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

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Abstract

In order to improve the safety of COVID-19 vaccines, there is an urgent need to unravel the pathogenesis of vaccine-induced immune thrombotic thrombocytopenia (VITT), a severe complication of recombinant adenoviral vector vaccines used to prevent COVID-19, and likely due to anti-platelet factor 4 (PF4) IgG antibodies. In this study, we demonstrated that 1E12, a chimeric anti-PF4 antibody with a human Fc fragment, fully mimics the effects of human VITT antibodies, as it activates platelets to a similar level in the presence of platelet factor 4 (PF4). Incubated with neutrophils, platelets and PF4, 1E12 also strongly induces NETosis, and in a microfluidic model of whole blood thrombosis, it triggers the formation of large platelet/leukocyte thrombi containing fibrin(ogen). In addition, a deglycosylated form of 1E12 (DG-1E12), which still binds PF4 but no longer interacts with Fcγ receptors, inhibits platelet, granulocyte and clotting activation induced by human anti-PF4 VITT antibodies. This strongly supports that 1E12 and VITT antibodies recognize overlapping epitopes on PF4. In conclusion, 1E12 is a potentially important tool to study the pathophysiology of VITT, and for establishing mouse models. On the other hand, DG-1E12 may help the development of a new drug that specifically neutralizes the pathogenic effect of autoimmune anti-PF4 antibodies, such as those associated with VITT.

Introduction

Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a rare but very serious complication of recombinant adenoviral vector vaccines used to prevent COVID-19 induced by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). VITT is associated with a high mortality rate, approaching 20-50% of affected patients,[1-3] and due to multiple and atypical thrombotic events, particularly cerebral venous sinus thrombosis (CVST) and splanchnic thrombosis (SVT), sometimes accompanied by disseminated intravascular coagulation.[1,3-8] Highly reactive anti-platelet factor 4 (PF4) immunoglobulin G (IgG) antibodies were detected in almost all reported patients, although they had not received heparin and likely play a central role in the pathogenesis of VITT.[9,10] These antibodies were found to activate platelets in the absence of heparin, by interacting with FcγRIIA receptors via their Fc part.[1,6] Although their characteristics strikingly resemble those of antibodies in "autoimmune" HIT,[11,12] they remain to be further investigated, especially to better understand the predisposition of VITT patients to cerebral and splanchnic vein thrombosis.

Adenoviral vector-based vaccines are effective tools and were first used to fight the Ebola pandemic in Africa, and, unlike mRNA-based vaccines, they are currently affordable in many countries around the world.[13,14] VITT emerged after vaccination with ChadOx1 nCoV19 (AstraZeneca) and Ad26.COV2-S (Janssen, Johnson & Johnson), and other adenoviral vector-based vaccines might therefore trigger similar adverse events in the future. Thus, understanding the mechanisms of VITT has major implications beyond the current pandemic.

On the other hand, VITT is an exceptional side effect of

COVID-19 vaccination, and patient samples are rare and limited to a few milliliters. This severely limits mechanistic studies or the provision of positive controls for laboratory assays. All these reasons explain the urgent need to provide the scientific community with a monoclonal antibody to study the pathophysiology of VITT and to standardize *in vitro* diagnostic tests.

In this regard, we have recently developed a chimeric IgG1 anti-PF4 antibody named 1E12 with a human Fc fragment, which exhibits strong similarities to autoimmune HIT antibodies in terms of cellular effects.[15] Here we show that this antibody mimics VITT antibodies by comparing cellular activation of platelets and granulocytes induced by 1E12 and human VITT antibodies. Then, we evaluated the capability of deglycosylated 1E12, an inactivated form of the antibody, which is unable to bind FcγR, to inhibit cellular activation induced by VITT antibodies.

Methods

Material and antibodies

The chimeric anti-PF4 monoclonal IgG1, 1E12, has been co-developed with B-cell design (Limoges) as previously described.[15,16] The deglycosylated forms of 1E12 (DG-1E12) and cetuximab (Merck) tested as control antibody (DG-Ctrl Ab), were obtained after incubation overnight of each antibody (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).

VITT samples (plasma or serum) and HIT plasma samples were obtained from patients diagnosed in Tours or Greifswald. The study of serum and plasma from patients was approved by the local Ethics Board and conducted in accordance with the Declaration of Helsinki.

PF4-modified serotonin release assay

PF4-modified serotonin release assay (PF4-SRA) was performed as previously described.[6] Briefly, ^{14}C-serotonin labeled platelets were incubated with PF4 (10 µg/mL), plasma samples or 1E12 for 1 hour at room temperature. Then, the radioactivity was measured in supernatants. For competitive assays, washed platelets and PF4 (10 µg/mL) were pre-incubated for 10 minutes (min) with DG-1E12 or DG-Ctrl Ab before stimulation with either HIT or VITT plasma samples. For more details, see the *Online Supplementary Appendix*.

