Short-lived booster effect and stable CD8+ T cell memory after 3rd COVID-19 vaccine dose

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Abstract

Reports of waning immunity after COVID-19 vaccination (1-3) have recently led to large booster vaccination campaigns. Previous studies showed that basic immunization with two mRNA vaccine doses elicits a robust spike-specific CD8+ T cell response (4-6). The effect of mRNA booster vaccination on the spike-specific CD8+ T cell response remains, however, unclear. Indeed, very little is known about the efficacy, duration and effects on long-term immunity and recall responses in breakthrough infections. In this study, we show that spike-specific CD8+ T cells are immediately and vigorously activated and expanded in all tested individuals after the 3rd and 4th mRNA vaccine shots. However, this CD8+ T cell boost response is characterized by a steep contraction and lasts only for about 30-60 days compared to a prolonged contraction after natural infection. Booster vaccination did not affect long-term spike-specific CD8+ T cell immunity reflected by a stable stem cell memory pool that already reached maximum frequencies after basic immunization. Accordingly, rapid and full-fledged recall responses of boosted spike-specific CD8+ T cells were detectable after breakthrough infection with delta and omicron. Thus, in addition to the previously reported cross-reactivity (7-12) also a robust activation and effector response determines the efficacy of the CD8+ T cell response targeting emerging variants of concern. Neutralizing antibody responses displayed hardly any boost effect towards omicron, further highlighting the relevance of spike-specific CD8+ T cell immunity. In sum, these data will inform future vaccination strategies facing the next COVID-19 wave expected for late 2022/early 2023.

Introduction

More than two years after the outbreak of the SARS-CoV-2 pandemic, constantly emerging variants of concern (VOCs) still fuel the pandemic situation and vaccination is considered to be an important measure to accelerate the transition from the pandemic into the endemic stage[13]. mRNA vaccines against SARS-CoV-2 have been proven to rapidly induce protection against symptomatic COVID-19 and death by inducing robust antibody and T cell responses[4-6,14]. However, reports of waning humoral immunity[1-3] and high SARS-CoV-2 infection incidences with the VOC omicron (B.1.1.529) have recently led to large booster vaccination campaigns. Immediate beneficial effects of these booster immunizations were detectable with respect to the neutralization capacity of the humoral response[15]. The effect of mRNA booster vaccination on the spike-specific CD8+ T cell response remains, however, unclear. Indeed, there is little information available about the efficacy, duration and effects on long-term immunity and recall responses in breakthrough infections.

To address these important questions, we longitudinally traced and deeply profiled SARS-CoV-2 mRNA vaccine-boosted spike-specific CD8+ T cell responses on a single epitope level in vaccinees starting at baseline of prime vaccination until 4 months after 3rd or even 1-2 months after 4th vaccination. Additionally, we analyzed the spike-specific CD8+ T cell response in breakthrough infections with delta (B.1.617.2) and omicron after booster immunization. Moreover, we compared the kinetics of the spike-specific CD8+ T cell response with the neutralizing capacity of the humoral response. Our data show that

after the 3rd mRNA vaccine dose there is a substantial but short-lived booster effect on spike-specific CD8+ T cells without marked effect on the memory response and a minor boost effect on the neutralizing antibody response against omicron. These observations may advise future booster vaccination campaigns.

Results

Transient spike-specific CD8+ T cell booster responses

To assess the longevity of boosted adaptive effector responses, we longitudinally collected sera and peripheral blood mononuclear cells (PBMCs) from 13 volunteers (Supplementary Table 1) before and after 3rd mRNA vaccination with Comirnaty/Pfizer-Biontech or Spikevax/Moderna (Extended Data Fig. 1a). All volunteers included in this cohort did not have a history of SARS-CoV-2 infection and received their vaccinations with a three-week interval between 1st and 2nd and six-to-nine-months interval between 2nd and 3rd dose. First, we analyzed the kinetics of the spike-specific CD8+ T cell response targeting the A*01/S_{865} (n=7) or A*02/S_{269} epitope (n=8). A*01/S_{865}- and A*02/S_{269}-specific CD8+ T cells are dominant within the overall spike-specific CD8+ T cell response after mRNA vaccination[5,10] and target epitopes that are highly conserved in all VOCs including the omicron variants BA.1 and BA.2 (Extended Data Fig. 1b) but not in common cold coronaviruses[5]. To increase the detection sensitivity of spike-specific CD8+ T cells targeting single epitopes, we performed peptide-loaded MHC-class I tetramer-based enrichment[5,16]. Spike-specific CD8+ T cell frequencies had already reached their set point approx. 60 days after the 2nd dose and were rapidly boosted with peak expansion one week after the 3rd dose in all tested donors (Fig. 1a). Robust boosting of the spike-specific CD8+ T cell response by the 3rd dose was also reflected by high CD38, Ki-67 and T-BET expression and thus by rapid activation, strong proliferation and substantial induction of an effector cell program (Fig. 1b and Extended Data Fig. 1c). However, within 30-60 days the circulating spike-specific CD8+ T cell frequency, activation, proliferation and effector cell differentiation decreased rapidly to a similar level as before the 3rd dose (Fig. 1a/b and Extended Data Fig. 1c), thus following the same kinetics as after the 2nd dose. Similarities between the spike-specific CD8+ T cell response after the 2nd and 3rd dose, respectively, are also supported by t-distributed stochastic neighborhood embedding (t-SNE) and pseudotemporal diffusion analysis of T cell phenotypes including CD38, T-BET, BCL-2, PD-1, and TOX expression (Fig. 1c and Ext. Data Fig. 1d/e). Phenotypic differences (e.g., in T-BET and TOX expression) were detectable between the spike-specific CD8+ T cell response after the 1st dose compared to the 2nd and 3rd doses and only minor differences were apparent with respect to the targeted epitopes A*01/S_{865} or A*02/S_{269} (Fig. 1c and Extended Data Fig. 1e/f). With respect to the neutralizing antibody response, boosting efficacy clearly depended on the VOC (Fig. 1d and Extended Data Fig. 1g; $p<0.0001$). In particular, neutralization was only slightly increased against the omicron variant BA.1 (B.1.1.529) by the 3rd dose while neutralization of the parental B.1 strain and delta (B.1.617.2) peaked after approx. 3 weeks followed by a decrease to baseline neutralization after 4 months (Fig. 1d). Of note, after the 4th dose similar kinetics (Fig. 1e) and dynamics (Extended Data Fig.

