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Brief communication

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Neutralization of SARS-CoV-2 KP.1, KP.1.1, KP.2 and KP.3 by human and murine sera

Kun Xu[1,5], Yaling An[2,5], Xueyuan Liu[3,5], Haitang Xie[4,5], Dedong Li[2], Ting Yang[3], Minrun Duan[2], Yuanzhuo Wang[1], Xin Zhao[2], Lianpai Dai[2] & George F. Gao[1,2]

We report SARS-CoV-2 KP.1, KP.1.1, KP.2 and KP.3 neutralizing antibody titers. They displayed increased immune evasion compared to JN.1, especially KP.1 and KP.3, for participants who experienced BF.7/BA.5.2 breakthrough infection or received bivalent (delta/BA.5) vaccine boosting. Second XBB sub-variants breakthrough infection enhanced the neutralization responses. HK.3-JN.1 RBD-heterodimer induced balanced and potent neutralizing responses against recently-circulating SARS-CoV-2 sub-variants in mice, supporting to replace the COVID-19 antigen containing JN.1 or its sub-variants.

Currently, several SARS-CoV-2 JN.1 descendants variants emerged and circulated globally, including KP.1 (JN.1.11.1.1), KP.1.1 (JN.1.11.1.1.1), KP.2 (JN.1.11.1.2) and KP.3 (JN.1.11.1.3). As of 3 May 2024, the World Health Organization (WHO) classified KP.2 and KP.3 as a Variant Under Monitoring (VUMs). Their prevalence is increasing rapidly. Compared to the spike protein receptor binding domain (RBD) of JN.1, KP.1 contains an extra F456L mutation, and KP.3 contains F456L and Q493E mutations (Fig. 1a, b, Supplementary Fig. 1). KP.1.1 and KP.2 contain R346T and F456L mutations, which are called "FLiRT" variants (Fig. 1a, b, Supplementary Fig. 1). Therefore, their immune escape potential is highly concerned[1,2].

First, we studied the neutralization evasion of KP.1, KP.1.1, KP.2 and KP.3 with the 52 human sera using a panel of SARS-CoV-2 pseudotyped viruses. These participants were divided into four groups according to their SARS-CoV-2 vaccine inoculation and breakthrough infection (BTI) status (Supplementary Table 1). SARS-CoV-2 inactivated vaccines (CoronaVac and BBIBP-CorV) and protein subunit vaccine ZF2001 (containing RBD-dimer immunogen) are widely used in China and many other countries[3–5]. Our results indicated that vaccination with three doses of inactivated vaccines with BF.7/BA.5.2 BTI[6] (Group 1) induced neutralizing antibodies against BA.5 with a geomean titer (GMT) of 410 (Fig. 1c). The cross-neutralizing activities against XBB.1.5, BA.2.86 and JN.1 significantly decreased to GMTs of 29, 55 and 17, respectively (Fig. 1c and Supplementary Fig. 2). However, compared to JN.1, KP.1, KP.2 and KP.3 showed further lower neutralizing GMTs (Fig. 1c). Three doses of ZF2001 vaccination with BF.7/BA.5.2 BTI (Group 2) elicited a similar neutralization profile to Group 1. The neutralizing GMT against BA.5 was as high as 1823 and decreased to 53 against JN.1 significantly ($p = 0.0005$). In addition, KP.1, KP.1.1, KP.2 and KP.3 showed further increased immune evasion compared to JN.1 with GMTs of 24, 44, 43 and 37, respectively (Fig. 1d). Significant statistical differences were observed between titers against JN.1 and KP.1 ($p = 0.002$) (Fig. 1d and Supplementary Fig. 2). The third group of participants had a second BTI during the wave of XBB and its sub-variants and had higher neutralizing antibody responses than Group 1. Their GMTs against XBB.1.5 and JN.1 were 286 and 53, respectively, with significant statistical difference ($p = 0.0005$). Compared to JN.1, GMTs against KP.1 and KP.3 further decreased to 43 ($p = 0.2754$) and 50 ($p = 0.7695$) (Fig. 1e and Supplementary Fig. 2). Specially, we observed that neutralizing GMTs against KP.1.1 (GMT, 75) and KP.2 (GMT, 67) were slightly higher than JN.1 with 1.4- and 1.3-fold with significant statistical differences. p-values were 0.0059 and 0.0098, respectively (Fig. 1e, Supplementary Table 2). This was probably contributed by the R346T mutation, which is present in BF.7, XBB.1.5, KP.1.1 and KP.2, but not JN.1 (Supplementary Fig. 1). The fourth group participants received three doses of inactivated vaccines and a booster dose of bivalent ZF2202-A vaccine, containing Delta-BA.5 RBD-dimer immunogen. This group showed potent neutralizing responses against BA.5 (GMT, 5149). In comparison, the GMT against JN.1 significantly ($p < 0.0001$) decreased to 206. Compared to JN.1, the cross-neutralizing GMTs against KP.1, KP.1.1, KP.2 and KP.3 further decreased to 151 ($p = 0.0063$), 195 ($p = 0.8603$), 178 ($p = 0.0155$) and 168 ($p = 0.0131$), respectively (Fig. 1f). In addition, the ZF2202-A vaccine induced significantly higher neutralizing responses

