Original Research

Highlight article

Absence of antibody responses to SARS-CoV-2 N protein in COVID-19 vaccine breakthrough cases

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Impact statement

Although it is well established that the presence of antibodies to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) N protein indicates the previous infection, we found that vaccination prevented robust antibody responses to viral N protein. Therefore, our finding suggests that the utilization of antibodies to N protein to determine the infection rate of populations likely misses a substantial number of infection cases among fully vaccinated individuals. In this context, the finding urges us to adapt current coronavirus disease 2019 (COVID-19) serology surveillance strategies and develop more accurate methods to assess prior infectivity and viral circulation.

Abstract

Understanding the risk factors for breakthrough coronavirus disease 2019 (COVID-19) (BC19) is critical to inform policy. Herein, we assessed Delta (Lineage B.1.617.2) variant-specific effectiveness of the BNT162b2 (Pfizer) vaccine and characterized Delta-driven BC19 cases (fully vaccinated individuals who get infected) with known-time-since-vaccination. In this longitudinal prospective study (January 21–October 30, 2021), 90 naïve and 15 convalescent individuals were enrolled at the initiation of vaccination. Samples from 27 unvaccinated individuals with previous laboratory-confirmed COVID-19 diagnosis were collected at a single time point. Longitudinal serology profile (antibodies against severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2] S and N proteins) and live-virus-based neutralization capacities were assessed while controlling for age. Sex, age, history of reactions to the COVID-19 vaccine, and viral neutralization capacities were identified as significant risk factors for breakthrough COVID-19. At 8 months postvaccination, male sex, individuals $\geqslant$65 years of age, and individuals who experienced noticeable side effects with the COVID-19 vaccine were at 5.47 (p-value = 0.0102), 4.33 (p-value = 0.0236), and 4.95 (p-value = 0.0159) fold greater risk of BC19 as compared to their peers, respectively. Importantly, every five-fold increase in viral neutralization capacities (by live-virus-based assays) was significantly associated with ~4-fold reduction in the risk occurrence of breakthrough COVID-19 (p-value = 0.045). Vaccine boosting remarkably increased these viral neutralization capacities by 16.22-fold (p- value = 0.0005), supporting the importance of the BNT162b2 booster in efforts to control the incursion of future variants into the population at large. Strikingly, BC19 cases exhibited a delayed/absent antibody response to the N protein, suggesting limited exposure to the virus. Since antibodies against N protein are widely used to evaluate the extent of virus spread in communities, our finding has important implications on the utility of existing serological diagnostic and surveillance for COVID-19.

Keywords: COVID-19 breakthrough, booster, SARS-CoV-2, and N protein

Introduction

The fourth wave of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic was driven by the Delta (Lineage B.1.617.2) variant in many countries, including the United States[1,2] where just over 54% of the population is fully vaccinated.[3] The Delta variant was first identified in Louisiana in late June 2021 and, by August 2021 accounted for most cases in the state and across the Southern region of the United States.[1,2,4] While unvaccinated individuals comprised the vast majority of hospitalizations and deaths, it quickly became evident that some minor proportion of vaccinated individuals could contract emerging variants and could develop mild COVID-19.[5,6] The neutralization

capacities of sera from fully vaccinated individuals and convalescent individuals against the Delta variant were shown to be significantly reduced as compared to other variants.[7] However, the association between the reduced neutralizing activity and the role in the development of breakthrough COVID-19 is unclear. Also, whether the reduction in the ability of the vaccine to block infection is a result of time-since-vaccination or age has been partly confounded as older age groups received the vaccine first. Importantly, most neutralization assays have been performed using proxy systems, such as pseudo-neutralization assays.[8–11] While we have benefited from these studies, pseudo-virus-based entry inhibition assays have been shown to be discrepant from those obtained from live-virus-based assays and may not be a true reflection of the ability to prevent infection.[12,13] Herein, we investigate the ability of sera from vaccinated individuals with known-times-since-vaccination across all age groups through 8 months postvaccination and from unvaccinated individuals with previous laboratory-confirmed COVID-19 diagnosis(8–16 months postinfection) to protect against the infection from the basal B.1 strain (i.e. the original lineage of SARS-CoV-2) and the Delta variant. In addition, in our cohort, there were 16 cases of mild-to-moderate breakthrough COVID-19 attributed to the Delta variant (BC19 cases), allowing us to interrogate factors associated with breakthrough disease risk.

