

# Neutralization of Omicron BA.1, BA.2, and BA.3 SARS-CoV-2 by 3 doses of BNT162b2 vaccine

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The newly emerged Omicron SARS-CoV-2 has several distinct sublineages including BA.1, BA.2, and BA.3. BA.1 accounts for the initial surge and is being replaced by BA.2, whereas BA.3 is at a low prevalence at this time. Here we report the neutralization of BNT162b2-vaccinated sera (collected 1 month after dose 3) against the three Omicron sublineages. To facilitate the neutralization testing, we have engineered the complete BA.1, BA.2, or BA.3 spike into an mNeonGreen USA-WA1/2020 SARS-CoV-2. All BNT162b2-vaccinated sera neutralize USA-WA1/2020, BA.1-, BA.2-, and BA.3-spike SARS-CoV-2s with titers of >20; the neutralization geometric mean titers (GMTs) against the four viruses are 1211, 336, 300, and 190, respectively. Thus, the BA.1-, BA.2-, and BA.3-spike SARS-CoV-2s are 3.6-, 4.0-, and 6.4-fold less efficiently neutralized than the USA-WA1/2020, respectively. Our data have implications in vaccine strategy and understanding the biology of Omicron sublineages.

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The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Omicron variant has recently emerged as the fifth variant of concern (VOC) after the previous Alpha, Beta, Gamma, and Delta VOCs. As of 12 March 2022, the Omicron variant includes 3 sublineages: BA.1, BA.2, and BA.3. After initial identification in South Africa in November 2021, the Omicron sublineage BA.1, and its derivative BA.1.1 (containing an extra spike R346K substitution), became dominant worldwide. Subsequently, sublineage BA.2 sharply increased its prevalence in many countries, including Denmark, the Philippines, South Africa, and Belgium. In the United States, the prevalence of BA.2 increased from 0.4% on January 22 to 23.1% on March 12, 2022 (<https://covid.cdc.gov/covid-data-tracker/#variant-proportions>). Studies have also shown that BA.2 may be approximately 30% more transmissible than BA.1<sup>1</sup>, but it does not appear to cause more severe disease<sup>2</sup>. Compared with the BA.1 and BA.2 sublineages, the prevalence of BA.3 has remained low, with a total of 613 BA.3 sequences in the GISAID database (<https://www.gisaid.org/>) as of 12 March 2022. Recent studies showed that BA.1 evades vaccine- and non-Omicron infection-elicited neutralization<sup>3,4</sup>, which, together with increased transmissibility,

may partially account for the variant replacement from the previous Delta to the current Omicron.

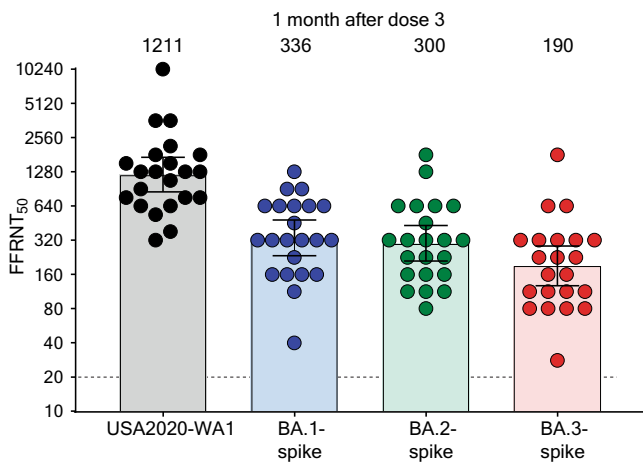
BNT162b2 is an mRNA vaccine that encodes a stabilized pre-fusion full-length spike glycoprotein from the original SARS-CoV-2 Wuhan-Hu-1 isolate. BNT162b2 mRNA has been approved for vaccination of people  $\geq 16$ -year-old and has also been authorized under emergency use provision for immunization of children 5- to 15-year-old by the US Food and Drug Administration. Since BA.1, BA.2, and BA.3 have distinct sets of mutations in their spike glycoproteins (Fig. S1a), laboratory results are urgently needed to evaluate the susceptibility of the three Omicron sublineages to vaccine-elicited neutralization. In this work, we show the neutralization of BNT162b2-vaccinated sera (collected 1 month after dose 3) against the three Omicron sublineages.

