

## CORONAVIRUS

# Two doses of the SARS-CoV-2 BNT162b2 vaccine enhance antibody responses to variants in individuals with prior SARS-CoV-2 infection

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Understanding the impact of prior infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) on the response to vaccination is a priority for responding to the coronavirus disease 2019 (COVID-19) pandemic. In particular, it is necessary to understand how prior infection plus vaccination can modulate immune responses against variants of concern. To address this, we sampled 20 individuals with and 25 individuals without confirmed previous SARS-CoV-2 infection from a large cohort of health care workers followed serologically since April 2020. All 45 individuals had received two doses of the Pfizer-BioNTech BNT162b2 vaccine with a delayed booster at 10 weeks. Absolute and neutralizing antibody titers against wild-type SARS-CoV-2 and variants were measured using enzyme immunoassays and pseudotype neutralization assays. We observed antibody reactivity against lineage A, B.1.351, and P.1 variants with increasing antigenic exposure, through either vaccination or natural infection. This improvement was further confirmed in neutralization assays using fixed dilutions of serum samples. The impact of antigenic exposure was more evident in enzyme immunoassays measuring SARS-CoV-2 spike protein-specific IgG antibody concentrations. Our data show that multiple exposures to SARS-CoV-2 spike protein in the context of a delayed booster expand the neutralizing breadth of the antibody response to neutralization-resistant SARS-CoV-2 variants. This suggests that additional vaccine boosts may be beneficial in improving immune responses against future SARS-CoV-2 variants of concern.

## INTRODUCTION

Clinical trials of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines have collectively involved thousands of participants, providing evidence to support expedient and widespread vaccination globally (1–4). In the United Kingdom, the first vaccine to be given emergency authorization was the Pfizer-BioNTech stabilized spike-based mRNA vaccine, now marketed as BNT162b2. The vaccines developed to this point are proving to be effective in reducing the infection rate and morbidity and mortality associated with SARS-CoV-2 infection (2, 5, 6). However, there are concerns about the impact of antigenic viral variants that have recently emerged with spike protein variations, presumably in the face of

suboptimal immunity in areas of high virus circulation (7). One of these variants, first reported in South Africa (8) and known as 501Y.V2 (lineage B.1.351) or the  $\beta$  variant, has been shown to effectively escape from neutralization by convalescent serum samples, monoclonal antibodies, and vaccine-elicited serum samples (9–12), and poor neutralization may lead to vaccine breakthrough (13). Given the potential for antigenic variation to lead to vaccine escape, consideration is being given to provide additional boosts, either with the vaccines containing the original lineage A (Wuhan) viral spike or with antigens matched to emerging variants (14). Although increased antigenic exposure is expected to elevate antibody titer and potentially expand the epitope repertoire, previous studies of other respiratory viruses, for example, influenza A, have shown limits to the extent to which repeated antigenic exposure can boost antibody concentrations (15).

To investigate the potential for increased antigenic exposure to produce antibody-mediated immunity with increased neutralizing potency against SARS-CoV-2 variants, we analyzed serum samples obtained from a prospective, longitudinal cohort of health care workers (HCWs) collected at regular intervals through the period of pandemic. We analyzed serum samples collected after first and second vaccine doses from individuals previously infected with SARS-CoV-2 and seronegative individuals for the presence of spike-reactive or virus-neutralizing antibodies against lineage A and lineage B.1.351 virus. We also performed neutralization assays against P.1 ( $\gamma$ ) variant pseudotypes using available post-boost serum samples.

