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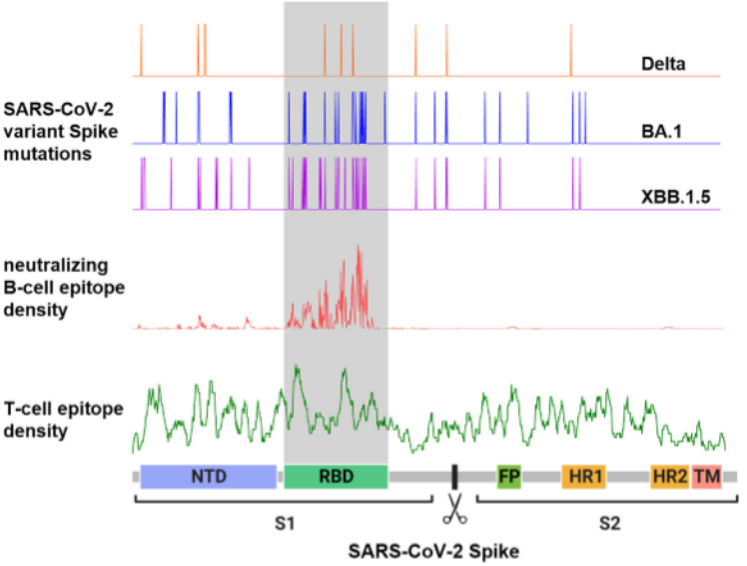
Progressive loss of conserved spike protein neutralizing antibody sites in Omicron sublineages is balanced by preserved T cell immunity

Graphical abstract



NTD, N-terminal domain; RBD, Receptor-binding domain; FP, Fusion peptide; HR1, Heptad repeat 1; HR2, Heptad repeat 2; TM, Transmembrane domain; S1, S1-fragment; S2, S2-fragment

Authors

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In brief

Muik et al. show that although recent SARS-CoV-2 Omicron sublineages exhibit an increasingly effective evasion of neutralizing B cell immunity, spike-protein-specific T cell responses remain largely unaffected. Their observations indicate that preserved T cell immunity may mitigate diminished neutralizing antibody activity in preventing or limiting severe COVID-19.

Highlights

- Omicron evades vaccine-elicited immunity by altering neutralizing B cell epitopes
- XBB.1.5 even escapes neutralization by Omicron-convalescent vaccinee sera
- Most T cell spike epitopes are conserved across the Omicron lineage, including XBB.1.5
- T cell immunity of vaccinated individuals against Omicron remains largely intact

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Progressive loss of conserved spike protein neutralizing antibody sites in Omicron sublineages is balanced by preserved T cell immunity

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SUMMARY

Evolution of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Omicron variant has led to the emergence of sublineages with different patterns of neutralizing antibody evasion. We report that Omicron BA.4/BA.5 breakthrough infection of individuals immunized with SARS-CoV-2 wild-type-strain-based mRNA vaccines results in a boost of Omicron BA.4.6, BF.7, BQ.1.1, and BA.2.75 neutralization but does not efficiently boost BA.2.75.2, XBB, or XBB.1.5 neutralization. *In silico* analyses showed that the Omicron spike glycoprotein lost most neutralizing B cell epitopes, especially in sublineages BA.2.75.2, XBB, and XBB.1.5. In contrast, T cell epitopes are conserved across variants including XBB.1.5. T cell responses of mRNA-vaccinated, SARS-CoV-2-naive individuals against the wild-type strain, Omicron BA.1, and BA.4/BA.5 were comparable, suggesting that T cell immunity against recent sublineages including XBB.1.5 may remain largely unaffected. While some Omicron sublineages effectively evade B cell immunity, spike-protein-specific T cell immunity, due to the nature of polymorphic cell-mediated immune responses, may continue to contribute to prevention/limitation of severe COVID-19 manifestation.

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Omicron variant of concern (VOC), which emerged in November 2021, contains over 30 amino acid alterations in its spike (S) glycoprotein, as compared to the original Wuhan-Hu-1 (wild-type) strain, that mediate partial escape from previously established humoral immunity.[1–3] Omicron sublineages BA.1, BA.2, BA.4, and BA.5 consecutively dominated the pandemic landscape. The capacity to escape vaccine- and infection-elicited neutralizing antibodies became more pronounced in the more recent sublineages, prompting the development and leading to the authorization of BA.1- and BA.4/BA.5-adapted vaccines.[4,5] While BA.5 was the globally dominant sublineage during the second half of 2022, Omicron BA.2.75, BA.2.75.2, BA.4.6, BF.7, BQ.1.1, and XBB have locally increased in late 2022 and early 2023.[6–8] More recently, XBB.1.5 displaced other sublineages and became the next globally dominant variant.[7–9]

Omicron BA.2.75 differs in five amino acids within the N-terminal domain from its parental sublineage BA.2 and previous Omicron VOCs (Figure S1). BA.2.75.2 differs from BA.2.75 in the receptor-binding domain (RBD) alterations R346T and F486S. Omicron BA.4.6 and BF.7 are identical in their S glycoprotein sequence (called Omicron BA.4.6/BF.7 S in this study), which differs from that of their respective parental sublineages, BA.4 and BA.5 (called Omicron BA.4/BA.5 S in this study due to sequence identity) by a single R346T amino acid alteration. BQ.1.1 in turn is distinguished from BA.4.6/BF.7 by the K444T and N460K

alteration. The S protein of XBB, which emerged through recombination between two BA.2-descendant sublineages,[10] is altered at seven amino acid positions including R346T relative to Omicron BA.1, BA.2, and BA.4/5. Its descendant XBB.1.5 has acquired the additional G252V alteration and S486P in relation to XBB. Importantly, BQ and XBB sublineages are not neutralized by any authorized therapeutic antibody.[10,11] Also, the combination of cilgavimab or tixagevimab (Evusheld), which is used for pre-exposure COVID-19 prophylaxis in immunocompromised patients, is not effective against the current VOCs.[11–14]

While vaccine- or infection-elicited neutralizing antibodies are a critical component of sterilizing immunity against SARS-CoV-2, T cell responses are known to contribute to the adaptive anti-viral immune response as well and constitute an important additional layer of protective immunity.[15,16] Cytotoxic $CD8^+$ T cells that recognize HLA class I-presented peptide epitopes play a critical role in reducing susceptibility to severe COVID-19 disease, hospitalization, and death.[17,18] HLA class II-dependent $CD4^+$ helper T cells orchestrate the immune response by their pleiotropic functions and have an important role in the prevention of severe disease.[19,20]

It is to be expected that SARS-CoV-2 evolution will continue. Real-time understanding of the transmissibility, pathogenicity, and immune-evasion properties of new variants and variant sublineages in conjunction with dynamic immunity patterns in the human population that are shaped through repeated infections with different VOCs and different primary and booster vaccination cycles will continue to be critical in assessing the level of risk to public health going forward.