Flow cytometry analysis

The flow cytometric assay was performed as a modified version of the PF4-induced flow cytometry-based platelet activation test (PIFPA), which was recently described.[17] For more details, see the *Online Supplementary Appendix*.

Enzyme-linked immunosorbant competition assay

The ability of 1E12 Fab'$_2$ fragment to inhibit binding of VITT or HIT Ab to modified PF4 was performed by using PF4 IgG assay (Immucor). For more details, see the *Online Supplementary Appendix*.

Microfluidic whole blood thrombosis model

Whole blood (WB) collected on 0.129 M sodium citrate from healthy donors was incubated with VITT plasma, 1E12 or ALB6 Ab (Beckman Coulter). Then, blood samples were recalcified to 5 mM CaCl$_2$ and perfused at a shear rate of 20 µL/min (500 s^{-1}) in microfluidic channels (Vena8 Fluo1, Cellix) precoated overnight at 4°C with 160 µg/mL purified human von Willebrand factor (LFB). For competitive assays, WB was pre-incubated for 10 min with DG-1E12 or DG-Ctrl Ab before adding VITT plasma samples or ALB6. For more details, see the *Online Supplementary Appendix*.

Effect of antibodies on NETosis

The formation of neutrophilic extracellular traps (NET) ("NETosis") induced by VITT sera or 1E12 (10 µg/mL) was measured in a microplate assay in the presence of purified neutrophils, platelets and PF4 (10 µg/mL). In addition, the inhibitory effect of DG-1E12 and DG-Ctrl Ab (100 µg/mL) on NETosis was evaluated. For more details, see the *Online Supplementary Appendix*.

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 8.0.1 software. Mann-Whitney *U* test and Wilcoxon signed-rank test were performed to compare data obtained after DG-1E12 or DG-Ctlr Ab treatment under different conditions. *P*<0.05 was considered statistically significant.

Results

1E12 mimics vaccine-induced immune thrombotic thrombocytopenia antibody-induced platelet activation

Using a PF4-SRA, we demonstrate that 1E12 strongly activates platelets, with a pattern very similar to that of human VITT plasma samples, i.e., in a PF4-dependent manner and without the addition of heparin (Figure 1). Moreover, platelet activation induced by 1E12 (5 and 10 µg/mL) was inhibited by a low heparin concentration (0.5 IU/mL), an effect we also observed with several VITT samples. Moreover, platelet activation induced by 1E12 was fully inhibited by IV.3, a monoclonal antibody blocking FcγRIIa receptors, and IdeS (IgG-degrading enzyme derived from *Streptococcus pyogenes*), a bacterial protease that cleaves the hinge region of IgG and thus suppresses the binding of pathogenic IgG antibodies to FcγRIIa receptors (Figure 1B). This inhibitory effect of IV.3 and IdeS,

