Towards Understanding ChAdOx1 nCov-19 Vaccine-induced Immune Thrombotic Thrombocytopenia (VITT)

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Abstract

Background

SARS-CoV-2 vaccine ChAdOx1 nCov-19 rarely causes vaccine-induced immune thrombotic thrombocytopenia (VITT) that—like autoimmune heparin-induced thrombocytopenia—is mediated by platelet-activating anti-platelet factor 4 (PF4) antibodies.

Methods

We investigated vaccine, PF4, and VITT patient-derived anti-PF4 antibody interactions using dynamic light scattering, 3D-super-resolution microscopy, and electron microscopy. Mass spectrometry was used to analyze vaccine composition. We investigated the mechanism for early post-vaccine inflammatory reactions as potential co-stimulant for anti-PF4 immune response. Finally, we evaluated VITT antibodies for inducing release of procoagulant DNA-containing neutrophil extracellular traps (NETs), and measured DNase activity in VITT patient serum.

Results

Biophysical analyses showed formation of complexes between PF4 and vaccine constituents, including virus proteins that were recognized by VITT antibodies. EDTA, a vaccine constituent, increased microvascular leakage in mice allowing for circulation of virus- and virus-producing cell culture-derived proteins. Antibodies in normal sera cross-reacted with human proteins in the vaccine and likely contribute to commonly observed acute ChAdOx1 nCov-19 post-vaccination inflammatory reactions. Polyphosphates and DNA enhanced PF4-dependent platelet activation by VITT antibodies. In the presence of platelets, PF4 enhanced VITT antibody-driven procoagulant NETs formation, while DNase activity was reduced in VITT sera, with granulocyte-rich cerebral vein thrombosis observed in a VITT patient.

Conclusions

ChAdOx1 nCoV-19 vaccine constituents (i) form antigenic complexes with PF4, (ii) EDTA increases microvascular permeability, and (iii) vaccine components cause acute inflammatory reactions. Antigen formation in a proinflammatory milieu offers an explanation for anti-PF4 antibody production. High-titer anti-PF4 antibodies activate platelets and induce neutrophil activation and NETs formation, fueling the VITT prothrombotic response.

Introduction

Vaccination against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the most important countermeasure to fight the ongoing Covid-19 pandemic. Two vaccines approved by the European Medicines Agency are adenoviral vector-based vaccines (recombinant human adenovirus type 26 vector encoding SARS-CoV-2 spike glycoprotein, Covid-19 Vaccine Janssen; and recombinant chimpanzee adenoviral [ChAdOx1-S] vector encoding the spike glycoprotein of SARS-CoV-2, COVID-19 Vaccine AstraZeneca [Vaxzevria]).[1] ChAdOx1-S is propagated using T-REx HEK293 cells, a transformed human embryonic kidney cell line. The product is nuclease-treated and further purified (Supplementary material 1).[2]

To date more than 62 cases of cerebral venous sinus thrombosis (CVST) and 24 cases of splanchnic vein thrombosis in combination with moderate to severe thrombocytopenia were reported in Germany in healthy individuals under 60 years of age within 5 to 20 days of ChAdOx1 nCov-19 vaccination.[3] This novel disorder, “vaccine-induced immune thrombotic thrombocytopenia (VITT)”, is associated with high titers of immunoglobulin G class antibodies directed against the cationic platelet chemokine, platelet factor 4 (PF4; CXCL4).[4] These antibodies potently activate platelets via platelet FcγIIa receptors, with platelet activation greatly enhanced by PF4.[4,5] Another vector-based vaccine, Covid-19 Vaccine Janssen, also appears to be associated with formation of anti-PF4 antibodies,[6,7] currently under investigation of the European Medical Agency and the U.S. Food and Drug Administration.[8]

PF4 opsonizes polyanionic surfaces of pathogens, facilitating the binding of anti-PF4 antibodies.[9] This is likely an evolutionary old immune defense mechanism, as anti-PF4 producing B-cells can be found in nearly all individuals, including even in newborn cord blood.[10–12] However, a strong anti-PF4 antibody response, when misdirected, underlies the thromboembolic disorder, immune heparin-induced thrombocytopenia and its most severe presentation, autoimmune heparin-induced thrombocytopenia.[13,14] Autoimmune heparin-induced thrombocytopenia is characterized by formation of platelet-activating anti-PF4 antibodies with very high avidity that are reactive even in the absence of heparin.[15] Severe heparin-induced thrombocytopenia also features “pancellular” activation (platelets, neutrophils, monocytes, endothelium).[16,17] VITT closely mimics autoimmune heparin-induced thrombocytopenia both clinically and serologically. One of the major risk factors for formation of anti-PF4 antibodies is inflammation and tissue trauma, which substantially increase the risk for forming pathogenic anti-PF4 antibodies.[18]

Based on our detailed understanding of autoimmune heparin-induced thrombocytopenia, we systematically analyzed whether similar mechanisms underlie the pathogenesis of VITT. We focused on elucidating potential triggering and downstream prothrombotic events. Our specific aims were: (a) to assess whether constituents of the ChAdOx1 nCov-19 vaccine bind to PF4, forming complexes and potentially neo-antigen(s); (b) to find evidence for proinflammatory signals during the peri–vaccination period; (c) to test whether antibodies in VITT sera activate platelets in a PF4-dependent manner facilitated by polyanions such as DNA; and (d) to examine whether VITT antibodies trigger neutrophil activation and release of prothrombotic neutrophil extracellular traps (NETs).

Our findings help explain some aspects of the potential for immune-mediated adverse effects induced by the ChAdOx1 nCov-19 vaccine, and provide insights into modulating these risks.

Material And Methods

All materials and methods are described in detail (Supplementary material).

Interaction of PF4 and ChAdOx1 nCov-19 vaccine

Binding of vaccine components to PF4 (Chromatec, Greifswald, Germany) was analyzed by dynamic light scattering (DLS), 3D-super-resolution microscopy and transmission electron microscopy (TEM). In some experiments, high concentration of heparin (100 IU/mL) was added to disrupt charge-related complex formation; or antibodies against PF4 and adenovirus (affinity-purified from VITT patient sera; mouse monoclonal [B025/AD51] against hexon polypeptide, Abcam, Cambridge, UK). PF4 used for TEM was biotinylated on a heparin column to protect the polyanion binding site (Supplementary material 2.1-2.3).

