Boosting compromised SARS-CoV-2-specific immunity with mRNA vaccination in liver transplant recipients

Hendrik Luxenburger, David B. Reeg, Julia Lang-Meli, Matthias Reinscheid, Miriam Eisner, Dominik Bettinger, Valerie Oberhardt, Elahe Salimi Alizei, Katharina Wild, Anne Graeser, Vivien Karl, Sagar, Florian Emmerich, Florian Klein, Marcus Panning, Daniela Huzly, Bertram Bengsch, Tobias Boettler, Roland Elling, Robert Thimme, Maike Hofmann, Christoph Neumann-Haefelin

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Supplementary methods

PBMC isolation

Samples of venous blood were collected in EDTA-anticoagulated tubes. PBMCs were isolated with lymphocyte separation medium by density gradient centrifugation (Pancoll separation medium, PAN Biotech GmbH). PBMCs were stored at −80 °C. Frozen PBMCs were thawed in complete medium (RPMI 1640 supplemented with 10 % fetal calf serum, 1 % penicillin/streptomycin and 1.5 % HEPES buffer 1 M (all additives from Thermo Scientific) containing 50 U ml^{-1} benzonase (Sigma).

In vitro expansion and intracellular IFNγ staining with overlapping peptides

We tested a total of 182 overlapping peptides (OLPs) spanning the entire SARS-CoV-2 spike protein sequence (Gene Bank Accession code MN908947.3), synthesized as 18-mers overlapping by 11 amino acids with a free amine NH_2 terminus and a free acid COOH terminus with standard Fmoc chemistry showing a purity of > 70% (Genaxxon Bioscience). To perform the *in vitro* expansion with OLPs, we stimulated 20 % of the PBMCs with a pool of all 182 SARS-CoV-2 spike OLPs (10 µg ml^{-1}) for 1 h at 37 °C. Next, the cells were washed and co-cultured with the remaining PBMCs in RPMI medium supplemented with recombinant IL-2 (20 U ml^{-1}). On day 10, we performed intracellular IFNγ staining with pooled OLPs. Cells were re-stimulated with 45 OLP pools (50 µM) containing four peptides each (dimethylsulfoxid (DMSO) as negative control and phorbol 12-myristate 13-acetate (PMA) as well as ionomycin (50 ng ml^{-1} and 1 µg ml^{-1}) as positive control) in the presence of both brefeldin A (GolgiPlug, 0.65 µl ml^{-1}) and IL-2 (0.05 U ml^{-1}). Cells were stained for surface markers (CD8, CD4), viability (Viaprobe) and intracellular markers (IFNγ) after 5 h of incubation at 37 °C. On day 12, the single overlapping peptides of all positive pools were tested by intracellular cytokine staining. The viral amino acid sequences of positive individual OLPs were then screened for previously described minimal epitopes or the best HLA-matched predicted candidate using the Immune Epitope Database website (www.iedb.org) combined with two prediction algorithms ANN 4.0 and NetMHCpan EL 4.123 for 8-mer, 9-mer and 10-mer peptides with half-maximal inhibitory concentration (IC_{50}) of < 500 nM.

Magnetic bead-based enrichment of spike-specific CD8+ T cells

Enrichment of spike-specific CD8+ T cells was performed as described previously [1]. Briefly, 1 × 10^7–2 × 10^7 PBMCs were labelled with APC-coupled peptide-loaded HLA class I tetramers for half an hour. Next, the enrichment was performed using anti-APC beads with MACS technology (Miltenyi Biotec) according to the manufacturer's instructions. Subsequently, analysis of the enriched spike-specific CD8+ T cells was conducted by multiparametric flow cytometry and frequencies of spike-specific CD8+ T cells were calculated as described before [1]. Importantly, only samples including ≥ 5 spike-specific CD8 T cells were used for further subset gating and phenotypic analyses (detection limit of 5 × 10^{-6}).

SARS-CoV-2 pseudovirus neutralization assay

Pseudovirus neutralization assays against SARS-CoV-2 wild-type (Wuhan-Hu-1) and the Omicron variants BA.4/5 were performed on a selection of 47 samples as previously described [2-4]:

Cell lines: HEK293T cells and 293T-ACE2 cells

HEK293T cells and HEK293T-ACE2 cells [5] were grown in DMEM (Gibco) supplemented with 10 % FCS, 0,5 % Ciprofloxacin, 1 mM L-Glutamine and 1 mM Sodium pyruvate. Cells were maintained on tissue culture treated dishes in a T75 flask at 37 °C in a 5 % CO_2 humidified incubator.

Generation of spike pseudotyped lentiviral particles

For lentivirus production, adherent HEK 293T cells in T75 flasks were transfected with single plasmids encoding a fluorescent reporter protein (pHAGE-Luc-IRES-ZsGreen), a set of lentiviral proteins necessary for virion formation (pHDM-Hgpm, pHDM-Tat, pHDM-Rev) and the variant-specific spike protein (SARS-CoV-2 strain Wuhan-Hu-1 and Omicron VOC BA.4/5). For transfection, we used FuGENE 6 transfection reagent (Promega) according to manufacturer instructions. After 48 and 72 hours of incubation, we harvested the virus supernatant of the cell culture. Virus supernatants were centrifuged (400 g, 10 °C for 5 min), then filtered (0.45 µm filter) and stored in 1 ml aliquots at -80 °C until use.

Each viral batch was titrated by infection of 293T-ACE2. After incubation for 48 hours at 37 °C and 5 % CO2, the infectious titers of pseudovirus supernatants were assessed by adding luciferin/lysis buffer (10 mM $MgCl_2$, 0.3 mM ATP, 0.5 mM coenzyme A, 17 mM IGEPAL CA-630 (all Sigma-Aldrich), and 1 mM D-Luciferin (GoldBio) in Tris-HCL). Relative light units (RLUs) were measured with a microplate reader (TECAN SPARK).

SARS-CoV-2 pseudovirus neutralization assay

To quantify the SARS-CoV-2 neutralizing activity of serum samples, serial dilutions of serum (heat-inactivated at 56 °C for 30 minutes) were incubated with pseudovirus supernatants at 37 °C for 1 hour. 293T-ACE2 cells were added after the incubation period. Firefly luciferase activity was measured after 48 hours of incubation. The 50 % inhibitory dose (ID50) was defined as the serum dilution that resulted in a 50 % RLU reduction compared with the RLU signal from pseudovirus-infected cells without human serum (minus background luminescence). The serum samples were measured in technical duplicates and the average ID50 values are reported. ID50 values were determined in Prism 9 by plotting a dose-response curve.

References for Supplementary Methods

Author names in bold designate co-first authorship.

[1] Alanio C, Lemaitre F, Law HK, Hasan M, Albert ML. Enumeration of human antigen-specific naive CD8+ T cells reveals conserved precursor frequencies. Blood 2010;115:3718-3725.
[2] Gruell H, Vanshylla K, Tober-Lau P, Hillus D, Sander LE, Kurth F, et al. Neutralisation sensitivity of the SARS-CoV-2 omicron BA.2.75 sublineage. Lancet Infect Dis 2022;22:1422-1423.
[3] **Habermann E, Gieselmann L**, Tober-Lau P, Klotsche J, Albach FN, Ten Hagen A, et al. Pausing methotrexate prevents impairment of Omicron BA.1 and BA.2 neutralisation after COVID-19 booster vaccination. RMD Open 2022;8.
[4] Vanshylla K, Di Cristanziano V, Kleipass F, Dewald F, Schommers P, Gieselmann L, et al. Kinetics and correlates of the neutralizing antibody response to SARS-CoV-2 infection in humans. Cell Host Microbe 2021;29:917-929 e914.
[5] Crawford KHD, Eguia R, Dingens AS, Loes AN, Malone KD, Wolf CR, et al. Protocol and Reagents for Pseudotyping Lentiviral Particles with SARS-CoV-2 Spike Protein for Neutralization Assays. Viruses 2020;12.

