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Supplementary appendix

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Supplementary appendix related to the correspondence

Divergent dynamics of humoral and T cell immunity after two and three BNT162b2 vaccinations in adults aged >80 years

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Authors' contributions

AJRO and ARS organized and performed experiments, analyzed data, and created the figures. CK and SH provided data for the figures. DDG, SH, and VH collected blood. HH and ARS performed flow cytometry analysis. AJRO, DS, BC, SH, prepared cells. SH, CM, SS were involved in antibody analyses. HRC performed statistical analysis. VK, HMK, and KV performed serological assays. HEM, CK, and ML design the study, recruited the study population. HEM, CK, AJRO, ARS, DDG, and ML critically discussed the data. ML wrote the manuscript. HEM, CK, AJRO, and ARS revised the manuscript.

Ethics committee approval and patient consent

The study of patients with COVID-19 and vaccinations against COVID-19 was approved by the ethics committee of the medical faculty of the Philipps-University Marburg (study number 40/21-12032021). All donors provided informed consent to participate in the study.

48 **Table S1. Summary of vaccinated donors' characteristics**

Group	Total donors	Sex	Age range	Age mean / SD
W0, W3	*n*=52	female: 31 (60%) male: 21 (40%)	80–97 years	84·0 / 3·7
W5	*n*=49	female: 30 (61%) male: 19 (39%)	80–97 years	84·0 / 3·7
W24	*n*=37	female: 23 (62%) male: 14 (38%)	80–97 years	83·4 / 3·7
W26	*n*=34	female: 22 (65%) male: 12 (35%)	80–97 years	83·4 / 3·6
W40	*n*=15	female: 11 (73%) male: 4 (27%)	80–97 years	84·7 / 4·6

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50 **Table S2. Baseline characteristics of vaccinated participants**

DoNor ID	Age (years)	Sex	Exclusion (reason)	Smoking	Chronic lung disease	Diabetes	Charlson Comorbidity Index (CCI)
1	81	m		No	No	No	4
2	86	m		No	No	No	4
3	81	f		No	No	No	4
4	84	m		No	No	No	4
5	81	m		No	No	No	6
6	83	f		No	No	No	4
7	83	m		No	No	Yes	7
8	87	f		No	Chronic asthma	Yes	5
9	83	f		No	No	Yes	5
10	81	m		Ceased, 10py	No	No	4
11	86	f		No	No	No	5
12	82	f		No	No	No	7
13	82	m		Ceased, 20py	No	No	6
14	82	f		Ceased	COPD, bronchial asthma	No	4
15	84	f		No	No	Yes	4
16	82	m		No	No	No	6
17	95	m		No	No	No	4
18	85	f		No	No	Yes	5
19	85	f		No	No	No	4
20	81	f		No	Spastic bronchitis	No	5
21	86	f		No	No	No	4
22	82	m		No	No	No	5
23	82	f		No	No	No	4
24	87	m	Death due to accident				
25	81	f	Own decision				
26	80	m		Yes	No	No	4
27	86	m		No	Mild bronchitis	No	4
28	89	f		No	No		4
29	85	f		No	No	No	5
30	81	f		No	No	No	5
31	82	m		No	COPD		9

32	88	f		No	No	No	4
33	86	f		No	COPD	No	5
34	86	m	Previous COVID-19				
35	83	f		No	Allergic asthma	No	4
36	80	f		No	No	No	4
37	91	f		No	No	No	4
38	81	f		No	No	No	4
39	80	m		Ceased	COPD	Yes	6
40	84	f		Yes	No	No	4
41	97	f		No	No	No	5
42	88	f		No	No	No	4
43	80	m		No	No	No	4
44	86	m		No	Lung fibrosis	No	7
45	87	f	Own decision				
46	83	f		No	No	No	4
47	83	f		40py	Dry cough	No	4
48	81	m		40py	No	Yes	5
49	84	f		No	No	Yes	5
50	91	m		No	No	Yes	6
51	80	m		Ceased	No	Yes	7
52	89	f		10py	No	No	4
53	81	f		No	No	No	4
54	83	m		30-40py	No	No	4
55	81	m		No	COPD	No	5
56	82	f		No	No	No	4

