Supplemental Material and Methods

Thrombocytopenia and splenic platelet directed immune responses after intravenous ChAdOx1 nCoV-19 administration

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VITT sera MAIPA assay

Sera of patients with confirmed thrombocytopenia (platelet count <150*10^9/L) in temporal relationship to ChAdOx1 nCoV-19 vaccination sent to the platelet laboratory of the University Medicine Greifswald in April 2021 and SeCo study participants were screened for platelet-specific antibodies by the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay1,2. Briefly, 20*10^6 platelets were incubated with 20 µl patient serum for 30 min at 37 °C. Following washing, platelets were incubated with either a mouse anti-human glycoprotein-specific antibody against GPIIbIIIa (clone P2; Jackson ImmunoResearch), GPIbIX (clone Gi 9; Jackson ImmunoResearch), or GPIaIIa (clone FMC-25; AbD Serotec). After incubation for 30 min at 37 °C, platelets were washed three times and lysed with solubilization buffer (Tris buffered saline containing 1% Triton X-100). Lysis at 4 °C for 30 min was followed by centrifugation at 15 000 x g for 30 min to remove particulate material. The lysates were resuspended with TBS wash buffer (Tris buffered saline containing 0.5% Triton X-100 and 0.5% Tween-20) and incubated for 90 min at 4 °C in wells of microtiter plates precoated with goat antimouse immunoglobulin G (IgG). Four times washing with TBS wash buffer was followed by 120 min incubation at 4 °C with 100 µl peroxidase-labeled goat antihuman IgG (Jackson ImmunoResearch) and five washes with TBS wash buffer. Glycoprotein-bound IgG was visualized by addition of 100 µl substrate solution (in the dark for 15 min at room temperature). After stopping the reaction with 50 µl 2 N H2SO4, the extinction was determined at 492 nm with a photometer.

Flow cytometry

Flow cytometry to characterize platelet profiles was performed as previously described3. Briefly, incubation with panels at 1:200 per antibody of either washed platelets or whole blood, at least 5x the amount of FACS Lysing Solution (BD) was added to fixate platelets and lyse blood. Flow cytometry was performed on a BD LSRFortessa and BD FACSCanto. Flowjo (BD) was used for flowcycmtometric analysis, gating strategies are shown in Supplemental Figure 2. Platelet extracellular vesicles were measured through flow cytometry, and identified as CD41+ particles smaller than platelets, with gating adjusted to
beads of approximately 0.5µm. Platelets leukocyte aggregates were assessed by the number of platelet (CD41)-positive CD45+ leukocytes, or CD45+, GR1+CD11b+ myeloid leukocytes. For adenovirus binding, as well as IgM and IgG binding a positive gate was used, for MFIs the MFI of each marker per data point was normalized by dividing it by the highest MFI of that marker and experimental run.

Antibodies used for Flow Cytometry:

| Color     | Target | Species reactivity | Catalogue # and company            |
|-----------|--------|-------------------|------------------------------------|
| AF700     | CD44   | Mouse             | #103026, biolegend                 |
| APC       | GPIb   | Mouse             | X649, emfret Analytics             |
| APC/Fire 750 | CD31 | Mouse             | #102433, biolegend                 |
| BV421     | CD41   | Mouse             | #133932, biolegend                 |
| BV510     | AnV    | Mouse             | #640937, biolegend                 |
| BV711     | CD107a | Mouse             | #121631, biolegend                 |
| PE-Cy7    | CD62p  | Mouse             | #148309, biolegend                 |
| PE-Dazzle | CD9    | Mouse             | #124821, biolegend                 |
| PerCP-Cy5.5 | CD154 | Mouse             | #106513, biolegend                 |
| Cy3       | IgG    | Mouse             | #M30010, ThermoFisher              |
| PE        | GP2b3a | Mouse             | M023-2, emfret Analytics           |
| FITC      | IgM    | Mouse             | A21042, life technologies          |
| FITC      | GPIb   | Mouse             | X488, emfret Analytics             |
| APC       | GR1 (Ly-6G/C) | Mouse | 108412, biolegend |
| PE-Cy7    | CD11b  | Mouse             | 101216, biolegend                 |
| PerCP-Cy5.5 | CD45 | Mouse             | 103132, biolegend                 |
| FITC      | Hexon  | Adenovirus        | ab87333, abcam                    |
| BV650     | CD63   | Human             | #353026, biolegend                 |
| FITC      | CLEC-2 | Human             | #372007, biolegend                 |
| PE        | CD162/PSGL1 | Human | #328805, biolegend |
| PERCP-Cy5.5 | CD36 | Human             | #336224, biolegend                 |
| AF 647    | PAC-1  | Human             | #362806, biolegend                 |
| APC-Cy7   | CD184 (CXCR4) | Human | #306528, biolegend |
| BV510     | CD42b  | Human             | #303933, biolegend                 |
| Antibody   | Target          | Species  | Catalog #       |
|------------|-----------------|----------|-----------------|
| BV 711     | CD154 (CD40L)   | Human    | #310837, biolegend |
| AF 700     | CD41            | Human    | #133926, biolegend |
| BV421      | CD62P           | Human    | #304926, biolegend |
| PE-Dazzle  | CD31 (PECAM)    | Human    | #303130, biolegend |
| PE-Cy7     | CD284 (TLR4)    | Human    | #312805, biolegend |
| BV650      | CD63            | Human    | #353026, biolegend |

**Histology and thrombosis assessment**

Organs were harvested directly after sacrifice, fixed at 4% PFA for 1h and subsequently 30% sucrose overnight, then embedded in OCT and stored at -80°C. Sections were stained with antibodies against F4/80 (Biolegend, Cat. No. 123110), CD45R/B220 (Biolegend, Cat. No. 103212), ki67 (Abcam, Cat. No. ab15580), Lectin PNA (Thermofisher, Cat. No. L32458). Secondary antibodies and nucleic acid stain included FITC goat anti mouse (Thermofisher, Cat. No. A11029) and Cy3 goat anti rabbit (Jackson Immunoresearch, Cat. No. 111-165-003) and Hoechst33342. For bone marrow histology embedded bones were cut vertically, removed from OCT and washed twice with PBS. The bone marrow whole mounts were stained overnight with CD41 (Biolegend, Cat. No. 133904) and TER-119 (Invitrogen, Cat.No. 12-5921-83), washed and counterstained with Hoechst33342. Micrographs were taken on a Zeiss LSM 880 confocal microscope in airyscan mode and a Leica LAS X epifluorescence microscope. To analyze megakaryocyte density, a 3D-reconstruction of the z-stack bone marrow images was analyzed with Imaris software. Scarified mice were assessed for thrombosis both macroscopically (by examining all major blood vessels for thrombosis) and microscopically in the liver, by measuring the number of occluded vessels per field of view (FOV). D-Dimer measurement in the mouse plasma was performed using an ELISA according to manufacturer specifications (NBP3-08100, Novus Biologicals).

**Aspiration injection**

To assess if aspiration of blood could potentially prevent intravenous injection, the mouse hindlimb musculature of anesthetized mice was exposed and a 30-gauge needle (BD) mounted on transparent tubing to visualize blood return upon aspiration was either placed in the local vasculature or muscle. After placement and assessment of blood return into the tube injection of Evans Blue (1%, 100 µl) through a 1 ml syringe (BD) was realized. After 10 minutes, systemic Evans Blue distribution was assessed by photographing the front paws and mouth.
## Supplemental Tables

Suppl. Table 1 – Platelet counts and results of the MAIPA assay of patients with confirmed thrombocytopenia (platelet count <150*10⁹/L) after ChAdOx1 nCov-19 vaccination:

| Sample ID | VITT§ | Platelet count x10⁹/L | Mean OD GPIIb/IIIa* | Mean OD GPIb/IX* | Mean OD GPIa/IIa* | Anti-platelet antibodies |
|-----------|-------|-----------------------|--------------------|----------------|----------------|--------------------------|
| 2         | positive | 22              | 0.095             | 0.11           | 0.09           | negative                 |
| 3         | positive | 18              | 0.095             | 0.095          | 0.07           | negative                 |
| 5         | positive | 36              | 0.145             | 0.12           | 1.915          | positive                 |
| 7         | positive | 14              | 0.105             | 0.11           | 0.13           | negative                 |
| 8         | positive | 6.8             | 0.1               | 0.1            | 0.09           | negative                 |
| 13        | positive | 32              | 0.1               | 0.07           | 0.06           | negative                 |
| 14        | positive | 24              | 0.19              | 0.245          | 0.09           | positive                 |
| 16        | positive | 50              | 0.25              | 0.305          | 0.44           | positive                 |
| 19        | positive | 37              | 0.17              | 0.135          | 0.14           | negative                 |
| 22        | positive | 87              | 0.09              | 0.08           | 0.075          | negative                 |
| 27        | positive | 51              | 0                 | 0.1            | 0.075          | negative                 |
| 30        | positive | 55              | 0.105             | 0.1            | 0.09           | negative                 |
| 31        | positive | 35              | 0.095             | 0.09           | 0.08           | negative                 |
| 32        | positive | 45              | 0.095             | 0.1            | 0.095          | negative                 |
| 38        | positive | 149             | 0.12              | 0.08           | 0.075          | negative                 |
| 40        | positive | 99              | 0.2               | 0.21           | 0.235          | positive                 |
| 42        | positive | 94              | 0.18              | 0.265          | 0.34           | positive                 |
| 43        | positive | 82              | 0.055             | 0.065          | 0.065          | negative                 |
| 44        | positive | 20              | 0.095             | 0.09           | 0.07           | negative                 |
| 47        | positive | 61              | 0.05              | 0.05           | 0.045          | negative                 |
| 52        | positive | 86              | 0.155             | 0.175          | 0.105          | negative                 |
| 55        | positive | 72              | 0.055             | 0.07           | 0.085          | negative                 |
| 56        | positive | 46              | 0.305             | 0.215          | 0.305          | positive                 |
| 60        | positive | 12              | 0.265             | 0.235          | 0.3            | positive                 |
| 62        | positive | 32              | 0.09              | 0.075          | 0.07           | negative                 |
| 64        | positive | 21              | 0.12              | 0.105          | 0.1            | negative                 |
| 65        | positive | 21              | 0.685             | 0.645          | 2.2            | positive                 |
| 4         | negative | 12              | 0.07              | 0.095          | 0.065          | negative                 |
| 6         | negative | 110             | 0.78              | 2.105          | 2.195          | positive                 |
| 9         | negative | 92              | 0.105             | 0.11           | 0.09           | negative                 |
Suppl. Table 2 – Sera from healthy employees of the University medicine Greifswald vaccinated with ChAdOx-1 nCoV were screened for anti-platelet antibodies by the MAIPA assay and anti-PF4 antibodies by EIA.

| Sample ID | VITT§ | mean OD GPIIb/IIIa* | mean OD GPIb/IX* | mean OD GPIa/IIa* | Anti-platelet antibodies |
|-----------|-------|---------------------|------------------|------------------|-------------------------|
| 47        | negative | 0.09                | 0.05             | 0.07             | negative                |
| 48        | negative | 0.12                | 0.09             | 0.105            | negative                |
| 49        | negative | 0.105               | 0.09             | 0.1              | negative                |
| 50        | negative | 0.13                | 0.14             | 0.07             | negative                |
| 51        | negative | 0.075               | 0.09             | 0.06             | negative                |
| 52        | negative | 0.09                | 0.1              | 0.1              | negative                |

§VITT assessment was according to the results of the PF4 heparin enzyme-linked immunosorbent assay (ELISA) and the PF4-dependent platelet activation assay. See Materials and Methods.