Supplementary figures

Figure S1

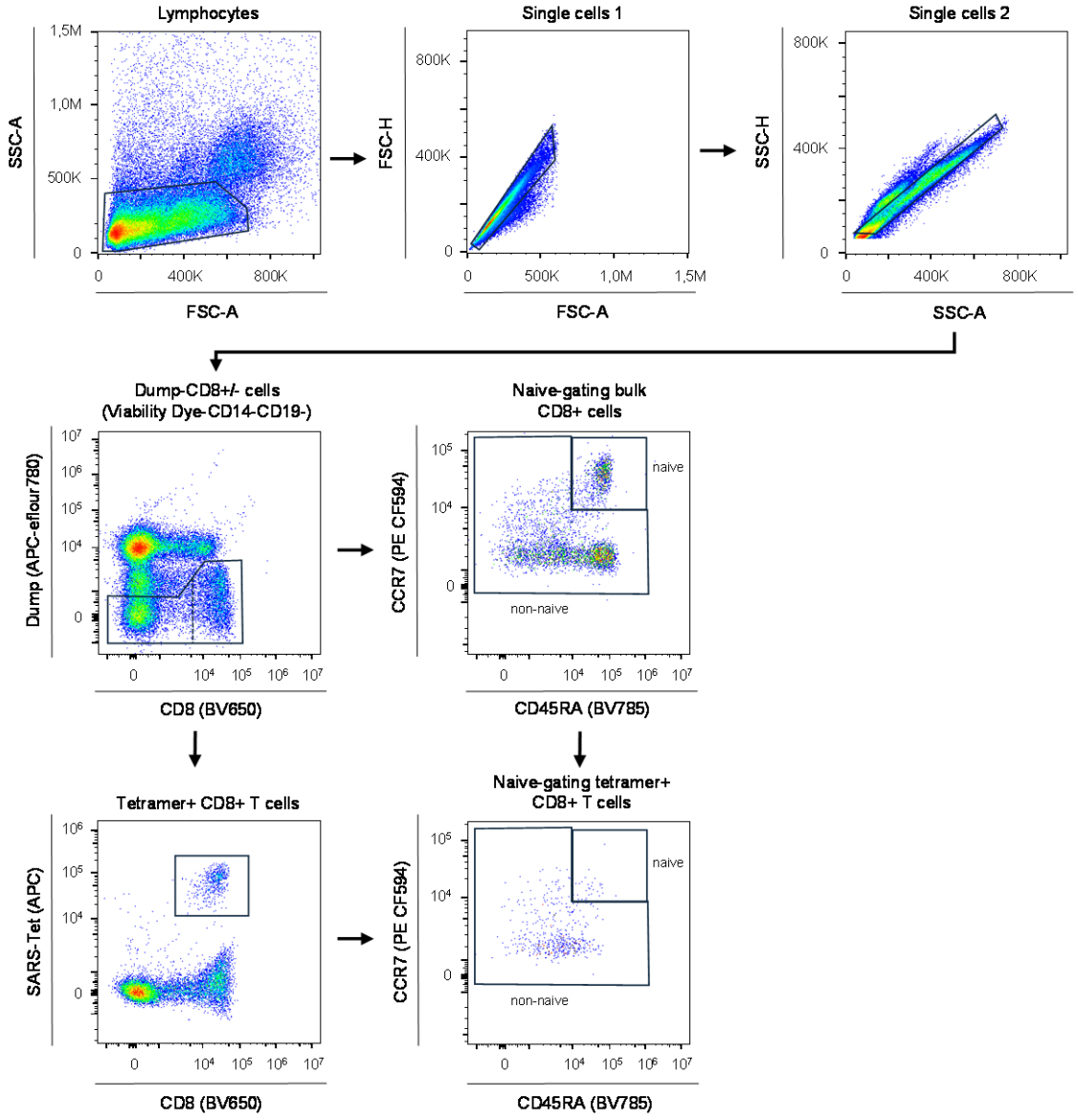

Fig. S1: Gating strategy peptide/MHC I tetramer enrichment.

Gating strategy of flow cytometry data to specify spike-reactive CD8+ T cells after peptide/MHC class I tetramer-enrichment

