Superior cellular and humoral immunity towards SARS-CoV-2 and alpha and beta VOC strains in COVID-19 convalescent as compared to the prime boost BNT162b2 vaccinated dialysis patients

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Table S1: Characteristics of convalescent and vaccinated hemodialysis patients

Patient groups	Convalescent	Vaccinated with BNTb162b
No. of patients	18	22
Age in years; Median (range)	62 (39 – 78)	67 (43 – 80)
Sex (male/female)	10/8 (55.6%/44.4%)	14/8 (63.6%/36.4%)
Time since last antigenic contact (COVID-19 convalescence or vaccination) in days; Median (range)	158 (61 – 390)	60 (17-441)
Hemodialysis	18 (100.0%)	22 (100.0%)
Immunosuppressive medication	0 (0.0%)	0 (0.0%)
Severity of COVID-19		
Mild (no, few symptoms)	15 (83%)	0
Moderate (hospitalization)	3 (17%)	0
Severe (hospitalization)	0 (0%)	0
Cause of ESRD		
Diabetic nephropathy	4 (22.2%)	11 (50.0%)
Hypertensive kidney disease	4 (22.2%)	5 (22.7%)
Glomerulonephritis including vasculitis	5 (27.8%)	1 (4.5%)
Other	4 (22.2%)	4 (18.2%)
Not specified	1 (5.6%)	1 (4.5%)

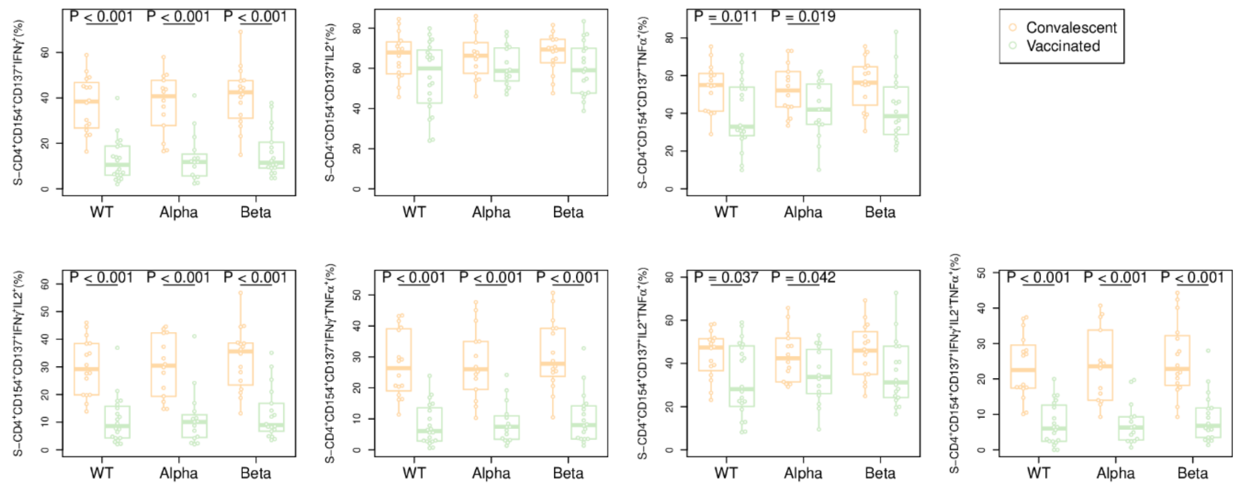

Figure S1: Frequency of IFNγ-, IL2-, and TNFα-producing cells among S-protein reactive CD4⁺CD154⁺CD137⁺ T cells in convalescent and vaccinated hemodialysis patients

Cytokine expression by activated CD4⁺ T cells was evaluated in PBMC after stimulation with OPP of S-WT, alpha, and bate, only samples with a frequency of activated T cells over 0.01% are considered in this analysis. The upper row indicates the expression of IFNγ (left), IL2 (middle) and TNFα (right). The lower row indicates the frequencies of bi-functional CD4⁺CD154⁺CD137⁺ T cells that produce (from left to right): IL2 and IFNγ, IFNγ and TNFα, IL2 and TNFα as well as trifunctional CD4⁺CD154⁺CD137⁺ T cells that produce IFNγ, IL2, and TNFα. Frequencies of multicytokine-producing T cells were identified by Boolean gating.