Materials and methods

Study approval

Our prospective study was conducted at Woman's Hospital and Louisiana State University (LSU), Baton Rouge, LA, USA. The study protocol was reviewed and approved by Woman's Hospital Foundation Institutional Review Board protocol# RP-20-029, approved on 22 January 2021 with reliance from Louisiana State University's Institutional Review Board and was conducted in accordance with relevant guidelines and institutional policies. The authors confirm that informed consent was obtained from all enrolled participants. In addition, the authors vouch for the accuracy of the data reported. All study sites worked under their approved biosafety protocols for handling SARS-CoV-2 specimens.

Study participants

There were 105 vaccinated participants. Of these, 90 had no evidence of previous infection: including negative either for antibodies to N protein or viral RNA and no COVID-19 symptoms (self-reported) at the time of enrollment. Fifteen individuals had laboratory-confirmed SARS-CoV-2 infection and/or COVID-19 symptoms before vaccination. Saliva, blood samples, and self-reported clinical symptoms were recorded and collected from all participants receiving the Pfizer BNT162b2 vaccine at specific time points at Woman's Hospital. Our studies had four time points (T1, T2, T3, and T4). T1 was 2 weeks after the first dose of the Pfizer vaccine; T2 was 2 weeks after the second dose of the Pfizer vaccine; T3 and T4 were 5 and 8 months postvaccination, respectively (Figure 1). The SARS-CoV-2 infection was laboratory-confirmed by quantitative reverse transcription

polymerase chain reaction (qRT-PCR) using Food and Drug Administration (FDA)-approved Luminex SARS-CoV-2 panel 2 and/or enzyme-linked immunosorbent assay (ELISA) for total antibodies against SARS-CoV-2 N protein. The titers of antibodies to S protein were evaluated at T1, T2, and T3, while inhibition capacities (blocking RBD–ACE2 interaction) were assessed at T1 and T2. Live-virus-based microneutralization assay (described below) were performed using serum collected at T3 and T4. In addition, we acquired longitudinal samples from one COVID-19 patient (referred to as MI) infected with SARS-CoV-2 during the Delta surge. MI possessed no neutralizing antibody responses to the vaccine at the time of infection; therefore, this case was classified as Delta-infected control individual without preexisting neutralizing antibodies.

Additional blood samples were collected from participants who received a vaccine booster and developed breakthrough COVID-19. These samples were collected at 2–12 weeks postsymptom onset and 2–3 weeks postbooster for the virus-based microneutralization assay, respectively.

Finally, we acquired samples from 27 unvaccinated individuals with previous laboratory-confirmed COVID-19 diagnosis (convalescent). The sera were obtained 8–16 months postsymptom onset or after the first lab-confirmed SARS-CoV-2 infection.

SARS-CoV-2 propagation and plaque assay

Isolate hCoV-19/USA/PHC-658/2021 (Lineage B.1.617.2; Delta variant) SARS-CoV-2 from BEI Resources (Cat# NR-55611) was kindly provided by Dr Weishan Huang, hereafter referred to as Delta variant or B.1.617.2. Isolate hCoV-19/USA/LA-GBCL-26791723/2020 (basal B.1 lineage D641G, GSAID accession ID: EPI_ISL_485342), hereafter referred to as B.1-isolate, was isolated from the nasopharyngeal swab sample of a COVID-19 patient during March 2020.

The viruses were propagated using Vero E6 (VE6) cells line (ATCC#CCL-81) as previously described,[14] and titers were determined using plaque assays and recorded as plaque-forming unit (PFU)/mL also as previously described.[15]

Antibody and RBD–ACE2-binding inhibition assay

Sera, collected at different time points for all participants, were aliquoted and stored at –80°C to avoid repeated freeze-thaw cycles. Complement components and potential infectious viruses in serum samples were heated-deactivated at 56°C for 30 min immediately before use.

