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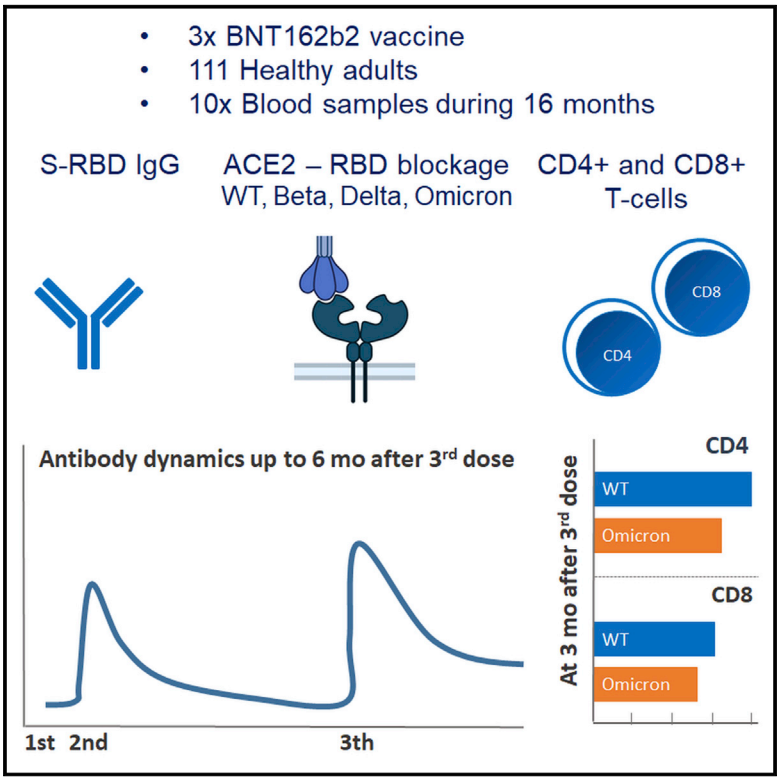
Protective antibodies and T cell responses to Omicron variant after the booster dose of BNT162b2 vaccine

Graphical abstract



Highlights

- After the third BNT162b2 dose, the antibody levels decline slower than after the second
- The protective antibodies persist for at least 3 months after the third dose
- 81% of individuals have T cell responses to Omicron at 3 months after third dose

Authors

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In brief

Naaber et al. monitor individuals vaccinated with BNT162b2 up to 6 months after the third dose. The S-RBD IgG levels decline but slower after the third than second dose. The booster restores the serum ability to block wild-type and Omicron RBD interaction with the ACE2 and boosts cellular immunity.

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Protective antibodies and T cell responses to Omicron variant after the booster dose of BNT162b2 vaccine

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SUMMARY

The high number of mutations in the Omicron variant of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes its immune escape. We report a longitudinal analysis of 111 vaccinated individuals for their antibody levels up to 6 months after the third dose of the BNT162b2 vaccine. After the third dose, the antibody levels decline but less than after the second dose. The booster dose remarkably increases the serum ability to block wild-type or Omicron variant spike protein's receptor-binding domain (RBD) interaction with the angiotensin-converting enzyme 2 (ACE2) receptor, and these protective antibodies persist 3 months later. Three months after the booster dose, memory $CD4^+$ and $CD8^+$ T cells to the wild-type and Omicron variant are detectable in the majority of vaccinated individuals. Our data show that the third dose restores the high levels of blocking antibodies and enhances T cell responses to Omicron.

INTRODUCTION

The Omicron variant of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused breakthrough infections worldwide among previously vaccinated individuals, showing that the antibody levels formed after the vaccination are not sufficient to inhibit its transmission.[1] The variant has more mutations compared with previous variants of concern (VOCs), which has enabled an escape from neutralizing antibodies and added to its transmission efficiency in communities.[2] Many of the mutations are in spike protein that is needed for the virus entry to human cells and is targeted by most of the currently available vaccines.[3]

Despite increased infectivity, the risk for severe disease and hospitalization with Omicron among vaccinated individuals is reduced compared with earlier variants, including Delta.[4] For one reason, animal studies have shown that Omicron was limited to the upper respiratory tract, causing less damaging infection in the lungs, and these findings have been supported by human *ex vivo* studies.[5] Secondly, the high number of mutations in spike protein has added to its ability to escape from neutralizing antibodies and develop resistance to therapeutic antibodies in clinical use.[6–11] The variant efficiently escapes from the neutralizing antibodies in individuals who have been vaccinated with two doses of mRNA vaccine.[7,10] However, the third dose of mRNA vaccine has been reported to prominently induce neutralizing antibodies against the Omicron variant, inhibiting the loss of neutralizing activities.[10] Furthermore, the breakthrough infection by non-Omicron variants robustly elicits Omicron-neutralizing antibodies in vaccinees who have received mRNA vaccines,[12] suggesting that three contacts with the viral antigen either in form of vaccination or breakthrough infections are sufficient to avoid severe coronavirus disease 2019 (COVID-19). Thus, hospitalization with the Omicron variant was found to be 65% and 80% lower for those who received 2 or 3 doses, respectively, when compared with those who had not received any vaccination.[13–15] Apart from the neutralizing antibodies, vaccination and previous infections with SARS-CoV-2 induced robust $CD4^+$ and $CD8^+$ T cell responses that largely cross-reacted with Omicron despite its high rate of spike gene mutations.[16–18]

In addition to the number of vaccinations, another critical factor influencing the immune response to SARS-CoV-2 is the time since mRNA vaccination.[19] As the antibody levels wane in vaccinated individuals over time, these two factors need to be considered in intraindividual and longitudinal analyses of vaccinated cohorts

Table 1. Summary results at different time points

	Study groups/time points										
	B1D	B2D	1wA2D	6wA2D	3mA2D	6mA2D	9mA2D	2wA3D	3mA3D	6mA3D	6mA3D + COVID
Antibodies to S-RBD											
IgG (AU/mL) median/IQR (n)	1.25/0.3–2.5 (88)	1,246/666–2,582 (111)	24,534/13,985–36,616 (106)	12,752/8,225–17,348 (89)	5,226/3,097–6,924 (90)	1,383/893–2,463 (84)	739/419–1,359 (73)	32,899/17,914–46,718 (60)	13,119/6,149–21,767 (51)	8,367.5/3,137.5–15,598.5 (28)	36,849/19,428–66,319 (31)
Inhibition of spike RBD-ACE2 interaction (relative OD)											
SARS-CoV-2 (wild-type) median/IQR (n)	0.97/0.95–0.99 (49)	–	0.33/0.13–0.46 (49)	–	0.76/0.64–0.83 (49)	–	0.92/0.86–0.96 (71)	0.13/0.07–0.33 (56)	0.55/0.31–0.70 (51)	–	–
Beta (B.1.351) median/IQR (n)	1.00/0.97–1.01 (49)	–	0.64/0.50–0.71 (49)	–	0.86/0.79–0.91 (49)	–	0.90/0.86–0.96 (71)	0.23/0.14–0.42 (56)	0.47/0.26–0.6 (51)	–	–
Delta (B.1.617.2) median/IQR (n)	0.99/0.97–1.02 (49)	–	0.46/0.29–0.58 (49)	–	0.80/0.68–0.84 (49)	–	0.89/0.86–0.95 (71)	0.16/0.09–0.36 (56)	0.59/0.41–0.73 (51)	–	–
Omicron (B.1.1.529) median/IQR (n)	0.96/0.92–1.03 (49)	–	0.79/0.68–0.85 (49)	–	0.90/0.81–0.97 (49)	–	0.87/0.82–0.97 (71)	0.44/0.31–0.58 (56)	0.64/0.44–0.73 (51)	–	–
T cells											
Spike-specific $CD8^+$ T cells (percentage from $CD8^+$ median/IQR) (n)	–	–	–	–	0.070/0.008–0.153 (79)	–	0.045/0–0.24 (68)	0.31/0.13–0.45 (51)	0.11/0.04–0.27 (43)	–	–
Spike-specific $CD4^+$ T cells (percentage from $CD4^+$ median/IQR) (n)	–	–	–	–	0.245/0.008–0.510 (79)	–	0.185/0.06–0.38 (68)	0.49/0.3–0.82 (51)	0.34/0.23–0.61 (43)	–	–

(*Continued on next page*)

Table 1. *Continued*

	Study groups/time points										
	B1D	B2D	1wA2D	6wA2D	3mA2D	6mA2D	9mA2D	2wA3D	3mA3D	6mA3D	6mA3D + COVID
Omicron-specific $CD8^+$ T cells (percentage from $CD8^+$ median/IQR) (n)	–	–	–	–	–	–	–	–	0.06/0.01–0.16 (43)	–	–
Omicron-specific $CD4^+$ T cells (percentage from $CD4^+$ median/IQR) (n)	–	–	–	–	–	–	–	–	0.14/0.06–0.42 (43)	–	–

B1D, before the first dose; B2D, before the second dose; 1wA2D, 1 week after the second dose; 6wA2D, 6 weeks after the second dose; 3mA2D, 3 months after the second dose; 6mA2D, 6 months after the second dose; 9mA2D, 9 months after the second dose; 2wA3D, 2 weeks after the third dose; 3mA3D, 3 months after the third dose; 6mA3D, 6 months after the third dose; 6mA3D + COVID, 6 months after the third dose and diagnosed with COVID-19 after the third dose.

to identify the antibody and T cell responses elicited after the vaccinations. However, most studies have analyzed the immune response shortly after the booster, and there is limited data on the durability of the effect of the third dose on Omicron neutralization.