Constituents of PF4 used in the assays and of the ChAdOx1 CoV-19 vaccine Composition of PF4 and ChAdOx1 CoV-19 vaccine was analyzed by mass spectrometry and ^{1}H-nuclear magnetic resonance spectroscopy (NMR), 1D-SDS PAGE electrophoresis, and proteomics (LC-MS, Supplementary material 3.1-3.3, Table S2). Results of PF4 reagent analysis are given in detail in Supplementary material S3, Table S1, Table S3.

Effects of EDTA in the vaccine

The vaccine contains ethylenediaminetetraacetic acid (EDTA),[2] which is known to increase vascular permeability.[19] We analyzed whether ChAdOx1 nCoV-19 increases microvascular leakage in mice using the Miles edema model. We assessed platelet activation and alpha granule release by 0.00001-0.02 mM EDTA by whole blood flow cytometry (Supplementary material 4).

Preformed antibodies and proinflammatory signal occurring close to vaccination

Preformed antibodies in normal human serum and VITT patient serum against human proteins in the vaccine were assessed by the Peggy Sue Simple Western Assay (ProteinSimple, Santa Clara, CA, USA; Supplementary material 5).

Vaccine reactions in vaccinated employees of the Department of Transfusion Medicine, Greifswald, were assessed by interview. Other symptoms were obtained from spontaneous patient-initiated reports.

Interaction of anti-PF4 antibodies from VITT patients with platelets

Platelet activation by antibodies from VITT patient sera was measured by a functional test using washed platelets,[4] in the absence or presence of PF4 (10 µg/mL), low-molecular-weight heparin (reviparin, 0.2 anti-factor Xa units/mL), synthesized double-stranded DNA (20-mer; 0.1 µg/mL), polyphosphate (PP70, 0.2 µg/mL), pentosan polysulfate (2 µg/mL, BenePharma, Munich, Germany), and EDTA (0.0001-0.02 mM). For some experiments, sera were diluted with saline (Supplementary material 6).

Formation of NETs by anti-PF4 antibodies and DNase activity in the sera of VITT patients

Formation of NETs (“NETosis”) was measured in a microplate assay in the presence or absence of VITT anti-PF4 antibodies, and PF4. DNase activity in VITT patient serum samples was measured by single radial enzyme diffusion (SRED) assay [20] (Supplementary material 7).

Results

Interaction of ChAdOx1 nCov-19 vaccine with PF4

Addition of PF4 (diameter, 5 nm)[21] to ChAdOx1 nCoV-19 vaccine increased the size of vaccine particles from 105.5 ± 2.8 nm to 185.2 ± 44.5 nm (mean diameter ± SD; **Figure 1A** and **Supplementary Figure S1**). Addition of mouse monoclonal anti-PF4 IgG (mAb RTO) or affinity-purified anti-PF4 IgG from VITT patient sera further increased the size of PF4/ChAdOx1 nCoV-19 vaccine complexes to 288.4 ± 10.4 nm and to 288.5 ± 13.9 nm, respectively. Addition of DNA further increased the size of PF4/ChAdOx1 nCoV-19 vaccine/VITT patient anti-PF4 IgG complexes (to 710.4 ± 183 nm; **Figure 1A** and **Supplementary Figure S2C**), while 100 IU/mL heparin dissociated 98.5% of complexes (**Figure 1A** and **Supplementary Figures S1C, S2D**).

3D-super-resolution immunofluorescence microscopy confirmed close proximity between PF4 molecules, platelets and adenovirus capsid protein positive particles (**Figure 1B** and **Supplementary Figures S3** and **S4**). Furthermore, transmission EM visualized aggregates after incubation of the vaccine with PF4. These aggregates stained positive for both adenovirus hexon polypeptide and PF4 (**Figure 1C-E** and **Supplementary Figure S5**). Virus particles in the untreated vaccine were covered with proteins, which were removed from the particle surfaces by high concentrations of heparin (**Supplementary Figure S6**)**.** Together, these results show vaccine components binding to virions and charge-driven (sensitive to heparin) PF4 binding to vaccine components, leading to complex formation and subsequent VITT anti-PF4 antibodies binding.

ChAdOx1 nCoV-19 vaccine contains EDTA and cell culture-derived human proteins

Using ^{1}H-NMR spectrometry, we identified known additives (sucrose, EDTA [approximately 100 µM], histidine) within the ChAdOx1 nCov-19 vaccine (**Supplementary Figure S8**). We found 70-80 µg protein/mL vaccine in four lots tested. Silver staining of ChAdOx1 nCoV-19 vaccine separated on SDS-PAGE showed numerous protein signals (**Supplementary Figure S9**). Proteomics identified vector proteins (blue dots), human proteins (grey dots)—including human membrane proteins (green dots)—and the SARS-CoV-2 spike protein (red dot) in the ChAdOx1 nCov-19 vaccine (**Figure 2, Supplementary Figure S10** and **Supplementary Table S3**).

Effects of EDTA in the vaccine

EDTA (0.0002 mM) induced platelet activation in whole blood of healthy volunteers assessed by flow cytometry (**Supplementary Figure S11**). Intradermally-injected ChAdOx1 nCoV-19 vaccine increased vascular leakage in a Miles skin edema mouse model. Edema formation was likely due to EDTA in the carrier solution as EDTA alone triggered edema and vascular leakage to a similar extent as the vaccine (**Supplementary Figure S12**).

Proinflammatory signals occurring close to vaccination

Preformed antibodies against proteins in the vaccine

Western blot analysis showed cross-reactivity of endogenous immunoglobulins in the serum of healthy, non-vaccinated volunteers with vaccine proteins (**Figure 3** and **Supplementary Figure S13**). Cross-reactivity with vaccine components was substantially stronger in sera of VITT patients; it could not be determined whether such strong cross-reactivities were present prior to vaccination or were boosted as a consequence of recent vaccination.

