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Received: October 5, 2021.
Accepted: January 5, 2022.

Citation: Stephan Michalik, Florian Siegerist, Raghavendra Palankar, Kati Franzke, Maximilian Schindler, Alexander Reder, Ulrike Seifert, Clemens Cammann, Jan Wesche, Leif Steil, Christian Hentschker, Manuela Gesell-Salazar, Emil Reisinger, Martin Beer, Nicole Endlich, Andreas Greinacher, and Uwe Völker. Comparative analysis of ChAdOx1 nCoV-19 and Ad26.COV2.S SARS-CoV-2 vector vaccines.

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Comparative analysis of ChAdOx1 nCoV-19 and Ad26.COV2.S SARS-CoV-2 vector vaccines

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Competing interest statement: Andreas Greinacher reports personal fees and non-financial support from Aspen, grants from Ergomed, grants and non-financial support from Boehringer Ingelheim, personal fees from Bayer Vital, grants from Rovi, grants from Sagent, personal fees from Chromatec, personal fees and non-financial support from Instrumentation Laboratory, grants and personal fees from Macopharma, grants from Portola, grants from Biokit, personal fees from Sanofi-Aventis, grants from Fa. Blau Farmaceutics, grants from Prosensa/Biomin, grants and other from DRK-BSD NSTOB, grants from DRK-BSD Baden-Württemberg/Hessen, personal fees and non-financial support from Roche, personal fees from GTH e.V. In addition, Andreas Greinacher reports having a patent, Application no. 2021032220550000DE, pending.
Running heads: Comparative analysis of SARS-CoV-2 vector vaccines

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Data sharing statement: The mass spectrometry proteomics data have been deposited to ProteomeXchange (dataset identifier PXD027344).

Word count: 3344

Acknowledgments: We thank Katrin Schoknecht for support in the proteomics analyses, Mandy Jörn for graphical design of EM micrographs. The study has been funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) grants: 374031971 - A06 and A11-TRR240, 398967434 - SFB/TR261, A11 - SFB877, P6 - KFO306, B8 - SFB841, and INST 2026/13-1 FUGG, the Ministerium für Wirtschaft, Arbeit und Gesundheit Mecklenburg-Vorpommern (project COVIDPROTECT), „Structure and Function of the Proteasome System in Platelets“ GR2232/8_1 and SE 885/2-1 (DFG, German Research Foundation), Leibniz WissenschaftsCampus – ComBioCat – W10/2018, by the Federal Ministry of Education and Research (BMBF, grant 01GM1518B, STOP- FSGS), the Südmeier fund for kidney and vascular research (“Südmeier-Stiftung für Nieren- und Gefäßforschung”), the Dr. Gerhard Büchtemann fund, Hamburg, Germany and the PeNe_C19 study by the Ministerium für Wirtschaft, Arbeit und Gesundheit Mecklenburg-Vorpommern.
Abstract

Vector-based SARS-CoV-2 vaccines have been associated with vaccine-induced thrombosis with thrombocytopenia syndrome (VITT/TTS), but the causative factors are still unresolved. We comprehensively analyzed ChAdOx1 nCoV-19 (AstraZeneca) and Ad26.COV2.S (Johnson & Johnson). ChAdOx1 nCoV-19 contains significant amounts of host cell protein impurities, including functionally active proteasomes, and adenoviral proteins. In Ad26.COV2.S a much lower amount of impurities was found. Platelet factor 4 (PF4) formed complexes with ChAdOx1 nCoV-19 constituents, but not with purified virions from ChAdOx1 nCoV-19 or with Ad26.COV2.S. Vascular hyperpermeability was induced by ChAdOx nCoV-19 but not by Ad26.COV2.S. These differences in impurities together with EDTA-induced capillary leakage might contribute to the higher incidence rate of VITT associated with ChAdOx1 nCoV-19 compared to Ad26.COV2.S.
Introduction

Vaccination is key for the control of the SARS-CoV-2 pandemic. Adenoviral vector-, mRNA encapsulated in lipid nanoparticles-, and antigen-based vaccines are currently in use, all encoding the spike protein.\(^1\)\(^2\) Since February 2021 the rare but severe adverse reaction of vaccine-induced immune thrombotic thrombocytopenia (VITT; synonym thrombosis with thrombocytopenia syndrome (TTS)) has been observed in individuals vaccinated against SARS-CoV-2. VITT/TTS occurs 5-20 days (occasionally later) after vaccination with the ChAdOx1 nCoV-19 vaccine (AstraZeneca) and the Ad26.COV2.S vector vaccine (Janssen / Johnson & Johnson). The incidence rate of VITT/ TTS seems to be higher for ChAdOx1 nCoV-19. In the United States, 0.355 cases of VITT/TTS per 100,000 people have been reported for Ad26.COV2.S\(^3\), compared to 1 per 50,000 - 100,000 people vaccinated with ChAdOx1 nCoV-19 in the UK.\(^4\) In Germany both vaccines were used and within this medical system, 0.56 suspected cases per 100,000 vaccine doses for Ad26.COV2.S (3,186,297 vaccine doses administered) and 1.49 suspected cases per 100,000 vaccine doses for ChAdOx1 nCoV-19 (12,692,700 vaccine doses administered) were reported.\(^5\) TTS/VITT involves high-affinity, platelet-activating anti-platelet factor 4 (PF4) antibodies\(^6\)-\(^8\), but the mechanisms triggering these anti-PF4 antibodies are still unresolved. VITT/TTS shows striking similarities with another PF4-mediated adverse drug effect, heparin-induced thrombocytopenia (HIT) and autoimmune HIT. In HIT, polyanions form complexes with PF4, inducing a conformational change, which triggers anti-PF4 antibodies. This immune response is pronounced in patients with tissue trauma and inflammation. We have shown that one or more constituents of the ChAdOx1 nCoV-19 vaccine interact with PF4, forming complexes which contain PF4 and the adenovirus hexon protein. This might trigger conformational changes in the positively charged chemokine PF4 leading to the formation of a neoantigen and then subsequent activation of B-cells in a pro-inflammatory environment.\(^9\) These activated B-cells then produce high avidity anti-PF4 antibodies that bind PF4 and trigger an activation cascade of platelets and granulocytes, leading to NETosis and massive thrombin production. However, it is not known which vaccine components, beside the hexon protein, interact with PF4 and which additional factors influence this interaction.

Both vaccines (ChAdOx1 nCoV-19 and Ad26.COV2.S) are produced in human cell lines, T-REx-293 cells (human embryonic kidney cell, a HEK293 derivate) for ChAdOx1 nCoV-19 and PER.C6 TetR cells (human embryonic retinal cells) for
Ad26.COV2.S, respectively. We and others have previously shown that the vaccine ChAdOx1 nCoV-19 contains a large number of host cell proteins (HCP). Here we report the results of a comprehensive, comparative analysis of the ChAdOx1 nCoV-19 and Ad26.COV2.S vaccines, using proteomics, transmission electron microscopy, dynamic light-scattering, single-molecule light microscopy, and an in vivo capillary leakage assay.

Our data reveal substantial differences in composition and functional properties between both vaccines, which may contribute to the different incidences of TTS/VITT.

Methods

Comprehensive Material and Methods are described in the Supplementary Material. All experiments were performed in accordance with local and national ethics standard and German animal protection law overseen by the “Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Rostock” of the federal state of Mecklenburg - Western Pomerania.

Sample preparation and LC-MS/MS analysis.

Vaccines were precipitated using salt-acetone precipitation. Adenovirus particles were purified using subsequent sucrose-cushion and sucrose-gradient ultracentrifugation. Protein was digested with trypsin as described by Blankenburg et al. LC-MS/MS experiments were performed on an Orbitrap ExplorisTM 480 mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to an UltimateTM 3000 RSLCnano HPLC (Dionex/ Thermo Scientific, Waltham, MA, USA). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE18 partner repository with the dataset identifier PXD027344.

SDS gel and Western blot analysis

For protein separation, one-fiftieth of one vaccine dose, as well as dilutions of HEK293 total protein lysate, were loaded to a precast NuPAGE™ 4 to 12 % gel. Electrophoresis was performed at 150 V. The Western blots were prepared using the Trans-Blot Turbo Transfer System from BioRad and the transfer of proteins to the PVDF membrane was verified and documented using LICOR's Revert Total Protein Stain protocol (Doc # 988-19494). The detection of the specific proteins was per-
formed with primary and secondary antibodies described in the Supplementary Materials and Methods.

**Proteasome activity assays**

Chymotrypsin-like activity was assessed in vaccine or HEK293T cell lysate using 0.2 mM fluorescently tagged Suc-LLVY-AMC (Bachem, Bubendorf, Switzerland) quantified with a fluorometer using a 380/460 nm filterset. To confirm proteasomal activity 100 nM Bortezomib\(^{14}\) or 200 nM Carfilzomib (Selleckchem, Houston, US)\(^{15}\), was added.