We here followed a cohort of individuals vaccinated with BNT162b2 for induced antibodies until 6 months after the third dose. In addition, we longitudinally measured their serum capacity to block spike's receptor-binding domain (RBD) with interaction with angiotensin-converting enzyme 2 (ACE2) receptor and the prevalence of spike-specific $CD4^+$ and $CD8^+$ T cells to wild type (WT) and the Omicron variant after the third dose.

RESULTS

Study group

We studied a cohort of diagnostics lab personnel vaccinated with three doses of the BNT162b2 vaccine, which we have earlier followed for their second-dose vaccine response.[20] The details of the study groups, vaccinations, and sample collection are given in Table 1 and Figure S1. Briefly, the cohort received the first and the second vaccination doses in January 2021 and the third 9 months after the start of the trial in October 2021. The collected samples included ten different time points collected before and after the three-dose vaccination; these were before the first dose (B1D), before the second dose (B2D), 1 week after the second dose, 6 weeks after the second dose, 3 months after the second dose, 6 months after the second dose, 9 months after the second dose, 2 weeks after the third dose, 3 months after the third dose, and 6 months after the third dose.

SARS-CoV-2 antibody dynamics after three-dose vaccination

Before the third vaccination, which was given 9 months after the second dose, the antibody levels to spike protein RBD (S-RBD immunoglobulin G [IgG]) were significantly declined (median: 739 AU/mL; interquartile range [IQR]: 419–1,359; $p < 0.0001$) compared with the peak value after second dose of vaccination (1 week after the second dose) (Figure 1; Table 1). Two weeks after the third dose, S-RBD IgG levels increased to a median of 32,899 AU/mL (IQR: 17,914–46,718; $p < 0.0001$) compared with the pre-vaccination (9 months after the second dose) time point. The IgG increase due to the third dose was a median of 37.91 times (IQR: 23.94–57.36), which is higher than the increase after the second dose (median: 15.37 times; IQR: 8.45–26.82; $p < 0.001$). However, 3 months after the third dose, the S-RBD IgG levels were trending lower (median: 13,119 AU/mL; IQR: 6,149–21,767) and were further declined at 6 months after the third dose in the persons who were not diagnosed with COVID-19 until this time point (median: 8,367.5 AU/mL; IQR: 3,137.5–15,598.5; $n = 28$). Between the third vaccination and time point 6 months after the third dose, 31 persons from the vaccinated cohort got a breakthrough infection with SARS-CoV-2, most likely by Omicron as a dominant circulating variant in spring 2022 (see details of monitoring the infections in STAR Methods). In these persons, IgG levels were significantly higher compared with non-infected individuals (median: 36,849; IQR: 19,428–66,319; $p < 0.001$; 6 months after the third

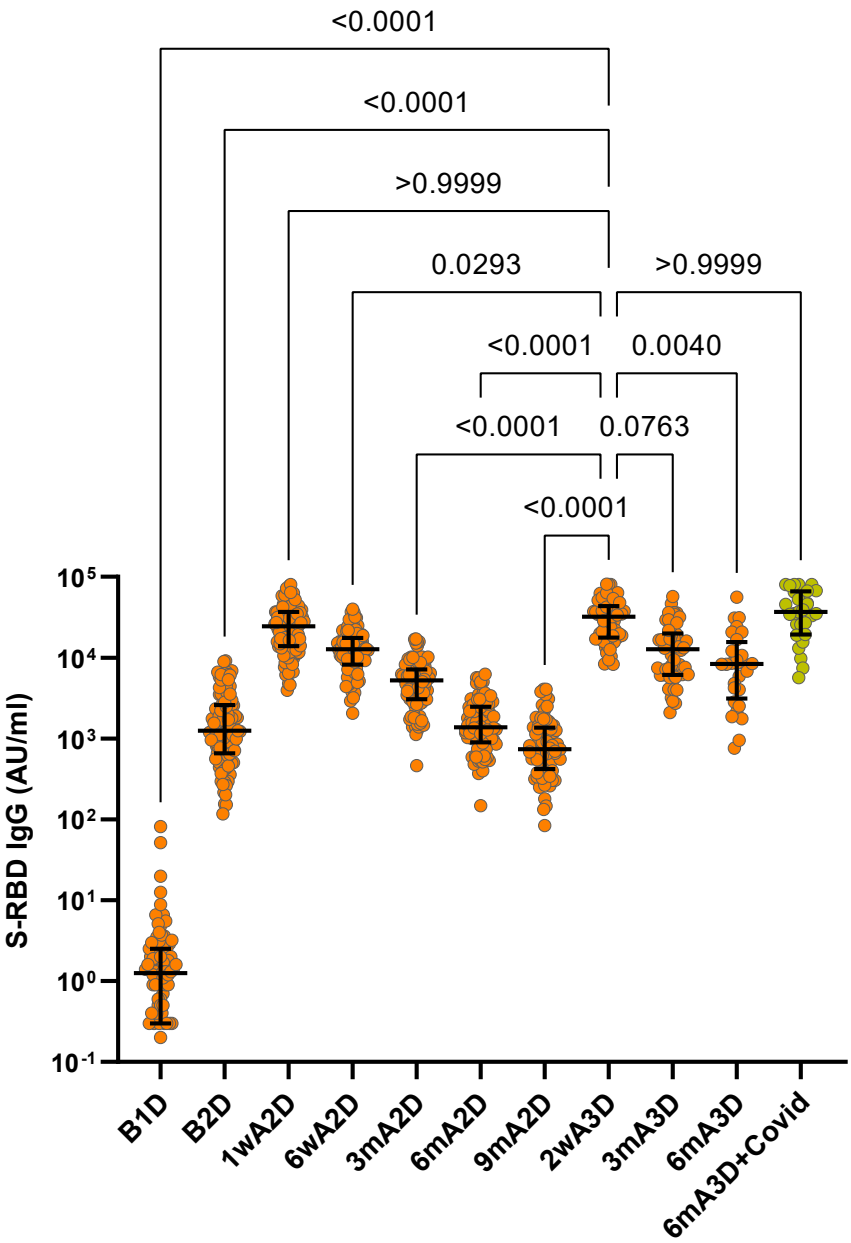

Figure 1. Antibody responses in BNT162b2 vaccinated individuals up to 6 months after the third dose

S-RBD IgG levels (AU/mL) before vaccination (B1D; n = 88), after the first vaccination dose (B2D; n = 111), 1 week (1wA2D; n = 106), 6 weeks (6wA2D; n = 89), 3 months (6mA2D; n = 84), and 9 months (9mA2D; n = 73) after the second vaccination dose, and 2 weeks (2wA3D; n = 60), 3 months (3mA3D; n = 51), and 6 months (6mA3D; n = 28) after the third vaccination dose (all shown in orange). Additional group in green shows S-RBD IgG levels at 6 months after the third dose in individuals who had COVID-19 after the third dose (6mA3D + COVID; n = 31). The data comparisons are shown relative to peak level after the third dose (2mA3D) and were performed with the Kruskal-Wallis test and Dunn's multiple testing correction; p values >0.0001 are reported as exact numbers. Median and interquartile range is shown on top of each scatterplot.

dose + COVID group; Figure 1; Table 1) and were similar to the peak level at 2 weeks after the third dose.

