μM; Invitrogen) and Alexa Fluor 647-fibrinogen (40 mg/mL; Invitrogen), respectively. Leukocytes were labeled using a specific DNA dye (Hoechst 33342; Invitrogen). Images were acquired after 8 minutes using an Axio Observer 7 microscope (Zeiss) and an LD Plan-Neofluar 20×/0.4 Ph2 objective equipped with an ORCA-Flash 4.0 LT plus C11440 digital CCD camera (Hamamatsu) controlled by Zen 2.6 2018 image-capture software. The area covered by aggregates (>100 μm^2) was measured using ImageJ software after analyzing 30 different fields of view (size 600 × 100 μm) for each condition.

Whole blood stimulation and quantitative reverse transcription PCR

Whole blood from good responders to 5B9 and 1E12 was collected on 0.129 M sodium citrate and incubated with 5B9 and/or 1E12 and/or UFH. After lysis of red blood cells with 0.13 M of ammonium chloride, circulating cells were homogenized in TRIzol reagent (Life Technologies). Total RNA was extracted using the chloroform/isopropanol/ethanol method; 500 ng of total RNA was reverse transcribed to complementary DNA using High-Capacity complementary DNA Reverse Transcription Kit (Thermo Fisher Scientific). Samples were heated for 10 minutes at 25°C and then incubated for 1 hour at 37°C, followed by 5 minutes at 85°C.

Quantitative polymerase chain reaction (PCR) was performed using TaqMan Universal PCR Master Mix and TaqMan probes (tissue factor [TF], Hs00175225_m1; ABL, Hs01104728_m1; Applied Biosystems). The expression of the TF transcript was normalized to ABL expression, used as a housekeeping gene. The relative increase in TF messenger RNA (mRNA) levels was quantified after incubation of HIT Abs and heparin compared with the unstimulated whole blood condition and using the $2^{-\Delta\Delta Ct}$ method.

In vivo effects of 5B9 and 1E12 in HIT mouse model

Studies in transgenic HIT mice (human FcγRIIA, human G6b-B, human PF4-transgenic, mouse PF4 knockout) were approved by the French Ministry of Research, in accordance with the guidelines of the Regional Committee for Ethics in Animal Experimentation of Strasbourg (CEEA-35). Mice were generated by crossing human FcγRIIA/human PF4 mice[13] with human G6b-B mice.[14] G6b-B is a coinhibitory immunoreceptor that contains a tyrosine-based inhibition motif and inhibits signaling from immunoreceptor tyrosine-based activation motif–containing receptors, including FcγRIIA.[15] PCs were measured on day 0, and mice were injected intraperitoneally with 5B9 and/or 1E12 on day 1. UFH (1.4 U/g) was then injected subcutaneously on days 1, 2, 3, and 4. Platelets were counted 4 hours later and on days 1, 2, 3, 4, and 8 using an automatic cell counter (Scil Animal Care, Altorf, France). On day 8, mice were euthanized and the lungs removed, rinsed with 1 mL phosphate-buffered saline (PBS), and incubated in 4% paraformaldehyde overnight. The fixed lungs were then embedded in paraffin, sectioned at 4 μm, and mounted on microscope slides. Slides were deparaffinized and rehydrated; subjected to hematoxylin, phloxine, and saffron staining; and examined by light microscopy. Slides were also washed with PBS/0.1% Triton X-100, blocked with 3% bovine serum albumin in PBS, and probed with anti-CD42b Ab (ab183345, Abcam) overnight at 4°C, followed by incubation with secondary Abs (goat anti-rabbit Alexa Fluor 555, ab150078; Abcam) for 2 hours. Glass coverslips were mounted on the slides using mounting medium containing DAPI (4′,6-diamidino-2-phenylindole; ProLong Gold antifade reagent, P36935; Invitrogen) and imaged by fluorescence microscopy to visualize intravascular thrombi.

Statistical analysis

The statistical analysis was performed with GraphPad Prism software (version 10.2.1). The quantitative variables were analyzed using a Mann-Whitney *U* test. A *P* value <.05 was considered statistically significant.

Results

A cooperative effect between anti-PF4 and anti-PF4/H Abs promotes platelet activation and aggregation

As expected, anti-PF4/H mAb 5B9 (10 μg/mL) induced platelet activation in the presence of therapeutic concentrations of UFH (0.1 IU/mL), whereas no activation was observed without UFH (mean of serotonin release, 68% vs 7.3%, respectively). However, when 5B9 was coincubated without UFH with a very low and nonactivating concentration of 1E12 (0.5 μg/mL), significant serotonin release was observed (mean, 41.6% of serotonin release; $P < .001$; Figure 1A). Similar results were obtained after coincubation of 5B9 with 1C12 or 2E1 (0.5 μg/mL), 2 other anti-PF4 mAbs (supplemental Figure 1A-B). Furthermore, a synergistic effect of 1E12 was also demonstrated toward KKO (20 μg/mL), another anti-PF4/H mAb,[16] whereas it was not observed when 5B9 (20 μg/mL) was coincubated with KKO (supplemental Figure 2A). Notably, the cooperative effect of 5B9 and 1E12 was enhanced by low concentrations of UFH (≤0.1 IU/mL) and no longer significant with heparin concentrations of ≥0.5 IU/mL (Figure 1A; supplemental Figure 2B). Moreover, it was completely inhibited by the anti-FcγRIIA mAb IV.3 and was PF4-specific given that no activation was measured when a control Ab was coincubated with 5B9 or 1E12.

