Arteriosclerosis, Thrombosis, and Vascular Biology

TRANSLATIONAL SCIENCES

PAD4-Induced NETosis Via Cathepsin G–Mediated Platelet-Neutrophil Interaction in ChAdOx1 Vaccine-Induced Thrombosis—Brief Report

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BACKGROUND: Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a rare syndrome characterized by platelet anti-PF4 (platelet-activating antiplatelet factor 4)–related thrombosis. Platelet-neutrophil interaction has been suggested to play a role, but the underlying mechanism has not been fully elucidated.

METHODS: The study included 10 patients with VITT after ChAdOx1 (chimpanzee adenovirus Oxford 1) nCoV-19 (Oxford-AstraZeneca) vaccine administration, 10 patients with ischemic stroke (IS), 10 patients with acute deep vein thrombosis, and 10 control subjects in whom blood levels of neutrophil extracellular traps (NETs), soluble TF (tissue factor), and thrombin generation were examined. Furthermore, we performed in vitro studies comparing the effect of serum from patients and controls on NETs formation. Finally, immunohistochemistry was performed in cerebral thrombi retrieved from a patients with VITT and 3 patients with IS.

RESULTS: Compared with patients with IS, patients with deep vein thrombosis, controls, and patients with VITT had significantly higher blood values of CitH3 (citrullinated histone H3), soluble TF, D-dimer, and prothrombin fragment 1+2 ($P<0.0001$). Blood CitH3 significantly correlated with blood soluble TF (Spearman rank correlation coefficient=0.7295; $P=0.0206$) and prothrombin fragment 1+2 (Spearman rank correlation coefficient=0.6809; $P<0.0350$) in patients with VITT. Platelet-neutrophil mixture added with VITT plasma resulted in higher NETs formation, soluble TF and thrombin generation, and platelet-dependent thrombus growth under laminar flow compared with IS and deep vein thrombosis plasma; these effects were blunted by PAD4 (protein arginine deiminase 4) and cathepsin G inhibitors, anti-FcγRIIa (Fc receptor for IgG class IIa), and high doses of heparin. Immunohistochemistry analysis showed a more marked expression of PAD4 along with more diffuse neutrophil infiltration and NETs formation as well as TF and cathepsin expression in VITT thrombus compared with thrombi from patients with IS.

CONCLUSIONS: Patients with VITT display enhanced thrombogenesis by PAD4-mediated NETs formation via cathepsin G–mediated platelet/neutrophil interaction.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: extracellular traps ■ ischemic stroke ■ neutrophil ■ thrombocytopenia ■ thrombosis

Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a rare syndrome observed between 4 and 30 days after receiving the Vaxzevria (Astra Zeneca) vaccine and in few cases after Ad26.COV2. S (Johnson & Johnson) and caused by PF4 (platelet-activating antiplatelet factor 4) IgG antibodies.[1,2] Clinical and laboratory read-outs of VITT are characterized by thrombosis in unusual sites such as cerebral venous sinus or portal vein circulation and coexistence with thrombocytopenia and high D-dimer.[3] The mechanism accounting for VITT has not been

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Nonstandard Abbreviations and Acronyms

CitH3	citrullinated histone H3
DVT	deep venous thrombosis
F1+2	prothrombin fragment 1+2
IS	ischemic stroke
MPO	myeloperoxidase
NE	neutrophil elastase
NETs	neutrophil extracellular traps
PAD4	protein arginine deiminase 4
PAR4	protease-activated receptor 4
PF4	platelet-activating antiplatelet factor 4
PRP	platelet-rich plasma
TF	tissue factor
VITT	vaccine-induced immune thrombotic thrombocytopenia

fully understood. A recent immunohistochemistry analysis of cerebral sinus sagittal superior thrombus retrieved from a patient with VITT revealed extensive formation of neutrophil extracellular traps (NETs), suggesting a major role for inflammatory cells in the thrombosis by VITT.[4] Thus, NETs comprise DNA and histones and are released on activation of PAD4 (protein arginine deiminase 4).[5]

