The deglycosylated form of 1E12 inhibits platelet activation and prothrombotic effects induced by VITT antibodies

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METHODS

Material and antibodies

The chimeric anti-PF4 monoclonal IgG1, 1E12, have been produced and purified by ArkAb (Limoges). Cetuximab, a chimeric IgG1 anti-EGFR therapeutic antibody (Merck) was used as a negative control in our experiments. The deglycosylated forms of 1E12 (DG-1E12) and cetuximab (DG-Ctrl Ab) were obtained after incubation overnight of each antibody (1mg/mL) with 40 U of N-Glycosidase F (Sigma-Aldrich), followed by removal of the enzyme using a Vivaspin 50 kDa column (Sartorius). The Fab'$_2$ fragment of 1E12 have been obtained by using FragIT kit (Genovis). The murine IgG1 anti-CD9 antibody ALB6 (Beckman Coulter), which activates platelets via FcγRIIa receptors{Rollin, 2012 #73} was used as a positive control in some experiments. The monoclonal anti-FcγRIIa antibody IV.3 (Stemcell) was also used to block FcγRIIa-dependent platelet activation. IdeS, *IgG-degrading enzyme derived from Streptococcus pyogenes* was obtained from Genovis, and unfractionated heparin (UFH; Heparin Choay) was purchased from Sanofi.

VITT samples (plasma or serum) were obtained from patients diagnosed in Tours (n = 13) or Greifswald (n = 5) based on a suggestive clinical context and detection of platelet-activating PF4-specific IgG antibodies using immunoassays and PF4-modified platelet function tests (PF4-SRA or PIFPA, as described below). Nine plasma samples from patients with definite HIT, with both positive PF4-specific immunoassay and SRA, were also studied.

PF4-modified serotonin release assay

Platelets were labeled with 14C-serotonin (14C-5HT, PerkinElmer) for 45 minutes at 37°C (0.4 µCi per mL PRP). After washing, platelets (300 x 10^9/L, 75 µL) were incubated with PF4 (10 µg/mL), plasma samples or 1E12 (20 µL) and various concentrations of UFH (0-0.1-0.5 or 10 IU/mL, 5 µL) for 1 hour at room temperature. For competitive assays, washed platelets and PF4

(10µg/mL) were pre-incubated for 10 min with 50µg/mL (final concentration) of deglycosylated 1E12 (DG-1E12) or deglycosylated cetuximab (DG-Ctrl Ab) before stimulation with either HIT or VITT plasma samples.

Platelet activation was stopped with PBS-EDTA buffer (0.1 mol/L, 250 µL), followed by centrifugation (2000 g, 4 minutes, 20°C). The radioactivity (β−) was measured in supernatants (Tri-Carb® 3180TR/SL liquid scintillation analyzer, Perkin Elmer), and results were expressed as a percentage release of serotonin (%) according to the basal radioactivity of the supernatants (Tyrode buffer) and maximal radioactivity released by platelets (0.5% Triton X-100). A result was considered positive in case of serotonin release >20% with 0, 0.1, or 0.5 IU/mL UFH, and strongly inhibited at 10 IU/mL heparin (release <20% or 50% inhibition).

Flow cytometry analysis

The flow cytometric assay was performed as a modified version of the PF4-induced flow cytometry-based platelet activation test (PIFPA). Briefly, citrated whole blood (WB) was obtained from three healthy donors who had not taken anti-platelet medication or non-steroidal anti-inflammatory drugs (NSAIDs). From each donor, 396 µL WB were spiked with 4 µL Hirudin 5400 U/mL (Canyon Pharmaceuticals). Hirudin-spiked WB was then incubated with 200 µg/mL (final concentration) DG-1E12 or DG-Ctrl Ab or PBS for 10 min at room temperature. Next, 5 µL WB were incubated for 20 min at 37°C with 2.5 µL of PBS and 2.5 µL undiluted sera from VITT patients were added to each tube and incubated for further 20 min at 37 °C. Each sample was incubated with 1 µL PE mouse anti-human CD61 (clone SZ21, Beckman Coulter) and 1 µL -PE-Cy5 mouse anti-human CD62P (Becton Dickinson) for 10 min at room temperature in the dark, followed by a fixation step of 20 min at room temperature with 2% PFA (Morphisto). After washing, the supernatant was discarded, the pellet was suspended

in 500 µL 1x BD FACS Lysing Solution (Becton Dickinson), and incubated at least 10 min before starting flow cytometry using a Cytomics FC500 (Beckman Coulter).

