Supplementary information

Materials and Methods

Production of mRNA-LNP vaccines

Tandem repeat prototype SARS-CoV-2 (2019-nCoV strain IVDC-HB-01/2019, GISAID: EPI_ISL_402119) RBD-dimer, tandem repeat SARS-CoV-2 Omicron (BA.1) variant (GISAID: EPI_ISL_6795848) RBD-dimer and SARS-CoV-2 Delta-Omicron BA.1 (GenBank: OK091006.1 and GISAID: EPI_ISL_6795848) chimeric RBD-dimer are comprised of 5' cap1 structure, 5' UTR, coding sequence, 3' UTR and ploy- adenyl tail. The mRNA was generated using T7 RNA polymerase (Novoprotein) from linearized plasmids, with 1-N-Methyl-pesudouridine 5'-triphosphate (Hongene Biotech) instead of UTP. Capped mRNA was produced using Cap1 Capping System Kit (Novoprotein) and purified by LiCl precipitation at -20^{o}C, followed by centrifuging, washing with 75% ethanol and resuspending in nuclease-free water. LNPs are composed of ionizable lipid, cholesterol, DSPC and PEG-lipid at molar ratio of 50:38.5:10:1.5. The lipid mixture was added into 50 mM acetic buffer (pH 4.3) containing mRNA using a microfluidic mixer (Precision Nanosystems, Vancouver). Formulations were tested for particle size (ZetasizePro, Malvern Panalytical), RNA encapsulation (Quant-iTTM RiboGreenTM RNA Assay Kit, Invitrogen) and polydispersity index (ZetasizePro, Malvern Panalytical).

In the head-to-head comparison experiment shown in Supplementary information, Fig. S3, the RBD-dimer and RBD-trimer (BNT162b1) vaccines were produced by BioNTech, Mainz, Germany. The RNA specifications, production and LNP formulation are described elsewhere[1].

Antigen expression

HEK 293T cells, pre-plated in a 12-well plate, were transfected with mRNA

encoding prototype, Omicron, or Delta-Omicron RBD-dimer using TransIT-mRNA Transfection Kit (Mirus) according to manufacturer's instructions. After incubation for 1 or 2 days, culture supernatants were collected. The expression of antigen proteins was analyzed by sandwich enzyme-linked immunosorbent assays (ELISA) and Western blot.

For ELISA, 96-well plate was precoated with 0.3 μg/mL recombinant hACE2 protein (Sino Biological) for 12 hours (h) at 4°C, followed by blocking with 5% skim milk. Then the plates were incubated with cell culture supernatants for 3 h, then S309[2] antibody (10 μg/mL) for 2 h, then goat anti-human IgG horseradish peroxidase for 1.5 h, and then TMB substrate and reaction stop buffer (2 M hydrochloric acid). The absorbance was measured at 450 nm using a microplate reader.

For Western blot assay, the supernatant was mixed with loading buffer. Samples were separated by SDS-PAGE and proteins were transferred to hybridization nitrocellulose filter (Merck Millipore). Target proteins was probed by rabbit anti-SARS-CoV-2 RBD polyclonal antibodies. Goat anti-rabbit IgG-horseradish peroxidase (1:2000) was used as the secondary antibodies. The membranes were developed by the SuperSignalWest Pico chemiluminescent substrate (Thermo Fisher Scientific).

Immunization

Female BALB/c mice at 6–10 weeks of age were immunized with vaccine candidates through the intramuscular route. PBS, 300 mM sucrose-supplemented PBS or LNP-GFP were used as sham control. The sera were collected as indicated in figures and figure legends. For mice primed with RBD-dimer protein subunit vaccine, 100 μL (10 μg) of SWE (produced by SEPPIC) adjuvanted RBD-dimer was injected to each mouse.

Antigen-specific IgG titration

ELISA were performed as previously described[1,3], with some modifications. Briefly, 96-well plates were precoated with antigen protein, blocked and incubated in turn with diluted murine serum, anti-mouse secondary antibody, substrate and stop buffer. The absorbance was measured at 450 nm using a microplate reader. The endpoint titers were defined as the highest reciprocal dilution of serum to give an absorbance higher than 2.5-fold of the background values. Antibody titer below the limit of detection was determined as half the limit of detection. For data shown in Supplementary information, Fig. S3, 96 well MaxiSorp plates (Thermo Fisher Scientific) were precoated with 0.1 μg/mL recombinant RBD (Sino Biological) in sodium carbonate buffer. Data collection was performed using a BioTek Epoch reader. For concentration analysis, samples were tested in different dilutions and linear signal range was identified. A mouse IgG isotype control (Southern Biotech) was used in parallel generating a standard curve and used for calculation.