We then assessed whether this cooperative effect of 1E12 could also be observed with human anti-PF4/H Abs from typical HIT samples. Therefore, SRA was performed after the addition of a low concentration of 1E12 without heparin to plasma samples from 18 patients with typical HIT (ie, without anti-PF4 Abs), and significant platelet activation (with serotonin release between 28% and 63%) was observed in 7 of them (Figure 1B). In contrast, no potentiating effect of 1E12 (or a very weak effect with serotonin release <25% in 3 cases) was observed with plasma samples from patients without HIT with nonpathogenic anti-PF4/H Abs (Figure 1C).

Anti-PF4/H IgG levels were similar in HIT plasmas whether an effect of 1E12 or not was observed (mean OD_{405nm} values, 2.14 vs 2.55, respectively; $P = .25$), and no differences in other clinicobiological characteristics were observed between the 2 groups of patients.

The synergistic effect of anti-PF4 Abs was further studied by adding 1E12 (2 μg/mL) in whole blood containing 5B9 (20 μg/mL), and a significant platelet aggregation was induced in the absence of UFH in 13 of the 23 donors tested (Figure 1D). As expected, each Ab alone or the control Ab tested in combination with 5B9 or 1E12 did not induce any significant platelet aggregation.

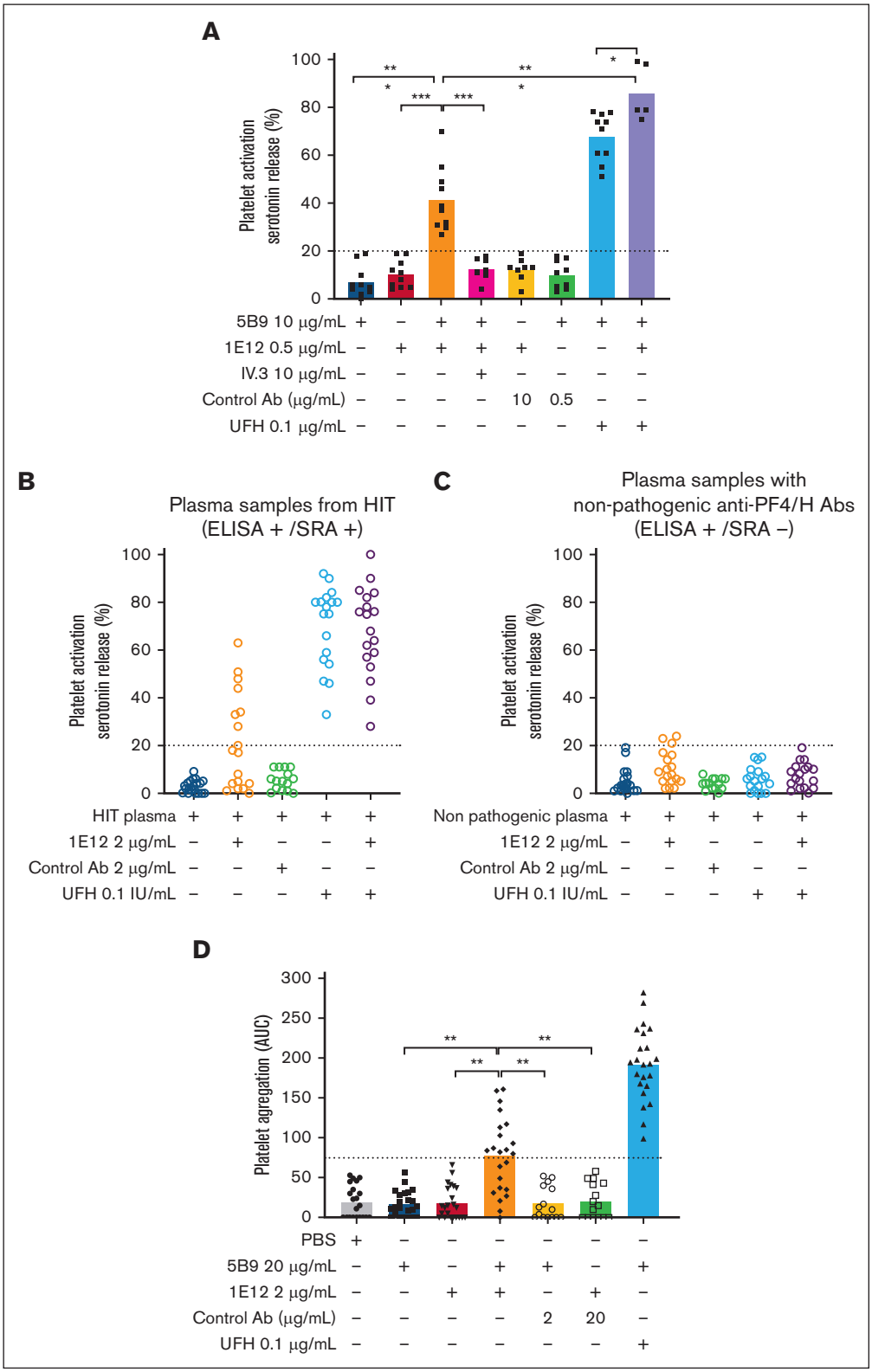