Titers of S protein antibodies and the presence of N protein antibodies were determined using Ortho Anti-SARS-CoV-2 total immunoglobulin (Ig) (Vitros, Cat# 6199922) and Platelia SARS-CoV-2 Total Ab (Biorad, Cat# 72710), respectively. In addition, the serum capacities of inhibiting RBD–ACE2 binding were determined using SARS-CoV-2 surrogate virus neutralization kits (Genscript, Cat# L00847).

Virus-based microneutralization assay

Neutralizing steps. The virus-based microneutralization assay using B.1.617.2 and B.1.D614G was modified from a previously published protocol.[14] Briefly, heat-inactivated
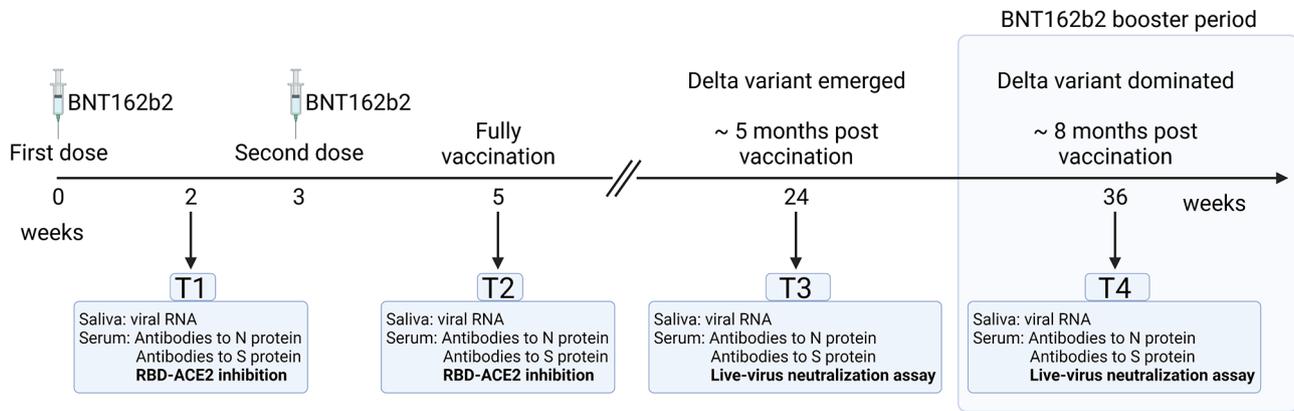
Figure 1. Study design and sampling time points. Participants included naïve and convalescent individuals given either two or three doses of Pfizer vaccine; a convalescent individual given the J&J vaccine; and unvaccinated convalescent individuals. Viral RNA, antibodies against S or N proteins, and inhibition capacities against the viral receptor-binding domain (RBD) of SARS-CoV-2 were evaluated at T1 (2 weeks post the first dose) and T2 (2 weeks post the second dose) in individual participants. At T3 (24 weeks post the first dose or ~5 months postvaccination) and T4 (36 weeks post the first dose or ~8 months postvaccination), viral RNA, antibodies against S or N proteins of SARS-CoV-2, and neutralizing antibodies were evaluated by live-virus-based microneutralization assay. Since the Delta variant emerged (around T3), we evaluated neutralization capacities against the basal B.1 virus (original SARS-CoV-2) and the Delta variant. The Delta variant became the dominant variant (≥ 99%) as most of our participants were reaching T4, thus, we mainly focused on the Delta variant at this time point. Participants gradually received BNT162b2 booster shots after T3 until the endpoint of our study.

serum samples were serially diluted with infection medium (minimum essential medium [MEM], 2% fetal bovine serum [FBS], 1% penicillin–streptomycin, 0.21% bovine serum albumin); mixed with diluted viruses at a 1:1 ratio, and allowed to incubate at room temperature (RT) for 1 h. The virus–serum mixture was then applied to VE6 monolayers a 96-well plate and incubated at 37°C in a humidified incubator with 5% CO_2 for 1 h. After 1 h, all virus–serum mixtures were removed from VE6 monolayers, and 100 μL of diluted serum sample and an equal volume of infection medium was added to each well. After 48 h of incubation, the medium was removed, and the cells were fixed with 10% formaldehyde. Neutralization activities were calculated as the half-maximal effective concentration (EC50).

