Long-term follow-up of SARS-CoV-2 convalescents reveals distinct magnitude of spike-specific immunity after viral re-exposure and vaccination

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Extended data Figures 1-8

Legends to extended data Figure 1-8

Materials and Methods

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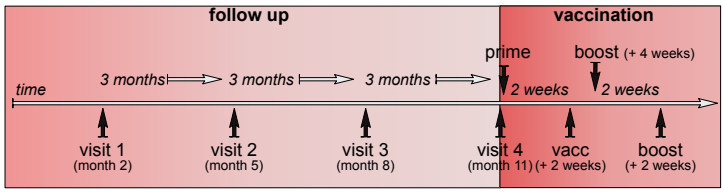
a



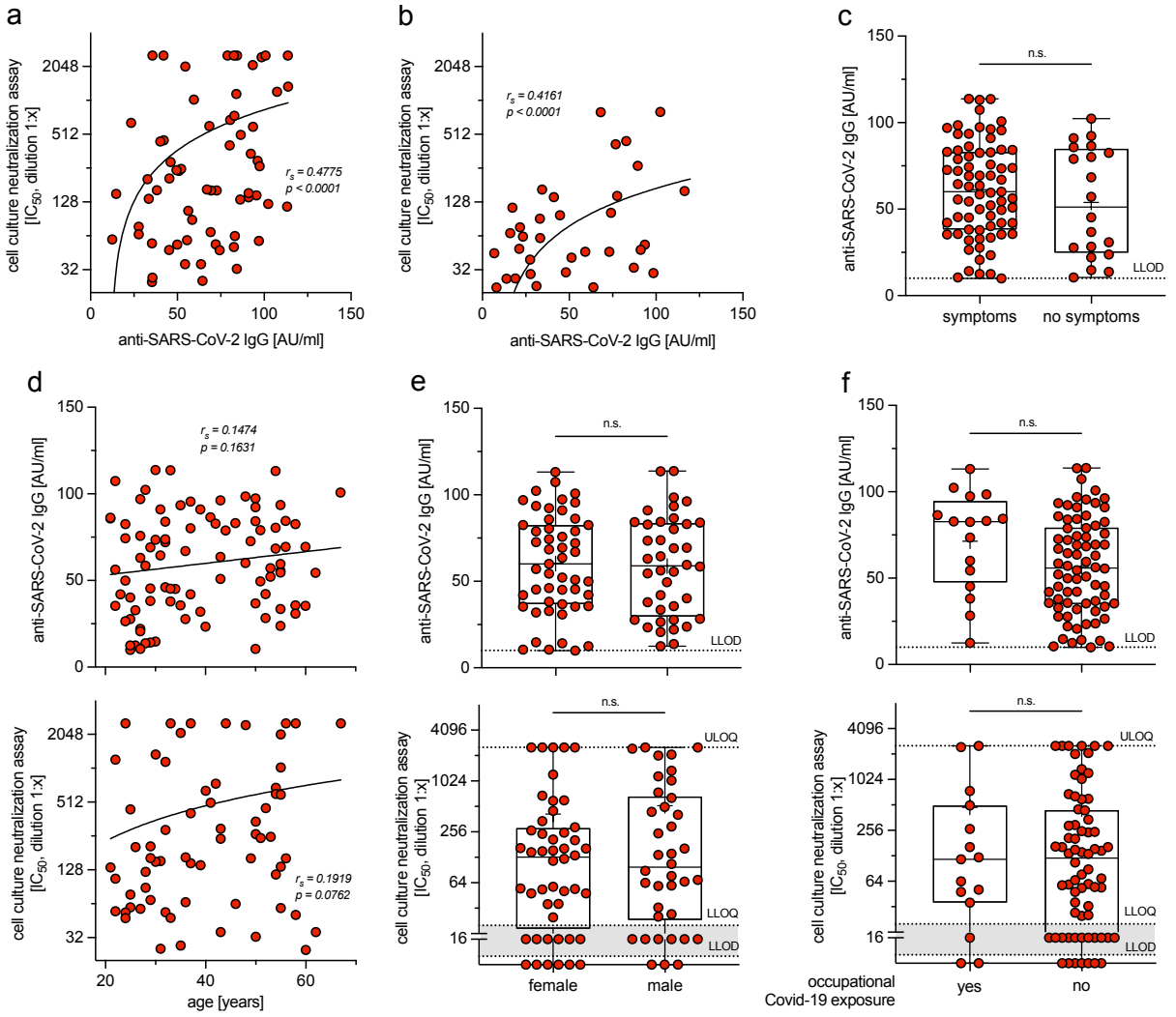
b

study visits	Estimated mean time after infection	study steps	evaluated parameters	convalescent individuals	naïve individuals
visit 1	month 2	serological screening	anti-SARS-CoV-2 IgG (CLIA, YHLO)	108	4446
		participants with written informed consent for further participation in study and confirmation of infection by complementary testing	convalescents: ≥ 2 positive test results for anti-SARS-CoV-2 detection by CLIA using assays from Roche, Abbott, Euroimmun, and immunoblots using RecomLine, virus-neutralization activity by cell culture neutralization assay (IC_{50})	91	——
		participants with written informed consent for further participation in study and paired matching of naïve controls to 91 convalescents	naïve individuals: seronegative, paired matching (age, sex, occupation, exposure to COVID-19 patients)	——	53
visit 2	month 5	evaluation of SARS-CoV-2 specific immunity	anti-SARS-CoV-2 IgG, virus-neutralization activity by cell culture neutralization assay (IC_{50}), surrogate virus-neutralization activity, (Fluorospot analysis, intracellular cytokine staining when samples available)	91 (88)	53 (52)
visit 3	month 8	evaluation of SARS-CoV-2 specific immunity	anti-SARS-CoV-2 IgG, virus-neutralization activity by cell culture neutralization assay (IC_{50}), surrogate virus-neutralization activity	82	45
visit 4	month 11	evaluation of SARS-CoV-2 specific immunity	anti-SARS-CoV-2 IgG, virus-neutralization activity by cell culture neutralization assay (IC_{50}), surrogate virus-neutralization activity, (Fluorospot analysis when samples available)	59 (49)	36 (39)
prime vaccination (vacc)	14 days after prime vaccination	evaluation of SARS-CoV-2 specific immunity	surrogate virus-neutralization activity, (Fluorospot analysis when samples available)	82 (52)	51 (50)
boost vaccination (boost)	14 days after boost vaccination	evaluation of SARS-CoV-2 specific immunity	surrogate virus-neutralization activity, (Fluorospot analysis, intracellular cytokine staining when samples available)	33 (23)	49 (40)