* Monoclonal antibody-specific immobilization of platelet antigens (MAIPA); The cutoff for a negative result is OD <0.2. See materials and methods. Yellow cells signify anti-platelet antibody detection.
|    | negative |   0.085 |   0.11 |    0.1 | negative |
|----|----------|---------|--------|--------|----------|
| 53 | negative |  0.085  |  0.11  |  0.1   | negative |
| 54 | negative |  0.095  |  0.1   |  0.09  | negative |
| 55 | negative |   0.08  |  0.07  |  0.065 | negative |
| 56 | negative |  0.065  |  0.085 |  0.08  | negative |
| 57 | negative |  0.075  |  0.08  |  0.085 | negative |
| 58 | negative |  0.045  |  0.09  |  0.085 | negative |
| 59 | negative |  0.13   |  0.1   |  0.085 | negative |
| 60 | negative |  0.09   |  0.08  |  0.09  | negative |
| 61 | negative |  0.09   |  0.09  |  0.07  | negative |
| 62 | negative |  0.08   |  0.09  |  0.08  | negative |
| 63 | negative |  0.065  |  0.085 |  0.08  | negative |
| 64 | negative |  0.075  |  0.135 |  0.105 | negative |
| 65 | negative |  0.09   |  0.12  |  0.12  | negative |
| 66 | negative |  0.06   |  0.065 |  0.06  | negative |
| 67 | negative |  0.085  |  0.095 |  0.115 | negative |
| 68 | negative |  0.075  |  0.095 |  0.115 | negative |
| 69 | negative |   0.1   |  0.09  |  0.105 | negative |
| 70 | negative |  0.105  |  0.095 |  0.115 | negative |
| 71 | negative |   0.13  |  0.125 |  0.18  | negative |
| 72 | negative |  0.075  |  0.085 |  0.095 | negative |
| 73 | negative |  0.105  |  0.065 |  0.095 | negative |
| 74 | negative |  0.085  |  0.06  |  0.09  | negative |
| 75 | negative |  0.065  |  0.065 |  0.085 | negative |
| 76 | negative |   0.19  |  0.125 |  0.13  | negative |
| 77 | negative |  0.085  |  0.075 |  0.09  | negative |
| 78 | negative |  0.065  |  0.085 |  0.085 | negative |
| 79 | negative |   0.08  |  0.08  |  0.09  | negative |
| 80 | negative |  0.085  |  0.065 |  0.065 | negative |
| 81 | negative |  0.075  |  0.07  |  0.07  | negative |
| 82 | negative |  0.075  |  0.07  |  0.065 | negative |
| 83 | negative |   0.13  |  0.175 |  0.135 | negative |
| 84 | negative |  0.075  |  0.085 |  0.08  | negative |
| 85 | negative |  0.075  |  0.075 |  0.075 | negative |
| 86 | negative |   0.07  |  0.055 |  0.075 | negative |
| 87 | negative |   0.05  |  0.055 |  0.07  | negative |
| 88 | negative |   0.1   |  0.14  |  0.09  | negative |
| 89 | negative |   0.07  |  0.065 |  0.065 | negative |
| 90 | negative |   0.08  |  0.09  |  0.085 | negative |
| 91 | negative |   0.09  |  0.105 |  0.085 | negative |
| 92 | negative |  0.055  |  0.06  |  0.045 | negative |
| 93 | negative |   0.15  |  0.155 |  0.115 | negative |
|   |   |   |   |   |
|---|---|---|---|---|
| 94 | negative | 0.05 | 0.06 | 0.065 | negative |
| 95 | negative | 0.135 | 0.195 | 0.135 | negative |
| 96 | negative | 0.17 | 0.065 | 0.075 | negative |
| 97 | negative | 0.085 | 0.075 | 0.08 | negative |
| 98 | negative | 0.065 | 0.09 | 0.065 | negative |

§VITT assessment was according to the results of the PF4 heparin enzyme-linked immunosorbent assay (ELISA) and the PF4-dependent platelet activation assay. See Materials and Methods.