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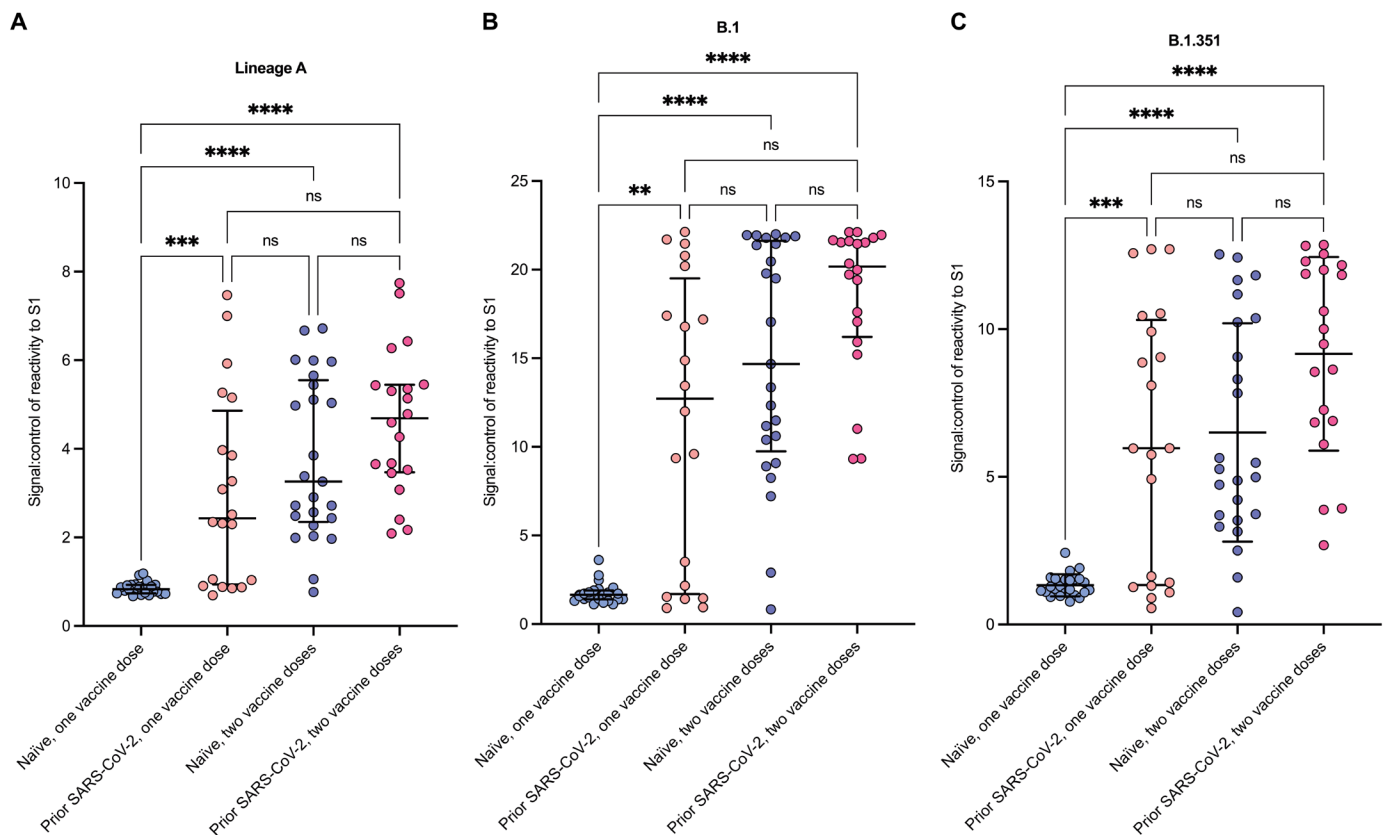
## RESULTS

**Repeated SARS-CoV-2 antigenic exposure, through vaccination or natural infection, results in increased and more broadly reactive spike-specific antibody responses**

The PANTHER (Pandemic Tracking of Healthcare workers) vaccine cohort consists of frontline HCWs, of which 18% had serological or polymerase chain reaction (PCR)-confirmed past SARS-CoV-2 infection before vaccination (16, 17). Because of occupational risk, and to reduce nosocomial transmission in hospitals, a subset of these people was included in the first-priority group for coronavirus disease 2019 (COVID-19) vaccination. For this study, we identified a total of 45 individuals who had received both doses of the BNT162b2 vaccine with complete serology and prioritized for vaccination and divided them into two groups: those with past infection and those with no evidence of past infection (table S1). The two groups did not differ in terms of age or body mass index (fig. S1), although more females ( $n = 36$ ) than males ( $n = 9$ ) were recruited into the study cohort. Before assessing the S1-specific antibody response, we validated our in-house S1-specific antibody assay against the commercially available assay (Roche) and showed good correlation between the two assays (fig. S2). We then went on to measure the S1-specific antibody response, using the validated in-house enzyme immunoassay, in serum samples obtained 2 days before and then at least 2 weeks after the second (booster) vaccine dose. After the first vaccine dose, concentrations of antibodies specific to the lineage A (Fig. 1A) and lineage B.1 (Fig. 1B) S1 proteins were

significantly higher in individuals with evidence of prior infection compared to those who were uninfected (median absorbance ratio of 2.43 versus 0.83;  $P = 0.0008$  and 12.72 versus 1.65;  $P = 0.0045$ , respectively). Antibody concentrations in individuals with past infection and a single dose of vaccine were comparable to uninfected individuals after two doses of vaccine. The booster dose significantly increased both lineage A-specific and lineage B.1-specific antibody concentrations in both groups (lineage A, no infection, two doses versus 1 dose,  $P < 0.0001$ ; B1, no infection, two doses versus one dose,  $P < 0.001$ ). Although most individuals showed an increase in S1 antibody concentrations to all variant S1 proteins, before and after plots showed that, in a small proportion (5 of 45 or 11%) of individuals, the antibody concentration decreased after boosting (fig. S3). One individual who had no prior exposure to SARS-CoV-2 generated a potent antibody response from a low baseline value after a single dose of vaccine comparable to that seen in individuals with prior infection. The before and after plots also showed that antibody reactivity before and after boost to the S2 sub-unit of spike was relatively low, when compared to S1, in most serum samples (median absorbance ratio of 1.157 versus 1.779 after boost), although they did show a significant increase in the individuals who did not have a natural infection ( $P = 0.01205$ ). As expected, reactivity to nucleocapsid (0.815 versus 0.787 after boost) was also low (fig. S3).