In the face of this dynamic situation, we have set up a COVID-19 pandemic preparedness and rapid response strategy. Our approach includes screening emerging and circulating variants through an artificial intelligence-/machine-learning-based early warning system,[21] testing prototypical mRNA vaccines adapted to these variants in mouse studies,[16] and mapping SARS-CoV-2 T cell epitopes recognized by the human T cell repertoire.[19,20] Further, studying the neutralizing antibody activity of individuals following breakthrough infections with the latest circulating variants allows us to detect potential immune escape patterns early and informs on the need for rapid vaccine adaptation strategies.[16–18,22,23]

As part of this work, our current study assessed Omicron sublineages BA.4.6, BF.7, BA.2.75, BA.2.75.2, BQ.1.1, XBB, and XBB.1.5, as these sublineages have collectively displaced BA.5.

RESULTS

Omicron BA.4/BA.5 breakthrough infection of triple-mRNA-vaccinated individuals mediates partial cross-neutralization of some Omicron sublineages, yet BA.2.75.2, XBB, and XBB.1.5 largely evade neutralization

We investigated neutralizing activity of immune sera from individuals who received three or four doses of SARS-CoV-2 wild-type S-glycoprotein-based mRNA COVID-19 vaccines (BNT162b2/mRNA-1273 homologous or heterologous regimens) with or without subsequent breakthrough infection by different Omicron sublineages. Specifically, the following cohorts were investigated: BNT162b2-triple-vaccinated, SARS-CoV-2-naive individuals ($BNT162b2^3$; n = 18; age <55 years), BNT162b2-quadruple-vaccinated, SARS-CoV-2-naive elderly (>60 years) individuals ($BNT162b2^4$; n = 15), and triple-mRNA-vaccinated (BNT162b2 or mRNA-1273) individuals who experienced breakthrough infection with Omicron BA.1 ($mRNA\text{-}Vax^3$ + BA.1; n = 14), BA.2 ($mRNA\text{-}Vax^3$ + BA.2, n = 19), or BA.4/BA.5 ($mRNA\text{-}Vax^3$ + BA.4/BA.5, n = 17) (Table S1). With a fourth vaccine dose being recommended for the elderly, and given the high frequency of breakthrough infections with Omicron sublineages, we considered this study population to be representative for a substantial proportion of the European and North American population. Serum neutralizing activity was tested in a well-characterized pseudovirus neutralization test (pVNT)[22–24] by determining 50% pseudovirus neutralization (pVN_{50}) geometric mean titers (GMTs) for pseudoviruses bearing the S glycoproteins of the SARS-CoV-2 wild-type strain or relevant Omicron sublineages.

In the triple-/quadruple-vaccinated individuals without breakthrough infection, pVN_{50} GMTs against Omicron BA.4/BA.5 were 5- to 6-fold lower than GMTs against the wild-type strain (GMTs against BA.4/BA.5 were 69 and 121, respectively) (Figure 1A). GMTs against BA.4/BA.5 were similarly reduced in BA.1 convalescent individuals (GMT 263, 5-fold lower than wild type), whereas in the BA.2- and BA.4/BA.5-convalescent cohorts, titers against BA.4/BA.5 remained higher (GMTs of 386 and 521, respectively; 3- and 2-fold lower than wild type).

In all three convalescent cohorts, neutralizing antibody titers against Omicron BA.4.6/BF.7 and BA.2.75 were higher than those of triple-/quadruple-vaccinated, SARS-CoV-2-naive individuals (GMTs 239–525 compared with 55–139, respectively). Among convalescent individuals, Omicron BA.4.6/BF.7 and BA.2.75 GMTs were largely comparable with no significant differences from those against Omicron BA.4/BA.5. In contrast, pVN_{50} titers against Omicron BQ.1.1, BA.2.75.2, XBB, and XBB.1.5 were significantly lower than those against BA.4/BA.5 across cohorts. Titers against BQ.1.1 were overall very low in the SARS-CoV-2-naive, vaccinated cohorts and BA.1 convalescent individuals (GMTs $\leq$ 38) and moderately higher in the BA.2- and BA.4/BA.5-convalescent cohorts (GMTs 100 and 154, respectively). Titers against BA.2.75.2, XBB, and XBB.1.5 were low across cohorts (GMTs $\leq$88, $\leq$33, and $\leq$49, respectively).

To assess neutralization breadth irrespective of magnitude, we normalized the Omicron sublineage pVN_{50} GMTs against those for the wild-type strain. GMT ratios for all Omicron subvariant pseudoviruses were comparable between the $BNT162b2^3$ and $BNT162b2^4$ cohorts (Figure 1B). Hence, both neutralizing antibody titers and variant cross-neutralization were broadly similar in quadruple-vaccinated elderly individuals and triple-vaccinated younger individuals. GMT ratios were in the range of 0.09–0.22 for BA.4/BA.5, BA.4.6/BF.7, and BA.2.75 and $\leq$0.05 for BQ.1.1, BA.2.75.2, XBB, and XBB.1.5 in both cohorts.

Cross-neutralization of BA.4/BA.5 and BA.4.6/BF.7 was significantly ($p < 0.05$) higher in sera from BA.2 convalescent individuals as compared to triple-vaccinated individuals (GMT ratios: 0.37 vs. 0.17 for BA.4/BA.5 and 0.23 vs. 0.12 for BA.4.6/BF.7) and even more so in BA.4/BA.5 convalescent individuals for both the BA.4/BA.5 pseudovirus (GMT ratio: 0.48, $p < 0.01$ vs. $BNT162b2^3$) and the BA.4.6/BF.7 pseudovirus