c

	convalescent individuals	naïve individuals
	n = 91	**n = 53**
sex		
male	40 (44.0 %)	20 (37.7 %)
female	51 (56.0 %)	33 (62.3 %)
mean age ± SD	39 ± 13 years	42 ± 20 years
field of work		
physician	15 (16.5 %)	6 (11.3 %)
nursing	18 (19.8 %)	11 (20.8 %)
medical assistance	5 (5.5 %)	2 (3.8 %)
research / laboratory	10 (11.0 %)	11 (20.8 %)
IT	10 (11.0 %)	5 (9.4 %)
administration	7 (7.7 %)	8 (15.1 %)
student	16 (17.6 %)	7 (13.2 %)
other	10 (11.0 %)	6 (11.3 %)
patient contact	49 (53.8 %)	27 (50.9 %)
COVID-19 patient contact	16 (17.6 %)	14 (26.4 %)
risk factors		
smoking	7 (7.7 %)	11 (20.8 %)
pre-existing conditions	19 (20.9 %)	17 (32.1 %)
respiratory conditions	7 (7.7 %)	3 (5.7 %)
cardiovascular conditions	4 (4.4%)	4 (7.5 %)
diabetes mellitus	5 (5.5 %)	0 (0.0 %)
immune deficiency	0 (0.0 %)	1 (1.9 %)
immune suppression	0 (0.0 %)	2 (3.8 %)
other	4 (4.4%)	8 (15.1 %)
symptoms	71 (78.0 %)	18 (34.0 %)
fever	49 (53.8 %)	5 (9.4 %)
coughing	46 (50.5 %)	6 (11.3 %)
dyspnea	21 (23.1%)	4 (7.5 %)
cold symptoms	67 (73.6 %)	14 (26.4 %)
loss of smell / taste	45 (49.4 %)	4 (7.5 %)
fatigue / exhaustion	62 (68.1 %)	13 (24.5 %)
headache	45 (49.4 %)	8 (15.1 %)
diarrhea	19 (20.9 %)	3 (5.7 %)

Extended Data Figure 1 | Characteristics of anti-SARS-CoV-2 IgG seropositive study participants
(a) time axis of long-term follow-up in SARS-CoV-2 convalescents. (**b**) overview of the prospective study. (**c**) individual characteristics of study participants with ≥ two positive anti-SARS-CoV-2 IgG assays and reports on symptoms during SARS-CoV-2 infection.



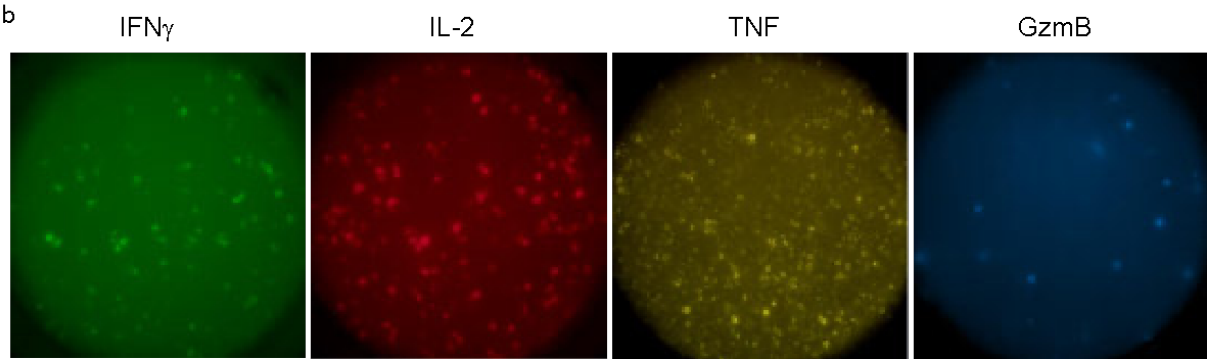
Extended Data Figure 2 | Characteristics of the dynamics of anti-SARS-CoV-2 IgG levels and virus-neutralization activities in convalescent individuals

(**a,b**) correlation of anti-SARS-CoV-2 IgG with virus-neutralization activity of convalescents at month 2 (a) and month 5 (b) after SARS-CoV-2 infection, 21 samples (a) or 57 samples (b) were below the quantification limit and are not shown. (**c**) reporting of symptoms in convalescents and anti-SARS-CoV-2 IgG levels. LLOD – lower limit of detection. (**d**) correlation of anti-SARS-CoV-2 IgG levels and virus-neutralization activity with age of convalescents. 21 samples were below the quantification limit and are not shown (lower row). (**e,f**) anti-SARS-CoV-2 IgG levels and virus-neutralization activity in convalescents according to age, sex or occupational exposure to COVID-19 patients. LLOQ – lower limit of quantification; ULOQ – upper limit of quantification. Data are shown as median of results and interquartile ranges, statistical analysis by Mann-Whitney (c,e,f), and by Spearman correlation (a,b,d); r_s denotes Spearman correlation coefficient; n.s. denotes not significant.