Figure 1. Synergistic platelet activation and aggregation induced by 5B9 and 1E12. (A) SRA performed with 5B9 and 1E12 incubated alone or together, without or with UFH, IV.3, or a control Ab. Data are the mean of serotonin release (n = 10). (B) SRA was performed with plasma samples from patients with HIT (n = 18) or with (C) nonpathogenic anti-PF4/H Abs (n = 18) and washed platelets from 4 healthy donors, without or with 1E12, control Ab, or UFH. (D) Whole blood platelet aggregation induced by 5B9 and 1E12 alone or together, without or with UFH or control Ab. Data are the mean of AUC (n = 23). Mann-Whitney *U* test was used to compare the different conditions tested. *$P < .05$; **$P < .01$; ***$P < .001$. The dotted line represents the cutoff value for SRA in panel A, B and C or whole blood platelet aggregation in panel D. AUC, area under the curve; ELISA, enzyme-linked immunosorbent assay.

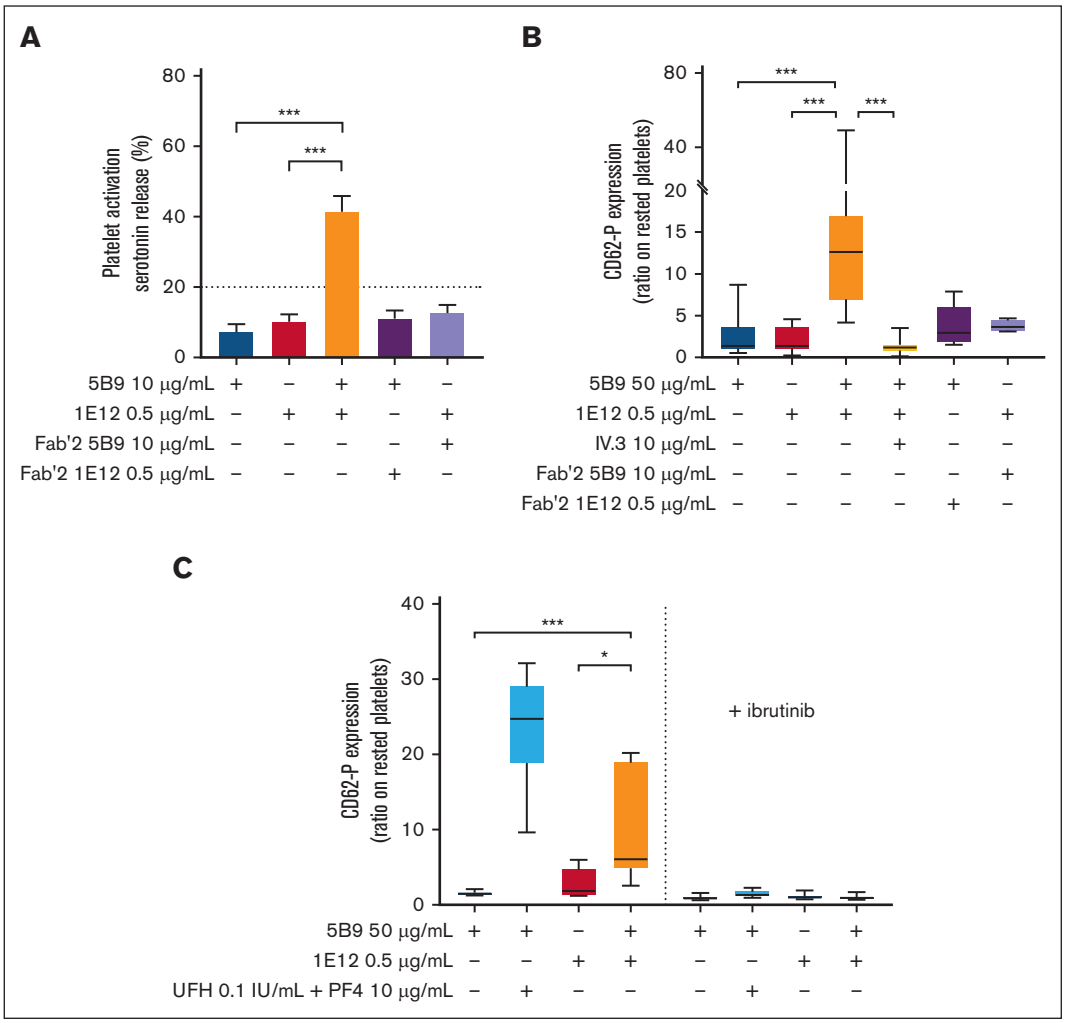

Figure 2. Cross-linking of 5B9 and 1E12 with FcγRIIA is essential for their synergistic Ab effect. (A) Platelet activation measured by SRA (n = 10) and (B) flow cytometry assay (CD62P expression, n = 13). 5B9 and 1E12 were tested alone or together, without or with IV.3 (10 µg/mL) or their F(ab')$_2$ fragments. (C) Platelet activation (CD62P expression, n = 4) induced by 5B9 and 1E12 alone or together, without or with ibrutinib. 5B9 with UFH and PF4 was tested as a positive control. Mann-Whitney *U* test was performed to compare the different conditions tested. *$P < .05$; **$P < .01$; ***$P < .001$.

