Supplementary Material

Significantly reduced abilities to cross-neutralize SARS-CoV-2 variants by the sera from convalescent COVID-19 patients infected by delta or earlier strains

Ting Pan[1,2,6], Zhongwei Hu[3,6], Fengyu Hu[3,6], Yiwen Zhang[1,6], Bingfeng Liu[1], Changwen Ke[4], Qiumin She[3], Xin He[1,*], Xiaoping Tang[3,*], and Hui Zhang[1,5,*]

[1]Institute of Human Virology, Key Laboratory of Tropical Disease Control of Ministry Education, Guangdong Engineering Research Center for Antimicrobial Agent and Immunotechnology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong, 510080, China
[2]Center for Infection and Immunity Study, School of Medicine, Sun Yat-sen University, Shenzhen, Guangdong, 518107, China
[3]Guangzhou Eighth People's Hospital, Guangzhou Medical University, Guangzhou, Guangdong, 510080, China.
[4]Guangdong Provincial Center for Disease Control and Prevention, Guangzhou, Guangdong, 511430, China
[5]National Guangzhou Laboratory, Bio-Island, Guangzhou, Guangdong, 510320, China
[6]These authors contributed equally to this work

*Corresponding authors.
Xin He; E-mail: hexin59@mail.sysu.edu.cn
Xiaoping Tang; E-mail: xiaopingtangcn@163.com
Hui Zhang; E-mail: zhangh92@mail.sysu.edu.cn

Methods

Ethics statements

The Ethics Review Boards of Sun Yat-sen University and Guangzhou 8th People's Hospital approved this study. Ten sera samples from healthy participants were obtained from Guangzhou Blood Center. We did not have any interaction with the healthy participants or protected information. Therefore, no informed consent was required. Twenty-four early sera samples and thirty Delta sera samples from convalescent COVID-19 patients were obtained from Guangzhou 8th People's Hospital. All the participants were given written informed consent with approval of the Ethics Committees.

Plasmid constructions.

Different spike protein-expressing plasmids, which were derived from eight SARS-CoV-2 lineages, were constructed to package pseudotyped SARS-CoV-2 S/HIV-1 viruses[1]. The gene encoding the spike protein of the original D614 virus (Wuhan-Hu-1, GISAID: EPI_ISL_402125) was codon-optimized, followed by cloning into pcDNA3.1-MCS-IRES-eGFP-WPRE vector. The gene encoding the spike protein of the G614 virus (SYSU-IHV, EPI_ISL_444969) was generated by introducing the D614G mutation into the spike protein of the D614 virus utilizing site-directed mutagenesis. Genes encoding spike proteins of B.1.1.7 (GISAID: EPI_ISL_581117), B.1.351 (EPI_ISL_678597), P.1 (EPI_ISL_792683), B.1.617.1 (EPI_ISL_1372093), B.1.617.2 (EPI_ISL_2131531) and C.37 (EPI_ISL_3372605) were constructed by multiple rounds of overlapping polymerase chain reaction (PCR) to introduce specific mutation combinations. The sequences of all the spike protein-expressing plasmids were verified by Sanger sequencing.

Pseudotyped virus neutralization assay.

Pseudotyped virus neutralization assay was performed as previously described[1].

Pseudotyped SARS-CoV-2 S/HIV-1 viruses were packaged in HEK293T cells by co-transfecting with a lentiviral construct pHIV-Luciferase (Addgene plasmid # 21375), a packaging construct psPAX2 (Addgene plasmid # 12260) and a plasmid expressing spike protein of various variants which included D614 virus, G614 virus, B.1.1.7, B.1.351, P.1, B.1.617.1, B.1.617.2, and C.37[2]. The luciferase gene which was integrated in the pHIV-Luciferase construct can be expressed upon pseudotyped virus infection. The culture medium was exchanged with fresh DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin 6 h post transfection. The supernatant which contained pseudotyped viruses was collected 48 h post transfection and filtered through 0.45 μm filters. The pseudotyped virus-containing supernatant was stored at -80℃ and tested in titration assays to determine the dilution of pseudotyped virus in neutralization assays.

For detection of neutralizing antibodies against pseudotyped SARS-CoV-2 and corresponding mutants, serially diluted sera from heathy individuals, and convalescent individuals were mixed with each virus and incubated at 37 ºC, 5% CO2 for 1 h. Sera/virus mixtures were added into wells which were seeded with 1×10^4 hACE2-HEK293T cells and went on culturing for 48 h. Cells were lysed with passive lysis buffer (Promega) 48 h post infection. The lysate was measured for relative luminescence units with luminometer (Promega). Neutralizing antibodies titers of sera against the indicated pseudotyped viruses were analyzed with GraphPad Prism 8.0 software using non-linear regression to measure the NT50 titer. The NT50 titer was defined as the reciprocal of sera dilution at which nAbs caused 50% inhibition of infection.