A similar analysis against S1 protein representing the neutralization-resistant B.1.351 S1 protein revealed more variability in recognition



**Fig. 1. Multiple exposures increase antibody reactivity to SARS-CoV-2 variants.** (A to C) Ratio of reactivity signal to control (Signal:control) of lineage A S1 (A), B.1 S1 (B), and B.1.351 S1 (C) at a 1:600 serum dilution. The number of vaccine doses and infection history is indicated. Plots show median and interquartile ranges (IQRs). Differences between groups were analyzed using a Kruskal-Wallis test with Dunn's multiple comparisons correction. \*\*\* $P < 0.01$ ; \*\*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ; ns, nonsignificant.

after multiple exposures to S1 antigen (18), although, in general, increasing antigenic exposure through either natural infection or vaccination resulted in increased reactivity. The average reactivity in uninfected individuals after a single dose of vaccine was relatively low initially but increased significantly after the vaccine boost (median absorbance ratio of 1.24 and 5.27, respectively,  $P < 0.0001$ ; Fig. 1C). A single vaccine dose in previously infected people elicited significantly higher antibody reactivity (median absorbance ratio = 5.97,  $P = 0.0006$ ) than the prime-dose response observed in uninfected individuals. Last, we revalidated the in-house assay against the Roche commercial spike antibody assay using samples collected after vaccine boost, and again, there was a strong correlation between the two assays (fig. S2).

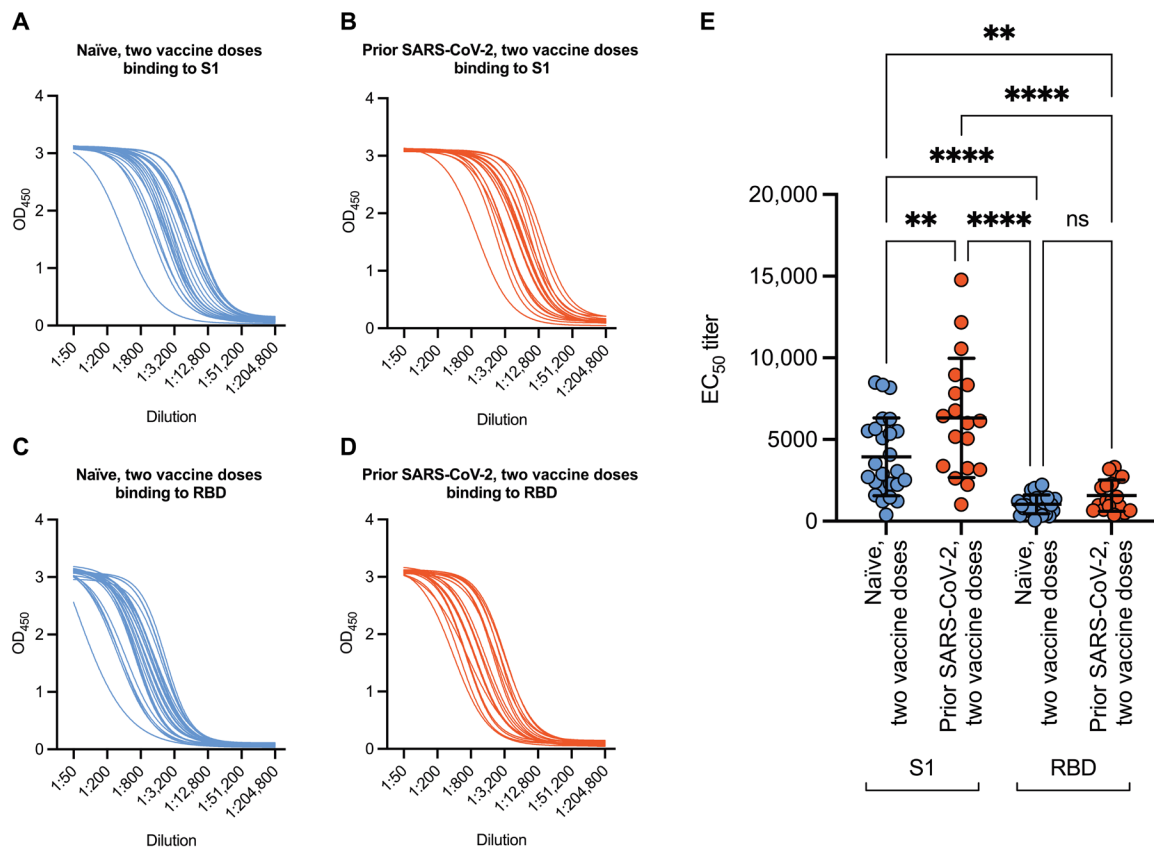
### Increasing antigenic exposure preferentially increases targeting of epitopes outside of the receptor binding domain