Fc fragments of both 5B9 and 1E12 are essential for Ab synergy

We then investigated whether Fab and Fc fragments of both PF4-specific Abs contributed to their synergistic effect on platelet activation. As demonstrated, coincubation of the 2 intact Abs induced significant platelet activation (mean serotonin release, 41.6%; $P < .007$), but not when 5B9 or 1E12 was incubated with the F(ab')$_2$ of the other Ab (mean serotonin release, 10% and 11% respectively; Figure 2A). This result was confirmed by flow cytometry, given that the increase in P-selectin expression induced by 5B9 with 1E12 in the absence of UFH was no longer observed when experiments were done with the F(ab')$_2$ fragment of 1 of these 2 Abs (Figure 2B). In addition, platelet activation resulting from the cooperation between 5B9 and 1E12 was inhibited by IV.3 (Figure 2B) or ibrutinib, an inhibitor of Bruton tyrosine kinase (Figure 2C), supporting that the FcγRIIA pathway and Fc fragments of both Abs are critical in their synergistic effect.

5B9 and 1E12 mutually reinforce their binding to the platelet surface

The Fc-independent and Fab-dependent platelet binding of 5B9 and 1E12 was evaluated using deglycosylated (to prevent FcγRIIA binding) and labeled forms of these Abs. A significant increase in the binding of DG-5B9-AF488 to platelets was observed with 1E12 compared with the basal condition (median MFI ratio, 1.65; Figure 3A). In addition, 5B9 also increased the binding of DG-1E12-AF488 to platelets to a greater extent (median MFI ratio, 4.01; Figure 3C). However, this effect was no longer observed with F(ab')$_2$ fragments or when FcγRIIA receptors were blocked with IV.3 (Figure 3A-C). Similarly, ibrutinib abolished the increase in DG-5B9-AF488 or DG-1E12-AF488 platelet binding dependent on the other PF4-specific Abs, supporting that FcγRIIA signaling pathway is crucial for cooperation between the Abs (Figure 3B-D). As expected, ibrutinib did not modify the binding of DG-5B9-AF488 to the platelet surface in the presence of PF4 and UFH.