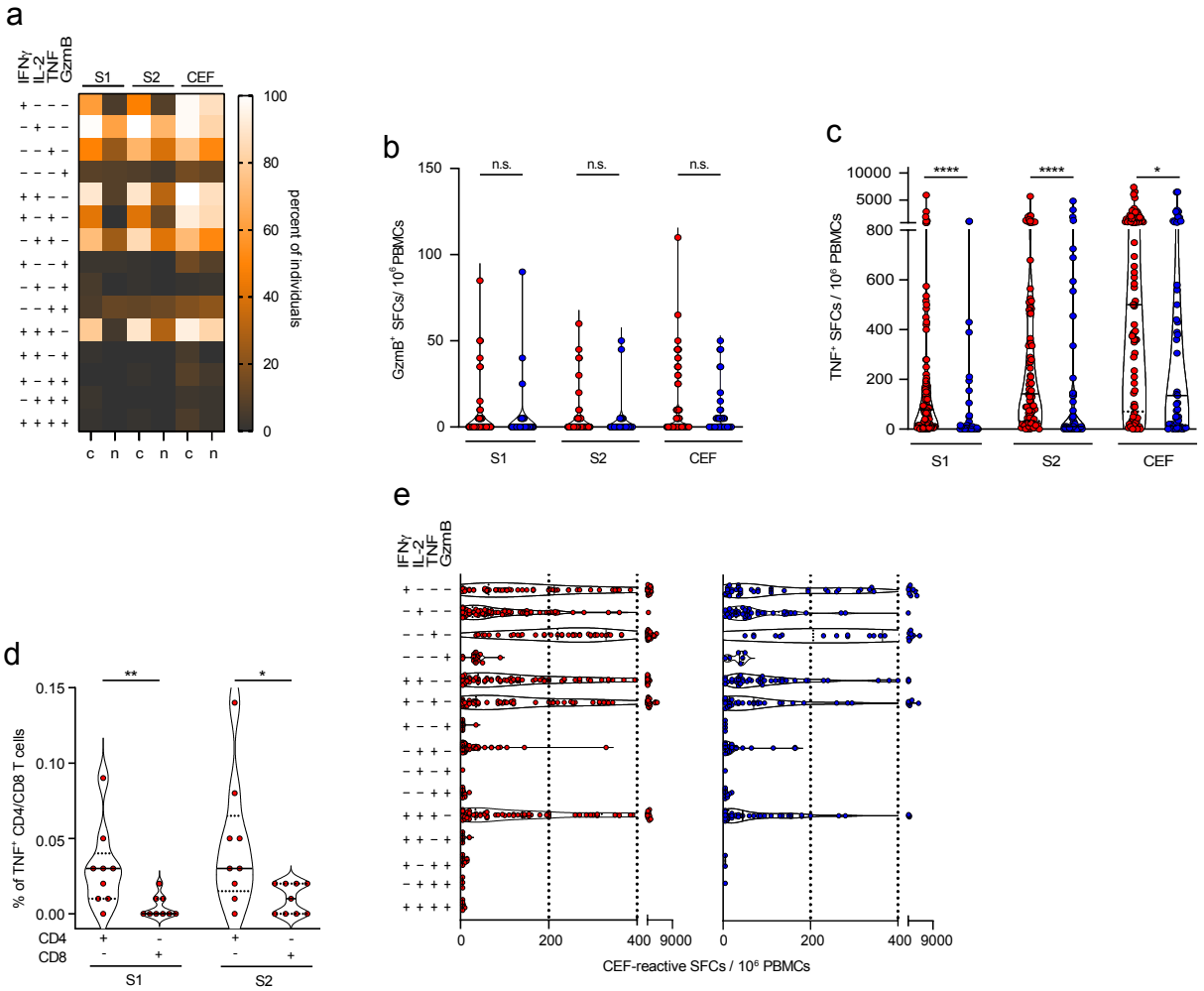
a

	CV (%)			
Parameter	**IFNγ**	**IL-2**	**TNF**	**GzmB**
Intra-assay variation (well to well; 4 replicates)	4.9	6.6	5.5	13.6
Inter-assay variation (plate to plate)	3.1	2.7	5.0	8.1
Inter-assay variation (day to day)	5.5	8.4	8.4	10.8
Inter-operator	6.6	11.1	4.8	6.7

b

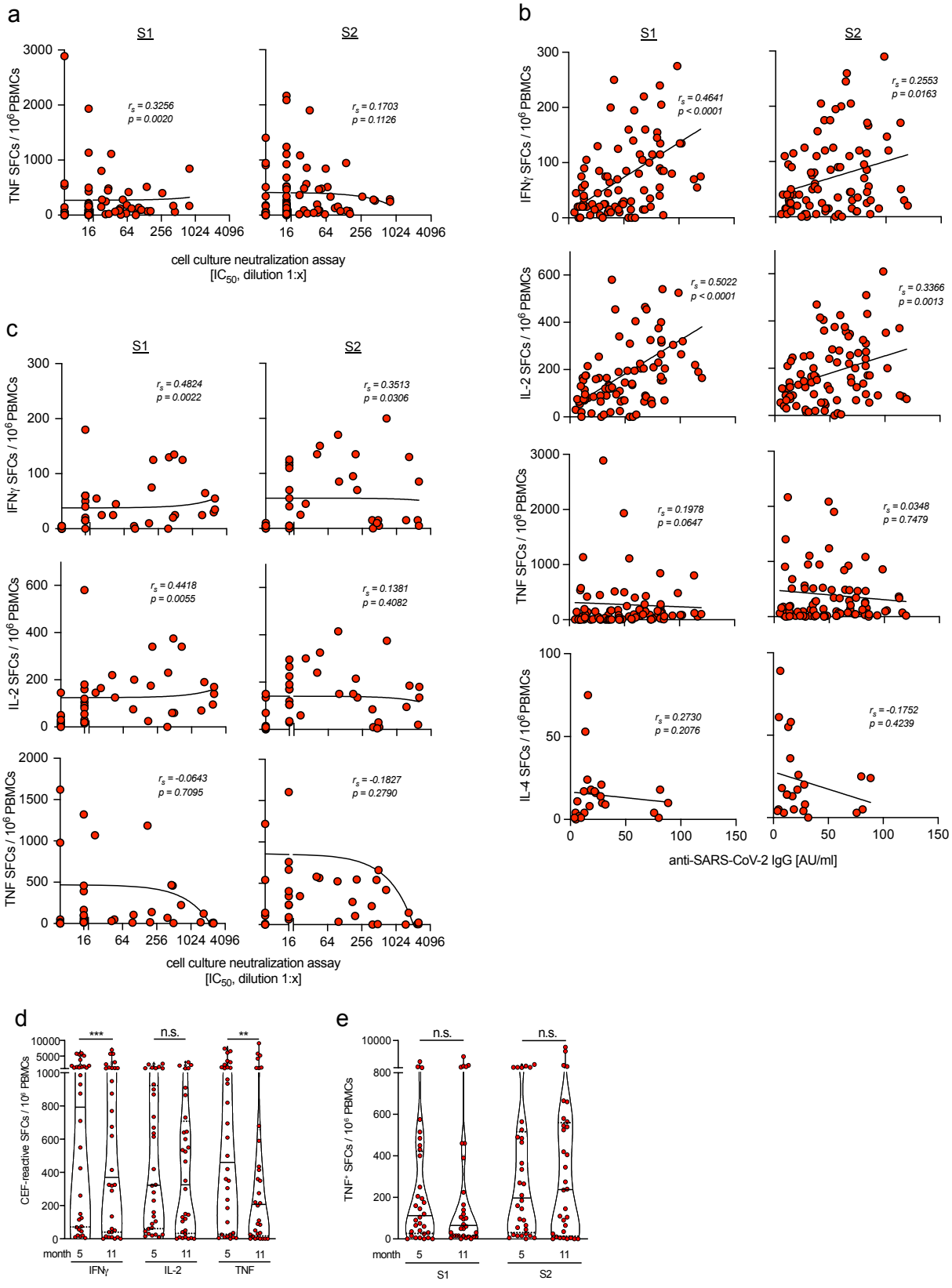


Extended Data Figure 3 | Performance of the four-color Fluorospot assay to detect spike-reactive cytokine-secreting cells directly ex vivo in 10^6 peripheral blood mononuclear cells (PBMCs)
(a) report of the Fluorospot assay characteristics. (**b**) representative images of the Fluorospot assay detecting frequencies of cytokine secreting cells among 10^6 PBMCs from a convalescent individual stimulated with the S1-peptide pool.



