Inactivated vaccine fueled adaptive immune responses to Omicron in 2-year COVID-19 convalescents

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There is NO Competing Interest.
Inactivated vaccine fueled adaptive immune responses to Omicron in 2-year COVID-19 convalescents

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Abstract

For nearly three years, humans have experienced multiple rounds of global transmission of SARS-CoV-2 and its variant strains. In addition, the widely used vaccines against SARS-CoV-2 involve multiple strategies of development and inoculation, globally. Thus, the acquired immunity established among humans is quite complicated, and there is still a lack of understanding within a panoramic vision. Here, we provide the special characteristics of the cellular and humoral immune responses in 2-year convalescents after the inoculation of inactivated vaccine, in parallel to vaccinated COVID-19 naïve persons and unvaccinated controls. The decreasing trends of the IgG, IgA, and neutralizing antibodies (NAb), but not IgM of the convalescents were reversed by the vaccination. Both cellular and humoral immunity to SARS-CoV-2 in convalescents after vaccination were higher than the vaccinated COVID-19 naïve persons. Notably, the inoculation of inactivated vaccine fueled the NAb to Omicron BA.1, BA.2, BA.4, and BA.5 in the 2-year convalescents, much higher than the NAb during 6 months and 1 year after symptoms onset. And no obvious T cell escaping to the S protein was observed after the inoculation in the 2-year convalescents. The study provides insight into the complicated features of acquired immunity of humans to SARS-CoV-2 and variants in the real world, and indicated that promoting vaccine inoculation is an essential way to achieve herd immunity against emerging viral variants for the ongoing COVID-19 pandemic, including the convalescents.

Keywords: Omicron, SARS-CoV-2, COVID-19, convalescents, inactivated vaccine, T cell, antibody.
1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic has been wreaking havoc for nearly three years, with more than 647 million infected cases worldwide as of December 2022 (https://covid19.who.int/). The herd immunity raised by the vaccination or natural infection has been recognized as the barrier to cut off the transmission of the virus. Thus, the immune memory characteristics of the convalescents and the vaccinees, including cellular and humoral immunity have raised wide concern. Cellular immune memory can last up to 1 year after the COVID-19 patient's recovery, while for humoral immunity, neutralizing antibodies (NAb) are still detectable 16 months after symptoms onset, but the neutralizing activity of antibodies has marked declined over time. But the immune statuses of convalescents become complicated in consideration of the application of vaccines.

In the past three years, to effectively prevent COVID-19 and achieve herd immunity with minimal loss of life, different types of vaccines have been developed and widely used globally, such as mRNA, inactivated, polypeptide subunit, and adenovirus vector vaccine. It was indicated that immune memory waned quickly after a single vaccine dose, but was boosted and became more sustained following the second and third doses. Studies also showed that the breakthrough infection among the vaccinees showed special immune characteristics. Prior vaccination substantially restrains pneumonia development, reduces cytokine storms, and facilitates clinical recovery. However, the features of the humoral and cellular immune memory are still largely unknown, especially among COVID-19 convalescents in the first wave during the pandemic who later received two or three doses of inactivated vaccines.

The emergence of different severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) variants, notably Delta and Omicron, has led to successive waves of pandemics, posing enormous public health challenges. Studies showed that Omicron is more transmissible than other variants. The Omicron variant harbors 32 mutations in spike glycoproteins (S), the mediator that binds to host cell receptors and the main target of neutralizing antibodies. As many as 15 of these mutations occurring within the receptor-binding domain (RBD), raised concern over the
Omicron escaping from immunity induced by natural SARS-CoV-2 wild type (WT) infection or vaccination. Studies have shown that sera from COVID-19 convalescents or individuals who were vaccinated exhibited substantially diminished neutralizing activity against Omicron \(^{16-21}\). To reveal the cellular immunity to Omicron, studies have shown that mRNA-1273, BNT162b2, Ad26.COV2.S, and NVX-CoV2373 vaccines elicit highly conserved cellular immunity to SARS-CoV-2 Omicron \(^{22-26}\). The memory status of T-cell immunity and the cross-reactivity to Omicron among inactivated vaccines were still unexplored, especially in terms of the vaccines recovered from COVID-19.

In this study involving COVID-19 and control cohorts, we assessed the antibody and T-cell responses to SARS-CoV-2 WT and Omicron strains in COVID-19 convalescents who have later inoculated with inactivated vaccines, and healthy individuals after routine two- or three-dose vaccination. This study will expand knowledge of the immune features and the memory persistence in vaccinated COVID-19 convalescents.
2. Methods

2.1 Study design and participants

The trail of healthy controls and COVID-19 convalescents who have received inactivated vaccines (CoronaVac/BBIBP-CorV/WIBP-CorV) was initiated two years after the infection, with a focus on immunity levels and cross-reaction to Omicron (Fig. 1). The demographic and clinical characteristics of all individuals, such as sex, severity, and vaccination status, were retrieved (Table 1). Participants included 51 COVID-19 vaccinated convalescents who recovered for two years after the infection in Macheng where the first case was imported from Wuhan in January 2020 during the first wave of COVID-19 (2Y-Vac). Among the 51 2-year convalescents, 23 blood samples were also kept before the vaccination during the followed up at 6 months(6m) and 1 year(1Y) after symptoms onset. Healthy control groups who were not infected by COVID-19 included 35 healthy vaccinated controls (HC-Vac) in Macheng and 26 healthy unvaccinated controls (HC-Unvac) in Beijing, China. The ethics of the study was approved by the Ethics Committee of National Institution for Viral Disease Control and Prevention, China CDC. All participants in the trial provided written informed consent.

2.2 Sera and peripheral blood mononuclear cells (PBMCs) collection

For sera collection, whole blood was collected by venipuncture in promoting coagulating tubes and centrifuged at 2500 rpm for 10 min. Supernatant was separated, aliquoted, and stored at -80°C until using. PBMCs were separated from anticoagulated blood by density gradient centrifugation with Human Lymphocyte Separation tubes (DAKEWE, China), centrifuged for 25 min at 800 g at room temperature. Then the PBMCs buffy coat was washed two times with RMPI1640, and frozen with fetal bovine serum (FBS) containing 10% DMSO immediately. Isolated PBMCs were frozen at -80°C overnight and then store in liquid nitrogen before use.