2a-c) of spike-specific CD8+ T cell and neutralizing antibody responses were detectable. Hence, after booster vaccination, there is a transient mRNA vaccine-associated booster response of spike-specific CD8+ T cells and neutralizing antibodies of about 2 and 4 months, respectively.

Stable memory T cell response irrespective of booster immunization

To investigate the effect of booster immunization on long-term spike-specific CD8+ T cell immunity, we comparatively analyzed the spike-specific CD8+ T cell response after three and nine months post 2nd dose versus three months after the 3rd dose. The memory T cell subset distribution (Fig. 2a and Ext. Data Fig. 3a/b) and activation/differentiation footprint (Fig. 2b and Ext. Data Fig. 3c) of spike-specific CD8+ T cells were similar at the tested time points, indicating stable long-term memory features without a clear effect of the 3rd dose. To more precisely quantify and characterize the T cell memory compartment, we analyzed on the one hand spike-specific CD8+ T cells highly expressing BCL-2 (BCL-2^{hi}) to map the overall memory pool irrespective of certain subsets and on the other hand focused on stem cell memory T cells (T_{SCM}, defined by expression of CD45RA, CCR7, CD27, CD28 and CD95; Ext. Data Fig. 3a) that are essential for long-term T cell immunity[17]. Spike-specific BCL2^{hi} CD8+ T cells and CD8+ T_{SCM} cells were induced after the 1st dose and their frequencies remained stable after the 2nd dose throughout the subsequent vaccination (Fig. 2c/d). t-SNE analysis revealed that spike-specific BCL-2^{hi} CD8+ T cells showed minor phenotypic differences such as higher T-BET expression 3 months post 2nd and 3rd dose compared to 9 months after the 2nd dose (Fig. 2e and Ext. Data Fig. 3d) probably reflecting a more resting state at later time points post immunization. In contrast, phenotypic characteristics of CD8+ T_{SCM} remained completely stable, indicated by a complete intermingling within the t-SNE analysis (Fig. 2f). Furthermore, the spike-specific CD8+ T cell memory pool was also conserved during and after the 4th dose (Ext. Data Fig. 4a-c). Thus, these results indicate that booster immunizations give rise to a rapid effector T cell response that is based on a stable memory T cell pool as illustrated by a subset of spike-specific CD8+ T cell expressing high levels of BCL-2 in parallel to T-BET^{hi}, PD-1^{hi}, TOX^{+} subsets at peak expansion after booster immunization (Ext. Data Fig. 2a).

Conserved spike-specific CD8+ T cell recall capacity

Next, we analyzed the recall capacity of spike-specific CD8+ T cells after the 3rd dose. For this, we stained for TCF-1 expression, a transcription factor indicative of the proliferative capacity of T cells[18]. The frequencies of TCF-1^{+} spike-specific CD8+ T cells were stable suggesting a conserved pool of proliferative competent T cells throughout the 2nd and 3rd dose (Fig. 3a). Accordingly, the peptide-specific *in vitro* expansion capacity of spike-specific CD8+ T cells was also conserved before and after the 3rd dose (Fig. 3b and Ext. Data Fig. 5a/b). Furthermore, after two weeks of expansion, spike-specific CD8+ T cells produced similar amounts of IFNg and TNF, including co-production irrespective of whether the input cells were obtained before or after the 3rd dose (Fig. 3c and Ext. Data Fig. 5b-d). Spike-specific production of IFNg and TNF were assessed in relation to the frequency of spike-specific CD8+ T cells after expansion as a measure of the effector function per cell. In order to estimate the cytotoxic potential of spike-specific

CD8+ T cells after *in vitro* expansion, we analyzed degranulation as indicated by CD107a expression in relation to the frequency of spike-specific CD8+ T cells (Fig. 3d and Ext. Data Fig. 5e) and Granzyme B (Fig. 3e) and Perforin (Fig. 3f) expression of spike-specific CD8+ T cells. Similar to cytokine production, the cytotoxic potential was also largely conserved with almost all spike-specific CD8+ T cells expressing Granzyme B and Perforin. Thus, recall capacity tested *in vitro* comprising expansion capacity, differentiation to cytokine-producing and cytotoxic effector cells is robust and stable before and after 3rd vaccination.

Recall response in breakthrough infections

To determine the recall capacity *in vivo* and to compare booster responses after vaccination versus infection, we next analyzed the spike-specific CD8+ T cell response and the neutralizing antibody capacity in breakthrough infections. Specifically, we compared the adaptive effector immune response after the 4th antigen contact, either by the 4th vaccine dose (n=5) or by breakthrough infection with omicron (n=12) or delta (n=2) after three mRNA vaccine doses. The spike-specific CD8+ T cell response was rapidly and robustly induced and similar after the 4th vaccine dose versus an omicron or delta breakthrough infection (Fig. 4a). Spike-specific CD8+ T cells peaked in a classical effector cell response with pronounced expression of CD38, Ki-67 and T-BET (Fig. 4b and Ext. Data Fig. 6a-c). Variations within the proportion of spike-specific CD8+ T cells expressing these markers may be due to differences in infection time-point or time point of symptom onset, which is especially relevant in breakthrough infections with omicron that are often characterized by mild symptoms in vaccinated individuals. However, within one to two months after breakthrough infection and 4th vaccine dose, a fully functional early T cell memory was present with similar reactivation capacities comprising expansion, cytokine production and degranulation (Fig. 4c and Ext. Data Fig. 6d). Furthermore, phenotypic characteristics of early spike-specific memory CD8+ T cells were similar after breakthrough infections and 4th vaccine dose as depicted by t-SNE analysis including CD38, CCR7, TCF-1 and BCL-2 (Fig. 4d) and memory subset distribution with transitional and effector memory subsets being dominant (Ext. Data Fig. 6e). Again, the frequencies of the BCL-2^{hi} memory pool within the spike-specific CD8+ T cells was stable before and after the 4th antigen contact irrespective of breakthrough infection or 4th vaccine dose (Fig. 4e). Hence, vaccine-elicited spike-specific CD8+ T cell immunity exhibited a substantial recall capacity *in vivo* even towards VOCs such as omicron. However, the neutralizing antibody response after the 4th antigen contact differed from the spike-specific CD8+ T cells in the following aspects (Fig. 4f): First, neutralization capacity depended on the targeted SARS-CoV-2 variant regardless of whether the 4th antigen exposure was through vaccination or breakthrough infection. Second, the neutralizing antibody response depended on the 4th antigen contact with differences in vaccination and breakthrough infections. Third, the neutralizing antibody response exhibited a prolonged decay after breakthrough infections compared to vaccination. Thus, in contrast to spike-specific CD8+ T cells, recall of the neutralizing antibody response was less efficient with respect to VOCs.

