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Immune complexes, innate immunity, and NETosis in ChAdOx1 vaccine-induced thrombocytopenia

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See page 4073 for the editorial comment for this article 'Thromboinflammatory findings and clinical predictors of mortality in vaccine-induced immune thrombotic thrombocytopenia', by J.M. Connors, https://doi.org/10.1093/eurheartj/ehab585.

Aims We recently reported five cases of vaccine-induced immune thrombotic thrombocytopenia (VITT) 7–10 days after receiving the first dose of the ChAdOx1 nCoV-19 adenoviral vector vaccine against corona virus disease 2019 (COVID-19). We aimed to investigate the pathogenic immunological responses operating in these patients.

Methods and results We assessed circulating inflammatory markers by immune assays and immune cell phenotyping by flow cytometry analyses and performed immunoprecipitation with anti-platelet factor (PF)4 antibody in plasma samples followed by mass spectrometry from all five patients. A thrombus was retrieved from the sinus sagittal superior of one patient and analysed by immunohistochemistry and flow cytometry. Precipitated immune complexes revealed multiple innate immune pathway triggers for platelet and leucocyte activation. Plasma contained increased levels of innate immune response cytokines and markers of systemic inflammation, extensive degranulation of neutrophils, and tissue and endothelial damage. Blood analyses showed activation of neutrophils and increased levels of circulating H3Cit, dsDNA, and myeloperoxidase–DNA complex. The thrombus had extensive infiltration of neutrophils, formation of neutrophil extracellular traps (NETs), and IgG deposits.

Conclusions The results show that anti-PF4/polyanion IgG-mediated thrombus formation in VITT patients is accompanied by a massive innate immune activation and particularly the fulminant activation of neutrophils including NETosis. These results provide novel data on the immune response in this rare adenoviral vector-induced VITT.

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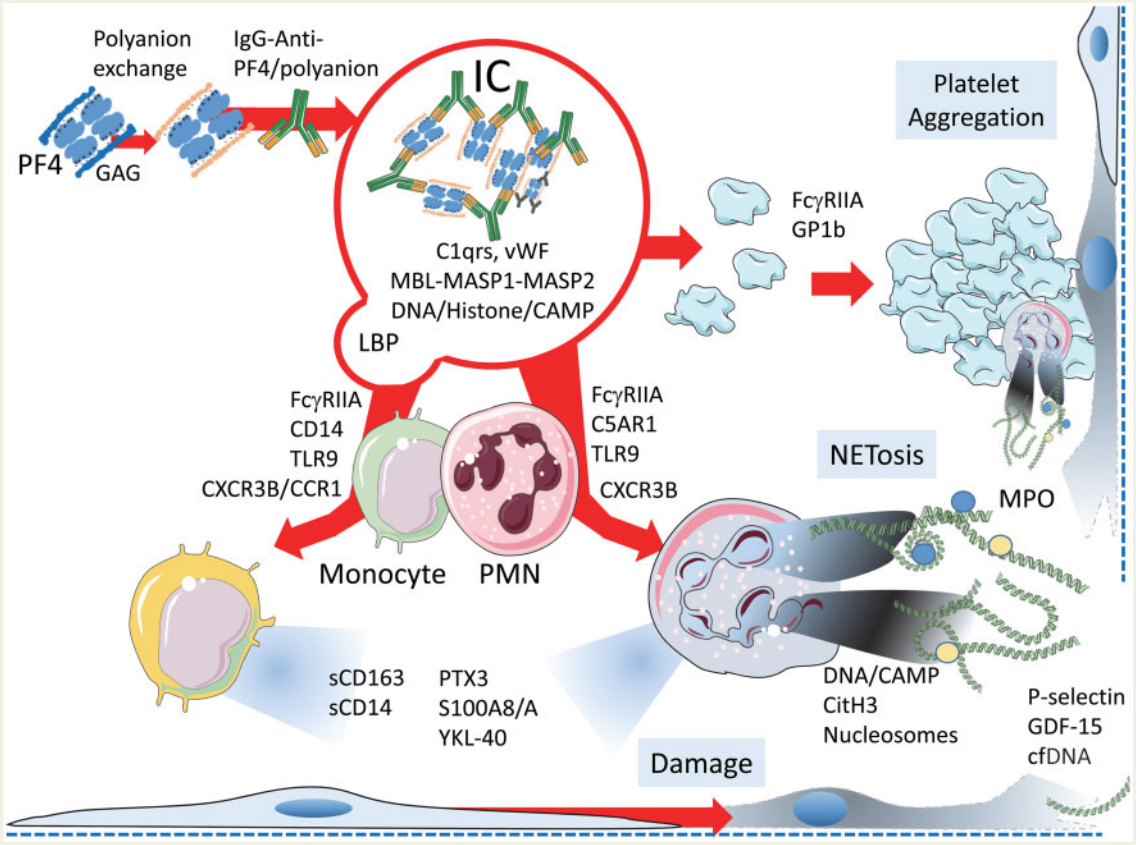
Graphical Abstract