Pseudotyped virus neutralization assay

The pseudotyped viruses displaying SARS-CoV-2 (prototype and variants) S protein express GFP in infected cells and were prepared as previously described[4]. Serially diluted mice sera were incubated with pseudotyped viruses at 37°C for 1 h, then the sera-virus mixtures were transferred to pre-plated Vero cell monolayers in 96-well plates. After incubation for 18 h, the transducing unit numbers were calculated on a CQ1 confocal image cytometer (Yokogawa). Neutralization titer was determined by fitting nonlinear regression curves using GraphPad Prism and calculating the reciprocal of the serum dilution required for 50% neutralization of infection. Neutralization titer below the limit of detection was determined as half the limit of detection. In the head-to-head comparison experiment shown in Supplementary information, Fig. S3, the

pVNT assay was performed as described elsewhere[1].

IFNγ ELISpot assays

To detect antigen-specific T lymphocyte responses, an IFNγ-, IL-2- and IL-4-based ELISpot assays were performed using the mouse IFNγ, IL-2 and IL-4 ELISpot kits (Mabtech) following the manufacturer's instructions. Briefly, spleens of vaccinated BALB/c mice were harvested at 2 weeks after boosting and splenocytes were isolated. MultiScreen HTS IP Filter Plates (Millipore Sigma #MSIPS4W10) were precoated with anti-mouse IFNγ, IL-2 or IL-4 antibodies overnight at 4°C, washed twice with RPMI 1640 medium plus 10% FBS and blocked for 1 h with RPMI 1640 medium plus 10% FBS at room temperature. Mouse splenocytes were plated and mixed with the peptide pool (10 μg/mL) consisting of 15–18-mers (overlapping by 11 amino acids) and spanning the SARS-CoV-2 RBD. The cells were incubated for 36 h at 37°C. The cells were removed, and the plates were processed in turn with biotinylated detection antibody, streptavidin-ALP conjugate, and substrate. Spotnumbers were determined using an automatic ELISpot reader and image analysis software (Cellular Technology Ltd.).

Intracellular cytokine staining (ICS) and flow cytometry

ICS assays were performed as previously described[5], with some modifications. Briefly, mouse splenocytes were stimulated with the peptide pool (10 μg/mL) for 3 h at 37°C. The cells were incubated with GolgiStop (BD Biosciences) for an additional 6 h at 37°C. Then, the cells were harvested and stained with anti-CD3 (BioLegend), anti-CD4 (Bio Legend), and anti-CD8α (BioLegend) surface markers. The cells were subsequently fixed and permeabilized in permeabilizing buffer (BD Biosciences) and stained with anti-mouse anti-IFNγ (BioLegend), anti-TNFα (BioLegend), anti-IL-2 (BioLegend) and anti-IL-4

(BioLegend) antibodies. Data were analyzed on a LSR Fortessa flow cytometer (BD Biosciences).

Animal protection against virus challenge

To evaluate the protection efficacy of the vaccine candidates against SARS-CoV-2, a human ACE2-expressing BALB/c mouse model was used. Immunized BALB/c mice were intranasally infected with 8×10^9 vp of a recombinant adenovirus expressing human ACE2 (Ad5-hACE2). Five days later, the transduced mice were challenged with 5×10^5 $TCID_{50}$ of SARS-CoV-2 (hCoV-19/China/CASB001/ 2020, GISAID No. EPI_ISL_514256-7) via the intranasal route. Three days post challenge, mice were euthanized and necropsied. Lung tissues were collected and split into two parts for virus titration and pathological examination. All animal experiments with SARS-CoV-2 challenge were conducted under animal biosafety level 3 (ABSL3) facility in Institute of Microbiology, Chinese Academy of Science (IMCAS). All animal experiments were approved by the Committee on the Ethics of Animal Experiments of the IMCAS, and conducted in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the IMCAS Ethics Committee.

Virus titration and histopathology analysis

SARS-CoV-2-specific quantitative reverse transcription-PCR (qRT-PCR) assays were performed as previously described[6]. Briefly, mouse lung tissues were weighed and homogenized. Virus RNA was isolated from 50 μL supernatants of homogenized tissues using a nucleic acid extraction instrument MagMAX™ Express Magnetic Particle Processor (Applied Biosystems, USA). SARS-CoV-2-specific qRT-PCR assays were performed using a FastKing One Step Probe RT-qPCR kit (Tiangen Biotech, China) on a CFX96 Touch real-time PCR detection system (Bio-Rad, USA) according to the manufacturer's protocol.