## Results and discussions

To determine the susceptibility of Omicron sublineages to BNT162b2-elicited neutralization, we engineered the complete BA.1 (GISAID EPI\_ISL\_6640916), BA.2 (GISAID EPI\_ISL\_6795834.2), or BA.3 (GISAID EPI\_ISL\_7605591) spike into the mNeonGreen (mNG) reporter USA-WA1/2020, an SARS-CoV-2 strain isolated in January 2020 (Fig. S1a). The mNG gene was inserted into the open-reading-frame-7 (ORF7) of the viral genome to enable the development of a fluorescent focus reduction neutralization test (FFRNT)<sup>4</sup>. Our historical data set indicate that the high-throughput FFRNT produces comparable neutralization results as the gold standard plaque-reduction neutralization test (PRNT; using SARS-CoV-2 without the mNG reporter) when analyzing BNT162b2-vaccinated human sera (Fig. S2). Thus, FFRNT can reliably be used to measure antibody neutralization.

Characterization of the recombinant BA.1-, BA.2-, and BA.3-spike mNG SARS-CoV-2s showed that all viruses produced infectious titers of  $>10^6$  focus-forming units per milliliter (FFU/ml), similar to the wild-type USA-WA1/2020 mNG virus. Although the recombinant viruses formed different sizes of fluorescent foci in the order of wild-type USA-WA1/2020  $>$  BS.2-spike  $\approx$  BA.3-spike  $>$  BA.1-spike mNG SARS-CoV-2 (Fig. S1b), all viruses showed equivalent viral RNA genome/FFU ratios when analyzed on Vero E6 cells (Fig. S1c), suggesting equivalent specific infectivities of the viral stocks. All recombinant viruses were sequenced to ensure no undesired mutations.

Using a panel of human sera collected at 1-month post dose 3 (PD3) of BNT162b2 vaccine<sup>3</sup> (Fig. S3), we determined the 50% fluorescent focus-reduction neutralization titers (FFRNT<sub>50</sub>) against recombinant USA-WA1/2020 and Omicron sublineage-spike mNG SARS-CoV-2s. We chose the PD3 sera, rather than post dose 2 sera, because (i) two doses of BNT162b2 did not elicit robust neutralization against Omicron BA.1<sup>3</sup> and (ii) many individuals have already received 3 doses of BNT162b2. The PD3 sera neutralized USA-WA1/2020, BA.1-, BA.2-, and BA.3-spike mNG viruses with geometric mean titers (GMTs) of 1211, 336, 300, and 190, respectively (Fig. 1). Although all PD3 sera neutralized recombinant viruses with titers of  $>20$ , the neutralizing GMTs against BA.1-, BA.2-, and BA.3-spike mNG viruses were 3.6-, 4.0-, and 6.4-fold lower than the GMT against the mNG USA-WA1/2020, respectively (Fig. 1). The results support two conclusions. First, BA.1 and BA.2 spikes evade the neutralization of PD3 sera to comparable degrees. Similar reduced neutralization of BA.1 and BA.2 sublineages was reported<sup>5,6</sup>; however, the reduction folds varied among studies, likely due to different serum specimens and neutralization assay protocols. These results may also imply that the observed increasing prevalence of BA.2 over BA.1 among circulating strains was not driven by the difference in antibody neutralization after vaccination, but by other factors, such as differences in viral replication<sup>7</sup> and transmission<sup>8</sup>, or other potential immune evasion mechanisms.