A

B
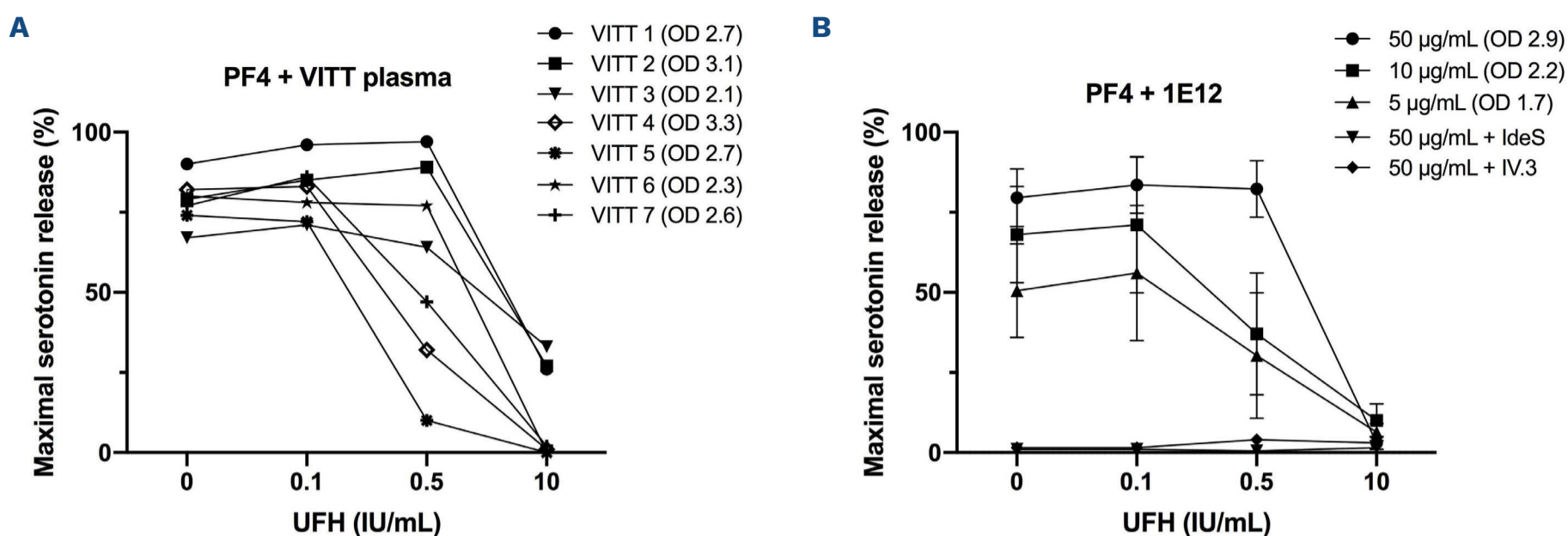
Figure 1. 1E12 mimics human vaccine-induced immune thrombotic thrombocytopenia antibodies to activate platelets. (A) Platelet activation induced by vaccine-induced immune thrombotic thrombocytopenia (VITT) plasma samples (n=7) and (B) 1E12 (5, 10, 50 µg/mL) in serotonin release assay performed in the presence of exogenous human PF4 (10 µg/mL) and increasing concentrations of heparin. The effects of the immunoglobulin G (IgG) cleaving protease IdeS and the monoclonal FcγRIIa blocking antibody IV.3 (10 µg/mL) on platelet activation induced by 1E12 (50 µg/mL) are also presented in (B). Data are the percentage of platelet activation, with mean (+/- standard error of the mean) of 4 independent experiments for (B). Optical density (OD) values measured in enzyme-linked immunsorbant assay (Immucor) and reflecting the levels of IgG antibodies to PF4 in all samples tested are indicated in brackets.

which we previously demonstrated with VITT samples,[6] strongly supports that interaction between pathogenic anti-PF4 IgG and FcγRIIa plays a central role in the pathogenesis of VITT.
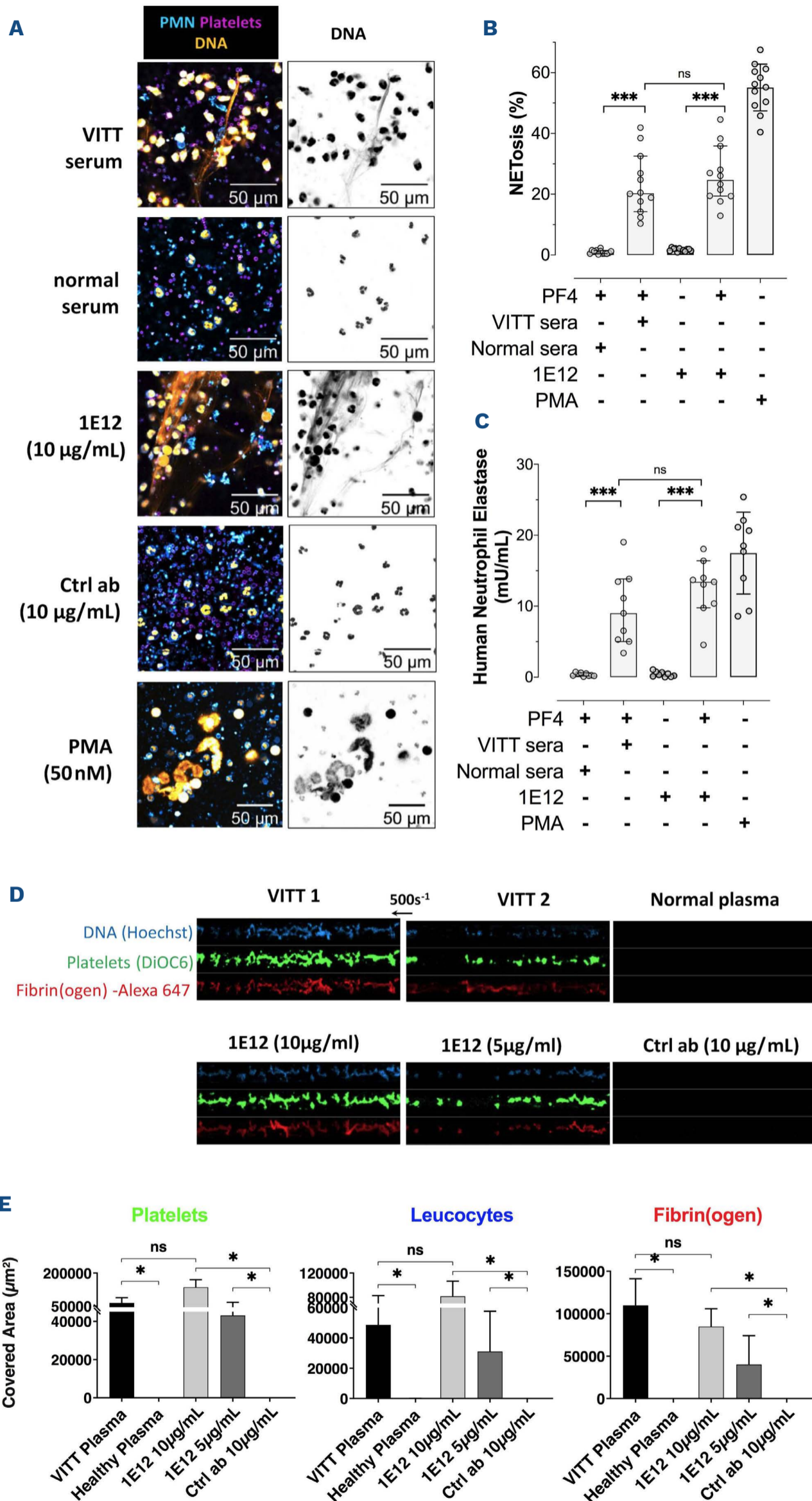
1E12 mimics vaccine-induced immune thrombotic thrombocytopenia antibody-induced prothrombotic cellular effects