2.3 Enzyme-linked immunosorbent assay (ELISA)

Sera IgG and IgM antibodies binding to SARS-CoV-2 were assessed by an ELISA kit (WANTAI BioPharm, China) as described previously. Briefly, ELISAs were performed by 96-well plates coated with anti-IgG/IgM antibodies, with three
negative control, two positive control, and one blank control on each plate. Reference substances or sera samples (10 μL) diluted in 100 μL sample dilution buffer were added to the plates and incubated for 30 min at 37 °C. After incubation, the samples mentioned above were discarded and wells were washed five times with the washing buffer. Plates were incubated with SARS-CoV-2 spike protein receptor binding domain (RBD) conjugated to horseradish peroxidase (HRP) for 30 min at 37 °C except for blank control and then were washed five times with cleaning solution. 96-well plates were developed by the addition of color solution including TMB at 37 °C for 15 min. The reaction was terminated by a 50 μL stop solution containing H2SO4 at a concentration of no more than 2 M, and OD values at 450-630 nm were measured via a microplate reader within 10 min. The cutoff level for IgG was defined as OD value ≥ 0.16 + the mean OD of three negative controls (if the mean of three negative controls is < 0.03, take it as 0.03), whereas the cutoff level for IgM was the mean OD of three negative controls × 2.1 (if the mean of three negative controls was < 0.05, it was defined as 0.05).

2.4 Competitive enzyme-linked immunosorbent assay (cELISA)

Neutralizing antibodies against SARS-CoV-2 S protein RBD of WT, Delta, Omicron BA.1, BA.2, and BA.4/5 strains, were measured using competitive ELISA (Vazyme, China)27 (Supplementary Fig. 4). Sera samples at a dilution of 1:10 in diluent buffer were co-incubated with equal volume HRP-RBD solution at 37 °C for 30 min, and then the solution was transferred to 96-well ELISA plates coated with human ACE2 protein for 20 min incubation at 37 °C. Plates were washed 4× with cleaning fluids and developed with 50 μL TMB substrate solution, after 15 min, 50 μL stop buffer was used to terminate the reaction. Signal was read at 450 nm immediately on a microplate reader, and neutralizing antibody inhibition rates = (1 − OD450 value of sample/average OD450 value of four negative controls) × 100%, where the positive thresholds of inhibition rates were 20%. The neutralizing antibody inhibition rate was proportional to the titer of neutralizing antibodies.

2.5 Magnetic chemiluminescence enzyme immunoassay (MCLIA)

In addition to ELISA, the titer of anti-SARS-CoV-2 IgG, IgM, and IgA were
measured by MCLIA (Bioscience based on indirect immunoassay, Tianjin, China). As described previously, IgG/IgM/IgA in sera was conjugated with antigens spike protein and the nucleoprotein and immobilized on magnetic beads coated with anti-FITC antibody. The secondary antibody labeled alkaline phosphatase was the detection antibody and catalyzed substrate. The signal was calculated as relative light units (RLU) on an automated magnetic chemiluminescence analyser (Axceed 260, Bioscience) and positively associated with the level of antibody concentration. The anti-SARS-CoV-2-RBD antibody titers were expressed as the luminescence value of each sample divided by the cut-off (S/CO), the cut-off = the RLU mean of two positive controls × 0.1 + the RLU mean of two negative controls. If the S/CO value is yielded more than 1, it will be considered positive, and otherwise, it is seen as negative.

2.6 Peptide pools

For the detection of T cell responses to SARS-CoV-2 WT (IVDC-HB-01, GISAID: EPI_ISL_402119), 15- to 18-mer peptides with 10 amino acids of overlap were synthesized and covered the full length of S, M, and N proteins, as described previously. Meanwhile, the RBD peptide pool spanning residues 319-541 of WT S protein was also used. Spike peptides were combined with two pools S1 and S2, including 92 and 93 peptides, respectively. To further determine T cell cross-recognition to Omicron variant BA.1 (GISAID: EPI_ISL_7138045), the pools spanned Omicron spike protein carrying the selective 34 mutations, containing RBD, S1, and S2. All individual peptides were dissolved in DMSO at a concentration of 20 mg/mL and used at an ultimate concentration of 2 μg/mL for a single peptide in the pool.

2.7 Enzyme-linked immunospot (ELISpot) assay

IFN-γ ELISpot assay kits (BD, USA) were used to test SARS-CoV-2-specific T cell reaction in health controls and 2-year COVID-19 convalescents, as described in our previous study. Fresh PBMCs after thawing were used to determine the responses to different SARS-CoV-2 peptide pools (S1, S2, M, and N). Then the left PBMCs were plated at 2.5~3.5 × 10^6 cells/well in 24-well plates and rested for 3 h in
RPMI1640 supplemented with 10% FBS and 1% Penicillin-Streptomycin Solution, stimulated with four SARS-CoV-2 structural peptide pools, respectively. After cultivation for 9 days under the addition of IL-7 (25 μg/mL) and IL-2 (200 U/mL), 1 x 10^5 cells were added per well to ELISpot plates pre-coated with the capture antibody at 4 °C overnight, with the addition of peptides, PMA as positive control, and medium as negative control. After incubation in a 5% CO₂ incubator for 18 h at 37 °C, the plates were washed with deionized water, PBS-T, and PBS buffers and dyed with detection antibody (Biotinylated Anti-human IFN-γ), following the enzyme conjugate (Streptavidin-HRP). Spots in air-dry plates were counted using an ELISpot reader (CTL-Immunospot S5 versa Analyzer). If the spot forming cells (SFCs) /10^6 PBMCs of negative control wells are < 20, a positive response will be considered when the spot had ≥ 40; If the SFCs/10^6 PBMCs of negative control wells are > 20, we will define the results as positive which are > 2-fold of negative control wells.

2.8 Intracellular cytokine staining (ICS) and flow cytometry

After the 9-day culture, PBMCs were collected and washed twice with complete medium. 1 x 10^6 cells in each tube were incubated with or without 10 μg/mL WT (GIASID: EPI_ISL_402119)/Omicron (GIASID: EPI_ISL_7138045) peptide pools for 1 h in a 5% CO₂ atmosphere at 37°C, and cultured for 9-12 h after blocking the IFN-γ secretion with Golgiplug (BD, USA). Then PBMCs were first stained with Zombie NIR™ Dye (Biolegend, 1:1000 dilution), followed by staining with surface antibodies CD3-FITC (Biolegend, Clone: UCHT1, 1:100 dilution), CD8-BV510 (BD Horizon, Clone: SK1, 1:100 dilution), CD4-PerCP5.5 (Biolegend, Clone: OTK4, 1:100 dilution) for 30 min on ice. Cells were thereafter washed with FACS buffer containing 0.5% FBS in PBS and fixed with fixation and permeabilization solution (BD Bioscience), and then staining was performed on ice for 30 min with intracellular cytokine antibodies IL-2-APC (Biolegend, Clone: MQ1-17H12, 1:100 dilution), IFN-γ-PE-Cy7 (BD Pharmingen, Clone: B27, 1:100 dilution), TNF-α-PE (Biolegend, Clone: Mab11, 1:100 dilution). After the final wash, all events were measured on a BD LSR Fortessa flow cytometer (BD Bioscience) and analyses were performed by using FlowJo v.10.6.2. All samples had blank control, which was processed similarly
except for peptide pool stimulation.