To provide insight into the type of antibody response generated by multiple exposures, we titrated serum samples from both groups after two doses of vaccine and evaluated their reactivity against lineage A S1 and receptor binding domain (RBD) of the same strain (Fig. 2, A to D). The median effective concentration ( $EC_{50}$ ) titers against the S1 region of the spike protein were significantly higher

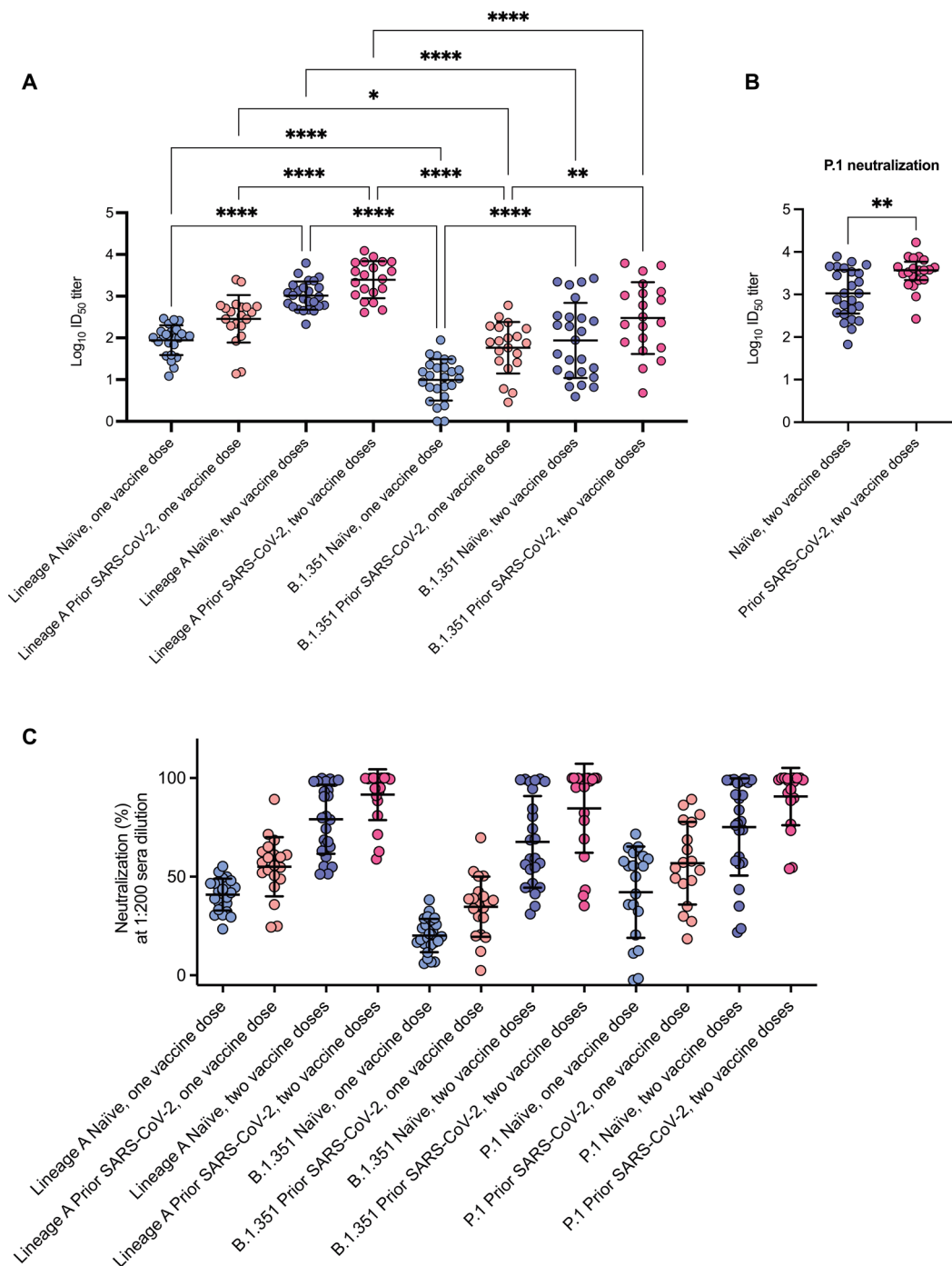
in individuals with two vaccine doses and natural infection compared to individuals who had two doses of the vaccine and no prior history of infection (6328 versus 3936,  $P = 0.0034$ ; Fig. 2E). The  $EC_{50}$  against RBD was lower than against full S1 in both cohorts, but no difference was observed between two doses with and without natural infection [1568 versus 1039, nonsignificant (ns); Fig. 2E]. These data suggest that the response elicited by multiple exposures to spike protein is being directed toward epitopes outside of the RBD.