Experimental and clinical studies suggested a key role for NETs in the thrombotic process via activation of platelets, TF (tissue factor)-dependent clotting activation, and endothelial perturbation.[6] Greinacher et al[7] showed that isolated human neutrophils and platelets incubated with serum from patients with VITT, but not healthy control serum, triggered neutrophil activation and NET formation that was significantly increased by the addition of PF4. Also, Leung et al[8] recently demonstrated that total IgG from plasma of patients with VITT induced NETosis and thrombosis and that the anti-PF4 IgG activated platelets and neutrophils. Moreover, Leung et al[8] demonstrated that murine VITT models (*FcγRIIa+/hPF4+*) deficient in PAD4 (PAD4$^{-/-}$), a key enzyme for NETs formation,[9] treated with VITT IgG, had a dramatic reduction in clot formation compared with control VITT mice; these results were paralleled by a significant reduction of thrombosis compared with control animals in animals treated with VITT IgG plus GSK484, an inhibitor of NETosis. Taken together these studies would suggest a potential interplay between platelets and neutrophils in the formation of NETs, but the underlying mechanism is still to be clarified. Also, experiments conducted in PAD4 knockout suggested a role for this enzyme in eliciting NETosis in VITT mice, but further data are necessary to support the role of PAD4 in human thrombosis.

Previous study performed in human thrombi and in vitro demonstrated a role for cathepsin G, as mediator of platelet-neutrophil interaction, in the formation of platelet-neutrophil aggregates and thrombus growth.[10] Cathepsin G is a single-chain glycoprotein of about 28.5 kDa, characterized by an N-glycosylation site and 3 disulfides. It is a serine protease stored in the azurophilic granules of neutrophils released after activation of the cells and participates in the intracellular degradation of pathogens. It has been demonstrated that neutrophil-derived cathepsin G activates platelets via PAR4 (protease-activated receptor 4),[11] that in turn interacts with both cathepsin G and thrombin with high specificity to complete platelet activation, including shape change, glycoprotein IIb-IIIa activation, and secretion. Cathepsin G blockade reduced platelet aggregation induced by NETs[12] and formation of platelet-neutrophil aggregates.[10]

To explore the role of cathepsin G in the interplay between platelets and neutrophils, we performed in vitro experiments to evaluate its role in the formation of NETs using plasma from patients with VITT compared with plasma taken from 10 patients with ischemic stroke (IS), 10 patients with acute deep venous thrombosis (DVT), and 10 healthy subjects. Furthermore, we performed an immunohistochemistry analysis to measure NETosis, PAD4, and cathepsin G expression in cerebral thrombi retrieved from 3 patients with ischemic stroke (IS) and a patient with VITT.

Highlights

- Patients with vaccine-induced immune thrombotic thrombocytopenia display enhanced thrombogenesis.
- Immuno-mediated platelet/neutrophil interaction elicits neutrophil extracellular traps formation via PAD4 (protein arginine deiminase 4) activation.
- PAD4 activation elicits thrombus growth via tissue factor activation and thrombin generation.

METHODS

We compared 10 patients with VITT after ChAdOx1 (chimpanzee adenovirus Oxford 1) nCoV-19 (Oxford-AstraZeneca) vaccine administration with 10 patients with IS, 10 patients with acute DVT, and 10 healthy subjects. The diagnosis of VITT was based according to the American Society of Hematology.[2] The diagnosis of IS and DVT was done according to the current guidelines from the American Heart Association/American Stroke Association[13] and American Society of Hematology,[14] respectively. Clinical and demographic characteristics of patients and controls are reported in Table S1. At the time of enrollment, patients with IS, patients with DVT, and controls were not taking antiplatelet drugs, as per exclusion criteria. To improve study power, data from males and females were combined and to minimize the effect of demographic characteristics on the results, groups were balanced for age and sex (Table S1). Written informed consent was obtained from all participants according to Italian regulations, and the experimental procedure was approved by the Institutional Review Board at Sapienza University of Rome and was conducted in accordance with the Declaration of Helsinki.

Data Availability

The data that support the findings of this study are available from the corresponding author on reasonable request.

Platelet Preparation

Blood samples added with sodium citrated (3.8%, 1/10 [v:v]) or EDTA were taken from healthy subjects in fasting conditions. To obtain PRP (platelet-rich plasma), plasma samples added with sodium citrated were centrifuged for 15 minutes at 180*g* at room temperature, as previously described.[15]

Washed platelets were isolated from PRP treated with ACD (acid citrate dextrose; 10/1 v/v), centrifugated (10 minutes at 300*g* at room temperature), and resuspended in Tyrode's buffer 137 mmol/L NaCl, 2.7 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 20 mmol/L HEPES, 0.35% w/v bovine serum albumin and 5.6 mmol/L glucose, pH 7.35 (Sigma Aldrich, St. Louis, MO) at 2×10^5 platelets/µL. Prostaglandin E1 (1 µmol/L; Santa-Cruz Biotechnologies, Dallas, TX) was added to prevent platelet activation.