Focus Reduction Neutralizing Test (FRNT) with authentic SARS-CoV-2 viruses

FRNT assay was performed as previously described[2]. Vero E6 cells were seeded in 96-well plates at a density of 2×10^4 cells per well. Cell-seeded plates were placed in cell incubator until cells reached 90% to 100% confluent. Sera of convalescent individuals was 10-fold serially diluted. Two authentic SARS-CoV-2 strains were used. A G614 strain was isolated by our group in April 2020[2]; while a Delta strain was

isolated in May 2021 by Guangdong Center of Diseases Control and transferred to the BSL-3 lab in Sun Yatsen University. Four hundred FFU of authentic SARS-CoV-2 viruses were mixed with the diluted sera in a ratio of 1:1. The sera/virus mixture was incubated at 37°C for 1 h. Cell culture medium was removed from the 96-well plate, followed by the incubation with virus/sera mixture. Plates were then incubated for 1 h at 37°C. The supernatant was removed and cells were overlaid with DMEM medium containing 1.6% CMC. The 96-well plates were placed in incubator and incubated for 24 h. At the next day, supernatant was removed completely. Cells in each well were fixed with 200 μL of 4% paraformaldehyde for 12 h at 4°C. Fixation solution was removed, followed by washing with 200 μL PBS for 3 times. Cells were subsequently incubated with 100 μL of PBS containing 0.2% Triton X-100 and 1% BSA for 30 min. After washing with 200 μL of PBS for 3 times, cells were incubated with 50 μL of diluted primary antibody against SARS-CoV-2 nucleocapsid (N) (Sino Biological) which was 1:1000 diluted with PBS containing 1% BSA at 37°C for 1 h. After primary antibody incubation, cells within each well were washed three times with 200 μL of PBS/T (0.1% Tween-20). HRP-conjugated secondary antibody against rabbit IgG (Sino Biological) was 1:2000 diluted with PBS containing 1% BSA and incubate with cells at 37°C for 1 h. The secondary antibody was removed and washed with 200 μL PBS/T for 3 times. After washing, 50 μL of TrueBlue (KPL) was added to each well and incubated for 5 min while shaking. The supernatant was removed, followed by washing with ddH2O twice. The residual ddH_2O was removed and imaged with ImmunoSpot microanalyzer.

The FRNT50 titer was defined as the reciprocal of sera dilution at which nAbs caused 50% inhibition of infection of cells. The inhibition was represented by the decrease of number of SARS-CoV-2-infected cells in the sample wells compared to virus control wells. Reduction rates of the serial dilution assay were analyzed by Graphpad Prism 8.0 using non-linear regression to measure the FRNT50 titer.

Sera IgG detection.

Sera IgG detection assay was performed as previously described[3]. Full length His-

tagged SARS-CoV-2 S1+S2 ECD proteins (Sino Biological Inc., 40589-V08B1 and 40589-V08B16) (5 μg/ml) were coated on a Costar Stripwell™ Microplate at 4 °C overnight (50 μl/well). The plates were blocked with 5% non-fat milk in PBS for 2 hours at 37 °C. Subsequently the plates were washed three times with PBS containing 0.1% v/v Tween-20 (PBS-T), followed by incubation with 10-fold serially diluted sera from mice in PBS-T for 1 hour at 37 °C. After washing three times with PBS-T, A 1:10000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody was incubated in each well for 1 h at 37 °C. The plates were then washed four times with PBS-T, followed by adding 100 μl of tetramethylbenzidine (TMB) substrate (Invitrogen) at room temperature in the dark. After 10 minutes of incubation, the reaction was stopped with 100 μl 2 M H_2SO_4 solution. The absorbance of each well was measured at 450 nm.

Statistical analysis.

All the measurements have been performed for at least three times by at least two laboratory technicians. Detailed statistical information for specific experiments, including statistical tests, number of samples, mean values, standard errors of the mean (SEM) and p-values derived from indicated test, had been annotated in the main text, figure legends and showed in the figures. Statistical analysis was conducted with Graphpad Prism 8.0 or Microsoft Excel. Data were presented as mean ± SD. A value of $p \geq 0.05$ was considered to be not statistically significant and represented as "ns". A value of $p < 0.05$ was considered to be statistically significant and represented as asterisk (*). Value of $p < 0.01$ was considered to be more statistically significant and represented as double asterisks (**). Value of $p < 0.001$ was considered to be the most statistically significant and represented as triple asterisks (***). Value of $p < 0.0001$ was considered to be the extremely statistically significant and represented as quadruple asterisks (****). For comparing mean differences between groups which were split on one independent variable, one-way ANOVA with Tukey's multiple comparison test was used. For comparing mean differences between groups which were split on two independent variables, two-way ANOVA with Tukey's multiple comparisons test was

used.

References

1、Li, R., *et al.* Differential efficiencies to neutralize the novel mutants B.1.1.7 and 501Y.V2 by collected sera from convalescent COVID-19 patients and RBD nanoparticle-vaccinated rhesus macaques. *Cell Mol Immunol* **18**, 1058-1060 (2021).