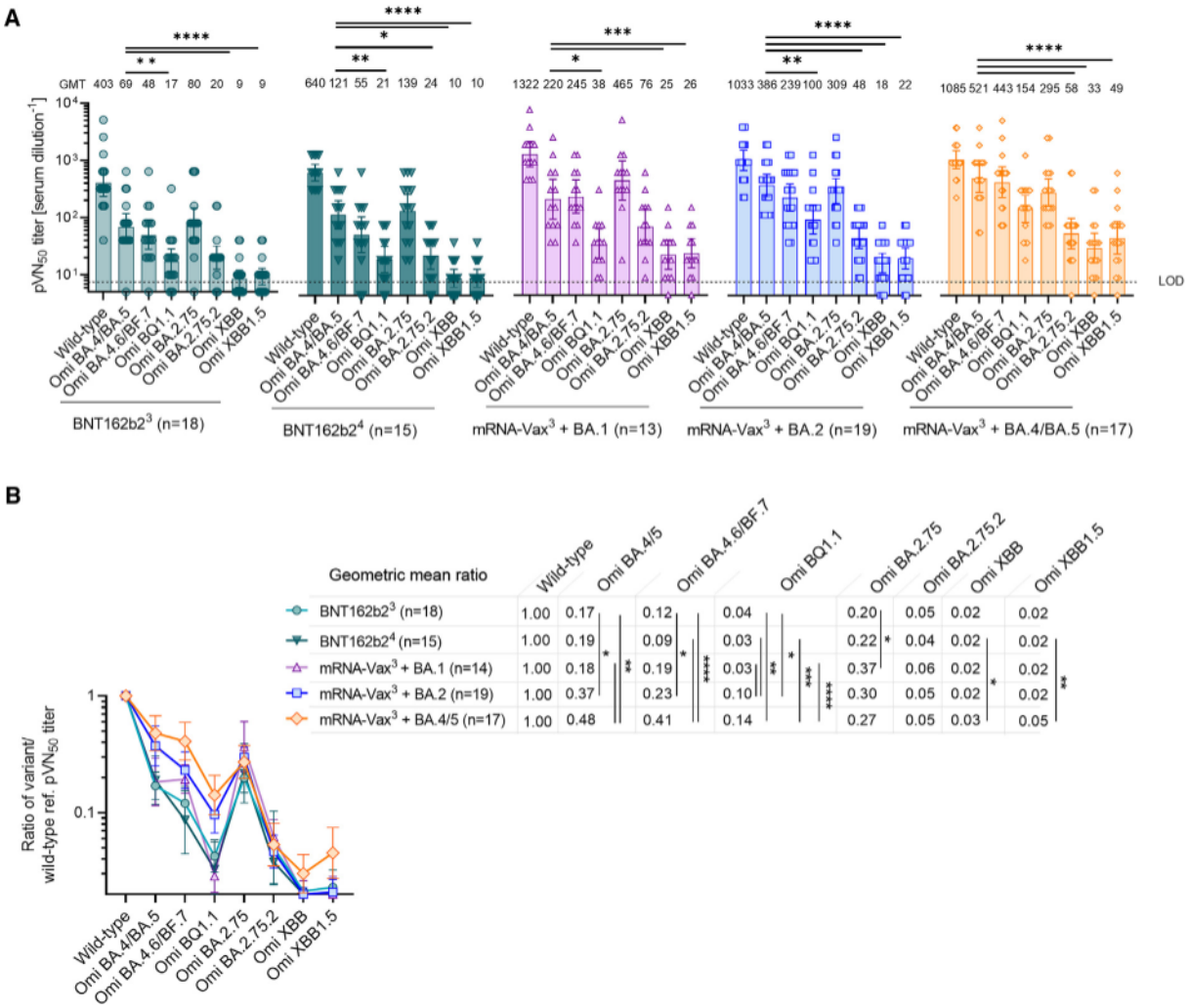

Figure 1. Omicron BA.4/BA.5 breakthrough infection of triple-mRNA-vaccinated individuals mediates partial cross-neutralization of some Omicron sublineages, yet BA.2.75.2, XBB, and XBB.1.5 largely evade neutralization

Cohorts and serum sampling as shown in Table S1.

(A) 50% pseudovirus neutralization (pVN_{50}) geometric mean titers (GMTs) against the indicated SARS-CoV-2 wild-type strain or Omicron variants of concern (VOCs). Values above bar graphs represent group GMTs. For titer values below the limit of detection (LOD), LOD/2 values were plotted. Each data point represents the geometric mean of two technical replicates. The non-parametric Friedman test with Dunn's multiple comparisons correction was used to compare neutralizing titers against the Omicron BA.4/BA.5 pseudovirus with titers against the other pseudoviruses. Multiplicity-adjusted p values are shown.

(B) SARS-CoV-2 VOC pVN_{50} GMTs normalized against the wild-type strain pVN_{50} GMT (ratio VOC to wild type). Group geometric mean ratios with 95% confidence intervals are shown. The non-parametric Kruskal-Wallis test with Dunn's multiple comparisons correction was used to compare the VOC GMT ratios between cohorts. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

(GMT ratio: 0.41, $p < 0.0001$). Cross-neutralization of BA.4.6/BF.7 was also significantly ($p < 0.0001$) stronger in BA.4/BA.5 convalescent individuals compared with quadruple-vaccinated individuals. While BQ.1.1 was cross-neutralized less efficiently than BA.4/BA.5 in all cohorts (GMT ratios $\leq$ 0.14), cross-neutralization in BA.4/BA.5 and BA.2 convalescent individuals remained significantly stronger compared with SARS-CoV-2-naive, triple- or quadruple-vaccinated and BA.1-breakthrough-infected cohorts. Cross-neutralization of BA.2.75, BA.2.75.2, XBB, and XBB.1.5 pseudoviruses was broadly comparable across cohorts. GMT ratios were in the range of 0.20–0.37 for BA.2.75 and $\leq$0.06 for BA.2.75.2, XBB, and XBB.1.5.

Together, these data show that partial neutralization of some Omicron sublineages is retained, being broadest in BA.4/BA.5-convalescent individuals. In contrast, sublineages BA.2.75.2, XBB, and XBB.1.5 have evolved to largely evade neutralizing antibody responses in vaccinated individuals and in those who experienced breakthrough infections with previous Omicron sublineages.

S glycoprotein neutralizing antibody epitopes are progressively lost in the Omicron lineage

To assess the degree of B cell epitope conservation across VOCs, we analyzed a total of 454 unique neutralizing B cell epitopes

