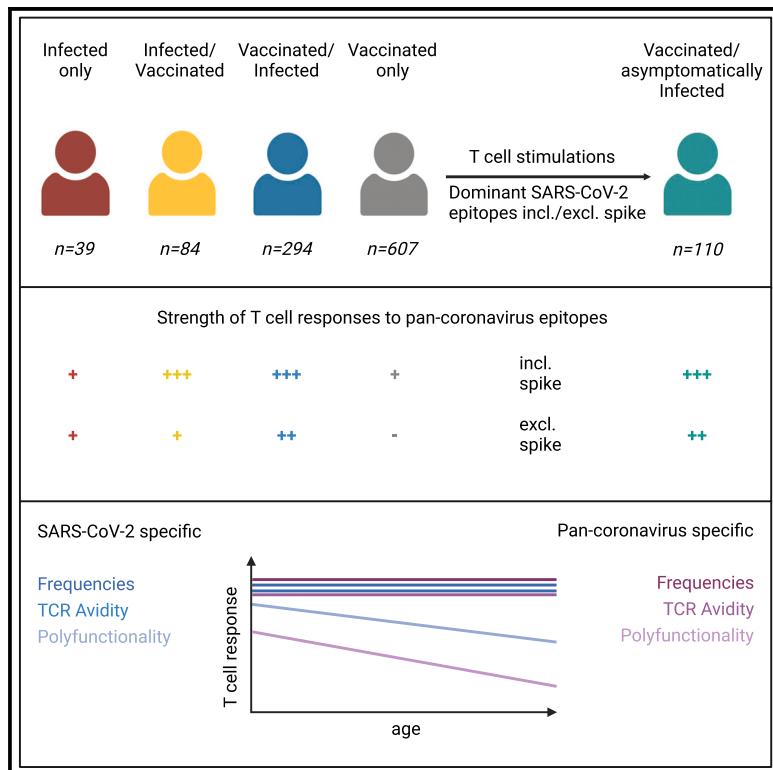


Hybrid immunity-based induction of durable pan-endemic-coronavirus immunity in the elderly

Graphical abstract



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In brief

Loyal et al. show that 30 pan-endemic-coronavirus spike-specific and 31 non-spike epitopes with low mutation rates in SARS-CoV-2 generate strong T cell immunity even in the elderly. Hybrid immunity from infection and vaccination enhances these responses, providing insights for the development of vaccines focusing on T cell and pan-coronavirus immunity.

Highlights

- Low mutation rate in epitopes shared between SARS-CoV-2 and endemic coronaviruses
- Hybrid immunity most effectively boosts pan-endemic-coronavirus-specific immunity
- Targeting 30 epitopes provides effective pan-endemic-coronavirus immunity also in the elderly
- T cell responses identify asymptotically infected individuals lacking NCAP antibodies



Article

Hybrid immunity-based induction of durable pan-endemic-coronavirus immunity in the elderly

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SUMMARY

Repeated vaccinations and infections have led to diverse states of hybrid immunity against SARS-CoV-2 in the global population. However, age and comorbidities can compromise protection against severe disease, and antibody-mediated immunity is undercut by viral immune escape mutations. Whether and to what extent durable T cell responses compensate for reduced humoral immunity, particularly in the elderly, have not been investigated. Here, we utilize SARS-CoV-2-specific and pan-coronavirus-derived peptide pools, including or excluding spike glycoprotein-derived epitopes, to measure vaccination and infection induced pan-human endemic coronavirus (PHEC)-directed T cell immunity. In contrast to vaccinated individuals, hybrid immunity induced by vaccination and SARS-CoV-2 infection comprises high frequencies of PHEC-reactive T cells with comparable frequencies and functional TCR avidities across all age groups. With waning humoral immunity and vulnerability to escape mutations, PHEC-reactive T cells may provide critical protection. Our findings underscore the importance of incorporating pan-coronavirus T cell epitopes in future vaccine strategies.

INTRODUCTION

Adaptive immunity is the key to control severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causing coronavirus disease 2019 (COVID-19).¹ Humoral and cellular immunity provided by preexisting immunity and thereafter shaped and maintained by repeated antigen exposure through vaccinations and infections have contributed to greatly reduce COVID-19-associated hospitalization rates.^{2–4} However, durable sterile immunity could not be induced because of the rapid waning of neutralizing antibodies at mucosal surfaces, a key to prevent reinfection.^{5–7} Additionally, immune escape mutations in most emerging SARS-CoV-2 variants reduced the neutralization by serum antibodies.^{8,9} T cell-based cellular immunity seems to be less susceptible to viral escape due to a broader epitope coverage also including conserved pan-human endemic coronavirus (PHEC)

epitopes—in other words, conserved peptide motifs of the human endemic coronavirus strains HKU1, NL63, OC43, 229E, and SARS-CoV-2.¹⁰ However, whether and to what extent long-term durability of responses to pan-SARS-CoV-2 epitopes is induced and maintained in a population including the elderly are not known. Understanding the level of protective immunity in a population requires complementing antibody screenings with robust and detailed T cell analyses.

The emergence of SARS-CoV-2 was just the latest of the zoonotic coronavirus spillovers, including previously contained outbreaks of MERS-CoV and SARS-CoV, as well as the four circulating endemic coronaviruses (HKU1, OC43, NL63, 229E) that used to cause up to one-third of the seasonal cold symptoms annually.¹¹ The ongoing high transmission pressure on *Coronaviridae*, and the immune evasion mutations of currently circulating SARS-CoV-2 variants demand a better understanding of



the role of pan-coronavirus immunity to drive intelligent vaccine design. Most first-generation vaccines relied on anti-spike glycoprotein immunity and have subsequently been adapted to emerging variants of concern. As a result, current cellular immunity consists of pan-coronavirus-specific T cells induced in previous infections with endemic coronaviruses and *de novo* SARS-CoV-2-specific T cells generated in response to SARS-CoV-2 infection and/or vaccination.^{3,4,12–15} SARS-CoV-2 consists of 11 open reading frames (ORFs), of which ORF1a/b encodes for 16 non-structural proteins (NSPs). Spike (S), nucleocapsid (N), membrane (M), and, to a lesser extent, ORF3a, ORF7a, ORF9b, ORF9c, NSP2-3, and NSP12-14 contain immunodominant antigens in SARS-CoV-2 infection.^{4,15} The endemic coronaviruses only share S, N, M, envelope (E), and ORF1a/b with SARS-CoV-2, with S, NSP2-4, and NSP12-14 being the most immunogenic proteins in cross-reactive pan-coronavirus responses.^{4,15} We and others have previously demonstrated that cross-reactive T cell clones derived from previous infections with endemic coronaviruses contributed to rapid responses and even abortive infections in SARS-CoV-2-naïve individuals.^{4,16–18} However, we also observed that T cells specific for endemic coronaviruses and the dominant S816-830 spike PHEC epitope wane from the T cell pool in older individuals.^{4,19} Some previous studies reported generally weaker cellular and humoral responses with lower induction and higher CD4⁺ T cell contraction rates in the elderly, questioning the quality of PHEC immunity in this cohort at risk.^{7,20,21} However, these studies did not specifically delineate the level, quality, and role of PHEC-specific T cell immunity.

