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Supplementary appendix

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Supplement to: Arora P, Nehlmeier I, Kempf A, et al. Lung cell entry, cell–cell fusion capacity, and neutralisation sensitivity of omicron sublineage BA.2.75. *Lancet Infect Dis* 2022; published online Sept 15. https://doi.org/10.1016/S1473-3099(22)00591-6.

Appendix

Omicron sublineage BA.2.75 shows increased lung cell entry, cell-cell fusion capacity and reduced neutralisation sensitivity compared to BA.2

Content

Figure

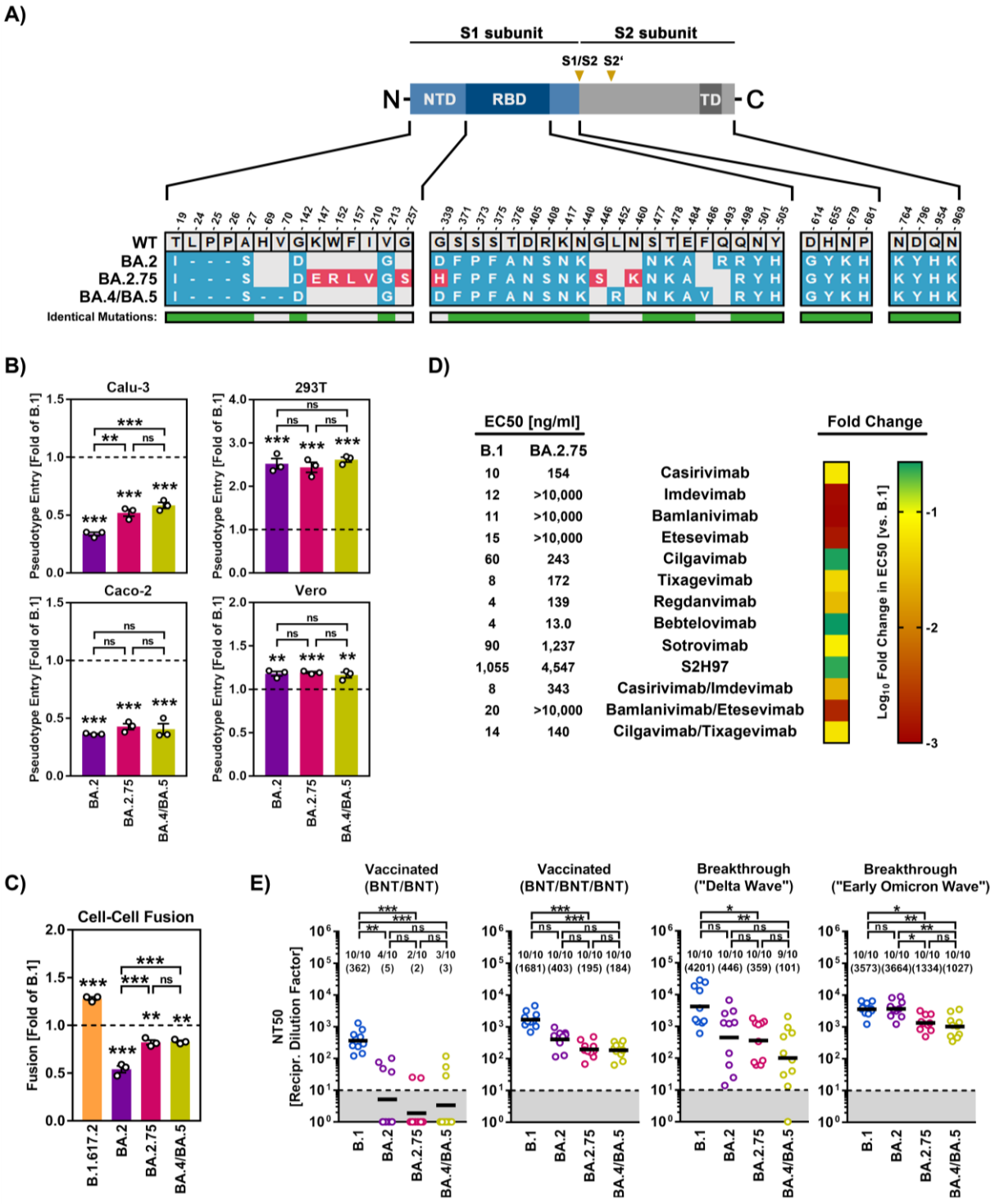

Figure: Host cell entry efficiency, cell-cell fusion capacity and neutralisation sensitivity of Omicron sublineage BA.2.75.