[1]Research Network of Immunity and Health (RNIH), Beijing Institutes of Life Science, Chinese Academy of Sciences (CAS), Beijing, China. [2]CAS Key Laboratory of Pathogen Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences (CAS), Beijing, China. [3]School of Public Health, Cheeloo College of Medicine, Shandong University, Jinan, Shandong Province, China. [4]Yijishan Hospital of Wannan Medical College, Wuhu, Anhui Province, China. [5]These authors contributed equally: Kun Xu, Yaling An, Xueyuan Liu, Haitang Xie. e-mail: gaof@im.ac.cn

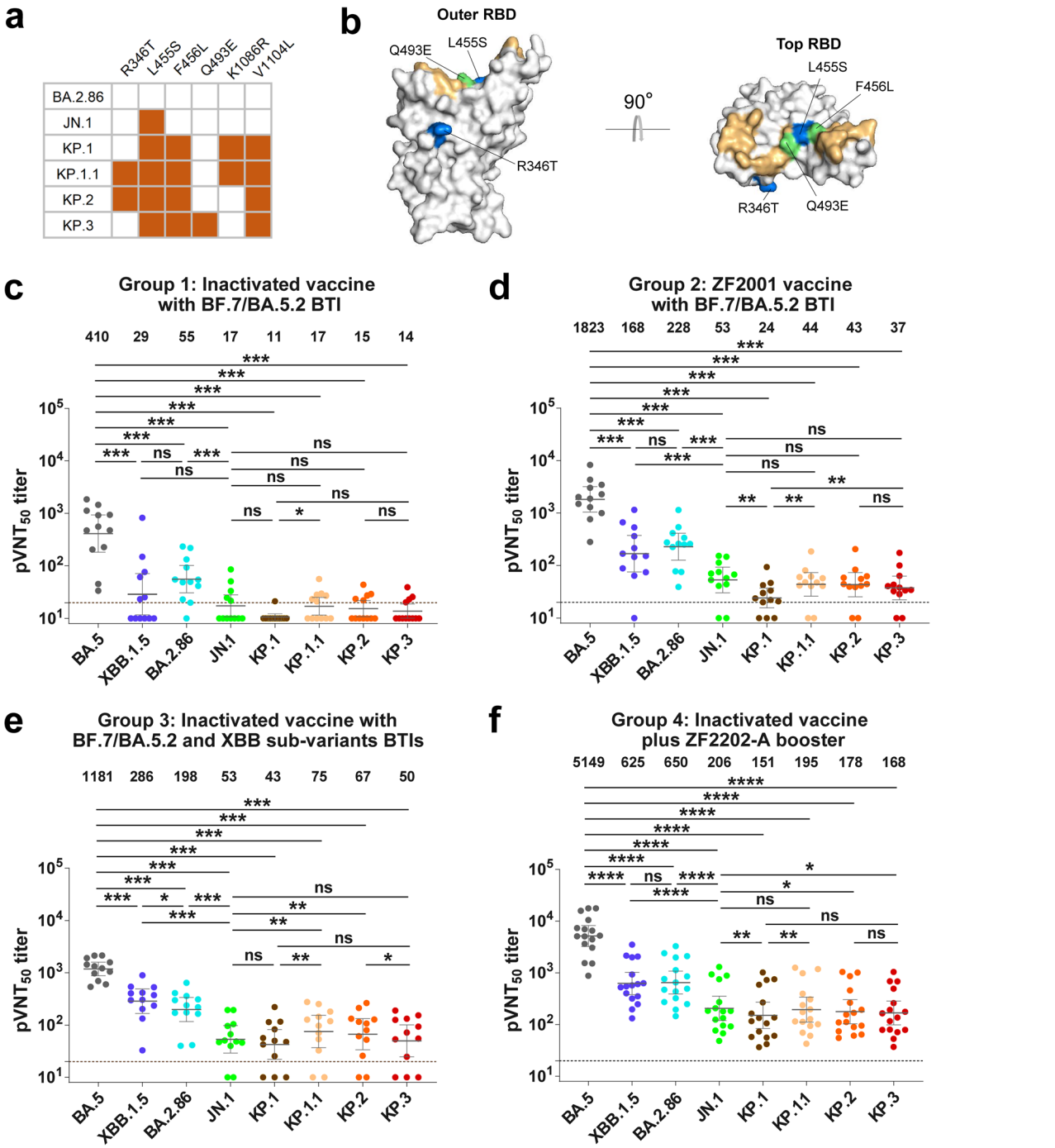

Fig. 1 | Mutations of SARS-CoV-2 KP.1, KP.1.1, KP.2 and KP.3 and evaluation of their immune evasion by human sera. a Mutations in the spike protein of SARS-CoV-2 JN.1, KP.1, KP.1.1, KP.2 and KP.3 compared to BA.2.86. The amino acid differences are highlighted in brown. The amino acid in variant sequences identical to those in the BA.2.86 are maintained as white. **b** Mutation mapping of JN.1, KP.1, KP.1.1, KP.2 and KP.3 on BA.2.86 RBD (PDB: 8QSQ). The interface of the BA.2.86 RBD binding with hACE2 is colored in light orange. **c**–**f** Neutralizing activities against SARS-CoV-2 variants in human sera. The participants were divided into four groups. The first (**c**) and second (**d**) groups of participants had breakthrough infections during the wave of late-2022 Omicron sub-variants BF.7 and BA.5.2 after three doses of inactivated vaccines or protein