Discussion

Here, we mapped the dynamics of mRNA vaccine- versus SARS-CoV-2 infection-boosted spike-specific adaptive effector immunity. By analyzing the dominant A*01/S_{865}- and A*02/S_{269}-specific CD8+ T cells responses, we observed a rapid and robust expansion of spike-specific CD8+ T cells with a similar slope and amplitude after the 3rd and 4th vaccine dose versus breakthrough infections with SARS-CoV-2 delta and omicron variants. Notably, kinetics and magnitude were comparable to the spike-specific CD8+ T cell response after the 2nd vaccine dose with a remarkably high recruitment of spike-specific CD8+ T cells to the effector pool that is reflected by high expression of activation and proliferation markers, like CD38 and Ki-67. This observation highlights the effectiveness of mRNA vaccination to elicit rapid and functional CD8+ T cell responses. Thus, given the important role of CD8+ T cells to control SARS-CoV-2 infection and protect from severe COVID-19[19-22], mRNA booster vaccination represents a valuable tool to immediately react to high viral burden in the population in order to e.g., protect vulnerable groups and to reduce the risk of overwhelming the public health system.

Importantly, however, the spike-specific CD8+ T cell booster responses after 3rd and 4th mRNA vaccine only lasted approximately 1-2 months until it decreased to a similar level as prior booster vaccination. This rapid contraction of the spike-specific CD8+ T cell response has to be taken into account when booster vaccination strategies are planned especially for risk groups but most likely also for broader populations this fall. Furthermore, the steep decline of the spike-specific CD8+ T cell boost response is in stark contrast to a more prolonged contraction evident after SARS-CoV-2 breakthrough infection irrespective of the infecting variant. This prolonged contraction has also been reported for non-spike epitope-specific CD8+ T cells after SARS-CoV-2 infection[16] indicating a more general difference in virus-specific CD8+ T cells induced by SARS-CoV-2 infection versus mRNA vaccination. Possible explanations include differences in antigen half-life, innate immunity, co-stimulation, cytokines, antigen presentation and CD4+ T cell responses that are all required for coordinating CD8+ T cell responses[6,20,21] after mRNA vaccination versus SARS-CoV-2 infection. Hence, a better understanding of the interplay between the different immune components in viral infection versus vaccination is required to adapt mRNA vaccines prolonging antigen-specific CD8+ T cell booster responses in the context of SARS-CoV-2 but also of other targets.

A durable and functionally competent spike-specific memory CD8+ T cell response was already detectable after the 2nd dose of mRNA vaccine and thus after completed basic immunization[5,6,8]. Here, we now show that this well-established durable spike-specific CD8+ T cell memory response is not tremendously affected by the 3rd dose. In particular, a pool of T_{SCM} cells that have been reported to maintain long-term CD8+ T cell immunity after yellow fever virus (YFV) vaccination[17,23] and that were reported to be detectable after basic immunization with SARS-CoV-2 mRNA vaccination[24] were already induced within spike-specific CD8+ T cells after prime vaccination. The spike-specific CD8+ T_{SCM} cell pool reached final size after the 2nd dose and remained constant throughout the 3rd and 4th dose. Thus, 3rd mRNA vaccinations do not amplify long-term CD8+ T cell immunity and also do not drive the CD8+ T cell memory pool into relevant senescence. This is in line with a recent report showing that repeated antigen

exposure does also not induce T cell exhaustion of spike-specific CD8+ T cells[25]. It rather appears that the spike-specific CD8+ T cell booster response is an effector response based on a stable memory pool. Importantly, spike-specific memory CD8+ T cells were capable to efficiently mount recall responses *in vitro* and *in vivo* after breakthrough infections with delta or omicron. This recall efficiency is explained by the previously described cross-reactivity of the vaccine-elicited spike-specific CD8+ T cell response[7-12], diversification of the T cell repertoire[25] and of the rapid kinetics and effector differentiation dynamics shown in this study. It is, however, important to note that our study is limited to healthy, rather young individuals without higher risk to develop severe COVID-19[21] and with the capability to establish robust vaccine-elicited immune responses[4-6,8,14]. Future studies have to show whether individuals above the age of 60 develop comparable immunity after mRNA vaccination. Still, our observations challenge the discussion about the necessity to frequently apply mRNA booster vaccination to healthy individuals that are not compromised in their immune response in three-to-six-months intervals.

In contrast to spike-specific CD8+ T cells, boosting the neutralizing antibody response by currently applied, non-adapted mRNA vaccines clearly depends on the targeted SARS-CoV-2 variant. In particular, neutralizing capacity against omicron is hardly increased. Of note, neutralizing capacity against omicron is also only moderately increased after breakthrough infections with omicron probably reflecting antibody escape. Similar to the spike-specific CD8+ T cell response, neutralizing capacity declines slower after infection versus vaccination. Yet, it appears that neither after vaccination nor after infection the neutralization capacity of the humoral response is sufficient to protect from omicron infection as indicated by the high numbers of breakthrough infections in vaccinees and convalescents. In sum, our study highlights that mRNA vaccines are potent inducers of a robust, functionally competent and durable spike-specific CD8+ T cell immunity already after completed basic immunization. This contributes to protection from symptomatic disease that is especially relevant for at-risk individuals and has also important implications for vaccine development targeting other CD8+ T cell-controlled infectious agents and cancers.