(A) Schematic overview of the SARS-CoV-2 spike (S) protein domain structure (top) and summary of the mutations found in the different Omicron sublineages (bottom, numbering according to the S protein of SARS-CoV-2 Wuhan-Hu-01). S protein mutations found in Omicron sublineages BA.2, BA.2.75 and BA.4/BA.5 are indicated, with those that are specific to BA.2.75 being highlighted in pink (identical

mutations found in all three Omicron sublineages are indicated). Abbreviations: NTD = N-terminal domain; RBD = receptor-binding domain; TD = transmembrane domain; S1/S2 and S2' = cleavage sites in the S protein. (B) Pseudovirus particles bearing the indicated S proteins were inoculated onto Vero (African green monkey, kidney), 293T (Human, kidney), Caco-2 (Human, colon), or Calu-3 (Human, lung) cells and cell entry was analysed at 16-18 h postinoculation by measuring the activity of virus-encoded firefly luciferase in cell lysates. Presented are the mean data from three biological replicates, each performed with four technical replicates. Error bars indicate the standard error of the mean (SEM). Data were normalised against cell entry of $B.1_{pp}$ (set as 1, dashed line). Statistical significance was assessed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (not significant [ns], $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$). Please also see Supplementary Information Figure S1 for more information. (C) Effector cells expressing the indicated S protein (or no S protein) together with the 293T effector cells transfected to co-express the indicated S proteins (or no S protein) and the beta-galactosidase alpha fragment were co-cultured with 293T target cells transfected to co-express ACE2 and the beta-galactosidase omega fragment. After an incubation period of 18 h, cells were incubated for 90 min in the presence of beta galactosidase substrate, before fusion was analysed by measuring the activity of reconstituted beta galactosidase. Presented are the mean data from three biological replicates, each performed with four technical replicates. Error bars indicate the SEM. Data were normalised against fusion induced by B.1 S (set as 1, dashed line). Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparisons test (not significant [ns], $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$). Please also see Supplementary Information Figure S1 for more information. (D) Neutralisation of BA.2.75 by monoclonal antibodies. Pseudovirus particles bearing B.1 or BA.2.75 S protein were preincubated with monoclonal antibody (or irrelevant human IgG) dilutions before being inoculated onto Vero cells. Presented are the mean data from three biological replicates, each performed with four technical replicates. Data were normalised against samples that did not include antibody in order to calculate the relative inhibition. Please also see Supplementary Information Figure S2 for more information. (E) Neutralisation of BA.2.75 by antibodies induced upon vaccination with or without breakthrough infection. Pseudovirus particles bearing the indicated S proteins were preincubated with serum/plasma dilutions of people vaccinated with two (n = 10) or three doses (n = 10) of the BNT162b2/Comirnaty mRNA vaccine (BNT), or vaccinated individuals that

experienced breakthrough infections during the “Delta” (n = 10) or early “Omicron” wave (n = 10) in Germany, before being inoculated to Vero cells. Relative inhibition of pseudovirus entry was calculated using particles incubated in the absence of serum/plasma as control (= 0% inhibition) and NT50 (neutralizing tire 50) values were calculated using a non-linear regression model. Samples that yielded NT50 values below 10 were considered negative and manually assigned an NT50 value of 1. Presented are the geometric mean NT50 data (indicate by black lines and numerical values in brackets) from a single experiment, performed with four technical replicates. The proportion of samples with detectable neutralizing activity are indicated. Statistical significance was assessed by Kruskal-Wallis analysis with Dunn’s multiple comparison test (ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$). Please also see Supplementary Information Figure S3 for more information.