Keywords Vaccine-induced immune thrombotic thrombocytopenia • Thrombus • Immune activation • Neutrophils

Introduction

On March 11, after 132 686 individuals in Norway had received the first dose of the ChAdOx1 nCoV-19 adenoviral vector vaccine against COVID-19,[1] this vaccination was stopped following reports from Denmark of a possible connection between the vaccine and fatal cases of thrombosis.[2] Within 10 days after vaccination with ChAdOx1 nCov-19, five healthcare workers from 32 to 54 years of age were admitted to Oslo University Hospital with severe cases of thrombosis, thrombocytopenia, remarkably high titres of anti-platelet factor (PF)4/polyanion IgG, and atypical, hyperactive platelet aggregation assays. Three of the five patients died, of what has been termed as vaccine-induced immune thrombotic thrombocytopenia (VITT) in two previous reports.[3,4] More recently, Scully *et al.*[5] reported 23 similar cases with severe thrombotic episodes 6–24 days following ChAdOx1 nCoV-19 vaccination. Based on reported cases of VITT from nine countries with moderate-to-high data quality, the estimated risk of developing VITT after the first dose of ChAdOx1 nCoV-19 ranges from 1 case per 26 500 to 1 case per 148 200.[6] All these studies have identified a pathogenic anti-PF4/polyanion-dependent syndrome, unrelated to the use of heparin therapy. However, the molecular triggers are not fully elucidated, and detailed examination of the thrombus has been lacking. Here, we describe immune complexes (ICs) that incorporated multiple triggers of innate immunity, and inflammatory and thrombogenic profiles in five VITT patients admitted to Oslo University Hospital. This includes the composition of sinus venous thrombi retrieved from one of the patients with fatal outcome.

Methods

Patients

In brief, the patients were five healthcare workers from 32 to 54 years old: one man and four women. They were admitted to Oslo University Hospital with thrombosis and thrombocytopenia 7–10 days after vaccination with ChAdOx1 nCoV-19. Four of the patients had major cerebral haemorrhage and three of the patients died. Clinical data are summarized in Supplementary material online, *Table S1* and have previously been

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described.[3] Blood samples from health careworkers of both sexes, in the same age group as the patients, were used as controls for plasma studies (unvaccinated $n = 11$, ChAdOx1 nCov-19 vaccinated $n = 8$). Controls for flow cytometry analysis of blood cells included samples from non-vaccinated blood bank donors ($n = 11$) and vaccinated healthcare workers ($n = 8$).

Ethical considerations

Written informed consent for publication was obtained from all patients and donors. The study and all control and reference material were approved by Regional Committees for Medical and Health Research (2014/2078; 2020/135924, 2020/175588, 2021/235424). In the event that the patient was not able to provide consent, family guardians provided written informed consent.

Further description of methodology is found in the Supplementary material online.

Results

Analysis of immune complexes

The spontaneous aggregation of platelets by anti-PF4/polyanion IgG patient samples[3] demonstrated that circulating IC could activate platelets directly in the absence of added heparin. We precipitated IC with anti-PF4 antibodies and analysed for protein content. Analysis revealed the expected antibodies and PF4, and principal component analysis of the mass spectrometry data showed that the patient group clearly separated from the healthy vaccinated or non-vaccinated controls (*Figure 1A*). In addition, the IC contained multiple innate immune pathway activators (*Figure 1B*) that included complement pathway (classical pathways: C1s, C2, lectin pathway; MBL: MASP2), the acute phase C-reactive protein (CRP) that also can activate the classical complement pathway, nucleosomes/chromatin [histones and toll-like receptor (TLR)9 cofactor cathelicidin antimicrobial peptide (CAMP)], lipopolysaccharide-binding protein (LBP), a co-activator of CD14, and the platelet activator von Willebrand factor (VWF) that is released upon endothelial cell activation. The IC thus included ligands for innate immune response pathways including FcγR, PF4 receptors, complement receptors, CD14, and platelet thrombosis—glycoprotein Ib (GPIb). Proteoglycan 4 contains glycosaminoglycan (GAG) chondroitin sulphate and keratan sulphate polyanions and may potentially serve as a PF4 ligand. Leucine-rich alpha-2-glycoprotein is expressed mainly in granula of neutrophils. Kinesin-like protein (KIF25) is a cytosolic microtubule-dependent motor protein that would require release via necrosis or potentially also neutrophil extracellular trap (NET)osis.