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Declarations

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Author contributions

M.R., H.L., V.K., A.G., S.G., K.C. and D.R. planned, performed and analyzed experiments, interpreted data and contributed to writing the manuscript with the help of V.O., K.H., N.G. and C.B.. M.R., H.L., V.K., A.G.,

S.G., K.C. and D.R. contributed equally to this work. H.L., A.G., N.R., J.L.-M., S.R., C.S., S.B. and D.S. were responsible for donor recruitment. F.E. performed four-digit HLA-typing by next generation sequencing. B.B. and T.B. contributed to data interpretation. M.S. and G.K. provided virological expertise, supervised virological work and interpreted data. R.T., C.N.-H. and M.H. designed and supervised the study, interpreted data, wrote the manuscript and contributed to experimental design and planning. M.H., C.N.-H. and R.T. are shared last authors.

Declaration of interest

The authors have nothing to declare.

Online Methods

Study cohort

In total, 38 individuals receiving SARS-CoV-2 vaccinations were recruited at the Freiburg University Medical Center, Germany. Of those, blood was collected from 31 individuals vaccinated three times with the mRNA vaccines bnt162b/Comirnaty or mRNA-1273/Spikevax, with 5 individuals receiving a fourth vaccination, without a SARS-CoV-2 infection history and 13 individuals with SARS-CoV-2 breakthrough infections after a third mRNA vaccination. Breakthrough infections were confirmed by positive PCR-testing from oropharyngeal swab. All 13 individuals with breakthrough infections included in this study had mild symptoms without respiratory insufficiency (according to WHO guidelines[1]). Characteristics of the participants are summarized in Supplementary Table 1 including the results of the HLA-genotyping performed by next-generation sequencing.

Ethics

Written informed consent was obtained from all study participants. The study was conducted in accordance to federal guidelines, local ethics committee regulations (Albert-Ludwigs-Universität, Freiburg, Germany; vote: 322/20, 21-1135 and 315/20) and the Declaration of Helsinki (1975).

PBMC isolation

PBMCs were isolated from venous blood samples collected in EDTA blood collection tubes by density centrifugation with lymphocyte separation medium (Pancoll separation medium, PAN Biotech GmbH). PBMCs were stored at -80°C until further processing. The cells were thawed in pre-warmed RPMI cell culture medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1.5% 1M HEPES (all purchased from Thermo Scientific) and 50 U/mL Benzonase (Sigma).

Sequence alignment

Sequence homology was analysed in Geneious® 11.0.5 (https://www.geneious.com/) using Clustal Omega 1.2.2 alignment with default settings[2]. Reference genome of human ancestral SARS-CoV-2

(MN908947.3) was obtained from NCBI database. Genome sequences of SARS-CoV-2 variants of concern (VOCs) B.1, B.1.1.7, B.1.351, P.1, B.1.617.2, B.1.1.529 BA.1 and B.1.1.529 BA.2 were identified via CoVariants (https://covariants.org/). Spike epitopes in ancestral strain and all VOCs were aligned according to their homology on an amino acid level.

Peptides and tetramers for T cell analysis

Peptides were manufactured with an unmodified N-terminus and an amidated C-terminus with standard Fmoc chemistry (Genaxxon Bioscience). All peptides showed a purity of >70%. To generate tetramers, SARS-CoV-2 spike peptides (A*01/S_{865}: LTDEMIAQY, A*02/S_{269}: YLQPRTFLL) were loaded on biotinylated HLA class I (HLA-I) easYmer® (immunAware) according to manufacturer's instructions. Subsequently, peptide-loaded-HLA class I monomers were tetramerized with phycoerythrin (PE)-conjugated streptavidin according to the manufacturer's instructions.

In vitro expansion of spike-specific CD8+ T cells and assessment of effector function

1.5 x 10^6 PBMCs were stimulated with the spike protein-derived peptides A*01/S_{865} or A*02/S_{269} and anti-CD28 monoclonal antibody (0.5µg/mL) for 14 days in RPMI cell culture medium supplemented with rIL-2 (20 IU/ml, StemCell Technologies). At day 4, 7 and 11, 50% of the culture medium was exchanged with freshly prepared medium containing 20 IU/mL rIL-2. After 14 days, PBMCs were stimulated with peptides again, and stained for CD107a for 1 h at 37 °C to analyse degranulation. Subsequently, brefeldin A (GolgiPlug, 0.5 µl/mL) and monensin (GolgiStop, 0.5 µl/mL) (all BD Biosciences) were added and incubation continued for four more hours, followed by surface and intracellular staining with anti-IFNy, anti-TNF and anti-IL-2-specific antibodies. For calculation of the expansion capacity and to assess the cytotoxic capacity of the expanded cells, peptide-loaded HLA class I tetramer staining was performed together with intracellular staining of Granzyme B, Granzyme K, Perforin and Granulysin.

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

CD8+ T cells targeting spike epitopes were enriched as described previously[3]. In brief, 5 x 10^6 to 20 x 10^6 PBMCs were stained with PE-coupled peptide-loaded HLA class I tetramers for 30 min at room temperature followed by incubation with magnetic anti-PE microbeads. Subsequent positive selection of magnetically labelled cells was achieved by using MACS technology (Miltenyi Biotec) according to the manufacturer's protocol. The enriched spike-specific CD8+ T cells were analysed using multicolor flow cytometry. Cell frequencies were calculated as previously described[3]. Of note, only samples with ≥ 5 virus-specific CD8+ T cells were included in subsequent analyses. Accordingly, the detection limit of virus-specific CD8+ T cells in this study was: 0.25 – 1 x 10^{-6}, depending on the initial cell input.