**Fig. 1 Serum neutralization of Omicron BA.1-, BA.2-, and BA.3-spike mNG SARS-CoV-2s and USA-WA1/2020 after three doses of BNT162b2.** A panel of 22 human sera collected 1 month after dose 3 of the BNT162b2 vaccine were tested for the 50% fluorescent focus-reduction neutralization titers (FFRNT<sub>50</sub>) against recombinant USA-WA1/2020 (black circles), Omicron BA.1- (blue circles), BA.2- (green circles), and BA.3-spike (red circles) mNG SARS-CoV-2s. The serum collection scheme is described in Fig. S3 as previously reported<sup>18</sup>. The BA.1-, BA.2-, and BA.3-spike mNG SARS-CoV-2s were produced by engineering the complete Omicron spike genes into the mNG USA-WA1/2020<sup>19</sup>. Each data point represents the geometric mean FFRNT<sub>50</sub> obtained with a serum specimen against the indicated virus, as detailed in Tables S1. The neutralization titers for BA.1-, BA.2-, and BA.3-spike mNG SARS-CoV-2s were determined in duplicate assays; the FFRNT<sub>50</sub>s for USA-WA1/2020 mNG SARS-CoV-2 were determined in two independent experiments, each with duplicate assays. Data are presented as the geometric mean of titer (GMT) with 95% confidence intervals (CI). The bar heights and the numbers above indicate GMTs. The whiskers indicate 95% CI. The dotted line indicates the limit of detection of FFRNT<sub>50</sub>. Statistical analysis was performed with the use of the Wilcoxon matched-pairs signed-rank test. The statistical significances of the differences between geometric mean titers against the USA-WA1/2020 and Omicron BA.1-, BA.2-, or BA.3-spike SARS-CoV-2 are all  $p$  (two-tailed)  $<0.0001$ . The statistical significances of the differences between geometric mean titers against the BA.3 and Omicron BA.1-, or BA.2-spike SARS-CoV-2 are  $p$  (two-tailed)  $<0.0001$ , and  $p$  (two-tailed) = 0.004, respectively.

Second, BA.3 spike evades the BNT162b2-elicited neutralization more efficiently than BA.1 and BA.2 spikes. If the viral replication of BA.3 is not attenuated (which remains to be determined), the BA.3 sublineage may have the potential to expand and elongate the current Omicron surges. Thus, we should closely monitor the prevalence of BA.3 during surveillance.

Despite the reduced neutralization of Omicron sublineages, other immune effectors, such as T cells and non-neutralizing antibodies that mediate antibody-dependent cytotoxicity, also contribute to the protection against severe COVID-19. The majority of T cell epitopes after vaccination or natural infection are preserved against Omicron spikes<sup>9</sup>. Indeed, 3 doses of BNT162b2 have conferred efficacy against Omicron disease; however, recent real-world effectiveness data showed a waning of protection against symptomatic infection caused by Omicron. The waning of protection was more modest against severe disease caused by Omicron, with overall efficacy remaining high 3–6 months after dose 3<sup>10–14</sup>. Studies are underway to determine the extended durability of the PD3 neutralization against Omicron sublineages and future variants. These laboratory investigations, together with real-world vaccine effectiveness data, will continue to guide 4th dose vaccine strategy for optimal breadth and duration of protection.

## Methods

**Ethical statement.** All virus work was performed in a biosafety level 3 (BSL-3) laboratory with redundant fans in the biosafety cabinets at The University of Texas Medical Branch at Galveston. All personnel wore powered air-purifying respirators (Breathe Easy, 3M) with Tyvek suits, aprons, booties, and double gloves.

**Cells.** Vero E6 (ATCC® CRL-1586) was purchased from the American Type Culture Collection (ATCC, Bethesda, MD), and maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, South Logan, UT) and 1% penicillin/streptomycin at 37 °C with 5% CO<sub>2</sub>. All culture media and antibiotics were purchased from ThermoFisher Scientific (Waltham, MA). The cell line was tested negative for mycoplasma.

**Human serum.** Serum samples were collected from BNT162b2 vaccinees participating in the phase 1 portion of the ongoing phase 1/2/3 clinical trial (ClinicalTrials.gov identifier: NCT04368728). The protocol and informed consent were approved by institutional review boards for each of the investigational centers participating in the study. The study was conducted in compliance with all International Council for Harmonisation Good Clinical Practice guidelines and the ethical principles of the Declaration of Helsinki. The primary outcomes for phase 1 were reported previously<sup>15,16</sup>. BNT162b2-vaccinated serum (n = 22) was collected on the day of the third dose of BNT162b2, which was administered at 7.9–8.8 months after the second dose; and sera collected 1 month after the third dose were used in the present study for neutralization testing. Table S1 summarizes the patient information (e.g., age and gender) and sample collection time points. The information for the serum panels were reported previously<sup>17,18</sup>.