**Dynamic light scattering and zeta potential**

All dynamic light scattering (DLS) measurements were performed in a fixed scattering angle Zetasizer Nano-S system (Malvern Instruments Ltd., Malvern, UK). The hydrodynamic diameter (nm) was measured at 25°C, and light scattering was detected at 173°. Surface zeta potential was performed in folded capillary zeta cell (DTS1070, Malvern Instruments Ltd., Malvern, UK). Data analysis was performed using Zetasizer software, Version 7.13 (Malvern Instruments Ltd., Malvern, UK).

**Immunoelectron and transmission electron microscopy**

Vaccines or the purified adenovirus particles were incubated with biotinylated PF4 and transferred to formvar-coated TEM grids. After washing, samples were labeled with an anti-Adenovirus mAb detected by a gold conjugate. Same samples were labeled with a streptavidin-gold conjugate to detect PF4-biotin. Grids were stained with 1% phosphotungstic acid and analyzed with a Tecnai-Spirit transmission electron microscope (FEI, Eindhoven).

**Super Resolution Single Molecule Light Microscopy**

Diluted vaccine or purified virions were incubated with human PF4 and immobilized on cleaned coverslips. After fixation and blocking, PF4 and adenoviral hexon were visualized using secondary (PF4) and primary (hexon) immunofluorescence detected by Alexa Fluor 488 and Cy5. Coverslips were mounted in Everspark dSTORM buffer (Idylle Labs, France)\(^{16}\) and blinking sequences imaged on a Zeiss Elyra PS.1 super resolution system. SMLM data was processed in FIJI using NanoJ core\(^{17}\) and Thunderstorm\(^{18}\) and analyzed using custom FIJI\(^{19}\) scripts.
Zebrafish vascular permeability assay

A novel zebrafish-based in vivo assay was developed to determine local changes of vascular permeability following intramuscular injections: Transgenic zebrafish at 5 days past fertilization expressing a 78 kDa GFP-tagged plasma protein\textsuperscript{20,21} were injected intramuscularly with 1 nl of native vaccine, purified virions, 100 µM EDTA or 0.9% NaCl. Fluorescence intensity ratios (intravascular vs. intramuscular) were measured in the direct vicinity of the injection site at t=0 and t=10 min.

Results

Comparative profiling of ChAdOx1 nCoV-19 and Ad26.COV2.S vaccines

Comparative profiling of ChAdOx1 nCoV-19 and Ad26.COV2.S (three different lots each) consistently revealed significant differences: i) the total protein concentration of the ChAdOx1 nCoV-19 vaccine was approximately 3.4-times higher than that of the Ad26.COV2.S vaccine (mean: 102 ng/µl vs. 29.8 ng/µl) (Figure 1A), ii) silver-nitrate staining of SDS-PAGE separated vaccines displayed a markedly more complex protein pattern than expected for pure virions for ChAdOx1 nCoV-19 compared to Ad26.COV2.S (Figure 1B). iii) mass spectrometric analysis (Supplementary Table 1) identified a much higher proportion (44.5 % to 59.2 % vs. only 0.26 % to 0.96 %; Figure 1D, Supplementary Figure 1, 2) and number (N\textsubscript{ChAdOx1 nCoV-19} = 1571±31 vs. N\textsubscript{Ad26.COV2.S} = 59±14; two-sided t-test p-value = 8.709e-06; Supplementary Figure 2) of host cell-derived human proteins (HCPs). A dilution series of a laboratory HEK293 cell line lysate, which was used instead of the cell line used for vaccine production, confirmed the quantities of HCPs (54 % for ChAdOx1 nCoV-19 and 1.5 % for Ad26.COV2.S; Supplementary Figure 3). None of the top 10 most abundant human proteins in ChAdOx1 nCoV-19 was detected in Ad26.COV2.S (Supplementary Table 2). Adenoviral proteins comprised 40.8 - 55.5 % (ChAdOx1 nCoV-19) and 99.04 - 99.74 % (Ad26.COV2.S) of total ion intensity, while the SARS-CoV-2 spike protein was detected only in the ChAdOx1 nCoV-19 vaccine (n = 3 different lots; Supplementary Figure 1). Western blot analysis confirmed the significant abundance of eleven selected HCP-proteins in the ChAdOx1 nCoV-19 vaccine, which even exceeded the abundances detected in the HEK293 cell line. None of these proteins was detected in
the Ad26.COV2.S vaccine (Figure 1C, Supplementary Figure 4).

In summary, per vaccine dose (500 µl) of ChAdOx1 nCoV-19 we detected 19.1 - 33.8 µg HCP and 23.3 - 26.3 µg chimpanzee adenovirus proteins and for the Ad26.COV2.S vaccine 0.04 - 0.19 µg HCP and 10.2 - 19.2 µg adenoviral proteins. Since the ca. 5x10e10 virions per vaccine dose weigh about 12.5 µg, both vaccines contain unassembled virus proteins, mostly hexon proteins. However, the amount of approximately 10-14 µg unassembled virus proteins in ChAdOx1 nCoV-19 is again much larger compared to 0-6.5 µg in Ad26.COV2.S.

Proteasome activity in the different vaccines

Proteasome subunits were identified by mass spectrometry and verified by Western blot analysis (Figure 2A, Supplementary Figure 5). Chymotrypsin-like activity associated with the proteasomal beta-5 subunit showed lot-dependent high levels in ChAdOx1 nCoV-19, while in Ad26.COV2.S only minimal proteasome activity was found in one of three lots (Figure 2B and C). Substrate turnover varied in the different lots according to the proteasome subunit expression level (Figure 2A and C). Inhibition of the proteasome activity by 100 nM bortezomib\(^\text{14}\) or 200 nM carfilzomib\(^\text{15}\) confirmed assay specificity (Supplementary Figure 6).

PF4-vaccine cluster formation

PF4 is the key protein involved in the immune response causing VITT. We assessed the interaction of PF4 with the native vaccines and the purified adenoviral particles of ChAdOx1 nCoV-19, which were obtained by sucrose cushion and gradient ultracentrifugation. The purity of isolated ChAdOx1 nCoV-19 virions was confirmed by transmission electron microscopy (TEM) and silver-staining of 1D SDS-PAGE (Supplementary Figure 7 and 8).

DLS confirmed PF4-induced clustering of the non-purified ChAdOx1 nCoV-19 vaccine (Figure 3A, left panel). The hydrodynamic diameter increased from 88 ± 2.4 nm up to 151 ± 12 nm and 320 ± 45 nm with 10 µg/mL and 50 µg/mL PF4, respectively (Figure 3A, PF4 dose-dependent size change is shown in Supplementary Figure 9). This complex formation was reversible upon the addition of unfractionated heparin (UFH) and can be attributed to weakened electropositive surface potential of PF4 in the presence of highly negatively charged UFH thus decreasing its ability to electrostatically interact with vaccine components. In contrast, the addition of PF4 (50
only marginally increased the particle size when incubated with purified virions from ChAdOx1 nCoV-19 (from 79.6 ± 10.3 nm to 86.7 ± 6.22 nm; p=0.2404) (Figure 3A, middle panel) or the Ad26.COV2.S vaccine (from 85.7 ± 2.2 nm to 91.3 ± 2.83 nm; p=0.0620) (see particle size-frequency distribution plots for Supplementary Figure 10).

Complex formation of PF4 with the ChAdOx1 nCoV-19 vaccine was charge-dependent, as ChAdOx1 nCoV-19’s negative charge (zeta potential -27.5 ± 4.7) was neutralized by PF4. In comparison, both, purified ChAdOx1 nCoV-19 virions (zeta potential -1.7 ± 4.7) and the untreated Ad26.COV2.S vaccine (zeta potential -4.5 ± 5.7) showed only a minimal negative charge (Figure 3B).

Consistent with DLS and as described before⁶, PF4 induced the formation of electron-dense aggregates with ChAdOx1 nCoV-19 (Figure 4A), which contained unassembled hexon protein (Supplementary Figure 11). In contrast, no comparable aggregates were detected after incubation of PF4 with purified virions from ChAdOx1 nCoV-19 (Figure 4B) or the Ad26.COV2.S vaccine (Figure 4C).

PF4 single-molecule density analysis using single-molecule localization microscopy (SMLM; Supplementary Figure, 12 and 13) revealed that PF4 clusters formed on or in close vicinity of ChAdOx1 nCoV-19 adenoviral hexon proteins (PF4 single-molecule density ratio 7.09±1.38, Figure 4D and G, arrowheads in Supplementary Figure 12), but not on Ad26.COV2.S (1.13±0.14, p<0.0001) or purified ChAdOx1 nCoV-19 virions (2.33±0.44, p=0.0115, mean±SEM, Figure 4D-G; p-values refer to comparison with ChAdOx1 nCoV-19).