High frequency of early reactions to vaccination

ChAdOx1 nCov-19 vaccine inoculation frequently induced inflammatory reactions. Of 22 healthy healthcare workers, who received the ChAdOx1 nCoV-19 vaccine, 12 reported adverse-effects, including fever, chills, and pain in large joints, starting 6-12 hours after vaccination, usually resulting in one to two days of sick leave. Sporadically patients reported spontaneously vaccine-associated lymphadenopathy or dermal lesions that appeared similar to “serum sickness” or symptoms of capillary leakage. An example is shown in **Figure 3 B**, including a description of symptoms using the patient’s own words.

Interaction of VITT sera with blood cells in vitro

Consistent with previously published data,[4] all (8/8) sera of VITT patients and their respective affinity-purified antibody fractions showed strong reactivity in the PF4/heparin ELISA (optical densities >3.00 units; reference value, <0.50). The PF4 preparation used in the experiments contained only traces of EDTA (which had no effect on antibody binding; **SupplementaryFigure S14**), formate and acetate in ^{1}H-NMR analysis. It was free of contaminating proteins as shown by proteome analysis but some PF4 variant-1 (1.5-2.0%) was present (Supplementary material, **Figure S7, Table S1, S3**).

Addition of PF4 amplified platelet activation stimulated by VITT sera (**Figure 4**). Serum spiked with polyphosphate (a platelet-derived inorganic polymer, 0.2 µg/mL), or DNA (1 µg/mL) similarly activated platelets; however, stimulation was weaker compared with addition of PF4. In contrast, addition of highly sulfated polyanions heparin and pentosan polysulfate did not affect VITT patient serum-stimulated platelet activation (not shown).

Anti-PF4 antibodies induce NETs formation and extracellular DNase activity is reduced in VITT patient sera

Incubation of purified neutrophils with PF4 and VITT patient serum triggered formation of procoagulant NETs (NETosis; **Figure 5A**). Similarly, the respective affinity-purified anti-PF4 antibodies activated neutrophils (not shown). PF4/VITT serum-stimulated NETosis was strongly enhanced in the presence of platelets (**Figure 5B**), while healthy control sera were inactive (**Supplementary Figure S15**). NETs are degraded by extracellular DNases. We measured DNase activity in sera of 10 patients with severe VITT and found it reduced by ~40% compared to healthy control levels (**Figure 5E**). High numbers of granulocytes in a cerebral vein thrombus of a VITT patient support a role for neutrophils in VITT-driven CVST (**Figure 5D**).

Discussion

From our data, the following sequence of events appears to mediate VITT (Fig. 6).

In Step 1, a neo-antigen is generated: following intramuscular injection, vaccine components and platelets come into contact, resulting in platelet activation. ChAdOx1 nCov-19 vaccine activates platelet by multiple mechanisms including platelet interaction with adenovirus, cell-culture derived proteins (currently, it is unknown which of the > 1,000 proteins identified in the vaccine are involved in platelet activation), and EDTA. Activated platelets release PF4. As shown by TEM, released PF4 binds to constituents of the vaccine forming multimolecular aggregates, which also include virus proteins, resulting in particles formation of ≥ 120 nm size.

Step 2 generates an inflammatory co-signal that further stimulates the immune response: EDTA in the vaccine increases capillary leakage at the inoculation site, likely by endothelial (VE)-cadherin disassembly.[19] Proteins found in the vaccine include virus proteins, but also proteins originating from the human kidney-derived production cell line T-REx HEK-293. Increased vascular permeability facilitates dissemination of these proteins into the blood. Blood dissemination of vaccine components is not unique to ChAdOx1 nCov-19. A ChAdOx1 vector variant (with a hepatitis B vector insert) was detectable by PCR in multiple organs, including liver, heart, and lymph nodes at days 2 and 29 after intramuscular injection in mice.[22]

Within the circulation, vaccine constituents including its complexes with PF4 are recognized by preformed natural immunoglobulin G antibodies,[23] presumably resulting in immune complexes. This contributes to clinical symptoms within 8 to 24 hours following inoculation that are reminiscent of systemic inflammation (fever, chills, large joint arthralgia, occasionally skin lesions, probably reflecting a similar process as known in serum sickness or serum sickness-like illness[24]). Such symptoms have also been observed as acute vasculitis like reaction when a column used for immunoadsorption leaked protein A with bound antibodies.[25] This inflammatory response likely provides an important co-signal that stimulates antibody production by preformed B-cells capable of producing anti-PF4 antibodies, as is known to occur in the pathogenesis of “classical” HIT [26.12] Multimolecular complexes containing PF4 also activate the complement system.[27,28] Complement bound to the aggregates subsequently allows binding of the complexes to B-cells via their complement receptor.[27]

Step 3 leads to prothrombotic reactions: high avidity anti-PF4 antibodies among the anti-PF4 antibodies in VITT patient blood bind and cluster PF4 on the platelet surface, likely involving polyanions such as cell surface chondroitin sulfate or exposed polyphosphate.[29] Clustering of PF4 by high-avidity

autoantibodies is also crucial for platelet activation in autoimmune heparin-induced thrombocytopenia.[15] The resulting PF4/IgG immune complexes activate platelets, which release additional PF4 and polyphosphate. Crosstalk of PF4, activated platelets and antibodies with neutrophils subsequently leads to NETosis. Extracellular DNA in NETs binds PF4 and resulting DNA/PF4 complexes further recruit anti-PF4 antibodies with lower avidity,[30,31] which require the polyanion cofactor (DNA).[26] This culminates in massive Fcγ receptor-dependent activation of neutrophils, platelets and, most likely (by analogy with HIT), monocytes and endothelial cells (not shown in the present study; for review[17]).