**2.9 Statistical analysis**

The statistical analyses were conducted with IBM SPSS Statistics 26.0. A comparison of antibodies and T-cell reaction between groups were analyzed using the Mann-Whitney U-test and Paired Wilcoxon Signed-Rank Test. The difference in positive rate was performed by the Chi-square test. Details of statistical analysis are illustrated in the figure legend of each trial. The figures were done with GraphPad Prism 8.0.2. The statistical significance of these results was defined as * p < 0.05, ** p < 0.01, and *** p < 0.001.

**2.10 Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All data supporting the findings of this study are included in the article and its Supplementary Information files. Source data are provided with this paper.
3. Results

3.1 SARS-CoV-2 antibody features in 2-year convalescents after inactivated vaccines

To increase the vaccination rate coverage of SARS-CoV-2 and construct herd immunity, the 2-year COVID-19 convalescents in Macheng were vaccinated in the second half of 2021. Herein, to assess SARS-CoV-2-specific antibody features of COVID-19 convalescents, who had recovered for 2 years after inactivated vaccines (CoronaVac or BBIBP-CorV or WIBP-CorV), sera from participants was evaluated for the presence of IgG, IgM, IgA, and NAb together with the vaccinated healthy control donors in the same town. We found that over 96% of 2-year convalescents after inactivated vaccines have a positive IgG response to SARS-CoV-2, as presented by the double positive for ELISA and MCLIA tests (Table 2). No significant difference was observed in the positive proportion of IgG between the healthy individuals (85.7%) and 2-year convalescents (96.1%) after the inoculation of inactivated vaccines (Table 2). However, IgG levels of 2-year convalescents after inactivated vaccines were higher compared to healthy individuals with either vaccinated (p < 0.0001) or unvaccinated (p < 0.0001, Fig. 2A, B). 2-year convalescents after vaccination exhibited IgG titers that were 1.2-fold more than healthy individuals after vaccination (Fig. 2A, B). Certain levels of IgM could be detected in both vaccinated 2-year convalescents and healthy individuals but with no significant difference (31.4% vs 28.6% in ELISA; 23.5% vs 20.0% in MCLIA) (Fig. 2C, D and Table 2).

To reveal the dynamic antibody responses within the convalescents, we retrieved the paired sera at the 6-month and 1-year follow-up visits of the convalescents. The 2-year convalescents had higher IgG antibodies following vaccination in contrast with the 1-year visit, with 50% (9/18) of employees increased more than 1-fold (p = 0.005 for ELISA and p = 0.004 for MCLIA), but with no difference with the 6-month visit (Fig. 2E, F). However, a significant reducing trend was observed for IgM levels among the convalescents from 6 months to 2-year recovery, although the convalescents were vaccinated before the 2-year visit (Fig. 2G, H). We next analyzed
whether severity affected the levels of IgG/IgM, and no differences were observed among vaccinated 2-year convalescents with different disease severities (Fig. 2I-L).

We also tested the IgA antibody within the sera of the donors. 58.8% (30/51) of 2-year convalescents had positive IgA responses to SARS-CoV-2 S protein RBD, which were significantly higher compared with the vaccinated individuals without prior infection (Fig. 2M). For a longitudinal analysis of the IgA antibodies among the convalescents, the avidity of IgA antibodies decreased from 6-month to 1-year visits but increased significantly during the 2-year visit in the convalescents (Fig. 2N).

To demonstrate the potential influence of the vaccination dose on the antibody levels among the convalescents and the healthy individuals, we compared the antibodies from healthy individuals after 3 doses and 2-year convalescents vaccinated with 2 doses. The IgG, IgA, and NAb levels of 2-year convalescents after 2 doses of inactivated vaccines were even higher than healthy individuals with 3 doses of vaccination (Supplementary Fig. 1A, B, E, F). No IgM differences were observed between the two groups (Supplementary Fig. 1C, D). Overall, these results suggest that 2-year convalescents after inoculated inactivated vaccines possibly develop and maintain stronger IgG, IgA, and NAb but not IgM antibodies responses to the WT stain than vaccinated healthy individuals.

### 3.2 T cell reactivity to SARS-CoV-2 in 2-year convalescents after inactivated vaccines

We also compared the SARS-CoV-2-specific T cell responses among the 2-year convalescents and healthy controls after inactivated vaccines, assessed by ELISpot and ICS assays (Fig. 3A). In the ex vivo ELISpot assay based on freshly-isolated PBMCs, both two groups have low-frequency antigen-specific T cell responses, with no obvious difference between 2-year convalescents and healthy controls (Fig. 3B). After incubating with viral S1, S2, M, and N peptides in vitro, the positive proportion with IFN-γ responses in the population showed a noteworthy increase (Fig. 3C). 92.2% (47/51) of the 2-year convalescents had positive T-cell response memory to at least one of these four antigens, which was markedly higher than the corresponding proportion (54.3%) of vaccinated healthy controls (Fig. 3F and Table 4). Meanwhile,
the altitudes of T cell responses to M and N peptide pools, as well as S1 and S2, were
greater in the 2-year convalescents (median 500 SFU/10⁶ PBMC [IQR 190-1582.5]
for M; median 485 SFU/10⁶ PBMC [IQR 200-1487.5] for N) than in the healthy
controls following vaccination (median 20 SFU/10⁶ PBMC [IQR 0-105] for M;
median 20 SFU/10⁶ PBMC [IQR 0-222.5] for N) (Fig. 3C). We also compared the T
cell immune responses between the four peptide pools and found that proteins M and
N overlapping peptide pools-specific T cell responses were much higher than S
protein among both the 2-year convalescents after vaccination (Fig. 3C). 2-year
convalescents after 2 doses immunization can produce even significantly higher IFN-γ
responses than healthy controls inoculated with the 3 doses vaccination, which further
underlined the significance of memory T-cell responses induced by the previous
infection, and boosted by the following vaccination (Supplementary Fig. 2A, B). But
there were no significant differences in T cell immune responses between the 2-year
convalescents with different severity during the acute infection (Fig. 3D, E).