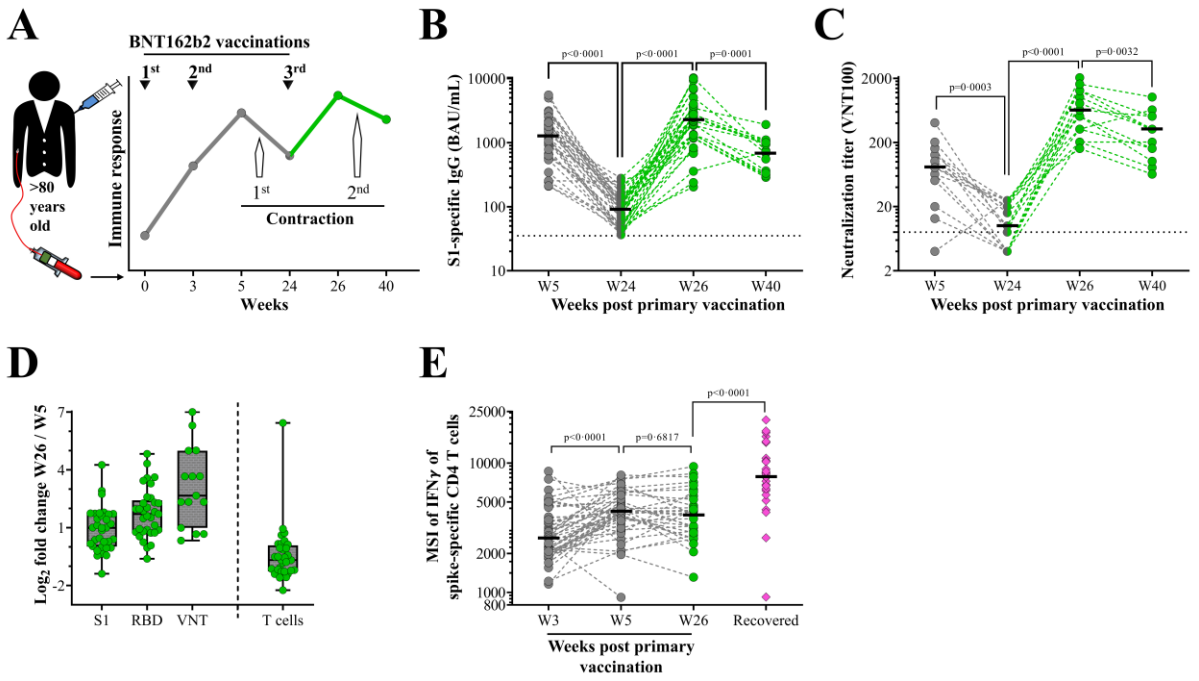

Figure S1. Humoral and cellular SARS-CoV-2 immunity in >80 year-old donors after receiving a third dose of the BNT162b2 vaccine.

Study overview (A). Immune response kinetics were followed in older adults (Table S1 and S2) in the course of vaccinations with BNT162b2. Green color indicates data related to the third dose of BNT162b2. Vaccinations (black arrow heads) and blood samplings (dots) are indicated. SARS-CoV-2 spike (S1-subunit)-specific serum IgG levels (B) and serum titers of 100% virus neutralization for SARS-CoV-2 wild type B·1 (VNT 100) (C) were analyzed in donors aged >80 years at the indicated time points after primary vaccination with BNT162b2. *n*=35, 36, 34, 15 donors were analyzed on W5, W24, W26, W40, respectively in (B); *n*=15 donors on all time points in (C). Log2 of the fold change in peak response values for spike (S1 subunit)- (*n*=33) and RBD-specific (*n*=33) IgG, neutralizing antibody titers (*n*=15), and spike-specific T cell frequencies (*n*=32) in participants after 2nd and 3rd vaccination (D). Comparison of the median staining intensity (MSI) of interferon (IFN)γ of spike-specific CD4 T cells of vaccinated participants determined at W3 (*n*=51), W5 (*n*=51), and W26 (*n*=33), and of 80 year old donors recovered from COVID-19 (not vaccinated, *n*=30, magenta) (E). Control stimulations of PBMC with staphylococcal enterotoxin B (figure S3) confirmed the stability of the T cell stimulation conditions over time. Each symbol represents data of one donor at one time point. Horizontal lines indicate median values of data points in each column, horizontal dotted lines indicate the cutoff for antibody positivity at 35·2 BAU/mL (B) and the lower detection limit for the VNT 100 assay at a reciprocal titer of 8 (C). p values were determined using the two-tailed Wilcoxon matched-pairs signed rank test throughout except when comparing vaccinated (W26) vs. recovered groups (two-tailed Mann-Whitney test). VNT, virus neutralization test; RBD, receptor binding domain; BAU, binding antibody units.