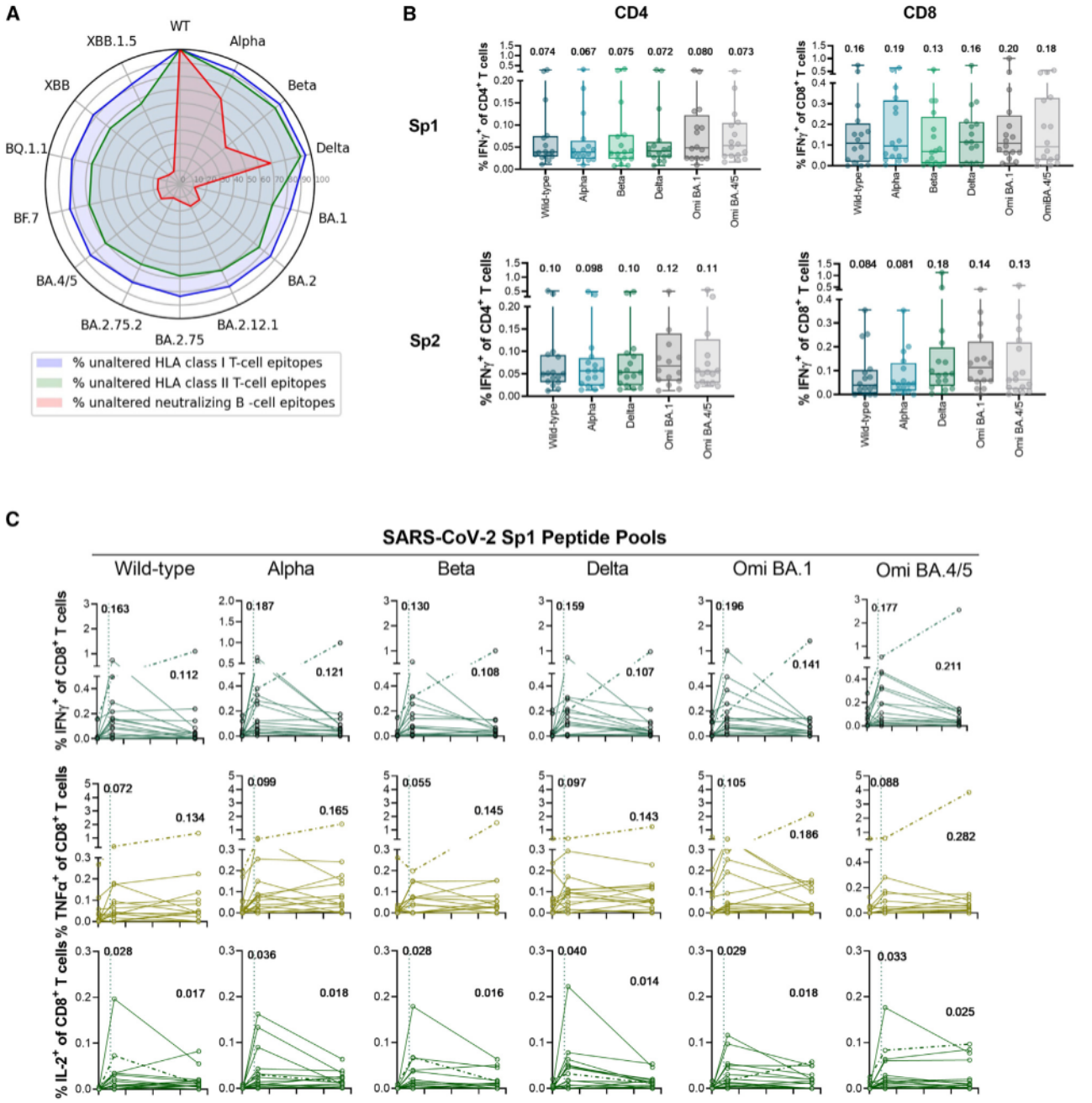

Figure 2. T cell epitopes of the wild-type SARS-CoV-2 S glycoprotein and T cell responses against S glycoprotein peptides are largely preserved across variants

(A) Percentages of unaltered neutralizing B cell epitopes (restricted to NTD and RBD) present in each variant strain compared to SARS-CoV-2 wild type are represented by the red shaded area (see also Table S2). Percentages of unaltered S glycoprotein linear HLA class I and class II T cell epitopes present in each variant strain as compared to SARS-CoV-2 S wild type are shown in blue (HLA class I) and green (HLA class II) shaded area (see also Table S3). The B cell epitopes were either retrieved from the IEDB on November 25, 2022, or were calculated from resolved antigen-antibody protein structures deposited in the Coronavirus Antibody Database (CoV-AbDab)[27] using a similar computational method to the one used in Beguir et al.[21] T cell epitopes were retrieved from the IEDB on November 29, 2022.

(B) IFN-γ cytokine response of $CD8^+$ and $CD4^+$ T cells after stimulation with SARS-CoV-2 S glycoprotein peptide pools corresponding to S glycoprotein aa 1–643 (Sp1) or 633–1,273 (Sp2) of different variant strains. Blood was drawn from n = 16 individuals 7 days after the second dose of BNT162b2 (cohorts and blood sampling as shown in Table S4). Each data point represents a single measurement per individual sample, with numbers above whiskers indicating the group arithmetic mean percentage of IFN-γ^+ cells. The non-parametric Friedman test with Dunn's multiple comparisons correction was used to compare IFN-γ cytokine responses after variant stimulation with those after SARS-CoV-2 wild-type S peptide pool stimulation. *$p < 0.05$.

(legend continued on next page)

within the S glycoprotein, consisting of experimentally confirmed epitopes from the Immune Epitope Database (IEDB) and epitopes computationally deduced from structures of SARS-CoV-2 neutralizing antibodies from the Coronavirus Antibody Database (CoV-AbDab) as described in Beguir et al.[21] To eliminate partial epitopes, we only took into account epitopes consisting of five amino acids or more. Of these 454 epitopes, 412 (91%) included a position that was altered in at least one of the analyzed variants.

We found that B cell epitopes were partially conserved in the earlier variants Alpha, Beta, and Delta (≥44%) (Figure 2A; Table S2), whereas most of these epitopes were altered in the Omicron BA.1 variant and remained so for subsequent Omicron sublineages (≤19% conservation). This was particularly the case in BA.2.75.2, XBB, and XBB.1.5 (≤12% conservation), explaining the gradual loss of cross-neutralization we had observed. We next analyzed the percentage of altered epitopes corresponding to neutralizing antibodies previously categorized through their capacity to block ACE2 binding and to bind the RBD in an "up" or "down" conformation.[25,26] RBD epitopes of all classes showed a low degree of conservation (≤25% of epitopes were conserved) across the Omicron lineage (Table S2). Essentially all RBD epitopes within the ACE2-binding site (corresponding to antibody classes 1 and 2), as well as epitopes corresponding to non-ACE2-blocking antibodies that bind the RBD in an "up" conformation (class 4), were altered. Conservation of N-terminal domain (NTD) epitopes was more variable between Omicron sublineages, with most epitopes being altered for BA.1, BA.2.75, BA.2.75.2, XBB, and XBB.1.5 (≤25% conservation). Epitopes of the other tested sublineages showed a higher degree of conservation (≥68%).

T cell epitopes display a high degree of conservation across the Omicron lineage and previous VOCs

Neutralizing antibodies are an important, but not the sole, component of immunity against SARS-CoV-2.[16] To estimate the impact of SARS-CoV-2 evolution on T cell immunity, we assessed the degree of conservation of T cell epitopes in the S glycoproteins of VOCs. To this aim, we filtered experimentally confirmed SARS-CoV-2 epitopes reported for HLA class I and II alleles from the IEDB database. In total, 594 and 559 unique epitopes were collected for HLA class I and class II, respectively. For stringency, we only retained minimal epitopes of either HLA class I or HLA class II experimentally confirmed in a human host, removed any inferred or predicted entries, and filtered epitopes by length (8–14 for HLA class I and 12–20 for HLA class II). 292 unique HLA class I epitope sequences remained, of which 260 were found in the wild-type strain S glycoprotein. Of these, 71 epitopes (27%) included a position reported to be mutated in at least one analyzed variant. Of 468 HLA class II epitopes found in the wild-type strain S glycoprotein, 230 (49%) included an alteration in at least one analyzed variant. HLA class I and class II epitopes post-filtering covered, in total, 40 unique alleles for both class I and class II.