We also found serine-protease inhibitors (serpin family A members) 10, 3, and 1 that may have anti-coagulative effects (see Discussion). The acute phase proteins, serum amyloid A-1 and A-2 proteins (SAA1, SAA2), bleomycin hydrolase, cysteine proteinase inhibitors (CST1 or 4), inter-alpha-trypsin inhibitor heavy chain H3 (a hyaluronan binder), cholesteryl ester transfer protein, and the apolipoprotein, apolipoprotein A-V (APOA5), have unclear consequence and/or function in terms of the IC. No peptides from the viral vector (chimpanzee adenovirus serotype Y25) and no SARS-CoV-2 spike peptide sequences were detected.

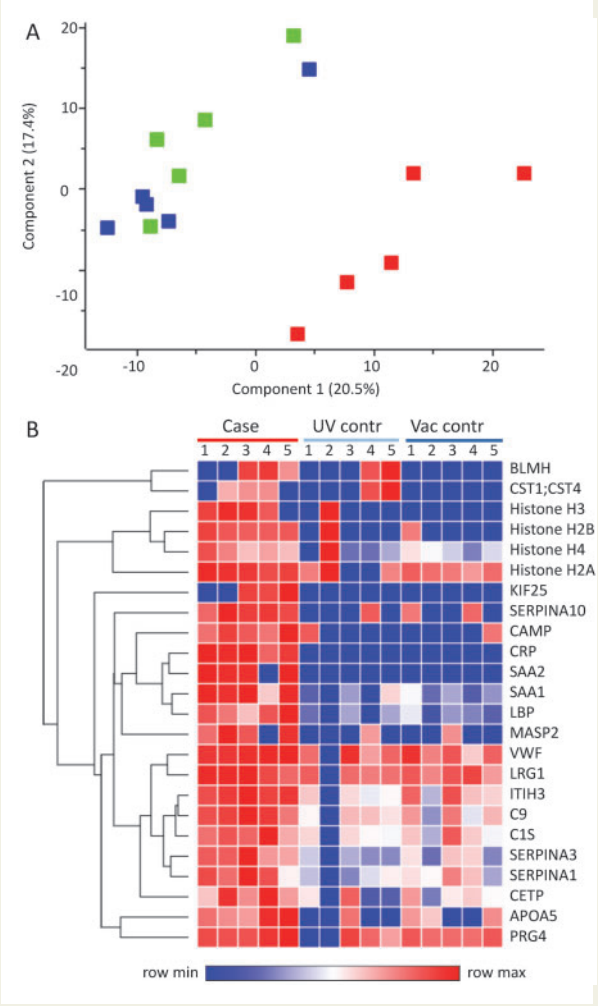

Figure 1 Analysis of immune complexes using immunoprecipitation and mass spectrometry. (*A*) Principal component analysis of the protein identification and quantification. Patient samples are marked with red, healthy vaccinated controls are marked with blue, and non-vaccinated controls are marked with green. **(***B*) Heatmap of the proteins that were more abundant ($P < 0.05$) in patient samples compared to vaccinated controls.

Inflammatory markers

To reveal the potential effects of the IC, we analysed inflammatory markers in all plasma samples. Patients had significantly elevated levels of proinflammatory cytokines [interleukin (IL)-6, IL-18], signs of monocyte activation (release of sCD163, sCD14), neutrophil degranulation [myeloperoxidase (MPO)] or both {pentraxin-related protein (PTX3), S100A8/A9, YKL-40 [chitinase-3-like protein 1 (CHI3L1)]}, endothelial cell activation (P-selectin), markers of tissue damage [growth differentiation factor (GDF)-15 and cfDNA], and systemic inflammation (LBP) compared to healthy vaccinated or non-vaccinated controls. Three markers of NETosis, citrullinated histone H3 (H3Cit), MPO–DNA complexes, and circulating dsDNA were also significantly increased (*Figure 2A*).