Figure S2

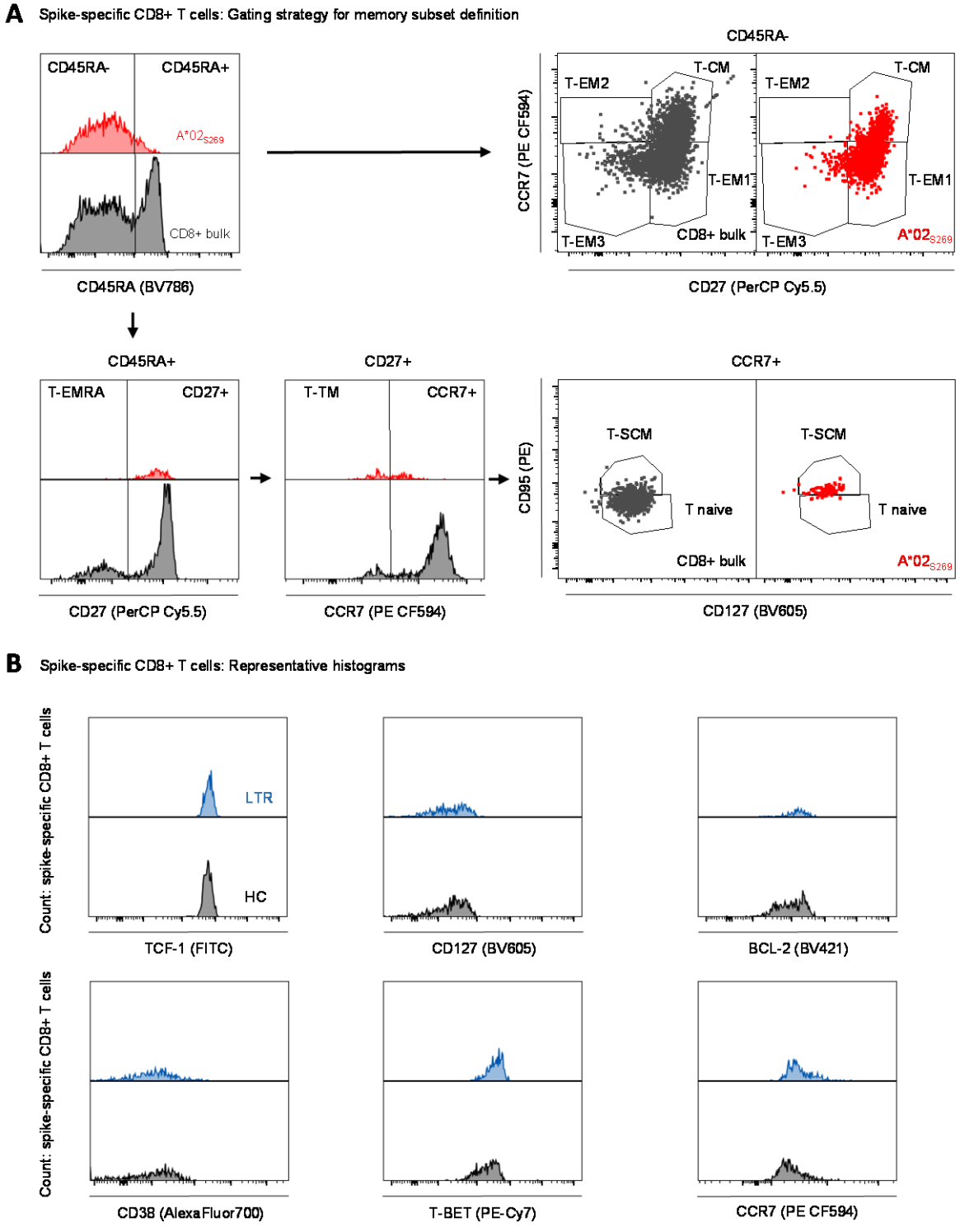

Fig. S2: Spike-specific CD8+ T memory cells.

(A) Gating strategy of flow cytometry data for memory subset specifications. This gating was applied to samples after *ex vivo* enrichment. **(B)** Representative histograms for indicated molecule expression on virus-specific CD8+ T cells after peptide/MHC I tetramer-enrichment in liver transplant recipients and healthy controls.

Figure S3

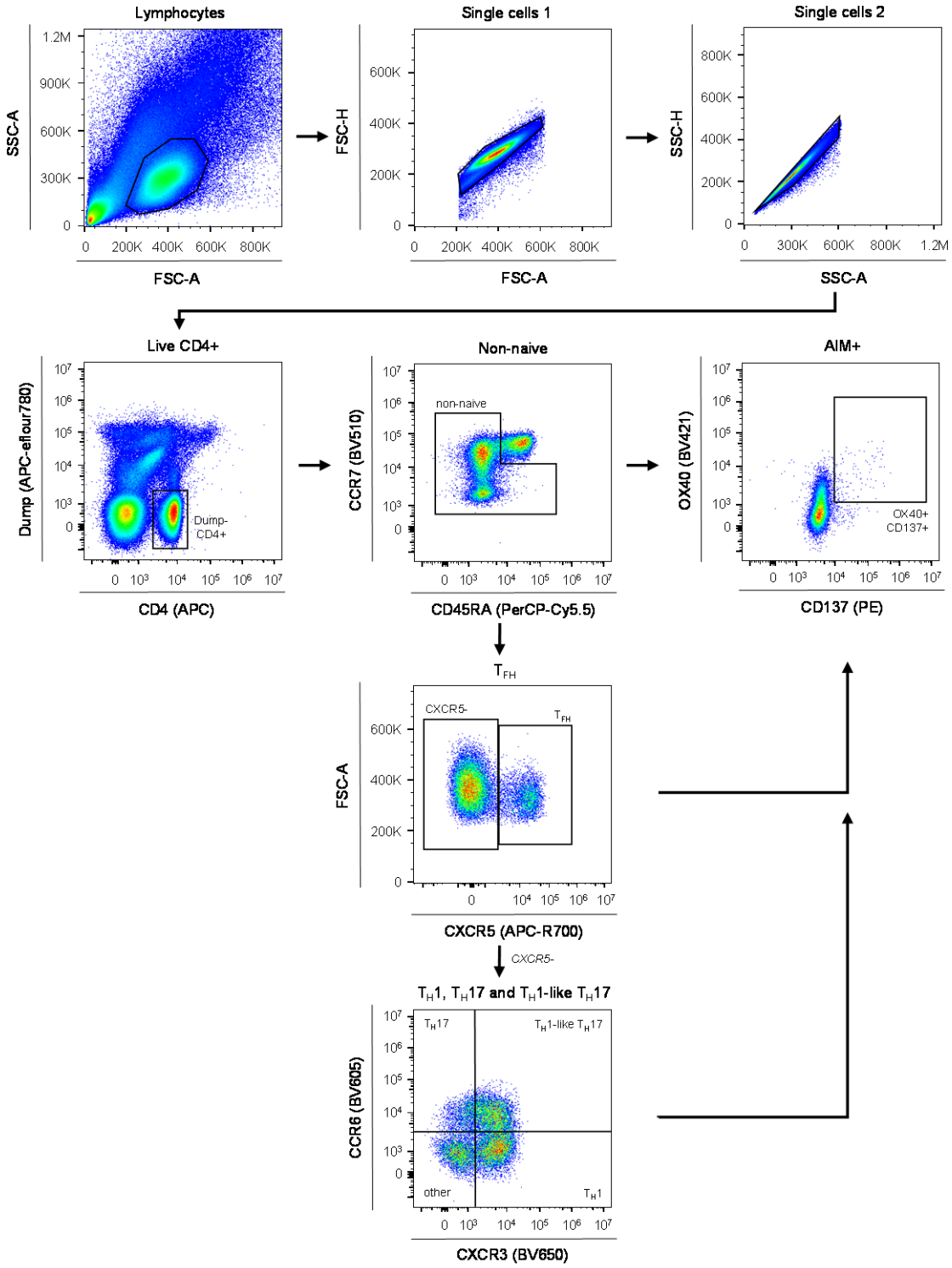

Fig. S3: Gating Strategy AIM-Assay.

Gating strategy to define AIM+ (CD137+ OX40+) non-naive CD4+ T cells and the respective CD4+ T cell subsets.

Figure S4

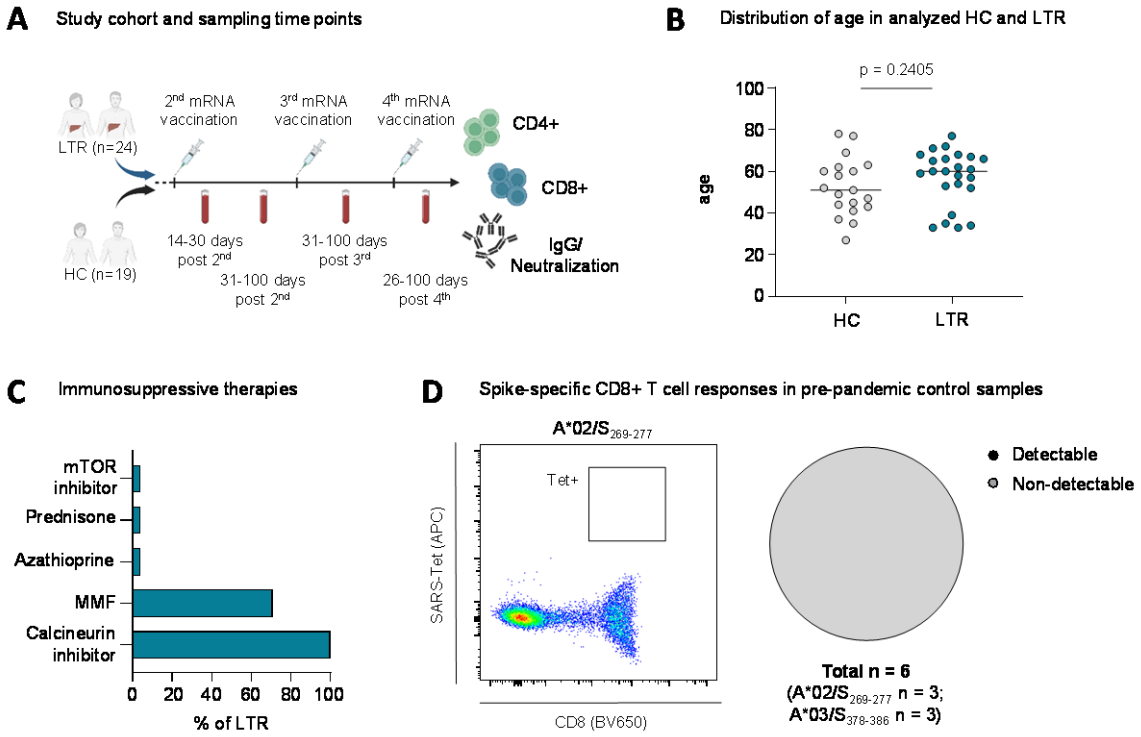

Fig. S4: Clinical parameters and spike-specific CD8+ T cell activation in LTR.