**Construction and characterization of recombinant Omicron sublineage spike mNG SARS-CoV-2s.** Recombinant Omicron BA.1-, BA.2-, and BA.3-spike mNG SARS-CoV-2s were constructed by engineering the complete spike gene from Omicron sublineages into an infectious cDNA clone of mNG USA-WA1/2020<sup>19</sup> (Fig. S1a). All spike mutations, deletions, and insertions were introduced into the infectious cDNA clone of mNG USA-WA1/2020 using PCR-based mutagenesis as previously described<sup>20</sup>. The BA.1, BA.2, and BA.3 spike sequences were based on GISAID EPI\_ISL\_6640916, EPI\_ISL\_6795834.2, and EPI\_ISL\_7605591, respectively. The full-length cDNA of viral genome containing the complete Omicron spike was assembled via in vitro ligation. The resulting full-length cDNA was used as a template for in vitro transcription of genome-length viral RNA. The in vitro transcribed viral RNA was electroporated into Vero E6 cells. On day 3 post electroporation, the original viral stock (P0) was harvested from the electroporated cells. The P0 virus was amplified for another round on Vero E6 cells to produce the P1 stock for neutralization testing. The infectious titer of the P1 virus was quantified by fluorescent focus assay on Vero E6 cells (Fig. S1b). We sequenced the complete spike gene of the P1 virus to ensure no undesired mutations. Only the P1 viruses were used for the neutralization test. The protocols for the mutagenesis of mNG SARS-CoV-2 and virus production were reported previously<sup>21</sup>. To determine the specific infectivity, we quantified the P1 virus stocks for their fluorescent focus units (FFU) and genomic RNA contents by fluorescent focus assay on Vero E6 cells and RT-qPCR, respectively. The methods for fluorescent focus assay and RT-qPCR

were reported previously<sup>4,22</sup>. The specific infectivity of each virus was measured by the genomic RNA-to-FFU ratios (genome/FFU).

**Fluorescent focus reduction neutralization test.** Neutralization titers of human sera were measured by FFRNT using the USA-WA1/2020, BA.1-, BA.2-, and BA.3-spike mNG SARS-CoV-2s. The details of the FFRNT protocol were reported previously<sup>4</sup>. Briefly,  $2.5 \times 10^4$  Vero E6 cells per well were seeded in 96-well plates (Greiner Bio-one™). The cells were incubated overnight. On the next day, each serum was 2-fold serially diluted in the culture medium with the first dilution of 1:20 (final dilution range of 1:20 to 1:20,480). The diluted serum was incubated with 100–150 FFUs of mNG SARS-CoV-2 at 37 °C for 1 h, after which the serum-virus mixtures were loaded onto the pre-seeded Vero E6 cell monolayer in 96-well plates. After 1 h infection, the inoculum was removed and 100 µl of overlay medium (supplemented with 0.8% methylcellulose) was added to each well. After incubating the plates at 37 °C for 16 h, raw images of mNG foci were acquired using Cytation™ 7 (BioTek) armed with 2.5× FL Zeiss objective with a wide-field of view and processed using the Gene 5 software settings (GFP [469,525] threshold 4000, object selection size 50–1000 µm). The foci in each well were counted and normalized to the non-serum-treated controls to calculate the relative infectivities. The FFRNT<sub>50</sub> value was defined as the minimal serum dilution that suppressed >50% of fluorescent foci. The neutralization titer of each serum was determined in duplicate assays, and the geometric mean was taken. All attempts at replication were successful. Table S1 summarizes the FFRNT<sub>50</sub> results. Data were initially plotted in GraphPad Prism 9 software and assembled in Adobe Illustrator.

**Statistics.** The nonparametric Wilcoxon matched-pairs signed-rank test was used to analyze the statistical significance in Fig. 1.

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

## Data availability

Source data are provided as a Source Data file with this paper. Source data are provided with this paper.

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## Author contributions

C.K., K.U.J., X.X., K.A.S., and P.-Y.S. conceived the study. C.K., J.Z., H.X., H.C., Q.Y., M.C., D.C., X.X., and K.A.S. performed the experiments. H.X., J.Z., M.C., D.C., A.M.,

K.U.J., X.X., K.A.S., and P.-Y.S. analyzed the results. C.K., J.Z., X.X., K.A.S., and P.-Y.S. wrote the manuscript. M.C., D.C., K.U.J., X.X., K.A.S., and P.-Y.S. supervised the project.

## Competing interests

X.X. and P.-Y.S. have filed a patent on the reverse genetic system of SARS-CoV-2. C.K., J.Z., H.X., X.X., and P.-Y.S. received compensation from Pfizer to perform the project. H.C., Q.Y., M.C., D.C., K.U.J., and K.A.S. are employees of Pfizer and may hold stock options. A.M. is an employee of BioNTech and may hold stock options. All other authors have no competing interests.

## Additional information

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