An important potential natural regulator of this process are extracellular DNases, which degrade NETs. DNase activity in VITT patients with thrombosis was markedly reduced, likely facilitating accumulation of NETs and DNA. Ultimately, ChAdOx1 nCov-19 vaccine-triggered VITT culminates in marked activation of the coagulation system.[4,5,32]

Broadened reactivity of antibodies in a boosted immune response is a hallmark of certain disorders besides VITT. For example, autoimmune heparin-induced thrombocytopenia features heparin-dependent reactivity that extends to include heparin-independent reactivity.[13] Similarly, post-transfusion purpura (PTP)[33] reflects a strong alloimmune response that progresses to include autoreactive properties.[34] In this regard, we identified an array of cell-culture derived human proteins in the vaccine, potentially predisposing to immune reactions against these antigens. If such proteins express a structures, e.g. by a genetic polymorphism not found in the corresponding endogenous protein of the vaccinated individual, a possible strong alloimmune response (with potential for autoreactivity) in a susceptible vaccine recipient should be considered.

Our study has limitations. The detailed specifications of the ChAdOx1 nCov-19 vaccine are not publicly available and potential impact of about 35–40 µg human cell culture proteins per vaccination dose remain to be assessed by the responsible regulatory agencies. Furthermore, we did not analyze the constituents of other adenovirus-based Covid-19 vaccines such as the Covid-19 Vaccine Janssen and the Sputnik V vaccine (these were not available to us). More importantly, quality control of vaccines requires the comprehensive methodological expertise of regulatory agencies.

Currently, we have not investigated the roles of B-cells or T-cells underlying the VITT immune response, nor the role of complement in initiating the immune response or in contributing to subsequent antibody-mediated immunothrombosis.[35] Our PF4 preparation contains a small amount of PF4variant-1 (which is also released by platelets in vivo); we cannot define its role in platelet or granulocyte activation.

We have provided evidence that VITT is not a consequence of antibodies directed against the SARS-CoV-2 spike protein (produced by all vaccines) cross-reacting with PF4 (preprint).[36] Those findings, together with our current study, indicate it is the adenovirus vector-based vaccines that are at risk of inducing VITT through adenovirus and/or other PF4-DNA interactions. The degree of acute inflammatory response induced by the vaccine components appears as an important—potentially remediable—cofactor that could be diminished by reducing impurities and omitting EDTA.

In summary, our study provides a mechanism by which an adenoviral vector vaccine can trigger an immune response leading to highly reactive anti-PF4 antibodies with downstream FcγIIa receptor-dependent amplification recruiting neutrophils and triggering NETosis with prothrombotic consequences.

Declarations

Ethics:

All studies using human material have been approved by the ethics board of the Universitätsmedizin Greifswald; animal studies have been approved by the respective board; the patient who's history is given as example for acute adverse reactions to vaccination has provided informed consent

Author contributions:

AG developed the concept, designed experiments, wrote the manuscript; JW, SH, RP, KA performed the platelet, granulocyte, DLS, ELISA, and some confocal microscopy experiments; ML, KM performed the NMR studies; UV, CH, SM, LSt performed the proteomic studies; LS, KS, TT characterized patients, contributed to the concept and wrote the manuscript; KF performed the electron microscopy studies; MB assessed results, discussed the concept, and wrote the manuscript; SK, LK first noticed impurities in the vaccine, and contributed to the study concept; FS, NE performed the high resolution microscopy; TEW contributed to the concept, discussed results, wrote the manuscript; RKM, CR, TR performed the DNase experiments, NET degradation and mouse edema studies and wrote the manuscript. All authors have critically revised and approved the final version of the manuscript.