At 6 months after the third dose, the antibodies in non-infected persons had declined (from time point 2 weeks to 6 months after the third dose) to a median of 4.06 (IQR: 3.07–6.55) times, which was significantly slower than the decline after the second dose during the same time interval (from 1 week to 6 months after the second dose: median: 14.86 times; IQR: 9.64–22.41; $p < 0.001$).

As in earlier time points,[20] the age of vaccinated individuals had a negative correlation with S-RBD IgG response before (9 months after the second dose: $r = -0.30$, $p = 0.009$) and after the third dose (2 weeks after the third dose: $r = -0.31$, $p = 0.015$; 3 months after the second dose: $r = -0.39$, $p = 0.004$; 6 months after the third dose: $r = -0.57$; $p = 0.0017$). Age was significantly associated with the rate of antibody decline after the third dose ($r = 0.48$, $p = 0.01$) but not after the second dose ($p < 0.05$).

Serum inhibition of trimeric spike RBD-ACE2 interaction

We next studied the effectiveness of two and three vaccination doses to induce antibodies capable of neutralizing cell entry of the Omicron variant compared with other VOCs. For this, we used an established experimental assay that measures the serum capacity to block the ACE2 receptor interaction with the SARS-CoV-2 trimeric S-RBD and thus indicates neutralizing capacity. Using the assay, we tested the longitudinal serum samples from the vaccinated individuals for their inhibition of SARS-CoV-2 Omicron, Delta, Beta, and WT spike protein interaction.

Expectedly, the samples from pre-vaccination time points did not inhibit the spike RBD-ACE2 interaction of WT, Omicron, or any other variant (Figures 2 and 3; Table 1). At 1 week after the second dose, most samples were able to block the WT SARS-CoV-2 (98%) and Delta (96%), and slightly less Beta (80%), VOCs; however, the capacity to inhibit the Omicron variant was remarkably less efficient (41%; $p < 0.0001$ compared with all other variants). Over time, the blocking capacity decreased and was lower for all variants at 3 months after the second dose, most prominently for Omicron (WT: 47%, Delta: 38%, Beta: 14%, and Omicron: 4%). Before the third dose, at 9 months after the second dose, less than 6% of serum samples were able to block ACE2 binding by WT and all variants, indicating that the length of the period after the second dose

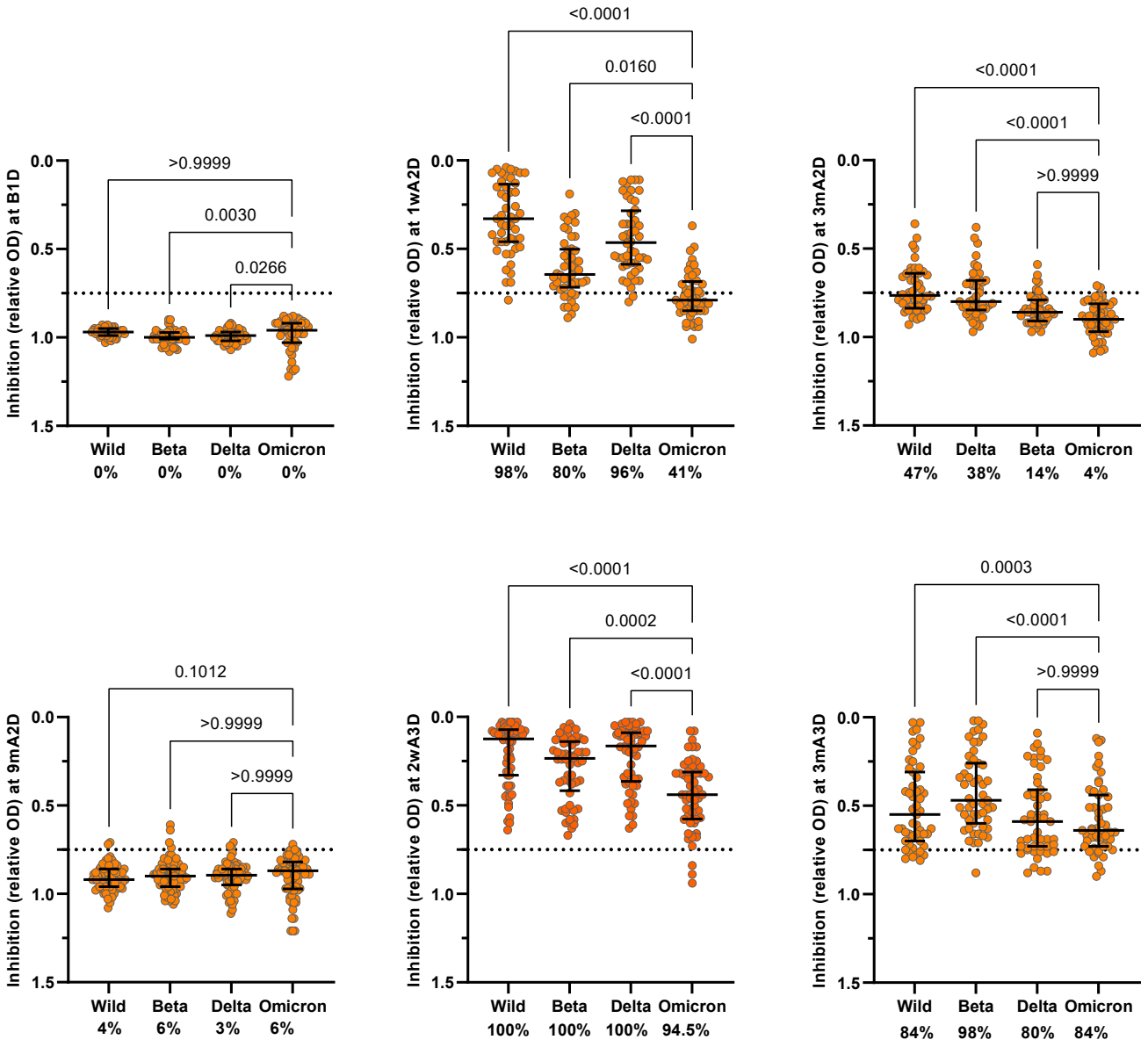

Figure 2. Inhibition of ACE2-trimeric spike interaction by vaccine-induced antibodies

Serum antibody capacities to block the interaction of ACE2 receptor and spike RBD of WT and Beta, Delta, and Omicron variants before the first dose (B1D; n = 49), at 1 week (1wA2D; n = 49), 3 months (3mA2D; n = 49), and 9 months (9mA3D; n = 71) after the second dose, and 2 weeks (2wA3D; n = 56) and 3 months (3mA3D; n = 51) after the third dose of vaccinations. The dotted line indicates the relative optical density 450 (OD450) value of 0.75, which is a threshold for sufficient blocking of ACE2 binding. The matched data analysis was performed with the Friedman test with Dunn's multiple testing correction; p values >0.0001 are reported as exact numbers. The percentage of samples that were able to reach the threshold of blocking activity is shown below each graph. Median and interquartile range is shown on top of each scatterplot.

had a strong negative effect on immune protection. The administration of the third dose (2 weeks after the third dose) restored the serum activity to inhibit the binding of the S-RBD of WT, Beta, and Delta VOCs to ACE2 and, importantly, induced neutralizing capacity toward Omicron variant in the majority of vaccinees. However, even with the three-dose vaccination, 5.5% of the sera did not achieve the blocking threshold for the Omicron variant (Figures 2 and 3). At 3 months after the booster, all SARS-CoV-2 variants, including Omicron, were still inhibited by the majority of immunized sera (≥80% of samples). However, compared with the peak levels at 2 weeks after the third dose, the blocking activity was diminished at 3 months after the third dose (medians range: 0.13–0.44 at 2 weeks after the third dose versus 0.47–0.64 at 3 months after the third dose; Figure S2). We also studied the inhibition dynamics for each variant separately. This showed a highly significant increase between the second (1 week after the second dose) and third dose (2 weeks after the third dose) for Omicron