Multiparametric flow cytometry for T cell analysis

The following antibodies were used for multiparametric flow cytometry: anti-CCR7-PE-CF594 (150503, 1:50), anti-CCR7-BV786 (G034H7, 1:50), anti-CD4-BV786 (L200, 1:200), anti-CD8-BUV395 (RPA-T8, 1:400),

anti-CD8-BV510 (SK1, 1:100), anti-CD8-BV421 (RPA-T8, 1:200), anti-CD11a-BV510 (HI111, 1:25), anti-CD28-BV421 (CD28.2, 1:100), anti-CD38-APC-R700 (HIT2, 1:400), anti-CD39-BV650 (TU66, 33:1), anti-CD45RA-BUV496 (HI100, 1:800), anti-CD45RA-BUV737 (HI100, 1:800), anti-CD107a-APC (H4A3, 1:100), anti-CD127-BUV737 (HIL-7R-M21, 1:50), anti-EOMES-PerCP-eF710 (WD1928, 1:10), anti-Granzyme B-PE-CF594 (GB11, 1:800), anti-Granzyme B-BV510 (GB11, 1:50), anti-IFN-γ-FITC (25723.11, 1:8), anti-PD-1-BV605 (EH12.1, 1:50), anti-PD-1-PE-Cy7 (EH12.2H7, 1:200), anti-TNF-PE-Cy7 (Mab11, 1:400), anti-HLA-DR-BUV395 (G46-6, 1:200), anti-CD137-BV650 (4B4-1, 1:100), anti-CD95-APC (DX2, 1:100) (BD Biosciences), anti-BCL-2-BV421 (100, 1:200), anti-CCR7-BV785 (G043H7, 1:50), anti-CD57-BV605 (QA17A04, 1:200), anti-CXCR3-PerCP-Cy5.5 (G025H7, 1:33), anti-IL-2-PerCP-Cy5.5 (MQ1-17H12, 1:100), anti-Ki-67-BV711 (CD28.2, 1:200), anti-Perforin-PerCP-Cy5.5 (B-D48, 1:200), anti-Granzyme K-PE-Cy7 (GM26E7, 1:25), anti-Granulysin-APC (DH2, 1:100) (BioLegend), anti-TCF-1-AlexaFluor488 (C63D9, 1:100) (Cell Signaling), anti-CD14-APC-eFluor780 (61D3, 1:400), anti-CD19-APC-eFluor780 (HIB19, 1:400), anti-CD27-FITC (O323, 1:100), anti-KLRG1-BV711 (13F12F2, 1:50), anti-T-BET-PE-Cy7 (4B10, 1:200), anti-TOX-eFluor660 (TRX10, 1:100) (Thermo Fisher). To discriminate live cells from dead cells, fixable Viability Dye (APC-eFluor780, 1:400) from Thermo Fisher or 7-AAD from BD Biosciences (ViaProbe, 1:33) was used. To facilitate staining of intranuclear and cytoplasmic targets, FoxP3/Transcription Factor Staining Buffer Set (Thermo Fisher) and Fixation/Permeabilization Solution Kit (BD Biosciences) were used, respectively. Finally, cells were fixed in 2% paraformaldehyde (Sigma) and samples were analysed on FACSCanto II or LSRFortessa with FACSDiva software version 10.6.2 (BD), or CytoFLEX (Beckman Coulter) with CytExpert Software version 2.3.0.84. Further analyses of the data were performed using FlowJo version 10.6.2 (Treestar).

Dimensional reduction of multiparametric flow cytometry data

For dimensionality reduction, flow cytometry data was analysed with R version 4.1.1 and the Bioconductor CATALYST package (release 3.13)[4]. Initially, viable and tetramer-positive CD8+ T cells (or subsets of those) were identified using FlowJo 10 in two separate multiparametric flow cytometry panels (activation panel: HLA-DR, BCL-2, PD-1, CD137, Ki67, TCF-1, EOMES, T-BET, TOX, CD38, CD45RA, CCR7; differentiation panel: CD45RA, CCR7, CD27, CD28, CD127, CD11a, CD57, CXCR3, CD95, CD57, CD39, KLRG1, PD-1). To facilitate visualization of the dimensionality reduction by *t*-SNE and diffusion map analysis, cell counts were sampled down to at least 20 cells per sample, and marker expression intensities were transformed by arcsinh-transformation with a cofactor of 150.

Serum IgG determination

Detection of anti-SARS-CoV-2 spike-IgG was performed using Anti-SARS-CoV-2-QuantiVac-ELISA (IgG) from Euroimmun according to the manufacturer's instructions (anti-SARS-CoV-2 S IgG; <35.2 BAU/mL: negative, ≥ 35.2 BAU/mL: positive).

Neutralization assay

Samples of vaccinated individuals and those with breakthrough infections were tested in a plaque reduction neutralization assay as previously described[5]. In brief, VeroE6 cells were seeded in 12-well

plates at a density of 4×10^5 cells per well. Serum samples were diluted at ratios of 1:16, 1:32, 1:64, 1:128, 1:256, 1:512 and 1:1024 in a total volume of 50 µl PBS. For each sample, a serum-free negative control was included. Diluted sera and negative controls were subsequently mixed with 90 plaque-forming units (PFU) of authentic SARS-CoV-2 (either B.1, B.1.617.2 (delta) and B.1.1.529 BA.1 (omicron)) in 50 µl PBS (1,600 PFU/mL) resulting in final sera dilution ratios of 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048. After incubation at room temperature for 1h, 400 µl PBS was added to each sample and the mixture was subsequently used to infect VeroE6 cells 24 h after seeding. After 1.5 h of incubation at room temperature, inoculum was removed and the cells were overlaid with 0.6% Oxoid-agar in DMEM, 20 mM HEPES (pH 7.4), 0.1% NaHCO3, 1% BSA and 0.01% DEAE-Dextran. Cells were fixed 72 h after infection using 4% formaldehyde for 30 min and stained with 1% crystal violet upon removal of the agar overlay. PFU were counted manually. Plaques counted for serum-treated wells were compared to the average number of plaques in the untreated negative controls, which were set to 100%. Calculation of PRNT50 values was performed using a linear regression model in GraphPad Prism 9 (GraphPad Prism Software).