Here, we utilized peptide pools containing dominant SARS-CoV-2- or PHEC-specific epitopes, including or excluding spike-derived epitopes as identified in Mateus et al.,¹⁵ to delineate SARS-CoV-2- and PHEC-specific T cell responses. We assessed a large cohort of 1,024 individuals with heterogeneous immunization background (infection[s] and/or vaccination[s]) including a substantial fraction of elderly individuals older than 70 years. Hybrid immunity derived from SARS-CoV-2 infection and vaccinations generated durable PHEC-reactive T cells with high frequencies and functional T cell receptor (TCR) avidities across all age groups. Moreover, recognized epitopes were rarely targeted by escape mutations. Our findings highlight the critical need to incorporate PHEC T cell epitopes into future vaccine strategies. This approach will enhance the breadth and durability of immune protection against SARS-CoV-2 and its variants, particularly in vulnerable populations. By targeting these epitopes, vaccines can generate robust and long-lasting T cell responses that are less susceptible to viral mutations, ensuring comprehensive and sustained immunity against current and potential future coronavirus outbreaks.

RESULTS

SARS-CoV-2 (excluding spike)-reactive CD4⁺ T cells identify asymptotically infected individuals

We assessed 1,024 individual measurements subclassified into SARS-CoV-2-infected (In, $n = 39$), infected-vaccinated (In/Vx, $n = 84$), vaccinated-infected (Vx/In, $n = 294$), and vaccinated-only (Vx, $n = 607$) donors, based on positive qPCR results and/or vaccination certificates recruited between December 2021

and December 2022 (for further details, see **Figure S1A** and table donor characteristics in **STAR Methods**). Peripheral blood mononuclear cells (PBMCs) were stimulated with a peptide pool containing dominant SARS-CoV-2-specific CD4⁺ and CD8⁺ T cell epitopes, including spike glycoprotein-specific peptides (dominant epitopes including spike [DE+S]) and a pool containing only the non-spike-derived peptides (dominant epitopes excluding spike [DE-S]). We assessed the performance of the peptide pools utilizing the activation-induced marker assay measuring activated, antigen-specific CD40L⁺4-1BB⁺ CD4⁺ or IFN- γ 4-1BB⁺ CD8⁺ T cells.³ CD4⁺ T cell responses to DE+S identified SARS-CoV-2 spike responders with a minimum 99% accuracy (background subtracted [bgsub] > 0.005) in all cohorts (**Figure 1A**). Most donors with previous infection (>86%) also displayed strong responses against non-spike SARS-CoV-2-specific peptides (DE-S), irrespective of their vaccination status prior to or after the infection (**Figure 1A**). However, 17% of the individuals of the Vx cohort with unreported infection also mounted a weak (bgsub 0.005–0.015) and 18% a strong (bgsub > 0.015) response to DE-S peptides (**Figure 1A**). Plotting DE+S against DE-S demonstrated a clear separation of infected from most vaccinated-only donors and a fraction of 110 double-positive donors with bgsub values > 0.015 in the Vx cohort (**Figures 1B** and **1C**). Among these, 84 individuals (76%) had measurable anti-nucleocapsid (NCAP) antibody titers, proving an undetected/unreported infection (**Figures 1C** and **1D**). This is in the range of the NCAP antibody detection rate (79%–84%) in qPCR-validated In individuals (**Figures 1D** and **1E**). Consequently, we classified the 110 DE+S and DE-S double-responsive individuals as asymptotically (subclinically) infected and vaccinated (aln/Vx). Their proportion among the Vx cohort followed the daily incidence in Germany²² (**Figure S1B**). Anti-SARS-CoV-2 S1 immunoglobulin G (IgG) titers increased strongly after second challenge (by either infection or vaccination), with aln/Vx S1 IgG titers closely resembling individuals who reported infection and vaccination (hybrid immunity) instead of the titers observed in the Vx cohort (**Figure 1E**). Anti-S1 IgG and anti-NCAP antibodies declined rapidly over time, with a reduction by half within 200 days (S1 IgG) and 100 days (NCAP antibodies), which becomes undetectable (**Figures S1C** and **S1D**). In the Vx cohort, 112 donors were longitudinally assessed with up to 3 follow-up measurements. While 98 donors remained DE-S negative, 2 donors became positive, and 12 donors were consistently positive throughout our measurements but did not report any symptoms of SARS-CoV-2 infection, which is consistent with our classification as asymptotically infected (**Figure 1F**). Antigenic challenges by infection or vaccination resulted in the selective expansion of antigen-specific T cells characterized by higher TCR avidity to the target antigen(s). We therefore utilized the CATCH assay, a direct cytometric TCR avidity assay, that we recently introduced. The assay determines TCR avidity by measuring the downregulation of CD3 expression (CD3^{lo}) among the pool of antigen-specific T cells.⁴ Frequencies of CD3^{lo} antigen-reactive T cells (indicating high-avidity T cells), demonstrated a reduced T cell population avidity in only-infected (non-vaccinated) donors, which mostly had experienced a single infection. The avidity increased with a second challenge (mostly the first vaccination) and remained stable thereafter (**Figures 1G** and