DLS experiments with ultracentrifugation-separated virions from the vaccines and the resulting supernatant confirmed the single-molecule density analysis of PF4 binding (Figure 5). Electron microscopy analysis revealed absence of intact virions in the supernatant fraction of both ChAdOx1 nCoV-19 and Ad26.COV2.S, while the pellet was enriched in virions (Figure 5A and 5B). Intriguingly, we observed amorphous electron-dense irregularly shaped particulate material in ChAdOx1 nCoV-19 supernatant that was absent in Ad26.COV2.S. No significant PF4 dependent complex formation was detected by DLS with the pellet fractions of ChAdOx1 nCoV-19 and Ad26.COV2.S, but the supernatant fraction of ChAdOx1 nCoV-19 showed clear PF4 complex formation potential (Figure 5B). However, it is important to note that under in vitro conditions, PF4 binds to both chimpanzee adenovirus Y25 (ChAdOx1) and human adenoviruses (HAdV-D26 and HAdV-C5) through weak electrostatic interactions.
that was abrogated in the presence of fondaparinux, an heparin pentasaccharide.\textsuperscript{22} Proteomic analysis showed that the HCP content in the pellet fraction was reduced compared to the non-fractionated vaccine and a large proportion of the ChAdOx1 nCoV-19 HCPs was located in the supernatant fraction. As expected, the viral proteins were enriched in the pellet fraction (Figure 5E, Supplementary Figures 14-16).

**ChAdOx1 nCoV-19 induced vascular hyperpermeability**

To study the effect of the two vaccines and EDTA (100 µM present in ChAdOx1 nCoV19 vaccine) on vascular permeability, we used \textit{in vivo} microscopy of transgenic zebrafish larvae expressing an eGFP-tagged plasma protein (gc-eGFP, 78kDa).\textsuperscript{20} Intramuscular injections (Supplementary Video 1) of 1 nL of 100 µM EDTA or ChAdOx1 nCoV-19 locally increased vascular permeability indicated by leakage of eGFP from the intravascular to the intramuscular compartment (p=0.0001) that was not observed after injection of purified ChAdOx1 nCoV-19 virions, Ad26.COVID.2.S or physiological saline (Figure 6).

**Discussion**

Our comprehensive analyses revealed major differences between ChAdOx1 nCoV-19 and Ad26.COVID.2.S vaccines. Confirming our previous observation, a high proportion of HCP (54 %) was found in the ChAdOx1 nCoV-19 vaccine, but only a very low level of HCP was found in Ad26.COVID.2.S (1.5 %). This observation suggests very different purification approaches and purification efficiencies for the two vaccines, with a more thorough purification of adenoviruses in Ad26.COVID.2.S. Such differences might be caused by the use of detergent treatment of the infected production cell culture for ChAdOx1 nCoV-19, which will make subsequent purification strategies more complicated.\textsuperscript{23} The SARS-CoV-2 spike protein was detected only in the ChAdOx1 nCoV-19 vaccine. This may be due to several reasons, since different cell lines and procedures were used for production and purification of the two vaccines, respectively. Both suppliers use systems in which the TetR repressor suppresses the expression of the SARS-CoV-2 transgene during the production of the recombinant adenoviral vectors. A different degree of leakage of repression might occur in the cell lines thus allowing different residual expression of the spike protein. Another reason could be depletion of
the spike protein during purification of Ad26.COV2 production. This can only be differentiated by in-process sampling at different production steps of the vaccines. The two vaccines display differences in their ability to interact with PF4. We confirm the previously observed complex formation of PF4 with ChAdOx1 nCoV-19. Consistent with a recent study using cryo-electron microscopy of ChAdOx1 nCoV-19\textsuperscript{22}, also in our study addition of PF4 to purified ChAdOx1 nCoV-19 virions or to Ad26.COV2.S resulted in a slight increase in particle size. This was reversed by addition of heparin, indicating charge-related binding of PF4 to the virions. This is again consistent with the data obtained by cryo-electron microscopy and \textit{in silico} modelling of ChAdOx1 nCoV-19, which established a possible electrostatic interaction of positively charged PF4 and negatively charged adenovirus hexon polypeptide.\textsuperscript{22} However, the interaction Kd of about 300 nmol was rather weak. This is likely the reasons that we could not demonstrate distinct complexes of PF4 with Ad26.COV2.S or purified ChAdOx1 nCoV-19 virion preparations. This further supports a role of impurities in ChAdOx1 nCoV-19 for the observed formation of large complexes in ChAdOx1 nCoV-19 after addition of PF4.\textsuperscript{9} We have shown that unassembled adenoviral hexon proteins are part of the PF4-complexes, but we can still not exclude contribution of additional constituents of the vaccine-supernatant.

Antibody formation against PF4 is enhanced by antigen presentation in an inflammatory environment. Recently, we have shown (collaboration with the laboratory of Prof Thomas Renne, Universitätsmedizin Hamburg Eppendorf, Germany) that intradermal injection of ChAdOx1 nCoV-19 leads to EDTA-induced capillary leakage in the Mile’s skin edema assay, thus increasing the vaccine’s intravascular distribution.\textsuperscript{9} Our zebrafish model allows intramuscular injections, which recapitulates the actual mode of vaccination. EDTA and ChAdOx1 nCoV-19 but not Ad26.COV2.S rapidly induced local vascular hyperpermeability. Such an increase of local capillary leakage might facilitate direct contact of the immune system with vaccine components, as does accidental intravascular administration of the vaccine.\textsuperscript{24} Inflammation early after vaccination may also be enhanced when HCP are recognized by endogenous natural IgG.\textsuperscript{25} These natural antibodies bind proteins of degrading cells and can form immune complexes.

Furthermore, proteasome activity was detectable in both vaccines, again with a remarkable difference between Ad26.COV2.S (only low proteasome activity and protein abundance in one lot) compared to substantially higher proteasome activities in
almost all lots of ChAdOx1 nCoV-19. This is of particular interest since Hauler et al. showed that adenoviral capsid proteins are intracellularly processed by proteasomal degradation which is mediated by the chaperone p97/VCP. In our proteomic analysis, we identified VCP as one of the top 5 most abundant proteins of the ChAdOx1 nCoV-19 vaccine (Supplementary Table 1, Supplementary Table 2). Proteasomal degradation of adenoviral components such as the hexon polypeptide and/or HCPs might lead to a reduction in vaccine efficiency. Whether proteasomal activity may also create potentially immunogenic or immunoreactive neoantigens remains unresolved.

VITT/TTS clinically occurs after ChAdOx1 nCoV-19 and Ad26.COV2.S and is mediated by platelet-activating anti-PF4 antibodies. Recently, we and others have shown that anti-spike protein antibodies and anti-PF4 antibodies react independently of each other, therefore making it unlikely that VITT is caused by cross-reacting anti-spike protein antibodies. The current data indicate that adenovirus particles and free hexon proteins are the common features of both vaccines. We only observed formation of larger complexes of PF4 with ChAdOx1 nCoV-19. This indicates that an additional cofactor is needed. This cofactor is present in the supernatant of the ChAdOx1 nCoV-19 vaccine. However, this does not exclude complex formation of PF4 and Ad26.COV2.S in vivo. Several studies have shown the interaction of different adenoviruses with platelets and on the platelet surface they may also interact with PF4. In addition, hexon proteins or the virions may form complexes with PF4 when they come into contact with additional lymphatic or plasma proteins. Beyond VITT/TTS, the potential for alloantibody formation by protein contaminants in vaccines might be a matter of concern since the PregSure® vaccine, used in cattle, induced alloantibody driven bovine neonatal pancytopenia (BNP). BNP is a vaccine-induced alloimmune disease that was observed in young calves of PregSure® vaccinated cows and is characterized by hemorrhage, pancytopenia, and severe destruction of hematopoietic tissue. The cows of affected calves had alloantibodies in their plasma, which were likely induced by alloantigen-expressing protein contaminants of the vaccine from the bovine kidney cell line used for vaccine production. Alloantibodies will only cause clinical effects in case of pregnancy or organ transplantation. Systematic screening of vaccinated individuals excluding or confirming such alloantibodies should be performed to clarify whether this theoretical concern requires further measures. Limitations of our study include its in vitro design. Further,
our findings do not exclude the contribution of certain cofactors (e.g., within the interstitial fluid, lymphatic system, plasma, or cell surfaces) for inducing the anti-PF4 immune response. We also did not investigate involvement of B-cell and T-cell populations. Moreover, since both adenoviral vector-based vaccines have been discontinued in Germany, we cannot compare the immune responses among vaccinated individuals. Finally, the lack of a suitable in vivo model for induction of VITT/TTS limits the ability to reach definite conclusions regarding in vivo mechanisms. In summary, we show that process-related impurities in the form of HCP, active proteases and unassembled hexon proteins differ in quality and quantity between ChAdOx1 nCoV-19 and Ad26.COV2.S SARS-CoV-2 vaccines. EDTA-induced capillary leakage and HCP impurities might further facilitate induction of an anti-PF4 immune response by intravascular translocation of vaccine constituents and induction of an early inflammatory response after vaccination. These factors might explain the higher incidence rate of VITT/TTS for ChAdOx1 nCoV-19 compared to Ad26.COV2.S vaccines. However, the authors would like to point out again that only the comprehensive vaccination of the human population can effectively contain the SARS-CoV-2 pandemic.