About 90% of $CD8^+$ and $CD4^+$ T cell epitopes of the wild-type S glycoprotein were fully conserved in the Alpha, Beta, and Delta variants (Figure 2A; Table S3), and over 80% of $CD8^+$ and ~70% $CD4^+$ T cell epitopes were conserved in Omicron sublineages. The degree of $CD8^+$ and $CD4^+$ T cell epitope conservation was marginally lower in the most recent sublineages BA.2.75.2, BQ.1.1, XBB, and XBB.1.5 (81%–82% for $CD8^+$, 66%–67% for $CD4^+$) and in previous ones (84%–86% for $CD8^+$, 68%–75% for $CD4^+$). Altogether, these data suggested that T cell responses against Omicron sublineages may remain largely intact in individuals immunized with wild-type-strain-based vaccines.

SARS-CoV-2 wild-type S mRNA-vaccine-elicited T cell responses are cross-reactive to Omicron sublineages

To test this assumption, we compared $CD8^+$ and $CD4^+$ T cell responses of BNT162b2 double-vaccinated individuals against the wild-type strain and the Alpha, Beta, Delta, Omicron BA.1, and BA.4/5 VOCs by analyzing T cell cytokine production upon stimulation with variant-specific S protein peptide pools representing the N-terminal (Sp1) and the C-terminal (Sp2) halves of the S protein. Seven days after the second vaccination, proportions of $CD8^+$ and $CD4^+$ T cells secreting interferon γ (IFN-γ) in response to Alpha, Beta, Delta, Omicron BA.1, or BA.4/5 S protein peptides were comparable to or slightly higher than those against wild-type S protein peptides (Figure 2B), as were proportions of T cells secreting interleukin-2 (IL-2) and tumor necrosis factor α (TNF-α) (Figures 2C and S2A).

While, on average, $CD8^+$ T cell responses contracted over time, they were still detectable 5.5 months after the second vaccination at levels comparable across all tested variants (Figure 2C). Contraction of S-glycoprotein-specific $CD8^+$ T cells was most prominent for individuals with a high initial response, whereas in individuals with a moderate initial response, cytokine production remained constant or slightly increased over the course of 5.5 months post-vaccination.

$CD4^+$ T cells showed a Th1-type cytokine profile characterized by IL-2 production in addition to IFN-γ and TNF-α, whereas proportions of cells secreting the Th2/Th17 cytokines IL-4 and IL-17 were much smaller (Figure S2B). Kinetics of Th1 $CD4^+$ T cell responses in the 5.5 months following the booster vaccination were comparable to those observed for S protein-specific $CD8^+$ T cells.

These findings show that the magnitude and quality of S-protein-specific T cell responses are similar across the main SARS-CoV-2 lineages including Omicron in BNT162b2-vaccinated individuals.

DISCUSSION

In summary, our findings provide insights into two mechanisms: first, the neutralizing activity of human sera against previously or currently circulating VOCs, and second, the potential of the

(C) Cytokine responses of $CD8^+$ T cells after stimulation as in (B) before the first dose, 7 days after the second dose, and 5.5 months after the second dose of BNT162b2. Each data point represents a single measurement per individual sample, with numbers above symbols indicating the group arithmetic mean percentage of cytokine-secreting cells per time point. The participant represented by the dashed line showed a SARS-CoV-2 nucleocapsid-specific T cell response at the 5.5-month assessment, indicative of a breakthrough infection. See also Figure S2 for cytokine responses of $CD4^+$ T cells after stimulation.

SARS-CoV-2 S-glycoprotein-directed $CD8^+$ and $CD4^+$ T cell repertoire to cross-recognize variant strains.

With regard to humoral immunity, breakthrough infection with SARS-CoV-2 variant strains is known to shape serum neutralizing activity of individuals immunized with wild-type-strain-based vaccines by eliciting memory B cell recall responses recognizing conserved epitopes. We show that breakthrough infection of vaccinated individuals with Omicron BA.4/BA.5 refocuses neutralizing antibody responses toward neutralization of BA.4/BA.5 itself, which goes along with partial cross-neutralization of BA.4.6/BF.7, BA.2.75, and, to a lower degree, BQ.1.1 while displaying poor activity against Omicron BA.2.75.2, XBB, and XBB.1.5.

That the Omicron BA.4.6/BF.7-neutralizing activity and even more so the neutralization of BQ.1.1 by sera from SARS-CoV-2-naive, vaccinated individuals and BA.2 convalescent individuals are further reduced as compared to their activity against BA.4/BA.5 suggests that the hallmark alterations R346T and N460K of these sublineages mediate further immune escape. Convergent evolution of the RBD at these critical residues in Omicron BA.4.6, BF.7, BQ.1.1, BA.2.75.2, XBB, and XBB.1.5[7,28] suggests that the resulting immune evasion may confer a growth advantage. Reduced neutralization of BA.2.75.2 compared with BA.2.75 confirms a prominent role of R346T as well as of alterations at the F486 residue in escape from neutralizing antibodies.[29]

Consistent with previous reports, we show that cross-neutralization of Omicron BA.2.75 and BA.4/BA.5 are broadly comparable,[13,30,31] indicating that factors other than immune evasion contribute to the growth advantage of BA.2.75 over BA.5. Minor differences in the sensitivity of Omicron BA.2.75 and BA.4/BA.5 to neutralization by BA.1- and BA.4/BA.5-convalescent sera may indicate amino acid changes with a context-dependent role in immune evasion.

While our findings are supportive for the suitability of Omicron BA.4/BA.5-adapted vaccines to boost neutralizing activity against some BA.4/BA.5-descendant variants, they also reveal that Omicron sublineages continue to accumulate mutations that disrupt critical B cell neutralization epitopes and that further boosters or variant adaptations may be required in future.

With regard to cell-mediated immunity, we showed that $CD8^+$ and $CD4^+$ T cell responses of vaccinated individuals against Omicron BA.1 and BA.4/5 are comparable to those against the wild-type strain. Given that HLA class I- and class II-presented T cell epitopes remained mostly unaltered across the entire evolution of SARS-CoV-2, T cell immunity against Omicron BQ.1.1, BA.2.75.2, XBB, and XBB.1.5 may be largely intact, despite profound neutralizing antibody evasion. This finding is consistent with previous published reports describing degenerate T cell epitopes located within conserved S protein regions[32] and showing that wild-type-strain-vaccinated individuals retain T cell immunity against Omicron BA.1.[33–35] A fundamental difference of T cells vs. antibodies is that that the T cell-mediated layer of immunity is more robust against population-level breaches by VOCs, due to the highly polymorphic nature of HLA molecules presenting linear epitopes.[36–38] Our observations indicate that T cell immunity may mitigate the lack of neutralizing antibody activity in preventing or limiting severe COVID-19 and further encourages development of vaccine formats that induce functional $CD8^+$ T cells against SARS-CoV-2 antigens concurrently to boosting antibody responses.