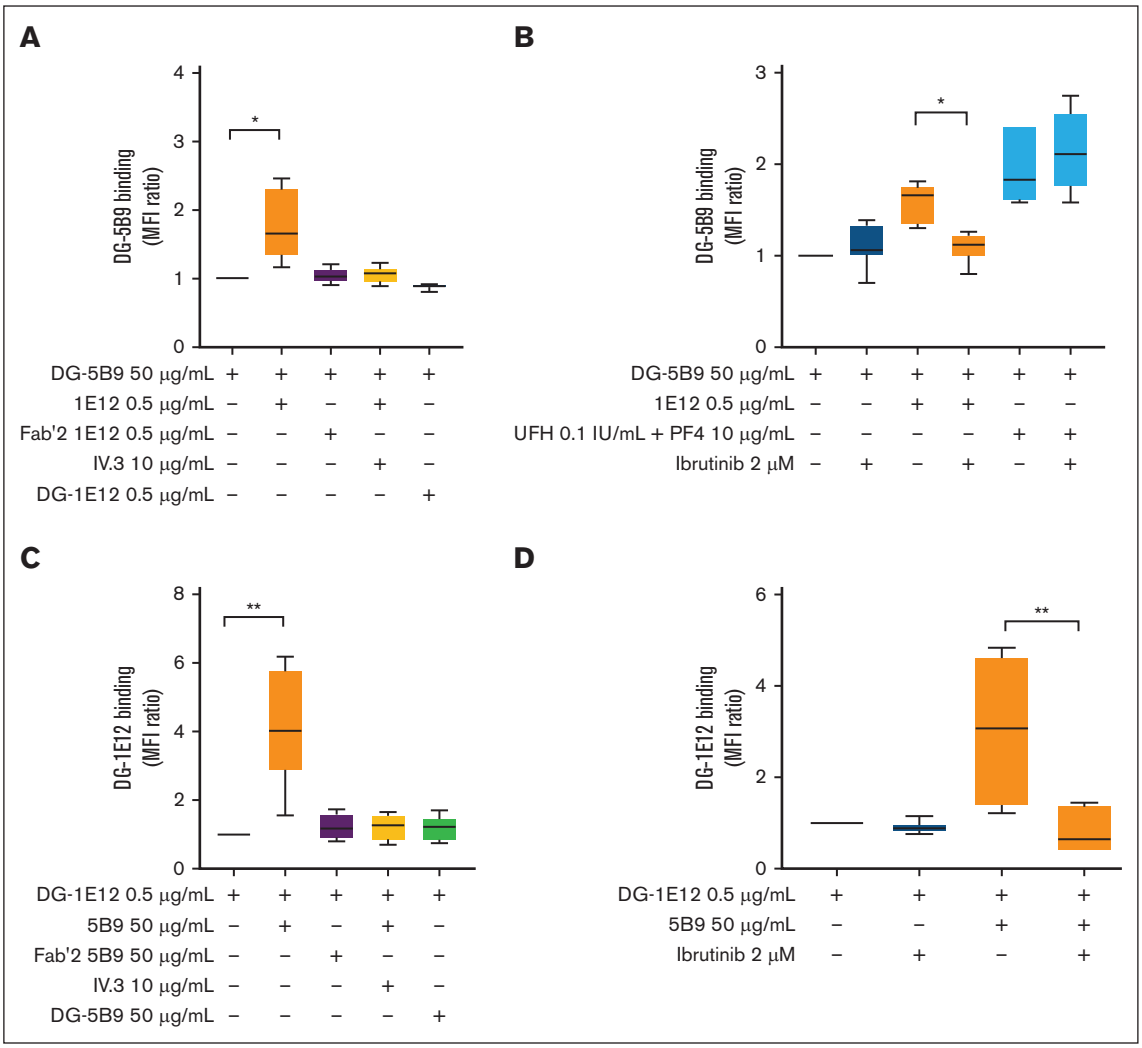

Figure 3. A 2-way cooperative effect of 5B9 and 1E12 enhances their binding to platelets. Platelet binding of (A) labeled-DG-5B9 (n = 5) and (C) labeled-DG-1E12 (n = 8) alone or in the presence of 1E12, 5B9, or their F(ab')$_2$ fragments or IV.3. Inhibitory effect of ibrutinib on the platelet binding (B) of labeled-DG-5B9 with or without 1E12 or UFH + PF4 (n = 7) (D) of labeled-DG-1E12 with or without 5B9 (n = 7). Data are the median (minimum to maximum) of the MFI ratio. Mann-Whitney *U* test was used to compare the different conditions tested. *$P < .05$; **$P < .01$; ***$P < .001$.

Synergistic effect of 1E12 and 5B9 promotes TF expression and thrombus formation in vitro

TF mRNA levels, measured in whole blood after incubation of both 5B9 and 1E12 without heparin, were higher than those quantified with only 5B9 or 1E12 (mean increase in TF mRNA level, 18.8-fold vs 10.2- and 2.9-fold respectively; Figure 4A). This TF mRNA synthesis induced by 5B9 and 1E12 was comparable with that observed with 5B9 and UFH. In addition, numerous fibrin-rich platelet-leukocyte aggregates formed when whole blood from healthy donors was perfused with 5B9 (100 µg/mL) and a non-activating concentration of 1E12 without UFH at 500 s^{-1} in capillaries coated with human von Willebrand factor (Figure 4B-C). These aggregates, similar to those observed with 5B9 and UFH (1 IU/mL), were absent when 5B9 or 1E12 were present alone without heparin or coincubated with the control Ab. Furthermore, aggregate formation induced by 5B9 with 1E12 was inhibited by IV.3 or therapeutic concentrations of IV immunoglobulins, or IdeS, that cleaves the hinge region of IgG (Figure 4C). Finally, coincubation of 5B9 with another anti–PF4-specific Ab, 1C12 or 2E1, also induced thrombus formation in vitro, as did the combination of 5B9 + 1E12 (supplemental Figure 3A-D).

Coinjection of 5B9 and 1E12 induces thrombocytopenia *in vivo*

To determine whether this cooperative effect also occurred in vivo, we first defined the minimal quantity of 1E12 or 5B9 that did not induce thrombocytopenia when injected alone without UFH in transgenic mice expressing the human forms of PF4, FcγRIIA, and G6b-B. The injection of 5B9 or 1E12 at a dose ≤1 µg/g did not induce a significant decrease in PC, but thrombocytopenia occurred when 1 µg/g of 5B9 was administered with UFH (supplemental Figure 4A). Profound thrombocytopenia was also evidenced when doses of 5B9 or 1E12 ≥3 µg/g were administered without UFH (supplemental Figure 4B).