Two sets of primers and probes were used to detect a region of the N gene of viral genome[7] and a region of the E gene of subgenomic RNA (sgRNA)[8] of SARS-CoV-2, respectively, with sequences as follows: gRNA-F, GACCCCAAAATCAGCGAAAT; gRNA-R, TCTGGTTACTGCCAGTTGAATCTG; gRNA-probe, FAM-ACCCCGCATTACGTTTGGTGGACC-TAMRA; sgRNA-F, CGATCTCTTGTAGATCTGTTCTC; sgRNA-R, ATATTGCAGCAGTACGCACACA; sgRNA-probe, FAM-ACACTAGCCATCCTTACTGCGCTTCG-TAMRA. Viral loads were presented on a $\log_{10}$ scale as viral copies/gram after calculation with a standard curve. Viral copy numbers below the limit of detection were set as half the limit of detection.

Mouse lung tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and then sectioned. Tissue sections (4 μm) were deparaffinized in xylene and stained with haematoxylin and eosin (H&E) for pathological examination, such as peribronchiolitis, interstitial pneumonitis, and alveolitis.

Electron microscopy of mRNA-LNP

The mRNA-LNP sample was deposited on a glow-discharged Lacey carbon grid (Electron Microscopy Sciences) and vitrificated using a Vitrobot Mark (Thermo Fisher Scientific). Cryo-EM imaging was conducted on a Titan Krios Equipped with a K2 camera, operated at 300 kV accelerating voltage.

Statistical analysis

Data are presented as geometric mean ± 95% confidence interval or mean ± standard error of mean (SEM). For all analyses, *P*-values were analyzed with two-tailed Mann Whitney test or one-way Dunnett's multiple comparisons test.

Supplementary references

1 Vogel, A. B. *et al.* BNT162b vaccines protect rhesus macaques from SARS-CoV-2. *Nature* **592**, 283-289 (2021).

2 Pinto, D. *et al.* Cross-neutralization of SARS-CoV-2 by a human monoclonal SARS-CoV antibody. *Nature* **583**, 290-295 (2020).

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4 Zhao, X. *et al.* Effects of a prolonged booster interval on neutralization of Omicron variant. *N Engl J Med* **386**, 894-896 (2022).

5 Xu, K. *et al.* Recombinant chimpanzee adenovirus AdC7 expressing dimeric tandem-repeat spike protein RBD protects mice against COVID-19. *Emerg Microbes Infect* **10**, 1574-1588 (2021).

6 Xu, K. *et al.* Protective prototype-Beta and Delta-Omicron chimeric RBD-dimer vaccines against SARS-CoV-2. *Cell* **185**, 2265-2278 e14 (2022).

7 Chandrashekar, A. *et al.* SARS-CoV-2 infection protects against rechallenge in rhesus macaques. *Science* **369**, 812-817 (2020).

8 Wolfel, R. *et al.* Virological assessment of hospitalized patients with COVID-2019. *Nature* **581**, 465-469 (2020).

9 Wei, Q. et al. Description of the first strain of 2019-nCoV, C-Tan-nCoV Wuhan Strain - National Pathogen Resource Center, China, 2020. China CDC Wkly 2, 81-82 (2020).

10 Gagne, M. et al. mRNA-1273 or mRNA-Omicron boost in vaccinated macaques elicits similar B cell expansion, neutralizing responses, and protection from Omicron. Cell 185, 1556-1571 e1518 (2022).

11 Lee, I.-J. et al. Omicron-specific mRNA vaccine induced potent neutralizing antibody against Omicron but not other SARS-CoV-2 variants. Preprint at bioRxiv https://doi.org/10.1101/2022.01.31.478406 (2022).