To further investigate the SARS-CoV-2-specific CD8⁺ and CD4⁺ T cells in
participants, we stimulated PBMCs with peptide pools of four antigens (S1, S2, M,
and N) and measured cytokine production (IFN-γ, IL-2, and TNF-α) using flow
cytometry (Supplementary Fig. 3). Though without statistical significance, the median
ratios of cytokine-secreting T cells against four peptide pools were higher in the 2-
year convalescents than in healthy controls who had been vaccinated (Fig. 3G, H).
Furthermore, specific T cells among both groups developed stronger cellular
immunity than individuals with neither infection nor vaccination (Fig. 3H).

3.3 The Omicron variant cross-recognizing feature of ancestral SARS-CoV-2-
specific T cell and antibody

We also explored whether the Omicron variant immune evasion exists for the T
cell responses and antibodies induced by the inactivated vaccine inoculated 2-year
convalescents. We performed a competitive ELISA assay to further evaluate the
SARS-CoV-2 S protein RBD-binding neutralizing antibodies against the WT strain as
well as Delta and Omicron (BA.1, BA.2, and BA.4/5). When comparing the titers at
three time points, i.e. 6 months, 1 year and 2 years, neutralizing capacities of sera
showed a decreasing trend between 6-month and 1-year cohorts against the WT as well as other variants (Fig. 4A). The inhibition rates of NAb to each strain were markedly raised in the vaccinated 2-year convalescents compared to their unvaccinated time points at 6 months and 1 year, with median inhibition rates against five strains (WT, Delta, BA.1, BA.2, and BA.4/5) in the 2-year visit that were 1.3, 1.4, 4.5, 2.0, and 2.9 times those for the 1-year visit (Fig. 4A). None of the individuals had detectable neutralizing antibodies in the unvaccinated cohort (Fig. 4B). In the sera of 2-year convalescents with vaccination, the median NAb inhibition rates against the WT, Delta, BA.1, BA.2, and BA.4/5 strains (94.5, 90.9, 39.3, 47.8, and 37.6%), were much higher than in healthy controls with vaccination against these strains (74.7, 54.7, 11.8, 16.7, and 14.0%) (Fig. 4B). The median anti-RBD NAb inhibition levels against WT, Delta, BA.1, BA.2, and BA.4/5 strains were higher by 1.3-, 1.7-, 3.3-, 2.9, 2.7-fold, respectively, in the vaccinated 2-year convalescents as compared to the vaccinated healthy controls (Fig. 4B). The NAb inhibition against WT and other variants was directly compared within 2-year convalescents and healthy controls after vaccination (Fig. 4C, D). The positive proportions of vaccinated 2-year convalescents sera against BA.1 (70.6%), BA.2 (78.4%), and BA.4/5 (82.4%) variants were lower than WT (94.1%) and Delta (94.1%) but mostly above the detection threshold (Fig. 4C). However, we found statistical differences among all five strains, except for BA.1 and BA.4/5 strains (p < 0.001, Fig. 4C and Table 3). For the 2-year convalescents who had a history of vaccination, non-reduction in the median ratio of neutralizing activity of Delta, a 2.4-fold reduction of BA.1, a 2.0-fold reduction of BA.2, and a 2.5-fold reduction of BA.4/5 was shown, compared with the neutralization of WT strain (Fig. 4C). Even lower proportions of healthy controls after vaccinations showed positive neutralization responses against BA.1 (34.3%), BA.2 (37.1%) as well as BA.4/5 (34.3%), and a markedly drop in comparison with WT (85.7%) and Delta (80.0%) was observed (Fig. 4D and Table 3). Notably, in the healthy controls, the cross-neutralizing antibodies were much lower than that for 2-year convalescents after vaccination. The drop level of NAb inhibition rates against Delta, BA.1, BA.2, and BA.4/5 was 1.4-, 6.3-, 4.5-, and 5.3-fold respectively, compared to WT (Fig. 4D).
Comparing the geometric mean ratio of four SARS-CoV-2 variants NAb inhibition rates normalized WT, these data indicate that 2-year vaccinated convalescents lead to more broadly cross-neutralization of BA.1 and BA.4/5 as compared with healthy individuals with vaccination, but not for Delta and BA.2 (Fig. 4E). We also calculated the seropositivity rates of NAb against all four SARS-CoV-2 strains, regarding cross-protection of neutralizing antibodies. We observed a higher proportion of antibodies neutralized overall stains in the convalescent participants (68.6%) compared to the healthy controls following vaccination (28.6%) (p = 0.001, Fig. 4F). These data show that 2-year COVID-19 convalescents with vaccination have more tolerance to the Omicron variant than healthy controls after vaccination, though both groups were inoculated with the inactivated vaccine for the WT strain.

Furthermore, we assessed the T cell responses from 2-year convalescents and healthy controls after vaccination to WT and Omicron BA.1 peptide pools (RBD, S1, and S2) (Fig. 5A). T cell reactivities in 2-year convalescents after inoculated inactivated vaccines were significantly higher than in healthy controls against WT and BA.1 peptide pools (p < 0.001) (Fig. 5A, B). Notably, we found no remarkable difference in IFN-γ levels between WT and Omicron BA.1 for 2-year convalescents (median 150 SFU/10^6 PBMC for S1; median 130 SFU/10^6 PBMC for S1-O) and healthy controls following immunization (Fig. 5B). Based on the profile of cytokine expression (IFN-γ, IL-2, TNF-α) after stimulation with either WT or Omicron BA.1 S1/S2 antigens among the 2-year convalescents and healthy controls after vaccination, we demonstrated that high cross-CD4+ and CD8+ T cell responses retained against Omicron BA.1 in both 2-year convalescents and healthy controls after vaccination (Fig. 5C, D). In conclusion, the spike-specific T cell reactivity produced by vaccination and/or previous infection is vastly preserved against Omicron BA.1.

4. Discussion

Since its emergence in 2019, the SARS-CoV-2 virus has continued to evolve into different mutant strains, posing continuous challenges to the control of COVID-19. To achieve the goal of herd immunity, a variety of vaccines have been introduced worldwide. One of the main vaccines promoted in China is the inactivated virus
vaccine, which has a high inoculation coverage among the population up to date. Thus, features of the immune memory triggered by natural infections of SARS-CoV-2 have become complicated, as several COVID-19 convalescents were vaccinated. At 2-year post-disease start visits, we only enrolled 8 unvaccinated convalescents. Thus in this study, we mainly focused on the SARS-CoV-2-specific humoral and cellular immunity and the ability to protect against Delta and Omicron variants after inoculated inactivated vaccines in COVID-19 convalescents and general healthy controls. Data from our study showed that SARS-CoV-2-specific IgG, IgA, and neutralizing antibodies were significantly higher in vaccinated convalescents than in vaccinated healthy controls. Though both groups had lower cross antibodies to Omicron variants, the vaccinated COVID-19 convalescents kept higher cross-antibody responses to Omicron compared to the vaccinated healthy controls. Meanwhile, the convalescents after vaccination carried robust T cell immune memory to SARS-CoV-2 and no T cell immune escape was observed for Omicron, and with a higher T cell immunity level than the vaccinated healthy controls.