Table

ID	Gender	Age (years)	Sample group	Vaccination	Time since last vaccination and sampling (days)	Documented SARS-CoV-2 infection? (yes/no, period, variant)	Time since first positive test and sampling (days)
4848	Male	29	Vaccinated	Yes (BNT/BNT)	25	No[1], n.a., n.a.	n.a.
4863	Female	58		Yes (BNT/BNT)	25	No[1], n.a., n.a.	n.a.
4866	Female	59		Yes (BNT/BNT)	27	No[1], n.a., n.a.	n.a.
4874	Female	26		Yes (BNT/BNT)	27	No[1], n.a., n.a.	n.a.
4877	Female	29		Yes (BNT/BNT)	27	No[1], n.a., n.a.	n.a.
4900	Female	54		Yes (BNT/BNT)	28	No[1], n.a., n.a.	n.a.
4883	Female	43		Yes (BNT/BNT)	34	No[1], n.a., n.a.	n.a.
4828	Female	23		Yes (BNT/BNT)	27	No[1], n.a., n.a.	n.a.
4864	Female	53		Yes (BNT/BNT)	26	No[1], n.a., n.a.	n.a.
4803	Female	35		Yes (BNT/BNT)	13	No[1], n.a., n.a.	n.a.
7543	Female	53		Yes (BNT/BNT/BNT)	41	No[1], n.a., n.a.	n.a.
7551	Male	59		Yes (BNT/BNT/BNT)	13	No[1], n.a., n.a.	n.a.
7690	Female	41		Yes (BNT/BNT/BNT)	32	No[1], n.a., n.a.	n.a.
7884	Female	34		Yes (BNT/BNT/BNT)	39	No[1], n.a., n.a.	n.a.
7960	Male	39		Yes (BNT/BNT/BNT)	24	No[1], n.a., n.a.	n.a.
7973	Female	53		Yes (BNT/BNT/BNT)	24	No[1], n.a., n.a.	n.a.
7974	Female	59		Yes (BNT/BNT/BNT)	45	No[1], n.a., n.a.	n.a.
8046	Female	52		Yes (BNT/BNT/BNT)	47	No[1], n.a., n.a.	n.a.
8047	Female	58		Yes (BNT/BNT/BNT)	16	No[1], n.a., n.a.	n.a.
8049	Female	63		Yes (BNT/BNT/BNT)	39	No[1], n.a., n.a.	n.a.
SD20	Female	82	Vaccinated & Breakthrough Infection[3]	3x (unknown[6])	unknown[7]	Yes[2], "Delta wave"[4], unknown	13
SD22	Male	78		3x (unknown[6])	unknown[7]	Yes[2], "Delta wave"[4], unknown	13
SD24	Male	39		3x (unknown[6])	85	Yes[2], "Delta wave"[4], unknown	3
SD26	Female	70		3x (BNT/BNT/BNT)	44	Yes[2], "Delta wave"[4], unknown	3
SD27	Female	86		3x (unknown[6])	unknown[7]	Yes[2], "Delta wave"[4], unknown	15
SD28	Male	92		3x (unknown[6])	>58[8]	Yes[2], "Delta wave"[4], unknown	4
SD30	Female	77		3x (BNT/BNT/BNT)	63	Yes[2], "Delta wave"[4], unknown	11
SD32	Male	74		3x (unknown[6])	unknown[7]	Yes[2], "Delta wave"[4], unknown	12
SD33	Female	87		3x (BNT/BNT/BNT)	unknown[7]	Yes[2], "Delta wave"[4], unknown	7

SD35	Male	82		3x (unknown[6])	unknown[7]	Yes[2], "Delta wave"[4], unknown	11
8647	Female	36		Yes (BNT/BNT/BNT)	165	Yes[2], "Omicron wave"[5], unknown	62
8618	Female	27		Yes (BNT/BNT/BNT)	149	Yes[2], "Omicron wave"[5], unknown	25
8619	Male	31		Yes (BNT/BNT/BNT)	139	Yes[2], "Omicron wave"[5], unknown	23
8648	Male	39		Yes (BNT/BNT/BNT)	146	Yes[2], "Omicron wave"[5], unknown	22
8680	Female	43		Yes (BNT/BNT/BNT)	123	Yes[2], "Omicron wave"[5], unknown	52
8693	Female	48		Yes (BNT/BNT/BNT)	149	Yes[2], "Omicron wave"[5], unknown	48
8742	Female	45		Yes (BNT/BNT/BNT)	174	Yes[2], "Omicron wave"[5], unknown	19
8744	Male	33		Yes (BNT/BNT/BNT)	187	Yes[2], "Omicron wave"[5], unknown	32
8765	Female	37		Yes (BNT/BNT/BNT)	152	Yes[2], "Omicron wave"[5], unknown	22
8770	Female	44		Yes (BNT/BNT/BNT)	167	Yes[2], "Omicron wave"[5], unknown	26

[1]: Vaccinees were anti-Spike IgG negative before vaccination and tested negative for anti-NCP IgG after their last vaccination

[2]: SARS-CoV-2 infection was confirmed by real-time reverse transcriptase polymerase chain reaction

[3]: The breakthrough infection groups were selected based on the timing of infection and the neutralisation profile in a pre-screen (with pseudovirus particles bearing the spike proteins of either B.1, B.1.617.2 [Delta variant] or BA.1 [Omicron variant]).

[4]: Germany, 12/2021 to 01/2022; samples were pre-screened for their specific neutralisation activity against $B.1_{pp}$, $B.1.617.2_{pp}$ and $BA.1_{pp}$.

[5]: Germany, 02/2022 to 05/2022, dominated by BA.1 and BA.2; samples were pre-screened for their specific neutralisation activity against $B.1_{pp}$, $B.1.617.2_{pp}$ and $BA.1_{pp}$.

[6]: No specific information on the type of vaccine(s) available.

[7]: No specific information on the date of last vaccination available (no vaccination within the last 14 days prior to sampling).

[8]: Third vaccination occurred in November 2021.