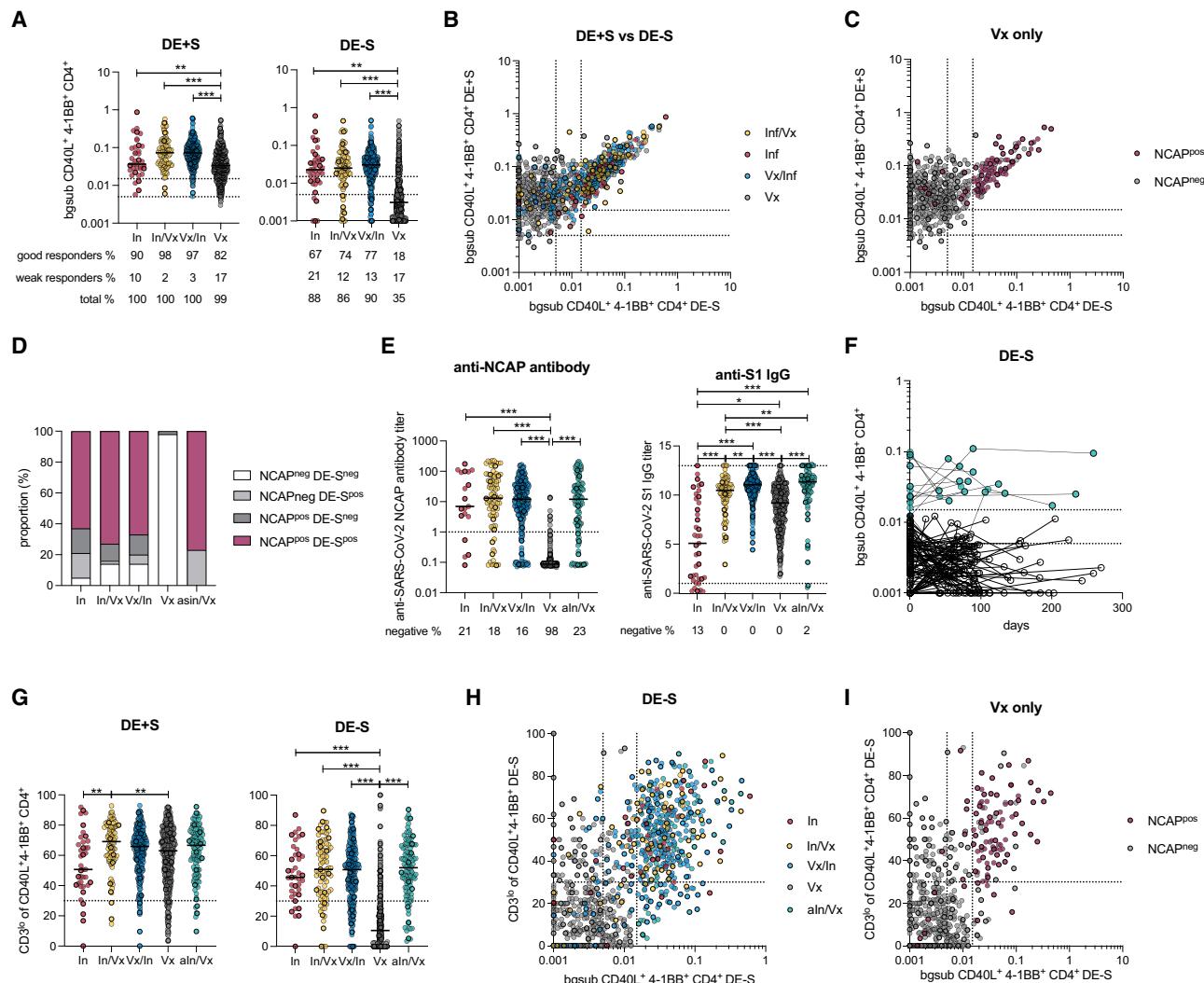


Figure 1. DE+S- and DE-S-specific CD4⁺ T cell responses

Ex vivo stimulation of PBMCs of infected (In, $n = 40$), infected-vaccinated (In/Vx, $n = 84$), vaccinated-infected (Vx/ln, $n = 294$), and vaccinated-only (Vx, $n = 607$, 111 of which were reclassified as asymptomatic In/Vx (asin/Vx) individuals, with peptide pools containing the dominant SARS-CoV-2-specific epitopes including (DE+S) or excluding (DE-S) spike epitopes.

(A–D) The percentage of CD40L*4-1BB* CD4⁺ T cells among stimulated PBMCs was subtracted (background subtracted [bgsub]) from the percentage of these cells among unstimulated PBMCs.

(A) Percentage of donors with weak (bgsub 0.005–0.015) DE+S- and DE-S-specific CD4⁺ T cells and proportion of good responders (bgsub > 0.015 as indicated by dotted lines). Bars represent the mean. Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and not significant for $p > 0.05$ (not indicated).

(B) Percentage of DE+S-specific CD4⁺ T cells plotted against percentage of DE-S-specific CD4⁺ T cells among indicated cohorts.

(C) Donors with anti-nucleocapsid (NCAP)-specific antibodies (red dots) among DE+S and DE-S antigen-specific CD4⁺ T cell responses in the Vx cohort.

(D) Proportion of NCAP antibody and/or DE-S positive donors in indicated cohorts.

(E) Anti-SARS-CoV-2 NCAP antibody and S1 IgG titer in indicated cohorts. Bars represent the mean. Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and not significant for $p > 0.05$ (not indicated).

(F) Longitudinal assessment of DE-S-specific T cells in the days since the first measurement. Transparent circles indicate donors that remain negative, black circles are measurements of donors that became DE-S positive, taupe circles indicate DE-S-positive measurements. Multiple measurements of the same donors are connected with lines.

(G) Frequencies of CD3^{lo} cells among DE+S- and DE-S-specific CD40L*4-1BB* CD4⁺ T cells. Bars represent the mean. Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and not significant for $p > 0.05$ (not indicated).

(H) Percentage of DE-S-specific CD4⁺ T cells plotted against percentage of CD3^{lo} cells among DE-S-specific CD4⁺ T cells.

(I) Donors with anti-NCAP-specific antibodies (red dots) among donors with high frequencies and avidities of DE-S-specific CD4⁺ T cell responses in the Vx cohort.

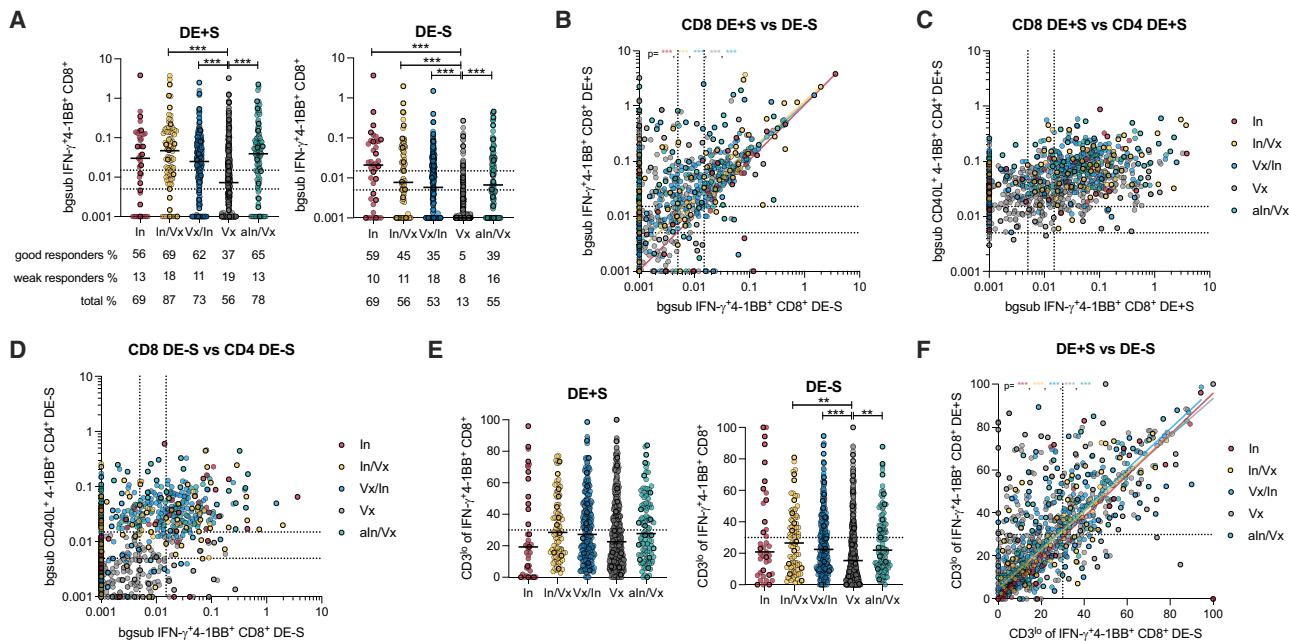


Figure 2. DE+S- and DE-S-specific CD8⁺ T cell responses

In vivo stimulation of PBMCs of In ($n = 40$), In/Vx ($n = 84$), Vx/In ($n = 294$), and Vx ($n = 607$), 111 of which were reclassified as aln/Vx individuals, with peptide pools containing the dominant SARS-CoV-2-specific epitopes including (DE+S) or excluding spike (DE-S) epitopes.

(A–D) The percentage of IFN- γ +4-1BB⁺ CD8⁺ T cells among stimulated PBMCs was subtracted (bgsub) from the percentage of these cells among unstimulated PBMCs.

(A) Percentage of donors with weak (bgsub 0.005–0.015) and good responses (bgsub > 0.015, as indicated by dotted lines) of DE+S- and DE-S-specific CD8⁺ T cells. Bars represent the mean. Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and not significant for $p > 0.05$ (not indicated).

(B) Percentage of DE+S-specific CD8⁺ T cells plotted against percentage of DE-S-specific CD8⁺ T cells among indicated cohorts. Two-tailed Spearman correlation. r and p values indicated in Table S1. * $p < 0.0332$; ** $p < 0.0021$; *** $p < 0.0002$ only indicated when significant. Lines display robust nonlinear regression (curve fit).

(C) Percentage of DE+S-specific CD8⁺ T cells plotted against percentage of DE+S-specific CD4⁺ T cells among indicated cohorts.