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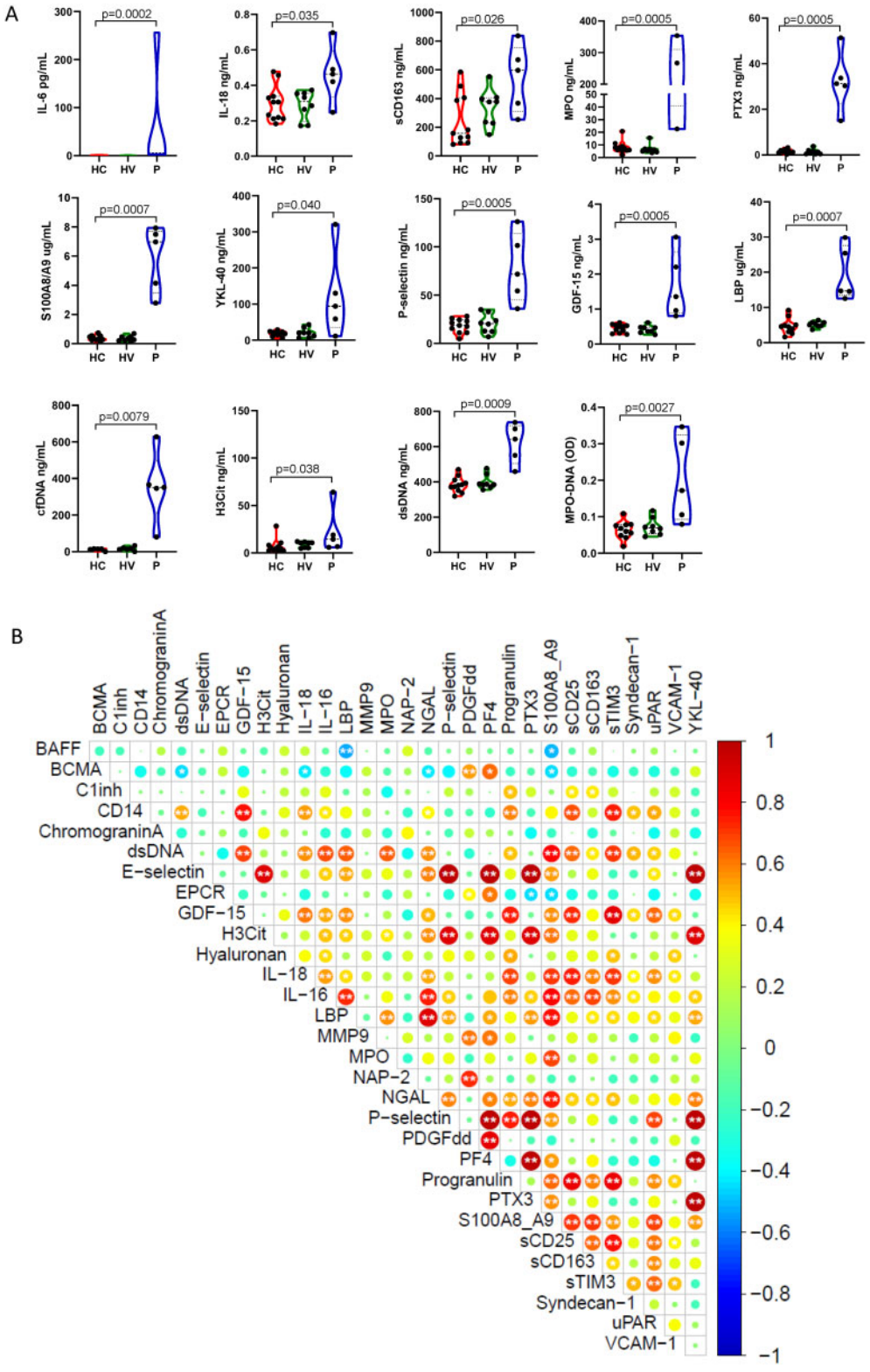

Figure 2 Characteristics of immune mediators in plasma from vaccine-induced immune thrombotic thrombocytopenia patients. Biomarkers in plasma from patients (P, $n = 5$), unvaccinated controls (HC, $n = 11$), and healthy vaccinated controls (HV, $n = 8$). (*A*) Violin plots of biomarkers, dashed lines indicate median and interquartile range. *P*-values indicate comparisons between HC and P. (*B*) Correlogram showing Pearson's correlation between all plasma molecules detected in non-vaccinated and healthy/non-healthy vaccinated donors. A heat scale where red colour shows positive linear correlation coefficients, and blue colour shows negative linear correlation. The size of the dot corresponds to the r correlation value and the significant correlations are indicated with stars (*$P < 0.05$, **$P < 0.01$, *P*-values were adjusted for multiple testing using the Bonferroni method).

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Multi-parameter analysis was performed to find significant correlations. Pearson's correlation analysis showed that the alarmins (S100A8/A9) significantly correlated with markers of platelet degranulation (PF4, platelet-derived growth factor-DD), endothelial damage (P-selectin) and NETosis (dsDNA and H3Cit; *Figure 2B*). We combined these parameters in a Volcano plot, which establish a hierarchy among our markers and highlighted the importance of the alarmins S100A8/A9, P-selectin, MPO (neutrophil marker), and PTX3 (potentiation of complement) for VITT (Supplementary material online, *Figure S1*). The same markers together with GDF-15 (associated with tissue damage) correlated significantly with fatal outcome among the hospitalized individuals (Supplementary material online, *Figure S1*).

Activation of blood leucocytes

Flow cytometry analysis of whole blood from the patients demonstrated an increased frequency of activated (HLA-DR$^+$ and CD38$^+$) and immature neutrophil subpopulations that had decreased expression of CD15, CD16, CD66b, CD101, and CD10[7,8] (*Figure 3A* and Supplementary material online, *Figure S2*). This expansion of immature neutrophil was not observed in healthy vaccinated donors. In two of the patients, intravenous immunoglobulin treatment was associated with normalized cell count, neutrophil/lymphocyte ratio, and neutrophil phenotype, as illustrated by the follow-up time points showing a similar profile as the healthy controls (*Figure 3B* and Supplementary material online, *Figure S3*). In contrast, the flow analyses did not reveal activation of T cells or isotype class switching for B cells in VITT patients compared to controls.