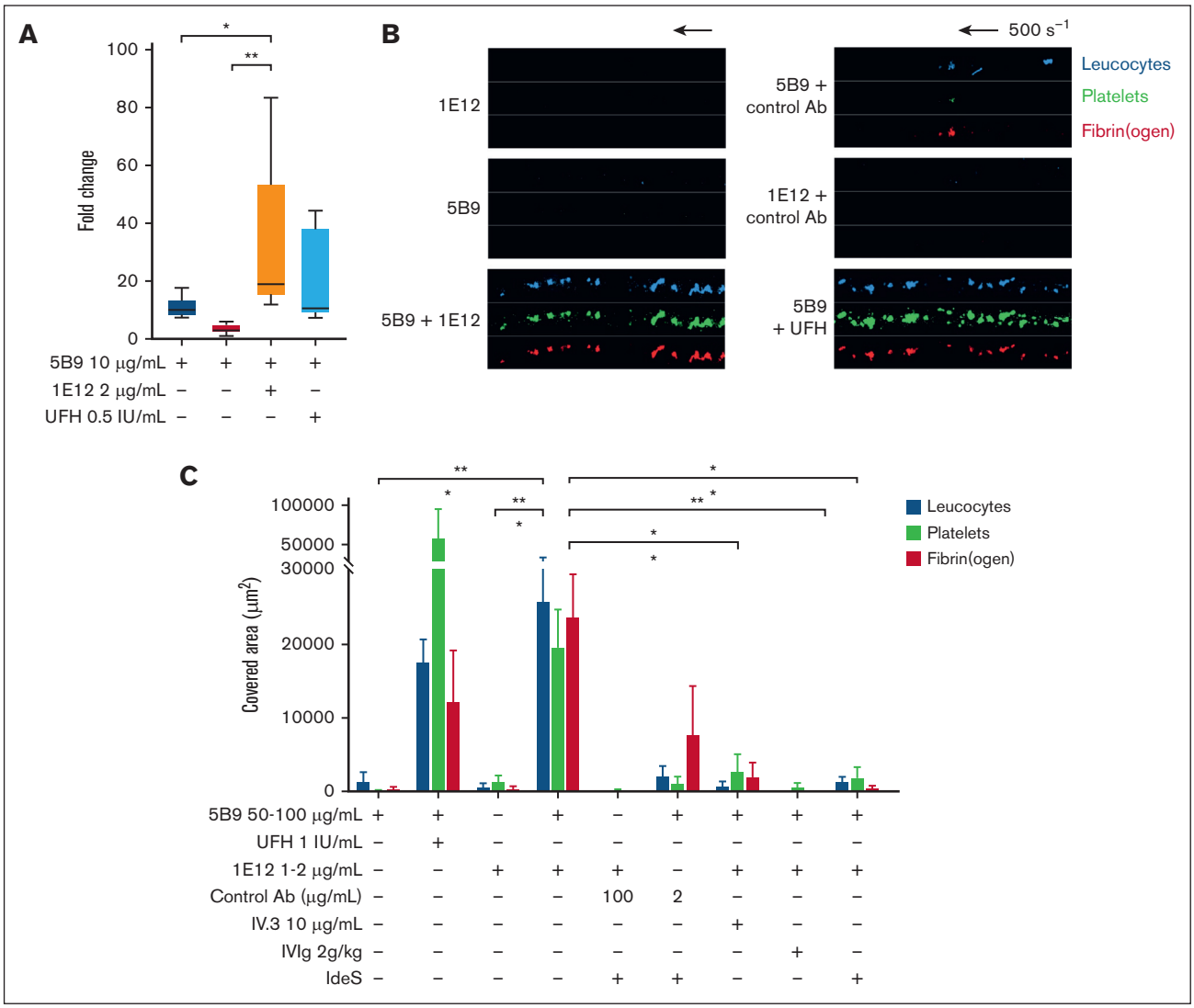

Figure 4. Synergistic effect between 5B9 and 1E12 on TF synthesis and thrombus formation in vitro. (A) Relative TF mRNA synthesis after addition of 5B9 and/or 1E12 or UFH to whole blood from healthy donors (mean ± standard error of the mean [SEM], n = 6 donors). (B) Representative images of thrombus formation in von Willebrand factor–coated microfluidic channels perfused (8 minutes, 500 s^{-1}) with recalcified whole blood incubated with 5B9 and/or 1E12 with or without control Ab or UFH. Images corresponding to areas of 0.1 mm^2 are shown with platelets in green (DiOC6), fibrin(ogen) in red (Alexa Fluor 647–labeled fibrinogen), and leukocytes in blue (Hoechst 33342, DNA dye). (C) The mean areas covered by platelets (green), leukocytes (blue), or fibrin(ogen) (red) were calculated for each condition (mean ± SEM; n = 12 independent experiments), by measuring using ImageJ software the surface covered by large aggregates (>100 $µm^2$) in 30 different areas. For each experiment, the highest concentration of 5B9 and 1E12 that did not induce thrombus formation when incubated alone in the absence of UFH was selected (50 or 100 µg/mL for 5B9 and 1 or 2 µg/mL for 1E12) and used for subsequent synergy experiments. Mann-Whitney *U* test was performed to compare the different conditions tested. *$P < .05$; **$P < .01$; ***$P < .001$. IVIg, IV immunoglobulins.

Three other mice were then injected with 1 µg/g of 5B9 and 1E12, and thrombocytopenia was observed in all, with a significant reduction in PC of 56% and 57% on days 1 and 2, respectively (Figure 5A). This thrombocytopenia was similar to that observed in mice treated with 5B9 and UFH (relative decrease of PC, 74% and 69% on days 1 and 2, respectively). In addition, a 53% transient decrease in PC was also observed on day 3 after the administration of 1 µg/g of 5B9 and 0.3 µg/g of 1E12. In contrast and as expected, when 5B9 or 1E12 were injected alone, PC changes were mild and not significant and similar to those observed after PBS injection. In contrast, examination of the lungs of the mice after sacrifice on day 8 revealed the presence of thrombi in one of the mice that had received 5B9 and 1E12 without heparin (Figure 5E,J). Similarly, pulmonary thrombi were found in 1 of the 3 mice injected with 5B9 and heparin (positive control, Figure 5F,K).

Discussion

The immune response underlying typical HIT is polyclonal, and in addition to IgG Abs specific for PF4/heparin complexes, some patients may also develop Abs that bind to unmodified PF4.[3,6] However, the consequences of the coexistence of these 2 types of PF4-specific Abs on the pathophysiology of HIT are not fully known.