subunit vaccine ZF2001, respectively. **e** The third group participants received three doses of inactivated vaccines, had breakthrough infections during the waves of BF.7/BA.5.2 and XBB sub-variants. **f** The fourth group participants received three doses of inactivated vaccine and a booster of ZF2202-A vaccine (ClinicalTrials.gov: NCT05850507). The 50% pseudovirus neutralization titers ($pVNT_{50}$) were measured. GMTs are shown at the top of each image. The error bars indicate 95% confidence intervals. The dashed horizontal line indicates the limit of detection (LOD). $pVNT_{50}$ below the LOD was determined as half the LOD. *p*-values were analyzed with two-tailed Wilcoxon matched-pairs signed-rank tests (ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

against all the tested pseudoviruses than those elicited by BF.7/BA.5.2 BTI (Fig. 1f, Supplementary Fig. 3).

Furthermore, we evaluated the neutralizing responses of murine sera. Groups of BALB/c mice were immunized with three doses of 2 μg immunogens, including Delta-BA.5, XBB.1-BQ.1, HK.3-JN.1 RBD-heterodimer and XBB.1.5 RBD-homodimer. These protein vaccines were adjuvanted by AddaVax. The results indicated that Delta-BA.5 RBD-dimer induced robust neutralizing activities against BA.5 pseudovirus with GTM of 31131, significantly decreasing to 4388 and 291 against BQ.1.1 ($p = 0.0313$) and XBB.1.5 ($p = 0.0313$), respectively (Fig. 2a). The neutralizing activities