References


Figure legends

Figure 1: Analysis of the protein composition of ChAdOx1 nCoV-19 and Ad26.COV2.S vaccines (A) Determination of the protein concentration of the two vaccines (3 different lots each). Protein concentration was determined with a quantitative bicinchoninic acid (BCA) assay. Protein concentration per 500 µl (vaccination dose) and 1 µl vaccine (secondary axis) of 3 lots of ChAdOx1 nCoV-19 or Ad26.COV2.S vaccine, respectively, are shown. Statistical testing was performed using a two-sided t-test. (B) Protein patterns of silver nitrate-stained SDS-PAGE of ChAdOx1 nCoV-19 or Ad26.COV2.S (3 lots each) vaccines along with a dilution series of a laboratory HEK293 cell line extract for comparison. HEK293 cell extract was loaded onto the gel at 1.5, 1.0, 0.5, or 0.25 µg per lane, and 10 µL (1/50th of a vaccine dose) were used for each vaccine. (C) Western blot analysis of HSP90-alpha protein, using the same gel loading scheme as for the silver nitrate-stained gel. (D) iBAQ protein intensities and theoretical molecular mass of identified proteins. Protein intensities of ChAdOx1 nCoV-19 or Ad26.COV2.S (exemplarily shown for lot 3) were calculated using the intensity-based absolute quantification (iBAQ) algorithm (minimum of three unique peptides per protein) and plotted against the theoretical molecular mass. Proteins are color-coded according to their respective class. Blue dots indicate vector proteins; gray dots represent human proteins; the red dot indicates the SARS-CoV-2 spike protein. Points highlighted with a cross indicate proteins additionally analyzed by Western blotting in Supplementary Figures 4-7.

Figure 2: Analysis of proteasome proteins and activity in the vaccines: (A) Western blot analysis of proteasome 20S subunit beta 5 (original Western blot image is provided in Supplementary Figure 4). (B) 50 µl (1/10th of vaccination dose) of different ChAdOx1 nCoV-19 (n=5) and Ad26.COV2.S (n=3) lots were analyzed for the chymotrypsin-like activity of the proteasome and compared with the activity of 0.25 µg of HEK293 cell lysate (Mean±SD of two technical replicates are shown). (C) Proteasomal activity was confirmed by inhibition with 100 nM Bortezomib. The mean of two technical replicates is shown.

Figure 3: DLS analysis of vaccine-induced PF4 clustering: Analysis of ChAdOx1 nCoV-19 vaccine, purified ChAdOx1 nCoV-19 virions and Ad26.COV2.S vaccine by dynamic light scattering (DLS) (A). The hydrodynamic diameter (mean ± SD, n=9) of ChAdOx1 nCoV-19 or Ad26.COV2.S particles before and after addition of 10 µg/ml or 50 µg/ml PF4 was determined. A dose-dependent increase in the size of ChAdOx1
nCoV-19 aggregates in the presence of PF4 was detected. This effect was markedly reduced in both purified ChAdOx1 nCoV-19 virions and Ad26.COV2.S. Addition of unfractionated heparin (1 IU/ml) dissociated complexes between PF4 and vaccine components. (B) The ζ-potential (mean ± SD, n=9) of ChAdOx1 nCoV-19 was lower than the purified ChAdOx1 nCoV-19 virions or of Ad26.COV2.S; and largely neutralized by PF4. In the presence of UFH, a charge reversal to net negative charge was observed in both vaccines and purified virions from ChAdOx1 nCoV-19 vaccine. Statistical analysis was performed by one-way ANOVA on ranks/Kruskal-Wallis followed by correction for multiple comparison by two-stage Benjamini, Krieger, & Yekutieli controlling the false discovery rate procedure. n=9 and alpha <0.05 was considered significant.

**Figure 4: Ultrastructural analysis of vaccine-induced PF4 clustering.** Panel (A)-(C) show representative micrographs of streptavidin-gold immunoelectron microscopy (arrow); biotinylated, human PF4 was incubated with either ChAdOx1 nCoV-19 (A), purified virions from ChAdOx1 nCoV-19 (B) or Ad26.COV2.S (C). Virions are exemplary labeled by asterisks, immunogold labeled aggregates by arrowheads; scale bars represent 200 nm. SMLM images in (D)-(F) show representative dual PF4 and hexon polypeptide reconstructions after incubation with either ChAdOx1 nCoV-19 (D), purified virions from ChAdOx1 nCoV-19 (D) or Ad26.COV2.S (F). As indicated by the green signal in close proximity to adenoviral particles in (D), PF4 was found in dense clusters after incubation with ChAdOx1 nCoV-19 whereas PF4 formed a more homogeneous layer on glass after incubation with purified ChAdOx1 nCoV-19 (E) or Ad26.COV2.S (F). For quantification, single-molecule particle analysis was performed and particle density ratios (on viral particles / on glass) analyzed (Supplementary Figure 10). Relative PF4 particle densities are plotted in (G) and showed a statistically significant affinity of PF4 to adenoviral particles predominantly on ChAdOx1 nCoV-19 but not on purified ChAdOx1 nCoV-19 virions or Ad26.COV2.S. Statistical analysis of n=92 particles was performed with Kruskal Wallis test with Dunn’s correction for multiple comparisons. Respective p-values are indicated in the plot, red lines and whiskers indicate mean±SD. Dashed line at y=1 (equal affinity of PF4 to adenoviral hexon and glass). Full-field of view images and single-channel reconstructions are provided in Supplementary Figure 9. Scale bars represent 100 nm.
Figure 5. Analysis of vaccine components prepared by ultracentrifugation.
Representative transmission electron micrographs of (A) ChAdOx1 nCoV-19 and (C) Ad26.COV2.S vaccine and their respective supernatants and pellets obtained after ultracentrifugation. Asterisks and arrows indicate virions and electron dense amorphous vaccine components, respectively. Scale bar represents 200 nm. Changes in the hydrodynamic diameter (in nm) of (B) ChAdOx1 nCoV-19 and (D) Ad26.COV2.S vaccine and their respective supernatants and pellets obtained after ultracentrifugation of vaccines before and after addition of 10 µg/ml or 50 µg/ml PF4 assessed by DLS. Dissociation of complexes between PF4 and the vaccine component was achieved by the addition of unfractionated heparin (10 IU/ml) (E) Western blot analysis of vaccine and pellet or supernatant fraction is shown. Primary antibodies for viral the vector (anti-hexon antibody) or human protein contaminants (anti-HSP90 antibody, anti-Epsilon 14-3-3 antibody, anti-PSMB5 antibody) were used. Statistical analysis was performed by one-way ANOVA on ranks/Kruskal-Wallis followed by correction for multiple comparison by two-stage Benjamini, Krieger, & Yekutieli controlling the false discovery rate procedure. n=9 and alpha <0.05 was considered significant.

Figure 6. Vascular hyperpermeability assay. Five days past fertilization Tg(fabp10a:gc-eGFP) zebrafish larvae were microinjected with either physiological saline, 100 µM EDTA, ChAdOx1 nCoV-19, purified ChAdOx1 nCoV-19 or Ad26.COV2.S in four adjacent myotomes. Local fluorescence intensities of the myotomes were measured at 0 min and 10 min p.i. (red asterisks) and normalized to the respective intravascular fluorescence (red arrows). Injection of 100 µM EDTA, as well as ChAdOx1 nCoV-19, resulted in a significantly elevated extravascular leakage of the 78 kDa gc-eGFP compared to the saline control (A, B, C, F). However, injection of Ad26.COV2.S, purified ChAdOx1 nCoV-19 or physiological saline did not cause an increase of local vascular permeability (A, D, E, F). Scale bar in E represents 200 µm without zoom and 100 µm with zoom.
Comparative analysis of ChAdOx1 nCoV-19 and Ad26.COV2.S SARS-CoV-2 vector vaccines