Neutrophil Isolation

Polymorphonuclear (PMNs) were isolated from fresh EDTA-blood taken from healthy volunteers by dextran enhanced sedimentation of red blood cells, Ficoll-Histopaque (Sigma Aldrich, St. Louis, MO) density centrifugation, lysis of remaining erythrocytes with distilled water and washing of cells with Hank's balanced salt solution (HBSS, Sigma Aldrich, St. Louis, MO) in the absence of any divalent cations.

Finally, the cell pellet was suspended in 1 mL of HBSS at the final concentration of 1×10^6 cells/mL and then treated with or without washed platelets at a concentration of 2×10^5 cells/µL in presence or <10% of plasma from patients with IS, patients with DVT, or patients with VITT.

We normalized the in vitro data by subtracting from the final concentration of each biomarker contained in 10% of added plasma. To assess if in our experimental set platelet-neutrophil mixture was equally responsive and not preactivated, we evaluated platelet aggregation and H_2O_2 (Abcam, Cambridge, United Kingdom) production by neutrophils in each subject before using the plasma as control. Before the incubation with plasma, neutrophils alone or neutrophils added with platelets were preincubated (20' at 37 °C) with GSK484 (10 µM; Abcam, Cambridge, United Kingdom), an inhibitor of PAD4, or high-dose heparin (100 IU/mL; Quimigen, Madrid, Spain) or FcγIIa receptor–blocking antibody IV.3, (10 µg/mL; Stemcell Technologies, Cambridge, United Kingdom), an inhibitor of FCγRIIa (Fc receptor for IgG class IIa) receptors, or CatGi (Cathepsin G inhibitor; 10 µM; Abcam, Cambridge, United Kingdom) an inhibitor of cathepsin G.

All inhibitors were dissolved and reached the final concentration in PBS. Therefore, as a negative control, the same percentage of inhibitors-diluent (1% PBS) was used.

Citrullinated Histone H3 Detection

Plasma and cell supernatant levels of CitH3 (citrullinated histone H3) were detected by ELISA Kit (Cayman Chemicals, Ann Arbor, MI). CitH3 values were expressed as ng/mL, and both intra-assay and interassay coefficients of variation were <10%.

Myeloperoxidase Detection

MPO (myeloperoxidase) concentration was detected in plasma and cell supernatant by ELISA kit (Boster Bio, Pleasanton, CA). The values of MPO were expressed as ng/mL, and both intra-assay and interassay coefficients of variation were <10%.

Neutrophil Elastase

NE (neutrophil elastase) was detected in plasma and cells supernatant by Commercial ELISA Kit (Abcam Cambridge, United Kingdom). Values were expressed as ng/mL, and both intra-assay and interassay coefficients of variation were <10%.

TF Production

TF concentration was detected in plasma and cell supernatants by a commercial ELISA kit (Elabscience, Houston, TX). Values were expressed as pg/mL; both intra-assay and interassay coefficients of variation were within 10%.

Prothrombin Fragment 1+2 Detection

Commercial ELISA Kit (Cusabio, Wuhan, China) was used for the quantitative determination of the prothrombin fragment 1+2 (F1+2) levels, a marker of thrombin generation, in plasma and supernatant of cells. F1+2 values were expressed as pmol/L, and both intra-assay and interassay coefficients of variation were <10%.

Western Blotting Analysis

To evaluate PAD4 expression, pellets from neutrophils alone or neutrophils added with platelets were lysed with RIPA (radioimmunoprecipitation) buffer in the presence of protease and phosphatase inhibitors cocktail (10 µg/mL; Thermo Fisher Scientific, Waltham, MA) and then centrifuged at 10 000*g* for 20 minutes to remove pellet residues. Afterward, the supernatants were collected, and the protein concentration was determined by Bradford assay.

Then, equal amounts of protein, 30 µg/lane, were solubilized in a 4X Laemmli buffer containing 2-mercaptoethanol and loaded in a denaturing sodium dodecyl sulfate (SDS) 10% to 12% polyacrylamide gel. Furthermore, membranes were incubated overnight at 4 °C with anti-PAD4 antibody (Abcam, Cambridge, United Kingdom) or with mouse monoclonal anti-β-actin (Santa-Cruz Biotechnologies, Dallas, TX). Thus, the membranes were incubated for 1 hour with horseradish peroxidase–conjugated secondary antibody (1:3000; Bio-Rad, Hercules, CA) and the immune complexes were detected by enhanced chemiluminescence substrate (ECL Substrates, Bio-Rad, Hercules, CA). Densitometry analysis of the bands was performed using Image J software and the value was expressed in arbitrary units.