2、Ma, X., *et al.* Nanoparticle Vaccines Based on the Receptor Binding Domain (RBD) and Heptad Repeat (HR) of SARS-CoV-2 Elicit Robust Protective Immune Responses. *Immunity* **53**, 1315-1330 e1319 (2020).

3、Liu, B., *et al.* Recovered COVID-19 patients with recurrent viral RNA exhibit lower levels of anti-RBD antibodies. *Cell Mol Immunol* **17**, 1098-1100 (2020).

Table S1 Characteristics of enrolled patients with COVID-19.

		Patients infected by Delta Strain			Patients infected by early strain
Number		Total	Vaccinated	Non-vaccinated	Non-vaccinated
		26	9 (34.6%)	17 (65.4%)	22
Gender	Male	15 (57.4%)	6 (66.7%)	10 (58.8%)	5 (22.7%)
	Female	11 (42.3%)	3 (33.3%)	7 (41.2%)	17 (77.3%)
Aged, Median (Range)		45 (22-83)	44 (25-58)	51 (22-83)	45 (32-77)
Disease duration (Days) Median (Range)		22 (15-28)	22 (17-24)	21(15-25)	26 (17-39)
Severity	Mild	4 (15.4%)	2 (22.2%)	2 (11.8%)	2 (9.1%)
	Moderate	22 (84.6%)	7 (77.8%)	15 (88.2%)	20 (90.9%)

Figure S1. Titrations of IgG targeting different S antigens in early convalescence sera and delta convalescence sera.

Normalized OD450 nm values of the anti-IgG to the different S proteins are compared between early convalescent sera (n=22) and delta convalescent sera (n=26) samples. The P value was calculated using unpaired two-way ANOVA with Tukey's multiple comparisons test. *p < 0.05, ***p < 0.001. Data was represented as the mean ± SD (n = 22 or 26).

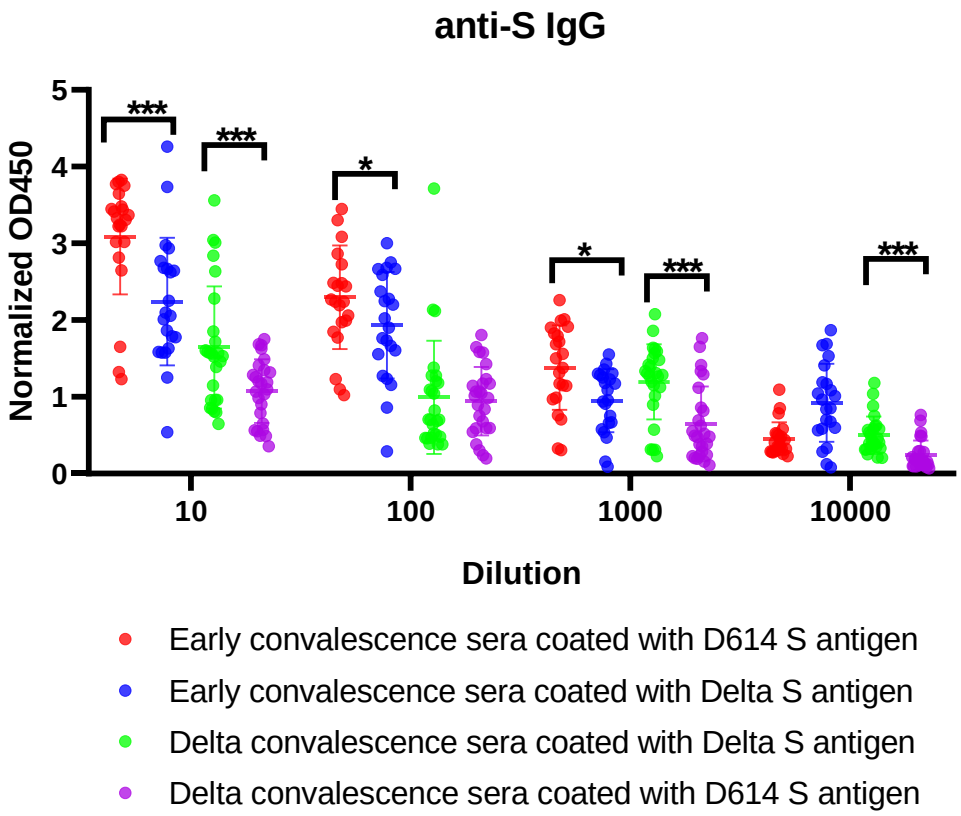

Figure S2. The impact of vaccination on the production of neutralizing antibodies. The analysis of SARS-CoV-2 neutralization of many variant pseudoviruses between non-vaccinated group and vaccinated group. The P value was calculated using unpaired two-way ANOVA with Tukey's multiple comparisons test. *p < 0.05, **p < 0.01. Data was represented as the mean ± SD (n = 9 or 17).