Supplemental Material

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Supplemental Figures

Supplementary Figure 1: Proteome profiling of ChAdOx1 nCoV-19 and Ad26.COV2.S vaccines revealed major differences in host cell protein content

iBAQ protein intensities and theoretical molecular mass of identified proteins. Protein intensities of vaccine lots 1-3 for ChAdOx1 nCoV-19 or Ad26.COV2.S vaccine were calculated using the iBAQ algorithm (min. 3 unique peptides per protein) and plotted against the theoretical molecular mass. Proteins were color-coded according to their respective class. Blue dots indicate vector proteins, gray proteins represent human proteins and the red dot indicates the SARS-CoV-2 spike protein. Points marked with a cross are those used in the Western blot analysis.
Supplementary Figure 2: Host cell protein count and HCP percentage

(A) Host cell protein (HCP) identification count for ChAdOx1 nCoV-19 or Ad26.COV2.S vaccines. Host cell proteins (HCP) with at least 3 peptides were counted per lot and displayed in a bar plot. (B) iBAQ protein intensities of human and vector proteins. For vaccine lots 1-3 for ChAdOx1 nCoV-19 or Ad26.COV2.S, iBAQ protein intensities were summed per protein class and resulting percentages were calculated and displayed in a stacked bar plot (grey = human proteins, blue = vector proteins).
Supplementary Figure 3: HCP DIA-MS analysis of the dilution series of ChAdOx1 nCoV-19 and Ad26.COV2.S vaccines

Normalized sum of identified HEK293 human ion signals per injection amount was used for the linear regression (black line = regression; single points = grey). The ion signals were used to estimate the amount of human signal in 500 ng of vaccine (dashed lines; ChAdOx1 nCoV-19 = blue, Ad26.COV2.S = red).
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Supplementary Figure 4: Western blot analysis

Western blot results (left panels) and corresponding Revert 700 total protein stains as a transfer control (right panels) of a dilution series of a laboratory HEK293 cell line extract [1.5, 1.0, 0.5 and 0.25 µg] for direct comparison to 10 µL (1/50th of a vaccine dose) of 3 lots of ChAdOx1 nCoV-19 and Ad26.COV2.S vaccines, respectively. Antibody specificity is indicated on the top left and the respective specific signal is indicated by a red arrowhead and the expected molecular weight (in kDa) of the target protein. Western blots of the target proteins are shown in the order HSP90-alpha, HSP90-AB1, GRP94, 14-3-3 epsilon, tubulin alpha-1B, YWHAZ, vimentin, histones H4, PSMB5-MB1, PSMB6 (beta-1 delta) as well as PSMA4.
Supplementary Figure 5: Western blot analysis of protein proteasome subunit abundances in vaccines

(A) Western blot shown for proteasome subunit beta type-5 protein (PSMB5 - MB1). (B) Western blot shown for proteasome subunit beta type-6 protein (PSMB6 - Beta-1 delta). ChAdOx1 nCoV-19 lot 6 was not included in the calculations because no proteasome activity measurement is available for this lot.
Supplementary Figure 6: proteasome inhibitor control experiments

(A) Chymotrypsin-like activity of standard proteasomes isolated from human erythrocytes was measured in assay buffer containing 30mM Tris pH 7.5, 5mM MgCl₂, 10mM KCl, 10% Glycerol and protease inhibitor cocktail Complete (Sigma-Aldrich) or in the presence of either 100 nmol/L bortezomib or 200 nmol/L carfilzomib (n = 3, technical replicates)

(B) Chymotrypsin-like activity of three lots ChAdOx1 nCoV-19 (#5334, 210072 and #210004, 1/10th of vaccination dose) was compared with the activity of 0.25 µg HEK293 cell lysate. As control for specific proteasome inhibition carfilzomib was added and displayed slightly more inhibition compared to bortezomib (n=3, technical replicate).
Supplementary Figure 7: Electron micrograph of purified adenovirus particles from ChAdOx1 nCov-19 vaccine in different magnifications

Asterisks exemplarily indicate adenoviral particles. Scale bars represent 300 nm and 100 nm from left to right panel.
Supplementary Figure 8: Protein pattern of the Ad26.COV2.S and the purified ChAdOx1 nCoV-19 vaccine

Supplementary Figure 9: Comparison of hydrodynamic diameter of ChAdOx1 nCov-19 vaccine and Ad26.COV2.S vaccine incubated with increasing concentrations of PF4

(A) ChAdOx1 nCov-19 vaccine and its supernatant incubated with PF4 shows a concentration dependent exponential increase in hydrodynamic diameter measured by DLS but not with (B) Ad26.COV2.S vaccine. Complexes formed between PF4 and vaccine or its components are disrupted by UHF (1 IU/mL).
Supplementary Figure 10: Representative percent intensity histogram of the hydrodynamic diameter from DLS

Changes in hydrodynamic diameter of (A) ChAdOx1 nCoV-19, (B) purified ChAdOx1 nCoV-19 and (C) Ad26.COV2.S vaccine incubated and in complex with PF4 (10 µg/mL and 50 µg/mL) and dissociation of the complexes after the addition of UFH (1 IU/mL)
Supplementary Figure 11: Purified ChAdOx1 nCov-19 vaccine and Ad26.COV2.S incubated with biotinylated PF4 and stained with anti-hexon mAb and streptavidin-gold conjugate

In immunoelectron microscopy the anti-hexon mAb binds to an epitope that is located inside the capsid of an intact virion. Binding events were only seen with defect virions or not assembled hexon protein. Arrows indicate single gold particles. Bars represent 200 nm.
Supplementary Figure 12: Dual-channel SMLM for hexon and PF4: Average shifted histograms

Full field of view of immuno-labeled PF4 and hexon polypeptide after incubation with either native ChAdOx1 nCoV-19 (A), purified ChAdOx1 nCoV-19 (B) or Ad26.COV2.S (C) acquired by SMLM and reconstructed as average shifted histograms. After incubation with ChAdOx nCoV-19 (A), PF4 can be visualized as dense clusters that colocalize with the hexon polypeptide (arrowheads). In contrast, after incubation with either purified ChAdOx nCoV-19 (B) or Ad26.COV2.S (C), PF4 formed a rather homogenous layer on glass slides and only single and small complexes were found.
Supplementary Figure 13: Dual-channel single-molecule scatterplots of the PF4 (green) and the adenoviral hexon polypeptide (magenta)

Herein, each spot represents one fluorophore localization. Clearly, distinct clustering of PF4 molecules can only be seen in native ChAdOx1 nCoV-19 (A) which shows a high PF4 density either directly on, or in direct vicinity of particles staining for hexon proteins. In contrast PF4 molecule density is very similar inside or outside hexon-localizations in purified ChAdOx1 nCoV-19 and Ad26.COV2.S (B, C). For comparative quantification of molecule density see main Figure 4, G.
Supplementary Figure 14: Comparison of summed iBAQ protein intensities per sub-proteome before and after purification of virions of ChAdOx1 nCoV-19 and Ad26.COV2.S

iBAQ protein intensities were summed per protein class and resulted intensity values were displayed in a barplot (grey = human proteins, blue = vector proteins, red = SARS-CoV-2 spike protein).
Supplementary Figure 15. Silver nitrate stained SDS-PAGE of fractionated vaccines

Protein patterns of silver nitrate-stained SDS-PAGE of 1/50th of a vaccine dose of the untreated vaccine as well as the supernatant and the pellet fraction after ultracentrifugation of the ChAdOx1 nCoV-19 (left) and Ad26.COV2.S (right) vaccines.
Supplementary Figure 16. Western blot analysis of the fractionated vaccines

Western blot results with their corresponding Revert 700 total protein stains as a transfer control of 1/50th of a vaccine dose of the untreated vaccine as well as the supernatant and the pellet fraction after ultracentrifugation of the ChAdOx1 nCoV-19 and Ad26.COV2.S vaccines. Antibody specificity A) Anti-Hexon, B) anti-HSP90-alpha, C) anti-Epsilon 14-3-3, D) anti PSMB5 and the specific target protein signals are indicated by red arrowheads and the expected molecular weight (in kDa).
Supplementary Table 1 on file Excel only
Supplemental table 1 contains the resulting iBAQ intensities of identified proteins of the comparison of ChAdOx1 nCoV-19 and Ad26.COV2.S.

Supplementary Table 2 on file Excel only
Top10 human protein impurities for ChAdOx1 and Ad26.COV2.S vaccine based on mean iBAQ protein intensity per vaccine. The red-white-green iBAQ color gradient shows the iBAQ intensities from high-middle-low abundant proteins respectively.

Supplementary Video 1
Supplementary video 1 shows intramuscular microinjections in adjacent myotomes of a zebrafish tail. Special care was taken not to injure the adjacent caudal vein or segmental arteries.
Supplementary Materials and Methods

Sample preparation for LC-MS/MS analysis

Vaccines were precipitated using a quantitative salt-acetone precipitation\(^1\) and the precipitate was resuspended in one-fifth of the initial volume using 20 mM HEPES buffer pH 8 containing 1 % (w/v) SDS. Determination of protein concentrations was performed using a microBCA assay according to the manufacturer’s instructions (Pierce Thermo Fisher, Bonn, Germany).