Our previous longitudinal study of COVID-19 convalescents demonstrated that SARS-CoV-2-specific humoral immunity was present within 95% of convalescents at 1-year post-disease onset, which was also concordant with other cohort studies. Recently, it was reported that NAbs against SARS-CoV-2 could persist for one and a half years among COVID-19 convalescents. However, most of the studies indicated a decreasing trend of the SARS-CoV-2-specific antibody levels with time among the convalescents. In our study, through the antibody titers decreased from 6 months to 1 year, a sharply increasing in the antibody titers can be observed among the 2-year convalescents after administration with inactivated vaccines, showing the robust antibody-inducing capacity of the inactivated vaccines, especially among the population after natural infection. The enhanced antibody level may play a positive role in the convalescents to avoid re-infection. Thus, vaccination should still be encouraged for the convalescents.

Although the IgG antibody could be obviously reinforced by the inactivated vaccine among the convalescents 2 years after symptoms onset, our data showed
positive rates of IgM antibody continued the decreasing trend even among the convalescents after the vaccination. This indicated that IgG among the convalescents could be more easily enhanced by the inactivated vaccine\textsuperscript{43}.

Previous studies showed that IgA dominates the early neutralizing antibody response to SARS-CoV-2 and may play an important role in the prevention of severe illness\textsuperscript{44,45}. And a higher concentration of IgA antibody can be detected in the convalescents who received booster vaccination of mRNA vaccine, but there is no significant increase in booster-vaccinated COVID-19 naïve people\textsuperscript{37}. Our studies indicated that the decreasing of IgA antibodies in follow-up sera from 6 months to 1 year after symptoms onset, and observed inactivated vaccines could also induce an increase of IgA antibody titer at 2 years post-infection.

The persistence of T-cell immunity stimulated by SARS-CoV-2 infection may play an important role in alleviating severe disease in the reinfection\textsuperscript{46}. Here we found that T cell immune memory among the 2-year convalescents after vaccination was much higher than the vaccinated even boosted healthy controls. This demonstrates that T cell immune memory by natural infection could persist up to 2 years after symptoms onset. Several studies show that mRNA COVID-19 vaccines could stimulate a certain level of virus-specific CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell immunity\textsuperscript{8,47,48}, while T-cell immunity induced by inactivated vaccines is far from being well-defined. However, unlike inactivated vaccines, mRNA vaccines are designed primarily to focus on the S protein, the cellular immunity that stimulates by this vaccine only targets the S protein\textsuperscript{8}. Our data showed that inactivated vaccines could stimulate T cell immunity against structural proteins S, M, and N in COVID-19 naïve people. Nevertheless, when evaluating the inactivated vaccine-related T cell responses, the potential baseline cross-T cell immunity against M or N proteins of common cold coronaviruses would be considered\textsuperscript{3}.

Despite extensive mutations-related neutralization escape against Omicron could be observed, the overall T cell responses induced by vaccination or infection could cross-recognize the variants\textsuperscript{26}. We verified that the neutralizing ability of the antibodies to Delta, Omicron BA.1, BA.2, and BA.4/5 decreased among vaccinated 2-
year convalescents and healthy vaccinees. However, the cross-neutralizing ability against the variants among the convalescents was significantly stronger than that of the vaccinated COVID-19 naïve people. This may indicate a special cross-reactive antibody-inducing feature by natural infection and the necessity for vaccination among the convalescents. Previous studies determined that cellular immunity elicited by the mRNA vaccine could cross-recognize the Omicron. We herein showed that the cellular immunity stimulated by inactivated vaccine also has a cross-response to Omicron. As it turns out, the vaccination of inactivated vaccine is beneficial to the population against the WT virus and its variants, and it is an effective method to realize herd immunity.

Our current study has several limitations. First, the number of subjects in our study, especially the number of subjects’ PBMCs available for the ICS test is limited, thus the statistical analysis has difficulties showing the differences between groups in the ICS test. However, our main conclusions are based on the statistical differences within the ELISpot assays and antibody tests. Second, we use the ELISA kit whose antigen is S-RBD as a surrogate for live virus neutralization assay, the detection results of the ability of antibodies to neutralize the virus may not be as accurate as the neutralization test, because other regions out of S-RBD could also be neutralizing. However, previous studies have applied the ELISA to detect neutralizing antibodies, and the data suggested that using RBD as an immunogen might be a better strategy for creating a robust neutralization Ab response compared with full-length S protein, and the ELISA results were highly responsive to neutralizing antibody levels.

In summary, we provide the special characteristics of the cellular and humoral immune responses in 2-year convalescents after the inoculation of inactivated vaccines. The decreasing trends of the IgG, IgA, and neutralizing antibodies, but not IgM of the convalescents were reversed by the vaccination. And both cross-reactive cellular and humoral immunity to Omicron variants in convalescents after vaccination were higher than in the vaccinated healthy controls. Thus, promoting vaccine inoculation is an essential way to achieve herd immunity during the ongoing COVID-19 pandemic, even for the convalescents.
Acknowledgements:
The study was supported by the National Key Research and Development Program of China (2022YFC2604105 and 2021YFC2301400), the National Natural Science Foundation of China (82161148008 and 81971501), and CAMS Research Units of Adaptive Evolution and Control of Emerging Viruses (2018RU009). W.J.L. is supported by the Excellent Young Scientist Program of the National Natural Science Foundation of China (81822040).

Author contributions

Competing interests
The authors declare no competing interests.


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BBIBP-CorV (Sinopharm) and BNT162b2 (Pfizer-biontech) vaccines using a homologous or heterologous booster vaccination strategy. Vaccines (Basel) 10, 539 (2022).


Table 1. Characteristics of participants in this study.