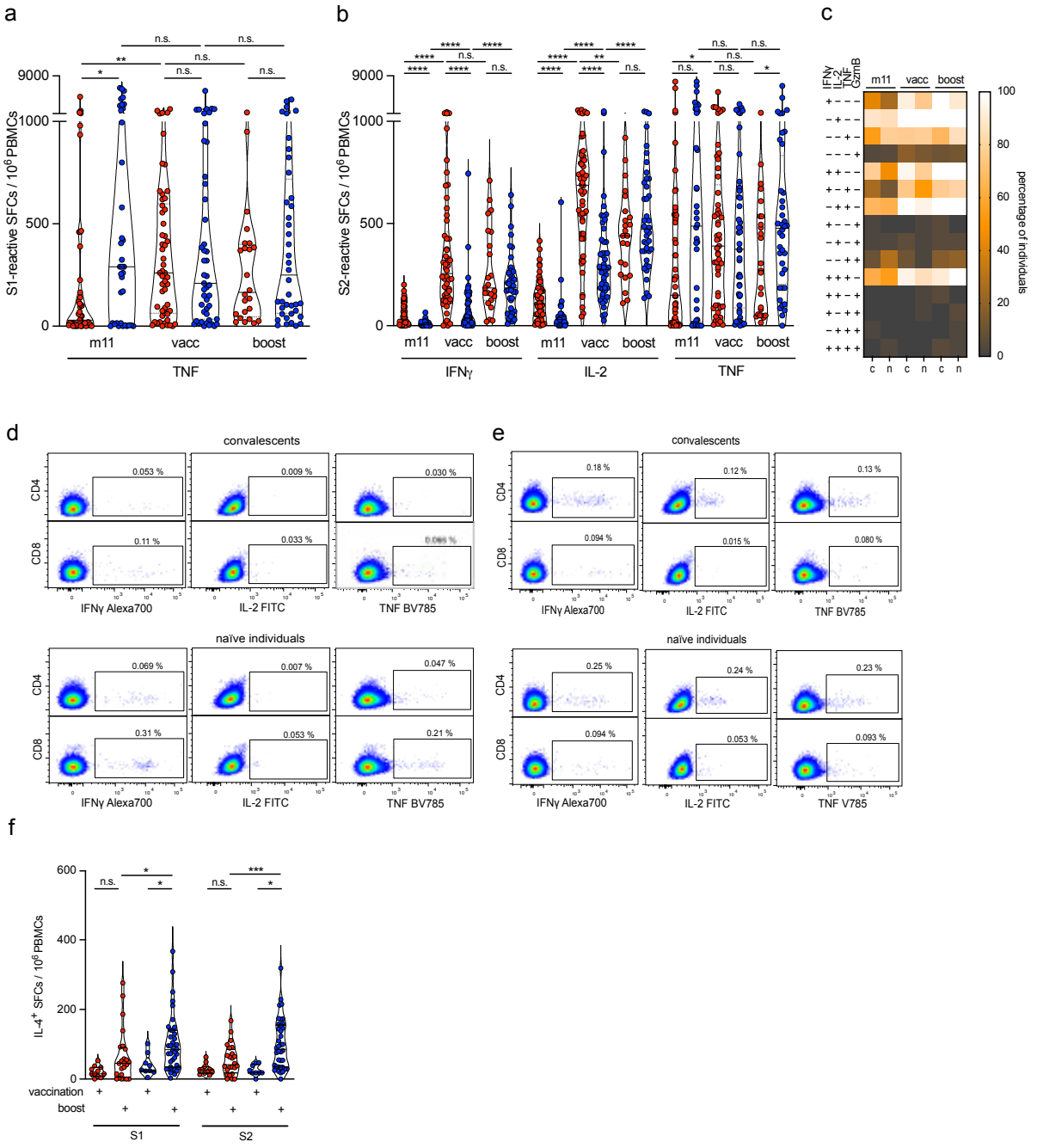
Extended Data Figure 4 | Characteristics of spike-reactive and CEF-reactive cytokine-secreting cells in convalescents and naïve individuals

(**a)** heatmap of individuals with mono- or polyfunctional S1-/S2-/CEF-reactive cytokine-secreting cells in convalescents (c) and naïve individuals (n) by direct *ex vivo* Fluorospot analysis at month 5 after SARS-CoV-2 infection**.** (**b**) frequencies of S1-/S2-/CEF-reactive GzmB-secreting cells. (**c**) S1-/S2-/CEF-reactive TNF-secreting cells by direct *ex vivo* Fluorospot analysis. (**d**) frequencies of S1- or S2-reactive TNF-secreting CD4 or CD8 T cells determined by direct *ex vivo* intracellular cytokine staining and flow cytometric detection. **(e**) frequencies of mono- and poly-functional CEF-reactive cytokine-secreting cells by direct *ex vivo* Fluorospot analysis. Analyses by Mann-Whitney and Wilcoxon signed-rank tests (c,d,e); n.s. denotes not significant; * $p<0.05$; ** $p<0.01$; *** $p< 0.001$; **** $p< 0.0001$.