Statistics

GraphPad Prism software version 9.3.1 was used for statistical analysis. Statistical significance was assessed by Kruskal-Wallis test, one-way ANOVA with mixed-effects model, two-way ANOVA with full model and main model. Statistical analysis was performed for A*01/S_{865} (n=7) and A*02/S_{269} (n=8) longitudinally analysed CD8+ T cell responses in Fig. 1A, 1B, 2C, 3A, 3B and Ext. Data. Fig 1C, 5C, 5D, 5E, for n=28 subjects longitudinally followed in Fig. 1D, for A*01/S_{865} (n=2) and A*02/S_{269} (n=3) T cell responses longitudinally followed in Fig. 1E, for n=26 subjects in Ext. Data. Fig 1G, for n=2 subjects in Ext. Data. Fig 2C, for n=7 at 3 months after 2nd vaccination, n=11 at 9 months after 2nd vaccination and n=11 at 3 months after 3rd vaccination in Fig. 2A and Ext. Data. Fig. 3B, for n=4 at 3 months after 2nd vaccination, n=8 at 9 months after 2nd vaccination and n=10 at 3 months after 3rd vaccination in Ext. Data. Fig. 3D, for A*01/S_{865} (n=7) and A*02/S_{269} (n=6) longitudinally analysed CD8+ T cell responses in Fig. 2D, for n=8 at 3 months after 2nd vaccination, n=12 at 9 months after 2nd vaccination and n=11 at 3 months after 3rd vaccination in Fig. 2B, for n=4 in Ext. Data. Fig. 4A, for A*01/S_{865} (n=2) and A*02/S_{269} (n=2) longitudinally analysed CD8+ T cell responses in Ext. Data. Fig. 4B, for n=10 at 3 months after 2nd vaccination, n=12 at 9 months after 2nd vaccination and n=11 at 3 months after 3rd vaccination in Fig. 3C, for n=10 at 3 months after 2nd vaccination, n=11 at 9 months after 2nd vaccination and n=11 at 3 months after 3rd vaccination in Fig. 3D, for n=6 at 3 months after 2nd vaccination, n=12 at 9 months after 2nd vaccination and n=10 at 3 months after 3rd vaccination in Fig. 3E, for n=6 at 3 months after 2nd vaccination, n=12 at 9 months after 2nd vaccination and n=11 at 3 months after 3rd vaccination in Fig. 3F, for omicron infection n=12, delta infection n=2 and 4th vaccination n=5 longitudinally analysed T-cell responses in Fig. 4A, for omicron infection n=11, delta infection n=2 and 4th vaccination n=4 analysed T cell responses in Fig. 4B and in peak response in Ext. Data. Fig. 6A, for omicron infection n=12, delta infection n=2 and 4th vaccination n=3 longitudinally analysed T cell responses in Fig. 4E, for omicron

infection n=11, delta infection n=1 and 4th vaccination n=3 in Fig. 4F, for omicron infection n=6, delta infection n=2 and 4th vaccination n=4 analysed T cell responses after 1 month in Ext. Data. Fig. 6A and Ext. Data Fig. 6E, for omicron infection n=6, delta infection n=2 and 4th vaccination n=2 analysed T cell responses in Ext. Data. Fig. 6D.

Data availability statement

Source data files will be provided with this manuscript. All requests for additional raw and analyzed data and materials are promptly reviewed by the University of Freiburg Center for Technology Transfer to verify if the request is subject to any intellectual property or confidentiality obligations. Patient-related data not included in the paper were generated as part of clinical examination and may be subject to patient confidentiality. Any data and materials that can be shared will be released via a Material Transfer Agreement.

Code availability statement

R code to reproduce the analyses of multiparametric flow-cytometry data is available at https://github.com/sagar161286/SARSCoV2_specific_CD8_Tcells.

Online references

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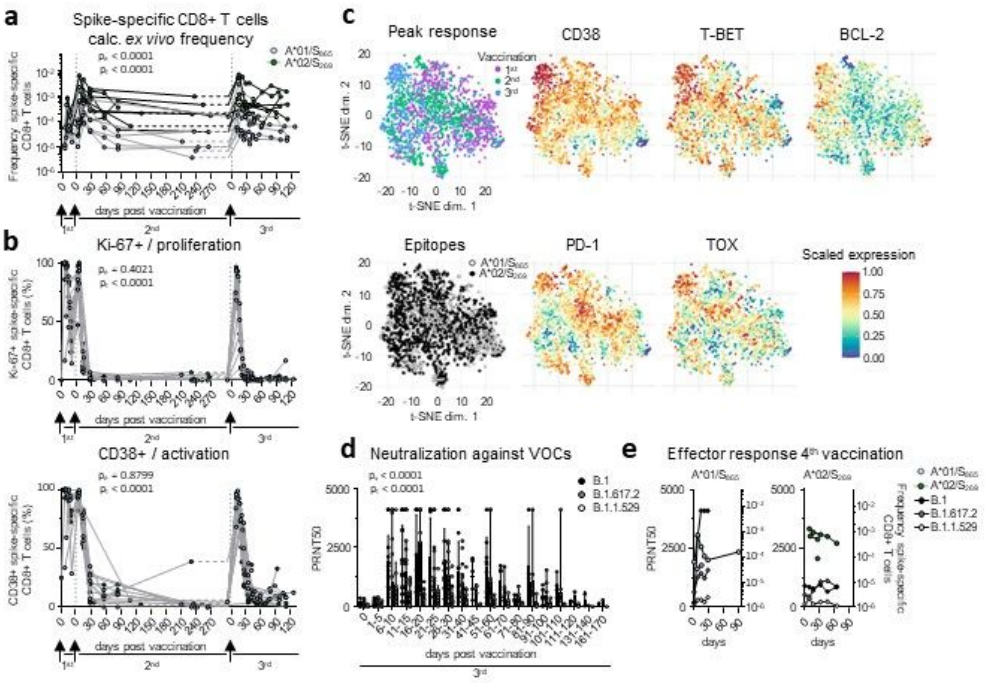
4 Crowell, H., Zanotelli, V., Chevrier, S. & Robinson, M. CATALYST: Cytometry dATa anALYSis Tools. R package version 1.16.2. (2021).