<table>
<thead>
<tr>
<th></th>
<th>Convalescents-6months(^a) (n=23)</th>
<th>Convalescents-1year(^a) (n=23)</th>
<th>Convalescents-2 year (Vaccination) (n=51)</th>
<th>Health control (Vaccination) (n=35)</th>
<th>Health control (Vaccination) (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>9(39.1%)</td>
<td>14(60.9%)</td>
<td>25(49.0%)</td>
<td>10(28.6%)</td>
<td>12(46.2%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Median (IQR)</td>
<td>Range</td>
<td>Median (IQR)</td>
<td>Range</td>
<td>Median (IQR)</td>
</tr>
<tr>
<td></td>
<td>57(45,69.5)</td>
<td>37-76</td>
<td>51(44,60)</td>
<td>21-68</td>
<td>37.5(26,52.5)</td>
</tr>
<tr>
<td>Severity</td>
<td>Asymptomatic</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1(4.3%)</td>
<td>9(39.1%)</td>
<td>10(43.5%)</td>
<td>3(13.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2(3.9%)</td>
<td>23(45.1%)</td>
<td>19(37.3%)</td>
<td>7(13.7%)</td>
<td></td>
</tr>
<tr>
<td>Vaccination</td>
<td>Unvaccination</td>
<td>1 dose</td>
<td>2 doses</td>
<td>3 doses</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5(9.8%)</td>
<td>36(70.6%)</td>
<td>10(19.6%)</td>
<td>—</td>
</tr>
<tr>
<td>Days since last dose (Median, IQR)</td>
<td></td>
<td>1 dose</td>
<td>2 doses</td>
<td>3 doses</td>
<td></td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>151(147,153)</td>
<td>124(106,175.5)</td>
<td>150(142.5,163)</td>
<td>62.5(52.75,65.25)</td>
<td>—</td>
</tr>
<tr>
<td>Days post after symptoms onset</td>
<td>Median (IQR)</td>
<td>177(172.5,179.5)</td>
<td>353(348.5,355.5)</td>
<td>696(691,699)</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\)23 of 51 2-year convalescents were also enrolled at the 6-month and 1-year follow-up visits.
IQR: interquartile range.
Table 2. The seropositivity of IgG and IgM antibodies in vaccinated 2-year convalescents and healthy controls with or without vaccination.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Group</th>
<th>Case Number</th>
<th>Positive Number</th>
<th>Positive Proportions (%)</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HC-Unvac</td>
<td>26</td>
<td>1</td>
<td>3.6</td>
<td>(0.1,19.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>HC-Vac</td>
<td>35</td>
<td>33</td>
<td>94.3</td>
<td>(80.8,99.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>51</td>
<td>49</td>
<td>96.1</td>
<td>(86.5,99.5)</td>
<td>1.000</td>
</tr>
<tr>
<td>ELISA-IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC-Unvac</td>
<td>26</td>
<td>0</td>
<td>0.0</td>
<td>NA</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>HC-Vac</td>
<td>35</td>
<td>30</td>
<td>85.7</td>
<td>(69.7,95.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>51</td>
<td>49</td>
<td>96.1</td>
<td>(86.5,99.5)</td>
<td>0.115</td>
</tr>
<tr>
<td>MCLIA-IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC-Unvac</td>
<td>26</td>
<td>0</td>
<td>0.0</td>
<td>NA</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>HC-Vac</td>
<td>35</td>
<td>10</td>
<td>28.6</td>
<td>(14.6,46.3)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>51</td>
<td>16</td>
<td>31.4</td>
<td>(19.1,45.9)</td>
<td>0.781</td>
</tr>
<tr>
<td>ELISA-IgM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC-Unvac</td>
<td>26</td>
<td>1</td>
<td>3.8</td>
<td>(0.1,19.6)</td>
<td>0.122</td>
</tr>
<tr>
<td></td>
<td>HC-Vac</td>
<td>35</td>
<td>7</td>
<td>20.0</td>
<td>(8.4,36.9)</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>51</td>
<td>12</td>
<td>23.5</td>
<td>(12.8,37.5)</td>
<td>0.698</td>
</tr>
<tr>
<td>MCLIA-IgM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC-Unvac</td>
<td>26</td>
<td>0</td>
<td>0.0</td>
<td>NA</td>
<td>0.503</td>
</tr>
<tr>
<td></td>
<td>HC-Vac</td>
<td>35</td>
<td>2</td>
<td>5.7</td>
<td>(0.7,19.2)</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>51</td>
<td>10</td>
<td>19.6</td>
<td>(9.8,33.1)</td>
<td>0.131</td>
</tr>
</tbody>
</table>

**Group**
- HC-Unvac: healthy controls without vaccination; HC-Vac: healthy controls with vaccination; 2Y-Vac: vaccinated 2-year convalescents.