2 µg of protein was reduced (2.5 mM DTT ultrapure, Invitrogen/Thermo Fisher, Bonn, Germany) for 30 minutes at 37 °C and alkylated (10 mM iodoacetamide for 15 minutes at 37 °C, Sigma Aldrich, Munich, Germany). Subsequently, protein was digested with trypsin (enzyme to protein ratio of 1:25) on SP3 beads as described by Blankenburg \(et\ al.\)^2

SDS gel and Western blot analysis

For protein separation, 2 µl of the three precipitated ChAdOx1 nCoV-19 (lot 1 – 1.14 µg, lot 2 – 1.05 µg, lot 3 – 0.86 µg) and Ad26.COV2.S vaccine samples (lot 1 – 0.39 µg, lot 2 – 0.3 µg, lot 3 – 0.2 µg) corresponding to one fiftieth of one vaccine dose as well as dilutions of HEK293 total protein lysate (1.5, 1.0, 0.5 and 0.25 µg) were adjusted to 10 µl sample volume with water and 4x SDS-PAGE sample buffer (Licor D00317-01) with added mercaptoethanol [10 %]. Accordingly, 0.5 µl of size standard (PageRuler Prestained Protein Ladder, Invitrogen/Thermo Fisher) was adjusted to a final volume of 10 µl with water and 4x SDS-sample buffer. Samples and standard were denatured for 2 min at 95 °C, chilled to room temperature and loaded to a precast NuPAGE™ 4 to 12 %, Bis-Tris Midi Gel 1.0 mm x 26 well (WG1403BX10, Invitrogen/Thermo Fisher). Electrophoresis was performed at 150 V using a Power Pack 200 (BioRad, Hercules, CA, USA) and a Criterion Cell (BioRad). All reagents and buffers were used according to the manufacturer’s instructions. For the pure visualization of protein bands, silver nitrate staining was performed, as previously described by Shevchenko \(et\ al.\)^3 Gel images were digitized using a digital camera. The Western blots were prepared using the Trans-Blot Turbo Transfer System from BioRad. Proteins were transferred onto midi-size LF PVDF membranes for 10 min at 2.5 A and 25 V. The proper transfer of proteins to the PVDF membrane was verified
and documented using LICOR's Revert Total Protein Stain protocol (Doc # 988-
19494). Control stains were scanned using the Odyssey® CLx Imaging System (LI-
COR Biosciences) in the 700 nm channel at 84 µm resolution, medium intensity and
auto adjust. All further steps were followed according to the Western blot protocol of
LI-COR (Doc # 988-19647). The detection of the specific proteins was performed
with primary and secondary antibodies indicated in Table 3 using the respective
incubation conditions. Detection of the specific signals was performed using the
Odyssey® CLx Imaging System in the 800 nm channel at 84 µm resolution, medium
intensity and auto adjust.

Supplemental Table 3: Used antibodies

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LC-MS/MS and data analysis
LC-MS/MS experiments were performed on an Orbitrap Exploris™ 480 mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to an Ultimate™ 3000 RSLCnano HPLC (Dionex/Thermo Scientific, Waltham, MA, USA).
Chromatographic separation of tryptic peptides was achieved by a 60 min linear gradient using a binary buffer system that consisted of: 0.1 % (v/v) acetic acid in HPLC-grade water; 100 % ACN in 0.1 % (v/v) acetic acid with increasing concentrations of acetonitrile (7-25% (v/v) in 0.1 % (w/v) acetic acid) on a reverse phase column (Accucore 150-C18, 25 cm x 75 μm, 2.6 μm C18, 150 Å), at a flow rate of 300 nL/min at 40 °C. The MS scans were carried out in a m/z range of 350 to 1200 m/z.
For data acquisition in data independent mode (DIA) (ChAdOx1 nCoV-19 or Ad26.COV2.S vaccine lot analysis), precursor scans were acquired at a resolution of 120,000 and fragments at a resolution of 30,000 in 66 windows with 13 m/z and a window overlap of 2 m/z. For detailed information see Supplementary Table 4. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE³ partner repository with the dataset identifier PXD027344.

Supplemental Table 4: Parameters for LC-MS/MS analyses

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<td>1:1000 / room temperature / overnight</td>
<td>Licor Goat anti-Mouse IRDye 800CW No. 926-32210</td>
<td>1:10000 / room temperature / 1 h</td>
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<tr>
<td>PSMB5</td>
<td>PSMB5 (D1H6B) Rabbit Monoclonal IgG (Cell Signaling Technology No:12919)</td>
<td>1:1000 / room temperature / overnight</td>
<td>Licor Goat anti-Rabbit IRDye 800CW No. 926-32211</td>
<td>1:10000 / room temperature / 1 h</td>
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<td></td>
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<tr>
<td>PSMB6</td>
<td>PSMB6 (E1K9O) Rabbit monoclonal IgG (Cell Signaling Technology No: 13267)</td>
<td>1:1000 / room temperature / overnight</td>
<td>Licor Goat anti-Rabbit IRDye 800CW No. 926-32211</td>
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<tr>
<td><strong>instrument</strong></td>
<td>Ultimate 3000 RSLC (Thermo Scientific)</td>
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<tr>
<td>---------------</td>
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<tr>
<td><strong>trap column</strong></td>
<td>75 μm inner diameter, packed with 3 μm C18 particles (Acclaim PepMap100, Thermo Scientific)</td>
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<tr>
<td><strong>analytical column</strong></td>
<td>Accucore 150-C18, (Thermo Fisher Scientific) 25 cm x 75 μm, 2.6 μm C18 particles, 150 Å pore size</td>
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<tr>
<td><strong>buffer system</strong></td>
<td>binary buffer system consisting of 0.1 % acetic acid in HPLC-grade water (buffer A) and 100 % ACN in 0.1 % acetic acid (buffer B)</td>
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<tr>
<td><strong>flow rate</strong></td>
<td>300 nl/min</td>
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<tr>
<td><strong>gradient</strong></td>
<td>linear gradient of buffer B from 2 % up to 25 %</td>
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<tr>
<td><strong>gradient duration</strong></td>
<td>60 min</td>
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<td><strong>column oven temperature</strong></td>
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</table>

**Mass spectrometry**

| **instrument** | Orbitrap Exploris\textsuperscript{TM} 480 |
| **electrospray** | Nanospray Flex\textsuperscript{TM} Ion Source |
| **operation mode** | data-independent |
| **Full MS** | |
| **MS scan resolution** | 120000 |
| **AGC target** | 3e6 |
| **maximum ion injection time for the MS scan** | 60 ms |
| **Scan range** | 350 to 1200 m/z |
| **RF Lens** | 50 % |
| **Spectra data type** | profile |
| **dd-MS2** | |
| **Resolution** | 30,000 |
| **MS/MS AGC target** | 3e6 |
| **maximum ion injection time for the MS/MS scans** | auto |
| **Spectra data type** | profile |
The data were analyzed with Spectronaut version 14.10.201222.47784 (Biognosis, Zurich, Switzerland) in directDIA mode using the human Uniprot database (version 01/2021) with added SARS-CoV-2 spike protein (YP_009724390.1) sequence and protein sequences of ChAdOx1 nCoV-19 vector consisting of chimpanzee adenovirus Y25 (NC_017825) with exchanged regions (E4ORF4, E4ORF6, E4ORF6/7) from human adenovirus 5 (AC_000008) for the ChAdOx1 nCoV-19 analysis. For the Ad26.COV2.S analysis we used a database comprised of human Uniprot database (version 01/2021) with added SARS-CoV-2 spike protein (YP_009724390.1) sequence and protein sequences of hAd26 (Uniprot taxon identifier ID 46928) with exchanged regions (E4ORF6/7) from human adenovirus 5 (AC_000008).

Identifications were based on a precursor Q-value cut-off of 0.001 and a FDRprotein of 0.01. The complete Spectronaut parameters are listed in Supplementary Table 3.