(A) Overview of study cohort and sampling time points. **(B)** Age of HC (n=19) and LTR (n=24) **(C)** Immunosuppressive therapy of LTR enrolled in this study (n=24). **(D)** Representative dot plot and percentage of pre-pandemic control samples with detectable/non-detectable response after tetramer-based enrichment. Statistics: Mann-Whitney test (B). HC: healthy control; LTR: liver transplant recipient; MMF: mycophenolate mofetil.

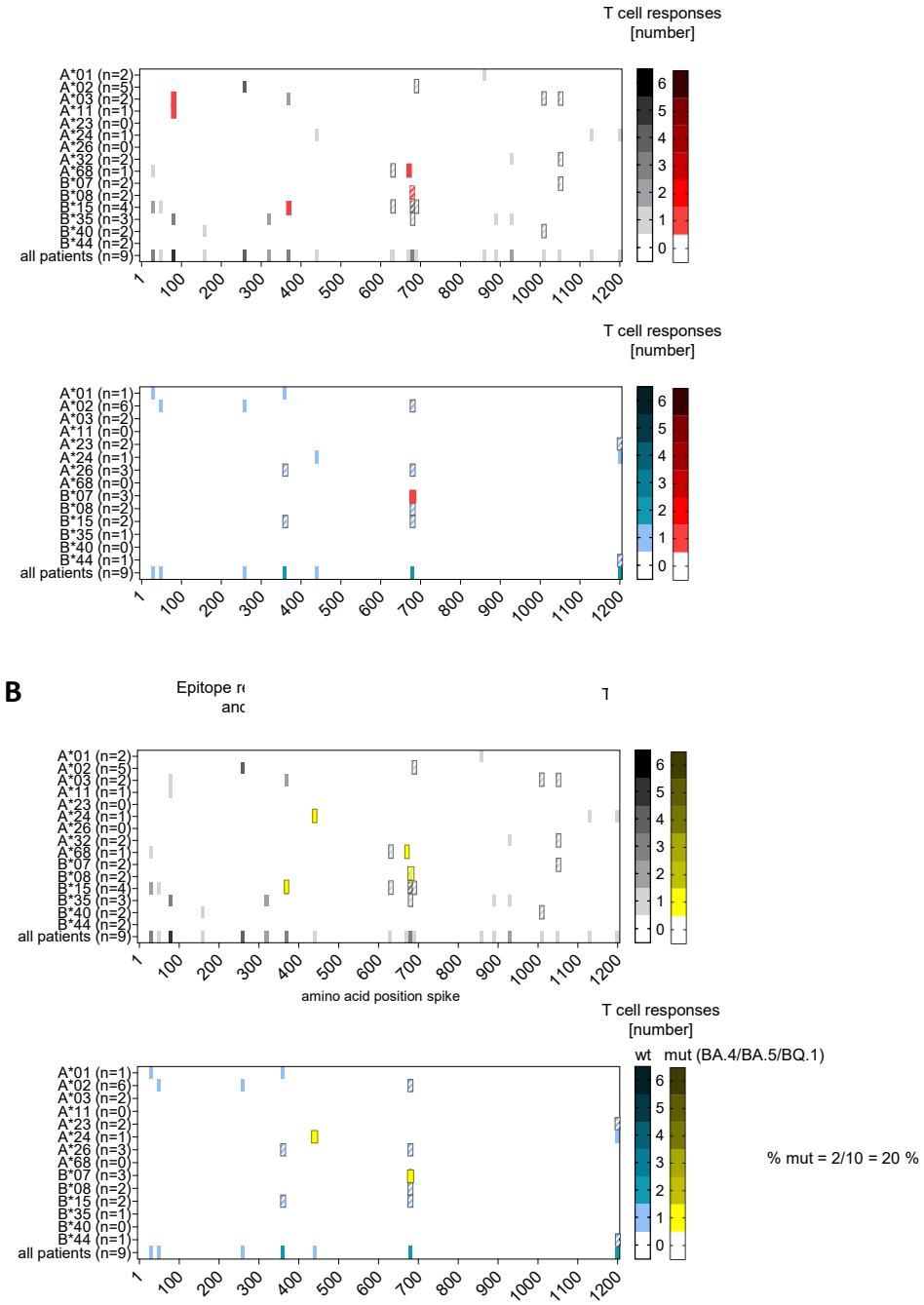

Fig. S5: CD8+ T cell responses targeting wild-type or variant spike-specific epitopes

Number and location of spike-specific CD8 + T cell responses to overlapping peptides (OLP) in HC and LTR after three vaccine doses. **(A)** Epitopes with amino acid sequence variations in Omicron BA.1 are marked in red. **(B)** Epitopes with amino acid sequence variations in Omicron BA.4/5 and BQ.1 are marked in yellow. Numbers of tested individuals (per HLA allotype and in total) and the percentage of total T cell responses targeting variant epitopes are indicated.

Figure S6

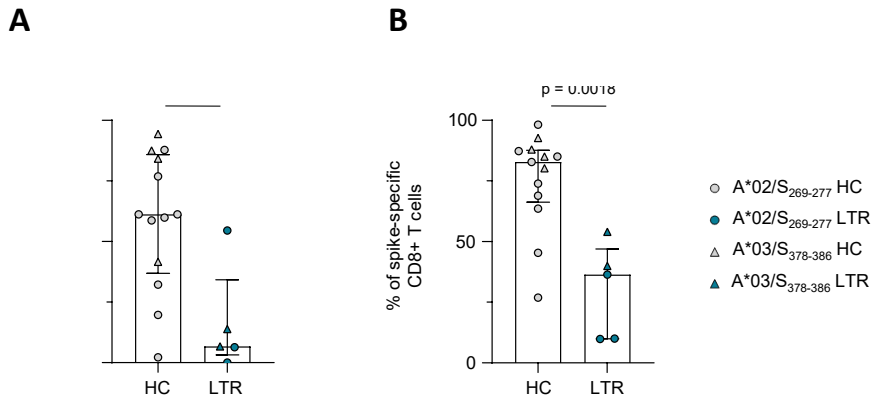

Fig. S6: Activation and effector differentiation of spike-specific CD8+ T cells after the 2nd vaccine dose.

(A) Percentage of spike-specific CD8+ T cells with T-BET^{hi} expression at 14-30 days post 2nd vaccine dose. **(B)** Percentage of CD38+ spike-specific CD8+ T cells at 14-30 days post 2nd vaccine dose. Statistics: Mann-Whitney test (A, B).