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References

1. Human regulatory: COVID-19 vaccines: authorised, European Medicines Agency, 2021, (https://www.ema.europa.eu/en/human-regulatory/overview/public-health-threats/coronavirus-disease-covid-19/treatments-vaccines/vaccines-covid-19/covid-19-vaccines-authorised#authorised-covid-19-vaccines-section), accessed 03/30/2021.
2. Vaxzevria (priviously COVID-19 Vaccine Astra Zeneca): EPAR - Product information, European Medicines Agency, 2021, 04/09/2021 (https://www.ema.europa.eu/en/medicines/human/EPAR/vaxzevria-previously-covid-19-vaccine-astrazeneca#product-information-section), accessed 04/15/2021.
3. AstraZeneca's COVID-19 vaccine: EMA finds possible link to very rare cases of unusual blood clots with low blood platelets, European Medicines Agency, 2021, April 07, 2021 (https://www.ema.europa.eu/en/news/astrazenecas-covid-19-vaccine-ema-finds-possible-link-very-rare-cases-unusual-blood-clots-low-blood), accessed 04/16/2021.
4. Greinacher A, Thiele T, Warkentin TE, Weisser K, Kyrle PA, Eichinger S. Thrombotic Thrombocytopenia after ChAdOx1 nCov-19 Vaccination. New Engl J Med. April 9, 2021 (Epub ahead of print) (https://www.nejm.org/doi/pdf/10.1056/NEJMoa2104840?articleTools=true) (10.1056/NEJMoa2104840).
5. Schultz NH, Sorvoll IH, Michelsen AE, et al. Thrombosis and Thrombocytopenia after ChAdOx1 nCoV-19 Vaccination. N Engl J Med. April 9, 2021 (Epub ahead of print) (https://www.ncbi.nlm.nih.gov/pubmed/33835768) (DOI: 10.1056/NEJMoa2104882).
6. Muir KL, Kallam A, Koepsell SA, Gundabolu K. Thrombotic Thrombocytopenia after Ad26.COV2.S Vaccination. N Engl J Med. Apr 14, 2021 (Correspondence to the Editor) (https://www.nejm.org/doi/full/10.1056/NEJMc2105869?query=featured_coronavirus) (DOI: 10.1056/NEJMc2105869).
7. Sadoff J, Davis K, Douoguih M. Thrombotic thrombocytopenia after Ad26.COV2.S vaccination—response from the manufacturer.. New Engl J Med. April 16, 2021 (Correspondence to the Editor) (https://www.nejm.org/doi/full/10.1056/NEJMc2106075?query=featured_coronavirus) (DOI: 10.1056/NEJMc2106075).
8. Meeting highlights from the Pharmacovigilance Risk Assessment Committee (PRAC) 6–9 April 2021, European Medicines Agency, 2021, (https://www.ema.europa.eu/en/news/meeting-highlights-pharmacovigilance-risk-assessment-committee-prac-6-9-april-2021), accessed 04/12/2021.
9. Krauel K, Potschke C, Weber C, et al. Platelet factor 4 binds to bacteria, [corrected] inducing antibodies cross-reacting with the major antigen in heparin-induced thrombocytopenia Blood 2011;117(4):1370–8.
10. Krauel K, Schulze A, Jouni R, et al. Further insights into the anti-PF4/heparin IgM immune response Thromb Haemost 2016;115(4):752–61.
11. Zheng Y, Zhu W, Haribhai D, et al. Regulatory T Cells Control PF4/Heparin Antibody Production in Mice J Immunol 2019;203(7):1786–1792.
12. Zheng Y, Wang AW, Yu M, et al. B-cell tolerance regulates production of antibodies causing heparin-induced thrombocytopenia Blood 2014;123(6):931–4.
13. Greinacher A, Selleng K, Warkentin TE. Autoimmune heparin-induced thrombocytopenia J Thromb Haemost 2017;15(11):2099–2114.
14. Greinacher A. Heparin-Induced Thrombocytopenia N Engl J Med 2015;373(19):1883–4.
15. Nguyen TH, Medvedev N, Delcea M, Greinacher A. Anti-platelet factor 4/polyanion antibodies mediate a new mechanism of autoimmunity Nat Commun 2017;8:14945.
16. Chong BH. Evolving concepts of pathogenesis of heparin-induced thrombocytopenia: Diagnostic and therapeutic implications Int J Lab Hematol 2020;42 Suppl 1:25–32.
17. Arepally GM, Padmanabhan A. Heparin-Induced Thrombocytopenia: A Focus on Thrombosis. Arterioscler Thromb Vasc Biol 2021;41(1):141–152.
18. Lubenow N, Hinz P, Thomaschewski S, et al. The severity of trauma determines the immune response to PF4/heparin and the frequency of heparin-induced thrombocytopenia Blood 2010;115(9):1797 – 803.
19. Gao X, Kouklis P, Xu N, et al. Reversibility of increased microvessel permeability in response to VE-cadherin disassembly Am J Physiol Lung Cell Mol Physiol 2000;279(6):L1218-25.
20. Jiménez-Alcázar M, Rangaswamy C, Panda R, et al. Host DNases prevent vascular occlusion by neutrophil extracellular traps Science 2017;358(6367):1202–1206.
21. Greinacher A, Gopinadhan M, Gunther JU, et al. Close approximation of two platelet factor 4 tetramers by charge neutralization forms the antigens recognized by HIT antibodies Arterioscler Thromb Vasc Biol 2006;26(10):2386–93.
22. Public Assessment Report Authorisation for Temporary Supply - COVID-19 Vaccine AstraZeneca, solution for injection in multidose container COVID-19 Vaccine (ChAdOx1-S [recombinant]). Medicines & Healthcare products Regulatory Agency. February 22, 2021 (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/963928/UKPAR_COVID_19_Vaccine_AstraZeneca_2 (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/963928/UKPAR_COVID_19_Vaccine_AstraZeneca_2

23. Pfueller SL, Logan D, Tran TT, Bilston RA. Naturally occurring human IgG antibodies to intracellular and cytoskeletal components of human platelets Clin Exp Immunol 1990;79(3):367–73.
24. Chary MA, Barbuto AF, Izadmehr S, Hayes BD, Burns MM. COVID-19: Therapeutics and Their Toxicities J Med Toxicol 2020;16(3):284–294.
25. Kabisch A, Kroll H, Wedi B, Kiefel V, Pralle H, Mueller-Eckhardt C. Severe adverse effects of protein A immunoadsorption Lancet 1994;343(8889):116.
26. Jaax ME, Krauel K, Marschall T, et al. Complex formation with nucleic acids and aptamers alters the antigenic properties of platelet factor 4 Blood 2013;122(2):272 – 81.
27. Khandelwal S, Ravi J, Rauova L, et al. Polyreactive IgM initiates complement activation by PF4/heparin complexes through the classical pathway Blood 2018;132(23):2431–2440.
28. Arepally GM, Cines DB. Pathogenesis of heparin-induced thrombocytopenia Transl Res 2020;225:131–140.
29. Cines DB, Yarovoi SV, Zaitsev SV, et al. Polyphosphate/platelet factor 4 complexes can mediate heparin-independent platelet activation in heparin-induced thrombocytopenia Blood Adv 2016;1(1):62–74.
30. Gollomp K, Kim M, Johnston I, et al. Neutrophil accumulation and NET release contribute to thrombosis in HIT JCI Insight 2018;3(18).
31. Perdomo J, Leung HHL, Ahmadi Z, et al. Neutrophil activation and NETosis are the major drivers of thrombosis in heparin-induced thrombocytopenia Nat Commun 2019;10(1):1322.
32. Scully M, Singh D, Lown R, et al. Pathologic Antibodies to Platelet Factor 4 after ChAdOx1 nCoV-19 Vaccination. N Engl J Med. Apr 16, 2021 (https://www.ncbi.nlm.nih.gov/pubmed/33861525) (10.1056/NEJMoa2105385).
33. Hawkins J, Aster RH, Curtis BR. Post-Transfusion Purpura: Current Perspectives J Blood Med 2019;10:405–415.
34. Bougie DW, Sutton J, Aster RH. Recapitulation of posttransfusion purpura by cross-strain platelet immunization in mice Blood Adv 2020;4(2):287–295.
35. Patel P, Michael JV, Naik UP, McKenzie SE. Platelet FcgammaRIIA in immunity and thrombosis: Adaptive immunothrombosis J Thromb Haemost 2021:Online ahead of print.
36. Greinacher A, Selleng K, Mayerle J, et al. Anti-SARS-CoV-2 Spike Protein and Anti-Platelet Factor 4 Antibody Responses Induced by COVID-19 Disease and ChAdOx1 nCov-19 vaccination. Research Square. 09 Apr, 2021 (Preprint) (https://www.researchsquare.com/article/rs-404769/v1) (DOI: 10.21203/rs.3.rs-404769/v1).