* Monoclonal antibody-specific immobilization of platelet antigens (MAIPA); The cutoff for a negative result is OD <0.2. See materials and methods. Yellow cells signify anti-platelet antibody detection.
Supplemental Figure Captions

Supplementary Figure 1 | a, Platelet surface marker expression of human platelets incubated with ChAdOx1 nCov-19 or BNT162b2. Normalized MFIs. Multiple t-tests with Holm-Sidak correction, non-significant. n=8 donors per group. b, Quantification of adenovirus platelet binding to mouse platelets. One-way ANOVA with post-hoc Tukey’s test. Comparison of ChAdOx1 nCov-19 to both controls. n=4 per group. c, Percentage of platelets binding ChAdOx1 nCov-19 after incubation with PGI, 100µM ADP or no incubation, compared to control without ChAdOx1 nCov-19. n=4 per group. Unpaired t-tests. d, Model of accidental i.v. injection. Placement of 30-gauge needle in the exposed hindlimb musculature either in the muscle or in the vasculature. Comparison of systemic Evans Blue distribution after no aspiration/aspiration of blood. Representative photographs of n=3 experiments. e, Platelet counts of mice over time after BNT162b2 injection. Multiple t-tests with Holm-Sidak correction of BNT162b2 i.v. and PBS i.v. is shown. n=4 per time point and group, not significant. f, Top: Sample flow-cytometric plot of ADV-004 (see methods) binding to platelets compared to control incubation. Bottom: Platelet counts of mice over time after intravenous or intramuscular ADV-004 injection. Multiple t-tests with Holm-Sidak correction of BNT162b2 i.v. and PBS i.v. is shown. n=4 per time point and group, not significant. Right: Cell count parameters of blood taken at 24h p.i. Two-way ANOVA with post-hoc Sidak’s test, all nonsignificant. n=4 per group. g, Cell count parameters of blood taken at 24h p.i. with either ChAdOx1 nCov-19 i.v. or i.m., BNT162b2 i.v. or PBS i.m.. Two-way ANOVA with post-hoc Tukey’s test, all nonsignificant. n≥5 per group. h, Quantification of megakaryocyte number and mean diameter in the bone marrow of ChAdOx1 nCov-19 iv or im injected mice. Left: Representative images of bone marrow micrographs of both groups are shown, scale bar is 100µm. Unpaired t-test, individual dots represent mice, n=4 per group. i, Comparison of platelet count at 24h p.i. with either ChAdOx1 nCov-19 i.v. or i.m. (same data as in Figure 1f) or low-dose ChAdOx1 nCov-19 i.v. administration. Unpaired t-tests, n≥4 per group. j, Time course of platelet counts right before and after ChAdOx1 nCov-19 i.v. administration of AID⁻/⁻slgM⁻/⁻ mice. Unpaired t-tests, n=4 per time point. k, Platelet counts of mice over time after repeat ChAdOx1 injection. One group was first injected with PBS, and then with ChAdOx1, while the other group was injected twice with ChAdOx1. Multiple t-tests with Holm-Sidak correction. n=4 per time point and group. l, Quantification of CD41⁺ extracellular vesicles in the blood of ChAdOx1 nCov-19 iv or im injected mice, normalized by platelet count. Unpaired t-test, n=4 per group. m, Percentage of platelet leukocyte aggregates (PLAs) and myeloid platelet leukocyte aggregates in the blood of ChAdOx1 nCov-19 iv or im injected mice. Unpaired t-tests, n=7 per group. Error bars are mean ±s.e.m. n.s. denotes non-significant, *p<0.05, **p<0.01, ***p<0.001.