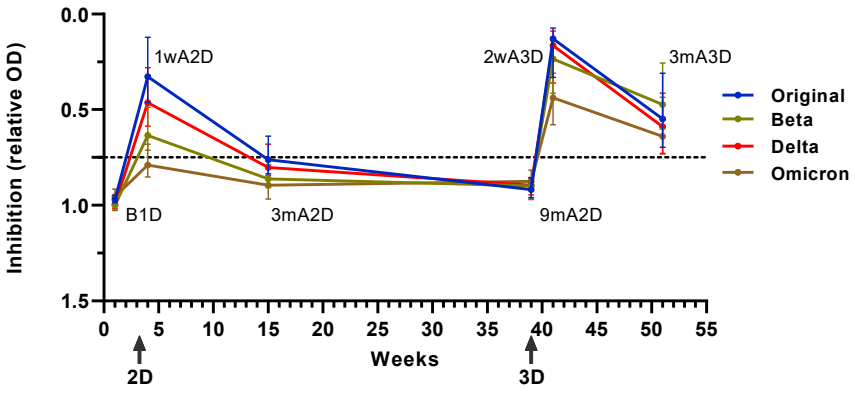

Figure 3. The dynamics of the inhibition of ACE2-spike RBD interaction

The ability of serum to inhibit the interaction of spike RBD of WT, Beta, Delta, and Omicron with ACE2 receptor is shown over time. Each line corresponds to one viral strain, and the data points show the median values and interquartile ranges of samples collected over time: before the first dose (B1D; n = 49), 1 week (1wA2D; n = 49), 3 months (3mA2D; n = 49), and 9 months (9mA2D; n = 71) after the second dose, and 2 weeks (2wA3D; n = 56) and 3 months (3mA3D; n = 51) after the third dose. The second and third vaccination points are shown at corresponding weeks on the x axis as 2D and 3D, respectively. The relative OD (OD450) values on the inverted y axis show the inhibition activity. The value over 0.75 relative OD450 is considered as the threshold of the serum sample to block ACE2-spike interaction.

($p < 0.0001$), less for Beta ($p < 0.005$) and not significantly different for original WT and Delta VOCs (Figure S2).

Our previous study showed a strong correlation between the S-RBD IgG levels and blocking antibodies in the pre-Omicron era.[20] The Spearman rank correlation analysis at each vaccination point showed that, in contrast to other variants, Omicron-blocking value had a moderate correlation with S-RBD IgG and other VOCs ($r = 0.57–0.63$) immediately after the second dose (Figure 4). However, when the antibody levels declined after the second dose (3 and 9 months after the second dose), the inhibition results with the Omicron VOC did not correlate any more with S-RBD IgG, indicating lower protection against the Omicron VOC at these time points. Strikingly, the correlation between Omicron variant's blocking values and S-RBD IgG was restored after the booster dose ($r = -0.85$ for IgG RBD and $r > 0.90$ for the inhibition experiments) and maintained at 3 months after the third dose ($r = -0.83$ for IgG RBD and $r > 0.92$ for the inhibition experiments). Together, these results demonstrate the benefit of three doses to gain protective antibodies against Omicron.

To confirm that our ELISA-based approach is comparable to standard neutralization analyses, we tested 30 serum samples from this study in Vero a E6 cell-based neutralization assay and a pseudovirus assay. We found that the cell-based neutralization assay of live virus correlated significantly with the ELISA analyses of SARS-CoV-2 WT ($r = -0.86$, $p < 0.0001$) and the Omicron variant ($r = -0.55$, $p < 0.01$; Figures S3A and S3B). The pseudovirus assay showed even stronger correlation with the ELISA ($r = -0.92$, $p < 0.0001$ for WT; $r = -0.52$, $p < 0.01$ and for the Omicron strain; Figures S3C and S3D). The lower correlation with the Omicron variant was expected, as the samples were from various time points: before and after the third vaccination. Thus, although spike RBD-ACE2 ELISA measures the inhibition of spike RBD and not the full-length protein, our ELISA results reflect well the neutralizing potential of serum antibodies.

T cell responses

Previous studies have reported stable or slightly diminished T cell responses to the Omicron VOC immediately after the booster dose. We here investigated the percentage of activation-induced marker (AIM)-positive $CD4^+$ and $CD8^+$ T cell compartments before and after the third dose as well as the Omicron-specific T cell responses 3 months after the booster dose. We found 84% and 53% of vaccinated individuals to have CD4 and CD8 WT spike-specific memory responses, respectively, before the third dose (9 months after the second dose) (Figure 5). Administration of the third dose (2 weeks after the third dose) increased these percentages to 100% (for CD4) and 90% (for CD8). In contrast, 2 out of 26 unvaccinated and SARS-CoV-2-unexposed individuals had modest T cell responses to spike (Figure 5). The frequency of spike-specific $CD4^+$ T cells was significantly higher after the third dose (median/IQR: 9 months after the second dose: 0.18/0.06%–0.38% versus 2 weeks after the third dose: 0.49/0.3%–0.82%; $p < 0.0001$). Similarly, the $CD8^+$ memory T cell frequency increased after the booster dose (median/IQR for 9 months after the second dose: 0.04/0%–0.24% versus 2 weeks after the third dose: 0.31/0.13%–0.45%; $p < 0.0001$). Three months later, at 3 months after the third dose, 97% of the vaccinated individuals still harbored AIM+ $CD4^+$ T cells (median/IQR: 0.34/0.23%–0.61%), and 77% had AIM+ $CD8^+$ T cells (median/IQR: 0.11/0.04%–0.27%). The frequency of AIM+ cells was not significantly different from 2 weeks after the third dose for $CD4^+$ T cells but slightly decreased for $CD8^+$ T cells. At 3 months after the third dose, the AIM+ $CD4^+$ T cell percentage correlated negatively with age ($r = -0.38$, $p = 0.01$) and positively with S-RBD antibody levels ($r = 0.34$; $p = 0.025$).

T cell responses induced by mRNA vaccines target WT spike, and this may affect T cell responses to the Omicron VOC, in which spike epitopes are highly mutated when compared with other variants.[21] To study the reactivity of T cells to spike regions that are mutated in Omicron, we performed T cell stimulations of the post-booster time point (3 months after the third dose) samples using the Omicron spike peptides. We found that from those individuals who responded to WT spike, the majority also had T cell responses (81% for $CD4^+$ and 63% for $CD8^+$ T cells) to Omicron spike epitopes.

Collectively, these results show efficient spike-specific memory T cell responses in all vaccinated individuals after the third dose. Three months later, the $CD4^+$ and $CD8^+$ memory T cell

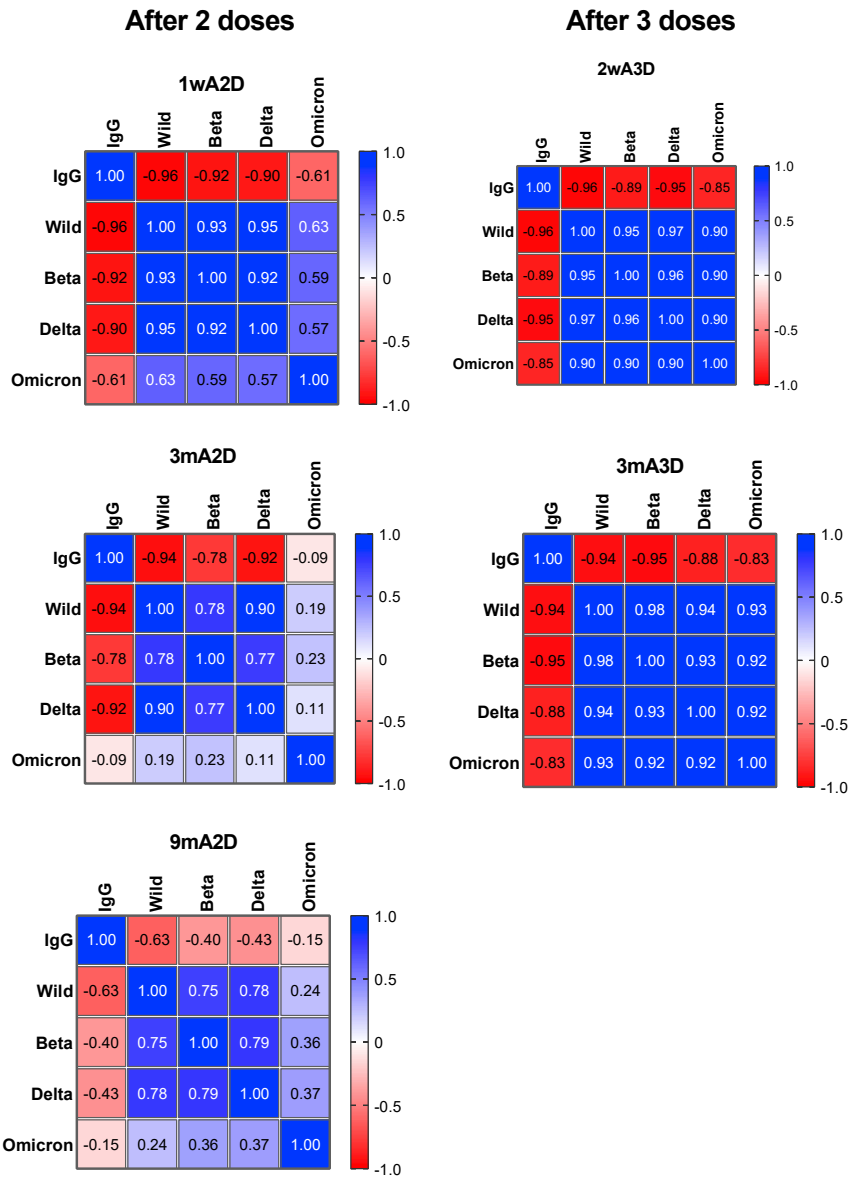

Figure 4. Correlation between serum S-RBD antibody levels and the inhibition capacity of ACE2-spike interactions