Abbreviations: ID, identifier; n.a., not applicable; BNT, BNT162b2/Comirnaty

Methods

Cell culture

All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. 293T (human, female, kidney; ACC-635, DSMZ; RRID: CVCL_0063), Vero cells (African green monkey kidney, female, kidney; CRL-1586, ATCC; RRID: CVCL_0574, kindly provided by Andrea Maisner) were grown in Dulbecco's Modified Eagle Medium (DMEM; PAN-Biotech). Calu-3 (human, male, lung; HTB-55, ATCC; RRID: CVCL_0609, kindly provided by Stephan Ludwig) were cultured in DMEM/F-12 medium (Gibco). Caco-2 cells (human, male, colon; HTB-37, ATCC, RRID: CVCL_0025) were cultured in minimum essential medium (Gibco). All media were supplemented with 10% fetal bovine serum (Biochrom), 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin (PAN-Biotech). Caco-2 and Calu-3 cells further received non-essential amino acid solution (1:100, from 100x stock; Gibco) and 1 mM sodium pyruvate (from 100 mM stock; PAN-Biotech). Cell lines were validated using STR analysis, amplification and sequencing of a cytochrome c oxidase gene fragment, microscopic investigation, and/or growth characteristics. Furthermore, cell lines were routinely tested for absence of mycoplasma.

Expression plasmids and sequence analysis

Expression plasmids pCAGGS-DsRed[1], pQCXIP-ACE2[2], pQCXIP-beta-galactosidase alpha fragment[3], pQCXIP-beta-galactosidase omega fragment[3], pCG1-SARS-CoV-2 B.1 SΔ18 (codon-optimised, C-terminal truncation of 18 amino acid residues, GISAID Accession ID: EPI_ISL_425259)[4], pCG1-SARS-CoV-2 B.1.617.2 (Delta variant) SΔ18 (codon-optimised, C-terminal truncation of 18 amino acid residues, GISAID Accession ID: EPI_ISL_1921353)[5], pCG1-SARS-CoV-2 BA.2 SΔ18 (codon-optimised, C-terminal truncation of 18 amino acid residues, GISAID Accession ID: EPI_ISL_8738174)[3] and pCG1-SARS-CoV-2 BA.4/BA.5 SΔ18 (codon-optimised, C-terminal truncation of 18 amino acid residues, GISAID Accession ID: EPI_ISL_11550739 and EPI_ISL_12029894)[6] have previously been described elsewhere. Gibson assembly was employed to generate expression plasmids for SARS-CoV-2 BA.2.75 SΔ18 (based on GISAID Accession ID: EPI_ISL_13692860) using overlapping DNA strings (Thermo Fisher Scientific), BamHI/XbaI-digested pCG1 plasmid and GeneArt™ Gibson Assembly HiFi Master Mix (Thermo Fisher Scientific). The manufacturer's instructions were followed for

preparing the reactions. The pCG1 expression plasmid was kindly provided by Roberto Cattaneo, Mayo Clinic College of Medicine, Rochester, MN, USA. A commercial sequencing service was used to confirm all PCR-amplified sequences (Microsynth SeqLab). The GISAID (Global Initiative on Sharing All Influenza Data) database was used to gather S protein sequences and the supporting data (collection date, location, https://www.gisaid.org/).

Pseudovirus particle production

Pseudovirus particles containing SARS-CoV-2 S proteins were produced as previously described[7]. After transfection using the calcium phosphate method, 293T cells expressing the respective S protein or DsRed (control) were infected with VSV-G-transcomplemented VSV*G(FLuc) (kindly provided by Gert Zimmer, Institute of Virology and Immunology, Mittelhäusern/Switzerland)[8]. The inoculum was removed after 1 h of incubation, and cells were washed with phosphate-buffered saline (PBS). The cells were further incubated for 16–18 h in medium containing anti-VSV-G antibody (culture supernatant from I1-hybridoma cells; ATCC no. CRL-2700; except for VSV-G-expressing cells). Finally, the pseudovirus particle-containing culture supernatants were harvested, separated from cellular debris by centrifugation (4,000 x g, 10 min), and stored at -80 °C until further use.

Transduction of target cells

Identical volumes of pseudotype particles were used to infect target cells, which were seeded in 96-well plates. Transduction efficiency was determined by measuring luciferase activity in cell lysates at 16–18 h postinoculation. Cells were lysed in PBS containing 0.5% triton x-100 (Carl Roth) for 30 minutes at room temperature. Afterwards, cell lysates were loaded in white 96-well plates and luciferase substrate (Beetle-Juice, PJK) was added. Luminescence was further quantified using a Hidex Sense plate luminometer (Hidex).