The thrombotic complications of VITT probably result from complex multicellular activation processes involving platelets and neutrophils. It has been recently shown that granulocytes produce large amounts of procoagulant NET in response to VITT antibodies.[18,19] Similarly, 1E12, incubated with granulocytes and isolated platelets in the presence of PF4 (10 µg/mL), induced NETosis with the release of elastase associated with DNA, at a level similar to that measured with VITT samples (Figure 2A to C). In addition, the presence of platelets was required for 1E12-induced NETosis (*Online Supplementary Figure S1*). When whole blood from healthy donors was incubated with 1E12 or VITT plasma and perfused it into capillaries coated with von Willebrand factor under shear stress (500 s⁻¹) mimicking venous conditions, numerous large fibrin(ogen)-containing platelet/leukocyte aggregates were observed (Figure 2D and E). As expected, thrombus formation induced by 1E12 was completely prevented by the addition of IdeS (0.1 U), IV.3 (10 µg/mL), or high concentrations of unfractionated heparin (UFH, 100 IU/mL) (*Online Supplementary Figure S2*). This *in vitro* thrombus formation did not require exogenous UFH or PF4, in contrast to thrombus induction by HIT antibodies.[20,21] These results strongly support that the specificity and affinity of VITT antibodies

are different from those of HIT antibodies since they recognize PF4 alone and activate cells without heparin.

Deglycosylated 1E12 inhibits vaccine-induced immune thrombotic thrombocytopenia antibody-induced platelet activation

Based on the assumption that 1E12 and VITT antibodies recognize overlapping epitopes on PF4, we performed competitive assays between VITT antibodies and an inactivated of 1E12 (DG-1E12) obtained after N-deglycosylation of its Fc fragment. We first confirmed that DG-1E12 was fully able to bind PF4 but no longer to activate platelets (*Online Supplementary Figure S3*) since the removal of the Fc glycan abolishes IgG binding to FcγR.[22] By incubating washed platelets with DG-1E12 (50 µg/mL), platelet activation induced by VITT antibodies we strongly reduced in PF4-SRA, and fully abrogated with nine of the 13 VITT samples tested (maximal serotonin release < 20%; Figure 3A). In contrast, DG-1E12 could only partially inhibit HIT antibody-induced platelet activation, with an effect less pronounced than those obtained with VITT plasma samples, since the serotonin release remained higher than 20% with all HIT plasma samples tested. In addition, the same inhibitory effect of DG-1E12 on VITT antibodies was demonstrated in whole blood using the recently described PF4-enhanced flow cytometry assay[17] (Figure 3B). As expected, no inhibitory effect of deglycosylated control antibody (DG-Ctrl Ab) on VITT or HIT antibody-induced platelet activation could be evidenced (Figure 2A and B). We then investigated whether the competitive effect of DG-1E12 was dependent on its Fab part, by inhibiting the binding of VITT antibodies to PF4. This hypothesis is likely since pre-incubation of the Fab'₂ frag-