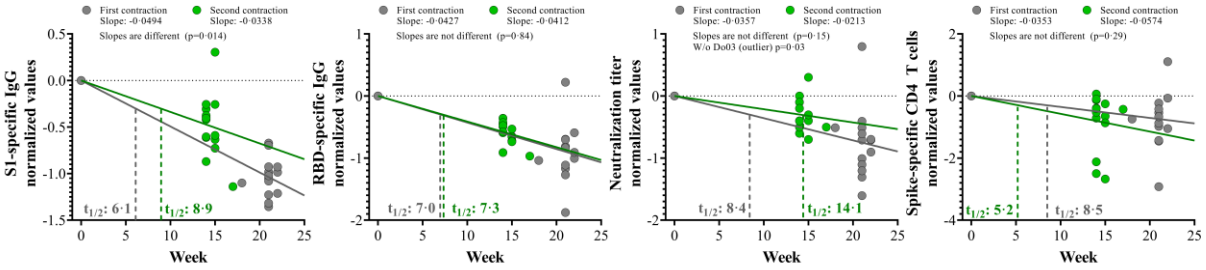

Figure S2. Contraction of SARS-CoV-2 spike-specific CD4 T cell and humoral immunity after second and third "booster" vaccination with BNT162b2.

Normalized slopes of serum SARS-CoV-2 S1-specific IgG, RBD-specific IgG, neutralization titers, and circulating SARS-CoV-2 spike-specific CD4 T cells, after the second dose (first contraction, grey dots) and after the third dose (second contraction, green dots) of BNT162b2. Weeks indicated on the x axis are weeks after respective peak responses. Slopes were calculated using a linear mixed effect model from log-transformed data and regression lines are shown as solid lines. Data for each donor and each contraction was normalized to zero at week five (first contraction) and week 26 (second contraction). The half-lives ($t_{1/2}$) of each modality are shown as dashed lines. The linear mixed effect models were calculated from 14 donors (neutralization titers, RBD- and S1-specific IgG serum responses) and 13 donors (spike-specific CD4 T cell responses. RBD, receptor binding domain.