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Supplementary Figure 1

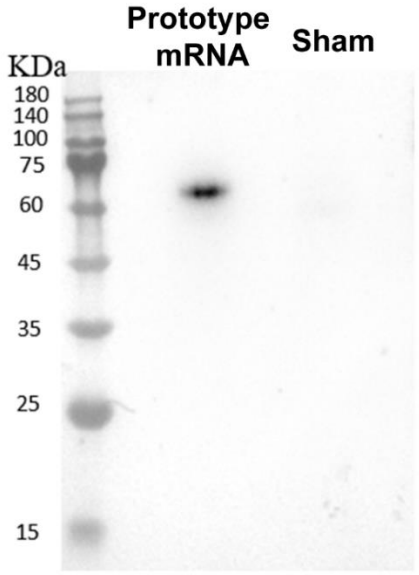
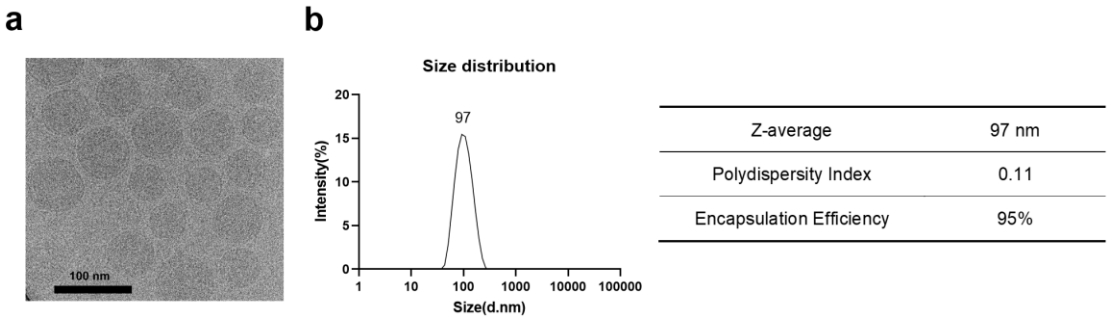


Fig. S1. Analysis of antigen expression by Western blot. HEK 293T cells were transfected with mRNA encoding prototype RBD-dimer or GFP (Sham). Western blot analysis was conducted to detect RBD in culture supernatants.

Supplementary Figure 2



Z-average	97 nm
Polydispersity Index	0.11
Encapsulation Efficiency	95%

Fig. S2. Characterization of the mRNA vaccine encoding RBD-dimer

(**a**) Representative cryo-EM image of LNP-encapsulated mRNA encoding the prototype RBD-dimer. (**b**) Particle size of LNPs as determined by dynamic light scattering. In the table, the mean hydrodynamic size of the ensemble collection of particles (z-average), polydispersity index (PDI), and the encapsulation effiiency are shown.