Limitations of the study

Our investigation is a retrospective analysis of cohorts of small sample size. These cohorts are not completely balanced with regard to demographic characteristics such as age and sex of individuals and are not fully aligned in terms of intervals between vaccine doses and between the last vaccine dose and infection as well as between the most recent antigen exposure and blood sampling. While most of these factors differ only mildly across cohorts, the large age difference between participants of the $BNT162b2^3$ and $BNT162b2^4$ cohorts resulting from the vaccination recommendations at the time of the trial may confound magnitude and breadth of neutralizing activity. While our study focused on neutralizing activity as a directly measurable and robust correlate of protection, Fc-mediated antibody effector functions were also shown to contribute to anti-viral immunity.[39,40] Hence, Fc-mediated effector functions may confer protective immunity along with T cell responses, especially when neutralizing antibody responses come up short due to immune evasion or decaying titers.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2023.112888.

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AUTHOR CONTRIBUTIONS

U.S., O.T., and A.M. conceived and conceptualized the work. A.M., J.Q., I.V., A.P., K.B., and B.G.L. planned and supervised experiments and epitope analyses. G.R.-Y., O.O., K.G., J. Grosser, N.K., S.H., and S.C. coordinated and conducted sample collection. K.G., Y.L., and G.R.-Y. coordinated sample shipments and clinical data transfer. B.G.L., J. Gordon, and M.B. performed experiments. A.M., B.G.L., J.Q., A.P., Y.F., and H.D. analyzed data. U.S., O.T., A.M., A.P., J.Q., A.T., and B.G.L. interpreted data and wrote the manuscript. All authors supported the review of the manuscript.

DECLARATION OF INTERESTS

U.S. and O.T. are management board members and employees at BioNTech SE. A.M., B.G.L., J.Q., M.B., A.T., J. Gordon, J. Grosser, I.V., and O.O. are employees at BioNTech SE. A.P. and H.D. are employees at BioNTech US. Y.F. and K.B. are employees of InstaDeep, Ltd. K.G., N.K., S.H., and S.C. are employees at the University Hospital, Goethe University Frankfurt. U.S., O.T., A.P., and A.M. are inventors on patents and patent applications related to RNA technology and COVID-19 vaccines. U.S., O.T., A.M., B.G.L., J.Q., M.B., A.T. A.P., H.D., J. Gordon, J. Grosser, I.V., and O.O. have securities from BioNTech SE. S.C. has received an honorarium for serving on a clinical advisory board for BioNTech SE.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-VSV-G (clone 8G5F11)	Kerafast Inc.	Cat#EB0010; RRID: AB_2811223
BD Horizon™ BV421 Mouse Anti-Human CD3 (clone UCHT1)	BD Biosciences	Cat#562426; RRID:AB_1115208
BD Horizon™ BV421 Mouse Anti-Human CD3 (clone RPA-T4)	BD Biosciences	Cat#746541; RRID:AB_2743832
BD Horizon™ BB515 Mouse Anti-Human CD8 (clone RPA-T8)	BD Biosciences	Cat#564526; RRID:AB_2744458
BD Horizon™ BB700 Mouse Anti-Human IFN-γ (clone B27)	BD Biosciences	Cat#566394; RRID:AB_2744484
BD Pharmingen™ APC Rat Anti-Human IL-4 (clone MP4-25D2)	BD Biosciences	Cat#554486; RRID:AB_398562
BD Pharmingen™ PE Rat Anti-Human IL-2 (clone MQ1-17H12)	BD Biosciences	Cat#554566; RRID:AB_395483
Bacterial and virus strains		
SARS-CoV-2 wild-type and Omicron BA.4/BA.5 lineage VSV pseudotypes	Muik et al.[23]	N/A
SARS-CoV-2 Omicron BA.4.6/BF.7, BQ.1.1, BA.2.75, BA.2.75.2, XBB and XBB.1.5 lineage VSV pseudotypes	This paper	N/A
Biological samples		
Serum samples from SARS-CoV-2 naive, triple-BNT162b2-vaccinated adults <55 years of age	BNT162 clinical trial NCT05004181	N/A
Serum samples from SARS-CoV-2 naive, quadruple-BNT162b2-vaccinated adults >60 years of age	BNT162 clinical trial NCT04955626	N/A
Serum samples from SARS-CoV-2 triple-mRNA vaccinated individuals with confirmed subsequent SARS-CoV-2 breakthrough infection during a period of Omicron BA.1, BA.2 or BA.4/5 lineage dominance	University Hospital, Goethe University Frankfurt Quandt et al.[41] Muik et al.[23] Muik et al.[42]	N/A
PBMC samples from SARS-CoV-2 naive, double-BNT162b2-vaccinated adults	BNT162 clinical trial NCT04380701	N/A
Chemicals, peptides, and recombinant proteins		
SARS-CoV-2 wild-type, Alpha, Beta, Delta, Omicron BA.1 and BA.4/5 S protein peptide pool Sp1 (encompassing aa 1–643)	JPT Peptide Technologies GmbH	Custom Orders: WT: 48886BAR-01; Beta: 47305BAR-01; Alpha: 48549BAR-01; Delta: 49114CHe-01; BA.1: 51849BAR-02; Off the Shelf Peptide Pool: BA.4/5: A10485-01 – Lot: 2452064
SARS-CoV-2 wild-type, Alpha, Beta, Delta, Omicron BA.1 and BA.4/5 S protein peptide pool Sp2 (encompassing aa 633-1,273)	JPT Peptide Technologies GmbH	Custom Orders: WT: 48886BAR-02; Alpha: 48549BAR-02; Delta: 49114CHe-02; BA.1: 51849BAR-03; Off the Shelf Peptide Pool: BA.4/5: A10485-01 – Lot: 2373881
Brefeldin A (BD Golgi Plug)	BD Biosciences	Cat#51-2301KZ
Fixable Viability Dye eFluor™ 780	eBioscience	Cat#65-0865-14
BD Horizon Brilliant Stain Buffer 1000 tests	BD Biosciences	Cat#566349

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
BD Horizon Brilliant Stain Buffer Plus	BD Biosciences	Cat#566385
Critical commercial assays		
Bright-Glo™ Luciferase Assay System	Promega	Cat#E2620
Fixation/Permeabilization Solution Kit (250 Test)	BD Biosciences	Cat#554714
Lipofectamine™ LTX with PLUS™ Reagent	Life Technologies	Cat#15338100
Deposited data		
Experimental models: Cell lines		
HEK293T/17	ATCC	CRL-11268; RRID:CVCL_1926
Vero 76	ATCC	CRL-1587; RRID:CVCL_0603
Recombinant DNA		
pcDNA3.1-SARS-CoV-2-S-BA.4.6/BF.7	This paper	N/A
pcDNA.3.1-SARS-CoV-2-S-BQ.1.1	This paper	N/A
pcDNA.3.1-SARS-CoV-2-S-BA.2.75	This paper	N/A
pcDNA.3.1-SARS-CoV-2-S-BA.2.75.2	This paper	N/A
pcDNA.3.1-SARS-CoV-2-S-XBB	This paper	N/A
pcDNA.3.1-SARS-CoV-2-S-XBB.1.5	This paper	N/A
Software and algorithms		
FlowJo Version 10.8.1	BD Biosciences	https://www.flowjo.com/
GraphPad Prism 9	GraphPad Software Inc.	https://www.graphpad.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ugur Sahin (Ugur.Sahin@biontech.de).