Thrombus Formation

Thrombus growth under laminar flow conditions was measured by thrombus formation analysis system (T-TAS01 apparatus, Fujimori Kogyo Co, Ltd, Japan) on PL chips (26 collagen-coated microcapillaries). Whole blood sample (400 µL) anticoagulated by benzylsulfonyl-D-argininyl-prolyl-4-amidinobenzylamide was incubated with 10% plasma from controls, patients with IS, patients with DVT, or patients with VITT with or without GSK484 (10 µM; Abcam, Cambridge, United Kingdom), an

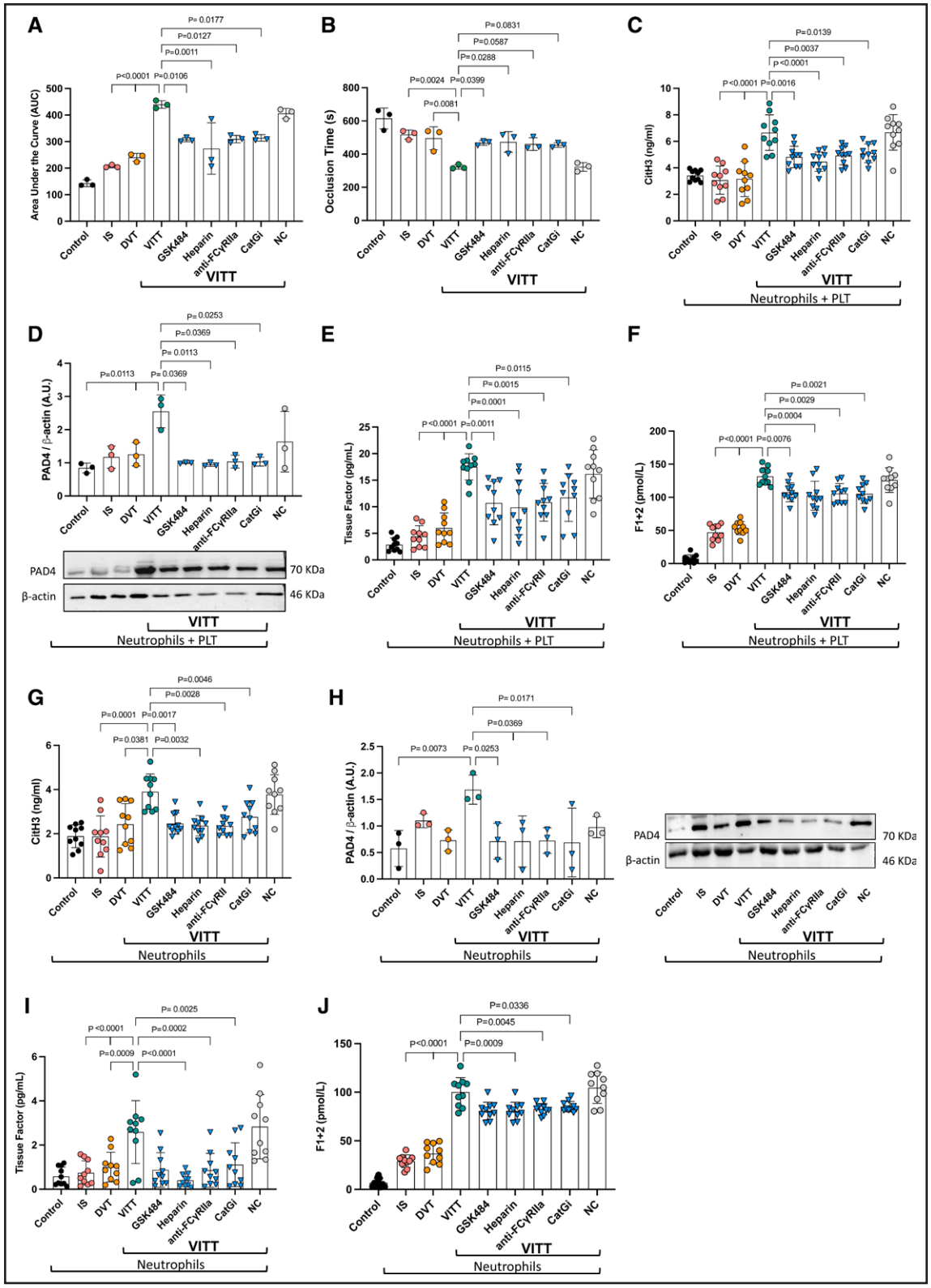

Figure 1. Effect of plasma from patients with ischemic stroke (IS), acute deep vein thrombosis (DVT), and vaccine-induced immune thrombotic thrombocytopenia (VITT) on platelet-dependent thrombus growth under laminar flow, neutrophil extracellular traps formation, and clotting activation.

Whole blood sample (400 µL) anticoagulated by benzylsulfonyl-D-argininyl-prolyl-4-amidinobenzylamide, neutrophils coincubated with platelets and neutrophils alone were incubated with 10% plasma from IS (pink symbols), DVT (orange symbols) or patients with VITT (green symbols) in the presence or not of GSK484 (10 µM; Abcam, Cambridge, United Kingdom), an inhibitor of PAD4 (protein arginine deiminase 4), or high-dose heparin (100 U/mL; Quimigen, Madrid, Spain) or anti-FCγRIIa (Fc receptor for IgG class IIa; 20 µg/ml; (*Continued*)

inhibitor of PAD4, or high-dose heparin (100 IU/mL; Quimigen, Madrid, Spain) or FcγIIa receptor–blocking antibody IV.3, (10 µg/mL; Stemcell Technologies, Cambridge, United Kingdom), an inhibitor of FCγRIIa receptors, or CatGi (10 µM; Abcam, Cambridge, United Kingdom), an inhibitor of cathepsin G, or the same percentage of inhibitor-diluent (1% PBS) as a negative control 20 minutes at 37 °C; then, 340 µL of samples were transferred to the PL-chip and analyzed. Growth, intensity, and stability of the formation of platelet clots were measured by the time needed to reach the occlusion pressure (occlusion time) and the area under the flow-pressure curve parameter, that is, an area under the pressure curve from the start of the test to a time of 10 minutes.[16]

Immunohistochemistry