Supplementary Figure 3

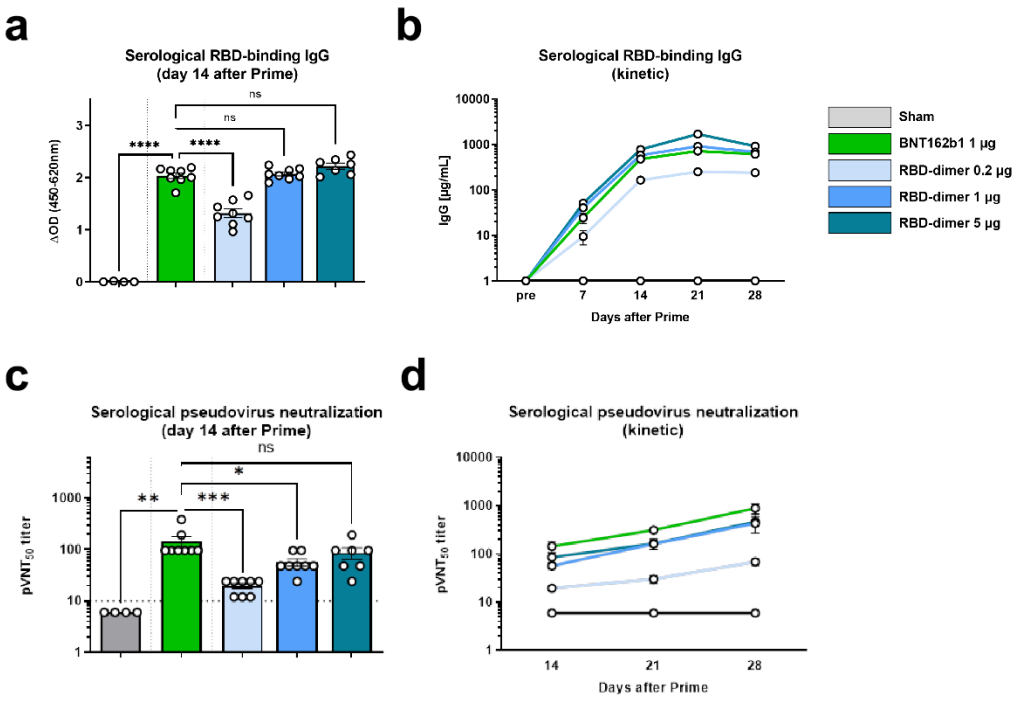


Fig. S3. Comparison of immunogenicity between the RBD-trimer mRNA vaccine BNT162b1 and the RBD-dimer mRNA vaccine. BALB/c mice (n = 8) were injected intramuscularly with a single dose of RBD-trimer mRNA vaccine BNT162b1 (1µg), RBD-dimer mRNA vaccine (0.2 µg/1 µg/5 µg) or buffer control (sham). Serum samples were collected 7, 14, 21 and 28 days post immunization (prime). For pre-values, a screening of randomly selected mice before prime was performed (n = 4). (**a**) Levels of RBD-specific IgG, determined by enzyme-linked immunosorbent assay (ELISA) were evaluated. Serum samples at day 14 after prime were diluted 1:2,700 and ΔOD-values are shown. (**b**) Anti-RBD IgG antibody concentrations over the study period of 28 days after prime. (**c**) Serological pseudovirus (prototype) neutralization at day 14 after prime. (**d**) Serological pseudovirus (prototype) neutralization over the study period of 28 days after prime. Data are group means ± SEM. For (**a**) and (**c**), *P*-values were determined with one-way Dunnett's multiple comparisons test

comparing means to the 1μg BNT162b1-immunized group mean (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Supplementary Figure 4

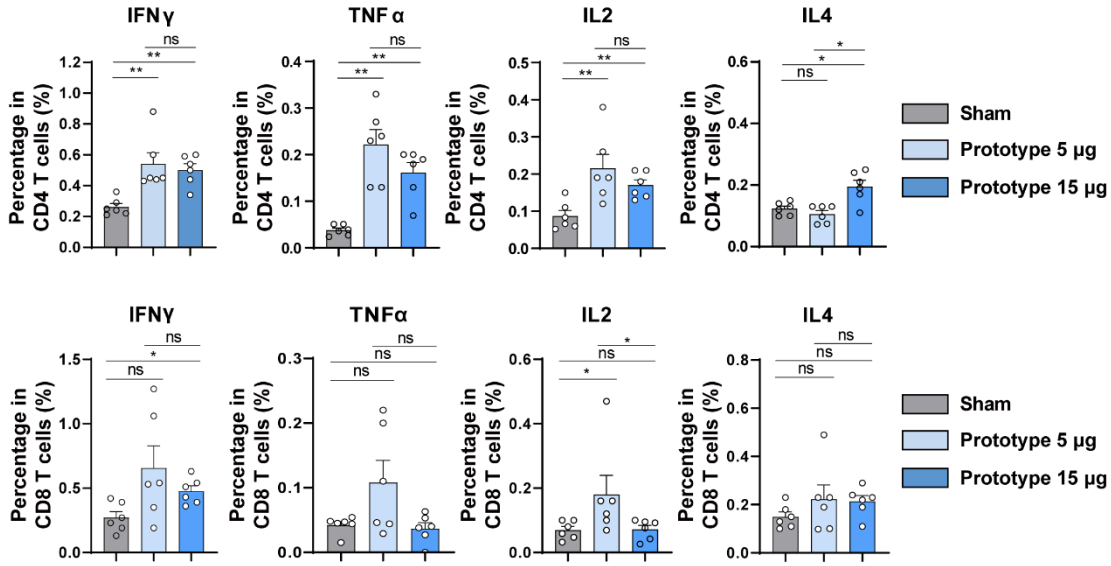


Fig. S4. Characterization of the cellular immune responses by ICS. Frequency of IFNγ-, TNFα-, IL-2- and IL-4-producing CD8+ T cells and CD4+ T cells of splenocytes from immunized mice. Data are means ± SEM. *P*-values were analyzed with two-tailed Mann Whitney test (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$).