Box plots depict the median, first and third quartile of a variable; the maximum length of the whiskers corresponds to 1.5 times the interquartile range.

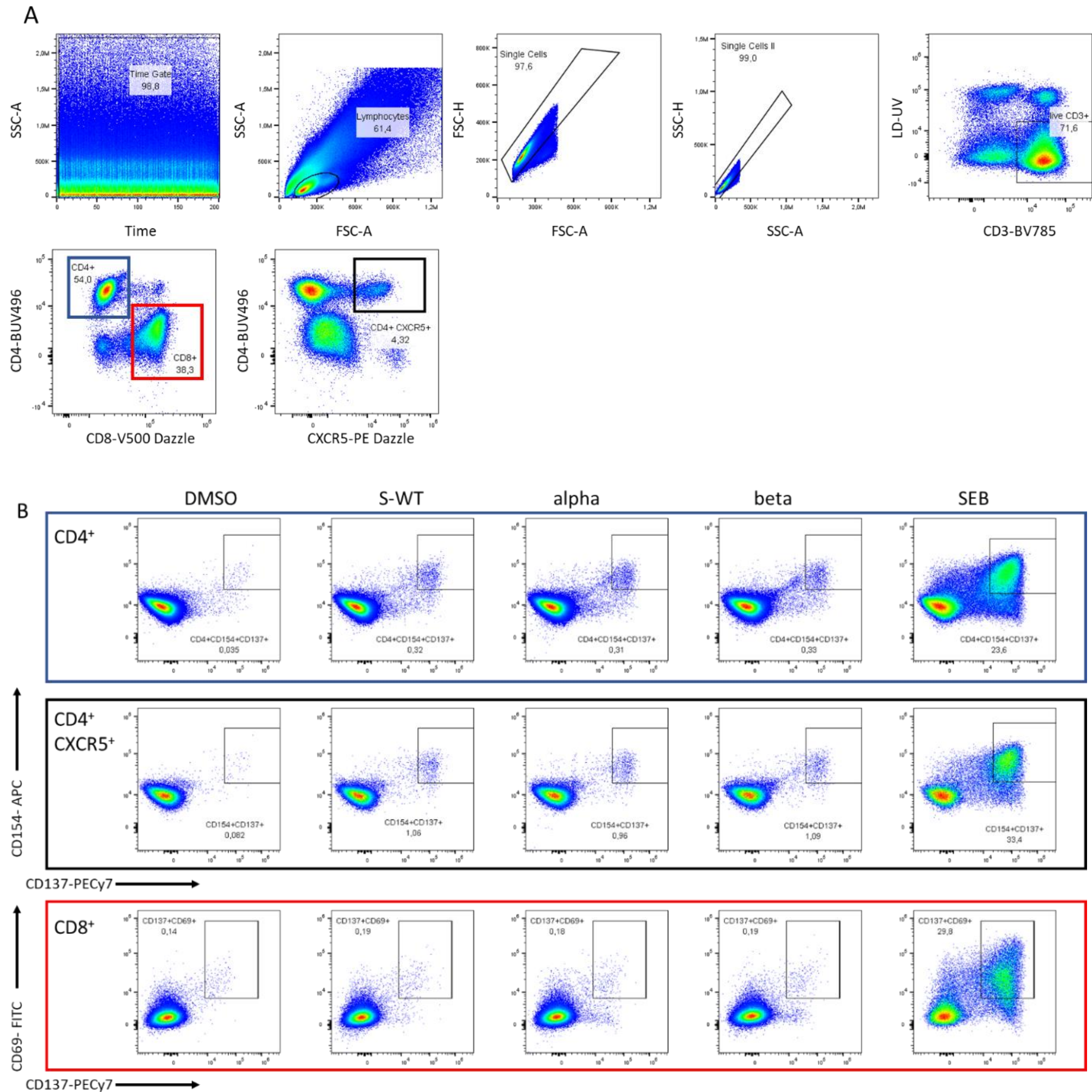

Figure S2: Gating strategy to identify S-protein reactive T cells among CD4⁺ T cells, CD4⁺ CXCR5⁺ T cells, and CD8⁺ T cells