Intracellular viral protein detection.   Fixed cells were washed with 200 μL phosphate-buffered saline (PBS) before being permeabilized with 100 μL of 0.1% PBS/Triton X-100/well (Sigma, Cat# 9036-19-5). After 15 min of incubation at RT, the cells were washed 1 time with 200 μL PBS, followed by blocking with 5% non-fat milk (AmericanBio, Cat# AB10109-01000) PBS in 1 h at RT. After the blocking, 100 μL of anti-SARS-CoV-2 N protein antibodies at 1:5000 dilution in 1% non-fat milk PBS (SinoBiological, Cat# 40143-R040), was added to each well and incubated for 1 h at RT. Unbound or non-specific-binding antibodies were removed by washing 3 times with 200 μL/well PBS. Next, 100 μL of secondary antibody anti-rabbit (IgG) horseradish peroxidase (HRP), at 1:1500 dilution in 1% non-fat milk PBS, was added to each well and incubated at RT for 1 h. After the incubation, cells were washed 3 times with 200 μL/well PBS, followed by adding 200 μL freshly prepared SigmaFast OPD (Sigma, Cat# P9187). The reaction was stopped by adding 50 μL/well 3N hydrochloric acid after 10 min of incubation. The presence of viral N protein was measured as optical density (OD) at 490 nm. The percent inhibition in each well is calculated using the following formula:

100 – ([OD value of each well – average OD of non-virus control wells] / [average OD of virus only control wells – average OD of non-virus control wells] × 100). Then, the neutralization capacities, presented as EC50, were determined using non-linear 4PL regression curves fix with top and bottom constraints at 100 and 0%.[14]

Viral RNA sequencing, mutation detection, and lineage classification

Viral RNA was extracted using MagMAX™ Viral/Pathogen II (Thermo Fisher, Cat# A48383). Viral RNA was detected by (qRT-PCR using FDA-approved Luminex SARS-CoV-2 panel 2 (IDT, Cat# 10006941). All the tests were performed in Clinical Laboratory Improvement Amendments (CLIA)-licensed diagnostic labs.

L452R and E484Q mutations were detected using end-point genotyping qRT-PCR with SARS-CoV-2 variant ValuPanel assay (Biosearch technologies, Cat# SCV-L452R and SCV-E484Q). The genotypes were determined using the fluorescent signal from each mutation-specific probe.

Viral RNA was also sequenced using methods based on the ARTIC network nCoV-2019 V3 primer scheme using two multiplexed primer pools to create overlapping 400 bp amplicon fragments in two PCRs. A detailed version of this protocol can be found here: https://andersen-lab.com/secrets/protocols/. Libraries were normalized and pooled in equimolar amounts at 2 nM. The 2 nM library pool was sequenced with the Illumina MiSeq using a MiSeq reagent kit V3 600 cycles (Illumina). Raw fastq files were processed on the FoxSeq® v.4.0 analytical pipeline, which automates the following steps: for each sample, raw reads were filtered using *Trimmomatic*[16] based on read length and mapped to the SARS-CoV-2 reference genome (NC045512.2/Wuhan-Hu-1) using Bowtie2.[17] Mapping quality statistics were generated using *picard* (http://broadinstitute.github.io/picard). Bases were called for each position using *bcftools mpileup* (https://github.com/samtools/bcftools) and filtered out if the depth

for that site was < 50 and/or the frequency of either the reference or the alternative allele was < 80%. A consensus sequence was generated for each sample using _bcftools consensus_. Pango lineages were assigned using Pangolin.[18] Alignment and neighbor-joining trees were conducted using Clustal Omega.[19] Delta variant sequences extracted from subjects P54, P94, and MI can be accessed from the GISAID database (accession#: EPI_ISL_4435367, EPI_ISL_4435360 and EPI_ISL_44353631, respectively).