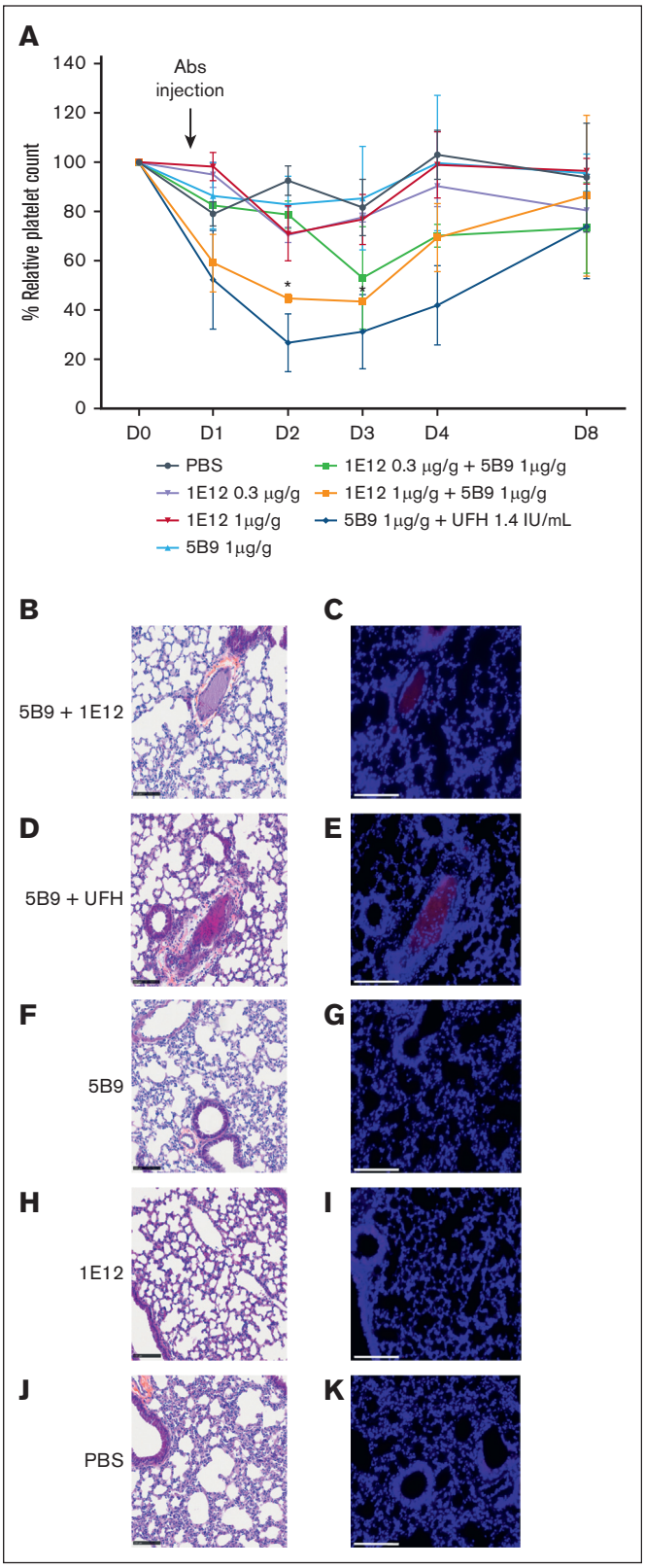

Figure 5. 5B9 and 1E12 induce thrombocytopenia and thrombosis in vivo. (A) Evolution of platelet count (PC) after injection of 5B9 (1 µg/g body weight; n = 4), 1E12 (0.3 or 1 µg/g; n = 3) or both Abs (n = 3) in transgenic HIT mice (human FcγRIIA, human G6b-B, human PF4 transgenic, mouse PF4 knockout). At days 1, 2, 3 and 4, 1.4 IU/g of UFH was injected in mice treated with 5B9 (n = 3). Data are represented as mean ± SEM. Mann-Whitney *U* test was performed to compare the different conditions tested. *$P < .05$. (B-E) Sections of lungs with intravascular thrombi from mice injected with 1E12 1 µg/mL + 5B9 1 µg/mL (B-C) or 5B9 1 µg/mL + UFH 0.1 IU/mL (D-E). (F-K) Sections of lungs without thrombi (controls) in mice injected with 5B9 1 µg/mL (F-G), 1E12 1 µg/mL (H-I), or PBS (J-K). Sections were stained using hematoxylin, phloxine, and saffron (HPS) in panels B,D,F,H,J or immunofluorescence in panels C,E,G,I,K. Scale bar, 100 µm (for HPS staining and 150 µm for immunofluorescence).

In the present study, we demonstrated that platelet activation was induced in the absence of heparin when a low "nonactivating" concentration of 1E12, an anti-PF4 mAb, was coincubated with 5B9 or KKO, 2 anti-PF4/H monoclonal IgGs that mimic the effects of human HIT Abs. Furthermore, a similar cooperative effect was also observed when 1C12 or 2E1, 2 other anti-PF4 mAbs with epitopes different from those recognized by 1E12,[17] was incubated with 5B9. On the contrary, 5B9 and KKO, which partially bind similar residues[7] but different from those recognized by 1E12, were unable to exert a reciprocal synergistic effect. Interestingly, 1E12, 1C12, and 2E1, like human anti-PF4 Abs, have a higher affinity for PF4 and form larger immune complexes than 5B9, KKO, and Abs against PF4/H developed in HIT.[8] This led us to hypothesize that anti-PF4 IgG may facilitate the formation of ultralarge and stable PF4 complexes on the platelet surface, which are critical for cell activation and the pathogenesis of HIT.[18] In this regard, a low concentration of 1E12 was shown by flow cytometry to promote the binding of 5B9 on the platelet surface, and inversely 5B9 facilitated the cellular binding of 1E12. Importantly, this Ab cooperative effect in terms of platelet binding was no longer observed with $F(ab')_2$ fragments or in the presence of IV.3, a mAb that blocks FcγRIIA or when FcγRIIA signaling was blocked by ibrutinib. Thus, the increase in Ab binding clearly involves the FcγRIIA pathway, but the exact mechanism remains to be fully elucidated. A low concentration of anti-PF4 Ab could sensitize platelets in an Fc-dependent manner in the absence of heparin or exogenous PF4, thereby favoring the release of PF4 molecules interacting with glycosaminoglycans, subsequent binding of anti-PF4/H IgG, and more potent cell activation. However, this hypothesis deserves further studies particularly to evaluate whether a change in platelet shape with increased membrane surface area may contribute to this synergistic effect.