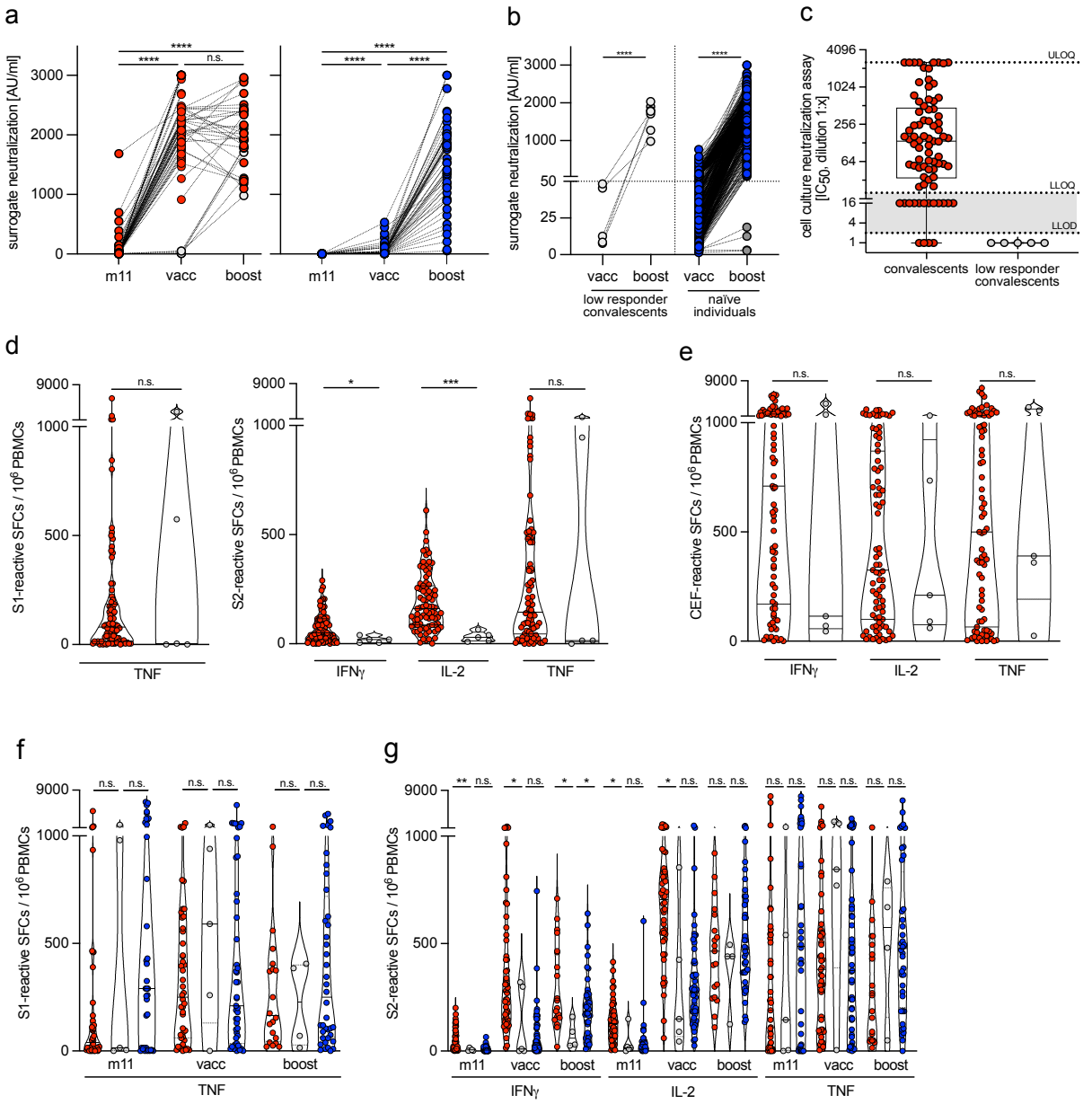
Extended Data Figure 5 | Correlation of spike-reactive cells with spike-specific antibodies and neutralization activity and dynamics of spike- and CEF-reactive cytokine-secreting cells over time

(a-c) correlation of S1- and S2-reactive cytokine-secreting cells from convalescents with serum anti-SARS-CoV-2 IgG levels or with cell culture virus-neutralization activity at month 5 (**a,b**) and 11 (**c**) after SARS-CoV-2 infection. r_s denotes Spearman correlation coefficient. (**d,e**) frequencies of CEF-reactive and spike-reactive cytokine-secreting cells at month 5 and 11 after SARS-CoV2-infection. r_s denotes Spearman correlation coefficient, Wilcoxon signed-rank test (d,e), n.s. denotes not significant; * $p<0.05$; ** $p<0.01$; *** $p< 0.001$; **** $p< 0.0001$.



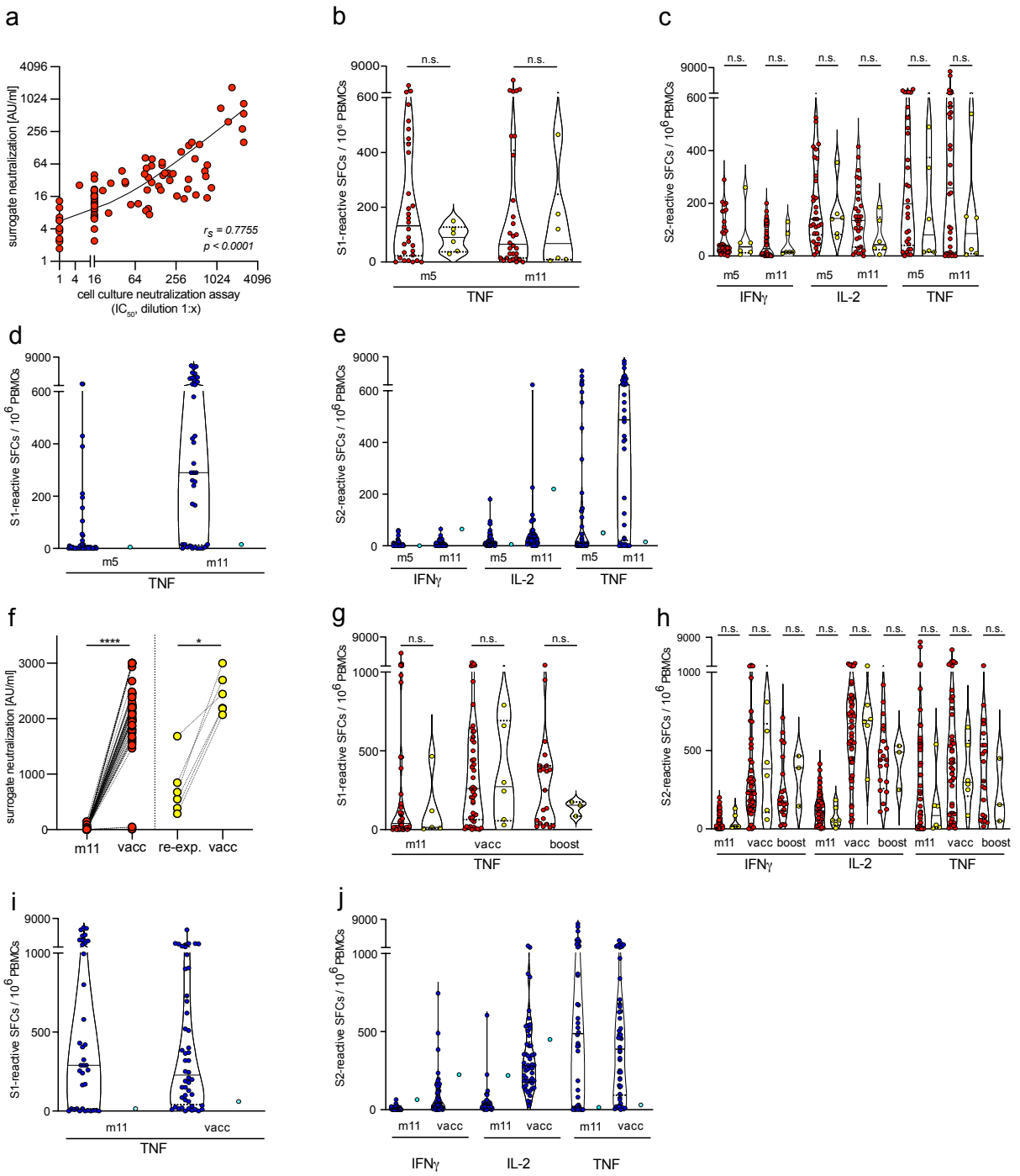
Extended Data Figure 6 | Frequencies of spike-reactive cytokine-secreting cells at month 11 after infection or after BNT162b2 mRNA prime and boost vaccination.