PBMC were incubated for 16 hours with overlapping peptide pools (OPP) of the SARS-CoV-2 strains wildtype (WT), alpha, and beta in the presence of brefeldin A, which was added 2 hours after the OPP. Stimulations with DMSO as peptide diluent and *Staphylococcus aureus* enterotoxin B (SEB) as polyclonal stimulus served as negative and positive controls, respectively. Cells were acquired using a Cytoflex LX flow cytometer. **(A)** Representative gating to identify $CD4^+$ T cells, $CD4^+$ $CXCR5^+$ T cells, which share features of T follicular helper cells (Tfh) and $CD8^+$ T cells. Top row from left to right: time gate followed by debris exclusion and lymphocyte identification; cell aggregated were excludes by SSC-H vs SSC-A and FSC-H vs FSC-A plots; live/dead (LD) and CD3 stainings identified live $CD3^+$ T cells; Bottom row, left: $CD4^+$ and $CD8^+$ T cell discrimination; right, $CD4^+$ $CXCR5^+$ T cells (TfH) identification. **(B)** Identification of T cells reactive against the wild-type S-protein (S-WT) and the S-proteins of the VOC alpha and beta among $CD4^+$ T cells (top, blue frame), $CD4^+$ $CXCR5^+$ T cells (middle, black frame), and $CD8^+$ T cells (bottom, red frame). Plots are representative for n=37 individuals that were either vaccinated against SARS-COV-2 or are convalescent after COVID-19.