(D) Percentage of DE-S-specific CD8⁺ T cells plotted against percentage of DE-S-specific CD4⁺ T cells among indicated cohorts.

(E) Frequencies of CD3¹⁰ cells among DE+S- and DE-S-specific IFN- γ +4-1BB⁺ CD8⁺ T cells. Bars represent the mean. Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and not significant for $p > 0.05$ (not indicated).

(F) Correlation of DE+S-specific CD3¹⁰ cells with DE-S-specific CD3¹⁰ cells. Two-tailed Spearman correlation. r and p values indicated in Table S1. * $p < 0.0332$; ** $p < 0.0021$; *** $p < 0.0002$ only indicated when significant. Lines display robust nonlinear regression (curve fit).

S1E–S1H). Moreover, infection resulted in increased frequencies of CD3¹⁰ DE-S-reactive T cells, further underlining the identification of undetected infections among the Vx cohort for 24% of individuals without detectable anti-NCAP antibodies (Figures 1H and 1I). Since DE+S and DE-S contain 7 and 5 potentially cross-reactive epitopes, respectively, we additionally analyzed 21 pre-pandemic donors (Figure S1I). Although one donor showed clear cross-reactivity (bgsub), the combination with TCR avidity allows the separation of cross-reactive from the SARS-CoV-2-infected donor

DE+S- and DE-S-specific CD8⁺ T cells

DE+S-specific CD8⁺ T cell responses were detected in 56%–87% of all measurements and with higher frequency in (additionally) infected compared to vaccinated individuals (Figure 2A). Responses against non-spike epitopes were pronounced after a single infection but waned in the Vx/In cohort over time (Figures 2A, S2A, and S2B). There was a strong positive correlation of spike-including (DE+S) and spike-excluding (DE-S) re-

sponses (Figure 2B). In line with this finding, DE+S CD8⁺ T cell responses were found only in individuals with robust CD4⁺ T cell responses, with only 5 out of 1,024 donors that did not mount any measurable T cell response (Figure 2C). Although generally stronger CD4⁺ responses compared to CD8⁺ T cell responses were detected, some donors responded to DE-S stimulation only with CD8⁺ T cells (Figure 2D). The different numbers and types of challenges resulted in comparable population avidity against spike and non-spike epitopes (Figures 2E, S2A, and S2B). Similar to the frequency of reacting T cells, the detected avidities against DE+S and DE-S SARS-CoV-2 epitopes showed donor-dependent variability (Figure 2F).

Hybrid SARS-CoV-2 challenges expand PHEC immunity

A set of human endemic coronavirus cross-reactive CD4⁺ T cell epitopes consisting of 31 spike- and 30 non-spike-specific epitopes has been identified for ancestral SARS-CoV-2 variants (listed in Table S2).¹⁵ However, emerging SARS-CoV-2 escape variants have accumulated several mutations potentially

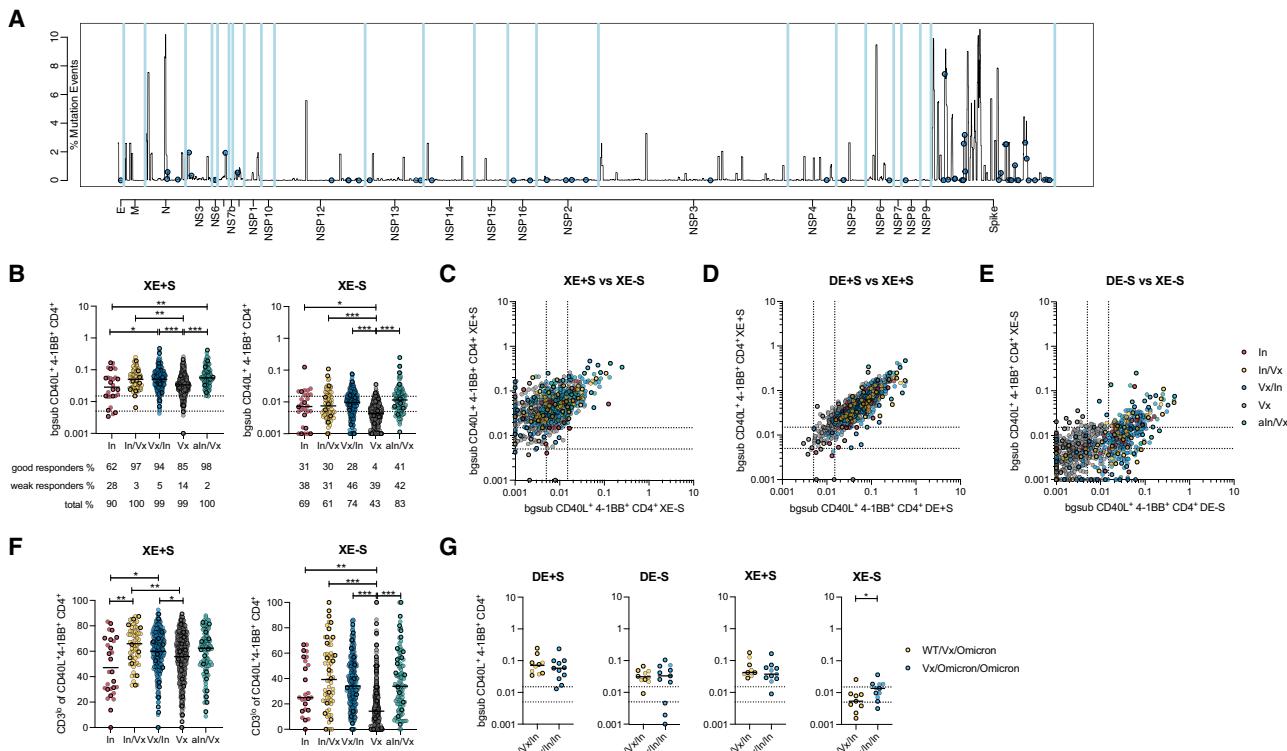


Figure 3. Pan-coronavirus epitopes are conserved throughout the SARS-CoV-2 evolution

(A) Mutations in pan-coronavirus epitopes (GISAID December 2023).

(B–E) *Ex vivo* stimulation of PBMCs of In ($n = 29$), In/Vx ($n = 63$), Vx/ln ($n = 281$), Vx ($n = 482$), and asln/Vx ($n = 103$) individuals, with peptide pools containing pan-coronavirus-specific epitopes including (XE+S) or excluding spike (XE-S) epitopes. The percentage of CD40L⁺4-1BB⁺ CD4⁺ T cells among stimulated PBMCs was subtracted (bgsu) from the percentage of these cells among unstimulated PBMCs.

(B) Percentage of donors with weak (bgsu 0.005–0.015) XE+S- and XE-S-specific CD4⁺ T cell responses and proportion of good responders (bgsu > 0.015, as indicated by dotted lines). Bars represent the mean. Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and not significant for $p > 0.05$ (not indicated).

(C) Percentage of XE+S-specific CD4⁺ T cells plotted against percentage of XE-S-specific CD4⁺ T cells among indicated cohorts.

(D) Percentage of XE+S-specific CD4⁺ T cells plotted against percentage of DE+S-specific CD4⁺ T cells among indicated cohorts.

(E) Percentage of XE-S-specific CD4⁺ T cells plotted against percentage of DE-S-specific CD4⁺ T cells among indicated cohorts.

(F) Frequencies of CD3^{lo} cells among XE+S- and XE-S-specific CD40L⁺4-1BB⁺ CD4⁺ T cells. Bars represent the mean. Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and not significant for $p > 0.05$ (not indicated).