### Prior infection and vaccine boosting increases neutralizing antibody titer

Having established that past infection and vaccine boosting increased S1-specific antibody concentrations, we used pseudotyped lentiviruses expressing either lineage A or B.1.351 spike protein to determine virus neutralization curves (fig. S4) after each dose of vaccine in the two groups and then compared the resulting median infective dose ( $ID_{50}$ ) values (Fig. 3A). In the analyses using lineage A pseudoviruses, the average serum  $ID_{50}$  neutralizing titers after a single dose of vaccine were higher in those who had a past infection with SARS-CoV-2 compared to those without previous infection (535.4 versus 114.7,  $P = 0.0008$ ; Fig. 3A). Similarly, mean reciprocal neutralizing antibody titers were higher in uninfected individuals after two vaccine doses compared to previously infected individuals (1405 versus



**Fig. 2. Prior history of SARS-CoV-2 infection increases binding to the S1 region of lineage A SARS-CoV-2 relative to those without prior history of infection.** (A and B) Binding of antibodies to lineage A S1 was measured in serum samples isolated from participants after two doses of vaccine without prior infection (A) or with prior infection (B). OD<sub>450</sub>, optical density at 450 nm. (C and D) Binding to the lineage A RBD was also evaluated in serum samples collected after two doses of vaccine without prior infection (C) or with prior infection (D). (E)  $EC_{50}$  titers are shown based on prior history of infection and binding. Because of sample constraints, the two doses with infection group  $n = 18$ . The plot in (E) shows mean  $\pm$  SD. Differences between groups in (E) were evaluated using one-way ANOVA with Tukey's multiple comparisons test. \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ .



**Fig. 3. Neutralization potency of SARS-CoV-2-specific antibodies increases with exposure to spike protein through infection or vaccination. (A)** Log<sub>10</sub> ID<sub>50</sub> of serum samples specific to lineage A or B.1.351 pseudoviruses. **(B)** Log<sub>10</sub> ID<sub>50</sub> of serum against P.1 variant pseudovirus for samples collected after two doses with ( $n = 18$ ) and without ( $n = 20$ ) prior history of SARS-CoV-2 infection. Sample sizes are smaller due to sample constraints. **(C)** Percent neutralization of serum samples diluted 1:200 for lineage A, B.1.351, and P.1 pseudoviruses. Samples are shown by number and type of exposure. Data are presented as mean  $\pm$  SD (A and C) or median and IQR (B) due to not passing the D'Agostino and Pearson normality test. Differences between groups in (A) and (C) were evaluated using two-way ANOVA with Tukey's multiple comparisons test. Differences between groups in (B) were evaluated using two-tailed Mann-Whitney test. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ . Significance values for (C) are shown in table S2.

535.4,  $P = 0.0002$ ) who had received a single dose. Vaccine boosting increased neutralizing antibody titers in both groups when compared to titers obtained after a single dose of vaccine. There was a 12-fold change in the mean reciprocal titer for uninfected individuals and a

7-fold increase for samples from previously infected individuals, and both increases were statistically significant ( $P < 0.0001$  for both).

We assessed the impact of antigenic variation using pseudoviruses bearing the lineage B.1.351 spike protein (Fig. 3A). Repeated



antigenic exposure resulted in an increase in neutralization of the B.1.351 variant. For example, in previously infected individuals, the mean ID<sub>50</sub> neutralizing titer increased from about 1:60 after the initial dose of vaccine to 1:300 after the booster dose ( $P = 0.0019$ ). Neutralizing titers for serum samples were consistently greater against pseudoviruses complemented with the lineage A variant spike protein as compared to the neutralization-resistant B.1.351 spike variant.

To determine whether the boosting effect of prior exposure was a general phenomenon, we determined the neutralizing potency of post-boost serum samples against pseudoviruses bearing a spike protein from a different variant of concern, the P.1 or  $\gamma$  variant first identified in Brazil. Comparison of the average reciprocal serum dilution ID<sub>50</sub> values showed significantly higher concentrations of neutralizing antibodies in serum samples obtained from individuals with prior SARS-CoV-2 infection compared to those with no known previous infection (3703 versus 1068,  $P = 0.0074$ ; Fig. 3B).

We then established the impact of antigenic variation on predicted vaccine effectiveness in the entire cohort by measuring pseudotype neutralization using a fixed 1:200 dilution of serum. We selected a 1:200 dilution because previous SARS-CoV-2 challenge studies in nonhuman primates have shown that serum samples yielding an in vitro IC<sub>50</sub> neutralization titer of at least 1:200 are protective (19). After a single dose of BNT162b2, the degree of neutralization in individuals without previous infection was significantly higher against the lineage A spike virus as compared to B.1.351 (40.9 versus 20.2%,  $P < 0.0001$ ). However, the average values were all less than 50% neutralization (Fig. 3C and table S2). Neutralizing antibodies against both variants increased with increasing antigenic exposure through either natural infection or vaccination. After the final vaccine boost, the ability of serum samples to neutralize virus was significantly lower for the B.1.351 variant compared to lineage A ( $P = 0.0345$ ) and P.1 ( $P = 0.0302$ ). However, no difference was observed after vaccine boost in previously infected individuals, with the average neutralization (lineage A 91.6% and B.1.351 84.7%) greater than 50% for both variants with all achieving effective neutralization against lineage A and 85% against B.1.351. Similarly, the average percentage neutralization observed for a fixed dilution of post-boost serum samples against the P.1 variant was 82% and 49% in previously infected versus SARS-CoV-2 naïve individuals, respectively ( $P < 0.0001$ ) (Fig. 3C and table S2). Last, we observed a positive correlation between total S1 antibody concentrations and neutralization titers (lineage A:  $r = 0.5583$ ,  $P < 0.0001$ ; B.1.351:  $r = 0.4452$ ,  $P < 0.0001$ ) (fig. S5).