5 Oberhardt, V. *et al.* Rapid and stable mobilization of CD8+ T cells by SARS-CoV-2 mRNA vaccine. *Nature* **597**, 268-273, doi:10.1038/s41586-021-03841-4 (2021).

Figures

Figure 1



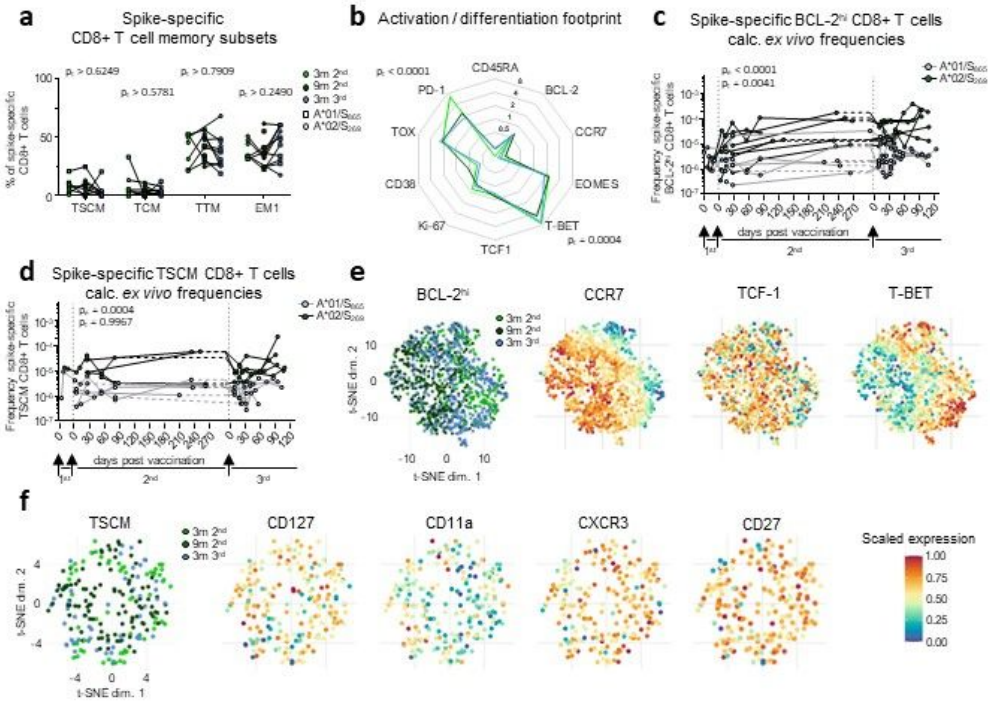
Cellular and humoral effector response after 3rd and 4th vaccine dose

a, Calculated *ex vivo* frequencies of spike-specific CD8+ T cells across 1st, 2nd and 3rd vaccination. **b**, Ki-67 and CD38 expression within spike-specific non-naïve CD8+ T cells. **c**, t-SNE representation of flow cytometry data comparing A*01/S_{865}- and A*02/S_{269}-specific CD8+ T cells at peak response after 1st, 2nd and 3rd vaccination. Expression levels of CD38, T-BET, BCL-2, PD-1 and TOX are depicted for all analyzed cells together. **d**, Antibody neutralization activity as 50% plaque reduction neutralization tests (PRNT50) for SARS-CoV-2 variants B.1, B.1.617.2 and B.1.1.529 after 3rd vaccination. Median values are depicted with 95% confidence interval error bars. **e**, Calculated ex vivo frequencies of A*01/S_{865}- (left) and A*02/S_{269}-specific (right) CD8+ T cells, with antibody neutralization activity after 4th vaccination. Statistical significance was determined by two-way ANOVA with main model (a, b, d) comparing the effects of targeted epitopes (p_e; a, b) or VOCs (p_v; d) and of the time course (p_t). No statistics were calculated for e due to limited sample size.

Figure 1

See image above for figure legend.

Figure 2



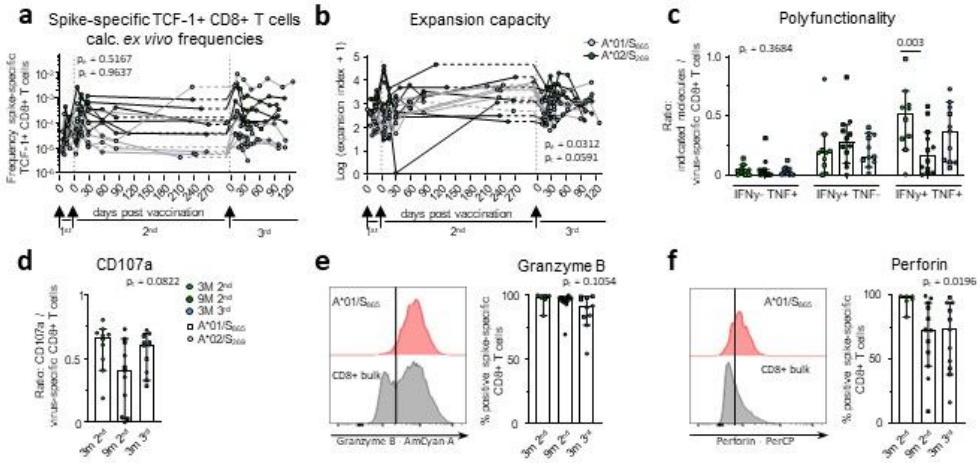
Spike-specific CD8+ memory T cells before and after 3rd dose vaccination