(G) Frequencies of antigen-specific cells in individuals with ancestral strain infection, vaccination, and Omicron infection ($n = 10$) compared to Vx individuals with two subsequent Omicron infections ($n = 11$). Bars represent the mean. Mann-Whitney t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and not significant for $p > 0.05$ (not indicated).

impairing the recognition by T cells.²³ We compared the mutations of the cross-reactive (PHEC epitopes) to mutations of other peptides from the same protein (GISAID November 2023). A high mutation rate in one epitope within spike (S131) was demonstrated, while the remaining epitopes displayed a low mutation likelihood (Figure 3A; Table S3). Utilizing the difference between the properties of the wild-type amino acid and the amino acid in the most frequent mutation in the respective peptide, we calculated a score reflecting the impact of the amino acid exchange on the peptides' characteristics (PAM30 similarity matrix, Table S3). For 9 out of 59 epitopes, the score predicted a disruptive mutation with a putative negative impact on T cell responsiveness. To assess the performance of pan-coronavirus-specific epitopes *ex vivo*, we generated peptide pools containing cross-reactive epitopes including (XE+S) or excluding (XE-S) spike and

measured 958 individual measurement time points subclassified into In ($n = 29$), In/Vx ($n = 63$), Vx/ln ($n = 281$), Vx ($n = 482$), and asln/Vx ($n = 103$). The response rate against the PHEC peptide pool XE+S increased from 90% after one challenge to 99% in donors with at least two challenges by infection(s) and/or vaccination(s) (Figures 3B, S3A, and S3B). Stimulations with the cross-reactive epitopes excluding spike (XE-S) demonstrated a repertoire of PHEC-specific T cells from non-SARS-CoV-2 infections in 43% of the Vx cohort, which increased to 61%–83% upon SARS-CoV-2 infection (Figure 3B). Plotting XE+S against XE-S demonstrated that spike epitopes dominated the CD4⁺ T cell response but that SARS-CoV-2 infection increased the overall frequencies of cross-reactive T cells (Figure 3C). Again, the amplitude of the T cell response was variable among donors. Those with favorable T cell reactivities also

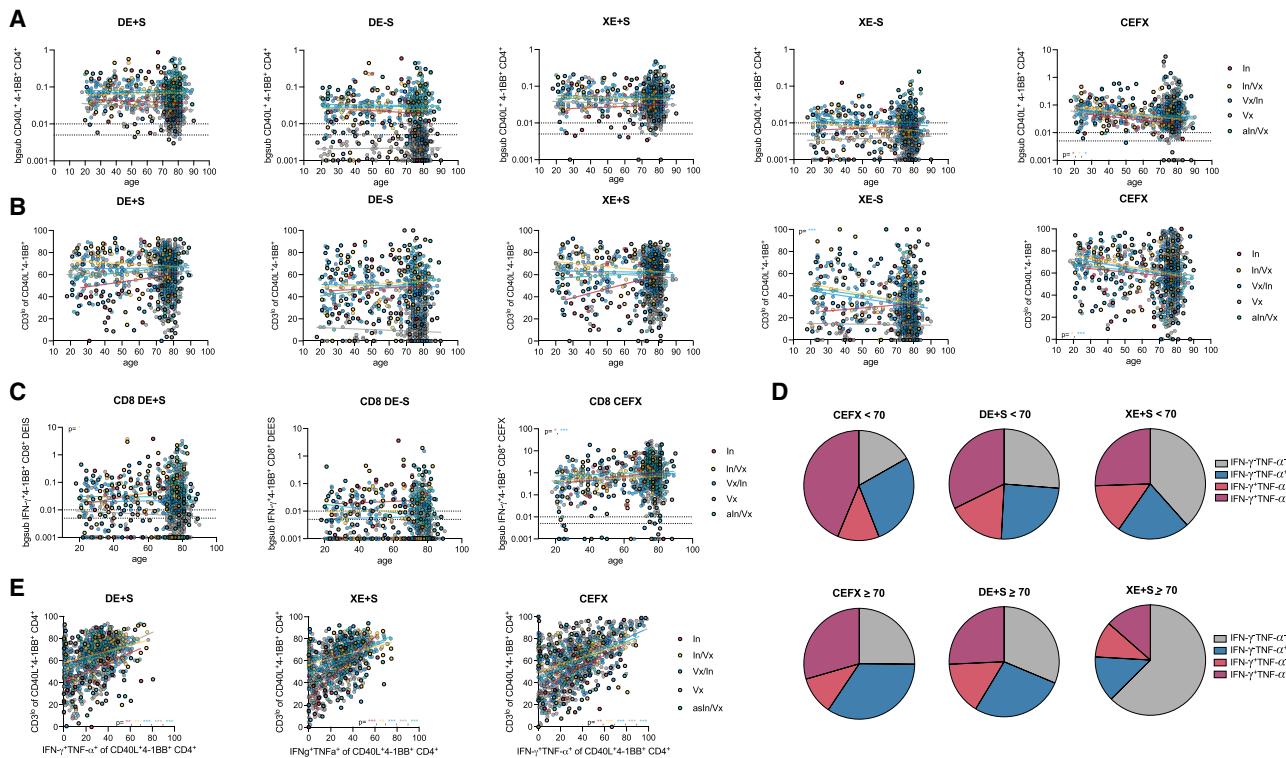


Figure 4. PHEC responses in the elderly

(A) Frequencies of antigen-specific CD40L^{+4-1BB+} CD4⁺ T cells of indicated specificity over age. Two-tailed Spearman correlation. r and p values indicated in Table S1. * p < 0.0332; ** p < 0.0021; *** p < 0.0002 only indicated when significant. Lines display robust nonlinear regression (curve fit).

(B) Frequencies of CD3^{lo} cells among antigen-specific CD40L^{+4-1BB+} CD4⁺ T cells of indicated specificity over age. Two-tailed Spearman correlation. r and p values indicated in Table S1. * p < 0.0332; ** p < 0.0021; *** p < 0.0002 only indicated when significant. Lines display robust nonlinear regression (curve fit).

(C) Frequencies of antigen-specific IFN- γ ^{+4-1BB+} CD8⁺ T cells of indicated specificity over age. Two-tailed Spearman correlation. r and p values indicated in Table S1. * p < 0.0332; ** p < 0.0021; *** p < 0.0002 only indicated when significant. Lines display robust nonlinear regression (curve fit).

(D) Proportion of IFN- γ and/or TNF- α -producing T cells among CD40L^{+4-1BB+} CD4⁺ T cells after stimulation with CEFX, DE+S, or XE+S in individuals younger than 70 years or 70 years or older.

(E) Frequencies of CD3^{lo} cells among antigen-specific CD40L^{+4-1BB+} CD4⁺ T cells against frequencies of IFN- γ ^{+TNF- α} CD40L^{+4-1BB+} CD4⁺ T cells. Two-tailed Spearman correlation. r and p values indicated in Table S1. * p < 0.0332; ** p < 0.0021; *** p < 0.0002 only indicated when significant. Lines display robust nonlinear regression (curve fit).

reacted in a superior way against PHEC epitopes (Figure 3D). Moreover, SARS-CoV-2 infection boosted non-spike PHEC-reactive T cells, also in previously vaccinated individuals (Figure 3E). SARS-CoV-2 infection increased the PHEC-reactive T cell populations' avidity and was qualitatively highest in individuals with a history of infection followed by vaccination(s) (Figure 3F). The response against these pan-coronavirus epitopes was durable and was not further boosted by repeated challenges either by additional infection(s) and/or vaccination(s) (Figures S3A and S3B). Since Vx individuals with previous ancestral strain infection have been shown to display weaker T cell responses against Omicron S1 protein,²⁴ we identified double-infected individuals in our cohort who had an ancestral strain infection prior to vaccination, followed by an Omicron variant infection or who were fully vaccinated (3–4 doses) and then infected with Omicron twice. We found no differences in DE+S, DE-S, and XE+S responses but stronger XE-S responses in double Omicron-infected individuals, despite the fact that all pools contained SARS-CoV-2 ancestral strain peptides, indi-

cating intact pan-coronavirus immunity against Omicron (Figure 3G).