## DISCUSSION

Using a well-characterized HCW cohort, we have been able to quantify the effect of a booster vaccination strategy on neutralizing or total immunoglobulin G (IgG) antibody responses to SARS-CoV-2 and variants of concern. We focused on IgG antibody concentrations because these are the predominant antibody class induced by intramuscular immunization and are associated with long-term protective immunity (20). We measured antibody responses in serum samples isolated from naïve individuals and individuals with prior history of SARS-CoV-2 infection at baseline and after vaccination with first and second doses. Although previous studies have shown that antibody responses in previously exposed individuals are greater after a single dose of a COVID-19 vaccine (21–23), this study shows

that the trend toward higher neutralizing antibody frequency in previously infected people continues after the second dose. Our results demonstrate that repeated antigenic exposure increases antibody and neutralizing responses to lineage A, B.1, B.1.351, and P.1 variants, suggesting that vaccination with boosters may be effective in combating variants of concern.

Vaccine effectiveness against neutralization-resistant SARS-CoV-2 variants, such as the B.1.351 variant, is of major public health concern (10, 24) [also reviewed in (25)]. This study confirms the neutralization-resistant phenotype of the B.1.351 variant. After one dose of vaccine in individuals with or without prior infection, spike protein-specific IgG and neutralizing antibody concentrations were lower against the B.1.351 variant than lineage A strains. However, increasing antigenic exposure in humans through natural infection followed by a two-dose BNT162b2 vaccination schedule increased IgG concentration and neutralization of the B.1.351 and P.1 variants to values comparable to the lineage A virus. Further characterization of the IgG response suggested that multiple antigenic exposure increases reactivity to epitopes outside of the RBD. This might contribute to the increased cross-reactivity of the antibody response and increased neutralizing activity. However, the relative contribution of repertoire expansion versus increased antibody affinity to increased antibody reactivity is unclear. Reactivity to the S2 region after vaccine boost showed only modest increases compared to S1, especially in individuals without prior exposure to SARS-CoV-2. Previous analysis of BNT162b2 antibody responses has shown that this vaccine elicits measurable S2-specific responses (26). Thus, it is unclear why robust S2 responses were not evident in all of our vaccine recipients. Future characterization of S1 and S2 neutralizing determinants, together with analysis of antibody affinity and avidity, will provide a better understanding of protective antibody responses and provide important insights for future vaccine development.

Recent studies have evaluated the durability of anti-SARS-CoV-2 neutralizing antibody titers through longitudinal sampling and modeling of phase 1 and 2 clinical data to estimate thresholds for protection against infection and against severe COVID-19. Independent of these specific thresholds, a lower degree of initial neutralization may result in a shorter time span before the titers are below the protective threshold. In the case of a 108-day half-life for titers, a 25% lower neutralizing titer for a given variant would mean 44.8 fewer days above a given protective threshold, which may be relevant in terms of public health in periods when viral transmission is high.

To evaluate virus neutralization, we used a lentivirus-based pseudovirus assay rather than live virus assays. This was chosen because of the superior tractability of the pseudotype assay compared to live virus neutralization assays. These assays are also less susceptible to cell culture adaptation that is frequently encountered during virus passage. In direct comparisons, the pseudotype assay was shown to produce data that were entirely comparable to more laborious live virus assays (27).

Our study has several limitations. Prior exposure in our cohort involves only exposure to the lineage A or B variants (28, 29), and natural infection by more antigenically diverse strains may skew the resultant immune potency and breadth. Unfortunately, we had too few positive PCR samples to try to elucidate any link between immunity and infecting lineage. Up to the start of the vaccination program, Nottingham and the surrounding region recorded a range of B.1-derived sublineages, with expansion of B.1.1.7 ( $\alpha$  variant)