We used a thrombus taken from sinus cerebral venous (VITT) and 3 arterial thrombi (IS). Immunohistochemistry was performed on sequential formalin-fixed paraffin-embedded thrombi sections of patients with VITT and control patients. The sections were deparaffinized and dehydrated through xylene and ethanol; antigen retrieval was performed in citrate buffer (pH 6.0) for 20 min in a microwave. Immunohistochemical staining was performed incubating antibodies directed against human PAD4 (Abcam, Cambridge, United Kingdom), CitH3 (rabbit polyclonal, 1:7500; Abcam, Cambridge, United Kingdom), TF (rabbit monoclonal, 1:400; Abcam, Cambridge, United Kingdom), and cathepsin G (rabbit polyclonal, 1:500; Novusbio Centennial, CO) for 1 hour at room temperature. Immunostaining was amplified using peroxidase-conjugated streptavidin complexes (Scytek, Logan, UT), and peroxidase was detected using DAB (Scytek, Logan, UT). Sections were lightly counterstained with hematoxylin, dehydrated, and mounted. The negative control was performed by omission of primary antibody.

For each antibody, the positive control was selected according to the instruction of the respective datasheet (PAD4–human lymphoma; CitH3H–human brain tissue; TF–human colon carcinoma; cathepsin G–human bone marrow tissue). The negative control was obtained by omitting the primary antibody and using immunohistochemistry universal negative control reagent (Figure S2).

The image analysis software FIJI/ImageJ was used to quantify the number of cells positive for PAD4 and cathepsin C and the areas positive for either CitH3 or TF, which were then expressed as a percentage of each field.

Statistical Analysis

Continuous variables were expressed as means±SD and categorical variables as number (n) and percentage (%).

To evaluate if variables have a normal distribution, a Shapiro-Wilk test was executed. *F* test of equality of variances was applied to compare sample variances.

Differences between percentages were analyzed by the χ^2 test. The analysis of differences between groups was obtained with *t* test for variables normally distributed and with nonparametric tests (Mann-Whitney *U* test) for those not-normally distributed. Comparison of the paired samples was obtained with the *t* test for paired data for variables normally distributed and with Wilcoxon matched-pairs signed-rank. test for those not-normally distributed.