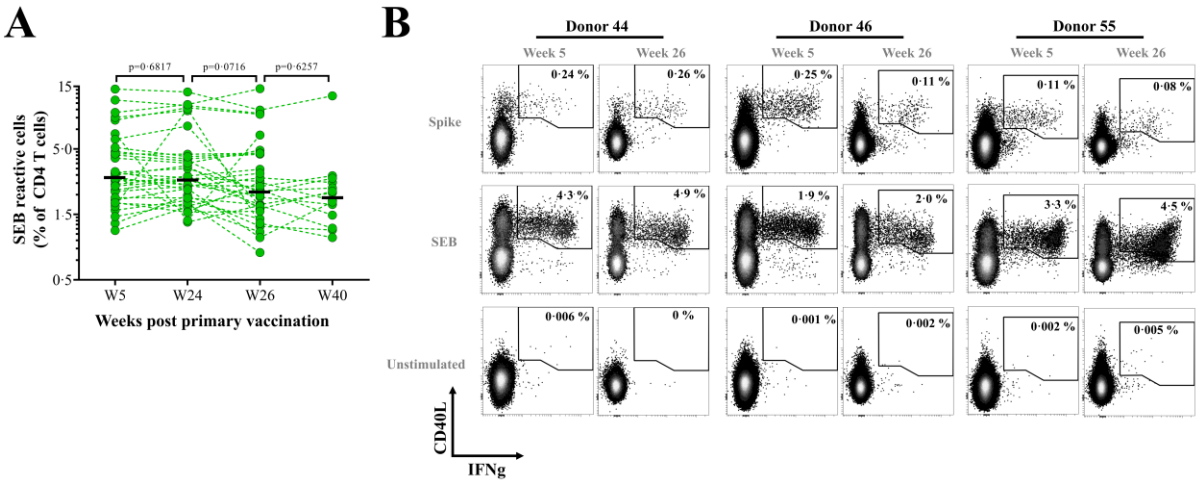

Figure S3. Blood CD4 T cell response to SEB in >80 year-old vaccinated donors and examples of flow cytometric analysis.

Blood was collected at the indicated time points post primary vaccination. Vaccinations with BNT162b2 were performed at week 0, week 3, and week 24 (figure 1A). Frequencies of CD40L+IFNγ+ CD4 T cells after culture of PBMC in the presence of the superantigen staphylococcal enterotoxin B (SEB) are shown (A). *n*=34, 35, 34, 14 donors were analyzed on W5, W24, W26, W40, respectively. p values were determined by two-tailed Wilcoxon matched-pairs signed rank test. Representative analysis of flow cytometry data depicting the detection of CD40L+IFNγ+ CD4 T cells in three donors at week 5 and week 26 are shown in (B). PBMC were stimulated in the presence or absence of spike protein peptide pool or SEB. Data of CD4 T cells are shown; frequencies of gated cells are indicated.

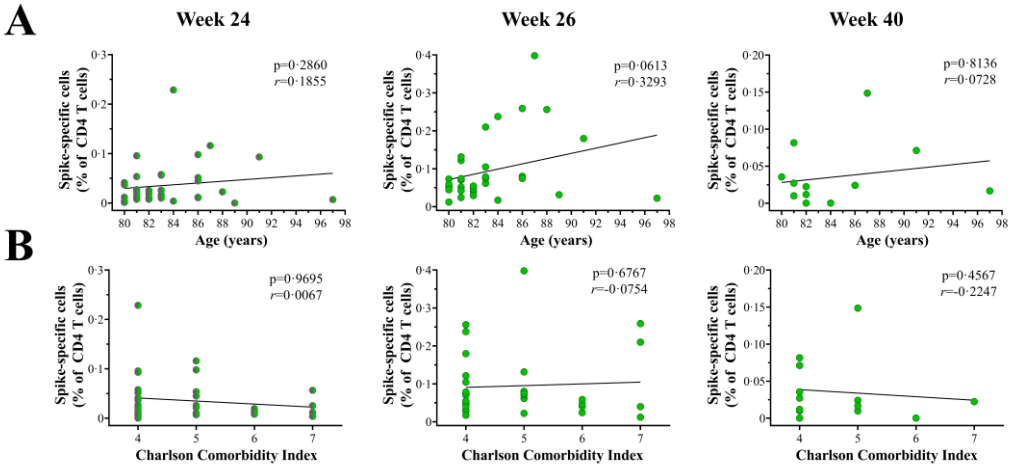

Figure S4. Lack of correlation between spike-specific CD4 T cell frequencies and age or comorbidities before and after a booster vaccination with BNT162b2.

Results of the correlation analysis between the indicated frequencies of SARS-CoV-2 spike-specific CD4 T cells and the age (A) or the Charlson Comorbidity Index (CCI) (B) of individual donors before (W24, *n*=35), two weeks later (W26, *n*=33), and average 16 weeks (W40, *n*=13) after a third "booster" BNT162b2 vaccination. Spearman's correlation test. Fitted linear regression lines are shown as black lines. Each dot represents one donor.