Figures

Figure 1

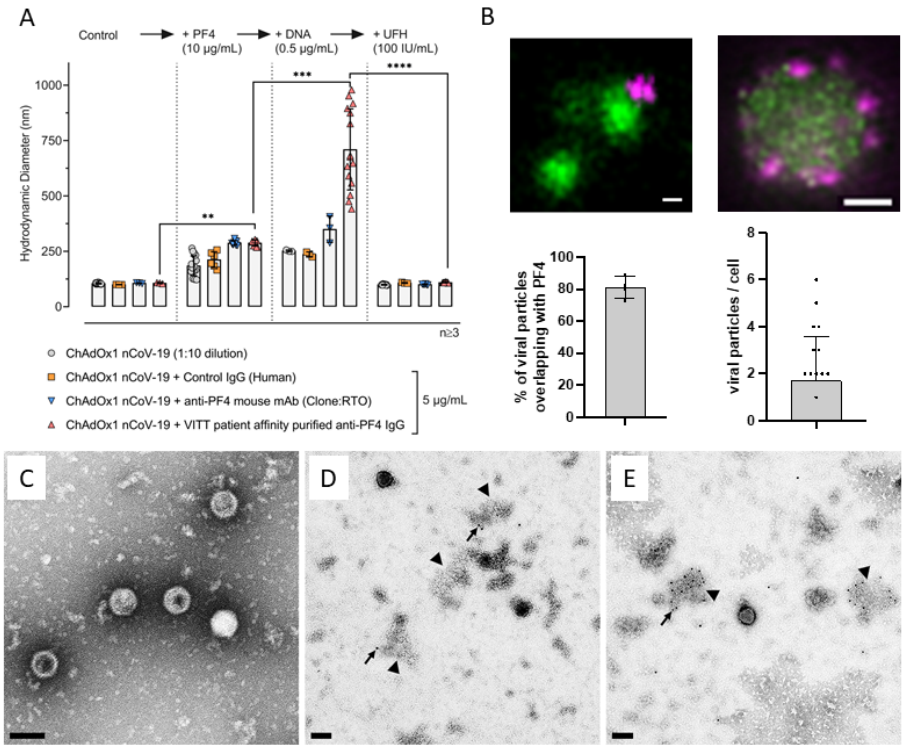

Figure 1

Interaction of PF4 with the vaccine. A) Hydrodynamic diameter of ChAdOx1 nCoV-19 vaccine (1:10 dilution in 0.9% NaCl, 0.4 mg/mL saccharose) was increased by the addition of PF4 (10 µg/mL). Addition of an anti-PF4 monoclonal antibody (Clone RTO) or affinity-purified anti-PF4 IgG from VITT patient sera further increased hydrodynamic diameters of vaccine components. The largest complexes were formed in the presence of DNA. Addition of heparin (100

IU/mL) dissociated formed complexes. Each data point represents 12 runs of n≥3 individual measurements. Statistical assessment by ordinary one-way ANOVA with Sidak's multiple comparisons test. **P < 0.01, ***P < 0.001, ****P < 0.0001. B) 3D-super-resolution microscopy shows binding of PF4 (green) to clusters of proteins of the ChAdOx1 virus (purple; upper left). Particle-/PF4 interaction was analyzed in 8005 aggregates of 4 individual images; 80.6% of particles stained positive for PF4. Upper right, adenoviral protein positive particles (purple) bound to platelets (green). Mean viral density of 1.7 stained particles per platelet. Platelets were incubated with 10 µg/mL PF4 or 1:10 diluted ChAdOx1 nCoV-19 vaccine. Scale: 1 µm. (Further details are given in supplementary material) C) -E) Electron micrograph of aggregates in the vaccine after addition of PF4 (Further details are given in supplementary material). C) vaccine without added PF4 shows no major aggregates but many amorphous small structures; D) aggregates (arrowhead) formed in the vaccine after addition of PF4. The adenovirus capsid protein is labelled. E) aggregates (arrowhead) formed in the vaccine after addition of PF4. Now the biotinylated PF4 is labelled with 10 nm gold particles (arrow):, Bars: 100 nm each.