Materials availability

Materials used in this study are available from the lead contact upon reasonable request under a material transfer agreement with BioNTech.

Data and code availability

- The published paper includes all data generated or analyzed during the study.
- 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.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Samples from human participants

The vaccination cohorts described in this report consist of SARS-CoV-2-naïve, double-BNT162b2-vaccinated adults (BNT162b2^2) 18–85 years of age, SARS-CoV-2-naïve, triple-BNT162b2-vaccinated adults <55 years of age (BNT162b2^3), and SARS-CoV-2-naïve quadruple-BNT162b2-vaccinated adults >60 years of age (BNT162b2^4). Individuals from the BNT162b2^2, BNT162b2^3 and BNT162b2^4 cohorts were participants in the Phase 1 trial BNT162-01 (NCT04380701), the Phase 2 trial BNT162-17 (NCT05004181) and the Phase 2 trial BNT162-16 Substudy F (NCT04955626), respectively. All participants provided written consent prior to participation in the respective study. All participants had no documented history of SARS-CoV-2 infection prior to vaccination. Participants were free of symptoms at the time of blood collection. Serum (cohorts BNT162b2^3 and BNT162b2^4) and PBMCs (cohort BNT162b2^2) were isolated from whole blood for downstream assays. Age, sex, SARS-CoV-2 status and intervals between vaccine doses and serum/blood draw of participants of this study are reported in Table S1 and Table S4.

The infection cohorts described in this report consist of triple-mRNA vaccinated individuals (BNT162b2 or mRNA-1273) who had a confirmed subsequent SARS-CoV-2 breakthrough infection which occurred either during a period of dominance of the Omicron BA.1 lineage (November 2021 to mid-January 2022; mRNA-Vax^3 + BA.1),[41] the Omicron BA.2 lineage (March to

May 2022; mRNA-Vax^3 + BA.2)[23] or the Omicron BA.4/BA.5 lineage in Germany (mid-June to mid-July 2022; mRNA-Vax^3 + BA.4/5).[42] Participants from the infection cohorts were recruited from University Hospital, Goethe University Frankfurt as part of a non-interventional study (protocol approved by the Ethics Board of the University Hospital [No. 2021-560]) of patients who had experienced Omicron breakthrough infection following vaccination for COVID-19. All participants provided written consent prior to participation in the study. Participants were free of symptoms at the time of blood collection. Serum was isolated from whole blood for downstream assays. Age, sex, SARS-CoV-2 status and intervals between vaccine doses, breakthrough infection and serum draw of participants of this study are reported in Table S1.

Serum was isolated by centrifugation of whole blood at 2000 x g for 10 min and cryopreserved until use. All sera were heat-inactivated in a 56°C water bath for 1 h prior to serological testing. Li-heparin blood samples were isolated by density gradient centrifugation using Ficoll-Paque PLUS (Cytiva). PBMCs were subsequently cryopreserved until use.

Cell lines

HEK293T/17 cells and Vero 76 cells were from ATCC (ATCC cat. no. CRL-11268, RRID:CVCL_1926, and CRL-1587, RRID:CVCL_0603, respectively). All cell lines were cultured using provider's guidelines and used as described in method details below.

METHOD DETAILS

Production of SARS-CoV-2 pseudovirus

Vesicular stomatitis virus (VSV) virions pseudotyped with SARS-CoV-2 S glycoprotein derived from either the wild-type strain (Wuhan-Hu-1, NCBI ref. 43740568), the Omicron BA.4/BA.5 variant (alterations: T19I, Δ24–26, A27S, Δ69/70, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, L452R, S477N, T478K, E484A, F486V, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K), the Omicron BA.4.6/BF.7 variant (alterations: T19I, Δ24–26, A27S, Δ69/70, G142D, V213G, G339D, R346T, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, L452R, S477N, T478K, E484A, F486V, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K), the Omicron BQ.1.1 variant (alterations: T19I, Δ24–26, A27S, Δ69/70, G142D, V213G, G339D, R346T, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, K444T, L452R, N460K, S477N, T478K, E484A, F486V, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K), the Omicron BA.2.75 variant (alterations: T19I, Δ24–26, A27S, G142D, K147E, W152R, F157L, I210V, V213G, G257S, G339H, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, G446S, N460K, S477N, T478K, E484A, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K), the Omicron BA.2.75.2 variant (alterations: T19I, Δ24–26, A27S, G142D, K147E, W152R, F157L, I210V, V213G, G257S, G339H, R346T, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, G446S, N460K, S477N, T478K, E484A, F486S, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K, D1199N), the Omicron XBB variant (alterations: T19I, Δ24–26, A27S, V83A, G142D, Δ144, H146Q, Q183E, V213E, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, N460K, S477N, T478K, E484A, F486S, F490S, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K), and the Omicron XBB.1.5 variant (alterations: T19I, Δ24–26, A27S, V83A, G142D, Δ144, H146Q, Q183E, V213E, G252V,G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, N460K, S477N, T478K, E484A, F486P, F490S, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K) were produced according to published pseudotyping protocols.[43] A diagram of S glycoprotein alterations in Omicron sublineages is displayed in Figure S1.

In brief, HEK293T/17 cells were transfected with Sanger sequencing-verified variant-specific SARS-CoV-2 S expression plasmid with Lipofectamine LTX (Life Technologies) following the manufacturer's instructions. At 24 h after transfection, the cells were infected at a multiplicity of infection (MOI) of three with VSV-G complemented VSVΔG vector. After incubation for 2 h at 37°C with 7.5% CO_2, cells were washed twice with phosphate buffered saline (PBS) before medium supplemented with anti-VSV-G antibody (clone 8G5F11, Kerafast Inc.) was added to neutralize residual VSV-G-complemented input virus. VSV-SARS-CoV-2-S pseudotype-containing medium was harvested 20 h after inoculation, passed through a 0.2 μm filter (Nalgene) and stored at −80°C. The pseudovirus batches were titrated on Vero 76 cells. The relative luciferase units induced by a defined volume of a SARS-CoV-2 wild-type strain S glycoprotein pseudovirus reference batch previously described in Muik et al., 2021,[24] that corresponds to an infectious titer of 200 transducing units (TU) per mL, was used as a comparator. Input volumes for the SARS-CoV-2 variant pseudovirus batches were calculated to normalize the infectious titer based on the relative luciferase units relative to the reference.