Platelets were positively gated using CD61-PE, and activation was determined by the degree of granule release measured using CD62P-PE-Cy5 antibody and given as mean fluorescence intensity (MFI) of the CD62P positive gated events multiplied by the percentage of gated platelets. The gate was set accordingly by a negative control containing PBS instead of serum (not shown). A final concentration of 20 µM thrombin receptor activating peptide (TRAP-6, Hart Biologicals) served as a positive control for each whole blood sample. Platelet activation for each serum was analyzed as the median activation result from the three whole blood samples.

ELISA competition assay

The Fab'2 fragment of 1E12 (10 µg/mL) was first incubated 10 minutes at room temperature (RT) in PF4/PVS coated wells. Then, diluted plasmas (1/50) were incubated for 50 min at RT. After washing, immunoassays were performed in accordance to the manufacturer instruction (HAT IgG, Immucor). Absorbances were measured at 450 nm.

Microfluidic whole blood thrombosis model

Whole blood (WB) collected on 0.129 M sodium citrate from healthy donors was incubated with VITT plasma (50 µL), 1E12 (5 or 10 µg/mL) or ALB6 antibody (20µg/mL). For competitive assays, WB was pre-incubated for 10 min with 100µg/mL (final concentration) of DG-1E12 or DG-Ctrl Ab before adding VITT plasma samples or ALB6. After 10 minutes, blood samples were recalcified to 5 mM CaCl2 and perfused at a shear rate of 20 µL/min (500 s-1; 20 dyn/cm2) in microfluidic channels (Vena8 Fluo1, Cellix) precoated overnight at 4°C with 160 mg/mL purified human von Willebrand factor (LFB). Platelets and fibrin deposition

were visualized by adding DiOC6 (10 µM) and AF647–fibrinogen (40 mg/mL; Invitrogen), respectively, to WB. Leukocytes were labeled by using a specific DNA dye (Hoechst 33342; Invitrogen). Images were acquired every 20 seconds for 8 minutes using an Axio Observer 7 microscope (Zeiss) and an LD Plan-Neofluar 20x/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 (> 100µm^2) was measured using ImageJ software and after analyzing 20 different areas for each experimental condition.

Effect of antibodies on NETosis

Isolation of neutrophils : EDTA-anticoagulated WB from healthy volunteers who had not taken anti-platelet medications or non-steroidal anti-inflammatory drugs (NSAIDs) during the previous ten days, was mixed with 5% dextran 500 solution (Serva Electrophoresis GmbH), and incubated for 30 minutes at 37°C to sediment red blood cells. The white blood cell-rich supernatant was transferred onto a separating solution (d = 1.077 g/mL; Biocoll, Biochrom AG), and centrifuged at 310 × g for 20 minutes at RT. After the supernatant was discarded, RBCs were lysed for 5 minutes on ice using chilled NH_4Cl lysis buffer. The cell suspension was then washed twice with PBS without Ca^{2+} and Mg^{2+} (Biochrom AG; 140 × g, 5 min at RT), neutrophils were resuspended in serum-free RPMI 1640 medium (Gibco), and cell count was adjusted as required. Neutrophils were stored on ice and used in experiments within 3 h of preparation.

Preparation of washed platelets: Platelets were isolated from acid citrate dextrose solution A (ACD-A) anticoagulated whole blood by differential centrifugation from healthy volunteers who had not taken antiplatelet medications or NSAIDs during the previous ten days. Platelet-rich plasma was washed, then suspended in Tyrode's buffer containing 0.35% bovine serum

albumin and 0.1% glucose, and platelet count was adjusted to 300,000/µL. Washed platelets were stored at 37°C and used in experiments within 3 h of preparation.