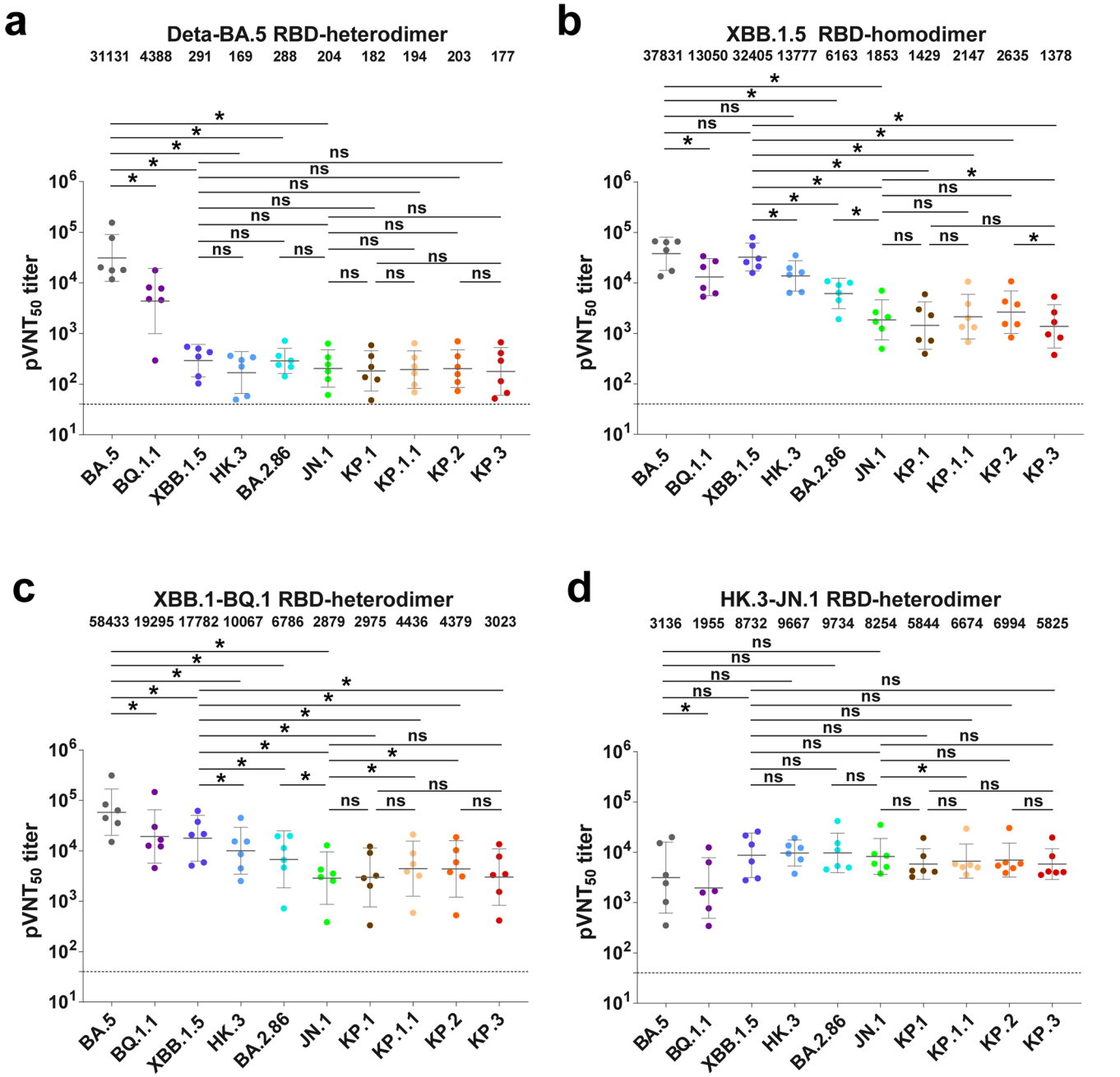

Fig. 2 | The RBD-dimer immunogens-elicited neutralizing antibodies against SARS-CoV-2 variants in murine sera. a–d Neutralizing activities against SARS-CoV-2 variants in murine sera. BALB/c mice were immunized with three doses of Delta-BA.5 RBD-heterodimer (**a**), XBB.1.5 RBD-homodimer (**b**), XBB.1-BQ.1 RBD-heterodimer (**c**) and HK.3-JN.1 RBD-heterodimer (**d**) using AddaVax as adjuvant. PBS plus adjuvant was given as the sham control. Sera were collected at 7 days post-third immunization. The $pVNT_{50}$ values of murine sera were measured. GMTs are shown at the top of each image. The error bars indicate 95% confidence intervals. The dashed horizontal line indicates the LOD. $pVNT_{50}$ below the LOD was determined as half the LOD. *p*-values were analyzed with two-tailed Wilcoxon matched-pairs signed-rank tests (ns, $p > 0.05$; $^*p < 0.05$).