Supplementary Figure 2 | a, Zoom-in of the crop outs in Figure 2a. Arrows show examples of single platelets in the splenic marginal zone, stars indicate GPIb⁺ agglomerations that are morphologically reminiscent of platelet remnants. Scale bars are 5µm. b, Illustration of intravital splenic imaging with transfused platelets and representative overview image of intravital microscopy. Scale bar 10µm. c, Z-projection of a 3D-stack intravital splenic microscopic image showing phagocytosed ChAdOx1 nCov-19 pretreated transfused platelets (white, arrow) in comparison to PBS treated platelets (green). Scale bar 5µm. d, Series of images from an intravital splenic microscopic video showing phagocytosis (upper arrow) and interactions (lower arrow) of ChAdOx1 nCov-19 pretreated transfused platelets. Time is shown on the upper left, scale bars 5µm. e, Spleen weights of animals 6d p.i. with 0.5µl ChAdOx1 nCov-19. Unpaired t-test. n=6 per group. f, Platelet activation marker expression after plasma incubation of mice with i.v. or i.m. ChAdOx1 nCov-19 administration. Normalized MFIs. Unpaired t-tests, non-significant. n=4 mice per
group. g, C1-E-Inh. complement blockade of anti-platelet antibody binding compared to control treatment as normalized binding ratio. Unpaired t-test. n=5 mice per group. h, Platelet bound IgG of TgH(KL25) mice injected iv or im with ChAdOx1, unpaired t-test, not significant. n=4 mice for iv and n=5 for im. (left). Comparison of iv injected TgH(KL25) with ChAdOx1 iv, im and AID<sup>−/−</sup>sIgM<sup>−/−</sup> iv injected mice, One-way ANOVA with Dunnett’s multiple comparisons test of ChAdOx1 iv. n=5 per group for i.v. or i.m., n=4 for AID<sup>−/−</sup>sIgM<sup>−/−</sup> (right, same data as Figure 4c). i, Observed thrombi of mice injected iv or im with ChAdOx1 nCov-19 in the liver (per FOV) or after macroscopic necropsy. Unpaired t-test. n=5 per group for liver, n=8 per group for necropsy. j, D-Dimer measurement in the plasma of iv or im ChAdOx1 nCov-19 injected mice. Unpaired t-test. n=5 mice per group. k, Anti-SARS-CoV-2 Spike Trimer antibody measurements in iv or im ChAdOx1 nCov-19 injected mice. Logarithmic y-axis. Mann-Whitney-U-tests. Error bars are mean ±s.e.m. *p<0.05, **p<0.01, ***p<0.001.

Supplementary Figure 3 | a, Gating strategy for human in-vitro platelet-adenovirus binding. Last gate was used to quantify platelet-adenovirus binding. b, Gating strategy for murine in-vitro platelet-adenovirus binding. Last gate was used to quantify platelet-adenovirus binding. c, Gating strategy for murine in-vivo platelet-adenovirus binding and platelet surface marker expression. Second gate was used to derive MFIs for platelet surface marker expression, last gate was used to quantify platelet-adenovirus binding. d, Gating strategy for transfused platelet tracking. Both of the last gates were used to quantify ChAdOx1 nCov-19 and BNT162b2 incubated transfused platelet fraction. e, Gating strategy for immunoglobulin binding of platelets incubated with plasma from vaccinated mice. Last gate was used to quantify either IgG (shown) or IgM binding. All gating is shown as contour plots with 2% counter and outliers shown.

Graphical abstract: Thrombocytopenia and splenic platelet directed immune responses after intravenous ChAdOx1 nCov-19 administration. Intramuscular injection of ChAdOx1 nCov-19 leads to normal vaccine response without platelet involvement. Accidental intravascular injection of ChAdOx1 nCov-19 leads to adenovirus-platelet binding and platelet activation. Platelets are cleared by professional phagocytes, particularly in the spleen. Trafficking and processing of platelet adenovirus-aggregates from the red pulp to splenic follicles leads to a B cell response with the emergence of platelet binding antibodies. These range from causing platelet opsonization and clearance (vaccine associated thrombocytopenia) to causing platelet activation and intravascular thrombus formation (VITT).
Suppl. References

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2. Kiefel V. The MAIPA assay and its applications in immunohaematology. *Transfus Med*. 1992;2(3):181-188.

3. Nicolai L, Leunig A, Brambs S, et al. Immunothrombotic dysregulation in COVID-19 pneumonia is associated with respiratory failure and coagulopathy. *Circulation*. 2020.
Supplementary Figure 2

(a) ChAdOx1-S im  ChAdOx1-S iv

(b) Intravital 4-color 4D splenic imaging with F4/80 and CD169 labelling

(c) CD169 F4/80 ChAdOx1-S Plts, Control Plts

(d) KL25 im  KL25 iv

(e) Spleen weight (mg)

(f) Normalized MFI

(g) Platelet bound IgG (normalized percentage)

(h) Platelet bound IgM (normalized percentage)

(i) Microthrombi per FOV in liver

(j) Macroscopic thrombi per mouse

(k) Anti-SARS-CoV-2 Spike IgG (pg/ml)
Supplementary Figure 3

a

b

c

d

e