**Positive Proportions (%)**

**95% CI**
- 95% confidence interval.

**P value**
- Statistical significance was determined using a two-tailed Chi-square test.

**Double-positive, i.e., IgG or IgM antibodies were detectable in both ELISA and MCLIA assays.**

**NA: not applicable.
Table 3. The seropositivity of neutralizing antibodies in vaccinated 2-year convalescents and healthy controls with or without vaccination.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Groupa</th>
<th>Case Number</th>
<th>Positive Number</th>
<th>Positive Proportions (%)</th>
<th>95% CI</th>
<th>P valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA-NAb</td>
<td>HC-Unvac</td>
<td>26</td>
<td>0</td>
<td>0.0</td>
<td>NA</td>
<td>HC-Unvac vs HC-Vac &lt;0.0001</td>
</tr>
<tr>
<td>(WT)</td>
<td>HC-Vac</td>
<td>35</td>
<td>30</td>
<td>85.7</td>
<td>(69.7,95.2)</td>
<td>HC-Unvac vs 2Y-Vac &lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>51</td>
<td>48</td>
<td>94.1</td>
<td>(83.8,98.8)</td>
<td>HC-Vac vs 2Y-Vac 0.347</td>
</tr>
<tr>
<td>ELISA-NAb</td>
<td>HC-Unvac</td>
<td>26</td>
<td>0</td>
<td>0.0</td>
<td>NA</td>
<td>HC-Unvac vs HC-Vac &lt;0.0001</td>
</tr>
<tr>
<td>(Delta)</td>
<td>HC-Vac</td>
<td>35</td>
<td>28</td>
<td>80.0</td>
<td>(63.1,91.6)</td>
<td>HC-Unvac vs 2Y-Vac &lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>51</td>
<td>48</td>
<td>94.1</td>
<td>(83.8,98.8)</td>
<td>HC-Vac vs 2Y-Vac 0.096</td>
</tr>
<tr>
<td>ELISA-NAb</td>
<td>HC-Unvac</td>
<td>26</td>
<td>0</td>
<td>0.0</td>
<td>NA</td>
<td>HC-Unvac vs HC-Vac &lt;0.0001</td>
</tr>
<tr>
<td>(BA.1)</td>
<td>HC-Vac</td>
<td>35</td>
<td>12</td>
<td>34.3</td>
<td>(19.1,52.2)</td>
<td>HC-Unvac vs 2Y-Vac &lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>51</td>
<td>36</td>
<td>70.6</td>
<td>(56.2,82.5)</td>
<td>HC-Vac vs 2Y-Vac 0.001</td>
</tr>
<tr>
<td>ELISA-NAb</td>
<td>HC-Unvac</td>
<td>26</td>
<td>0</td>
<td>0.0</td>
<td>NA</td>
<td>HC-Unvac vs HC-Vac &lt;0.0001</td>
</tr>
<tr>
<td>(BA.2)</td>
<td>HC-Vac</td>
<td>35</td>
<td>13</td>
<td>37.1</td>
<td>(21.5,55.1)</td>
<td>HC-Unvac vs 2Y-Vac &lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>51</td>
<td>40</td>
<td>78.4</td>
<td>(64.7,88.7)</td>
<td>HC-Vac vs 2Y-Vac &lt;0.0001</td>
</tr>
<tr>
<td>ELISA-NAb</td>
<td>HC-Unvac</td>
<td>26</td>
<td>0</td>
<td>0.0</td>
<td>NA</td>
<td>HC-Unvac vs HC-Vac &lt;0.0001</td>
</tr>
<tr>
<td>(BA.4/5)</td>
<td>HC-Vac</td>
<td>35</td>
<td>12</td>
<td>34.3</td>
<td>(19.1,52.2)</td>
<td>HC-Unvac vs 2Y-Vac &lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>51</td>
<td>42</td>
<td>82.4</td>
<td>(69.1,91.6)</td>
<td>HC-Vac vs 2Y-Vac &lt;0.0001</td>
</tr>
</tbody>
</table>

aHC-Unvac: healthy controls without vaccination; HC-Vac: healthy controls with vaccination; 2Y-Vac: vaccinated 2-year convalescents.
b95%CI: 95% confidence interval.
cStatistical significance was determined using a two-tailed Chi-square test.
Table 4. Proportion of donors with positive T cell responses to overlapping peptide pools of WT

<table>
<thead>
<tr>
<th>Peptide pool</th>
<th>Group(^a)</th>
<th>Positive Proportions (%)</th>
<th>95% CI(^b)</th>
<th>P value(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>HC-Vac</td>
<td>11.4(4/35)</td>
<td>(3.2,26.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>56.9(29/51)</td>
<td>(42.2,70.7)</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>HC-Vac</td>
<td>14.3(5/35)</td>
<td>(4.8,30.3)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>46.0(23/50)</td>
<td>(31.8,60.7)</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>HC-Vac</td>
<td>24.2(8/33)</td>
<td>(11.1,42.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>77.1(37/48)</td>
<td>(62.7,88.0)</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>HC-Vac</td>
<td>45.5(15/33)</td>
<td>(28.1,63.6)</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>75.0(36/48)</td>
<td>(60.4,86.4)</td>
<td></td>
</tr>
<tr>
<td>SARS-CoV-2</td>
<td>HC-Vac</td>
<td>54.3(19/35)</td>
<td>(36.6,71.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>2Y-Vac</td>
<td>92.2(47/55)</td>
<td>(81.1,97.8)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)HC-Vac: healthy controls with vaccination; 2Y-Vac: vaccinated 2-year convalescents.

\(^b\)95%CI: 95% confidence interval.

\(^c\)Statistical significance was determined using a two-tailed Chi-square test.
Fig. 1 Flow diagram of the study.

Schematic overview of workflow to detect the immune memory in vaccinated 2-year convalescents and healthy controls with vaccination. A total of 112 individuals were recruited for the analysis in Macheng and Beijing, and they were classified by whether they had prior exposure to SARS-CoV-2 and vaccination. 23 of 51 COVID-19 convalescents were enrolled in the follow-up study at 6 months, 1 year, and 2 years after symptoms onset. The SARS-CoV-2-specific IgG, IgM, IgA, and neutralizing antibodies of all participants were measured by ELISA and MCLIA. Intensity of T-cell responses to overlapping peptide pools from wild type (WT) or Omicron spike(S) and WT membrane(M) and nucleocapsid(N) proteins were analyzed by ELISpot and ICS in all 2-year convalescents and vaccinated healthy controls. Numbers for each group were displayed in the flow chart.
Fig. 2 Humoral responses to SARS-CoV-2 in vaccinated 2-year convalescents.

IgG, IgM, IgA, and neutralizing antibody levels were measured by MCLIA, ELISA, and cELISA in healthy individuals after being vaccinated (HC-Vac, green, n=35) or unvaccinated (HC-Unvac, blue, n=26), and in COVID-19 convalescents following at 6 months (6m, orange, n=23), 1 year (1Y, green, n=23) and 2 years with vaccination (2Y-Vac, red, n=51). A-D, Anti-SARS-CoV-2 IgG (A, B) or IgM (C, D) antibody titers determined by ELISA (A, C) or MCLIA (B, D). E-H, The longitudinally dynamic changes of IgG (E, F) or IgM (G, H) levels in convalescents at 6 months, 1 year, and 2 years in the same individuals after symptoms...
onset, using ELISA (E, G, n=23) or MCLIA (F, H, n=18). I-L, Correlation of IgG/IgM titers and different disease severity in 51 vaccinated 2-year convalescents (2Y-Vac), including Asym-Mild (Asymptomatic and Mild, red, n=25) and Mod-Sev (Moderate and Severe, dark green, n=26). Black bars denote median responses. M-N, IgA antibody levels measured by MCLIA were shown in HC-Vac and 2Y-Vac groups (M, HC-Vac: n=35; 2Y-Vac: n=51). The dynamic changes of IgA antibody responses during the 2-year follow-up (N, n=18). Each multi-colored dot or connective line indicates one individual. The dotted lines reflect the cut-off values for positive antibody titers. Significant differences in antibody titers were tested using A Mann-Whitney U-test (A-D, I-L, M). A Wilcoxon matched-pairs signed rank test was used to compare IgG/IgM/IgA antibody titers at three time points (E-H, N). * p < 0.05, ** p < 0.01, *** p < 0.001.
Fig. 3 Cellular immune responses to SARS-CoV-2 in vaccinated 2-year convalescents.
A. Cross-sectional T cell responses to the Wild type (WT) overlapping peptide pools

B.

C.