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ment of 1E12 almost fully suppressed the binding of VITT antibodies to PF4/PVS (median A_{450} 2.5 *vs*. 0.22). In contrast, it did not significantly modify the results obtained with all HIT samples tested (median A_{450} 1.9 *vs*. 1.5) (Figure 3C). In addition, the Fab'$_2$ fragment of 1E12 efficiently displaced all VITT IgG antibodies pre-bound to PF4/PVS complexes (*Online Supplementary Figure S4*).

Deglycosylated 1E12 inhibits vaccine-induced immune thrombotic thrombocytopenia antibody-induced prothrombotic cellular effects

When platelets and neutrophils were pre-incubated with DG-1E12, NETosis induced by VITT antibodies was completely inhibited (NETosis 3% *vs*. 23%, with or without DG-1E12, respectively) as well as the release of DNA-associated elastase (activity level 0.7 *vs*. 9 mU/mL) (Figure 4A to C). Using similar experimental conditions, DG-1E12 did not inhibit NETosis induced by ALB6, a murine monoclonal anti-CD9 antibody that also strongly activates platelets in an FcγRIIa-dependent manner (NETosis 15% *vs*. 14% and elastase activity 6.4 *vs*. 4 mU/mL, with or without DG-1E12, respectively) (Figure 4A to C). These data strongly suggested that DG-1E12 could prevent multicellular activation induced by VITT antibodies. This concept was reinforced by demonstrating that DG-1E12 fully abrogated VITT antibody-mediated thrombus formation in whole blood *in vitro* under vein flow conditions (Figure 4D and E). As expected and in accordance with the data obtained in NETosis experiments, DG-1E12 did not modify the size of platelet/leukocyte thrombi containing fibrin(ogen) induced by anti-CD9 antibodies. In addition, when the deglycosylated form of the irrelevant antibody (DG-Ctlr Ab) was tested in place of DG-1E12, no inhibitory effect on NETosis and thrombus formation induced by VITT antibody was observed (Figure 4B, C and E).

Discussion

In this study, we demonstrated that 1E12, a chimeric anti-PF4 antibody with a human Fc fragment, fully mimics the ef-

fects of human VITT antibodies, as it activates platelets to a similar level in the presence of PF4. Incubated with neutrophils, platelets, and PF4, 1E12 also strongly induces NETosis, and in a microfluidic model of whole blood thrombosis, it triggers the formation of large platelet/leukocyte thrombi containing fibrin(ogen). In addition, a deglycosylated form of 1E12 (DG-1E12), which still binds PF4 but no longer interacts with Fcγ receptors, inhibits platelet, granulocyte and clotting activation induced by human anti-PF4 VITT antibodies.

Using an alanine-scanning mutagenesis strategy, Huyhn *et al*. recently showed that amino acid residues involved in the epitopes recognized by VITT antibodies on PF4 are different from those required for HIT antibody binding to PF4/heparin complexes.[23] In addition, it was recently demonstrated that anti-PF4 antibodies in VITT are oligoclonal while those developed in HIT are polyclonal.[24] Our results obtained with an enzyme-linked ummunsorbant competitive assay strengthen this restricted specificity of VITT antibodies since the Fab'2 part of 1E12 strongly inhibits the binding of VITT IgG to PF4 without any significant effect on HIT antibody/PF4 interaction. Importantly, VITT antibody binding to PF4 was shown to be restricted to eight amino acids (R22, H23, E28, K46, N47, K50, K62, and K66), all of which are located in the heparin-binding site of PF4.[23] In this regard, we also showed by using a predictive docking model that six of these eight residues likely participate in the binding of 1E12 to PF4 (*Online Supplementary Figure S5*).[15] These results likely explain why we could inhibit binding of VITT antibodies or 1E12 to PF4, as well as cellular activation, by adding relatively low concentrations of heparin. In contrast, when low concentrations of heparin were added to HIT plasma samples or incubated with 5B9, a chimeric monoclonal IgG1 anti-PF4/H antibody mimicking human antibodies, HIT antibody binding and related cell activation were always enhanced.[16]