against JN.1, KP.1, KP.1.1, KP.2 and KP.3 were between 177 and 204. No significant statistical differences ($p > 0.05$) were observed between them (Fig. 2a). XBB.1.5 RBD-homodimer and XBB.1-BQ.1 RBD-heterodimer elicited potent neutralizing responses against BA.5, BQ.1.1, XBB.1.5 and HK.3 with GTMs of more than 10^4 (Fig. 2b, c). JN.1 and its descendants partially escaped the neutralizing responses, and showed decreased GTMs of about 10^3 (Fig. 2b, c). In addition, neutralization titers against KP.1, KP.1.1, KP.2 and KP.3 induced by XBB.1-BQ.1-heterodimer were higher than XBB.1.5-homodimer (Fig. 2b, c). Particularly, the XBB. 1.5 RBD-homodimer-induced neutralizing titers against KP.3 were significantly lower than those against JN.1 ($p = 0.0313$) and KP.2 ($p = 0.0313$) (Fig. 2b). In comparison, HK.3-JN.1 RBD-heterodimer induced balanced antibody responses. The GMTs against all the tested XBB sub-variants, BA.2.86 and its sub-variants were more than 5000 (Fig. 2d). In addition, compared to JN.1, the GMTs against KP.1 ($p = 0.0625$), KP.1.1 ($p = 0.0313$), KP.2 ($p = 0.4375$) and KP.3 ($p = 0.0625$) were slightly lower with less than 1.5-fold (Fig. 2d).

In summary, this study revealed that the immune evasion of KP.1, KP.1.1, KP.2 and KP.3 depended on the prior vaccination and BTI status. They displayed increased immune evasion compared to JN.1 for participants who experienced BF.7/BA.5.2 BTI. Furthermore, a second BTI with XBB sub-variants induced significantly higher neutralizing responses against KP.1.1 and KP.2 than JN.1, probably caused by the R346T mutation. Mutation on the hotspot site and their revertant can lead to the fluctuating effect on immune evasion[7]. Therefore, continuous surveillance of the

circulating SARS-CoV-2 is needed[8]. In addition, the ZF2202-A vaccine was demonstrated to induce higher neutralizing responses against KP.1, KP.1.1, KP.2 and KP3 than BTIs.

Li et al. demonstrated that SARS-CoV-2 S mutations R346T, L455S and F456L alter neutralization epitopes. They reported that sera from participants who received bivalent (prototype and BA.5) mRNA vaccine showed decreased KP.2 neutralization compared to JN.1 with 1.4-fold, which was similar to the participants who received ZF2202-A (delta and BA.5) boosting vaccination[9]. Yaku et al. studied the virological characteristics of the SARS-CoV-2 KP.3 and revealed that KP.3 showed increased immune evasion compared with the parental JN.1. In addition, KP.3 showed slightly higher neutralization resistance against all convalescent serum samples tested than KP.2, though without significant differences[10]. Our study also observed lower neutralization GMTs against KP.3 than KP.2 in all the tested participants groups. As of September 2024, KP.3 sub-variants became the dominant circulating variants. The strong immune evasion was probably a reason for their prevalence.