In vitro NETosis assay and analysis by confocal laser fluorescence microscopy: Neutrophils were suspended at 1×10^6 /mL in serum-free RPMI media (Gibco). *In vitro*, NETosis was studied by incubating neutrophils with PF4 (10 µg/mL) and platelets (1×10^6 /mL), in the presence of serum from healthy controls or VITT patients (1:50 diluted), or 1E12 (10 µg/mL). For competitive assays, neutrophils and platelets were pre-incubated for 10 min with 100µg/mL (final concentration) of DG-1E12 or DG-Ctrl Ab before adding VITT samples. As a positive control for NETosis, PMA (50 nM) was used. All incubations were performed on poly-L-lysin coated µ-Slide 18 Well-Flat imaging micro chambers (Ibidi) for 60 minutes at 37°C and 5% CO_2. Fixation was performed with 2% PFA for 30 minutes at RT, and slides were gently washed three times in PBS. For fluorescence microscopy, nuclear and extracellular DNA was stained with DRAQ5 (5 µM; Abcam). Platelets were labeled with FITC mouse anti-human CD61 (0.25 µg/mL; Clone RUU-PL7F12, BD Biosciences). To demarcate the plasma membrane, Lipilight 560 (20 nM; Membright, Idylle Labs) was used. All labeling procedures were performed in PBS for 30 minutes in the dark. Confocal laser microscopy was performed on a Leica SP5 confocal laser scanning microscope (Leica) equipped with HCX PL APO λ blue 40.0×/1.25 oil objective. For image acquisition, fluorophores (FITC, Membright 560, and DRAQ5) were excited with Argon-Krypton (488 nm), diode-pumped solid-state (DPSS, 561 nm), and Helium-Neon (HeNe, 633 nm) laser lines that were selected with an acousto-optic tunable filter (AOTF). Fluorescence emission was collected between 505-515 nm for FITC (detector HyD), 566-600 nm for Lipilight 560 (detector PMT), and 640-655 nm for DRAQ45 (detector HyD). Quantification of NETosis from fluorescent microscope images was performed using DNA Area and NETosis Analysis (DANA) software for ImageJ and Java.[1] Briefly, fluorescence microscopy images of channel for nuclear and extracellular DNA of neutrophils stained with

DRAQ5 were imported into DANA I module in ImageJ, thresholded and segmented automatically to generate a region of interests with grayscale intensities. The percent NETosis was computed from 12 individual images for each experimental condition acquired from n=3 replicate experiments in the DANA_II Java module.

Quantitative assay for *in vitro* human neutrophil elastase: To quantitatively assess the human neutrophil elastase associated with DNA, NETosis Assay Kit (Cat. No. ab235979, Abcam, Germany) was used following manufacturers protocols. Briefly, neutrophils were suspended at 1×10^6 /mL in serum-free RPMI media (Gibco) supplemented with 5% BSA. To assess the neutrophil elastase associated with DNA during the process of NETosis, neutrophils were incubated with PF4 (10 µg/mL) and platelets (1×10^6 /mL), in the presence of serum from healthy controls or VITT patients (1:50 diluted), or 1E12 (10 µg/mL). For competitive assays, neutrophils and platelets were pre-incubated for 10 min with 100µg/mL (final concentration) of DG-1E12 or DG-Ctrl Ab before adding VITT samples. As a positive control for NETosis, PMA (50 nM) was used. All incubations were performed in 24 well cell culture dishes at for 60 minutes at 37°C and 5% CO2. Neutrophils cells were gently rinsed twice with RPMI media (Gibco) supplemented with 5% BSA to wash away non-adherent cells and non-DNA-associated elastase. S7 Nuclease (30 U/mL) for 30 minutes at 37°C was used to release the DNA-associated elastase from neutrophils post-NETosis. The supernatant was collected by centrifuge at 300 x g for 5 minutes to pellet any cellular debris following EDTA (500 mM) treatment to inactivate the nuclease. Elastase activity assay was performed for 60 minutes at 37°C by using 100 µL of culture supernatant in the presence of 100 µL NET Assay Neutrophil Elastase Substrate per microtiter well as per manufacturer's guidelines. Absorbance was read at 405 nm in a microplate reader (Tecan, Männedorf, Switzerland). The human neutrophil elastase concentration was calculated using the equation of the line by performing a dilution series range

of neutrophil elastase at 36, 18, 9, 4.5, 2.25, 1.125, 0.56, and 0 mU/mL provided by the manufacturer.