a, Composition of spike-specific CD8+ T cell memory subsets at 3 and 9 months after 2nd, and 3 months after 3rd vaccination. **b**, Normalized molecule expression of spike-specific non-naïve CD8+ T cells at 3 and 9 months after 2nd, and 3 months after 3rd vaccination. **c**-**d**, Calculated *ex vivo* frequencies of BCL-2hi (c) and TSCM (d) spike-specific CD8+ T cells. **e**-**f** t-SNE representation of BCL-2hi (e) and TSCM (f) spike-specific CD8+ T cells at 3 and 9 months after 2nd, and 3 months after 3rd vaccination. Expression levels of CCR7, TCF-1 and T-BET are depicted for BCL-2hi, and CD127, CD11a, CXCR3 and CD27 are depicted for TSCM spike-specific CD8+ T cells. Statistical significance was calculated by two-way ANOVA with full model and Tukey's test for multiple comparison (a, b) to examine the effect of sampling time points (p_t) on memory subsets and marker expression, and two-way ANOVA with main model (c, d) to compare the effects of targeted epitopes (p_e) and time course (p_t).

Figure 2

See image above for figure legend.

Figure 3

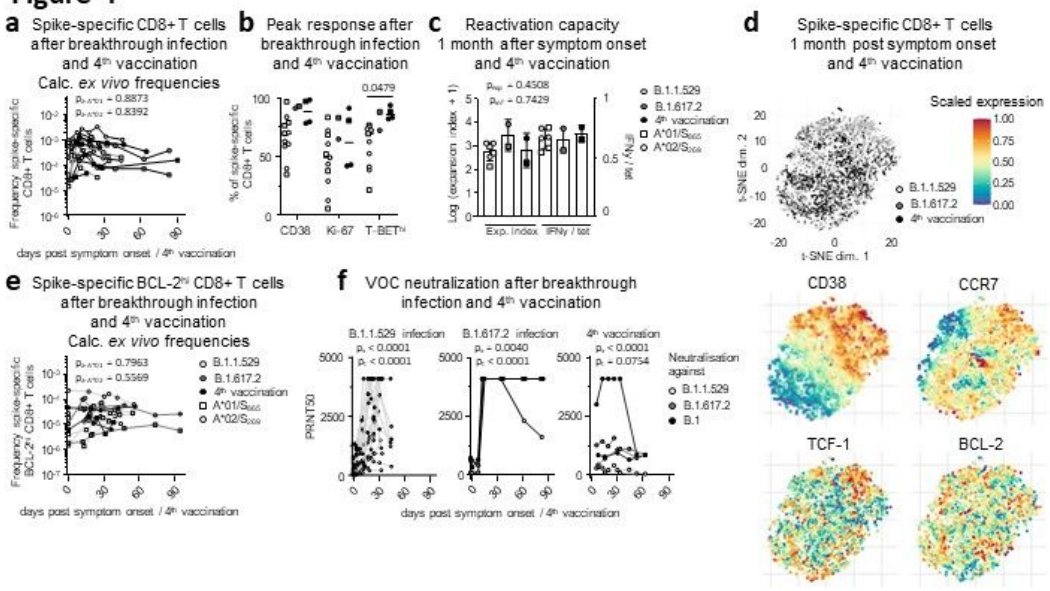


Reactivation capacity of spike-specific CD8+ T cells before and after 3rd vaccine dose

a, Calculated *ex vivo* frequencies of TCF-1+ non-naive spike-specific CD8+ T cells **b**, Expansion capacity of spike-specific CD8+ T cells over 14 days of *in vitro* expansion across 1st, 2nd and 3rd vaccination. **c**-**f**, Percentage of IFNγ and TNF (c), and CD107a (d) producing CD8+ T cells upon peptide stimulation related to the percentage of spike-specific CD8+ T cells from all CD8+ T cells at 3 and 9 months post 2nd, and 3 months post 3rd vaccination after *in vitro* expansion. Median values are depicted with 95% confidence interval error bars. **e**-**f**, Expression of Granzyme B (e) and Perforin (f) of spike-specific CD8+ T cells at 3 and 9 months post 2nd, and 3 months post 3rd vaccination after *in vitro* expansion with representative histograms. Statistical significance was calculated by two-way ANOVA with main model (a, b) to compare the effects of targeted epitopes (p_e) and time course (p_t), two-way ANOVA with full model and Tukey's multiple comparison test (c), and Kruskal-Wallis test (d-f) to examine the effect of sampling time on functionality (p_t).

Figure 3

See image above for figure legend.



Effector and memory CD8+ T cell response after breakthrough infection and 4th dose vaccination

a, Calculated *ex vivo* frequencies of spike-specific CD8+ T cells after breakthrough infection and 4th vaccination. **b,** Proportion of CD38+, Ki-67+ and T-BET^{hi} within non-naive spike-specific CD8+ T cells at peak expansion after breakthrough infection and 4th vaccination. **c**, Reactivation capacity of spike-specific CD8+ T cells 1 month after symptom onset and 4th vaccination. Median values are depicted with 95% confidence interval error bars. **d,** t-SNE representation of spike-specific CD8+ T cells 1 month after symptom onset and 4th vaccination. **e**, Calculated *ex vivo* frequencies of BCL-2^{hi} non-naïve spike-specific CD8+ T cells after breakthrough infection and 4th vaccination. **f,** Antibody neutralization activity as 50% plaque reduction neutralization tests (PRNT50) for SARS-CoV-2 variants B.1, B.1.617.2 and B.1.1.529 after breakthrough infection and 4th vaccination. Statistical significance was calculated by two-way ANOVA with main model (a, e, f) to compare the effects of antigen triggers (p_a) on epitope-specific T cell frequencies (a, e) or the effects of VOCs (p_v) and time course (p_t), two-way ANOVA with full model and Tukey's test for multiple comparison (b) to examine the effects of antigen triggers on activation marker expression, and Kruskal-Wallis test (c; due to limited sample size) to compare the effect of the antigen triggers on expansion (p_{exp}) and interferon production (p_{inf}).

Figure 4

See image above for figure legend.

Supplementary Files

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