occurring from January 2021. We did not include B.1.1.7 in our analysis because previous studies had shown that neutralizing antibody responses and vaccine effectiveness to this variant were similar to lineage A (30, 31). Instead, we focused on major variants of concern that had previously been shown to be resistant to neutralizing antibody responses (B.1.351 and P.1). However, other variants, such as the recently described B.1.617.2 ( $\delta$  variant first recorded India), might respond differently. Therefore, it will be important to further validate the effectiveness of vaccine-elicited serum on a variety of emerging variants of concern. For the antibody binding analysis, we relied on the use of recombinant protein from a commercial source that did not have the P.1 variant available. To ensure data comparability, we did not attempt to source the P.1 variant from an alternative supplier or to produce it in-house. A further limitation is that we have not been able to address the links between neutralization and T cell or B cell responses. Our analyses focused on predominantly female HCWs rather than more vulnerable older populations, and our cohort received only one of several vaccines being rolled out globally. Last, the U.K. vaccination policy at the time of our study dictated an extended interval between prime and boost vaccination of between 10 and 12 weeks. Therefore, it will be important to assess whether this affected immune response in comparison to the widely adopted 3- to 4-week schedule. Related to this, a recent study has indicated that increased antibody responses are associated with the extended dosing interval in those aged over 80 years (32). Despite the caveats outlined above, our data provide insight into the impact of multiple antigenic exposures and its effect on increasing antibody potency and breadth, and we expect this to be recapitulated across different vaccines and across people with different vulnerabilities. Certainly, the extensive clinical trial data and ensuing data captured during vaccine rollout suggest that approved vaccines have similar performance and that the response in different groups of recipients is also similar (4, 11, 33, 34).

Previous studies have shown that a single-dose vaccine can improve antibody titers and neutralizing potency in previously infected individuals (35–39). However, this study further explores the effects of prior infection on cross-reactive immunity induced by a complete, two-dose COVID-19 vaccine schedule with administration of the first and second doses separated by 10 weeks. Our results, therefore, capture the effect of a booster dose on neutralization after antibody titers have started to wane [around 30% assuming that they peak at 2 weeks and have a half-life of 108 days (40)]. Our data provide compelling evidence that neutralizing potency and breadth is further increased after a vaccine boost, and this effect is even greater if the vaccine recipient has had prior infection with SARS-CoV-2. This indicates that repeated antigenic exposure improves immunity, and in our cohort, there was no evidence for an apparent antibody ceiling, as has been reported previously for influenza A (15). This supports the notion that vaccine effectiveness, even against emerging neutralization-resistant variants, may be improved after an additional vaccine boost even if that vaccine boost contained the original lineage A spike protein.

## MATERIALS AND METHODS

### Study design

The PANTHER study is a longitudinal cohort study launched in April 2020 to follow up seropositivity to SARS-CoV-2 among HCWs in Nottingham for 10 weeks. Further serological follow-up

was performed in autumn 2020. The cohort is described in detail in the online published protocol (17) and in (16).

In January 2021, a subset of 45 participants from the above cohort who had already had the first dose of the BNT162b2 vaccine was enrolled and asked to give blood using a blood microsampling device ahead of their booster dose. They were then invited to attend a phlebotomy appointment between 14 and 21 days after their booster dose when a venous sample with serum collection was performed. Samples were collected and stored under a Human Tissue Authority license in the Nottingham Tissue Bank (license number 11035). The study protocol was approved by North West–Greater Manchester South Research Ethics Committee (reference 20/NW/0395). The objective of the present study was to assess differences in serological responses after vaccination among previously infected and infection-naïve individuals.

Participants who had taken part in the previous serological surveillance study were invited to take part in the post-vaccination study, and the first 20 previously infected with SARS-CoV-2 (as shown from their seropositivity status during 2020) and 25 individuals without prior infection were included in the present study. No randomization was performed. Samples were blinded for experiments and unblinded at the point of data assessment. All assays were performed in duplicate (serology) or triplicate (neutralization).

The sample size of  $n = 45$  pre-boost versus post-boost vaccination was powered (80% power with  $P < 0.05$ ) to detect differences of 0.43 SD in antibody or neutralization titers between the first and second vaccination doses. For the comparison between infection-naïve and previously infected individuals, the study was powered to detect (80% power  $P < 0.05$ ) differences of 0.87 SDs between groups, which was considered sufficient given that differences of over one order of magnitude in anti-RBD titers after the first vaccination dose (at least 2 SDs) between infection-naïve and preinfected individuals have been reported in a similar setting (41).

### Patient samples

We have leveraged the PANTHER study (ISCTRN: 15677965 ethics approved 27/10/2020 North West–Greater Manchester South Research Ethics Committee CPMS 47035, IRAS 285436), a longitudinal prospective observational study of HCWs in the East Midlands who underwent weekly quantitative serology testing from 20 April to 10 July 2020. This testing included quantitative measurements of IgG, IgM, and IgA, neutralization assays, and symptomatology (17). A predefined nested substudy to assess the effect of booster dose on antibody reactivity or neutralization to lineage A and two variants (B.1 and B.1.351) was carried out using samples isolated from 45 HCWs [36 women and 9 men, aged 47.5 years (SD = 11.4)] from the PANTHER cohort who had paired pre- and post-booster dose serology, 20 of whom had confirmed SARS-CoV-2 infection before the start of vaccination. Of those with previous SARS-CoV-2 exposure, 8 were diagnosed by PCR, whereas the rest were identified by serological testing in either the second (12 individuals), third (4 individuals), or final quarters (4 individuals) of 2020 (table S1). All participants had two doses of the BNT162b2 vaccine between December 2020 and February 2021 and a booster dose between late February and mid-March 2021. The booster immunization was administered between 10 and 12 weeks after the first vaccine, in accordance with current U.K. vaccine policy (which differs from the schedule of 3 to 4 weeks adopted in other countries). Baseline serum samples were taken and analyzed before the vaccine program

commenced. Then, further samples were taken and analyzed on average 68 days after the first vaccine dose (and 2 days before the boost) and 18 to 19 days after the boost for both groups of volunteers (table S1).