Analysis of cerebral sinus sagittal superior thrombus

We next analysed the retrieved thrombus from one of the patients who died (Patient 5) by immunohistochemistry (IHC) and flow cytometry. Microscopic and flow analyses revealed unusual thrombi with very high cellularity, massive polymorphonuclear leucocytes (PMN) infiltrates (*Figure 4A*), platelets and neutrophils, IgG deposits (*Figure 4B*), PMN degranulation (S100A8 and S100A9, *Figure 4C*), and extensive neutrophil activation characterized by decondensated chromatin (NETosis) and NET formation, as shown by the positive staining of citrullinated histone H3 (CitH3), and elastase (*Figure 4D*). No reliable level of spike protein was detected by IHC.

Flow cytometry analysis revealed that nearly 75% of infiltrating cells were PMN (in comparison to 10% in blood from the same patient). Among non-granulocytes, the main population detected in the clot was antigen-presenting cells (HLA-DR$^+$CD3$^-$). B cells were barely detectable in the clot, which was thus essentially composed of classical monocytes (CD14$^+$CD16$^+$). The relative proportion of T cells was decreased due to the increased frequency of APC in non-granulocytes derived from the clot, but the proportion of CD4 and CD8 or the activation, differentiation, and exhaustion status were not altered (*Figure 4E* and Supplementary material online, *Figure S4*).

Discussion

We here describe the composition of PF4-containing IC in patients with severe VITT, and demonstrate multiple ligands for innate immune receptors as illustrated in the *Graphical abstract*. This was linked

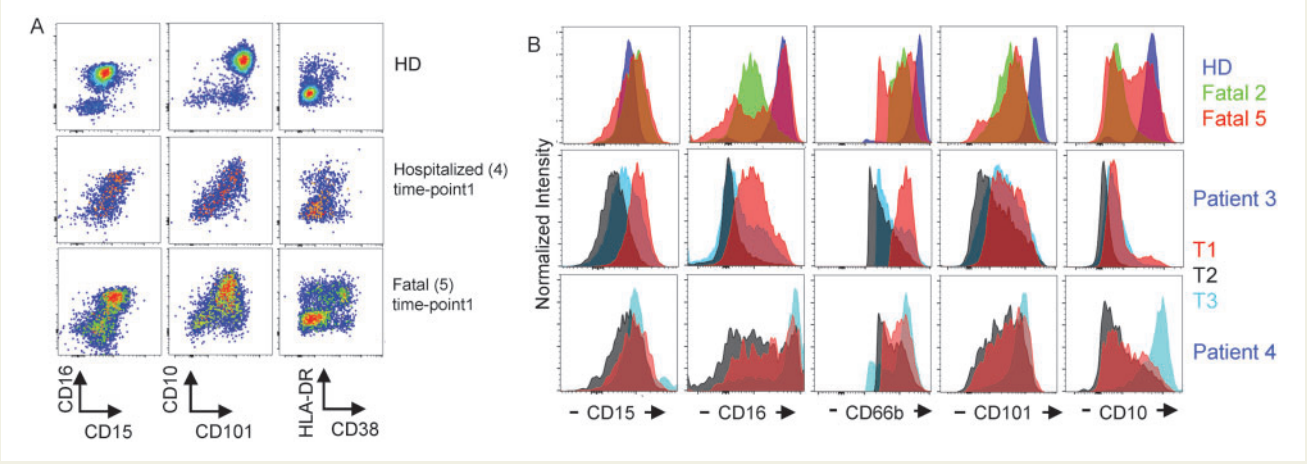

Figure 3 Immune phenotype of vaccine-induced immune thrombotic thrombocytopenia patients. Analysis of 5 cases and 12 vaccinated healthy control blood samples at three time points. (*A*) Peripheral blood of healthy donors and cryo-preserved PBMC from vaccine-induced immune thrombotic thrombocytopenia patients were surface stained to characterize low-density neutrophils. After the removal of debris and dead cells based on FSC-A and SSC-A, singlets were gated for haematopoietic cells. Granulocytes were defined as CD45$^+$SSC^{Hi}CD66b$^+$CCR3$^-$CD123$^-$ (see also Supplementary material online, *Figure S2* for gating). (*B*) Longitudinal follow-up of neutrophils in surviving vaccine-induced immune thrombotic thrombocytopenia patients. The expression of maturation markers on neutrophils was compared between healthy donors (HD, top panel) and fatal vaccine-induced immune thrombotic thrombocytopenia (Patients 2 and 5, top) or in longitudinal samples for non-fatal cases (Cases 3 and 4 at Day 7, Day 14 or 20 for Time-points 2 and 3, respectively). The intensity of each marker was normalized in the overlaid histograms. See also Supplementary material online, *Figure S4* for hierarchical clustering of all time points.