Figure 2

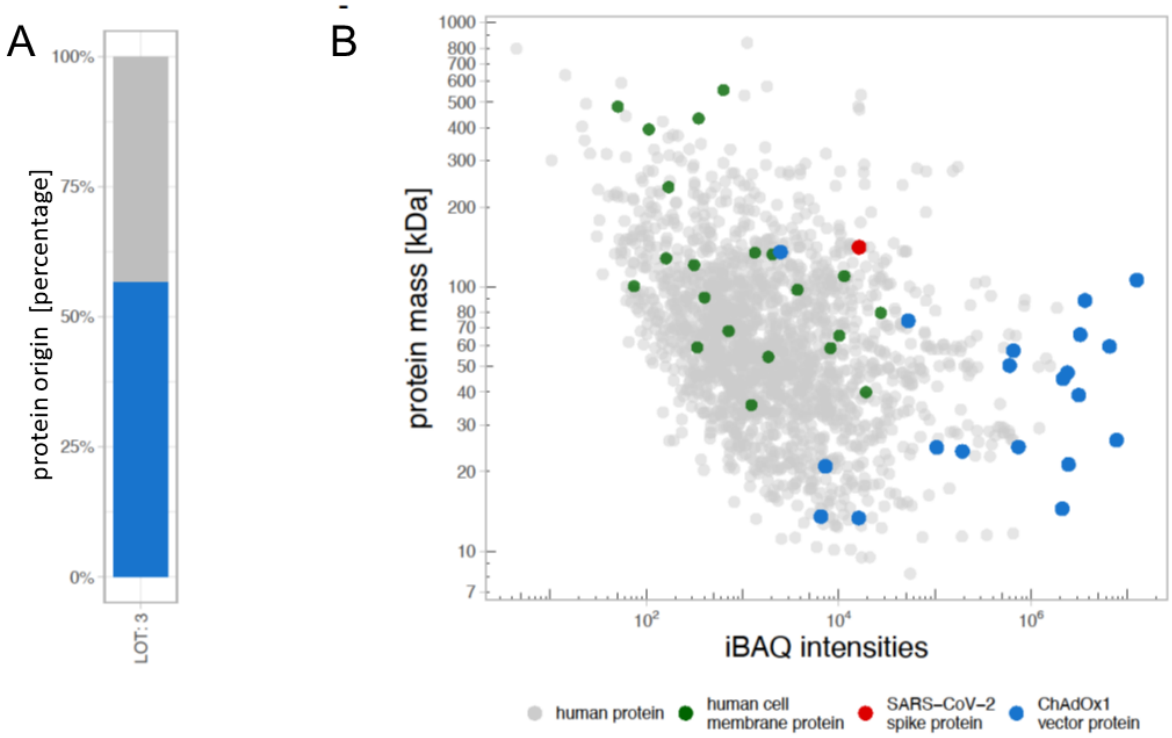

Figure 2

Proteome profiling of ChAdOx1 nCov-19 vaccine components. A) Intensity-based absolute quantification (iBAQ) of human and ChAdOx1 nCov-19 encoded proteins. For vaccine LOT3 iBAQ protein intensities were summed per protein class and relative amounts were calculated and plotted in a stacked bar plot (human proteins are given in grey, ChAdOx1 proteins in blue). B) iBAQ protein intensities and theoretical molecular masses of identified proteins. Protein intensities of vaccine LOT 3 were calculated using the iBAQ algorithm (>3 unique peptides per protein) and plotted against theoretical molecular mass. Proteins were colored according to their class with coding as above in A). Furthermore, green dots yielded human membrane proteins (UniProt annotation) and the single red dot shows the SARS-CoV-2 spike protein.

Figure 3

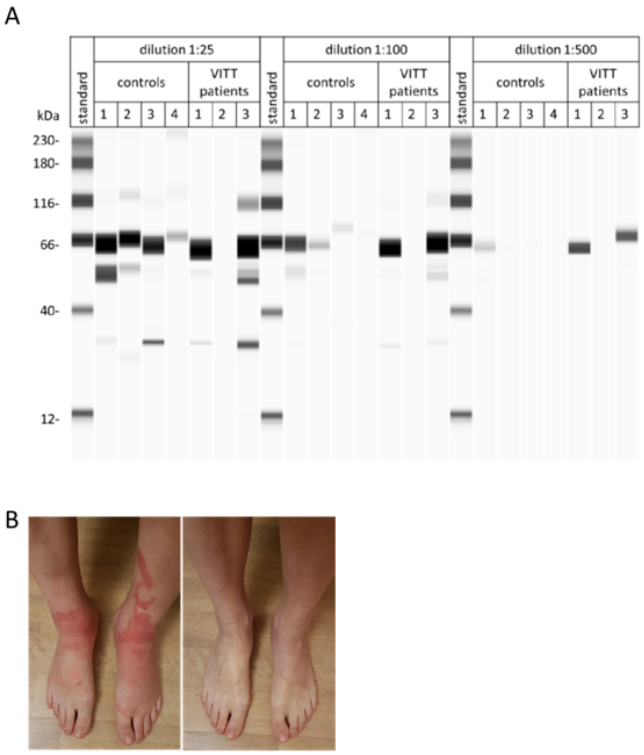

Figure 3

Binding of endogenous antibodies to ChAdOx1 nCov-19 vaccine components and serum sickness-like skin reaction in a vaccinated patient. A) Binding of IgG antibodies to ChAdOx1 nCoV-19 vaccine LOT 3 was determined in a Peggy Sue Simple Western Assay using sera from healthy control individuals and VITT patients at serum dilutions of 1:25, 1:100 and 1:500. Antibody reactivity was present at higher titers in VITT patients. B) Skin reaction after vaccination. Dermal abnormalities were first observed on day 1, reached maximum at day 4, and resolved by day 9. Photographs correspond to days 2 (left) and 14 (right). D-Dimer levels were high at day 4 and at day 6; thereafter symptoms in the legs improved. No test for anti-PF4 antibodies was performed. The text represents the verbatim description (translated to English) in a written log prepared by the vaccine recipient.