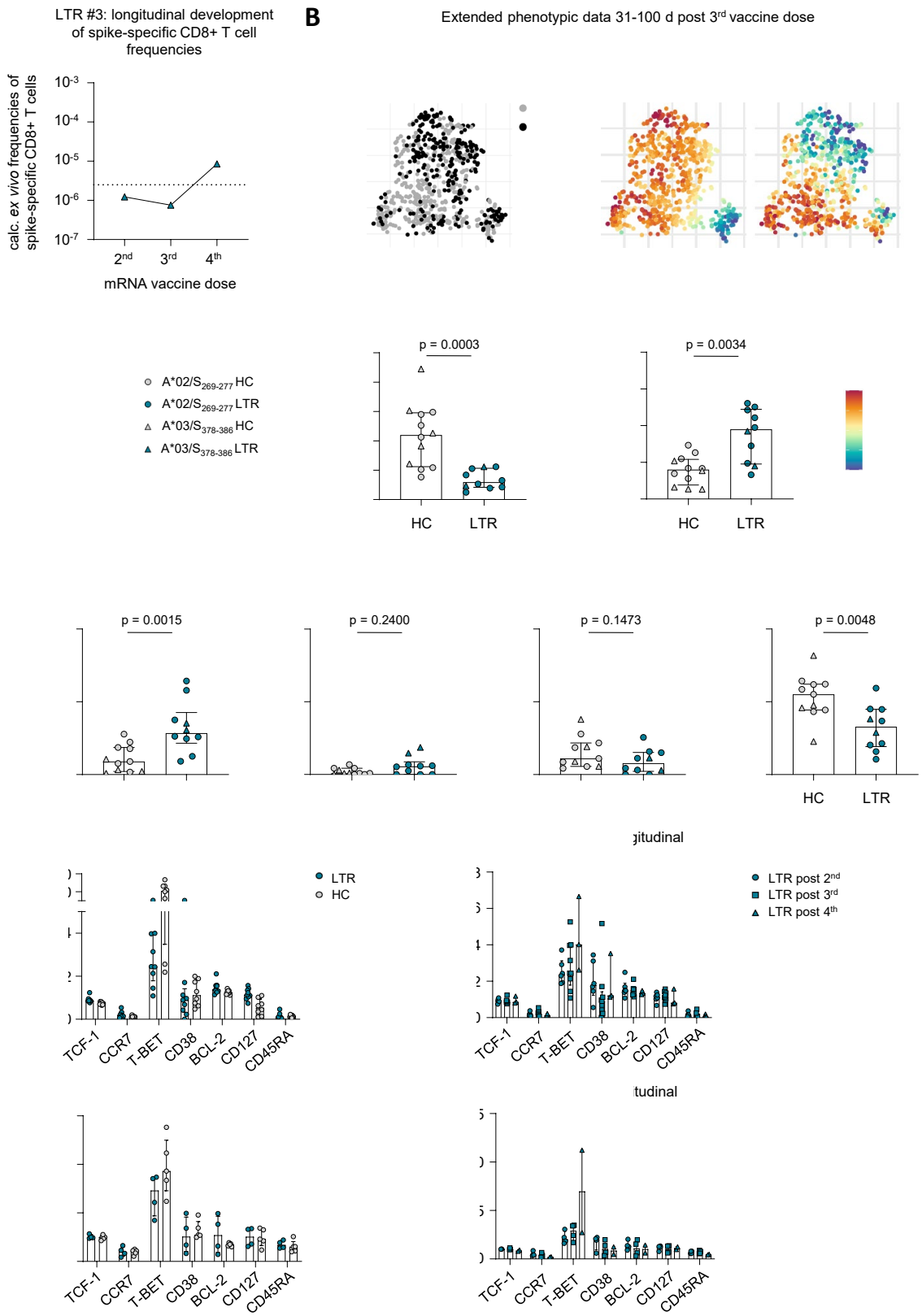
LTR #3: longitudinal development of spike-specific CD8+ T cell frequencies
calc. ex vivo frequencies of spike-specific CD8+ T cells
10-3
10-4
10-5
10-6
10-7
2nd
3rd
4th
mRNA vaccine dose
B
Extended phenotypic data 31-100 d post 3rd vaccine dose
A*02/S269-277 HC
A*02/S269-277 LTR
A*03/S378-386 HC
A*03/S378-386 LTR
p = 0.0003
p = 0.0034
HC
LTR
p = 0.0015
p = 0.2400
p = 0.1473
p = 0.0048
HC
LTR
gitudinal
LTR
HC
LTR post 2nd
LTR post 3rd
LTR post 4th
TCF-1
CCR7
T-BET
CD38
BCL-2
CD127
CD45RA
tudinal

Fig. S7: Marker expression and memory CD8+ T cell subset distribution in HC versus LTR.

(A) Calculated *ex vivo* frequencies of spike-specific CD8+ T cells for patient LTR#3 at 14-30 days after the 2nd, 31-100 days after the 3rd or 26-100 days after the 4th vaccine dose. **(B)** t-SNE representation or bar graphs of flow cytometry data comparing spike-specific CD8+ T cells with CD38, T-BET or CD127 expression derived from HC and LTR 31-100 days after the 3rd vaccine dose. **(C)** Spike-specific CD8+ T cells with central memory (T-CM; CD45RA-CCR7+CD27+), stem cell memory (T-SCM; CD45RA+CCR7+CD27+CD95+), transitional memory (T-TM; CD45RA+CCR7-CD27+) and effector memory (T-EM; CD45RA-CCR7-CD27+) phenotype in LTR versus HC at 31-100 days post 3rd vaccine dose (LTR n=10, HC n=11). **(D)** Individual fluorescence intensity (nMFI) of different markers within the subset of BCL-2^{hi} or T-SCM spike-specific CD8+ T cells in HC and LTR normalized to naïve CD8+ T cells; samples with ≥ 5 cells per subset are depicted. Statistics: Mann-Whitney test (B, C). HC: healthy control; LTR: liver transplant recipient.