Quantification of serum SARS-CoV-2 pseudovirus neutralizing antibody titers

Serum SARS-CoV-2 neutralizing capability was measured using a VSV-based pseudovirus neutralization (pVNT) assay as previously described.[24,42] Neutralizing titers detected in the pVNT assay strongly correlate with those from authentic live SARS-CoV-2 virus neutralization assays.[23] Vero 76 cells were seeded in 96-well white, flat-bottom plates (Thermo Scientific) at 40,000 cells/well in medium 4 h prior to the assay and cultured at 37°C with 7.5% CO_2. Human serum samples were 2-fold serially diluted in medium with dilutions ranging from 1:10 to 1:10,240. VSV-SARS-CoV-2-S particles were diluted in medium to obtain 200 TU in the assay. Serum dilutions were mixed 1:1 with pseudovirus (n = 2 technical replicates per serum per pseudovirus) for 30 min at room temperature

before being added to Vero 76 cells and incubated at 37°C with 7.5% CO_2 for 24 h. Supernatants were removed and the cells were lysed with luciferase reagent (Promega). Luminescence was recorded on a CLARIOstar Plus microplate reader (BMG Labtech), and neutralization titers were calculated as the reciprocal of the highest serum dilution that still resulted in 50% reduction in luminescence. Results for all pseudovirus neutralization experiments were expressed as geometric mean titers (GMT) of duplicates. If no neutralization was observed, an arbitrary titer value of half of the limit of detection [LOD] was reported.

T cell peptide stimulation and intracellular cytokine staining

Stimulation of PBMCs was performed using two pools of synthetic peptides (JPT Peptide Technologies GmbH) representing different portions of the wild-type and variant sequence of SARS-CoV-2 S protein encompassing either the NTD, RBD, and intermittent sequences (Sp1, aa 1–643) or the S2 domain and intermittent sequences (Sp2, aa 633-1,273). The peptide pools consisted of 15-mer overlapping peptides covering the respective S protein portion with 11 aa overlap.

PBMCs were rested for 4 h and restimulated in a round-bottom 96-well plate at 1×10^6 PBMCs/well with variant peptide pools (2 μg/mL/peptide) in the presence of GolgiPlug (BD) for 18 h at 37°C. Cells were then stained for viability (fixable viability dye eFluor 780) and extracellularly stained with fluorescently labeled antibodies for CD4, CD8, and CD3. Next, PBMCs were fixed and subsequently permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Intracellular staining of CD4, CD8, CD3, and of produced cytokines was performed in Perm/Wash buffer supplemented with Brilliant Stain Buffer Plus (according to the manufacturer's instructions) for 30 min at 4°C using fluorescently labeled, cytokine-specific antibodies detecting IFNg, TNF-α IL-2, IL-17, and IL-4. Samples were acquired utilizing a FACSLyric flow cytometer and analyzed with FlowJo software.

B-cell epitope conservation analysis

The B cell epitopes are collected from Immune Epitope Database (IEDB, https://www.iedb.org/) and Coronavirus Antibody Database (CoV-AbDab), http://opig.stats.ox.ac.uk/webapps/covabdab/).[27] The IEDB database was queried on November 25, 2022 using the following criteria: Organism: SARS-COV2; Antigen: Spike glycoprotein; Positive Assay; No T cell assays; No MHC assays; Host: Homo sapiens (human); B Cell Assays: neutralization | biological activity (neutralization). The resulting table was filtered by limiting epitopes to have a minimum number of 5 amino acids. Of the 381 unique epitope sequences obtained in this approach from IEDB, 307 were found in the wild-type strain S glycoprotein. Protein databank (PDB) structures and neutralization information were collected from CoV-AbDab (updated on October 3, 2022). The epitopes were calculated from the structures corresponding to SARS-CoV-2 neutralizing antibodies with human hosts, in a similar method to the one used in Beguir et al.[21] Precisely, given one or multiple antigen chains, the epitope consists of positions on the antigen chains that are in contact with antibody heavy or light chains. Two amino acids are in contact if the smallest Euclidean distance between their atoms was smaller than 4 Ångstroms and the largest Euclidean distance was smaller than 20 Ångstroms. The resulting epitopes were further pruned such that they do not target both NTD and RBD. Epitopes were also required to have a minimum number of 5 amino acids. Of the 290 unique epitope sequences obtained in this approach from CoV-AbDab, 229 were found in the wild-type strain spike glycoprotein. Merging structure-based epitopes and IEDB retrieved epitopes resulted in 454 unique epitopes. Of these, 412 epitopes (91%) included a position reported to be mutated in at least one of the variants investigated in our study. Each epitope is classified into one of the following classes: NTD, RBD class 1–4,[25,26] RBD class 5, and Non-NTD-RBD. Each epitope is classified to the dominant class of its amino acid residues, where each residue belongs to uniquely one class and all RBD residues not in class 1–4 are assigned to class 5.

T cell epitope conservation analysis

To estimate the rate of non-synonymous mutations in T cell epitopes in the spike glycoprotein, we obtained $CD4^+$ and $CD8^+$ T cell epitopes confirmed via experimental assays which were reported in the Immune Epitope Database (https://www.iedb.org/).[44] The experimental assays confirming the reactivity of these epitopes relied on multimer analysis, ELISpot or ELISpot-like assays, T cell activation assays, etc. The database was filtered using the following criteria: Organism: SARS-COV2; Antigen: Spike glycoprotein; Positive Assay; No B cell assays; No MHC assays; MHC Restriction Type: Class I (or II); Host: Homo sapiens (human). In order to restrict the dataset to confirmed minimal epitopes only, the resulting tables were filtered by removing entries that were deduced from a reactive overlapping peptide pool or relied on prediction to identify the minimal epitope. Only epitopes of length 8–14 amino acids (for HLA-I) or 12–20 amino acids (for HLA-II) were retained.

QUANTIFICATION AND STATISTICAL ANALYSIS

The statistical method of aggregation used for the analysis of antibody titers is the geometric mean and for the ratio of SARS-CoV-2 VOC titer and wild-type strain titer the geometric mean and the corresponding 95% confidence interval. The use of the geometric mean accounts for the nonnormal distribution of antibody titers, which span several orders of magnitude. The Friedman test with Dunn's correction for multiple comparisons was used to conduct pairwise signed-rank tests of group geometric mean neutralizing antibody titers and proportions of cytokine-secreting T cells with a common control group. The Kruskal-Wallis test with Dunn's correction for multiple comparisons was used to conduct unpaired signed-rank tests of group GMT ratios. All statistical analyses were performed using GraphPad Prism software version 9.