Pan-coronavirus-reactive T cells display no reduced frequencies or avidities but polyfunctionality in the elderly

Owing to thymic involution during adolescence, the numbers of naive T cells decrease with increasing age, causing a narrower T cell repertoire, which, together with inflamming, results in partially impaired T cell response in the elderly.²⁵ We had previously observed a decrease in SARS-CoV-2 spike cross-reactive cells in unexposed (no SARS-CoV-2 infection or vaccination) individuals with increasing age, and in line with the widely described reduced clonality, we noted a contraction of the immunodominant cross-reactive spike epitope (iCope)-specific T cells.^{4,19} To characterize PHEC cell immunity over age, we analyzed T cell reactivities in a large cohort of individuals aged 70 years or older (18% In, 33% In/Vx, 55% Vx/In, 85% Vx, and 86% aln/Vx). We did not observe quantitative or qualitative (TCR avidity)

differences in the older people's CD4⁺ or CD8⁺ T cell responses against SARS-CoV-2 epitopes or against pan-coronavirus epitopes (Figures 4A–4C), while overall T cell responses against a control peptide pool containing dominant epitopes of cytomegalovirus, Epstein-Barr virus, influenza, and of other pathogens (CEFX) were significantly decreased. However, we observed a general loss of polyfunctional T cells irrespective of the immunization and infection background in individuals older than 70 years of age (Figure 4D). Also, PHEC (XE+S)-specific T cells displayed a general, age-independent reduced polyfunctionality compared to SARS-CoV-2-specific clones (Figure 4D). Polyfunctionality correlated very strongly with the donors' individual T cell population avidity, demonstrating a specific deficiency rather than general impaired PHEC immunity in the elderly (Figure 4E). Altogether, these data suggested that boosting cellular immunity by vaccines comprising PHEC epitopes may improve the populations' resilience against further variants and outbreaks.

DISCUSSION

Here, we delineated SARS-CoV-2-specific T cell responses in a large cohort of adult and elderly individuals. Durable anti-SARS-CoV-2 T cell responses were mounted in nearly all (1,019 out of 1,024) individuals, allowing a specific delineation of hybrid and PHEC-reactive cellular immunity. By using specifically designed peptide pools containing dominant epitopes including and excluding spike glycoprotein epitopes, we could identify 110 (18.3%) asymptomatic SARS-CoV-2 infections among a large cohort of Vx donors that had not reported an infection ($n = 607$). Similar to other countries, Germany encountered multiple COVID-19 infection waves, which, during the time of our measurements, were caused mostly by the SARS-CoV-2 Delta variant, with a major peak in November 2021 (57,600 daily cases), followed by Omicron variants with several peaks in February 2022 (187,100 daily cases), April 2022 (226,900 daily cases), July 2022 (96,400 daily cases), and October 2022 (92,100 daily cases).²² In line with these data, the proportion of asymptomatic-infected among the vaccinated-only in our dataset increased from February to April 2022, and the ratio of putatively asymptomatic-infected among measured Vx was highest in July and October/November 2022 (Figure S1A). Similarly, biweekly qPCR tests of workers in daycare, childcare, schools, and some private companies showed a symptom-free positive rate of 14.7% mid-January to March 2022 in a region of south Germany, and a South African cohort reported 23% asymptomatic infections.^{26,27} We also assessed asymptomatic infection rates by analyzing serum antibody titers against NCAP. However, NCAP antibody analysis is challenged by several factors such as low sensitivity and short half-life of NCAP antibodies.^{28,29} Additionally, vaccination negatively impacts NCAP seroconversion,³⁰ while we could demonstrate here that T cell responses are not affected by vaccinations prior to infection. DE-S-specific T cell responses were detected in 86%–87% of individuals with an infection-based first challenge, and 90% of the donors with vaccinations prior to reported infection and their detection were robust up to 600 days after the infection. Others have previously demonstrated that exposed, asymptomatic family members without

seroconversion showed an increase in NCAP-specific T cell responses, indicating a symptom-free induction of T cell responses.³¹ We demonstrate here that T cell-based immunoassays are capable of delineating infection- and vaccination-induced as well as SARS-CoV-2-specific versus pan-coronavirus-specific T cell responses.^{32,33}

We confirm that hybrid challenges enhance SARS-CoV-2 T cell immunity with positive effects on pan-coronavirus-specific T cell immunity.³⁴ Although we detected stronger CD4⁺ T cell responses compared to CD8⁺ T cell responses in most donors, some donors responded to DE-S only with CD8⁺ T cells. Further long-term monitoring of such donors would be necessary to elucidate how CD8⁺ T cell-dominated immunity against SARS-CoV-2 impacts reinfections. Also, other studies have hypothesized that CD8⁺ T cells play a significant role in anti-SARS-CoV-2 mucosal immunity, with cross-reactive clones causing abortion of infections.^{17,35,36} In general, the quality (antigen-specific T cell population avidity) of T cell response correlated strongly with the quantity (antigen-specific T cell frequencies). This is in line with previous findings that good responders display an overall better response also on the humoral level.^{4,37}

PHEC-specific immunity holds great potential to contribute substantially to protection against infection and severe disease from future SARS-CoV-2 variants and coronavirus spillovers. Human epidemiological data suggested that endemic-corona-virus-infection-mediated, cross-reactive, HLA-B*15:01-specific T cell immunity for a certain epitope substantially increases the likelihood of asymptomatic SARS-CoV-2 infection.³⁸ In line with this, SARS-CoV-2 polymerase-targeting T cells can terminate the infection.^{17,36} In a murine experimental model, it could be validated that OC43-reactive T cells protect against SARS-CoV-2 infection and reduce lung damage.³⁹ T cells generated in response to coronavirus strains that can infect humans can cross-detect animal-restricted coronavirus strain-specific epitopes in unexposed and SARS-CoV-2-In individuals, demonstrating the potential of a pan-coronavirus immunity to protect against future spillovers.⁴⁰ Vaccinated and infected individuals showed a weakened but preserved T cell response against Omicron despite the introduction of more than 30 mutations in the Omicron variant.^{10,41} Utilizing recombinant S1 proteins, Reynolds et al. observed that Omicron infection increases immunity against previous variants of concern (VOCs) but not Omicron itself.²⁴ In five individuals with a previous ancestral strain infection, no T cell boost by Omicron infection could be observed. This unexpected result could not be reproduced in our dataset utilizing peptide pools also containing S2 epitopes and non-spike epitopes. Bioinformatic analyses revealed a comparatively low mutation rate of the used pan-coronavirus epitopes in all published SARS-CoV-2 sequences so far. Comparing T cell responses of 10 individuals with ancestral strain infection followed by vaccinations and Omicron infection to 11 individuals with 2 subsequent Omicron infections after their vaccinations, we found no differences in DE+S, DE-S, and XE+S responses. However, the latter cohort mounted a slightly stronger response against XE-S, which is most likely due to the low XE-S antigen-specific T cell frequencies, which dropped in the months after ancestral strain infection and are

boosted twice in a short time window in the double Omicron-In cohort.