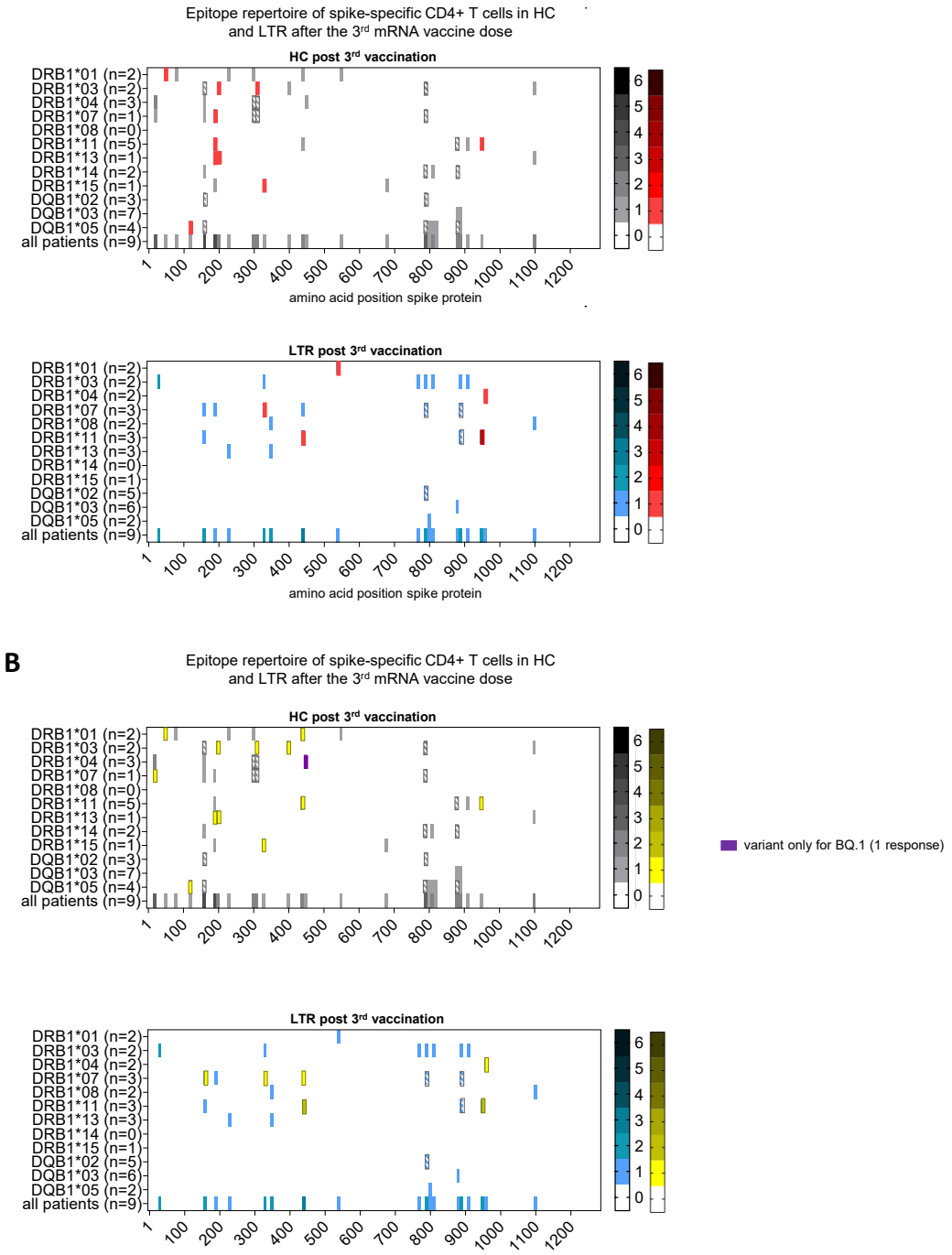

Fig. S8: CD4+ T cell responses targeting wild-type or variant spike-specific epitopes

Number and location of spike-specific CD4+ T cell responses to overlapping peptides (OLP) in HC and LTR after three vaccine doses. **(A)** Epitopes with amino acid sequence variations in Omicron BA.1 are marked in red. **(B)** Epitopes with amino acid sequence variations in Omicron BA.4/5 and BQ.1 are marked in yellow. Numbers of tested individuals (per HLA allotype and in total) and the percentage of total T cell responses targeting variant epitopes are indicated.

Figure S9

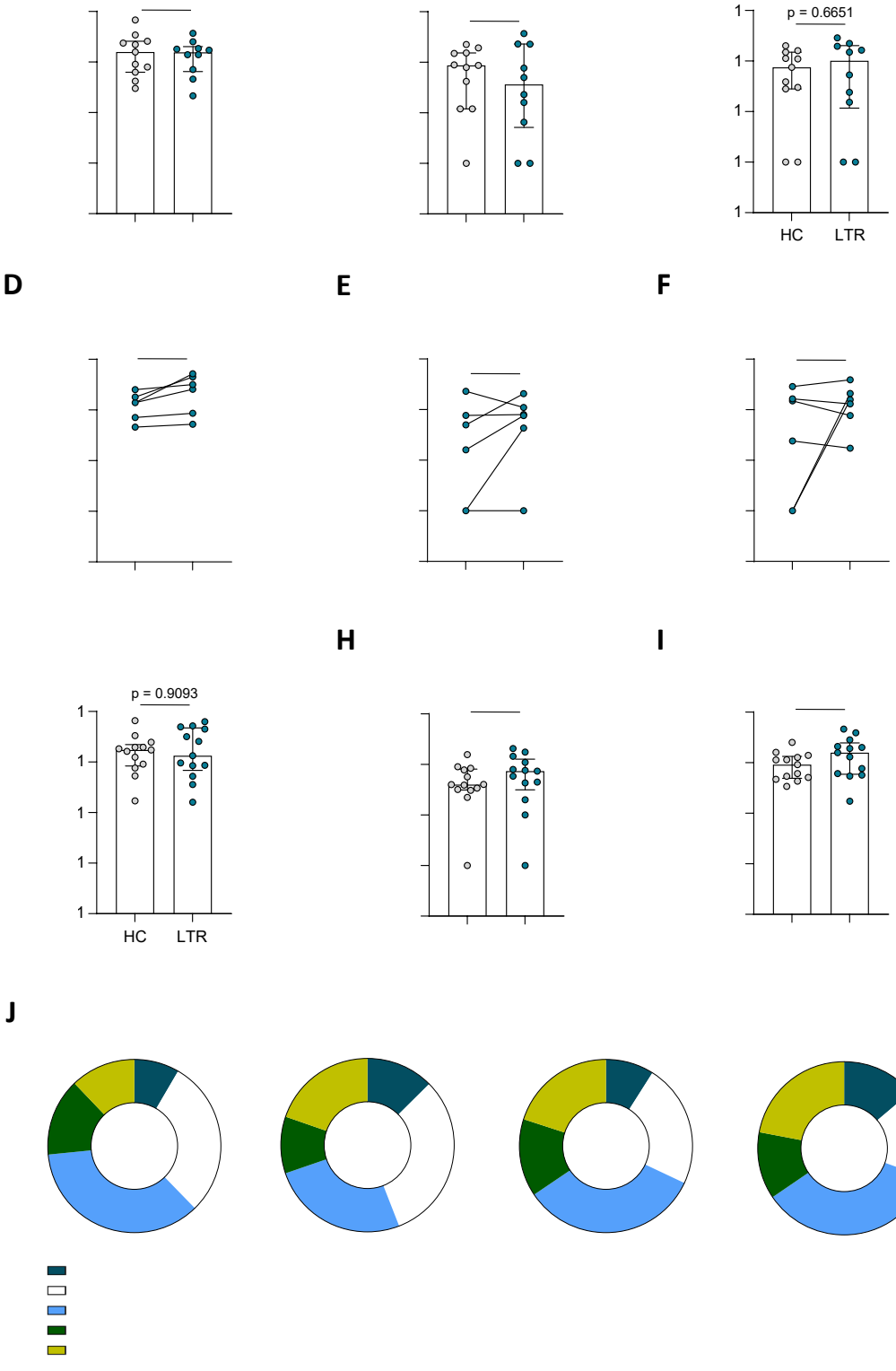

Fig. S9: Subset distribution of spike-specific CD4$^+$ T cells in HC and LTR.

(A-C) Frequencies of spike-specific T_H1 (A), T_H17 (B) and T_H1-like T_H17 (C) cells in HC (n=11) and LTR (n=10) after the 2nd vaccine dose. **(D-F)** Longitudinal development of the frequencies of spike-specific T_H1, T_H17 and T_H1-like T_H17 cells after the 2nd and 3rd vaccine dose in LTR (n=6). **(G-I)** Frequencies of spike-specific T_H1, T_H17 and T_H1-like T_H17 cells in HC (n=13) and LTR (n=13) after the 3rd vaccine dose. **(J)** Subset distribution of spike-specific CD4+ T cells in HC and LTR after the 2nd or 3rd vaccine dose. Statistics: Mann-Whitney test (A-C, G-I) and Wilcoxon matched-pairs signed rank test (D-F). HC: healthy control; LTR: liver transplant recipient.