Group comparisons were performed using 1-way ANOVA tests followed by Bonferroni post hoc test. The Spearman rank correlation coefficient was used for bivariate correlation.

Analyses were performed using GraphPad Prism 9 (GraphPad Software La Jolla, CA 92037). Only $P<0.05$ were regarded as statistically significant.

RESULTS

In patients with VITT, thrombosis occurred after an average of 9.20±5.07 days from the first dose of vaccine; all patients displayed positivity for anti-PF4 IgG antibodies and presented with concomitant thrombocytopenia (Table S1). Compared with patients with IS, patients with DVT, and controls, patients with VITT had significantly higher blood values of CitH3, MPO, and NE, all markers for NETs production in humans[17] (Figure S1A through S1C), soluble TF, D-dimer, and F1+2 (Table S1). Blood CitH3 significantly correlated with soluble TF (Spearman rank correlation coefficient=0.7295 P=0.0206) and F1+2 (Spearman rank correlation coefficient=0.6809; P=0.0350) in patients with VITT (Figure S1D and S1E).

In vitro, plasma from patients with VITT induced higher rate of thrombus growth compared with plasma from patients with IS and DVT and controls, as indicated by increased area under the flow-pressure curve and reduced occlusion time (Figure 1A and 1B). Thrombus growth by VITT plasma was reduced by GSK484, high-dose heparin, anti-FcγRIIa, or CatGi (Figure 1A and 1B). Also, incubation of normal platelet-neutrophil mixture with plasma from patients with VITT elicited a higher formation of CitH3 as compared with a mixture incubated with plasma from patients with IS and DVT and was associated with upregulation of PAD4 (Figure 1C and 1D); NETs overproduction was significantly inhibited in samples pretreated with GSK484 (Figure 1C and 1D). Analysis of clotting activation revealed that, compared with control plasma, plasma from VITT elicited higher TF activation as well as thrombin generation as depicted by higher levels of the F1+2 (Figure 1E and 1F). To investigate the contribution of platelets in the above-reported data, the experiment was conducted in presence of CatGi, which allows platelets to bind neutrophils[18]; the results showed a significant reduction but not abolishment of NETs formation, suggesting, however, a key role

Figure 1 Continued. Stemcell Technologies, Cambridge, United Kingdom), an inhibitor of FCRγIIa receptors, CatGi (Cathepsin G inhibitor; 10 µM; Abcam, Cambridge, United Kingdom) an inhibitor of cathepsin G or the same percentage of inhibitors-diluent (1% PBS) as a negative control (NC). Growth, intensity, and stability of the formation of platelet clots were measured in the whole blood sample (400 µL) by the analysis of (**A**) area under the curve (AUC) parameter and the time needed to reach occlusion pressure (**B**) the occlusion time. **C**, CitH3 (citrullinated histone H3), evaluated by ELISA method. **D**, PAD4 expression and representative Western blot bands (n=3). **E**, Tissue factor (TF) and (**F**) F1+2 (prothrombin fragment 1+2) ELISA levels were evaluated in neutrophils coincubated with platelets. **G**, CitH3 evaluated by ELISA measurement. **H**, PAD4 expression and representative Western blot bands (n=3). **I**, Tissue factor and (**J**) F1+2 ELISA levels were evaluated in neutrophils alone.

for platelets in the formation of NETs (Figure 1C through 1F). The immune-mediated nature of plasma-induced NETs formation and clotting activation was suggested by inhibition of NETs formation and clotting activation in cells pretreated with antibody blocking FcγRIIa or high doses of heparin (Figure 1C through 1F). The fact that interrupting platelet-neutrophil interaction did not abolish NETs formation prompted us to investigate if plasma from VITT was able to elicit NETs formation and clotting activation by neutrophils alone. The experiment showed again the formation of NETs and clotting activation even if the entity of this change was inferior compared with that observed with platelets and neutrophils (Figure 1G and 1H); thus, soluble TF and F1+2 were much higher in VITT plasma-treated platelet-neutrophil mixture versus neutrophils alone (17.51±2.47 versus 2.58±1.42 pg/mL; $P<0.0001$ for TF and 131,6±13,11 versus 100±14.87 pmol/L; $P<0.0001$) for F1+2, respectively. NETs formation and clotting activation were again accompanied by upregulation of PAD4 and blunted by GSK484 (Figure 1G through 1J): also, inhibition of NETs formation and clotting activation was observed after treating cells with an inhibitor of FcγRIIa and high doses of heparin, which mimicked the results above reported with platelets (Figure 1G through 1J). This finding is in keeping with previous data from heparin-induced thrombocytopenia patients showing that anti-PF4 IgG antibodies may react with neutrophils and give NETs formation via FcγRIIa.[17]