Figure 4

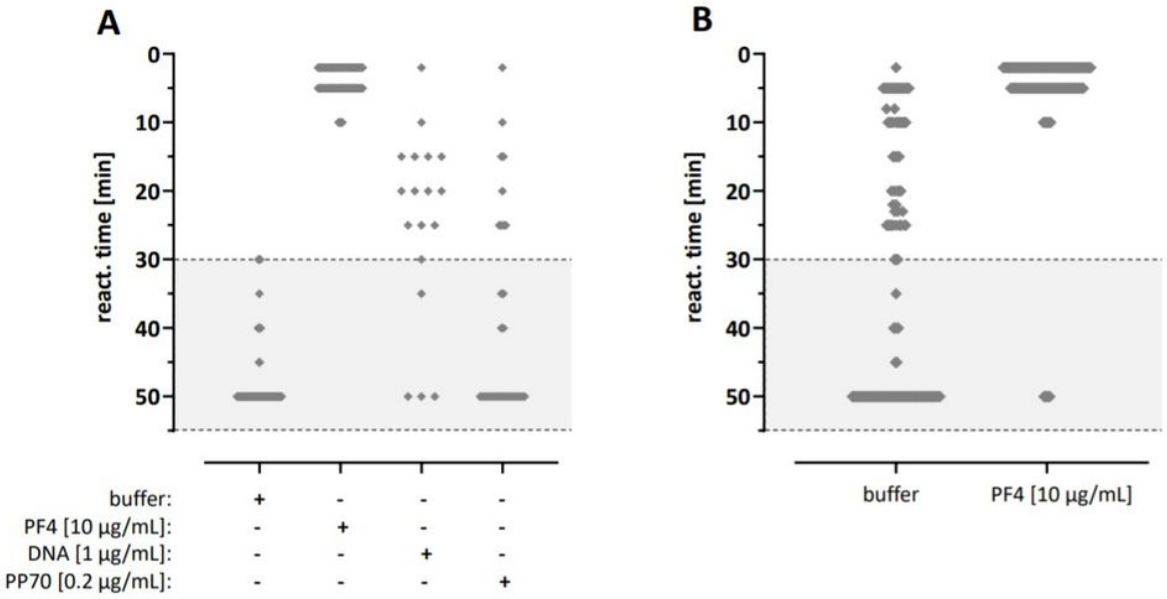

Figure 4

Interaction of VITT sera with platelets. A) Platelet activation assay was performed by adding 20 µL VITT patient serum to 75 µL washed platelets per well of a microtiter plate in the presence of buffer, PF4, DNA, or polyphosphates (PP70), as indicated. Reactivity is expressed semi-quantitatively as “reaction time“: shorter reaction times indicate stronger platelet-activating potency. Reaction time >30 min indicates background or clinically insignificant reactivity. B) VITT sera (n=8) were tested of activating healthy donor platelets in the presence of polyanions (buffer: n=60; PF4: n=78; DNA: n=18; PP70: n=34). For these experiments only sera were evaluated, which did not show activation of donor platelets in the presence of buffer (thus, permitting assessment of cross-reactivity). C) Platelet activation by VITT sera (n=14) in the presence of buffer or 10 µg/mL PF4 (n=121). Donor platelet dependency of reactivity in the absence of PF4 is modulated by currently unknow platelet cofactors.

Figure 5

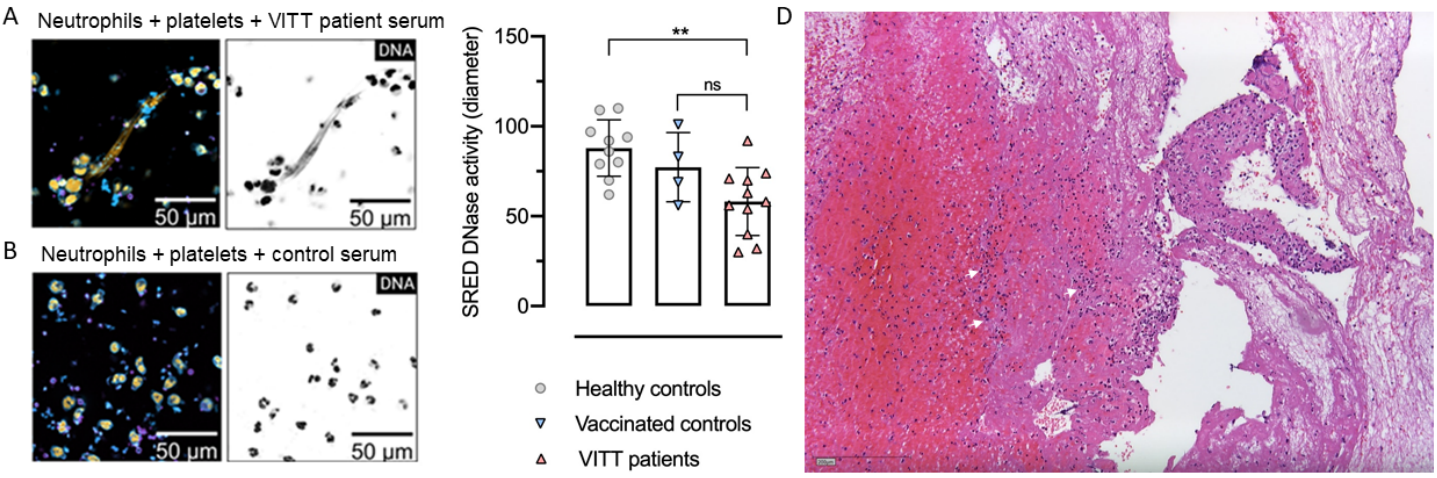

Figure 5

NETs and DNase and granulocyte-rich cerebral vein thrombus in VITT. A) Confocal laser scanning microscopy images of in vitro produced NETs following neutrophil stimulation by VITT patient serum in the presence of platelets. B) Combinations of platelets and healthy control serum did not induce NETosis (Additional controls in Supplementary Figure 15) C) Total DNase activity in VITT patients, vaccinated and healthy control serum samples analyzed by SRED assays. D) Hematoxylin and eosin stained histologic section of a cerebral sinus vein thrombus of a VITT patient shows many granulocytes (arrows indicate three of them). The thrombus was obtained by thrombectomy.

Figure 6

Parallels in Pathogenesis of VITT and autoimmune HIT

	VITT	aHIT
Anti-PF4 immunization (peri-vaccination)		
Antigen formation	PF4 binding to virus proteins and other anionic constituents in the vaccine => PF4/polyanion complexes	PF4 binding to heparin, bacterial polyanions, or DNA => PF4/polyanion complexes
Inflammatory co-signal	Vaccine EDTA-induced vascular leakage. Natural (preformed) antibodies bind to vaccinal viral and human proteins => immune complexes, inflammation, symptoms similar to serum sickness	Tissue trauma or infection Knee replacement surgery (tourniquet) (HIT pathogenesis: surgical > medical patients major > minor surgery)
=> IgG production ≥5 days later		
Immuno-thrombosis (≥5 days post-vaccination)	High-avidity anti-PF4 IgG autoantibodies induce platelet activation via FcγIIa receptors Anti-PF4 IgG-induced granulocyte activation =>NETosis (with DNase deficiency)	High-avidity anti-PF4 IgG autoantibodies induce platelet activation via FcγIIa receptors (no heparin needed) Anti-PF4 IgG-induced granulocyte activation =>NETosis (with DNase depletion)
Thrombosis	Polyanion-dependent and -independent anti-PF4 antibodies bind to PF4/DNA complexes in NETs => amplification	Polyanion-dependent and -independent anti-PF4 antibodies bind to PF4/DNA complexes in NETs => amplification

Figure 6

Parallels in Pathogenesis of VITT and autoimmune HIT

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FVMechanismofVITTSupplAppendix19042021.pdf
- FVSupplementaryTableS4iBAQproteinintensities.xlsx