**Supplementary Table 5: Spectronaut parameters.**

<table>
<thead>
<tr>
<th>Parameter level</th>
<th>Parameter</th>
<th>Setting</th>
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<tr>
<td>Peptides</td>
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<tr>
<td>Peptides</td>
<td>Min Peptide Length</td>
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<tr>
<td>Peptides</td>
<td>Max Peptide Length</td>
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<td>Peptides</td>
<td>Missed Cleavages</td>
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<td>Peptides</td>
<td>Digest Type</td>
<td>Specific</td>
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<tr>
<td>Peptides</td>
<td>Enzymes / Cleavage Rules</td>
<td>Trypsin/P</td>
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<td>Data Extraction</td>
<td>MS1 Mass Tolerance Strategy</td>
<td>Dynamic</td>
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<tr>
<td>Data Extraction</td>
<td>MS1 Mass Tolerance Strategy - Correction Factor</td>
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<tr>
<td>Data Extraction</td>
<td>MS2 Mass Tolerance Strategy</td>
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<tr>
<td>Data Extraction</td>
<td>MS2 Mass Tolerance Strategy - Correction Factor</td>
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<tr>
<td>XIC Extraction</td>
<td>XIC IM Extraction Window</td>
<td>Dynamic</td>
</tr>
<tr>
<td>XIC Extraction</td>
<td>XIC IM Extraction Window - Correction Factor</td>
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<tr>
<td>XIC Extraction</td>
<td>RT IM Extraction Window</td>
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</tr>
<tr>
<td>XIC Extraction</td>
<td>RT IM Extraction Window - Correction Factor</td>
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</tbody>
</table>
The Spectronaut unique protein iBAQ intensities (filtered for at least 3 peptides per protein with ion Qvalues < 0.001) were cleared for preparation contaminants (trypsin, keratin, dermicidin) used for a comparison of samples^{19}. Data analysis and generation of plots was carried out using R^{5} (version 4.0.2) depending on the tidyverse (version 1.3.0)^{6} and scales (version 1.1.1).
Purification or fractionation of adenovirus particles from the vaccine

The first step was a sucrose cushion ultracentrifugation. 1 to 6 ml vaccine were diluted in buffer (phosphate buffer) to 15 ml and loaded slowly over 2 ml 15 % sucrose solution (phosphate buffered; 17 ml tube, opaque, Beckman Coulter) forming a layer and centrifuged at 20,000 rpm, 12 °C for 2 hours with SW32Ti rotor in a Beckman Coulter Optima L-100XP. The supernatant was discarded and the resulting pellet was layered with buffer (1/10 of the starting volume of the vaccine) and incubated overnight at 4 °C.

The second step was a sucrose gradient ultracentrifugation. The resuspended pellet was loaded on a 5 to 50 % sucrose gradient (phosphate buffered; 17 ml tube, ultraclear, Beckman Coulter) that was prepared the day before for equilibration. For centrifugation, the same conditions were used as mentioned above. The resulting distinct and adenovirus-rich band was isolated.

The last step was ultracentrifugation for pelleting of adenovirus particles. The isolated band was diluted in buffer to 4 ml volume and centrifuged (4 ml tube, opaque, Beckman Coulter) at 12,000 rpm, 12 °C for 2 hours with SW60Ti rotor. The supernatant was discarded and the resulting pellet was layered with buffer (1/10 of the starting volume of the vaccine) and incubated overnight at 4 °C.

For the fractionation experiments, ultracentrifugation was performed at 20,000 rpm (=53750 x g, SW60 rotor), at 12 °C for 2 hours and the pellet was resuspended in 1/10 of the starting volume with PBS buffer. Phosphate buffer: 50 mM Na₂HPO₄*2H₂O pH 7.4, 150 mM NaCl.

Finally, the quality of each batch was analyzed by transmission electron microscopy (TEM) as described below.

Proteasome activity assays

ChAdOx nCoV-19, Ad26.COV2.S and HEK293 cell lysates were tested for proteasomal activity. HEK293 cells were gently lysed by repeated freeze-thaw cycles in buffer containing 10 mM Tris (pH 7.0), 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 1 mM DTT, 10 % glycerol, and protease inhibitor cocktail Complete (Sigma-Aldrich).

Chymotrypsin-like activity was assessed in 50 µl vaccine or HEK293 cell lysate (0.25 µg) using 0.2 mM fluorescently tagged substrate Suc-LLVY-AMC (Bachem, Bubendorf, Switzerland). To confirm proteasomal activity 100 nM Bortezomib
(Selleckchem, Houston, Texas, United States), an inhibitor of chymotrypsin-like activity\(^7\) or Carfilzomib\(^8\) (Selleckchem, Houston, Texas, United States), was added. The free AMC fluorescence was quantified with a fluorometer using a 380/460 nm filter set (Fluorescence spectrometer Infinite M200 Pro, software i-control1.7; TECAN, Männedorf, Switzerland). Increase of fluorescence was measured over 2 hours. Proteasome turnover was determined by calculating the rise of the linear slope at the beginning of the measurement (substrate turnover (Δ RFU/min)).

**Dynamic Light Scattering and Zeta Potential Measurements**

All dynamic light scattering (DLS) measurements were performed in a fixed scattering angle Zetasizer Nano-S system (Malvern Instruments Ltd., Malvern, UK). The hydrodynamic diameter (nm) was measured at 25 °C, and light scattering was detected at 173° and three repeating measurements consisting of 12 runs of 10 seconds each. Experimental data were collected from at least three independent experimental replicates. For all DLS measurements, non-purified ChAdOx1 nCoV-19, purified ChAdOx1 nCoV-19 and Ad26.COV2.S vaccine was diluted at a ratio of 1:10 in sterile-filtered 0.9 % NaCl supplemented with 4 mg/mL D(+) sucrose (RNase/DNase free; Cat. No. 9097.1, Carl Roth GmbH, Germany). Assessment of changes in the hydrodynamic diameter of ChAdOx1 nCoV-19 vector in the presence of PF4 was performed by incubating 10 and 50 µg/mL of human PF4 (Chromatec, Greifswald, Germany) with ChAdOx1 nCoV-19 vaccine at RT for five minutes before DLS measurements. For titration experiments, either 1:10 diluted ChAdOx1 nCoV-19 or Ad26.COV2.S vaccine was incubated with increasing concentrations of PF4 from 1, 5, 10, 15, 20, 25, and 50 µg/mL. Similarly, PF4 at a fixed concentration of 10 µg/mL was incubated with ChAdOx1 nCoV-19 vaccine with decreasing dilutions of 1:1000, 1:750, 1:500, 1:250, 1:100, 1:50. 1:25 and 1:10. Dissociation of complexes formed between ChAdOx1 nCoV-19 vector and added components was achieved by 1 IU/ml unfractionated heparin (UFH, Ratiopharm GmbH, Ulm, Germany). In a subset of experiments, undiluted vaccine, their supernatant and dilution adjusted pellet fractions were used and dissociation of complexes was achieved by 10 IU/ml UFH. Surface zeta potential (ζ, mV) was performed in folded capillary zeta cells (DTS1070, Malvern Instruments Ltd., Malvern, UK). It consisted of three runs, each with 20 measurements at a voltage set to 10 V.
Data analysis was performed using Zetasizer software, Version 7.13 (Malvern Instruments Ltd., Malvern, UK). Statistical analysis and data plots were prepared with GraphPad Prism version 9.0.0 for Windows. Differences between groups were considered significant after assessment by ordinary one-way ANOVA with Sidak’s multiple comparisons test, with Alpha set to 0.05.

**Immunoelectron microscopy and transmission electron microscopy**

For TEM, the vaccine or the purified adenovirus particles was incubated with biotinylated PF4 (10 ng/ml in phosphate buffer; PF4-biotin) for at least 1 hour at room temperature. After that, the sample was transferred to formvar coated TEM grids (400 mesh, Plano GmbH), washed with phosphate buffer and blocked with phosphate buffer containing 1 % BSA. On the one hand, samples were labeled with an anti-Adenovirus mAb (Abcam, ab7428, 1:500) for 1 hour at room temperature and an anti-mouse gold conjugate (BBI Solutions, GMHL10, 10 nm, 1:50) as secondary antibody. On the other hand, the same samples were labeled with a streptavidin-gold conjugate (Sigma, 10 nm, 1:10) for the staining of PF4-biotin for 45 min at room temperature.

All grids were stained with 1 % phosphotungstic acid at pH 7.4 and analyzed with a Tecnai-Spirit TEM (FEI, Eindhoven) at an accelerating voltage of 80 kV. The same procedure was used for preparing the controls, vaccine and PF4-Biotin.

For analysis of preparation quality after the purification of ChAdOx1 nCoV-19 the samples were transferred to formvar coated TEM grids (400 mesh, Plano GmbH), stained with 1 % phosphotungstic acid at pH 7.4 and analyzed with a Tecnai-Spirit TEM (FEI, Eindhoven) at an accelerating voltage of 80 kV.