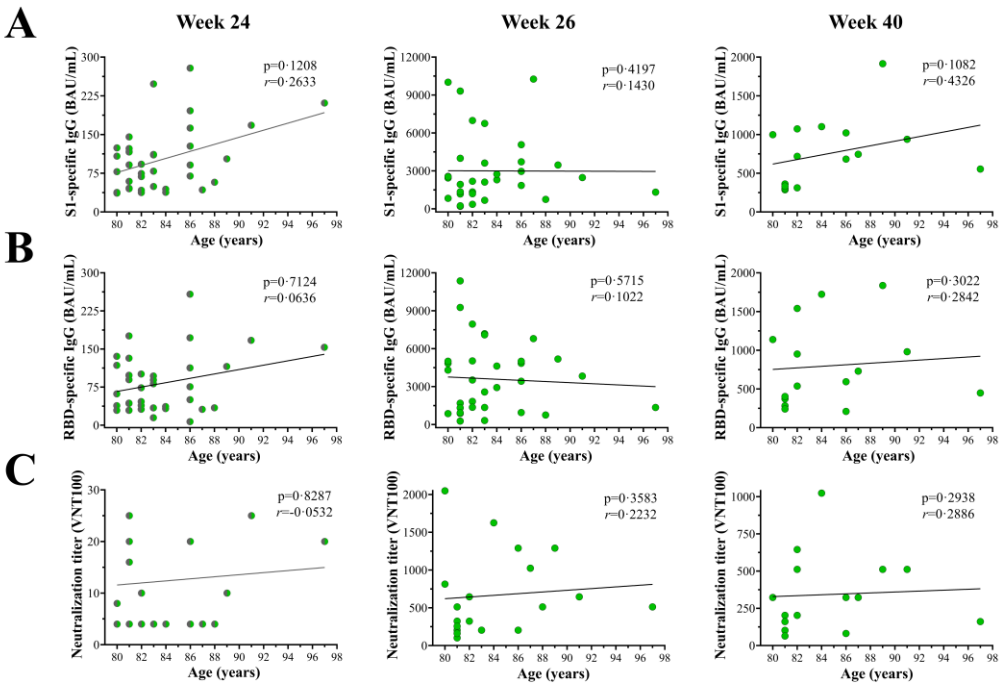

Figure S5. Lack of correlation between humoral SARS-CoV-2 immunity and age before and after a booster vaccination with BNT162b2.

Results of the correlation analysis between serum SARS-CoV-2 S1-specific IgG (A), RBD-specific IgG (B), or neutralization titer (C) and the age of individual donors before (W24), two weeks later (W26), and average 16 weeks (W40) after a third "booster" BNT162b2 vaccination. *n*=36, 34, 15 donors were analyzed on W24, W26, W40, respectively (A); *n*=36, 33, 15 donors were analyzed on W24, W26, W40, respectively (B); *n*=19, 19, 15 donors were analyzed on W24, W26, W40, respectively (C). Spearman's correlation test. Fitted linear regression lines are shown as black lines. Each dot represents one donor. RBD, receptor binding domain; BAU, binding antibody units; VNT, virus neutralization test.