Isolate and titrate replication-competent viruses from human specimens

Specimens (saliva from MI and nasopharyngeal swabs from P54 and P94) were diluted at 1:1 ratio with serum-free Dulbecco's Modified Eagle Medium (DMEM) before being filtered through 0.2 μm – pore microvolume filter units (MilliporeSigma, Cat# WHA67841302), the filtrates were then used to infect an 80% confluent monolayer of VE6 (ATCC, ATCC# CCl-81) using the absorption method as previously described. Viruses were harvested after 5 days of incubation at 37°C and 5% CO_2 The resulting supernatant was aliquoted and stored at 80°C until used. The titers were determined using Median Tissue Culture Infectious Dose assay (TCID50) and recorded as TCID50/mL as previously described.[20]

Statistical analyses

Data were analyzed with R 4.1.1, Prism 9.2 (GraphPad), and SPSS IBM version 25. Comparisons were performed using two-tailed Mann–Whitney _U_ (two-sided) tests, Kruskal–Wallis (two-sided) tests for continuous variables, and Fisher's exact test for qualitative and categorical variables. Normal distribution was examined using the Shapiro–Wilk test. The Spearman correlation coefficient presented the correlation between neutralization capacities against B.1.D164G and Delta variant (B.1.617.2). The two-stage step-up method of Benjamini, Krieger, and Yekutieli false discovery rate (FDR) was used to correct multiple comparisons[21] when appropriate. Rank analysis of covariance (ANCOVA) was used to evaluate neutralization capacities across study groups while controlling for the effects of continuous covariates, such as age. The rank ANCOVA results were presented at _F_-distribution with _p_-value. Rank ANCOVA was followed by Dunnett's (two-sided) post hoc tests to correct multiple comparisons. _p_-values ≤ 0.05 were considered significant.[22] Multivariable logistic regression was used to assess potential risk factors for the occurrence of breakthrough COVID-19.[23]

Results

Characterization of study cohort

From January 2021 to October 2021, we evaluated 133 participants in our study, including 105 fully vaccinated (received at least two doses of Pfizer BNT162b2 vaccine) and 27 unvaccinated individuals with previous laboratory-confirmed COVID-19 diagnosis and one Delta-COVID-19 patient (MI). At the time of vaccination, 90 out of 105 participants had no prior evidence of SARS-CoV-2 infection, while 15 had been laboratory diagnosed SARS-CoV-2 infection prior to

vaccination (Table 1). Age was not uniformly distributed across the three groups (Table 1). However, there was no difference between the three groups regarding gender, body mass index (BMI), or race (Table 1).

Following enrollment, specimens from vaccinated participants were tested for viral RNA and antibodies to viral proteins (S and N proteins) at four different time points: T1–T4 (Figure 1). N protein reactivity was used as evidence of prior SARS-CoV-2 exposure. Upon testing the naïve group for N protein reactivity, 7 out of 90 (7.77%) participants had evidence of SARS-CoV-2 exposure at T1, while another three (3.33%) individuals tested positive at T2, suggesting exposure prior to complete vaccination. All 10 individuals reported no symptoms. At T3 and T4, five asymptomatic individuals (5.55%) tested positive for N protein reactivity, hereafter referred to as breakthrough infection, and 16 (17.77%) reported mild-to-moderate COVID-19, hereafter referred to as breakthrough symptomatic COVID-19 cases (BC19) (Figure 2).

Thus, we stratified our cohort into four main groups for the downstream analysis (Figure 2): (1) vaccinated individuals with no history/evidence of natural infection before and during the study period: Vac_naive group (red throughout figures, _n_ = 59); (2) individuals with no history of infection at the time of vaccination, but who were found to be infected during the study period: Vac_infect group (light blue throughout figures, _n_ = 31); (3) vaccinated individuals with a history of natural infection prior to the initiation of vaccination (convalescent): Con_vac group (orange throughout figures, _n_ = 15); and (4) unvaccinated individuals with previous laboratory-confirmed COVID-19 diagnosis (convalescent, enrolled 8–16 months postinfection): Con_unvac group (green throughout figures, _n_ = 27) (Figure 2). Of the 105 vaccinated participants, 13 (12.38%) individuals received BNT162b2 booster vaccinations after T3. Of note, not all participants provided specimens at all time points. Therefore, the number of available data points for each analysis at each time point is annotated in all figures.