A critical factor of T cell-based anti-SARS-CoV-2 immunity in the elderly are the changes in T cell functionality observed here, including reduced polyfunctionality and TCR repertoire breadth.^{42,43} In parallel, multiple comorbidities may result in an overall higher risk in the elderly for severe COVID-19 disease progression.⁴⁴ Reports indicate that the response to vaccination can be dampened in older individuals, and the frequency of preexisting cross-reactive T cells in general decreases with increasing age.^{4,7,20,21} Nevertheless, in our large cohort of manifold challenged individuals the quantity and quality of anti-SARS-CoV-2 and PHEC T cell responses appeared to be surprisingly robust with age, in contrast to assessed responses against other well-known viral pathogens. We demonstrated a reduced polyfunctionality in the cohort older than 70 years accompanied by reduced TCR avidity in affected individuals, allowing for the identification of individuals with suboptimal responses, which may put them at greater risk of severe disease from future VOCs/spillovers. Overall, pan-coronavirus-reactive T cells boosted by hybrid challenges of SARS-CoV-2 vaccinations and infections serve as a hallmark of durable coronavirus-specific immunity even in a large cohort of elderly individuals.

Limitations of the study

The study utilizes an incomplete set of pan-coronavirus epitopes, with 31 spike- and 30 non-spike-derived peptides identified in SARS-CoV-2 unexposed individuals responsive to the ancestral SARS-CoV-2 strain. Within these pools, dominant epitopes and epitopes with high homology (>67%) to endemic coronaviruses were combined (6/25 in XE+S and 9/21 in XE-S).¹⁵ Also, our data analysis was challenged by the ongoing mutation of the virus, with different Omicron subvariants arising during the 12 months of sampling. We compared the response of Omicron-infected individuals to donors from the Pa-COVID-19 biobank infected with the Alpha and Delta variants and did not observe variant-dependent differences in the responses against the three different strains in line with the reported high conservation rate of epitopes.¹⁰ The recruited cohort was very heterogeneous with different administered vaccines, different vaccination and infection numbers, and different time points of challenges. Although our measurements identified a reliable proportion of probably asymptotically infected, we cannot exclude further infections missed in our settings. The DE+S and DE-S peptide pools contain 7 and 5 potentially cross-reactive epitopes, respectively. Data from 21 pre-pandemic donors suggest that combinations of antigen-specific T cell frequencies and TCR avidity allow separation between asymptotically infected and cross-reactive responses, but the pre-pandemic cohort is too small for absolute generalization. We therefore cannot exclude the possibility that individuals with quantitatively and qualitatively strong cross-reactive responses are falsely classified as asymptomatic infected. We tested the dataset against potential cofounding variables (age, time since last challenge/infection, types of vaccine, disease severity) to avoid misleading interpretations. Finally, a critical part of pan-coronavirus immunity lies in the mucosal response, which was not assessed in this study and warrants further investigation.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Andreas Thiel (andreas.thiel@charite.de).

Materials availability

Materials will be made available from the lead contact with a completed materials transfer agreement.

Data and code availability

- The accession number for the data of this paper can be found in the [key resources table](#).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.L.; data curation, L.L. and J. Braun; formal analysis, L.L. and J. Braun; funding acquisition, F.K., L.-E.S., and A.T.; investigation, L.L.; resources, U.R., L.M.-A., L.H., J.B., B.K., M.D., M.M., J. Behrens, P.T.L., J.M., K.S., H.W., W.J., V.D.C., A.N., F.K., and L.-E.S.; visualization, L.L., J. Braun, and U.R.; writing: L.L., L.-E.S., and A.T.

DECLARATION OF INTERESTS

U.R. was and K.S. is an employee of JPT Peptide Technologies GmbH. H.W. was the chief executive officer of JPT Peptide Technologies GmbH. L.L. and A.T. hold a patent regarding the usage of CD3 downregulation as a method for direct analysis of functional avidity of T cells.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
purified anti-CD28	BD	Cat# 555725, RRID AB_396068
CD3-FITC	Miltenyi Biotec	Cat# 130-113-138, RRID AB_2725966
CD4-VioGreen	Miltenyi Biotec	Cat# 130-113-230, RRID AB_2726041
CD8-VioBlue	Miltenyi Biotec	Cat# 130-110-683, RRID AB_2659239
CD137-PE	Miltenyi Biotec	Cat# 130-110-763, RRID AB_2654986
CD40L-PEVio770	Miltenyi Biotec	Cat# 130-113-614, RRID AB_2751148
TNF-α-BV605	Biolegend	Cat# 502936, RRID AB_2563884
IFN-γ-A700	BD	Cat# 557995, RRID AB_396977
Chemicals, peptides, and recombinant proteins		
Pan-SARS-CoV-2 Select	JPT	Cat# PM-Pan-SARS2select01-1
Pan-SARS-CoV-2 Select w/o S	JPT	Cat# PM-Pan-SARS2select02-1
XE+S peptide pool	JPT	custom made, Table S2
XE-S peptide pool	JPT	custom made, Table S2
CEFX Ultra SuperStim	JPT	Cat# PM-CEFX-1
Zombie Yellow	Biolegend	Cat# 423104
Critical commercial assays		
QIAamp Viral RNA Mini Kit	Qiagen	Cat# 52904
AgPath-ID™ One-Step RT-PCR Reagents kit	Thermo Fisher	Cat# AM1005
Anti-SARS-CoV-2 IgG ELISA	EUROIMMUN	Cat# EI 2606-9601 G
Elecsys® Anti-SARS-CoV-2	Roche	Cat# 09 203 095 190
Deposited data		
Flow cytometry raw data	Zenodo	https://doi.org/10.5281/zenodo.14391136
Software and algorithms		
Magellan Pro V7.4	Tecan	N/A
FlowJo 10.6	FlowJo LLC	N/A
GraphPad Prism 9	GraphPad	N/A
REDCap	Harris et al. ^{42,43}	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study participants

Patients were recruited within the Charité Corona Protect (CCP) study conducted at Charité – Universitätsmedizin Berlin. The study was approved by the Institutional Review board of the Charité (EA/152/20). Written informed consent was obtained from all included participants and the study was conducted in agreement with the declaration of Helsinki. All donors were assessed for age and gender (Table donor characteristics). Participants who had tested positive for SARS-CoV-2 RNA (RT-qPCR from nasopharyngeal swabs) and displayed SARS-CoV-2 related symptoms were classified as convalescent donors (In). The day of infection was set as

day –3 prior to reported symptom onset. Frozen PBMCs of 20 infected donors were retrieved from the Pa-COVID-19 biobank.⁴⁵ The vaccination (Vx) status was retrieved from official vaccination documents.