Cell-cell fusion assay

At 24 h post seeding, 293T effector cells were cotransfected with expression plasmids for the respective S protein or empty vector and the beta-galactosidase alpha fragment by calcium phosphate precipitation. At the same time, 293T target cells were cotransfected with expression plasmids for ACE2 and the beta-

galactosidase omega fragment by calcium phosphate precipitation. At 18 h posttransfection, cells were washed and further incubated with fresh medium for additional 6 h. Next, target cells were washed with PBS, resuspended in fresh medium and added on the effector cells. Cells were co-cultivated for 18 h, before beta-galactosidase substrate (Gal-Screen, Thermo Fisher Scientific) was added and luminescence was recorded after an incubation period of 90 min using a Hidex Sense plate luminometer (Hidex).

Serum/plasma samples

Serum/plasma were collected at the Hannover Medical School (Medizinische Hochschule Hannover, MHH) and University Medicine Göttingen (UMG). In total, four different cohorts were tested: (i) individuals vaccinated with two doses of the BNT162b2/Comirnaty vaccine without breakthrough infection (n=10, MHH, 2 males and 8 females, median age 39 years); (ii) individuals vaccinated with three doses of the BNT162b2/Comirnaty vaccine without breakthrough infection (n=10, MHH, 1 male and 9 females, median age 53 years); (iii) individuals vaccinated with three doses of the BNT162b2/Comirnaty vaccine with breakthrough infection during the "Delta wave" in Germany (n=10, UMG, 5 males and 5 females, median age 80 years); (iv) individuals vaccinated with three doses of the BNT162b2/Comirnaty vaccine with breakthrough infection during the "Omicron wave" in Germany (n=10, MHH, 3 males and 7 females, median age 38 years). Patient information is provided in Table 1. All samples were heat-inactivated at 56 °C for 30 min.

Ethics committee approval

The research ethics committee of the UMG (SeptImmun Study and PneumoSept Study) and institutional review board of MHH (8973 BO K 2020) gave its approval for the collection of samples. Written informed consent was obtained from each participant prior to the use of any plasma samples for research.

Neutralisation assay

Neutralisation assays were carried out according to an established protocol[6,9]. Briefly, pseudotype particles were pre-incubated (30 min at 37°C) with different concentrations (5, 0.5, 0.05, 0.005, 0.0005 µg/ml) of monoclonal antibody (Casirivimab, Imdevimab, Bamlanivimab, Etesevimab, Cilgavimab, Tixagevimab, Sotrovimab, Bebtelovimab, Regdanvimab, S2H97 or an unrelated human control

antibody [hIgG] or antibody cocktails [Casirivimab/Imdevimab, Bamlanivimab/Etesevimab, Cilgavimab/Tixagevimab]) or serum/plasma dilutions (1:50, 1:200, 1:800, 1:3,200, 1:12,800). Of note, for antibody cocktails each antibody was used at half the concentration in order to keep total antibody concentrations constant. After that, mixtures were added to Vero cells and incubated for 16–18 hours. Transduction efficiency was examined as described above. Pseudovirus particles incubated in medium without monoclonal antibody or serum/plasma were used as the reference (= 0% inhibition).

Statistical analysis

Microsoft Excel (part of Microsoft Office Professional Plus, version 2016, Microsoft Corporation) and GraphPad Prism version 8.3.0 were used to analyse the data (GraphPad Software). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (cell entry and cell-cell fusion studies), two-way ANOVA with Dunnett's post-hoc test (antibody neutralisation) and Kruskal-Wallis analysis with Dunn's multiple comparison test (plasma neutralisation) were used to determine statistical significance. Only p-values of 0.05 or lower were considered statistically significant (not significant [ns], $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$). A non-linear regression model was used to calculate the antibody concentrations (effective concentration 50, EC50) and plasma dilutions (neutralizing titre 50, NT50) resulting in half-maximal inhibition of pseudovirus entry.

Limitations of the study

Our research has some shortcomings. As a substitute model for the investigation of SARS-CoV-2 variant neutralisation, we used pseudotyped particles. Although these particles accurately simulate host cell entry and neutralisation of SARS-CoV-2 by antibodies [10], our findings await confirmation with authentic SARS-CoV-2 isolates, which are not available to us at present. Additionally, most sera/plasma were collected within the first two months following the last vaccination or a positive test result. Therefore, we cannot rule out Omicron sublineage-specific changes in the level of antibody evasion may become more or less pronounced at later time points. Finally, since only sera/plasma from BNT162b2/Comirnaty-vaccinated individuals were examined, it remains to be tested whether Omicron sublineages may be differentially neutralised in the context of different vaccines or vaccination regimes.