Spearman rank correlation analysis at each sample collection time after two and three vaccination doses. Neutralization data are in relative OD (OD450) values with inverted direction (lower values indicate stronger inhibition), and hence they are in negative correlation with S-RBD IgG (blue) and in positive correlation (red) between each other.

reactivity to the viral antigen persisted, and most of these individuals maintained T cell responses to Omicron spike epitopes.

Predictors of vaccine response

Altogether, 97% of participants reported adverse effects after the third dose. The frequency and severity of side effects after the third dose were similar to those after the second dose (total score median/IQR: 7/3–12 versus 6/2–12; p = 0.7). The common side effects after the third dose were pain or swelling at the injection site (90%), fatigue (69%), myalgia (51%), malaise (47%), headache (47%), and chills (36%) and resembled those after the second dose (Table S1).

We found a significant correlation between the severity of side effects after the second and third dose (r = 0.48, p = 0.0003). Thus, the individuals with pronounced side effects after the second dose also tended to have these after the third one. The total score of adverse effects after the third dose correlated with the antibody response to S-RBD (r = 0.53, p = 0.001) as it did after the second vaccine dose.[20] Detailed information is presented in Table S2.

DISCUSSION

Omicron has demonstrated its immune evasion among vaccinated individuals. We here investigated the samples of the vaccinated cohort for dynamics of S-RBD IgG antibodies and their capacity to inhibit ACE2-spike RBD interaction of original WT virus and Beta, Delta, and Omicron VOCs and to develop T cell responses.

Consistent with previous reports,[2,8,11] we found decreased serum capacity to block Omicron RBD in individuals who have received two vaccine doses. Less than half of the vaccinated sera were able to inhibit the reaction with Omicron, whereas the WT virus and Delta variant were blocked by more than 95% and the Beta variant by 80% of the samples. Over time, the blocking capacity declined, and at 9 months after the second dose, all samples had lost their blocking activity. Together with the significant waning of the S-RBD binding, this result suggests a remarkable decline of protective antibodies at 9 months after the two BNT62b2 vaccine doses. However, 2 weeks after the booster dose, the serum samples regained their blocking activity, which was in line with the elevated antibody levels to S-RBD.

Given the weaker serum activity generated against Omicron's spike protein, it was important to determine whether the antibody response stays durable in individuals who received three doses of mRNA vaccine. For this, we measured the S-RBD antibodies 6 months after the third dose. Compared with their peak values after the third dose, the antibodies to spike RBD were lower 6 months later. However, the 6 month decline was lower than after the second dose, and its median value (6 months after the third dose: 8,367.5 AU/mL) corresponded to median values between time points 1.5 and 3 months after the second dose

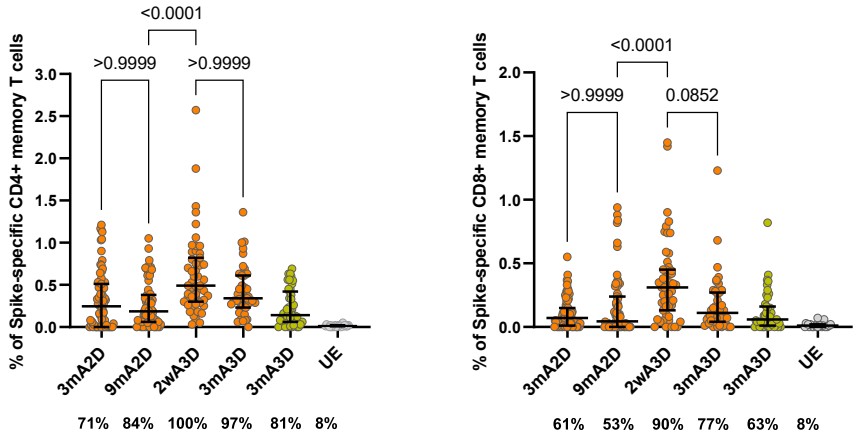

Figure 5. Spike-specific T cell responses to WT and Omicron in vaccinated individuals

Post-vaccination frequency (percentage of antigen-specific T cells from the respective T cell subpopulation) of spike-specific (left) $CD4^+$ and (right) $CD8^+$ T cells at 3 months (3mA2D; n = 79) and 9 months after the second dose (9mA2D; n = 68) and at 2 weeks (2wA3D; n = 51) and 3 months after the third dose (3mA3D; n = 43). On both graphs, the time points (3mA2D, 9mA2D, 2wA3D, and 3mA3D) in orange represent T cell responses to peptide pools of WT spike protein. Three months after the third dose (3mA3D) in green show T cell responses to peptide pool of Omicron spike protein. The T cell responses of unexposed (UE) individuals (pre-pandemic material) stimulated with T peptide pool are shown in gray (n = 26). The differences between T cell responses to WT spike peptides were analyzed with the Kruskal-Wallis test with Dunn's multiple testing correction. The percentages of samples that had spike-responsive $CD4^+$ or $CD8^+$ T cells are shown below the graphs.

(6 weeks after the second dose: 12,752 AU/mL versus 3 months after the second dose: 5,226 AU/mL). This shows that the antibodies also decline after the booster dose, albeit more slowly than after the second dose.

In the blocking assay, the individual samples collected after the third dose retained a strong correlation with S-RBD levels. This is in contrast to the inhibition activity that we saw after the second dose, where protection against Omicron did not correlate with S-RBD IgG antibody levels and was discordant with the inhibition of WT and Beta and Delta VOCs. The additional booster dose may elicit Omicron-neutralizing memory B cell subsets and expand the repertoire of the broadly neutralizing antibodies.[22] In this case, it is plausible that the Omicron-neutralizing clones were acquired as a result of affinity maturation of those with neutralizing activity to a previously recognized WT spike vaccine antigen.

The waning of antibody levels over time and the emergence of immune escape mutants have turned the focus toward antigen-specific T cells as important players in preventing serious COVID-19.[23] The durability of cellular response is one of the key parameters to consider in planning future booster vaccination strategies. mRNA vaccines have shown potent induction of spike-specific CD4 and CD8 responses by the first two vaccine doses; however, the number of circulating antigen-specific T cells contract during the first 3 months but stabilize thereafter.[19,24] We also saw this in our study, as the percentages of AIM+ $CD4^+$ and $CD8^+$ T cells were stable between 3 and 9 months after the second dose. However, we demonstrated here that the booster dose increased the percentages of spike-reactive T cells of both subtypes. This is in line with recent reports that measured immune responses shortly after the booster dose.[25] Importantly, we found that spike-specific CD4 T cell percentages were still elevated 3 months after the third dose of BNT162b2, and AIM+ CD8 T cells showed only a moderate decline compared with time point 2 weeks after the third dose.

Another important concern about the mRNA-vaccine-induced T cell responses is the cross-reactivity between VOCs. The Omicron variant has multiple spike mutations and, compared with previous VOCs, is associated with the fewest number of conserved spike epitopes (72% for CD4 and 86% for CD8).[21] Nevertheless, most T cell epitopes are unaffected by the Omicron spike mutations, and several studies have confirmed that T cell reactivity to the Omicron variant is well preserved in the majority of vaccinated individuals.[16,17,21,25,26] At 3 months after the third dose, we stimulated peripheral blood mononuclear cell (PBMC) samples in addition to overlapping peptide pools spanning the immunodominant regions of WT SARS-CoV-2 spike, also with a selected peptide pool containing only regions that are mutated in Omicron spike protein. Approximately 70% of these peptides are predicted to retain HLA class I binding[21] due to conservative substitutions or changes that do not affect HLA binding. In our study, the median responses to Omicron were lower and, with set cut-off levels, 16% and 14% of the studied individuals lost $CD4^+$ and $CD8^+$ T cell responsiveness toward the Omicron peptide pool at 3 months after the third dose, probably because their HLA alleles were not compatible with the mutated peptides.