(**a,b**) frequencies of spike-reactive cytokine-secreting cells determined directly *ex vivo* by Fluorospot analysis at month 11 after SARS-CoV-2 infection, at 2 weeks after BNT162b2 mRNA prime vaccination (vacc) or at 2 weeks after boost vaccination (boost), red - convalescents; blue - naïve individuals. (**c**) heatmap of frequencies of individuals bearing spike-reactive cytokine-secreting cells, c - convalescents, n - naïve individual. (**d,e**) intracellular cytokine staining of spike-reactive CD4 and CD8 T cells determined directly *ex vivo* by flow cytometry, time points as in (a). (**f**) frequencies of spike-reactive IL-4-secreting cells after BNT162b2 mRNA prime vaccination (vacc) or boost vaccination (boost). Statistical analyses by ANOVA, Mann-Whitney and Wilcoxon signed-rank tests (a, b, f); n.s. denotes not significant, * $p<0.05$; ** $p<0.01$; *** $p< 0.001$; **** $p< 0.0001$



Extended Data Figure 7 | Lack of spike-specific immunity in convalescents with a delayed response to BNT162b2 mRNA vaccination.

(**a**) individual courses of surrogate virus-neutralization activity in convalescents (red) after prime and boost BNT162b2 mRNA vaccination compared to naïve individuals (blue). **(b)** individual courses of surrogate virus-neutralization activity in naïve individuals after prime and boost BNT162b2 mRNA vaccination (right panel) demonstrating four individuals with a low response to vaccination (dark grey), compared to virus-neutralization activity in low responder convalescents after vaccination (left panel). (**c**) cell culture virus-neutralization activity at month 2 after SARS-CoV-2 infection in serum of convalescents with low response to vaccination. (**d,e**) spike- and CEF-reactive cytokine-secreting cells at month 5 after infection in convalescents (red) and convalescents with low response to vaccination. (**f,g**) frequencies of spike-reactive cytokine-secreting cells at month 11 after SARS-CoV-2 infection, 2 weeks after prime (vacc) and 2 weeks after boost vaccination (boost). Statistical analyses by ANOVA, Mann-Whitney and Wilcoxon signed-rank tests (a,b,d,e,f,g); n.s. denotes not significant; * $p<0.05$; ** $p<0.01$; *** $p< 0.001$; **** $p< 0.0001$



Extended Data Figure 8 | Characteristics of spike-specific immunity after SARS-CoV-2 re-exposure in convalescents compared to an individual after SARS-CoV-2 infection

(a) Correlation between cell culture neutralization assay (shown as dilution 1:x mediating a 50% inhibition of SARS-CoV-2 infection *in vitro* - IC50) with a competitive CLIA (YHLO) measuring surrogate neutralization (arbitrary units (AU)/ml) at month 5 after SARS-CoV-2 infection. (**b,c**) frequencies of S1- or S2-reactive cytokine-secreting cells in convalescents (red) or re-exposed convalescents (yellow) at month 5 and month 11 after SARS-CoV-2 infection. (**d,e**) frequencies of S1- or S2-reactive cytokine-secreting cells in naïve individuals (blue) or the infected individual (turquoise) at month 5 and month 11 after SARS-CoV-2 infection. (**f**) paired analysis of surrogate neutralization activity before and after BNT162b2 mRNA vaccination in convalescents (red) or re-exposed convalescents (yellow). (**g,h**) frequencies of S1 or S2-reactive cytokine-secreting cells in convalescents (red) or re-exposed convalescents (yellow) before vaccination (month 11), 2 weeks after vaccination (vacc) and 2 weeks after boost vaccination (boost). (**i,j**) frequencies of S1 or S2-reactive cytokine-secreting cells in naïve individuals (blue) or the infected individual (turquoise) before vaccination (month 11) and 2 weeks after vaccination (vacc). Statistical analyses by ANOVA, Mann-Whitney and Wilcoxon signed-rank tests (b,c,f,g,h), r_s denotes Spearman correlation coefficient; n.s. denotes not significant * $p<0.05$; ** $p<0.01$; *** $p< 0.001$; **** $p< 0.0001$

Materials and Methods

Study participants in seropositive and seronegative cohorts

In April/May 2020, 4554 employees of the University Hospital München rechts der Isar of the Technical University of Munich underwent SARS-CoV-2 IgG testing after the first wave of the SARS-CoV-2 pandemic. Seropositive and a matched group of seronegative individuals, who gave written informed consent, were included into a prospective follow up study (SeCoMRI) that was extended for further follow up after BNT162b2 mRNA vaccination (VaCoMRI). Both studies were approved by the local ethics committee of the Technical University of Munich (ethics vote 476/20S and 26/21S-SR).

We identified 108 individuals who tested positive for anti-SARS-CoV-2 IgG by an indirect chemiluminescence immunoassay (iFlash CLIA, YHLO Biotechnology, China; specificity 99.3 (98.3-99.7) %, sensitivity 94 (89.0-96.8) %) [1,2], among whom 94 individuals agreed to participate in the follow up study. Among these, positive anti-SARS-CoV-2 IgG testing was confirmed in 89 participants by at least two further commercial assay systems, and in one further assay system in two individuals, for which material was limited, resulting in a combined specificity of at least 99.94%. Confirmatory SARS-CoV-2 IgG tests were performed in the following order: Roche Elecsys (specificity 99.1 (96.7-99.7) %, sensitivity 92.7 (87.3-95.9) %) [1,3]; Abbott Architect (specificity 99.5 (98.5-99.8) %, sensitivity 90.0 (84.2-93.8) %) [1,4]; Mikrogen Diagnostics (Martinsried, Germany) *recom*Line blot (specificity 99.7%, sensitivity 95.2-100% according to the manufacturer); Euroimmune (Lübeck, Germany) anti-S1 IgG ELISA (specificity 99.2 (98.1-99.6) %, sensitivity 78.0 (70.7-83.9) %) [1]. Three individuals only tested positive for anti-SARS-CoV-2 IgG using the screening assay (Yhlo iFlash), and were therefore excluded from the study.