D.

E.

F.

G.

H.

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encompassing the S1, S2, N, and M proteins, which were detected using IFN-γ ELISpot assay in vaccinated 2-year convalescents (2Y-Vac, n=51) and vaccinated healthy controls without prior infection (HC-Vac, n=35). B-C, The magnitude of T cell responses in HC-Vac (blue, n=35) and 2Y-Vac (red, n=51) groups. Freshly isolated PBMCs (B) and cultured PBMCs (C) after 9 days of *in vitro* expansion were stimulated with one of four peptide pools (S1, S2, M, and N). D-E, Comparison of the magnitude of T cell responses in Asym-Mild (Asymptomatic and Mild, pink, n=25) and Mod-Sev (Moderate and Severe, dark green, n=26) convalescents for *ex vivo* (D) and *in vitro* experiments (E). F, The proportion of positive T cell responses in 2Y-Vac and HC-Vac individuals found in nil (0 response, white), one (1 response, pink), two (2 response, red), three (3 response, orange), or four (4 response, reseda) peptide pools. G, Representative dot plots showing IFN-γ production of CD4+T cell against S1, S2, M, and N peptide pools in HC-Vac and 2Y-Vac individuals. H, The proportion of mean protein-specific responses of CD4+ and CD8+ T cells secreting IL-2, TNF-α, or IFN-γ from individuals in groups HC-Unvac (blue, n=5), HC-Vac (green, n=21), and 2Y-Vac (red, n=29). Each dot reflects an individual donor. The solid lines represent the mean ± SEM. Statistical significance was performed with a Mann-Whitney U-test (B-E, H). * p < 0.05, ** p < 0.01, *** p < 0.001.
Fig. 4 Cross-responses of neutralizing antibodies to Omicron in vaccinated 2-year convalescents and healthy controls.

A, Competitive inhibition of anti-RBD neutralizing antibodies against Wild Type (WT) (red, n=23), Delta (blue, n=23), BA.1 (green, n=23), BA.2 (purple, n=23), and BA.4/5 (pink, n=23) was measured at 6-month, 1-year, and 2-year convalescent individuals after symptoms onset.

B, Comparison of the inhibition levels of neutralizing antibodies against the above five strains for HC-Unvac (yellow, n=26), HC-Vac (purple, n=35), and 2Y-Vac (red, n=51) groups.

C-D, Changes of neutralization capacity toward WT (red), Delta (blue), BA.1 (green), BA.2 (purple), and BA.4/5 (pink) were detected using sera from vaccinated 2-year convalescents (2Y-Vac, n=51) (C) and healthy controls after vaccination (HC-Vac, n=35) (C). Pie charts summarized the positive inhibition ratios of neutralizing antibodies (NAb) against different
strains. E, NAb inhibition rates against four SARS-CoV-2 variants (Delta, BA.1, BA.2, and BA.4/5) were normalized using the geometric mean ratio of NAb inhibition rates versus WT (HC-Vac: orange; 2Y-Vac: blue). F, The seropositivity of neutralizing antibodies, in 2Y-Vac and HC-Vac individuals, identified in naught (no response, white), one (WT, yellow), two (WT + Delta, purple), three (WT + Delta + BA.2, blue), four (WT + Delta + BA.1/2, green) or five (WT + Delta + BA.1/2/4/5, red) strains. Each dot represents a unique donor. Black dotted lines and bars represent the threshold for positive and medians of responders. Significance was assessed using the Wilcoxon matched-pairs signed rank test (A, C, D) and Mann-Whitney U-test (B). * p < 0.05, ** p < 0.01, *** p < 0.001.
Fig. 5 T cell cross-response to Omicron in vaccinated 2-year convalescents and healthy controls.

A. Representative ELISpot responses against RBD, S1, and S2 protein pools of wild type (WT) and Omicron BA.1. B. Comparative T cell responses in vaccinated 2-year convalescents (2Y-Vac, red, n=51) and healthy controls without COVID-19 infection (HC-Vac, blue, n=35) to peptide pools of WT and BA.1 RBD/S1/S2. C-D, Frequency of spike-reactive CD4⁺ and CD8⁺ T cells producing IL-2, TNF-α, or IFN-γ cytokines after PBMCs of vaccinated 2-year convalescents (C) and healthy controls (D) stimulated with S1 (C, n=5; D, n=7) and S2 (C, n=4; D, n=3) peptide pools from WT (red) and Omicron BA.1 (blue). Each dot represents an individual. A Mann-Whitney U-test was used to calculate statistical significance (B). * p < 0.05, ** p < 0.01, *** p < 0.001.
Supplementary Figure 1. Comparison of antibody response in 2-year convalescents after 2 doses and healthy individuals after 3 doses.

IgG (A, B), IgM (C, D), IgA (E), and neutralizing (F) antibody levels of healthy controls received 3 doses (HC-3doses, blue, n=32) and 2-year convalescents received 2 doses (2Y-2doses, red, n=36) with sera samples that were measured by ELISA (A, C), MCLIA (B, D, E), and cELISA (F). Dashed lines represent the lower limits of detection. Comparisons between the two groups were performed using a two-sided Mann-Whitney U-test (A-F). * p < 0.05, ** p < 0.01, *** p < 0.001.
Supplementary Figure 2. Comparison of T cell responses in 2-year convalescents after 2 doses and healthy individuals after 3 doses.

A-B, Dot plots summarizing the *ex vivo* (A) and *in vitro* (B) T cell responses against four peptide pools (S1, S2, M, and N) of wild type (WT) were shown in healthy controls received 3 doses (HC-3doses, blue, n=32) and 2-year convalescents received 2 doses (2Y-2doses, red, n=36). Each dot indicates one donor. The difference was calculated using a Mann-Whitney U-test (A-B). * p < 0.05, ** p < 0.01, *** p < 0.001.
Supplementary Figure 3. Gating strategy for cytokines (IL-2, TNF-α, and IFN-γ) expressing in SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells, enumerated by Intracellular cytokine staining (ICS).
Supplementary Figure 4. Alignment of the WT, Delta, BA.1, BA.2, and BA.4/5 RBD sequences.

Multiple sequence alignment of different SARS-CoV-2 RBDs (223aa), including wild type (WT) (GenBank: MN908947.3), Delta (GenBank: MZ724418.1), BA.1 (GenBank: OM212472.1), BA.2 (GenBank: OM172026.1), BA.4 (GenBank: ON395908.1), and BA.5 (GenBank: ON393428.1). The sequences of BA.4 and BA.5 RBD are the same. Non-consistent residues were marked as wathet or white.