References

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Figure S1

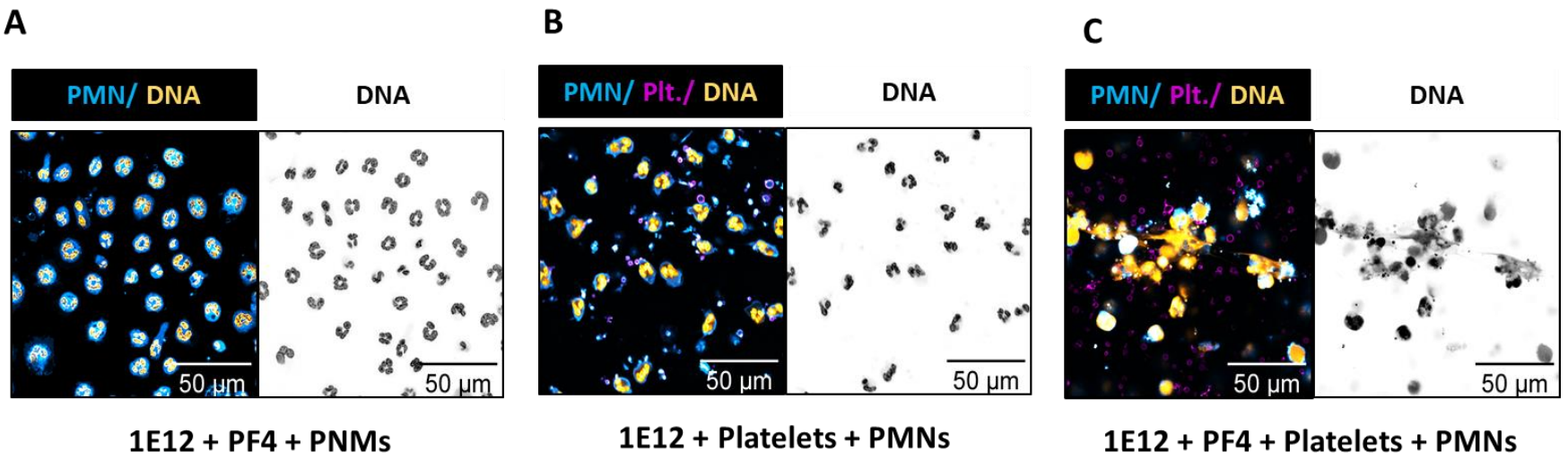
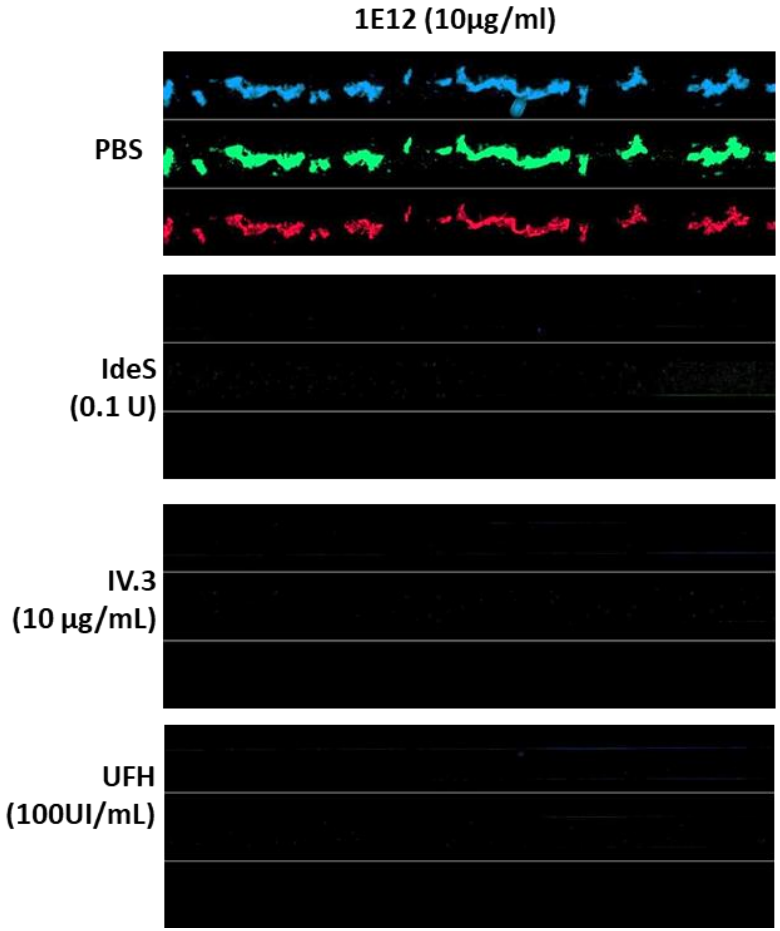


Figure S1: 1E12 induces NETosis when neutrophils are co-incubated with platelets and PF4.