Furthermore, we performed an extensive immunohistochemistry analysis, comparing a thrombus retrieved from a patient with VITT with 3 patients with IS of atherosclerotic origin. Both samples were made up of fibrin, platelets, and abundant neutrophils, consistent with fresh thrombus. A marked increase in the expression of PAD4, CitH3, TF, and cathepsin G was detected in the thrombus from patient with VITT as compared with non-VITT

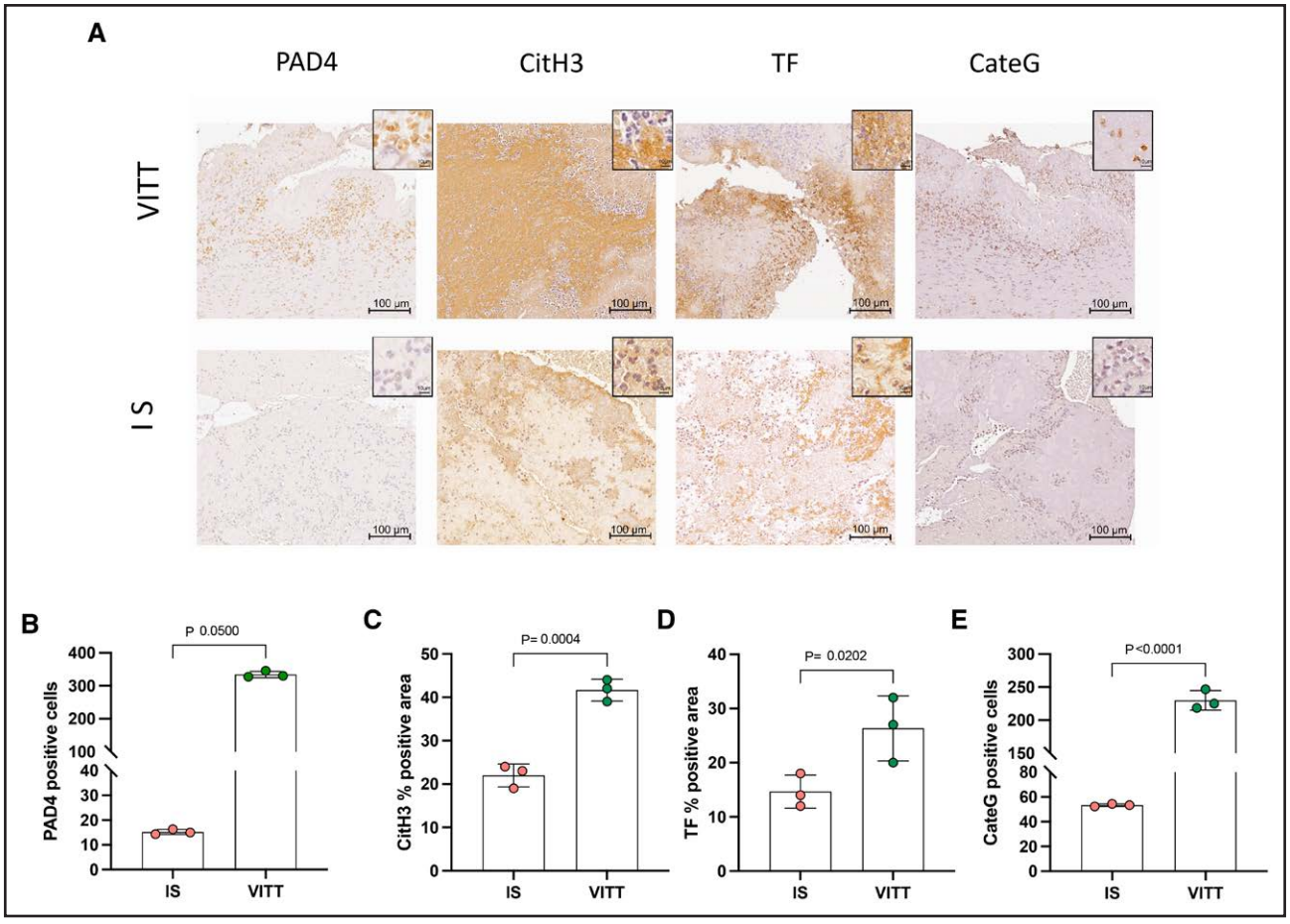

Figure 2. Immunohistochemistry analysis in thrombus of patients with vaccine-induced immune thrombotic thrombocytopenia (VITT) and ischemic stroke (IS).