Acknowledgements

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Supplementary references

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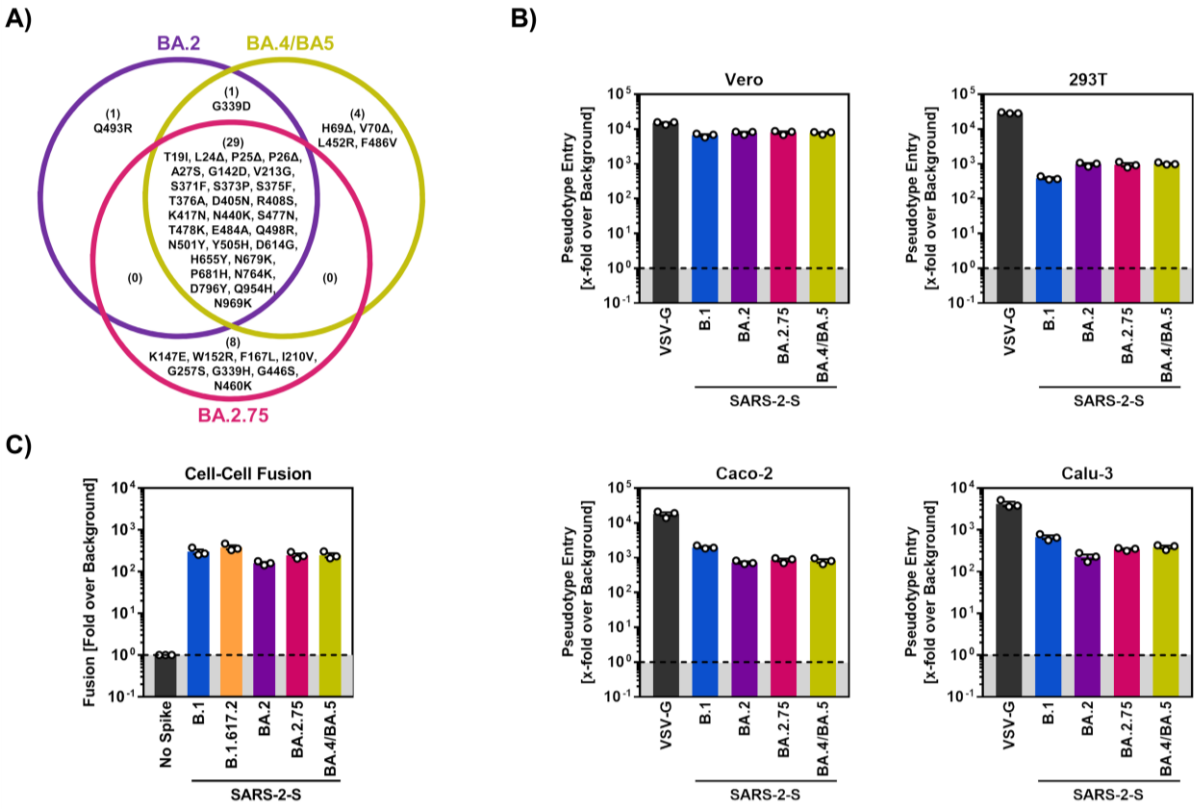
Supplementary figures



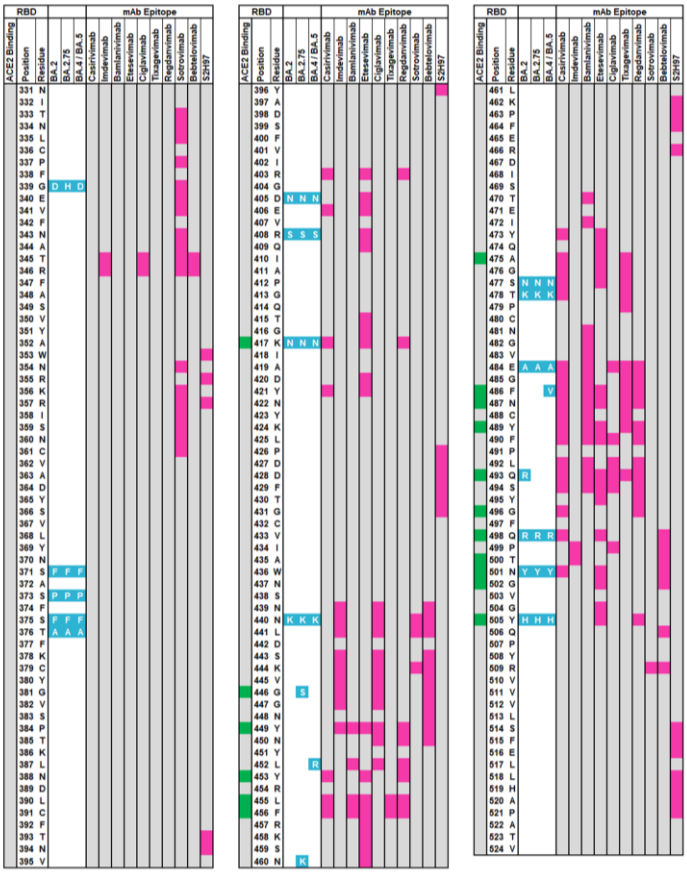
Supplementary figure 1: Omicron-specific S protein mutations and their impact on host cell entry.