In 34 / 91 seropositive individuals with at least two independent positive anti-SARS-CoV-2 IgG tests, the infection had been confirmed by PCR-testing for SARS-CoV-2 RNA (for details see **supplementary Table I**). Thus, 91 convalescents of mild SARS-CoV-2 infection were identified and included in the follow up study. The specific characteristics of this cohort of convalescents are shown in **Extended Data Fig. 1c**. A control cohort of naïve individuals for follow up studies was randomly selected from the 4446 seronegative individuals by matching for sex, age, working conditions and risk factors that were present in the seropositive cohort (**Extended Data Fig. 1c**). After follow up for 9 months, study participants were offered to continue follow up after BNT162b2 vaccination (VaCoMRI). A total of 82 convalescents and 51 naïve individuals gave informed written consent to participate in the follow up study. The first 23 convalescents received prime and boost BNT162b2 mRNA vaccination 28 days apart.

Due to a change in the national guidelines remaining convalescents received only a single shot of BNT162b2.

Serum SARS-CoV-2 neutralization activity

Serum neutralization activity was determined using an in-house cell culture-based infection inhibition assay and a surrogate neutralization assay based on the competition with binding of a labelled recombinant angiotensin-converting enzyme-2 to microparticle-coupled recombinant SARS-CoV-2 receptor binding domain (SARS-CoV-2 NAb, YHLO Biotechnology) used according to the manufacturer's instruction on the automated iFlash 1800 platform.

For infection inhibition, SARS-CoV-2 (D614G variant of the Wuhan strain) isolated from a nasal swab sample of one of the first patients in Germany in February 2020 was grown on VeroE6 cells. The full-length viral genome sequence has been uploaded into the GISAID database, accession ID: EPI_ISL_582134. For the infection inhibition assay, VeroE6 cells were seeded in supplemented Dulbecco's Modified Eagles medium (Thermo Fisher Scientific, Germany) at 15,000 cells per well in a 96-well plate one day before infection was started using SAR-CoV-2 at a multiplicity of infection (MOI) of 0.06 plaque-forming units (PFU) / cell. To detect virus-neutralization activity, serum samples were serially diluted 1:2 with DMEM up to a 2048 dilution and added to SARS-CoV-2 (900 PFU/15,000 cells/well) in a total volume of 50 µL at 37°C. After one hour of preincubation, the inoculum was transferred to the pre-seeded VeroE6 cells for another one-hour incubation at 37°C before the inoculum was replaced by supplemented DMEM. SARS-CoV-2 infection was terminated after 24 hours by adding 4% paraformaldehyde to fix the cells, and infection rate was analyzed by an in-cell ELISA. After fixation, cells were washed with PBS and permeabilized with 0.5% saponin (Sigma-Aldrich, Germany). Blocking buffer consisting of 0.1% saponin-10% goat serum (Sigma-Aldrich) in PBS was added and incubated for one hour on fixed cells to avoid unspecific binding of antibodies. Afterwards, viral double-stranded (ds) RNA inside cells were detected by anti-dsRNA J2 monoclonal antibody (Jena Bioscience, Jena, Germany; 1:500 diluted) overnight incubation at 4°C and followed by one-hour incubation under room temperature with secondary antibody goat anti-mouse IgG2a-HRP (Southern-Biotech, Alabama, USA; 1:2000 diluted). Washing steps with 0.01% Tween20-PBS buffer were included in between and after. Finally, signals were created by the interaction between HRP and the substrate and the reaction was stopped by addition of 2N sulfuric acid solution, continued by optical detection with a Tecan Infinite 200 reader (TECAN, Switzerland) at 450 nm wavelength. The inhibition curve of each

sample was analyzed by statistical analysis software Graph Pad Prism (GraphPad Software, USA), and 50% inhibitory concentration (IC_{50}) was determined using non-linear regression.

Isolation and cryopreservation of peripheral blood mononuclear cells (PBMCs)

Blood from study participants was drawn with the Vacutainer CPT™ System into sodium citrate CPT tubes (Becton Dickinson Biosciences, USA) and tubes were mixed five times before storing them upright at room temperature. Within two hours of blood collection, CPT tubes were centrifuged in a horizontal rotor (swing-out head) (1800g, 15 min, RT). Next, plasma was removed and PBMCs were transferred to 15 mL polystyrene Falcon tubes and mixed with 10 mL PBS by gently inverting the tubes for five times. PBMCs were centrifuged (300g, 10 min, RT) twice in 10 ml of PBS. For counting, cells were resuspended in CTL Test medium (CTL Europe GmbH, Germany) and 10 µL of the cell suspension was diluted 1:2 with CTL Live/Dead cell counting dye (CTL-LDC Live/Dead cell counting kit, CTL Europe, Germany). 10 µL of the stained cell suspension were pipetted into the counting chamber and cell counting was performed on an ImmunoSpot Ultimate UV Image analyzer (CTL Europe, Germany). PBMCs were either directly used in multiparameter Fluorospot assays and flow cytometry-based intracellular cytokine staining or 5 x 10^6 PBMCs were cryopreserved per vial in 1.8 mL cryotubes (Thermo Scientific, Denmark) at a concentration of 1 x 10^7 PBMC per 1 mL freezing medium (fetal calf serum (FCS) (Life Technologies, Germany), supplemented with 10% DMSO (Sigma-Aldrich, Germany), using a freezing container (Thermo Scientific, Denmark) and stored at -80°C. After 24h, PBMCs were stored in the vapor phase of a liquid nitrogen tank until further use.