High and early efficacy of Pfizer BNT162b2 vaccine to produce neutralizing antibodies inhibiting RBD–ACE2 interaction

Vaccinated participants were tested for successful induction of immune responses to SARS-CoV-2 through a combination of methods, including the production of antibodies against S protein at all time points and RBD–ACE2 inhibition at T1 and T2.[24,25] Since the RBD–ACE2 inhibition assay is unable to differentiate responses of neutralizing antibodies against different variants, sera collected at T3 and T4 were used in a live-virus microneutralization assay.

As expected, we found significantly enhanced RBD–ACE2 inhibition capacity from T1 to T2 in all but Con_vac group (Figure 3). Of note, the RBD–ACE2 inhibition capacities of Con_vac individuals at T1 were already near maximum (median = 94.81%, 95% CI: 95.7–91.49). The data indicate early and high efficiency of antibody responses in convalescent, vaccinated individuals. Also, we observed a progressive increase of S protein antibodies between T1 and T3 in all groups (Supplemental Figure 3S). Taken together, these

Table 1. Characteristics of study participants.

	No. of participants, *n* (%)		
	Pfizer vaccinated		Non-vaccinated convalescent
	Naïve	Convalescent	
	n = 90	*n* = 15	*n* = 27
Gender			
Female	75 (83.33)	14 (93.33)	23 (85.19)
Male	15 (16.67)	1 (6.67)	4 (14.81)
Missing data	0 (0)	0 (0)	0 (0)
Age (median, range)	50.5 (20–74)	47 (32–68)	33 (13-66)
<35	10 (11.11)	1 (6.67)	16 (59.25)
35–59	55 (61.11)	10 (66.66)	10 (37.03)
≥60	25 (27.78)	3 (20)	1 (3.7)
Missing data	0 (0)	1 (6.67)	N/A
BMI (median, range)	25.3 (14.8–42.7)*	26.7 (20.6–40.4)	
Normal (18–25)	41 (50)	2 (13.33)	N/A
Overweight (25.1–30)	24 (29.27)	5 (33.34)	N/A
Obesity (≥30)	17 (20.73)	6 (40)	N/A
Missing data	3 (1.17)	2 (13.33)	N/A
Race			
Caucasian	74 (82.22)	11 (73.33)	26 (96.3)
Africa American	13 (14.45)	4 (26.67)	1 (3.7)
Asian	3 (3.33)	0 (0)	0 (0)
Missing data	0 (0)	0 (0)	0 (0)

BMI: body mass index.
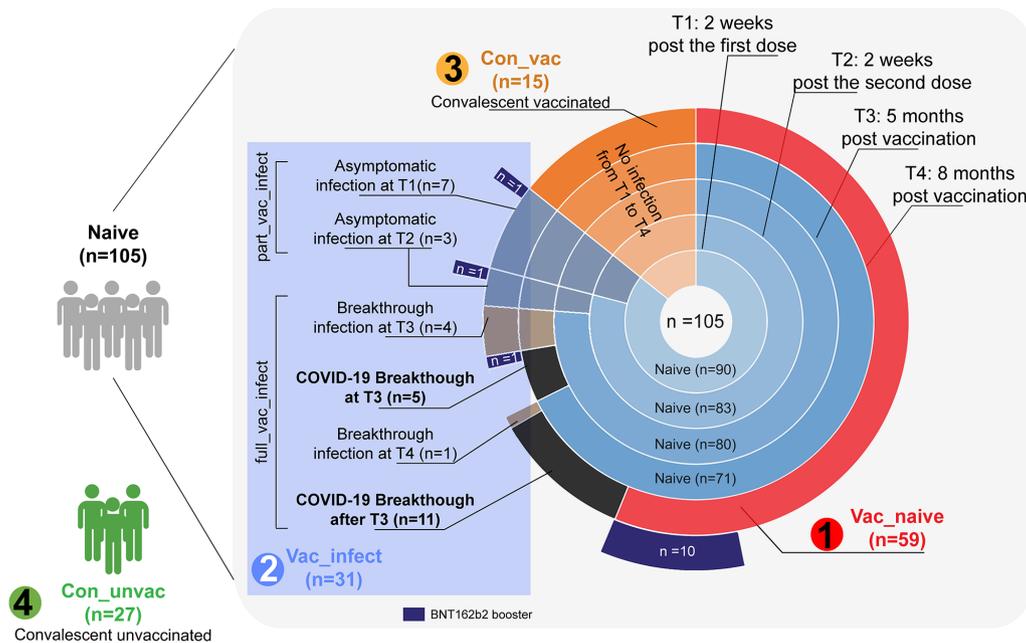*One participant with BMI < 18.