Functional cooperation between 5B9 and 1E12 has also been demonstrated by platelet aggregation tests performed in whole blood, but with a variable sensitivity of platelets from 1 individual to another. This variability may be related in part to several factors previously identified to influence the platelet response to HIT Abs, including gene polymorphisms involved in the FcγRIIA pathway[11,19] or plasma components such as normal IgG[11] and fibronectin.[20]

However, a variable response was also shown after the addition of a suboptimal concentration of 1E12 to plasma samples from 18 patients with HIT given that significant platelet activation in SRA in the absence of heparin was observed in only 7 of them, who had similar levels of anti-PF4/H Abs and no specific clinical or biological characteristics. In addition, a low concentration of 1E12 also induced a weak but significant platelet activation in 3 of 18 samples from patients without HIT but with significant levels of anti-PF4/H

(EIA$^+$ and SRA$^-$). These results suggest that the synergistic effect of anti-PF4 Abs may depend on different characteristics of anti-PF4/H Abs from 1 patient to another. The polyclonal nature of the immune response leading to HIT has been previously demonstrated,[21-24] and in some patients, low levels of high-affinity anti-PF4 Abs have been detected, which may cooperate with anti-PF4/H Abs to induce greater platelet activation.[25]

Actually, it has been suggested that anti-PF4 Abs, by interacting with PF4 via their Fab fragments, can cluster PF4 molecules and form antigenic complexes recognized by heparin-dependent anti-PF4 Abs in the absence of heparin,[6] and we had also shown that 1E12 behaves similarly by clustering PF4 and modifying its antigenicity.[8] 1E12 less efficiently potentiated the platelet-activating effect of anti-PF4/H Abs (typical HIT Abs or 5B9) than heparin, suggesting that the underlying mechanism involved is different, that is, without necessarily modifying the epitope exposure of PF4.

The coexistence of anti-PF4 and anti-PF4/H Abs also likely increases the thrombotic risk in HIT, as we have shown that 1E12 with 5B9 in whole blood and in the absence of heparin induced strong TF expression and the formation of large fibrin-rich platelet/leukocyte aggregates in our microfluidic thrombosis model. In addition, experiments with transgenic mice expressing the human forms of PF4, FcγRIIA, and G6b-B also confirmed that, in the presence of both categories of anti-PF4 Abs, a significant thrombocytopenia was observed, sometimes associated with vascular thrombi in the lungs, confirming that an Ab cooperative effect can be pathogenic in the absence of heparin in vivo. However, these effects were only observed when equal doses of anti-PF4/H and anti-PF4 Abs were injected, suggesting that the model that we used did not fully mimic what happens in humans. Recently, we reported that ~30% of patients with HIT develop both anti-PF4 and anti-PF4/H Abs and experience more severe thrombocytopenia with a longer time to PC recovery after heparin withdrawal than patients who develop anti-PF4/H Abs only,[4] and the results of the present study may explain this observation. Indeed, the cooperation between these 2 groups of Abs may explain why thrombocytopenia persists in some patients with HIT after heparin withdrawal and also why delayed-onset HIT is observed in only a few other cases.[26,27]

Anti-PF4 Abs are also key players in the pathophysiology of not only vaccine-induced immune thrombotic thrombocytopenia (VITT)[28,29] but also VITT-like syndromes, and the clinical importance of a specific assay for their detection has recently been highlighted.[5] Interestingly, VITT Abs can bind to 1 or 2 specific regions on PF4, and their specificity is associated with variable localization of thrombotic events.[30] Our study supports that screening for anti-PF4 Abs with a specific immunoassay might also be useful in patients with HIT, especially if a platelet activation without heparin is demonstrated. Indeed, a treatment other than prescribing an alternative anticoagulant after heparin withdrawal may be necessary in patients with HIT with anti-PF4 Abs, and in the most severely affected cases, high-dose IV immunoglobulins are likely to be more effective as in VITT.[31,32]

In conclusion, this study shows that a synergistic FcγRIIA-dependent effect of anti-PF4 and anti-PF4/H IgG can induce platelet activation, thrombocytopenia, and thrombosis without heparin. This supports a role for IgG Abs specific for unmodified PF4 in the pathophysiology of HIT, particularly in patients with severe and persistent thrombocytopenia and thrombosis, and their detection may be useful in clinical practice.

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Authorship

Contribution: S.B. performed the research, analyzed the data, and wrote the manuscript; C.V. performed and designed the research and analyzed the data; O.B., E.M.-S., N.C., A.D., L.C., and Y.A.S. performed the research and analyzed the data; C.P. designed the research and reviewed the manuscript; Y.G. and J.R. designed the research, analyzed the data, and wrote the manuscript; and all authors have critically revised and approved the final version of the manuscript.

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