In sum, our results suggest increased durability of humoral and cellular immune responses toward SARS-CoV-2 spike protein after the booster dose of the BNT162b2 vaccine and confirm the notion of an increase in the breadth of antibody response and preservation of T cell responses toward the Omicron variant.

Limitations of the study

Our study has limitations. We used an ELISA assay that measures the binding of the ACE2 to the RBD of the spike protein to analyze the dynamics of the neutralizing capacity of the vaccinated sera. The ELISA assay enables well-standardized and semi high-throughput analysis of neutralizing antibodies; however, it misses the antibodies interacting with spike epitopes outside of the RBD. It is likely that the neutralizing capacity of the studied sera was underestimated and does not reflect the complete activity of neutralizing antibodies. Nevertheless, to validate our approach, we confirmed the high correlation of ELISA with a Vero E6 cell-based assay that is considered a gold standard in SARS-CoV-2 neutralizing antibody testing and with a pseudovirus assay as a valid alternative to the surrogate and live-cell analyses. A recent analysis elsewhere supports

our observation on the good correlation between ELISA and the conducted alternative tests.[27] We also acknowledge limitations of the AIM assay. The $CD8^+$ T cell stimulation assay with 15-mer peptides is not optimal, as these are longer than standard peptides for the HLA class I binding groove. Also, we were not able to study T cell responses in our cohort before the first vaccination dose to test the presence of pre-pandemic SARS-CoV-2 spike cross-reactive T cells; nevertheless, only 8% of unexposed individuals had T cell responses in our assay.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100716.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.N. and P.P.; project administration, P.N. and P.P.; supervision, P.N., K. Kisand, and P.P.; formal analysis, P.N., P.P., and J.M.G.; validation, E.S.; resources, V.J.; investigation, V.J., E. Sepp, L.T., K. Kisand, K. Kangro, L.H., U.H., M.K., M.P., J.K., E. Sankovski, E.Z., J.M.G., A.P., and M.U.; methodology, L.T., K. Kisand, K. Kangro, L.H., A.P.R., R.M., U.H., M.K., M.P., J.K., E. Sankoviski, E.Z., J.M.G., A.P., and M.U.; writing – original draft, P.N., E. Sepp, K. Kisand, P.P., and J.M.G.; writing – review & editing, P.N., K. Kisand, J.M.G., and P.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Serum and cell samples	This study	N/A
Experimental models: Cell lines		
BHK cells expressing SARS-CoV-2 N protein and human ACE2 receptor plasmid DNA encoding SARS-CoV-2 infectious clones	kind gift from the research group of Prof. Alan Kohl (University of Glasgow)	N/A
HEK293 ACE2 cell-line	This study	N/A
Chemicals, peptides, and recombinant proteins		
SARS-CoV-2 trimeric S proteins	Icosagen AS	P-309-100; P-316-100; P-353-100; P-369-100
ACE2-hFc protein	Icosagen AS	P-308-100
plasmid pLV-ACE2	This study	N/A
plasmid pNL4-3	This study	N/A
plasmid pQ1TAR5-S_dC19_WU#29	This study	N/A
plasmid pQ1TAR5-SdC19_B.1.1.529#15	This study	N/A
Bacterial and virus strains		
SARS-CoV-2 (Original)	This study	N/A
SARS-CoV-2 (Omicron)	This study	N/A
Critical commercial assays		
SARS-CoV-2 IgG II Quant Reagent Kit	Abbott Laboratories	6S60
SARS-CoV-2 IgG Reagent Kit	Abbott Laboratories	6R86
TaqPath COVID-19, Flu A/B, RSV Combo Kit CE-IVD	ThermoFisher Scientific	A49867
MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit	ThermoFisher Scientific	A48383
IVD-CE SARS-CoV-2 Neutralizing Antibody ELISA kit	Icosagen AS	K5-002-096
EZ-Link™ NHS-PEG4 Biotinylation Kit	ThermoFisher Scientific	21455
Pierce™ High Sensitivity Streptavidin-HRP	ThermoFisher Scientific	21132
ELISA plates; Maxisorp F8 Nunc-Immunomodule	ThermoFisher Scientific	468667
7-AAD staining solution	Miltenyi Biotec	130-111-568
FcR Blocking Reagent, human	Miltenyi Biotec	130-059-901
PepTivator SARS-CoV-2 Prot_S (WT)	Miltenyi Biotec	130-126-700
PepTivator SARS-CoV-2 Prot_S B.1.1.529/BA.1 Mutation Pool	Miltenyi Biotec	130-129-928
CEFX Ultra SuperStim Pool	JPT Peptides	PM-CEFX-2
Ultra-LEAF™ Purified anti-human CD28 Antibody	Biolegend	302934
Ultra-LEAF™ Purified anti-human CD49d Antibody	Biolegend	304340
CD3 Brilliant Violet 650	Biolegend	317324
CD4 Alexa Fluor 700	Biolegend	317426
CD8 Brilliant Violet 605	Biolegend	301040
CCR7 Alexa Fluor 488	Biolegend	353206
CD45RA APC	Biolegend	304150

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD69 Brilliant Violet 510	Biolegend	310936
OX40 PE-Dazzle	Biolegend	350020
CD137 PE	Miltenyi Biotech	130-110-763
X-VIVO 15 cell culture medium	Lonza	BE02-060Q
BD Vacutainer® SST™ II Advance	BD Diagnostics	367957
BD Vacutainer® CPT™	BD Diagnostics	362780
Sample Preservation Solution	Jiangsu Mole Bioscience	P042T00901
FLOQSwabs	Copan	518C
ARCHITECT *i*2000SR/ Alinity i	Abbott Laboratories	N/A
LSR-Fortessa	BD Biosciences	N/A
ELISA reader (spectrophotometer) MultiSkan FC	ThermoFisher Scientific	N/A
KingFisher™ Flex	ThermoFisher Scientific	N/A
QuantStudio5	ThermoFisher Scientific	N/A
Software and algorithms		
GraphPad Prism v9.0	GraphPad Software Inc	https://www.graphpad.com/scientific-software/prism/
FCS Express 7	DeNovo Software	https://denovosoftware.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources or reagents should be directed to the Lead Contact, Dr. Paul Naaber (paul.naaber@synlab.ee).

Materials availability

IVD-CE SARS-CoV-2 Neutralizing Antibody ELISA kit, SARS-CoV-2 trimeric S proteins, ACE2-hFc protein used in this study are available either commercially from Icosagen Cell Factory or the lead contact with a completed Materials Transfer Agreement. Plasmids, cell lines, viral strains used in this study will be made available on request, but we may require a payment and/or a completed Materials Transfer Agreement if there is potential for commercial application.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report the 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 SUBJECT DETAILS

Human subjects

The study group and the blood sample collection procedures were also reported in our previous study.[20] All SYNLAB Estonia employees volunteering to be vaccinated (2–3 doses) with COVID-19 mRNA BNT162b2 (Comirnaty Pfizer-BioNTech) vaccine were invited to participate in the study. We only excluded the participants diagnosed with COVID-19 before the study. Briefly, starting January 2021, the first two vaccine doses were given three weeks apart, and the third dose was administered nine months after the second dose. The samples were taken before the first dose (B1D), before the second dose (B2D), one week after the second dose (1wA2D), six weeks after the second dose (6wA2D), three months after the second dose (3mA2D), 6 months after the second dose (6mA2D), 9 months after the second dose (9mA2D), two weeks after the third dose (2wA3D), 3 months after the third dose (3mA3D), and 6 months after the 3rd dose. The numbers of participants at each time point are presented in Table 1. In 9th follow-up month after the 2nd dose 73 persons volunteered to participate (median age=35, male/female ratio = 10/63), from these 60 decided to get 3rd booster dose and were available follow-up test (group 2wA2D: median age=35, male/female ratio = 8/52), 51 were available for follow-up tests after 3 months (3mA3D group: median age = 35, male/female ratio = 9/42) and 59 participated in follow-up 6 months after the 3rd dose (6mA3D and 6mA3D + COVID: median age = 35, male/female ratio 10/49). We planned the optimal cohort

size of 100 individuals for the study, however, during the study period of over 16 months we saw approximately 30% drop-out and 59 persons were probed at the last time point. The main reasons for the study dropout were non-declared personal reasons, non-availability during sampling dates and postponement or refusal of vaccine booster. Until time-point 3mA3D COVID-19 infected persons were also excluded (in total eight persons). At the last time-point (6mA3D) persons infected with SARS-CoV-2 after the 3rd dose were invited to participate, and data of infected (group 6mA3D + COVID, n = 31) and non-infected persons (group 6mA3D, n = 28) were analyzed separately. COVID-19 infection was detected by the following diagnostic tools: self-reporting of any symptom followed by PCR test (TaqPath COVID-19, Flu A/B, RSV Combo Kit CE-IVD, ThermoFisher); from January 2022 all personnel were tested by PCR twice per week; before 3rd dose and at 6mA3D timepoint all the participants were screened for SARS-CoV-2 nucleocapsid IgG (Abbott). As negative controls for T cell responses, we studied 26 unvaccinated and unexposed individuals, whose samples were stored from the pre-pandemic period and kept in liquid nitrogen.