Figure S10

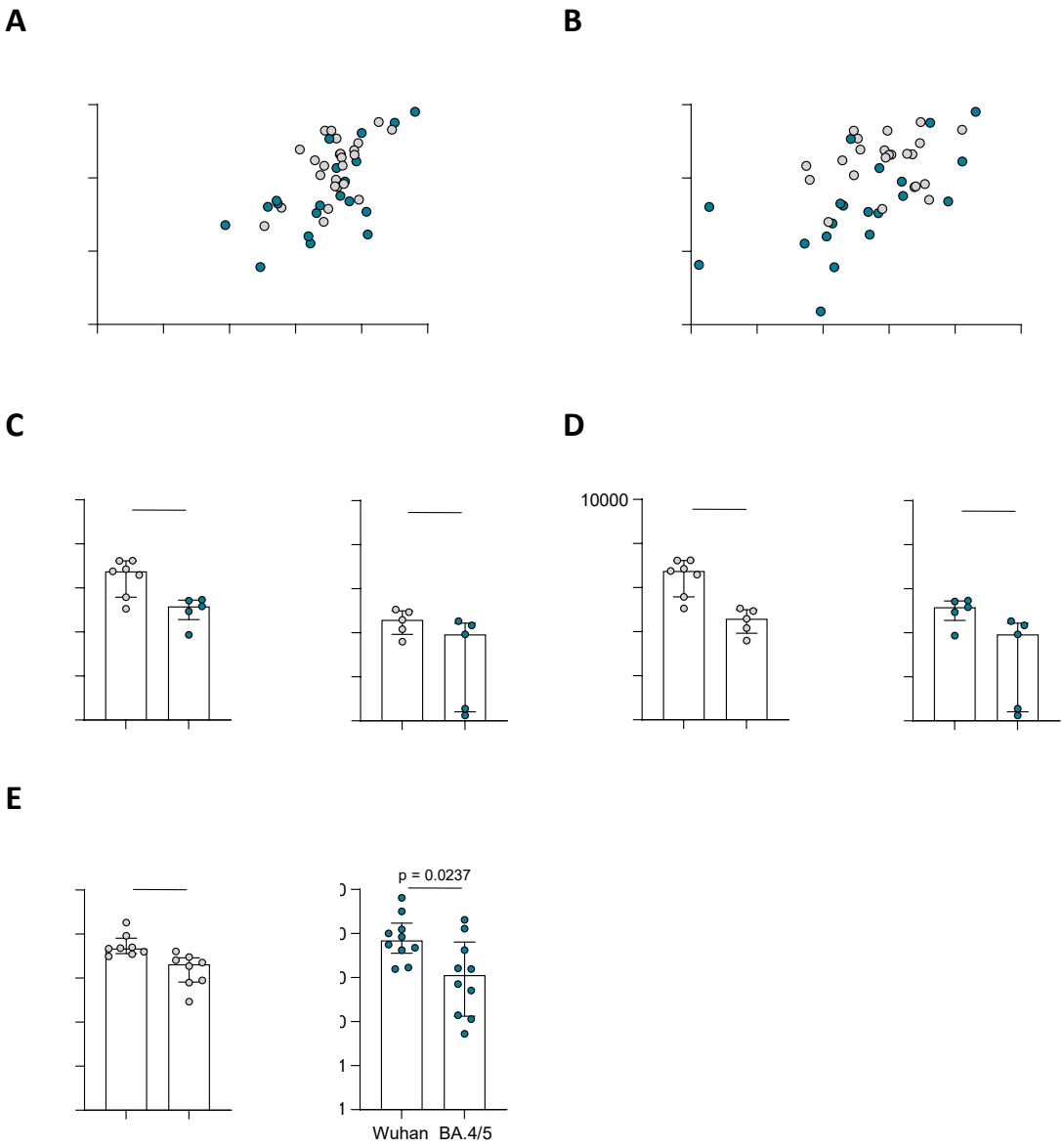

Fig. S10: Neutralizing capacity and spike-specific IgG in HC and LTR.

(A, B) Correlation between level of spike-specific IgG and neutralizing capacity against Wuhan (A) and Omicron BA.4/5 (B) variants. LTR and HC as well as all available vaccination time points were pooled for the correlation analysis. **(C, D)** Neutralizing capacity against SARS-CoV-2 Wuhan variant or Omicron BA.4/5 variants in HC (n=7 or 5, respectively) and LTR (n=5) after the 2nd vaccine dose. **(E)** Neutralizing capacity against SARS-CoV-2 Wuhan variant or Omicron BA.4/5 variant in HC (n=8) and LTR (n=10) after the 3rd vaccine dose. Statistics: Mann-Whitney test (C-E), Spearman correlation (A, B). HC: healthy control; LTR: liver transplant recipient

Table S1: Patient characteristics

Donor ID	Sex	Age	Years since transplantation	Etiology of liver disease	Immuno-suppression	HLA Type	1st vaccine dose	2nd vaccine dose	3rd vaccine dose	4th vaccine dose	OLP	Tested CD8+ T cell epitopes	AIM-Assay	Serum S-IgG1 assay
LTR 1	f	33	2	DILI	Tacrolimus, MMF	A*02:05, A*03:02, B*08:01, B*41:01	BNT162b2	BNT162b2	mRNA-1273	-	yes	-	yes	yes
LTR 2	f	60	9	other	Tacrolimus, MMF	A*02:01, A*24:02, B*07:02, B*15:17	BNT162b2	BNT162b2	BNT162b2	BNT162b2	yes	A*02/S269-277	no	yes
LTR 3	m	67	9	ALD	Ciclosporine, MMF	A*02:01, A*03:01, B*44:02, B*51:01	BNT162b2	BNT162b2	BNT162b2	BNT162b2	yes	A*03/S378-387	no	yes
LTR 4	m	58	4	ALD	Tacrolimus, MMF	A*02:01, A*26:01, B*13:02, B*39:01	BNT162b2	BNT162b2	BNT162b2	-	yes	A*02/S269-277	no	yes
LTR 5	m	66	25	ALD	Tacrolimus, MMF	A*03:01, A*29:02, B*07:02, B*27:05	BNT162b2	BNT162b2	BNT162b2	-	yes	A*03/S378-387	no	yes
LTR 6	m	68	7	viral hepatitis	Ciclosporine, MMF	A*02:02, A*23:01, B*41:01, B*44:03	BNT162b2	BNT162b2	BNT162b2	-	yes	-	no	yes
LTR 7	f	34	1	PSC	Ciclosporine, MMF	A*02:01, A*24:02, B*07:02, B*35:02	BNT162b2	BNT162b2	BNT162b2	-	yes	A*02/S269-277	yes	yes
LTR 8	m	57	1	NASH	Tacrolimus, MMF	A*02:01, A*30:01, B*07:02, B*13:02	BNT162b2	BNT162b2	BNT162b2	-	yes	A*02/S269-277	no	yes
LTR 9	m	61	23	other	Tacrolimus, MMF	A*01:01, A*02:01, B*08:01, B*27:05	BNT162b2	BNT162b2	BNT162b2	-	no	A*02/S269-277	yes	yes
LTR 10	m	39	2	NASH	Ciclosporine	A*03:01, A*11:01, B*07:02, B*35:03	BNT162b2	BNT162b2	BNT162b2	-	no	A*03/S378-387	no	yes
LTR 11	m	71	5	PSC	Tacrolimus, MMF	A*02:01, A*26:01, B*08:01, B*15:01	BNT162b2	BNT162b2	BNT162b2	BNT162b2	yes	A*02/S269-277	no	yes
LTR	m	33	5	PSC	Everolimus,	A*01:01, A*02:01,	BNT162b2	BNT162b2	mRNA-	-		A*02/S269-		