The risk of antibody-dependent enhancement (ADE) of SARS-CoV-2 infection by the non-neutralizing antibodies is a concern. In vitro studies found some SARS-CoV-2 neutralizing monoclonal antibodies targeting RBD can mediate enhancement of virus infection in specific cultured cells through Fc receptor-γ (FcγR), while some non-neutralizing N-terminal domain (NTD) antibodies mediated FcγR-independent in vitro infection enhancement. However, in vivo studies demonstrated that the RBD and NTD antibodies protected mice and monkeys against SARS-CoV-2[11,12]. In addition, the immune enhancement was not observed at the population level after SARS-CoV-2 infection or COVID-19 vaccine immunization[13]. Therefore, though the emerging SARS-CoV-2 variants showed immune evasion, there is a lack of robust evidence supporting the immune enhancement caused by infection or vaccination.

This study did not explore the correlation between protection and neutralization titers. Published articles have demonstrated that neutralizing titers were significant inverse correlates of risk of symptomatic COVID-19 occurrence in humans who received COVID-19 vaccines or experienced SARS-CoV-2 infection[14–16]. Therefore, we speculate that the higher neutralizing antibodies conferred by RBD-dimer vaccines provided stronger protection against COVID-19. The correlation will be explored in further clinical trials and real-world studies.

In April 2024, WHO suggested replacing the COVID-19 antigen with JN.1 or its sub-variants to enhance the prevention against the near future circulating of SARS-CoV-2. In August 2024, new 2024-2025 formulations of the mRNA COVID-19 vaccines (based on the KP.2 strain) manufactured by Pfizer/BioNTech and Moderna and the adjuvanted protein subunit COVID-19 vaccine (based on the JN.1 strain) manufactured by Novavax have been approved for use by the US FDA[17,18]. Our study also suggested that JN.1 and its sub-variants escaped the neutralizing responses induced by the XBB.1.5 vaccine, indicating the mutations on the immune epitopes. HK.3-JN.1 RBD-heterodimer vaccine induced more potent neutralizing responses against KP.1, KP.1.1, KP.2 and KP.3 than the XBB.1.5 RBD-homodimer, supporting updating the COVID-19 vaccine with JN.1 or its sub-variants. In comparison, Pfizer/BioNTech, Moderna and Novavax produced the monovalent 2024-2025 COVID-19 vaccine formulations, and our study revealed that the RBD-heterodimer vaccine also induced potent and balanced immune responses. This study will provide a timely risk assessment of currently-circulating SARS-CoV-2 sub-variants and a guide for updating COVID-19 vaccines. In addition, we call here for COVID-19 iterative (next-generation) vaccines by updating with newly emerged (sub) variants without further lengthy clinical trials as we are practicing with annual seasonal influenza vaccines.

Methods

Human participants

A total of 52 participants were enrolled in this study. The first group (10 females and 2 males, median age: 27.5) received three doses of inactivated vaccines[3,19] and had a breakthrough infection during a wave of BF.7/BA.5.2 circulation in China in December 2022[6], and their blood samples were collected on 13th Jan 2023. The second group (6 females and 6 males, median age: 30.5) received three doses of ZF2001 vaccine[4,20] and had a breakthrough infection during a wave of BF.7/BA.5.2 circulation in China in December 2022, and their blood samples were collected on 5th Jan 2023. The third group (9 females and 3 males, median age: 25.5) received three doses of inactivated vaccine and had two breakthrough infections during the waves of BF.7/BA.5.2 in December 2022 and XBB sub-variants in 2023, respectively. Their blood samples were collected on 26th Jan 2024. The fourth group (8 females and 8 males, median age: 48.5) received three doses of the inactivated vaccine, followed by a booster dose of the ZF2202-A vaccine. ZF2202-A is ZF2001 vaccine's next-generation COVID-19 vaccine with updated bivalent Delta-BA.5 RBD-heterodimer immunogen[8,21]. The COVID-19 inactivated vaccines BBIBP-CorV and CoronaVac are widely used in China and contain 4 and 3 μg inactivated prototype SARS-CoV-2, respectively. The ZF2001 and ZF2202-A vaccines contain 25 μg immunogen. All these vaccines are adjuvanted by aluminum hydroxide. Serum samples from the first, second and third groups were collected from the participants in real world, which were approved by the Ethics Committee of the Institute of Microbiology, Chinese Academy of Sciences. Serum samples from the fourth group were collected 3 weeks after boosting from a clinical trial (ClinicalTrials.gov: NCT05850507), which were approved by the clinical research ethics board of the First Affiliated Hospital of Wannan Medical College (Yijishan Hospital) ([2023]KY34). All participants signed the written informed consent. Detailed information about the participants is available in Supplementary Table 1.