**Immunofluorescence staining**

10 µg/ml human PF4 (Chromatek, Greifswald, Germany) was incubated with 1:10 diluted ChAdOx1 nCoV-19 (AstraZeneca, lots ABV5443, ABW0018, ABV5297) or Ad26.COV2.S (Johnson and Johnson, lots 21C11-01, 21C10-01, XD955) in 2 mg/ml sucrose in 0.9 % injection-grade NaCl. After 5 minutes incubation at room temperature, 10 µl were spread on beforehand washed (subsequent sonication in 99 % EtOH, 17 % HCl in 50 % MeOH, ultrapure water, 99 % EtOH) 22x22mm #1.5 high-precision coverslips (VWR, Germany). Slides were air-dried at room temperature and fixed with 2 % electron microscopy-grade paraformaldehyde
(Thermo Fisher Scientific) in 4 % sucrose-containing PEM-buffer (80 mM PIPES, 5 mM EGTA, 2 mM MgCl\textsubscript{2}) for 5 min at room temperature. Slides were blocked with 2 % fetal bovine serum, 2 % bovine serum albumin, 0.1 % cold fish gelatin and 2 % normal goat serum in 1x PBS pH 7.4 for 45 min at room temperature. For detection of PF4, a mouse monoclonal IgG2b (RTO clone, Thermo Fisher, MA5-17639) diluted to 2 µg/ml in 2 % bovine serum albumin in 1x PBS pH 7.4, was incubated for 30 min at room temperature under gentle agitation. Bound primary antibodies were detected after several washes in 1x PBS using AlexaFluor 488-conjugated secondary antibodies (A11001, Thermo Fisher Scientific) at 1:500 dilution for 30 min at room temperature. For detection of the hexon polypeptide, a custom Cy5-conjugated (Lightning-Link Cy5 conjugation kit according to manufacturer’s description (Novus Biologicals, 781-0010)) IgG2a antibody (abcam, ab7428) was incubated at 1 µg/ml in 2 % bovine serum albumin for 1 hour at room temperature. Slides were collected in 1x PBS and stored at 4 °C in the dark upon mounting and imaging. Antibody specificity was checked beforehand using secondary immunofluorescence with minus primary antibody control.

**Single-molecule light microscopy (SMLM): Direct stochastical optical reconstruction microscopy (dSTORM)**

Before imaging, coverslips were washed in ultrapure water and 100 nm multi-fluorescent Tetraspek Beads (Thermo Fisher) were added in a 1:800 dilution in ultrapure water for 5 min at room temperature. After one wash in electron microscopy-grade water, coverslips were inversely mounted on depression microscopy glass slides (VWR, Germany) in Everspark dSTORM buffer (Idylle Labs, Paris, France) containing deoxygenized 100 mM mercaptoethanolamin hydrochloride (MEA) in TRIS buffer pH 8\textsuperscript{23}. Coverslips were sealed airtight with TwinSil two-component dental silicone. Care was taken that mounting time did not exceed 30 seconds to minimize oxygenation of the buffer. Slides were imaged after between 1 hour and 1 week of incubation with the imaging buffer and were stored at 4°C in the dark.

For dSTORM imaging, a Zeiss Elyra PS.1 super-resolution system was used with a temperature-controlled chamber set to 30 °C. To equilibrate instruments and reduce drift, the system was switched on at least two hours before imaging. Samples were
equilibrated for at least 20 minutes in the imaging chamber. The objective used was a Zeiss, Apochromat, 63x, 1.4 NA TIRF objective, and emitted fluorescence was projected on an Andor iXon 897 EMCCD camera with a 512x512 pixel chip, resulting in an effective pixel size of 160 nm. Areas of interest with at least two fiducial markers per field of view were selected using the epifluorescence mode. Z-drift compensation was performed with the Definite Focus system set on continuous compensation. During continuous TIRF-HP-illumination, the laser power was gradually increased to bleach fluorophores until steady-state single-molecule photoswitching was observed. 256x256 pixels frames were acquired at a framerate of 55 Hz with a manual gain set to between 20 and 40. Subsequently, 14,000 frames image sequences of the same field of views of the Cy5 and AF488 fluorophores were recorded, saved as .czi files and imported to FIJI using the BioFormats importer. After import, raw data was saved as .nji files using the NanoJ core toolbox. Raw single-channel dSTORM data was initially drift-corrected with the built-in function of the NanoJ core plugin. Chromatic aberration between both channels was estimated on intensity-averaged frames of the drift-corrected data (to clearly visualize fiducial markers) and corrected on drift-corrected source data using the Channel Registration function of NanoJ core. After drift correction and sub-pixel channel registration, image sequences were cropped to 10,000 frames of 200x200 pixels before performing emitter-localization analysis. Cropped, drift-corrected and channel-registered data was exported with NanoJ core as .nji files. For both channels, blinking events were detected, and x-y localized with the Thunderstorm algorithm using normalized Gaussian fitting with a 3 px fitting radius, weighted least-squares fitting method, an initial Sigma of 1.6 px, and multi-emitter fitting analysis disabled. The resulting localization table was exported as .csv files and uncertainty- as well as sigma-filtered. Data was then density-filtered with a minimum distance radius of 50 nm with a minimum of 5 neighbors in the radius. The resulting localization data was visualized either using the built-in averaged shifted histograms function with 10x magnification or as well as the scatter plot function with 50x magnification (with herein hexon-localizations density-filtered with a minimum of 50 neighbors in a distance radius of 50 nm). Single-channel data was visualized using the inverted NanoJ-orange LUT or merged and exported using basic FIJI functions.
**Image analysis**

To analyze PF4-molecule aggregation on adenoviral capsid clusters, PF4 particle density in- and outside boundaries of adenoviral complexes were quantified. To automate this, an ImageJ1-macro script was developed. In brief, dual-channel 50x magnified single-molecule scatterplots were imported to FIJI, adenoviral clusters gaussian-blurred and thresholding-based binarized. A ring-like reference region outside (500 nm outside the adenoviral particles, width of 500 nm) of each complex was defined which did not contain adenoviral localizations. Then, PF4 localizations in each region of interest (ROI, inside adenoviral particles, and outside on glass as internal reference) were counted and normalized to the ROI area as particle density. To account for preparation-based differences in local molecule density, PF4 density ratios of the matching ROIs (inside/outside AV) were calculated and normalized to the median of every group outside ROI density ratio. ImageJ macro scripts are available on [http://www.github.com/siegerist](http://www.github.com/siegerist).

Statistical analysis and data visualization were performed with Prism 9.1.2 (GraphPad Software, San Diego, California USA): Normality was checked using Kolmogorov-Smirnov testing. For >2 groups and non-parametric data, differences between groups were checked using Kruskal-Wallis-test with Dunn’s multiple comparison test. P-values are indicated in the respective plots, where not indicated, p-values were >0.05.

**Zebrafish vascular permeability assay**

Zebrafish maintenance was performed as described previously. All experiments were performed in accordance with German animal protection law overseen by the “Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Rostock” of the federal state of Mecklenburg - Western Pomerania. In order to track vascular leakage, Tg(-3.5fabbp10a:gc-eGFP)13 (ZFIN-ID: ZDB-FISH-150901-8595) and Tg(-3.5fabbp10a:gc-eGFP); mitfa w2/w2 were used. Larvae at 5 days post fertilization were anesthetized with 0.02 % tricaine (MS-222, Sigma-Aldrich) and placed laterally on a custom-made agarose dish. As demonstrated in Supplementary Video 1, approximately 1 nl intramuscular injections of 0.9 % NaCl, 100 µM EDTA, ChAdOx1 nCoV-19 (lot: ABV5297) and Ad26.COV2.S (lot: XD955 and 21C10-01) were performed with glass capillaries (Femtotips I, Eppendorf AG, Hamburg, Germany)
attached to a Transjector 5246 (Eppendorf AG) into four adjacent myotomes caudal of the cloaca in each larva. Afterward, larvae were embedded laterally in 0.6 % agarose (LE agarose, Biozym, Hessisch Oldendorf, Germany) with the injection side facing up and covered with E3 medium containing 0.02 % tricaine. Imaging was performed with a P2-SHR Plan Apo 1x objective attached to an SMZ18 fluorescence stereomicroscope equipped with a motorized Z-drive (Nikon GMBH, Düsseldorf, Germany) and an X-Cite Xylis LED (Excelitas, Göttingen, Germany). Z-Stacks with 10 frames for each larva were acquired with 470 nm slice-to-slice distance with a 135x magnification in the caudal region at 0 and 10 min post-injection (p.i.). Z-Stacks were converted to maximum intensity projections (MIPs) and stacks of two MIPs for both timepoints were created. Fluorescence intensity measurements were performed with 4 custom ROIs for each injected myotome. The same ROIs were used to obtain the fluorescence intensity of the adjacent caudal vein in order to calculate a muscle to vessel ratio. The t=0 of each larva served as normalizer for the 10 min p.i. ratio and displays the leakage of eGFP into the musculature. Creation of MIPs, Stacks and measurements were performed with ImageJ (National Institutes of Health, Bethesda, MD, USA)). Statistical analysis was performed in Prism 9.1.2 (GraphPad Software, San Diego, California USA). After checking gaussian distribution with a Kolmogorov-Smirnov test, differences between groups were checked using Kruskal-Wallis-test with Dunn's multiple comparisons.
References