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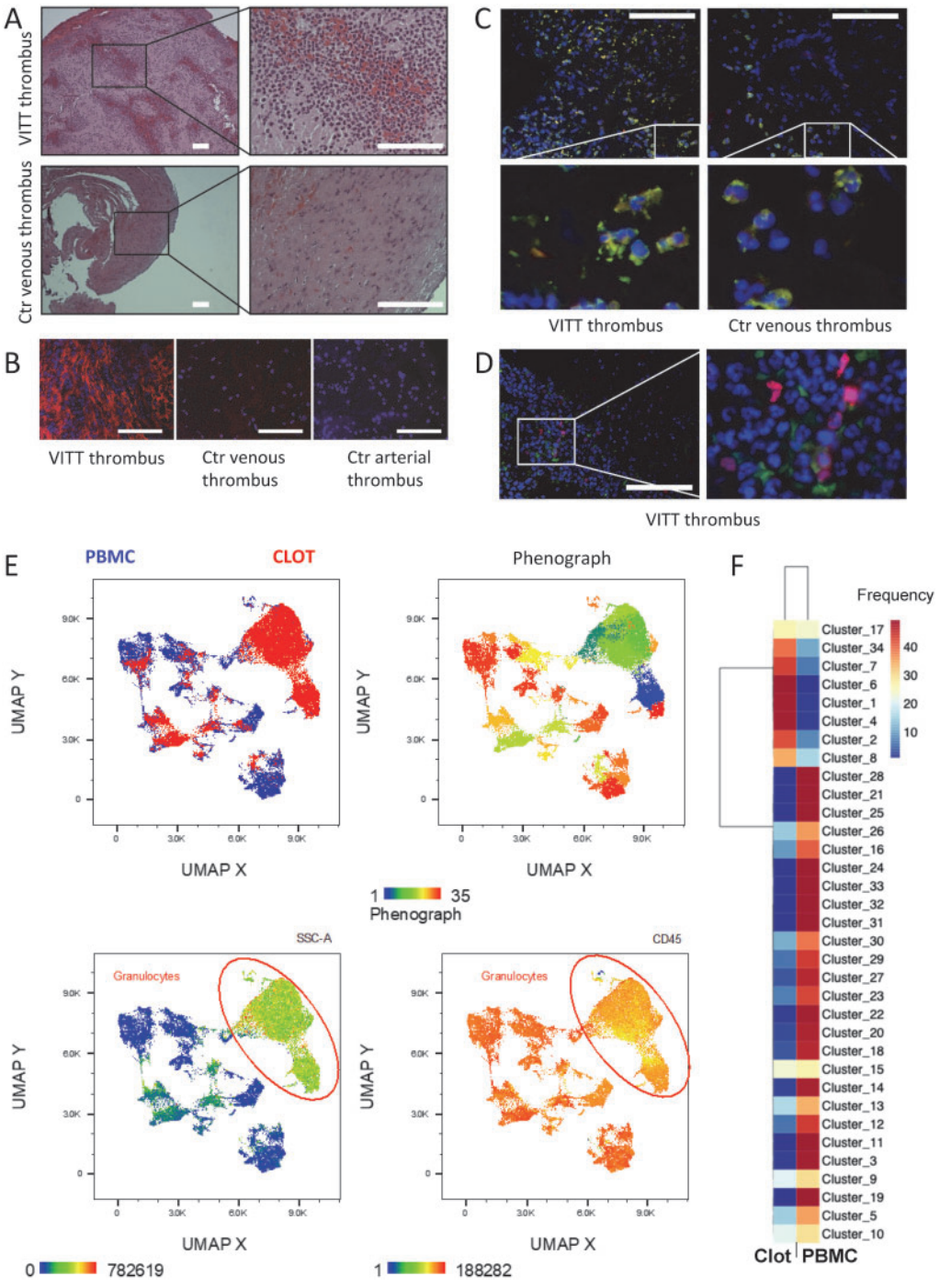