The thrombi sections were provided from patient with VITT (n=1, female, age 57 years), and patients with IS (n=3, 2 male and 1 female, mean age, 74.0±7.8 years). **A**, Representative picture of 3 thrombi sections of immunohistochemistry stains of the expression of PAD4 (protein arginine deiminase 4), CitH3 (citrullinated histone H3), TF (tissue factor), and CateG (cathepsin G) in the VITT thrombus (**upper**) as compared with IS patient's thrombus. Original magnification ×20, insert ×40. **B** through **E**, Positivity for PAD4, CitH3, TF, and CateG. The positive cells were analyzed in 3 fields in 3 different sections of patients with VITT and 3 different patients with IS and counted using FIJI/Imag cell counter tool (magnification, ×200). **C** and **D**, The image analysis software FIJI/ImageJ was used to quantify the CitH3 and TF expression. The percentage-stained area and mean intensity of the staining were calculated to obtain a final score representative of immunohistochemistry positive.

thrombi (Figure 2A through 2E). Also, PAD4 and cathepsin G were diffusely expressed by neutrophils, in VITT thrombus, whereas non-VITT thrombi showed only focal granulocyte expression (Figure 2A, 2B, and 2E). Analysis of 3 sections of VITT thrombus showed a significantly higher expression of PAD4, CitH3, TF, and cathepsin G compared with the 3 IS thrombi (Figure 2B through 2E).

DISCUSSION

The present study compared the behavior of platelet-neutrophil interaction in a patient with VITT versus patients with acute DVT as VITT is essentially complicated by acute venous thrombosis. Furthermore, as thrombus immunohistochemistry from VITT was of cerebral origin, we used, as comparator, thrombi taken from patients with IS. To best of our knowledge, this is the first report showing PAD4 overexpression in a thrombus from patient with VITT, suggesting a key role for this enzyme in thrombus growth, that is in keeping with a previous report showing impaired thrombosis in animals knockout for PAD4.[8,17] It is also of interest that PAD4 was more expressed in the VITT thrombus compared with thrombi from IS and patients suggesting a higher NETosis in case of immune-thrombosis compared with thrombosis from atherosclerotic origin. Even if this comparison should be wisely considered as analysis was performed in venous (VITT) versus arterial thrombosis (IS), in vitro experiments would suggest a major impact of immune-thrombosis in NETs formation; thus, plasma from VITT incubated with whole blood generated more NETs and significantly accelerated thrombus growth compared with patients with IS. We also analyzed the impact of neutrophils alone or platelet-neutrophils mixture in NETs formation; even if, in agreement with Leung et al,[8] plasma from VITT could induce small amount of NETs, the strongest stimulus occurred with platelet-neutrophil mixture reinforcing the concept that platelet-neutrophils cooperation has a key role in thrombus growth. Another novelty of the study is the identification of cathepsin G as a molecule implicated in the interaction between platelets and neutrophils. A previous study from our group demonstrated that cathepsin G was overexpressed in coronary thrombi suggesting its role in platelet-neutrophil interaction and eventually thrombus growth; this hypothesis was corroborated by in vitro experiments showing that cathepsin inhibition resulted in reduction of platelet-neutrophil aggregates.[10] The present study supports and extends this data showing a potentially even higher role of cathepsin G in the process of immune thrombosis as it is more expressed in a thrombus from patients with VITT compared with thrombi of nonimmune origin and participates in platelet-mediated NETs formation.

Analysis of only one VITT thrombus and indirect analysis of immuno-mediated clotting activation by VITT plasma in in vitro experiments are limitations of the study. However, the fact that an antibody blocking FcγRIIa prevented VITT plasma-induced PAD4, NETs formation, and clotting activation suggests that platelet/neutrophils interaction was immuno-mediated by anti-PF4 antibodies. The study implies that, as anticipated by interventional study in animals,[8] targeting PAD4 and cathepsin G may represent a tool to develop novel antithrombotic treatment in VITT or other immuno-mediated thromboses.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Table S1
Figures S1–S2

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