Figure 2. Study groups. Study participants were stratified into groups according to their vaccination and infection status. After T2 individuals are considered fully vaccinated and asymptomatic infection detected at T3 or T4 is thus referred to as "Breakthrough infection" (*n* = 5; 4 at T3 and *n* = 1 at T4). Individuals, who were infected after T2, and developed COVID-19, were considered as "COVID-19 Breakthrough" (black throughout figures, *n* = 16; 5 at T3 and *n* = 11 after T3). Participants who received a single BNT162b2 booster shot are shown in dark blue.
Participants were stratified into four main groups:
1. Individuals vaccinated with no history/evidence of natural exposure before and during the study period: Vac_naive group (red throughout figures, *n* = 59).
2. individuals with no history of infection at the time of vaccination, but who were found to be either infected or had evidence of infection (antibodies to viral N protein) at any time throughout the study (i.e. T1, T2, T3, or T4): Vac_infect group (light blue in subsequent figures, *n* = 31). This group includes partially vaccinated and infected individuals (asymptomatic infection at T1 and T2 – referred to as part_vac_infect), and fully vaccinated and infected individuals (breakthrough infection and COVID-19 breakthrough at T3 and T4 – referred to as full_vac_infect).
3. Vaccinated individuals with a history of natural infection prior to the initiation of vaccination: Con_vac group (orange throughout figures, *n* = 15).
4. Unvaccinated individuals with previous laboratory-confirmed COVID-19 diagnosis (enrolled 8–16 months postinfection): Con_unvac group (green throughout figures, *n* = 27).
Not all participants provided specimens at all time points. Therefore, the number of available data points for each analysis at each time point is annotated in all figures.
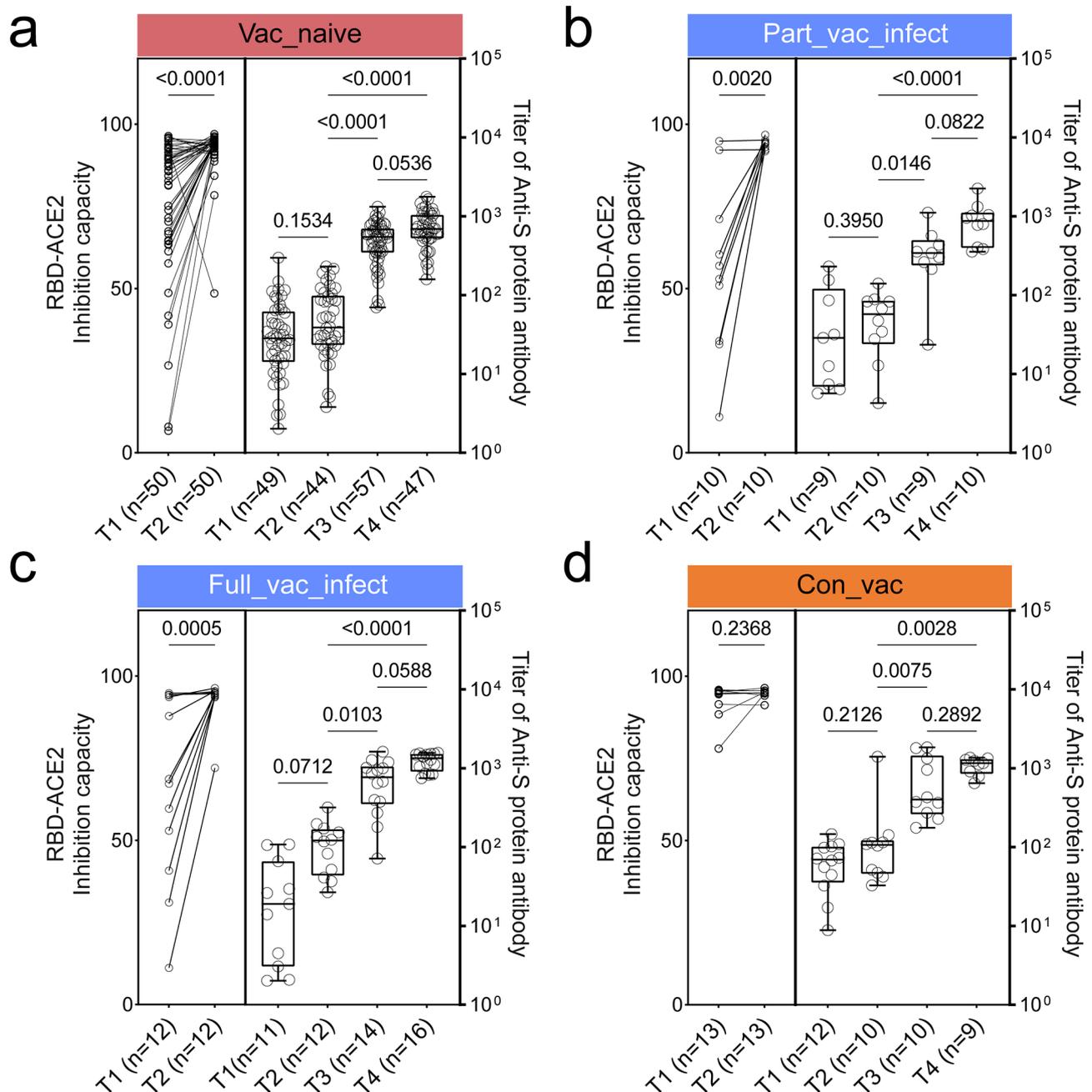