Mouse experiments

Specific pathogen-free (SPF) female BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (licensed by Charles River). All mice were allowed free access to water and a standard chow diet and provided with a 12-h light and dark cycle (temperature: 20 °C–25 °C, humidity: 40%–70%). All mice used in this study are in good health and are not involved in other experimental procedures. They were housed under SPF conditions in the laboratory animal facilities at IMCAS. The mice experiments conducted in IMCAS were approved by the Committee on the Ethics of Animal Experiments of the IMCAS, and performed in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the IMCAS Ethics Committee.

Antigen proteins were prepared as previously described[21]. They will be made available upon request. AddaVax adjuvant was purchased from InvivoGen. Groups of 6- to 8-week-old female BALB/c mice ($n = 6$) were intramuscularly immunized with three doses of 100 μL vaccines, containing 2 μg immunogens and AddaVax adjuvant. The interval between the first and second doses was 21 days. The interval between the second and third doses was 21 days. The serum samples were collected after anesthesia at 7 days post the last immunization. These mice were then euthanized in a box by carbon dioxide.

Pseudotyped virus neutralization assay

The methods for preparing pseudotyped viruses and neutralization assays were described previously[22,23]. Briefly, the spike protein with 18 amino acids deleted from the C-terminus of each variant was cloned into the pCAGGS vector, respectively. Codon optimization was performed to make the plasmid suitable for mammalian cell expression, and 30 μg of each construct was transfected into HEK-293T cells. VSV-ΔG-GFP pseudotyped virus was added 24 h after transfection. After 2 h of infection, the VSV-ΔG-GFP residues were removed by changing the medium to fresh complete DMEM medium containing anti-VSV-G antibody (I1Hybridoma, ATCC CRL-2700). After incubation at 37 °C for 30 h, the supernatants were collected, filtered through a 0.45 μm filter (Millipore, Cat#SLHP033RB), and then aliquotted and stored tor at −80 °C.

The pseudotyped viruses displaying SARS-CoV-2 spike protein express GFP in infected cells. The heat-inactivated (56 °C for 30 min) human and murine sera samples were 2-fold serial diluted starting from 1:20 and 1:40, respectively. Equivalent pseudovirus (1000 transducing units, TU) was incubated with the sera at 37 °C for 1 h, and the mixture was then added

onto pre-plated Vero E6 cells (ATCC CRL-1586) in 96 well plates. The TU numbers were calculated on a CQ1 confocal image cytometer (Yokogawa) after a 15 h incubation. The 50% pseudovirus neutralization titer ($pVNT_{50}$) was determined by fitting non-linear regression curves (log(inhibitor) vs. normalized response - Variable slope) using GraphPad Prism and calculating the reciprocal of the serum dilution required for 50% neutralization of infection. The model of the non-linear regression curve is Y = 100/(1 + 10^((Log$pVNT_{50}$-X)*HillSlope)). HillSlope describes the steepness of the family of curves. $pVNT_{50}$ below the limit of detection was determined as half the limit of detection.

Statistical analyses

p-values were analyzed with two-tailed Wilcoxon signed-rank tests or Dunn's multiple comparison tests. Significant levels are indicated by asterisks; $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. The details are indicated in the figure legends.

Data availability

The data generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

G.F.G. and K.X. conceived the study. Y.A., X.L., M.D. and Y.W. performed the neutralization experiments. H.X., D.L., and T.Y. collected human serum samples. K.X. and X.L. performed statistical analyses. K.X., Y.A., X.L., X.Z., L.D. and G.F.G. analyzed the data. K.X. wrote the first draft of the manuscript. G.F.G. reviewed the manuscript. K.X., Y.A. and G.F.G. edited the manuscript. All authors agreed to submit the manuscript, read and approved the final draft, and take full responsibility of its content.

Competing interests

K.X., Y.A., L.D., and G.F.G. are listed in the patent as the inventors of the RBD-dimer as a coronavirus vaccine. All other authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to George F. Gao.