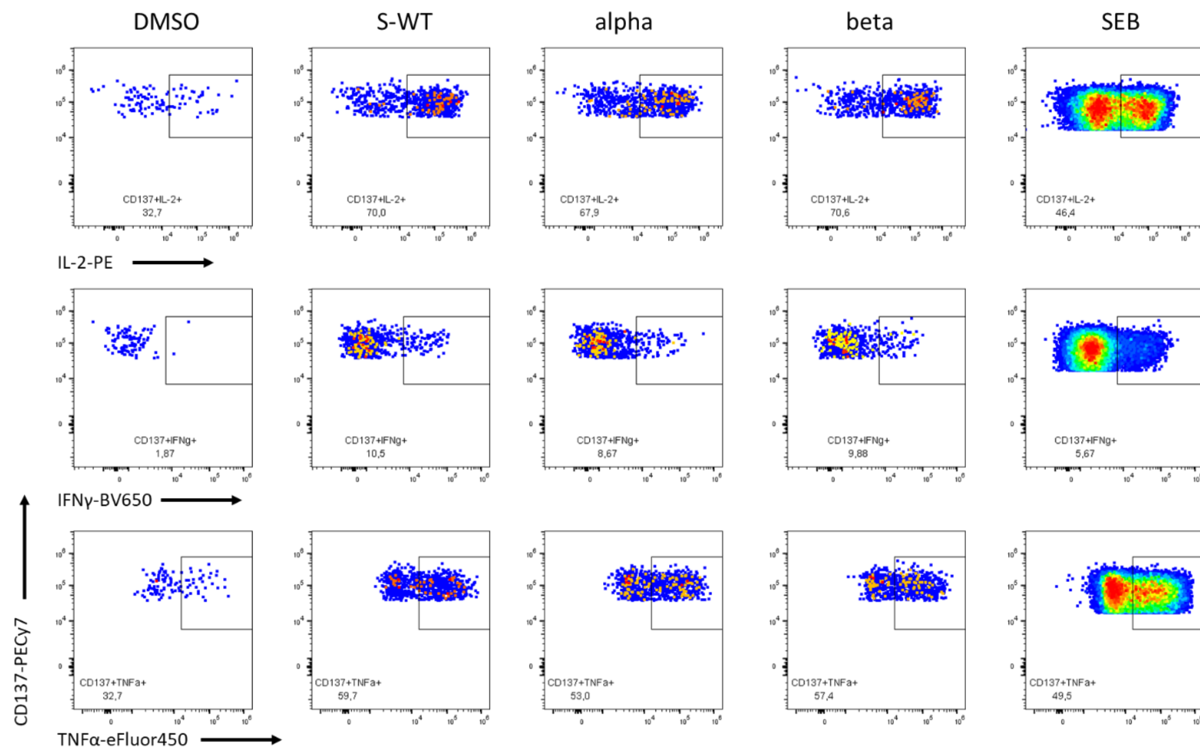

Figure S3: Representative dot plots illustrating expression of IL2, TNFα, and IFNγ by activated $CD4^{+}CD154^{+}CD137^{+}$ T cells. PBMC were treated as described above. $CD4^{+}CD154^{+}CD137^{+}$ T cells were identified as sown in Supplementary figure 2. Expression of IL2 (top), IFNγ (middle) and TNFα (bottom) in untreated (DMSO) PBMC and PBMC stimulated with OPP of S-WT, alpha, and beta. SEB served as positive control showing the overall capacity of the T cells to produce cytokines. Dot plots are representative for n=37 individual donors that were either vaccinated against SARS-COV-2 or are convalescent after COVID-19.

Supplementary Methods

Study population and design

For this study, 40 hemodialysis patients were recruited of whom 18 are convalescent and 22 were vaccinated two times with BNTb162b mRNA. Analysis was done in median 60 days post-vaccination (range 17-441 days) and 158 days (range 61-290 days) post infection in DP. The median age of DP was 64.8 years (range 39-80 years). Of the 40 participants, 24 (60%) were of male and 16 (40%) of female sex. The clinical characteristics of DP patients are summarized in Table S1. Most of the convalescent patients had a mild course of COVID-19 diseases. Only 3 out of 18 patients required hospitalization and short-term FiO2 supply without any further medical supportive measures. The study was approved by the ethical committee of the Ruhr-University Bochum (20-6886; 20-7126). Written informed consent was obtained from all participants.

SARS-CoV-2 IgG antibody titers

Serum was generated according to the manufacturer's instructions from blood collected into S-Monovette Z-Gel (Sarstedt). SARS-CoV-2 serum IgG levels were quantified in serum using the SARS-CoV-2 IgG kit (EUROIMMUN, Lübeck, Germany) according to the manufacturer's instructions. In brief, serum samples (n=39) were diluted 1:100 and added to plates pre-coated with recombinant SARS-CoV-2 S-WT protein. SARS-CoV-2 S1 protein-specific IgG was quantified using anti-human IgG conjugated to HRP, which catalyzed the conversion of 3,3′,5,5′-tetramethylbenzidine (TMB) to 3,3',5,5'-tetramethylbenzidine diimine, whose absorbance was measured at 450 nm with reference at 620 nm a spectrophotometer (Molecular Devices). IgG S1-ELISA reactivity was evaluated as the ratio of the absorbance of the sample to the absorbance of the internal standard.

SARS-CoV-2 WT, alpha and beta neutralization assay

The SARS-CoV-2 neutralization was performed using VeroE6 cells (kindly provided by C. Drosten and M. Müller), which were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, supplemented with 10 % (v/v) fetal calf serum (FCS), 1% (v/v) non-essential amino acids, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM L-Glutamine). VeroE6 cells were seeded at 1×10^4 cells/well one day prior the experiment. Sera from the hemodialysis patients (n=37) were heat-inactivated for 30 min at 56 °C and serially diluted 1:2 in a 96 well plate with an initial dilution of 1:10 in quadruplicates. In the next step, 200 TCID50 of either wildtype virus hCoV-19/Germany/BY-Bochum-1/2020 (GISAID accession ID: EPI_ISL_1118929), VOC alpha RKI-0026_B.1.1.7 (GISAID accession ID: EPI_ISL_751799), or VOC beta RKI-0029_B.1.351 (GISAID accession ID: EPI_ISL_803957) was added to each well and incubated for 1 hour at 37 °C. Afterwards, the virus/serum suspension was transferred onto VeroE6 cells and incubated for 72 hours at 37 °C. After incubation, VeroE6 cells were stained with crystal violet following and optically analyzed for cytopathic effects (CPE). Based on CPE values, the 50% neutralization capacity (ND50 value) was calculated using non-linear regression.