(A) Shared and unique S protein mutations between Omicron sublineages BA.2, BA.2.75 and BA.4/BA.5.

(B) The pseudovirus entry data were normalised against the assay background (luciferase activity obtained for particles bearing no viral surface protein, set as 1). Further, data for particles bearing vesicular stomatitis virus glycoprotein (VSV-G) are included.

(C) The cell-cell fusion data were normalised against the assay background (beta-galactosidase activity obtained for effector cells expressing no S protein mixed with target cells, set as 1).

A)



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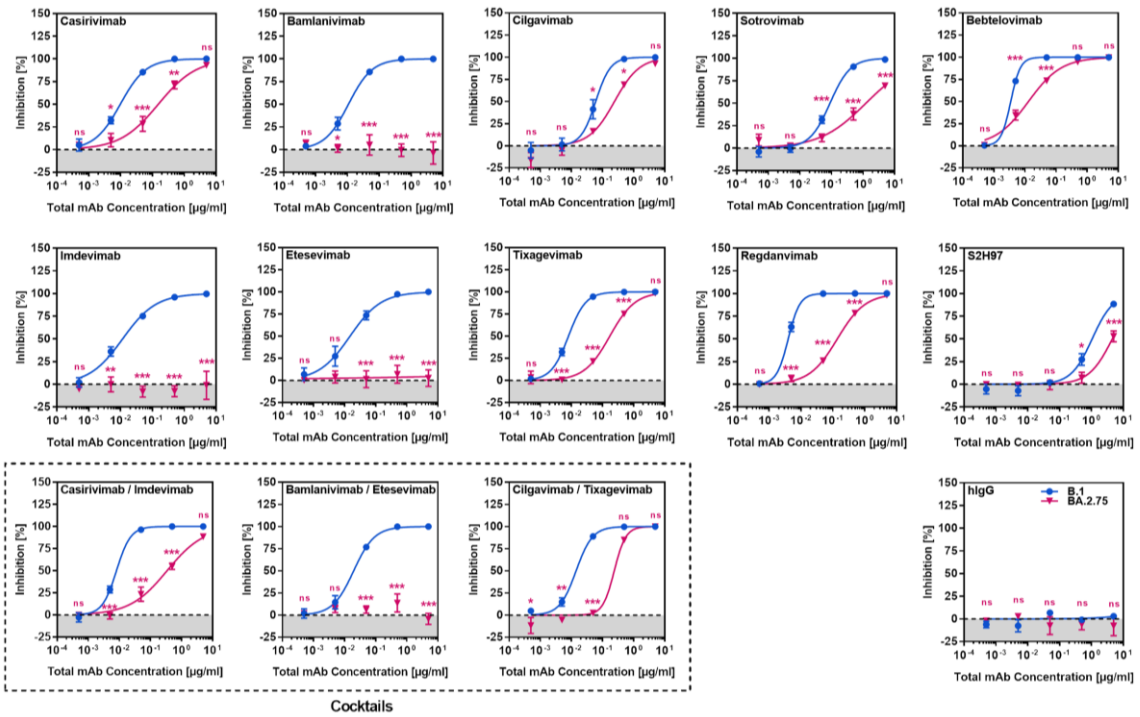
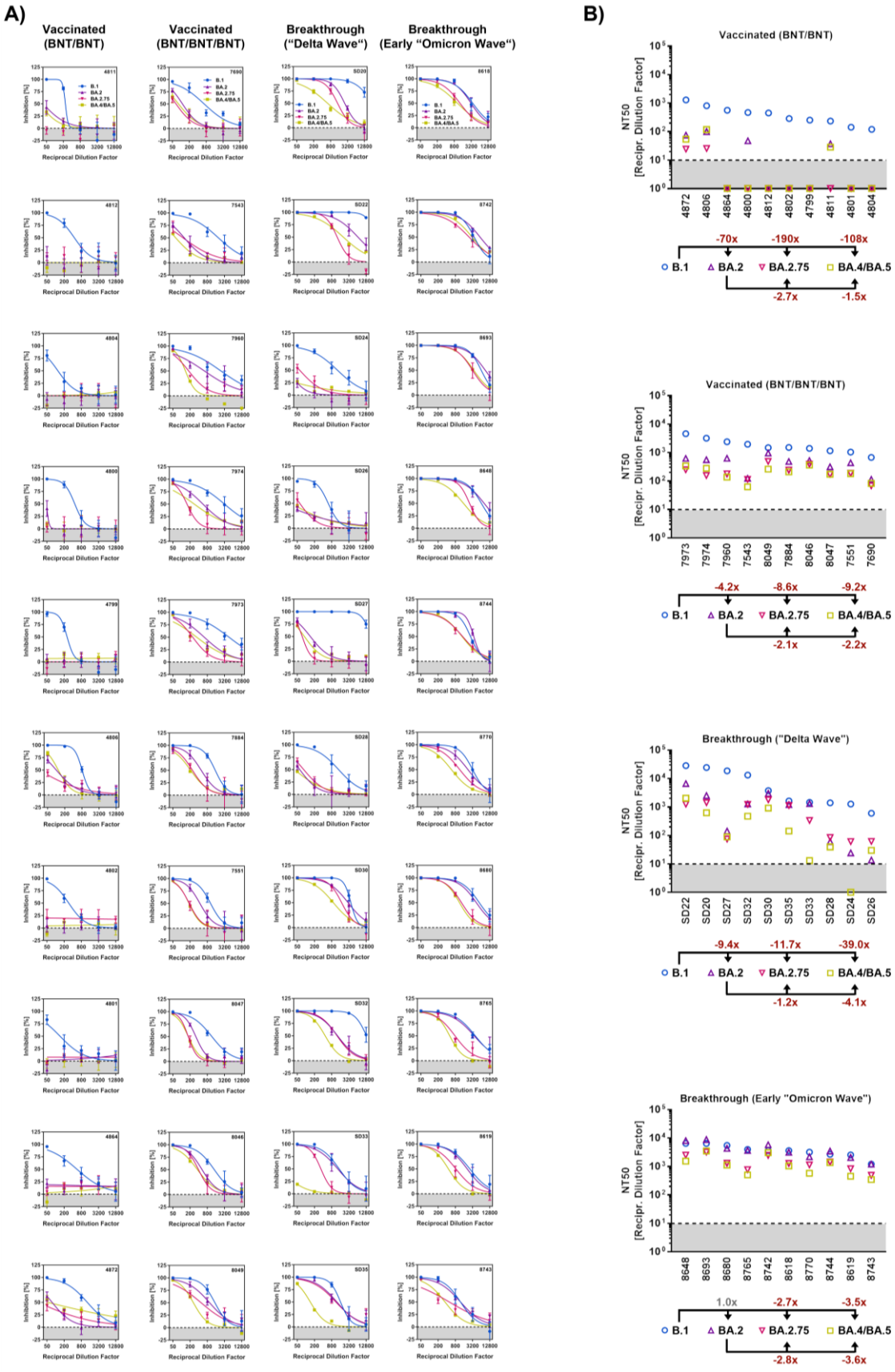