Confocal laser scanning microscopy images of *in vitro* NETosis following neutrophils stimulation by 1E12 10 µg/mL, in the presence or in the absence of platelets and human PF4 (10 µg/mL). Nuclear and extracellular DNA are shown in orange, platelets in purple, and PMN in blue.

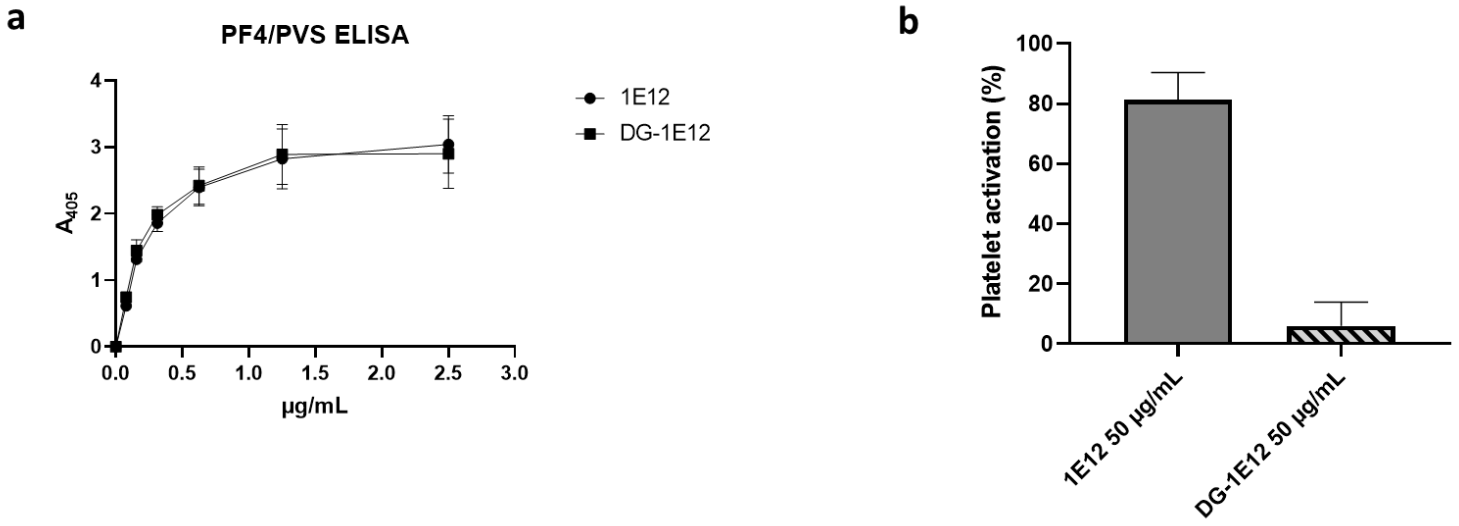
Figure S2



IdeS, IV.3 and heparin strongly inhibit *in vitro* thrombus formation induced by 1E12

In vitro thrombus formation in recalcified whole blood stimulated for 10 minutes with 1E12 (10 µg/mL), with or without pre-incubation for 10 minutes with the IgG degrading protease IdeS (0.1 U), the monoclonal anti-FcγRIIa antibody IV.3 (10 µg/mL), or unfractionated heparin (100 IU/mL). Fluorescence microscopy images represent areas of 0.1 mm2 vWF-coated microfluidic channels. Platelet, fibrin and leukocytes were visualized using DiOC6, AlexaFluor 647–labeled fibrinogen and Hoechst 33342, respectively. Images were acquired after 8 minutes of perfusion using an Axio Observer Z1 microscope (Zeiss) and an EC Plan-Neofluar 203/0.5 Ph2 M27 objective equipped