### Spike-specific enzyme immunoassay

His-tagged, human embryonic kidney (HEK) 293-expressed spike S1 variants lineage A (Wuhan), B.1 variant, lineage A spike S2 domain, and baculovirus-expressed nucleocapsid were produced by SinoBiologicals (Strattech Scientific UK). His-tagged, HEK293-expressed recombinant B.1.351 variant-stabilized S1 was obtained from The Native Antigen Company. Enzyme immunoassays were carried out as described previously (42). In brief, recombinant proteins were immobilized onto microtiter plate wells and used to capture spike-specific antibodies present in indicated dilutions of human serum samples. Captured IgG was detected using a human IgG-specific horseradish peroxidase-conjugated antibody followed by the addition of ultra-3,3',5,5'-tetramethylbenzidine substrate (Thermo Fisher Scientific). Absorbance at 450 nm was read using a GlowMax Discover spectrophotometer (Promega). Data were presented as the ratio of the absorbance (OD<sub>450</sub>) for the sample versus the mean absorbance of three separate pools ( $n = 21$ ) of negative control serum samples.

### Pseudovirus neutralization assay

Pseudovirus neutralization assays were performed as previously described (43). Briefly,  $1.5 \times 10^6$  HEK293T cells were seeded overnight in 8 ml of complete DMEM [Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% nonessential amino acids] in a 10-cm-diameter Primaria-coated dish (Corning). Transfections were performed with 2  $\mu$ g of murine leukemia virus Gag-Pol packaging vector (phCMV-5349), 2  $\mu$ g of luciferase-encoding reporter plasmid (pTG126), and 2  $\mu$ g of a plasmid encoding SARS-CoV-2 spike (lineage A, B.1.351, or P.1) with 24  $\mu$ l of polyethylenimine (Polysciences) in Opti-MEM (Gibco). A no-envelope control (empty pseudotype) was used as a negative control in all experiments. Supernatants containing pseudotypes were harvested at 72 hours after transfection and filtered through 0.45- $\mu$ m membranes. For neutralization assays,  $2 \times 10^4$  VeroE6 cells per well were plated in 100  $\mu$ l of complete DMEM in white 96-well tissue culture plates (Corning) and incubated overnight at 37°C. The following day, supernatants containing SARS-CoV-2 pseudoviruses were incubated for 1 hour at room temperature with heat-inactivated serum samples at a 1:200 dilution or serially diluted before adding the samples to the VeroE6 cells. VeroE6 cells were incubated with inocula for 4 hours, which was then discarded and replaced with 200  $\mu$ l of fresh complete DMEM. After 72 hours, the medium was discarded, cells were lysed with cell lysis buffer (Promega), and relative luciferase activity was measured using a FLUOstar Omega plate reader (BMG Labtech). Neutralizing activities were reported as reciprocal serum dilution corresponding to ID<sub>50</sub> values and were calculated by nonlinear regression (GraphPad Prism version 9.1.2) using lower and upper bounds (0 and 100% inhibition) as constraints to assist curve fitting. Each sample was tested in triplicate.

### Statistical analysis

Data are presented as scatter plots with mean and SD, unless otherwise stated in the figure legends. Data were tested for normality and lognormality with D'Agostino and Pearson tests. Serology data

were analyzed using Kruskal-Wallis with Dunn's multiple comparisons test. ID<sub>50</sub> data were log-transformed and analyzed with either one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test or two-way ANOVA with Tukey's multiple comparisons test. Proportion neutralization data were analyzed using one-way ANOVA with Tukey's multiple comparisons test. EC<sub>50</sub> data were analyzed using Mann-Whitney two-tailed test. Data analysis was blinded. Correlation between antibody concentrations and neutralizing ID<sub>50</sub> values was assessed using a Pearson correlation coefficient. Each experiment was performed once in either duplicate (serology) or triplicate (pseudovirus neutralization). Sample sizes were  $n = 25$  for uninfected and  $n = 20$  for infected, unless otherwise stated in the figure legends. Differences in means were considered statistically significant at  $P < 0.05$ . Significance levels are indicated as \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ; ns, nonsignificant. Analyses were performed using GraphPad Prism 9.1.0 software.

### SUPPLEMENTARY MATERIALS

[www.science.org/doi/10.1126/scitranslmed.abj0847](http://www.science.org/doi/10.1126/scitranslmed.abj0847)

Figs. S1 to S5

Tables S1 and S2

Data file S1

[View/request a protocol for this paper from Bio-protocol.](#)

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