We had also previously provided evidence that 1E12 likely exhibits a much higher affinity for PF4 and PF4/H complexes than the HIT monoclonal antibody 5B9.[15] This also parallels the characteristics of VITT antibodies recently obtained by Huyhn *et al*., who showed that VITT antibodies bind stronger to PF4 and PF4/H complexes than HIT anti-
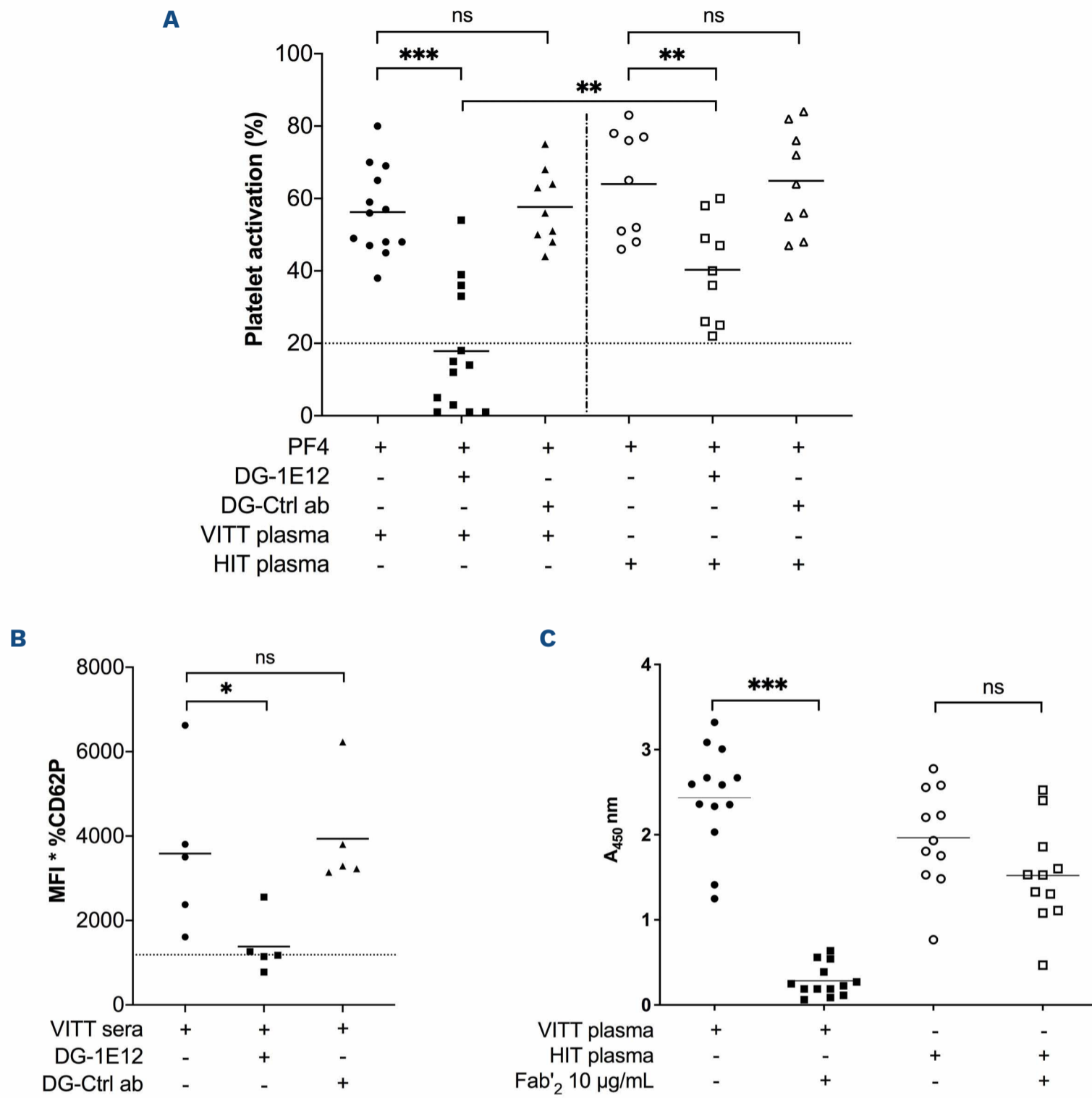
Figure 3. Deglycosylated 1E12 inhibits platelet activation induced by human vaccine-induced immune thrombotic thrombocytopenia antibodies. (A) Platelet activation induced by vaccine-induced immune thrombotic thrombocytopenia (VITT) (n=13) and HIT plasma samples (n=9) in PF4-modified serotonin release assay (PF4-SRA) with or without DG-1E12 or DG-Ctrl antibody (ab) (50 μg/mL). The maximal serotonin release values that were measured are expressed in percentages of (%), with means (horizontal lines). Experiments using DG-Ctrl ab (deglycosylated form of control antibody) was performed with all available VITT plasma samples (n=9) and HIT plasmas (n=9). All SRA experiments were performed in the presence of human PF4 (10 μg/mL). Platelet activation induced by VITT samples (n=5) added in whole blood with or without DG-1E12 or DG-Ctrl antibody (100 μg/mL), and measured by flow cytometry (B). CD62P expression is shown as the mean fluorescence intensity (MFI) of the gated events multiplied by the percentage of gated events. Each data point represents the averaged result from 3 different tests with 1 sample. Horizontal lines indicate the mean values obtained with the five VITT samples. Effects of Fab'2 fragment of 1E12 (10 μg/mL) on VITT (n=13) or HIT (n=11) antibody binding to PF4/PVS evaluated by competitive enzyme immunoassays (C).