Figure S3



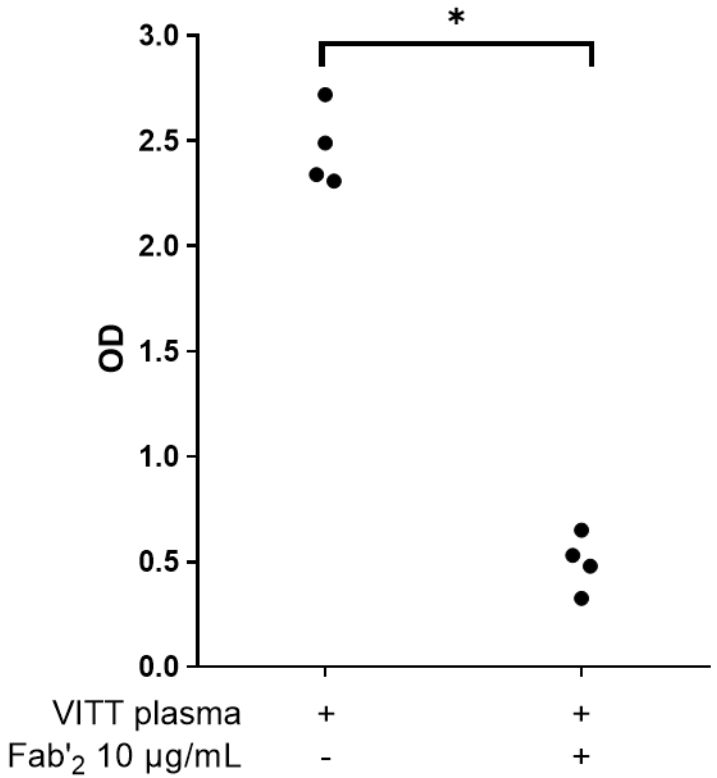
Deglycosylated 1E12 still binds to PF4 but no longer activates platelets

The deglycosylated form of 1E12 (DG-1E12) was obtained after incubation overnight with 40 U of N-Glycosidase F (Sigma-Aldrich). Then, the enzyme was removed using a Vivaspin 50 kDa column (Sartorius).

A) Binding of increasing concentrations of 1E12 and DG-1E12 to PF4 complexed with polyvinylsulfonate (PF4/PVS) in ELISA (Immucor). Data are mean optical density (OD) values ± SEM (n= 3 experiments).

B) Platelet activation induced by 1E12 and DG-1E12 (50 µg/mL) tested with the PF4-SRA as previously described {Vayne, 2017 #6}, without heparin. Data are mean percentage (%) ± SEM (n= 3 experiments).

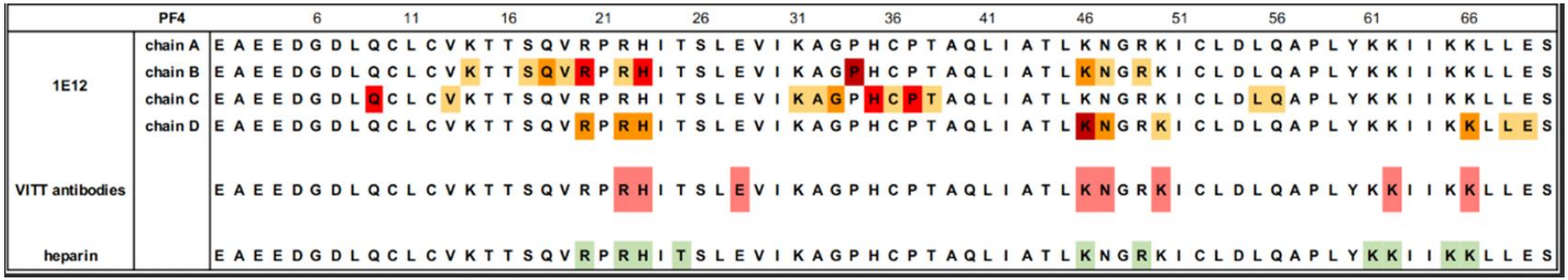
Figure S4



Effects of Fab'2 fragment of 1E12 (10 µg/mL) prebound VITT (n=4) antibody binding to PF4/PVS evaluated by competitive enzyme immunoassays.

Diluted plasmas (1/50) was first incubated 10 minutes at room temperature (RT) in PF4/PVS coated wells. Then, the Fab'2 fragment of 1E12 (10 µg/mL) were added and incubated for 20 min at RT. After washing, immunoassays were performed in accordance to the manufacturer instruction (HAT IgG, Immucor). Absorbances were measured at 450 nm.

Figure S5



1E12 and human VITT share similar epitopes on PF4

Amino acids involved in the binding of 1E2 to each of the four PF4 monomers (A, B, C and D) had been predicted using a docking model[1]. Residues are colored according to their probability of contributing to the epitopes, as given by MAbTope: dark red (very high probability), red (high probability), orange (medium probability), and yellow (low probability).

Amino acids critical for the binding of human VITT antibodies to PF4 were defined by Huynh et al using alanine scanning mutagenesis[2]; those involved in the interaction of heparin with PF4 were determined by Mayo et al using nuclear magnetic resonance spectroscopy and site-directed mutagenesis[3].

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