Preparation of Peripheral blood mononuclear cells (PBMCs) and stimulation with overlapping peptide pools from SARS-COV-2 S-protein VOC

PBMCs were isolated and stimulated as previously described.[1] Briefly, from whole blood, PBMC were obtained by gradient centrifugation and stimulated with 15mer overlapping

peptides pools (OPP) with an 11 amino acids overlaps from SARS-CoV-2 S-WT, B1.1.7, and B.1.3.5.1 S-Proteins (JPT, Germany).
All OPP were dissolved in DMSO according to the manufacturer's instructions and used at a final concentration of 1 µg/ml. For each condition $2.5x10^6$ PBMCs were plated in 96-UWell Plates in 200 µl RPMI media (Life Technologies, USA), supplemented with 1% Penicillin-Streptomycin-Glutamine (Sigma Aldrich, USA) plus 10% FCS (PAN-Biotech, Germany) and were treated with the diffent OPPS or DMSO alone as negative control for 16 hours. As a positive control, PBMC were stimulated with SEB (1 µg/ml, Sigma Aldrich). After incubation for 2 hours Brefeldin A (1 µg/ml, Sigma Aldrich, USA) was added. The SARS-CoV-2 OPP stimulated PBMC stained as previously described.[1]
Unless otherwise indicated, all antibodies used for flow cytometry were purchased from BioLegend, USA: Surface staining: Live-dead Fixable Blue Dead Cell Stain Kit (ThermoFisher, USA), CCR7 (CD197)-PerCP-Cy5.5; clone: G043H7, CD45RA-BV605; clone: HI100. CXCR5-PE-Dazzle594, clone: J252D4; CD3-BV785, clone: OKT3. Intracellular staining: CD4-BUV496, clone: SK3 (leu3a) (BD Biosciences, USA); CD8-V500; clone: RPA-T8 (BD Biosciences, USA); Granzyme B-AL700; clone: R712 (BD Biosciences, USA); IL2-PE; clone: MQ1-17H12; CD137 (4-1BB)-PE-Cy7; clone: 4B4-1; CD154 (CD40L)-APC/Cy7; clone: 24-31, TNFa-eFluor450; clone: MAb11 (eBioscience, USA), IFNγ-BV650; clone: 4S.B3; CD69-FITC, clone: FN50 (BD Biosciences, USA).
Immediately after antibody labelling samples were acquired on a CytoFlex LX flow cytometer (Beckman Coulter, USA). As a result of low cell counts after thawing, SARS-CoV-2 S-WT, S-alpha, and beta-reactive T cells could be measured in 36, 36, and 30 out of 40 DP, respectively. The threshold for an antigen-reactive T cell responses was set when the specific response after stimulation exceeded the background activation three times (stimulation index SI >3) as previously published.[1,2] For activated cells that are presented as frequency of $CD4^+$, $CD8^+$, or $CD4^+$ $CXCR5^+$ T cells, non-specific background in DMSO control was subtracted.

Statistical Analysis

Flow cytometry data was analyzed using FlowJo version 10.7.1 (BD Biosciences, USA); gating strategy and representative dot plots are shown in Figures S2-3. Statistical analysis was performed using R, version 4.0.4. Binary variables are depicted as bar plots, continuous variables are depicted as box plots; the maximum length of the whiskers corresponds to 1.5 times the interquartile range. In all cases, the sub-cohorts were compared only with respect to the same virus strain. Only two-sided tests were employed. Differences for a continuous variable between the sub-cohorts were analyzed using the Mann-Whitney U test. Significance threshold was set at 0.050. Since this study had an exploratory nature, we did not perform an adjustment for multiple testing.[3]

Supplementary References

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3. Bender R, Lange S. Adjusting for multiple testing - When and how? J Clin Epidemiol. 2001;54(4):343-349. doi:10.1016/S0895-4356(00)00314-0