bodies.[23] Under our conditions, VITT antibody-induced thrombus formation in flow did not require the addition of exogenous PF4, suggesting that a sufficient amount of this chemokine was released and present on cell surfaces. However, VITT is a thromboinflammatory syndrome with high PF4 plasma levels, and our study model probably does not entirely replicate the complex pathophysiological conditions present in most VITT patients.

Our data also support that the beneficial impact of DG-1E12 in preventing blood cell activation by VITT antibodies and thrombus formation likely depends on the competitive effect of its Fab part on antibody binding to PF4, without inhibiting FcγRIIa-mediated signaling. Hence, our results confirm that 1E12 has features very similar to those of human VITT regarding their specificity and cellular effects.
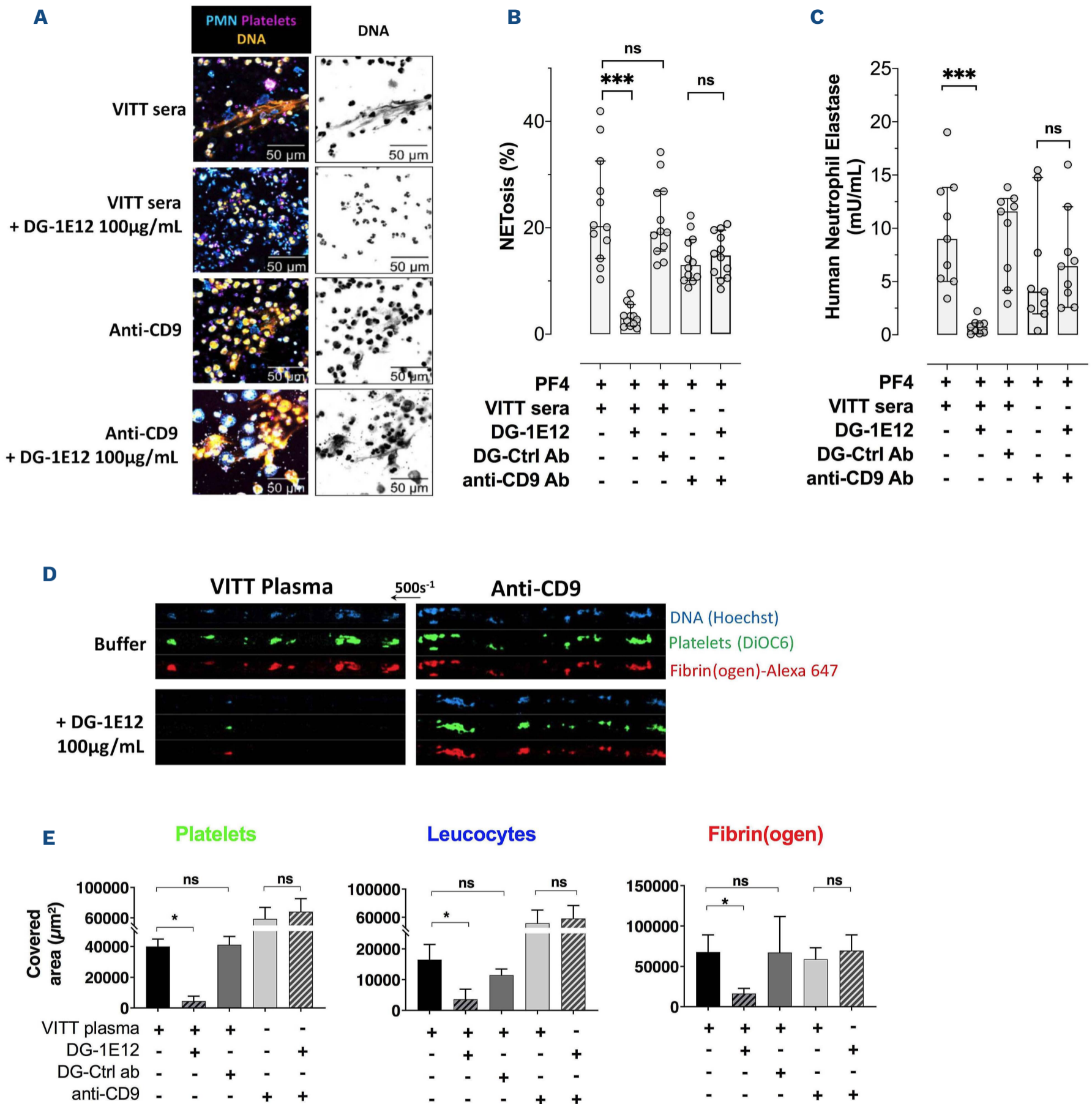
Figure 4. Deglycosylated 1E12 inhibits NETosis and thrombus formation induced by human vaccine-induced immune thrombotic thrombocytopenia antibodies. (A) Evaluation of *in vitro* NETosis by confocal laser scanning microscopy following neutrophils stimulation by vaccine-induced immune thrombotic thrombocytopenia (VITT) serum or anti-CD9 antibody (20 μg/mL), with or without DG-1E12 (100 μg/mL). Nuclear and extracellular DNA is shown in orange, platelets in purple, and polymorphonuclear neutrophils (PMN) in blue. (B and C) Quantification of NETosis by analysing 12 individual microscopy images (B) and by measuring levels of DNA-associated elastase activity in the supernatant after neutrophils stimulation (C). Four independent experiments were performed for each experimental condition. (D and E) Thrombus formation in von Willebrand factor (vWF)-coated microfluidic channels perfused with recalcified whole blood incubated for 10 minutes with VITT plasma or ALB6 (20 μg/mL), with or without DG-1E12 (100 μg/mL). Images corresponding to areas of 0.1 mm^2 are shown in (D) with platelets in green (DiOC6), fibrin(ogen) in red (Alexa Fluor 647–labeled fibrinogen), and leukocytes in blue (Hoechst 33342, DNA dye). The mean areas covered (E) by platelets (left), leukocytes (middle) or fibrin(ogen) (right) were calculated for each condition (n=5 independent experiments with 5 VITT plasmas), by measuring using ImageJ software the surface covered by large aggregates (>100 μm^2) in 20 different areas. Deglycosylated form of cetuximab was used as control antibody (DG-Ctrl ab). Wilcoxon signed-rank test was performed to compare the different conditions tested. *P<0.05, **P< 0.01, ***P<0.001.