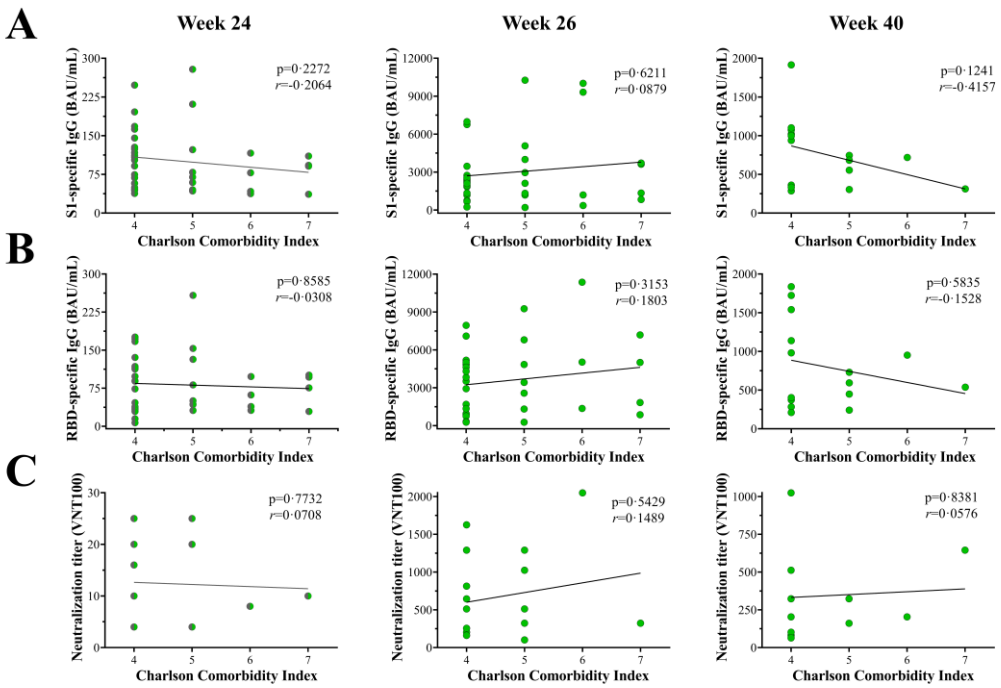

Figure S6. Lack of correlation between humoral SARS-CoV-2 immunity and comorbidities before and after a booster vaccination with BNT162b2.

Results of the correlation analysis between serum SARS-CoV-2 S1-specific IgG (A), RBD-specific IgG (B), or neutralization titer (C) and the Charlson Comorbidity Index (CCI) of individual donors before (W24), two weeks later (W26), and average 16 weeks (W40) after a third "booster" BNT162b2 vaccination. *n*=36, 34, 15 donors were analyzed on W24, W26, W40, respectively (A); *n*=36, 33, 15 donors were analyzed on W24, W26, W40, respectively (B); *n*=19, 19, 15 donors were analyzed on W24, W26, W40, respectively (C). Spearman's correlation test. Fitted linear regression lines are shown as black lines. Each dot represents one donor. RBD, receptor binding domain; BAU, binding antibody units; VNT, virus neutralization test.

Material and Methods

Study participants

Up to six blood samples were obtained from participants aged >80 years by venipuncture as shown in figure S1A. Samples were collected before first (week 0, W0), and three weeks (W3, right before 2nd vaccination), five weeks (W5), average 24 weeks (W24, before 3rd vaccination), average 26 weeks (W26), and 40 weeks (W40) after first vaccination by injection of Tozinameran (BNT162b2 vaccine, Comirnaty®) in the deltoid muscle, at a vaccination center in Marburg, Germany (Tables S1 and S2).

Analyses of vaccinated donors were performed between March 2021 and January 2022. In May 2021, blood samples were obtained by venipuncture from unvaccinated donors >80 years of age living in a retirement home, having recovered from COVID-19 after an outbreak with SARS-CoV-2 variant B·1·221 in January 2021. All donors provided informed consent to participate in the study. Few participants were later excluded from the study for reasons unrelated to the study (Table S2). Additionally, data of one donor was excluded from the whole T cell analysis due to strong erythrolysis (Polycythemia Vera) and data of one donor excluded on W40 due to sample clogging during the T cell assay. Number of donors analyzed and depicted on the different weeks in figure 1, S1B-S1E, and S3A was selected based on matched-pair comparisons.
The study of patients with COVID-19 and vaccinations against COVID-19 was approved by the ethics committee of the medical faculty of the Philipps-University Marburg (study number 40/21-12032021).

Sample processing and clinical lab

Blood serum was isolated from Serum Separator Clot Activator tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) according to the manufacturer's instructions, and stored frozen until analysis.
Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized whole blood by density gradient centrifugation over Pancoll human (Pan Biotech, Aidenbach, Germany) after dilution with an equal volume of PBS at room temperature. PBMC were washed twice (500xg, 10 minutes, 4°C) in cold PBS supplemented with 0·2% BSA, counted manually, and resuspended in RPMI 1640 media (Gibco, Life Technologies, Carlsbad, CA) supplemented with penicillin, streptomycin, and 10% human AB serum (all Sigma, St. Louis, MO) at $5x10^6$ cells/mL.