Supplementary figure 2: Neutralisation of BA.2.75 by monoclonal antibodies used for COVID-19 therapy.

(A) Schematic overview of the Omicron sublineage-specific RBD mutations in the context of binding sites for monoclonal antibodies. Mutations found in the RBDs of the Omicron sublineages (compared to the S protein of SARS-CoV-2 Wuhan-Hu-01) are highlighted in blue. RBD resides that make direct contact to ACE2 (green) or that are recognised as epitopes for therapeutic antibodies (pink) are highlighted.

(B) Individual data on BA.2.75 neutralisation by monoclonal antibodies. Particles bearing the indicated S proteins were preincubated with dilutions of single monoclonal antibodies used or developed for COVID-19 therapy or cocktails thereof before being added to Vero cells. An irrelevant antibody (hIgG) served as control. Of note, for antibody cocktails each antibody was used at half concentration in order to keep total antibody concentrations constant. S-protein-driven cell entry was analysed and normalised to samples without antibody (= 0% inhibition). Presented are the average (mean) data of three biological replicates, each performed with four technical replicates. Error bars indicate the SEM. Statistical significance was assessed by two-way analysis of variance with Dunnett's post-hoc tests (not significant [ns], $p > 0.05$] *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).



Supplementary figure 3: Neutralisation of BA.2.75 by antibodies induced upon vaccination or vaccination and breakthrough infection.

(A) Individual data on BA.2.75 neutralisation by antibodies induced upon vaccination or vaccination an and breakthrough infection. Pseudovirus particles bearing the indicated S proteins were preincubated

with serial dilutions of serum/plasma from people vaccinated with two or three doses of the BNT162b2/Comirnaty mRNA vaccine (BNT), or vaccinated individuals that experienced breakthrough infections during the “Delta” or early “Omicron” waves in Germany, before being inoculated to Vero cells. Pseudovirus particles incubated in the absence of serum/plasma served as control. Presented are the mean data from a single experiment, performed with four technical replicates. Error bars indicate the standard deviation. S-protein-driven cell entry was analysed and normalised to samples without serum/plasma (= 0% inhibition).

(B) Neutralizing titre 50 values for each serum/plasma (ranked according to the neutralizing activity against $B.1_{pp}$, from highest to lowest). The fold change in neutralisation (NT50) compared to either $B.1_{pp}$ or $BA.2_{pp}$ is indicated.