Multi-parameter IFNγ/IL-2/TNF/Granzyme B and IL-5/IL-4 Fluorospot assays for detection of the frequencies of spike-reactive PBMCs directly *ex vivo*

Human IFNγ/IL-2/TNF/Granzyme B four-color and human IL-5/IL-4 dual-color Fluorospot assays (CTL Europe, Germany) were performed according to the manufacturer's instructions for single parameter Fluorospot assays. One day before the Fluorospot assays were performed, the plates were activated by adding 70% ethanol for less than one minute. Followed by a washing step and addition of IFNγ/IL-2/TNF/Granzyme B or IL-5/IL-4 capture antibodies overnight, respectively. After decanting the plate, PBMCs were used directly after isolation and placed at 2 × 10^5 (4CFS) or 8 × 10^5 /well (2CFS) in a final volume of 200 µL/well. PBMCs were then stimulated for 22h (4CFS) or 48h (2CFS) with 1 µg/mL of overlapping peptide pools

(15mers overlapping by 11 aa) of the SARS-CoV-2 spike protein (PepMix™ SARS-CoV-2 (PM-WCPV-S), consisting of two peptide pools, i.e. S1 and S2 with 158 and 157 peptides, respectively) (JPT Peptide Technologies, Germany). The spike protein S1 peptide pool (158 15mer peptides) covered the N-terminal amino acid (aa) residues 1-643, and the S2 peptide pool (156 15mer peptides and one 17-mer at the C-terminus) encompassed the C-terminal aa residues 633-1273 ref[4]. As antigen-specific positive control, we used a CEF pool of in total 32 15mer peptides derived from Cytomegalovirus (5 peptides), Epstein-Barr virus (15 peptides), and Influenza virus (Flu) (12 peptides) proteins (National Institute for Biological Standards and Control (NIBSC), UK). After the stimulation period, the plates were washed and 80 µL of either anti-human IFNγ (FITC), anti-human IL-2 (Hapten2), anti-human TNF (Hapten1), and anti-human Granzyme B (Biotin) or anti-human IL-5 (Hapten3) and anti-human IL-4 (Biotin)detection antibody solution was added for additionally 2h. For the visualization of secreted cytokines and GzmB, plates were washed and a tertiary solution including either anti-FITC Alexa Fluor® 488 (visualizes IFNγ), anti-Hapten2 CTL-Red™ (visualizes IL-2), anti-Hapten1 CTLYellow™ (visualizes TNF), and Streptavidin eFluor® 450 (visualizes GzmB) or anti-Hapten3 Alexa Fluor® 488 (visualizes IL-5) and Strep CTL-Red™ (visualizes IL-4) was added for one hour. The staining procedure was stopped by washing the plate. After drying the plates for 24h on paper towels on bench top, multi-parameter Fluorospot plates were scanned using an automated reader system (ImmunoSpot Ultimate UV Image analyzer/ImmunoSpot 7.0.17.0 Professional DC Software, CTL Europe GmbH, Germany). Counting of spot forming cells (SFC) on Fluorospot plates was performed by adjusting the sensitivity, background balance, and the gates for the spot size using CTL software. Counting was performed in compliance with the guidelines for the automated evaluation of ELISpot assays[5]. All counts were reviewed and certified by a second person in a rigorous quality control process. Final results are represented as SFCs per 1×10^6 PBMCs. Positive reactivity to experimental stimulatory agents was given when the spot count in antigen-stimulated cells was greater than twice the spot count in unstimulated (background) wells.

Direct *ex vivo* detection of SARS-CoV-2 reactive T cells by flow cytometry using intracellular cytokine staining

1 x 10^6 freshly isolated PBMCs were transferred into 150 µL RPMI1640 medium supplemented with 10% FCS and 1% penicillin-streptomycin (PenStrep, Life Technologies, Invitrogen, Germany) (abbr.: RPMI-10) containing costimulatory antibodies to ensure effective T cell stimulation (1 µg/mL anti-CD28; BD Biosciences, Germany) in one well of a 96-well

polypropylene U-bottom microtiter plate. Cells were stimulated with the S1/S2 peptide pools (1 µg/mL) as mentioned above. After one hour of incubation at 37°C in 5% CO_2, 10 µg/mL of Brefeldin A (Sigma-Aldrich, Germany) was added to the cell suspension and incubated for 4h at 37°C in 5% CO_2, after which intracellular cytokine staining (ICS) was performed.

Stimulated PBMCs were labelled with the LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (Thermo Fisher Scientific, USA) in a total volume of 100 µL for 30 min at 4°C in the dark, and washed twice with 200 µL FACS buffer (BD Biosciences). After centrifugation (560g, 4°C, 5 min), PBMCs were fixed for 20 min at 4°C in the dark in 100 µL of an intracellular fixation buffer (Intracellular Fixation Buffer, Thermo Fisher Scientific, USA). After two wash steps with 200 µL/well Perm/Wash solution (Cytofix/Cytoperm Kit; BD Biosciences) and a centrifugation step (710g, 4°C, 5 min), PBMCs were stained with the antibodies listed in **supplementary data I** in a total volume of 80 µL Perm/Wash buffer including a brilliant violet buffer (BD Pharmingen Stain Buffer, BD Biosciences) for 30 min at 4°C in the dark. Single color compensation was performed using 25 µL of compensation beads (UltraComp eBeads and ArC™ Amine Reactive Compensation Bead Kit for LIVE/DEAD compensation, both from Thermo Fisher Scientific, USA) following the instructions of the manufacturer. Cells and beads were washed twice and finally re-suspended in 300 µL FACS buffer for acquisition. Cells were stored cold and in the dark until acquisition.

Acquisition of samples was performed within four hours after staining using a LSR2/LSR Fortessa flow cytometer and FACSDiva Software V.6.1.3 (Becton Dickinson, Germany). Photomultiplier voltages were adjusted with the help of unstained cells for all parameters. Analysis was performed on at least 1.5 x 10^5 living lymphocytes using the software FlowJo version 10.7.0 (FlowJo LLC, USA).

Gating strategy for flow cytometric analysis of *ex vivo* re-stimulated PBMCs is shown in the supplementary information (**supplementary data I**). Each gate was set in the negative control sample and then adjusted to peptide-stimulated cells with consideration of T cell receptor downregulation. Two independent audits were performed to control the gating. According to the differential expression of IFNγ, TNF, and IL-2, the CD4 and CD8 T cell subpopulations were defined, respectively.

After background subtraction, CD4 and CD8 T cell responses were calculated by summing up all events staining positive for expression of the cytokines IFNγ, TNF or IL-2 and their respective combinations. Raw data of all performed assays are available upon request.

Statistical analysis

All results were included in the analysis, and no attempt was made to exclude outliers. All tests were two-sided and conducted on exploratory 5% significance levels. Nonparametric statistical tests were applied in all cases. Unpaired Mann-Whitney tests were performed to compare distributions of relevant variables between different groups of study individuals. Wilcoxon signed-rank testing was employed to assess significance of change in values among different time points. One-Way ANOVA testing was applied for comparison of different time points within the study groups. In case of resulting p-values < 0.05, Mann-Whitney and Wilcoxon signed-rank tests were performed to assess significance of change in values between specific time points. Spearman correlation analysis was performed to evaluate the correlation of e.g. S1- or S2-reactive cytokine-secreting cells from convalescents with serum anti-SARS-CoV-2 IgG levels or with cell culture virus-neutralization activity. The software Graph Pad Prism 9.1.0 (GraphPad Software, La Jolla, California, USA) was used for statistical analyses.

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