Assessment of antigen-specific T cells (ART)

Antigen-reactive T cell responses were analyzed using a protocol based on previous work.[1] 500µL media containing $5x10^6$ PBMC were transferred into 12mL round-bottom tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and stimulated with either SARS-CoV-2 spike protein peptide mix (wildtype, Miltenyi Biotec), SEB (0·7µg/mL, kindly provided by Prof. Bernhard Fleischer, Bernhard Nocht Institute of Tropical Medicine, Hamburg, Germany), or with an equal volume of water as a control, in the presence of anti-CD28 (5µg/mL) and monensin (1µg/mL) for 12 hours under humid conditions in a 5% CO_2 atmosphere. Brefeldin A (1µg/mL) was added two hours after the start of the stimulation. The stimulation was stopped by

adding 2nM EDTA. PBMC were harvested, transferred to a new 15mL centrifuge tube, washed with 10mL PBS/0·2% BSA, and pelleted for ten minutes at 490xg, at 4°C. Dead cell labeling was performed by resuspending the cell pellet in 500µL PBS supplemented with 1:1000 amine-reactive Zombie Aqua™ Fixable Viability dye (Biolegend), incubated for 20 minutes in the dark at room temperature. PBS/0·2% BSA was added to quench the remaining reactive dye. After washing with 2mL PBS/0·2% BSA, and pelleting for ten min, at 490xg, at 4°C, PBMC were fixed for 20 minutes using 2% formaldehyde solution (Thermo Scientific, Germany) in the dark and washed twice with 2mL PBS/0·2% BSA (10 minutes, 700xg, 4°C). Cell pellets were resuspended in 200µL PBS/0·2% BSA, transferred into a V-bottom-96 well plate (Sarstedt), and centrifuged (700xg, 5 minutes, 4°C). After discarding the supernatants, pellets were resuspended in 50µL Brilliant Violet Staining Buffer (Biolegend) supplemented with antibodies including anti-CD4-PECy7 (1:400 dilution) for the detection of CD4 T cells, anti-CD8-BV570 (1:100 dilution), and anti-CD19/123/33-BV510 (all from Biolegend at 1:50,1:50, 1:400 dilution, respectively) for the exclusion of CD8 T cells, monocytes and other myeloid cells, B cells, basophils, and plasmacytoid dendritic cells, and incubated 30 minutes in the dark, at room temperature.

Afterwards, cells were washed once with 200µL PBS/0.2% BSA, and centrifuged (700xg, 5minutes, 4°C). Cell pellets were then washed twice in 200µL permeabilization buffer (diluted using Millipore water from 10x concentrated stock buffer, ThermoFisher, Waltham, MA), and finally resuspended in 50µL permeabilization buffer supplemented with antibodies targeting CD40L (1:100 dilution conjugated to BV421, Biolegend), and intracellular molecules including anti-IFNγ-R718 (1:100 dilution BD Biosciences, San Jose, CA), and incubated for 30 minutes at room temperature in the dark. Then, cells were washed once in 200µL permeabilization buffer, once in PBS/0.2% BSA, and stored at 4°C in PBS/0.2% BSA until acquisition on a MACSQuant 16 flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany).

FlowJo version 10 (BD, Ashland, OR) and OMIQ.ai (Santa Clara, CA) were used for analyzing flow cytometry data. Flow cytometry standard (FCS) files underwent quality control and, where applicable, anomaly removal by FlowAI,[2] and data of live CD4+ T lymphocytes were gated according to FSC and SSC parameters and their $CD4^{+}CD8^{-}CD19^{-}CD123^{-}CD33^{-}LD\text{-}Aqua^{low/-}$ phenotype. Cell aggregates were removed by gating according to FSC-H/FSC-A and SSC-H/SSC-A parameters. Antigen-reactive CD4 T cells (ART) were defined as CD40L+ IFNγ+ expressing cells after spike peptide stimulation, and their frequencies were determined among total CD4 T cells.

For background correction, frequencies of CD40L+IFNγ+ T cells among CD4 T cells from unstimulated control samples were subtracted from ART frequencies for each individual donor. Whenever this difference was equal to or below 0%, frequencies were set to 0·0001%, a value exceeded by all measurements in which ART were detectable, in order to allow the display of data on a logarithmic scale in figure 1.

Quantification of SARS-CoV-2-specific antibodies

Serum IgG antibodies against the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein were quantified using the automated SARS-CoV-2-IgG-II-Quant-Assay on the Abbott Alinity i analyzer (Abbott, Wiesbaden, Germany), following the manufacturer's protocol. Results obtained in arbitrary units/mL (AU/mL)

were converted into binding antibody units (BAU)/mL by multiplication with the factor 0·142, according to the manufacturer's instructions. Results in BAU/mL are calibrated against the "First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (NIBSC code: 20/136)". The lower cutoff for this assay is at 7·1 BAU/mL. Sera exceeding the detection range of the assay (40000 AU /mL) were automatically pre-diluted 1:2 by the device and measured again.