Cohort	In	In/Vx	Vx/In	Vx	asIn/Vx
number of donors	39	84	294	497	110
gender (n [%])					
female diverse	25 (64%)	49 (58%)	181 (62%)	286 (58%)	55 (50%)
diverse	– (0%)	– (0%)	– (0%)	1 (0.2%)	1 (1%)
mean age (years [SD])	52.05 (16.35)	56.14 (18.24)	61.33 (19.83)	73.11 (12.80)	72.24 (14.93)
>70 years (%)	7	33	55	85	86
symptoms (n [%])					
mild	39 (100%)	74 (88%)	294 (100%)	–	–
moderate	– (0%)	8 (9%)	– (0%)	–	–
severe	– (0%)	2 (2%)	– (0%)	–	–
mean time since infection (days [SD])	205.7 (156.1)	608.9 (267.5)	100.7 (73.80)	–	unknown
mean time since last challenge (days [SD])	205.7 (156.1)	158.1 (91.68)	100.7 (73.80)	139.6 (84.20)	unknown
number of challenges (n [%])					
1	37 (95%)	–	–	3 (0.6%)	unknown
2	2 (5%)	84 (100%)	8 (3%)	11 (2.2%)	unknown
3	–	–	21 (7%)	302 (61%)	unknown
4	–	–	204 (69%)	181 (36%)	unknown
5	–	–	59 (20%)	–	unknown
6	–	–	2 (0.7%)	–	unknown
vaccines					
BNT	–	70%	74%	75%	74%
Mo	–	7%	16%	16%	16%
AZ	–	23%	10%	8%	10%
others	–	0%	0%	1%	0%

Table donor characteristics: Inf, infected; Vx, vaccinated; BNT, BioNTech/Pfizer BNT162b2 vaccine; Mo, Moderna COVID-19 vaccine; AZ, AstraZeneca ChAdOx1 vaccine.

METHOD DETAILS

Coronavirus RT-qPCR

RNA was extracted from 140 µL of wet nasopharyngeal swabs (Copan mini UTM) using the QIAamp Viral RNA Mini Kit and QIAcube Connect (both Qiagen) with the manual lysis protocol. SARS-CoV-2 RNA detection was performed using a simultaneous two duplex one-step real-time RT-PCR assay with custom primers and probes (Metabion and Thermo Fisher Scientific) for SARS-CoV-2 E Gene and SARS-CoV-2 ORF1ab according to the RKI/ZBS1 SARS-CoV-2 protocol. Each one is duplexed with a control that either indicates potential PCR inhibition or proves the successful extraction of nucleic acid from the clinical specimen. As positive controls genomic SARS-CoV-2 RNA and genomic SARS-CoV RNA were used for the ORF1ab and the E-Gene assay, respectively, adjusted to the Ct values 28 and 32. PCR was conducted with the AgPath-ID One-Step RT-PCR Reagents kit (Applied Biosystems) using a Bio-Rad CFX96 or Bio-Rad Opus real-time PCR cycler.

Blood and serum sampling and PBMC isolation

Whole blood was collected in lithium heparin tubes for PBMC isolation and SSTII advance (all Vacutainer, BD) tubes for serology. SSTII advance tubes were centrifuged for 10 min at 1000g prior to removing serum. Serum aliquots were frozen at –20°C until further use. PBMCs were isolated by gradient density centrifugation according to the manufacturer's instructions (Leucosep tubes, Greiner; Biocoll, Bio&SELL).

SARS-CoV-2 IgG S1 ELISA

Anti-SARS-CoV-2 IgG ELISAs specific for spike subunit 1 (S1) were performed with a 1:100 serum dilution using the commercial kits (EUROIMMUN Medizinische Labordiagnostika AG) according to the manufacturer's instructions and measured at a Tecan infinite

M plex reader with Magellan Pro V7.4 software. The test results were considered positive within an OD ratio (defined as absorbance difference between control and study sample) of 1 with an upper detection limit at 13.

SARS-CoV-2 NCAP immunoassay

Antibodies targeting the nucleocapsid protein of SARS-CoV-2 were detected by the pan-immunoglobulin electrochemiluminescence immunoassay (ECLIA) Elecsys Anti-SARS-CoV-2 (Roche Diagnostics) using the Cobas 8000 (Roche Diagnostics). Samples ≥ 1 COI (cutoff index) were interpreted as reactive, according to the manufacturers' instructions.

Ex vivo T cell stimulation

PBMC were cultivated at a concentration of 5×10^6 PBMC/ml in AB-medium containing RPMI 1640 medium (Gibco) supplemented with 10% heat inactivated AB serum (Pan Biotech), 100 U/ml of penicillin (Biochrom), and 0.1 mg/mL of streptomycin (Biochrom). Stimations were conducted with PepMix overlapping peptide pools (15 aa length with 11 aa overlaps, JPT Peptide Technologies) covering the dominant SARS-CoV-2 Orfeome spanning peptides including (Pan-SARS-CoV-2 Select, 103 peptides, HLA-I and HLA-II restricted epitopes (DE+S)) or excluding spike epitopes (Pan-SARS-CoV-2 Select w/o S, 73 peptides, HLA-I and HLA-II restricted epitopes (DE-S)). Pools with pan-coronaviridae cross-reactive epitopes including (31 peptides, XE+S) or excluding (30 peptides, XE-S) spike epitopes as identified in Mateus et al.¹⁵ were custom generated (JPT Peptide Technologies, [Table S2](#)). All stimulations were performed at final concentrations of 1 μ g/mL per peptide. For negative control the stimulation peptide solvent DMSO diluted 1:1 in PBS was used at the same concentration as in peptide-stimulated tubes. CEFX Ultra SuperStim pool (1 μ g/mL per peptide) (JPT Peptide Technologies) were used as positive stimulation controls. For optimized costimulation, purified anti-CD28 (clone CD28.2, BD Biosciences) was added to each stimulation at a final concentration of 1 μ g/mL. Incubation was performed at 37°C, 5% CO₂ for 16 h in the presence of 10 μ g/mL brefeldin A (Sigma-Aldrich) during the last 14 h. CD4⁺ T cell activation is shown as background subtracted (bgsub) value = % of CD40L⁺4-1BB⁺ CD4⁺ T cells in the stimulation - % of CD40L⁺4-1BB⁺ CD4⁺ T cells in the unstimulated control. Dotted lines indicate a bgsub value of 0.005 (weakly positive) and 0.015 (strongly positive).

Flow cytometry

Stimulations were stopped by incubation in 2 mM EDTA for 5 min. Surface staining was performed for 15 min in the presence of 1 mg/mL of Beriglobin (CSL Behring) with the following fluorochrome-conjugated antibodies titrated to their optimal concentrations: anti-CD3-FITC (Miltenyi Biotec), anti-CD4-VioGreen (Miltenyi Biotec), anti-CD8-VioBlue (Miltenyi Biotec). During the last 10 min of incubation, Zombie Yellow fixable viability staining (Biolegend) was added. Fixation and permeabilization were performed with eBioscience FoxP3 fixation and PermBuffer (Invitrogen) according to the manufacturer's protocol. Intracellular staining was carried out for 30 min in the dark at room temperature with anti-4-1BB-PE (Miltenyi Biotec), anti-CD40L-PEVio770 (Miltenyi Biotec), anti-IFN- γ -A700 (Biolegend) and anti-TNF- α -BV605 (Biolegend). All samples were measured on a MACSQuant Analyzer 16 (Miltenyi Biotec). Instrument performance was monitored prior to every measurement with Rainbow Calibration Particles (BD Biosciences).

QUANTIFICATION AND STATISTICAL ANALYSIS

Study data were collected and managed using REDCap electronic data capture tools hosted at Charité – Universitätsmedizin Berlin.^{46,47} Flow cytometry data were analyzed with FlowJo 10.6 (FlowJo LLC) and statistical analysis conducted with GraphPad Prism 9. If not stated otherwise, data are plotted as mean. Comparison of datasets was performed with Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and not significant for $p > 0.05$ (not indicated). Correlations were analyzed with two-tailed Spearman correlation. r and p values are given in [Table S1](#). * $p < 0.0332$, ** $p < 0.0021$, *** $p < 0.0002$ only indicated when significant. Lines display robust nonlinear regression (curve fit).