Figure 4 Thrombus from vaccine-induced immune thrombotic thrombocytopenia patients is rich in neutrophils and stain positive for IgG and neutrophil extracellular traps. (*A*) Haematoxylin and eosin staining of vaccine-induced immune thrombotic thrombocytopenia and control thrombus at 100× and 400× magnifications. (*B*) Immunofluorescent staining of IgG (red) in vaccine-induced immune thrombotic thrombocytopenia—control venous thrombus and control cerebral arterial thrombus. (*C*) Double immunofluorescent staining of S100A8 (red) and S100A9 (green) in vaccine-induced immune thrombotic thrombocytopenia thrombus and control venous thrombus; the lower panels are magnifications of selected parts of the original image as indicated, showing the presence of the S100A8/A9 complex outside of cytosol in vaccine-induced immune thrombotic thrombocytopenia thrombus. (*D*) Double immunofluorescent staining of elastase (green) and citrullinated histone H3 (red) in vaccine-induced immune thrombotic thrombocytopenia thrombus; the image to the right is a magnification of the original image as indicated. DAPI serves as a nuclear DNA counterstain (blue). Scale bars indicate 100 μm. (*E*) Single-cell suspension was made from the blood clot and analysed by flow cytometry, presented as UMAP plots showing the clustering of all haematopoietic immune cells by tissue origin. Cells composing the clot occupy the upper right region of the two-dimensional map. Clustering is based on the expression of all phenotypic markers assessed. Granulocytes can be identified as SSC-A^{HI} CD45^{low} cells. Automatic clustering was generated by Phenograph. (*F*) Cluster quantification in samples derived from the fatal Case 5. A heat map represents the frequency of each cluster. See also UMAP phonograph, Supplementary material online, *Figure S4*.

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to a proinflammatory immune profile in all patients analysed, including cytokines that trigger innate immunity with massive activation of neutrophils in the circulation. We also found extensive neutrophil activation and NET formation in venous sinus sagittal superior thrombi from one of the patients with fatal outcome.

The present study highlights the role of innate immune responses in VITT that includes an unusual fulminant focal neutrophil activation in cell-rich thrombi as well as systemic activation of leucocytes and circulating cytokines, free nucleic acids, and acute phase reactants. Increased levels of alarmins S100A8 and S100A9 were observed both in circulation and in the sinus thrombus. These constitute 45% of cytosolar proteins in neutrophils[9] and after secretion are potent triggers of innate inflammatory cytokines such as IL-6 that we found to be increased in the plasma of VITT patients.

An important finding in this study was the massive NET formation at the site of thrombus formation in the one patient who died. All five patients also have indirect signs of NET formation in peripheral blood (H3Cit, dsDNA, MPO–DNA complex) as opposed to healthy controls and vaccinated healthcare workers without signs of thrombus formation. Neutrophil extracellular traps in interaction with platelets are involved in thrombus formation during severe COVID-19 disease and have also been seen during atherothrombus formation[10,11] and have been detailed in heparin-induced thrombocytopenia patients.[12,13] However, the massive immune infiltration including high numbers of neutrophils and related inflammatory molecules, as observed in these patients, is unusual.

Mass spectrometry analysis of immunoprecipitated PF4 from plasma identified PF4 in complex with proteins such as histones, complement pathway factors, GAG-binding lectins, and antibodies, suggesting several inflammatory pathways involved. These are ligands for a number of pathways that can explain the observations of marked inflammatory responses in these patients.

Although the mechanisms are not yet clear, adenovirus can activate platelets in a VWF and P-selectin[14] and alternative complement pathway dependent manner.[15,16] It is thus plausible that the adenoviral vector itself activated platelets and provided a first trigger for PF4 secretion. PF4/polyanion is a chemokine and can in itself activate leucocytes via the CXCR3-B splice variant of CXCR3 and CCR1.[17–19] Platelets express CCR1 that can trigger aggregation,[20] but the effect of PF4/GAG on platelet CCR1 is unclear. PF4 is secreted from α-granules of platelets as a tetrameric complex bound to serglycin GAG chains.[21] The GAG serve as polyanion ligands for cationic amine acid side chains of PF4. The complex includes four PF4 and two GAGs, these may be chondroitin sulphate or dermatan sulphate. After secretion, PF4 may bind other ligands with higher affinity, such as endothelial-derived perlecan heparan sulphate side chains,[22] or other polyanions.

It is thought that the polyanions allow conformational changes in PF4 exposing antigenic determinants for anti-PF4 IgG antibodies.[23] Our patients all had high titres of anti-PF4/polyanion IgG and platelet aggregation could not be abolished by high dose heparin in the majority of patients.[3] This indicated an unidentified non-heparin polyanion in the patients. The anti-PF4 IgG constitutes a second trigger as platelets express FcγRIIA (CD32a). FcγRIIA is a low-affinity Fc receptor for the constant region of IgG that recognizes ICs with high avidity and causes platelet aggregation and dense granule exposed release,[24] exposure of phosphatidylserine, complement factor deposition, and accelerated prothrombinase activity.[25] The IC included classical complement pathway initiator C1qrs, CRP and the lectin pathway activators MBL–MASP1–MASP2 that typically responds to lectins similar to the polyanion GAGs associated with PF4. MASP1 and MASP2 cleave prothrombin, convert fibrinogen to fibrin monomers, activate factor XIII, and promote fibrin cross-linking.[26] von Willebrand factor was also found in the IC and provides the ligand for GPIb on platelets, a receptor that triggers platelet aggregation and degranulation.