Serum IgG directed against the S1 subunit of the SARS-CoV-2 spike protein was quantified in BAU/mL using the Anti-SARS-CoV-2-QuantiVac ELISA (Euroimmun, Lübeck, Germany). Pipetting was performed on the automated EuroLab Workstation (Euroimmun), following the manufacturer's protocol. Primary data obtained in relative units per mL were converted into binding antibody units (BAU)/mL by multiplication with the factor 3·2, according to the manufacturer's instructions. Results in BAU/mL are calibrated against the "First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (NIBSC code: 20/136)". The lower cutoff for this assay is at 35·2 BAU/mL. Sera exceeding the detection range of the assay were manually pre-diluted 1:10 and/or 1:50 and measured again.

SARS-CoV-2 neutralization tests (VNT_{-100})

Human sera were heat-inactivated for 30 minutes at 56 °C and diluted in a two-fold dilution series in 96-well cell culture plates (1:4 to 1:512 for week 24 sera, 1:8 to 1:1024 for week 26 sera). One hundred plaque-forming units (PFU) of SARS-CoV-2 were added in the same volume to the serum dilutions. The German SARS-CoV-2 virus isolate BavPat1/2020 (European Virus Archive Global #026 V-03883, Genbank: MZ558051·1) was used. The sequence of the viruses was confirmed. Following incubation at 37°C for one hour, approximately 20000 Vero C1008 cells (ATCC, Cat. no. CRL-1586, RRID: CVCL_0574) were added. Plates were then incubated at 37°C in a 5% CO_2 atmosphere, and cytopathic effects were evaluated at day four post infection. Neutralization was defined as the absence of cytopathic effects in the serum dilutions. The reciprocal neutralization titer was calculated from the highest serum dilution without cytopathic effects as a geometric mean based on three replicates. The lower detection limit of the assay is 8 (reciprocal titer) for week 24 sera and 16 for week 26 sera, corresponding to the first dilution of the respective serum. Two positive controls were used as inter-assay neutralization standards and quality control for each test. Neutralization assays were performed in the BSL-4 laboratory of the Institute of Virology at Philipps University Marburg, Germany.

Statistical analysis details

Prism version 9 (GraphPad software, San Diego, CA) was used to display data, and perform descriptive statistics and significance testing. To determine differences between cohorts in figure 1F (vaccinated group W26 vs recovered group), a two-tailed Mann–Whitney test was applied to calculate the p value. For assessing donor-specific responses over time, the two-tailed Wilcoxon matched-pairs signed-rank test was used. To identify changes in the immune contraction after the 2^{nd} or 3^{rd} vaccination we used an exponential decay model with a decay constant for each of the two contractions. Since the response to the contractions showed a pronounced donor- and contraction-specific effect, we also included a donor-effect. We modeled the logarithm of the signals

using a linear mixed effect model, including the donor, the contraction and the time (in weeks) as well as interactions between donor and contraction and contraction and time as predictors. In this way we account for the donor-specific effect at time point zero of each contraction, the decay constant for the first contractions and the interaction term for the second contraction and time. We calculated the decay constant of the second contraction by adding the decay constant for the first contraction and the interaction term for the second contraction and time. We tested whether the difference between the decay constant in the first and second contraction was significantly different from zero using a t-test for the interaction term for the second contraction and time. Data for each donor and each contraction was normalized to 1 for week 5 (first contraction) and week 26 (second contraction), and donors selected to evaluate contractions (slopes) were those who participated also in W40 (14 donors, VNT, RBD and spike IgG serum responses; 13 donors, spike-specific CD4 T cell responses). All analysis have been performed in R (version 4.1.2).[3] Spearman's rank correlation analysis (two-tailed, 95% confidence interval) was employed in the correlation tests between cellular or humoral immune responses and age or CCI.

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Supplementary references

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