Supplementary Figure 5

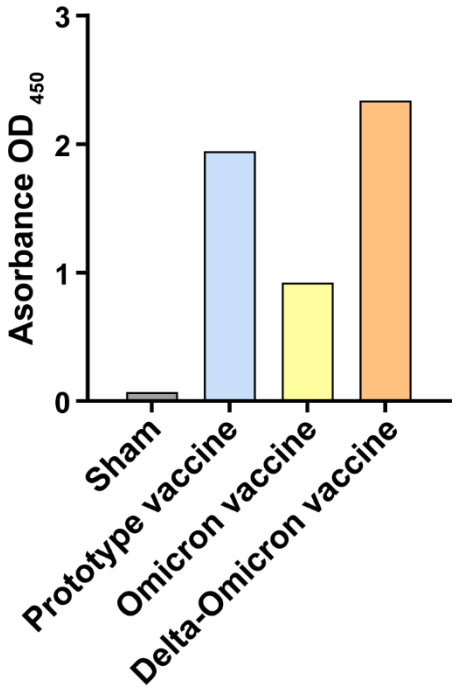


Figure S5. Analysis of antigen expression by ELISA. HEK293T cells were transfected with mRNA expressing prototype RBD-dimer, Omicron RBD-dimer, Delta-Omicron chimeric RBD-dimer or GFP (Sham). Secreted antigen proteins in supernatants were captured by hACE2 protein and detected by broadly reactive antibody S309.

Supplementary Figure 6

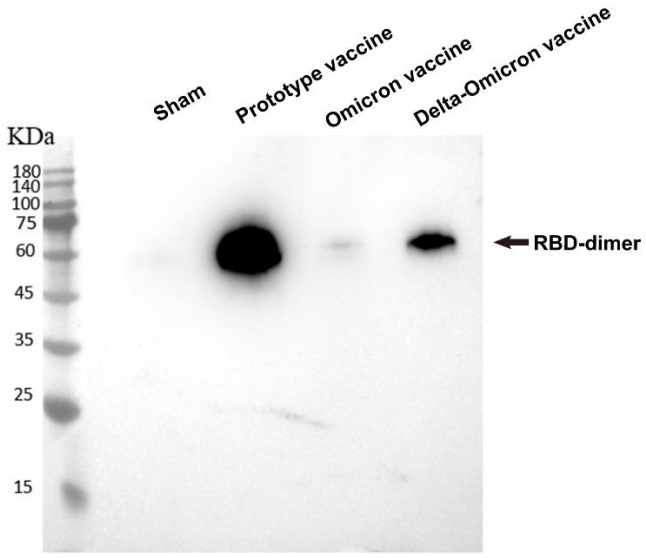


Fig. S6. Analysis of antigen expression by Western blot. HEK 293T cells were transfected with mRNA encoding prototype RBD-dimer, Omicron RBD-dimer, Delta-Omicron chimeric RBD-dimer or GFP (Sham). Western blot analysis was conducted to detect antigen epxresion (RBD-dimers) in culture supernatants using antisera from prototype RBD-dimer-immunized rabbit.

Supplementary Figure 7

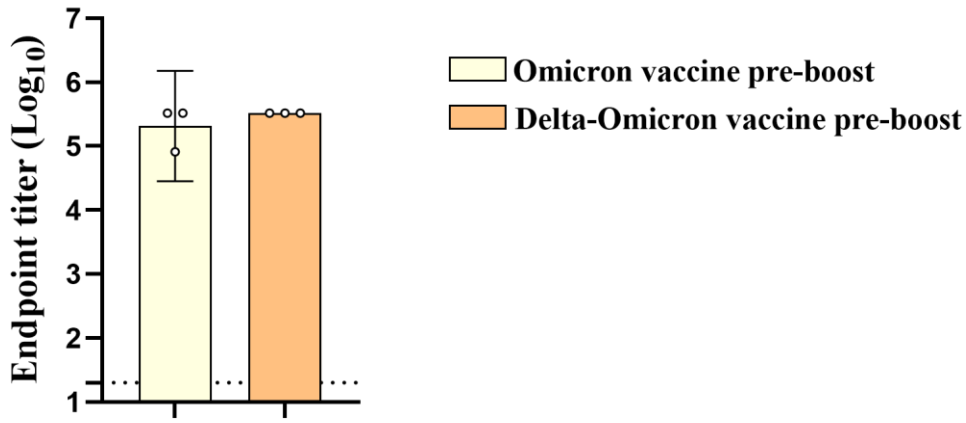


Fig. S7. Prototype RBD-binding IgG before heterologous boosting vaccination.
Related to Fig.1l, 6 mice received two doses of prototype RBD-dimer protein subunit vaccine, at a 21 days interval. Serum samples were collected at day 330 after priming and tested for prototype RBD-binding IgG. The animals were divided into two groups (n=3 each) with almost equivalent antibody levels and received a booster vaccination with either Omicron mRNA or Delta-Omicron mRNA.