The information about the presence of side effects after the third dose was collected as reported previously.[20] For this, the study participants filled in a questionnaire about the presence of side-effects after the second and the third dose and rated their side-effect severity with scoring from zero to three (Table S1 and S2). The total score of side effects was calculated as the sum of all self-rated side effect scores per patient.

Ethics statement

The study was approved by the Research Ethics Committee of the University of Tartu on February 15, 2021 (No 335/T-21). Participants signed informed consent before their recruitment into the study. The study was performed in accordance with Helsinki Declaration and followed Good Laboratory Practice.

METHOD DETAILS

SARS-CoV-2 spike and nucleocapsid antibody testing

Serum samples were analyzed for the IgG antibodies to SARS-CoV-2 Spike protein receptor-binding domain (S-RBD) IgG) and for SARS-CoV-2 nucleocapsid IgG using Abbott chemiluminescent particle microassays (SARS-CoV-2 IgG II QN and SARS-CoV-2 IgG) on Alinity i analyzer (Abbott Laboratories) as described previously.[20]

Spike RBD-ACE2 interaction blocking assay

The serum capacity to block the angiotensin-converting enzyme 2 (ACE2) receptor interaction with SARS-CoV-2 trimeric S protein receptor-binding domain (RBD) was tested using an IVD-CE SARS-CoV-2 Neutralizing Antibody ELISA kit (Icosagen, Icosagen Cat.-no. K5-002-096) and versions of this kit that encompass different VoCs.[20] 96-well microtiter plates (Maxisorp F8 Nunc-Immunomodule, Cat.-no 468667, Thermo Scientific) were coated with SARS-CoV-2 trimeric S proteins of either WT (Icosagen Cat.-no. P-309-100), Beta (B.1.351, Icosagen Cat.-no. P-316-100), Delta (B.1.617.2, Icosagen Cat.-no. P-353-100) or Omicron (B.1.1.529, Icosagen Cat.-no. P-369-100) at 2.5 μg/mL (100 μL per well) in 1xPBS, pH 7.4 for 16 to 24 h at 4 °C. Thereafter, the coating solution was aspirated and wells were washed 4 times with 300 μL wash-buffer (1xPBS, 0.05% Tween 20, pH 7,4) employing an automated ELISA plate washer. After removal of any residual wash buffer, plates were blocked by adding a blocking buffer (PBS, 1% Albumin BPLA, 2% sucrose, pH 7,4) 300 μL per well, covered and kept at room temperature for at least one and up to two hours. After that, the blocking buffer was aspirated and plates were kept at 35°C incubator for 15-16 h. Plates were vacuum sealed and kept at 4°C until further use. Before starting the analyses, all necessary reagents were brought to room temperature. Serum samples were diluted 1:50 into the analysis buffer 1xPBS, 0.5% BPLA, 2% sucrose, 0,1% Proclin 300) and added to the wells in duplicates at 50 μL per well. Plates were covered and incubated for 20 min on an orbital microtiter plate shaker at 450 rpm. After this, 50 μL of enzyme conjugate (1xPBS, 0.5% BPLA, 0.5 μg/mL ACE2-Fc-biotin (biotinylation with EZ-Link™ NHS-PEG4 Biotinylation Kit, ThermoFisher Scientific, Cat.-no. 21455; ACE2-Fc Icosagen Cat.-no P-308-100), 0.02 μg/mL Streptavidin-HRP (Pierce™ High Sensitivity Streptavidin-HRP, ThermoFisher Scientific, Cat.-no. 21132), 0.1% Proclin 300, pH 7.4) were added without touching the samples in the wells. Plates were covered and incubated on an orbital microtiter plate shaker at 450 rpm for 30 min. Thereafter, plates were aspirated and washed 4 times with 300 μL wash buffer as above. 100 μL TMB VII (Biopanda) were added to each well, the plates were covered and incubated for 10 min on an orbital microtiter plate shaker at 450 rpm. The reaction was terminated by the addition of 50 μL stop solution (0.5 M H_2SO_4). Plates were briefly shaken and incubated for 1-2 min and OD_{450} was measured with an ELISA plate reader (spectrophotometer).

The OD values of the measured samples were divided by the mean value of the three repeated samples of negative control (analysis buffer; composition as above) to obtain relative OD values. Samples with relative OD values of <0.75 were considered sufficient in blocking ACE2 binding. This threshold was previously determined during validation of the kit by comparison of negative sera and sera from COVID19-patients (measurements of level of blank (LoB) and level of detection (LoD) according to the CLSI standard EP17-A2).

SARS-CoV-2-induced cytopathic effect neutralization assay

The SARS-CoV-2 neutralization assay was performed using two viral strains: WT isolate (local Estonian isolate 3542) and a recombinant Omicron isolate (synthetic virus bearing amino acid substitutions in the SARS-CoV-2 Spike region characteristic to Omicron

strain; NCBI Reference sequence: NC_045512.2 with the following substitutions: A67V, del69–70, T95I, G142D, del143-145, del211, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F; this study).

For the rescue of SARS-CoV-2 recombinant viruses (WT and Omicron strains) from infectious clones, double knock-in baby hamster kidney (BHK) cells expressing SARS-CoV-2 N protein and human ACE2 receptor (kind gift from the research group of Prof. Alan Kohl, University of Glasgow) were pre-seeded onto T25 flasks to reach sub-confluency (~90-95%). Five micrograms of plasmid DNA encoding SARS-CoV-2 infectious clones (WT or Omicron strains) were mixed with 500 μL of OPTI-MEM medium (Thermo Fisher Scientific), and 5 μL of PLUS reagent (Thermo Fisher Scientific) were added to the mixtures. For each mixture, 7 μL of Lipofectamine LTX (Thermo Fisher Scientific) were mixed with 500 μL of OPTI-MEM. Both mixtures (DNA and Lipofectamine LTX) were combined and incubated for 5 min at room temperature. During incubation, the growth medium on pre-seeded BHK/SARS-CoV-2 N/hACE2 cells was replaced with 4 mL of viral growth medium (VGM, DMEM (Corning), 0.2% BSA (Sigma), 100 IU/mL Penicillin and 100 μg/mL Streptomycin (Sigma)). Transfected BHK cells were incubated at 37 °C and 5% CO2 in a humidified atmosphere for 24 h. Next, 1 mL of the medium was transferred to Vero E6 cells pre-seeded onto T25 flasks with 4 mL of VGM. Vero E6 cells were incubated at 37 °C and 5% CO2 in a humidified atmosphere until ~50% of the Vero E6 cells showed cytopathic effects (up to 10 days). Then, the viral stocks were harvested, clarified by centrifugation, and titrated using an immuno-plaque assay.