The therapeutic management of VITT patients is currently based on therapeutic dose anticoagulation with non-heparin anticoagulants, combined with high dose intravenous immunoglobulins (IVIG), which inhibit cell activation likely by competing with VITT antibodies.[25] Apart from being expensive and limited, IVIG is also problematic for managing severe CVST with intracranial hypertension as it may induce intracranial pressure, and the efficacy of this therapy may be transient in some VITT patients with persistently high levels of activating anti-PF4 IgG antibodies.[26] In this context, DG-1E12 combined with a non-heparin antithrombotic drug could be a new therapeutic approach for efficiently and safely treating the more severe cases of VITT. Interestingly, a similar strategy based on the use of an epitope-specific deglycosylated antibody was first successfully evaluated in a mouse model of fetal/neonatal alloimmune thrombocytopenia (FNAIT),[27] and then more recently in HIT.[28] In addition, we recently demonstrated that DG-1E12 completely inhibited cellular activation induced by a spontaneously synthesized anti-PF4 IgG antibody from a patient with monoclonal gammopathy whose paraprotein showed VITT-antibody-like activity and caused recurrend venous and arterial thrombosis.[29]

Recent guidelines clearly recommended performing functional assays for the diagnosis of VITT.[30] SRA and HIPA must be done in the presence of exogenous PF4,[1,6] but the diagnosis of VITT remains a challenge for many laboratories. In this context, the monoclonal antibody 1E12 could be useful as a positive control and external quality control to standardize the different functional assays usable for VITT diagnosis.

In conclusion, we demonstrate that 1E12 and VITT antibodies exhibit a similar capacity to activate platelets and neutrophils and to induce thrombus formation. Therefore, 1E12 is likely an excellent model antibody to further study the pathophysiology of VITT and in diagnostic assays. Our data also support that DG-1E12 may allow the development of a new drug neutralizing the pathogenic effect of autoimmune anti-PF4 antibodies, such as those associated with VITT.

Disclosures

Contributions

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Data-sharing statement

Data may be requested for academic collaboration from the corresponding author.

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