12					Tacrolimus, Prednison	B*07:02, B*51:01			1273		yes	277	yes	yes
LTR 13	f	52	28	other	Ciclosporine	A*03:01, B*35:03	BNT162b2	BNT162b2	mRNA-1273	-	no	A*03/S378-387	no	yes
LTR 14	f	60	14	PSC	Tacrolimus, Everolimus	A*01:01, A*03:01, B*08:01, B*40:01	BNT162b2	BNT162b2	BNT162b2	-	no	A*03/S378-387	no	yes
LTR 15	m	66	2	ALD	Tacrolimus, MMF	A*31:01, A*69:01, B*35:01, B*49:01	mRNA-1273	mRNA-1273	BNT162b2	BNT162b2	yes	-	yes	yes
LTR 16	m	62	8	PSC	Ciclosporin, MMF, Azathioprin	A*01:01, A*24:02, B*07:02, B*08:01	BNT162b2	BNT162b2	BNT162b2	-	no	A*01/S865-873	yes	yes
LTR 17	m	59	6	viral hepatitis	Ciclosporine, MMF	A*23:01, A*26:01, B*18:01, B*49:01	BNT162b2	BNT162b2	BNT162b2	-	no	-	yes	yes
LTR 18	f	77	9	viral hepatitis	Tacrolimus, MMF	A*32:01, B*35:01	BNT162b2	BNT162b2	BNT162b2	-	no	-	yes	yes
LTR 19	m	68	10	viral hepatitis	Tacrolimus, MMF	A*24:02, A*25:01, B*18:01, B*53:01	BNT162b2	BNT162b2	BNT162b2	-	no	-	yes	yes
LTR 20	m	65	4	NASH	Tacrolimus	A*02:01, A*68:01, B*27:05, B*51:01	BNT162b2	BNT162b2	BNT162b2	BNT162b2	no	A*02/S269-277	no	yes
LTR 21	m	72	13	NASH	Tacrolimus, MMF	A*02:01, A*68:01, B*44:02, B*44:03	BNT162b2	BNT162b2	BNT162b2	-	no	A*02/S269-277	yes	yes
LTR 22	m	53	21	SBC	Tacrolimus, MMF	A*02:01, B*41:01, B*51:01	BNT162b2	BNT162b2	BNT162b2	-	no	A*02/S269-277	yes	yes
LTR 23	m	54	1	PSC	Tacrolimus, MMF	A*02:01, B*08:01	BNT162b2	BNT162b2	-	-	no	A*02/S269-277	no	yes
LTR 24	m	35	8	other	Tacrolimus, MMF	A*01:01, A*02:01, B*13:02, B*40:01	BNT162b2	BNT162b2	BNT162b2	-	no	A*02/S269-277	no	yes
HC1	m	60	-	-	-	A*02:01, A*02:01, B*08:01, B*15:01	BNT162b2	BNT162b2	BNT162b2	BNT162b2	yes	A*02/S269-277	yes	yes
HC2	f	52	-	-	-	A*02:01, A*68:01, B*15:01, B*44:02	BNT162b2	BNT162b2	BNT162b2	-	yes	A*02/S269-277	yes	yes

HC3	m	47	-	-	-	A*02:01, A*24:02, B*27:05, B*51:01	BNT162b2	BNT162b2	BNT162b2	-	yes	A*02/S269-277	yes	yes
HC4	f	44	-	-	-	A*01:01, A*11:01, B*15:17, B*35:01	BNT162b2	BNT162b2	BNT162b2	mRNA-1273	yes	A*01/S865-873	yes	yes
HC5	m	43	-	-	-	A*03:01, A*32:01, B*07:02, B*40:02	BNT162b2	BNT162b2	mRNA-1273	-	yes	A*03/S378-387	no	yes
HC6	m	41	-	-	-	A*01:01, A*03:01, B*08:01, B*35:01	BNT162b2	BNT162b2	mRNA-1273	-	yes	A*03/S378-387	no	yes
HC7	f	35	-	-	-	A*01:01, A*03:01, B*07:02, B*57:01	BNT162b2	BNT162b2	BNT162b2	-	yes	A*03/S378-387	yes	yes
HC8	m	62	-	-	-	A*01:01, A*02:01, B*08:01, B*15:01	BNT162b2	BNT162b2	BNT162b2	-	yes	A*02/S269-277	no	yes
HC9	f	60	-	-	-	A*02:01, B*07:02, B*44:02	BNT162b2	BNT162b2	BNT162b2	-	yes	A*02/S269-277	yes	yes
HC10	m	69	-	-	-	A*02:01, B*13:02, B*15:01	BNT162b2	BNT162b2	mRNA-1273	-	yes	A*02/S269-277	yes	yes
HC11	m	77	-	-	-	A*31:01, A*32:01, B*35:01, B*40:02	BNT162b2	BNT162b2	BNT162b2	-	yes	-	yes	yes
HC12	f	78	-	-	-	A*02:01, A*03:01, B*07:02, B*18:01	BNT162b2	BNT162b2	BNT162b2	-	yes	A*03/S378-387	no	yes
HC13	m	37	-	-	-	A*02:01, A*68:01, B*15:01, B*51:01	BNT162b2	BNT162b2	BNT162b2	mRNA-1273	no	A*02/S269-277	yes	yes
HC14	f	63	-	-	-	A*02:01, A*03:01, B*27:05, B*37:01	BNT162b2	BNT162b2	BNT162b2	-	no	A*02/S269-277	yes	yes
HC15	f	49	-	-	-	A*03:01, A*69:01, B*35:08, B*51:01	BNT162b2	BNT162b2	BNT162b2	-	no	-	no	yes
HC16	f	51	-	-	-	A*03:01, B*07:02, B*27:05	BNT162b2	BNT162b2	-	-	no	A*03/S378-387	yes	yes
HC17	f	45	-	-	-	A*02:01, A*33:03,	BNT162b2	BNT162b2	-	-	no	-	yes	yes

						B*44:03, B*58:01								
HC18	f	27	-	-	-	A*02:01, A*02:02, B*41:01, B*57:01	BNT162b2	BNT162b2	BNT162b2	BNT162b2	no	A*02/S269-277	no	yes
HC19	m	58	-	-	-	A*01:01, A*03:01, B*08:01, B*35:01	BNT162b2	BNT162b2	BNT162b2	mRNA-1273	no	A*03/S378-387	no	yes

m: male; f: female; MMF: mycophenolate mofetil; S-IgG: spike-specific immunoglobulin G; DILI: Drug-induced liver injury; ALD: alcoholic liver disease; NASH: nonalcoholic steatohepatitis; PSC: primary sclerosing cholangitis; SBC: secondary biliary cholangitis; LTR: liver transplant recipient; HC: healthy control