Neutrophils express FcγRIIA and the complement receptor C5AR1 (for C5a) that activates degranulation and NETosis.[27] The IC also included histones, i.e. nucleosomes as well as CAMP, the cofactor for human DNA/chromatin binding of TLR9[28] required for inflammasome activation in myeloid cells. It should be noted that PF4 can bind and aggregate DNA (as a polyanion) and amplify TLR9 signalling by organizing fragmented DNA into liquid-crystalline ICs with inter-DNA spacings optimal for TLR9 amplification.[29] This suggests a positive feedback loop that would be further stabilized by anti-PF4 IgG. Thus, it is likely that the FcγRIIA, C5AR1, and TLR9 signalling converged in a massive activation of neutrophils in the patients.

NET and thrombus formation could potentially be induced by spike protein in the vaccine, but we could not detect any spike protein in plasma from the VITT patients or within the thrombus by IHC or IP of spike followed by targeted mass spectrometry.

Apart from reduced fibrinogen levels, the patients did not display convincing signs of disseminated intravascular coagulation. This may seem strange given the range of pro-coagulant activators described above. Immune complex, however, also included three serine-protease inhibitors (serpin family A members). SerpinA10 (protein Z-dependent protease inhibitor) binds heparin and together with protein Z inhibits coagulation protease factor Xa and Xia,[30,31] serpinA3 (alpha-1-antichymotrypsin) inhibits neutrophil cathepsin G (and thereby inhibits conversion of angiotensin-I to the active angiotensin-II). SerpinA1 (alpha-1-antitrypsin) has been described in NETs,[32] inhibits neutrophil elastase, and can also inhibit thrombin.[33]

In summary, we suggest that VITT is caused by a preliminary direct activation of platelets by the ChAdOx1 nCoV-19 adenoviral vector vaccine,[14–16] degranulation and PF4/GAG secretion, exchange of GAG with an unknown polyanion, generation of IgG against PF4/polyanion,[3–5] IgG-PF4/polyanion IC formation, secondary activation of platelets via IC IgG/FcγRIIA[24], addition of complement factors and VWF to IC, addition of DNA/nucleosomes/CAMP to IC, activation of neutrophils via multiple pathways[27–29], NETosis, and further massive platelet activation.[25,26]

However, the permissive factors that allow these events and why this is only seen in a minority of ChAdOx1 nCoV-19-vaccinated individuals are still not clear.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: N.H.S. has received honoraria from Pfizer, BMS, and Bayer for lectures about management of anticoagulation treatment and bleeding, and for participation in an advisory board on the same topic. B.H. has a licenced patent (sCD36 as biomarker of atherosclerosis and metabolic disorder) and a pending patent application (Cetoleic acids—health effects). P.A. and M.S. have a licenced patent (sCD36 as biomarker of atherosclerosis and metabolic disorder). M.S. has received meeting honoraria paid from Bayer in 2019 (not relevant for this manuscript). K.S. has received a personal fee from Bayer. L.A.M. has received honoraria from pharma companies for two lectures last 3 years and has no financial interests. H.K. was a shareholder and employee of ImmunoScape Pte Ltd in 2019–2020. G.L.G. has received honoraria from AbbVie, Biogen, Eli Lilly, Novartis, Pfizer, Roche, Sandoz, Orion Pharma, Celltrion, and Boehringer Ingelheim for lectures and advisory committees the last 3 years and has no financial interests. A.E.M. has stock ownership in Pfizer. P.A.H. has received research grants from Bayer, Pfizer, SOBI, Roche within area of bleeding disorders to institution (not personal), lecture honoraria, advisory boards in the area of bleeding disorders from Takeda, SOBI, Bayer, Pfizer, Roche, Octapharma, NovoNordisk, CSL, BMS, support for attending meetings from Takeda, Bayer, Roche, Pfizer, Octapharma, NovoNordisk, CSL, SOBI and is a member of executive committee of the ADVANCE group and ACHIEVE group, Bayer. All other authors declared no conflict of interest.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Translational perspective

Recently, Norway and Denmark stopped the ChAdOx1 nCoV-19 vaccination after several reported cases of vaccine-induced syndrome of severe thrombosis and thrombocytopenia with fatal outcome. Samples from vaccine-induced immune thrombotic thrombocytopenia (VITT) patients allowed us to investigate mechanisms in this severe syndrome and we report immune complexes (ICs) with multi-pathway triggers, innate immune response cytokines, activation of neutrophils in the blood, and extensive formation of neutrophil extracellular traps (NETs) surrounded by IgG in a thrombus ectomized from the sagittal sinus vein. Our results shed light on the underlying mechanisms in this rare adenoviral vector vaccine-induced syndrome of severe thrombosis and thrombocytopenia and suggest that antibody-mediated thrombus formation in VITT patients is accompanied by a massive innate immune activation with particular activation of neutrophils, at least partly induced by IC-mediated mechanisms with NET formation as a major pathogenic event.

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