Before the neutralization test, all sera samples were incubated at 56°C for 30 min with shaking (450 rpm/min) for inactivation of the blood complement system (to avoid non-specific virus inactivation in the assay). Sera were 2-fold serially diluted starting from 1:4 to 1:4096 in the viral growth medium (VGM, containing DMEM (Corning), 0.2% BSA (Sigma), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma). The dilutions were made in 96-well plates in duplicates in a final volume of 50 μL. The last column of each 96-well plate was left for negative (non-infected cells, not treated with sera) and positive controls (infected cells, not treated with sera). Viral stocks (WT strain 6×10^6 pfu/mL, Omicron strain 2.8×10^5 pfu/mL) stored at −80°C in 50 μL aliquots were diluted in VGM to obtain 100 pfu/50 μL (the amount needed for one well in the 96-well plate). The diluted viruses (50 μL/well) were added to the 96-well plates containing sera dilutions and incubated at 37°C for 1 h. During incubation, Vero E6 cells grown on T175 flasks were mounted with trypsin (PAN-BioTech) and trypsin was inactivated by the addition of DMEM (supplemented with 10% FBS (PAN-BioTech), and Pen-Strep mixture). The cells were collected by centrifugation at 200*g* for 5 min and resuspended in VGM to obtain cell suspension containing 4×10^4 cells per 100 μL. Hundred μl of Vero E6 cell suspension were added to each well on a 96-well plate with virus-sera mixture (including negative and positive control wells). The plates were incubated at 37°C, 5% CO_2 for 96 h.

The cytopathic effect (disturbance of cell monolayer, cell rounding and coming off the bottom) was evaluated microscopically. As a comparison, negative (healthy monolayer preserved) and positive (all cells are round, no monolayer, extensive cell debris) wells were used as controls. The last sera dilutions showing no cytopathic effect were considered as neutralization titer. Negative sera (pre-immune sera taken before vaccination) showed neutralization titers <1:4.

ACE2 expressing HEK293 cells and pseudovirus neutralization assay

For the construction of HEK293 cells expressing the human ACE2 receptor, the cDNA was cut from the plasmid pLV-ACE2 (10 μg) with PsiI/AleI restriction enzymes (Thermo Fisher Scientific). This resulted in three fragments 7405bp, 2286bp and 1859bp. The human ACE2 receptor cDNA sequence on the 7405 bp fragment was purified from an agarose gel (Zymoclean Gel DNA Recovery Kit, Zymo Research Corporation, Cat. No. D4008). Two μg of purified ACE2 DNA fragment was electroporated (GenePulser Xcell, Bio-Rad Laboratories) at 975 μF, 220V to HEK293 STF (ATCC CRL-3249) cells (2×10^6 cells in 250 μL DMEM +10% FBS), washed with 10 mL DMEM (Gibco, High glucose; 4.5 g/L, 41,966-029) containing 10% FBS (Gibco, A3160802) and Penicillin-Streptomycin 50,000 U (Gibco, 15,140-122). Cells were centrifuged 5 min at 200 g, the supernatant was removed and cells pellet was suspended in 1 mL DMEM (+10% FBS, + Pen/Strep) and seeded into a 100mm cell culture dish (Greiner Bio-One, 664,160). 48 h post-transfection 200 μg/mL Hygromycin (Roche, 10843555001) was added to the growth medium. Cells were grown under hygromycin selection for 2 weeks. The resulting HEK293 ACE2 cell-line was tested for ACE2 receptor by Western blot assay and subsequently used in the pseudovirus neutralization assay as the host cell line.

To conduct the pseudovirus neutralization assay, HEK293 STF (ATCC® CRL-3249) cells were seeded to 6-well plates (Greiner Bio-One 657160) at a density of 5×10^5 cells per well. On the following day, cells were transfected with DNA, using Lipofectamine 3000 (Thermo Fisher Scientific, L3000-015). For transfections, the first test-tube contained 250 μL DMEM without FBS, 1 μg plasmid pNL4-3, 60 ng vector pQ1TAR5-S_dC19_WU#29 or pQ1TAR5-SdC19_B.1.1.529#15 and 2 μL P3000 reagent. The second tube contained 250 μL DMEM without FBS and 8 μL Lipofectamine 3000. Tubes were combined and incubated for 12 minutes at room temperature. Next, the DNA-Lipofectamine mixture was added dropwise to wells harboring the HEK293 STF cells, while shaking the plate carefully at the same time to allow even and quick mixing of the transfection mixture with the growth medium.

After 72h the pseudovirus containing media were filtered through 0.45 μm filter (Ministart high flow syringe filter 0.45 μm, Sartorius, REF 16533) and used immediately to infect HEK293-ACE2 cells (see above). First, filtered pseudovirus containing media were divided into 96 well plates (Greiner Bio-One, 655,180), 140 μL media to the first row and 100 μL media to other wells. Then 10 μL of human serum was added to the first row and mixed by pipetting. For further dilutions, 50 μL of media + serum from the first row was transferred to the second row, mixed and transferred to the third row until all six rows were supplied with the serial dilution. The very first and the last rows (A and H) were left empty.

In parallel HEK293-ACE2 cells were prepared by seeding into 96-well plates (Cell culture microplate 96 well, Greiner Bio-One, 655098) at a density of 10 000 cells per well. After 48h the growth medium was removed from cells very carefully and replaced with media + sera from a replica plate (as described above). On the following day, media were exchanged for DMEM + FBS.

72h after infection 65 μL of the medium was removed from each well and 30 μL of Steady-Glo reagent (Steady-Glo Luciferase Assay System, Promega, E2520) was added. The plate was incubated for 5 minutes at room temperature and Renilla luciferase activity was detected with a GloMax reader (GloMax Explorer, Promega).

The luciferase assay reveals luminescence through integration of the vector into the host genome. The relative luciferase units are translated into percent of integration where close to 0% indicates no integration thus full neutralization and 100% full integration (no neutralization). Percentages were calculated from preparations without prior antibody incubation (as 100%). The results were plotted in an XY-graph and IC50 values were obtained from a non-linear fit with a four-parametric logistic regression model.

SARS-CoV-2 spike-specific CD4 and CD8 memory T cell responses

For CD4 and CD8 T cell response analysis, freshly isolated PBMCs (2×10^6 cells) were stimulated with two overlapping SARS-CoV-2 Spike protein peptide pools at final concentration 1 μg/mL). One of the pools contained immunodominant regions from the WT virus (124 peptides, 15-mers with 11 aa overlap) and the other only those which are mutated in the Spike protein of Omicron VOC (83 peptides, 15-mer sequences with 11 amino acid overlap). The cells were stimulated with anti-CD28 and anti-CD49d for 20 h in X-VIVO 15 culture medium. Negative control stimulation (diluent with costimulatory antibodies) and positive control stimulation (CEFX peptides) were run in parallel. After the stimulation T cells were blocked with FcR blocking reagent and stained with antibody mix in staining buffer (0.5% BSA, 2mM EDTA in PBS) for 30 min at 4°C in the dark. Dead cell discrimination dye 7-AAD was added before the acquisition of the samples on the flow cytometer. Antigen-specific cells were gated according to the upregulation of activation-induced markers (AIM) CD137 and CD69 in memory CD8 T cells and CD137, OX40, and CD69 in memory CD4 T cells (percentage calculated from total CD8 or CD4 cells respectively) as described previously[20] and shown in Figure S4. The percentage of AIM positive cells in the negative control sample (diluent with costimulatory antibodies) was subtracted from the value from the stimulated sample. The cut-off level for Spike-specific $CD4^+$ and $CD8^+$ T cell positivity was drawn to 90^{th} percentile of 26 well-defined negative pre-pandemic individuals. The 90^{th} percentile cut-off value was 0.023% and 0.039% of AIM positive cells for $CD4^+$ and $CD8^+$ T cells, respectively. Eight percent of unexposed controls showed pre-existing T cell responses, likely because of contact with seasonal coronaviruses.[28] The results were analyzed with FCS Express 7 (*DeNovo* Software). Due to different number of peptides in each of the pools, the percentages of responsive T cells are not directly comparable.

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad version 9 was used for statistical analyses and generation of box and whiskers plots and correlation plots. Variables of data (S-RBD IgG, T cell results, and Spike RBD-ACE2 interaction inhibition values, age, and the score of side effects) were considered non-normally distributed and are reported as medians and interquartile range (IQR). As we could not confidently assume that our measurement values were sampled from Gaussian distributions, we used nonparametric tests; Kruskal-Wallis test, Friedman test and Spearman rank correlation, for the statistical analyses. Kruskal-Wallis test with subsequent Dunn's multiple comparison testing was used to analyze the S-RBD IgG data and for T cell response data, Friedman test with Dunn's multiple comparisons for Spike RBD-ACE2 interaction inhibition assay results. The correlations between S-RBD IgG values and inhibition results and age or number of side effects were analyzed using Spearman's correlation with confidence intervals of 95%. For statistical analyses p values <0.05 were considered to be statistically significant and p values >0.0001 are reported as exact numbers. Statistical test used and sample size can be found in figure legends.