Figure 3. Vaccinated individuals exhibited robust antibody responses after two doses of Pfizer vaccine. The capacity to inhibit RBD–ACE2 interaction and the titers of antibodies to S protein in (a) Vac_naive, (b) partially vaccinated and infected individuals (part_vac_infect), (c) fully vaccinated and infected (full_vac_infect), and (d) vaccinated with history of infection at vaccination (Con_vac) groups were assayed. Lines connect longitudinal samples from the same individual over the sampling times as indicated. Significance was determined using two-sided Wilcoxon matched-pairs signed-rank for comparison of the RBD–ACE2 inhibition capacities between two groups. Two-sided Kruskal–Wallis tests followed by a two-stage-step-up Benjamini, Krieger, and Yekutieli false discovery rate method were used to compare the titer of anti-S protein antibodies between three groups. *p*-values ≤ 0.05 were considered statistically significant.

data confirm that the BNT162b2 vaccine induces long-term (~5 months postvaccination) and efficient antibody responses against S protein that inhibit RBD–ACE2 interaction.

Vaccinated individuals ≥ 60 years of age exhibited a significant reduction in neutralizing capacity against SARS-CoV-2 variants as compared to their younger peers at 5 months postvaccination

Due to the resurgence of COVID-19 during our study primarily driven by the Delta variant,[1,2,4] it became increasingly

important to determine if neutralizing antibodies generated in response to vaccination and/or natural infection/exposure were protective against the Delta variant. The neutralization capacities obtained from pseudovirus-based assays have been shown to be discrepant from those obtained from live-virus-based assays.[12,13] To avoid a potential overestimate of neutralization capacity from pseudovirus-based assays and to determine a true qualification of the ability of vaccine-induced antibodies to prevent infection, we employed a live-virus-based microneutralization against the basal B.1 lineage isolate (a previously dominant lineage in our study region,
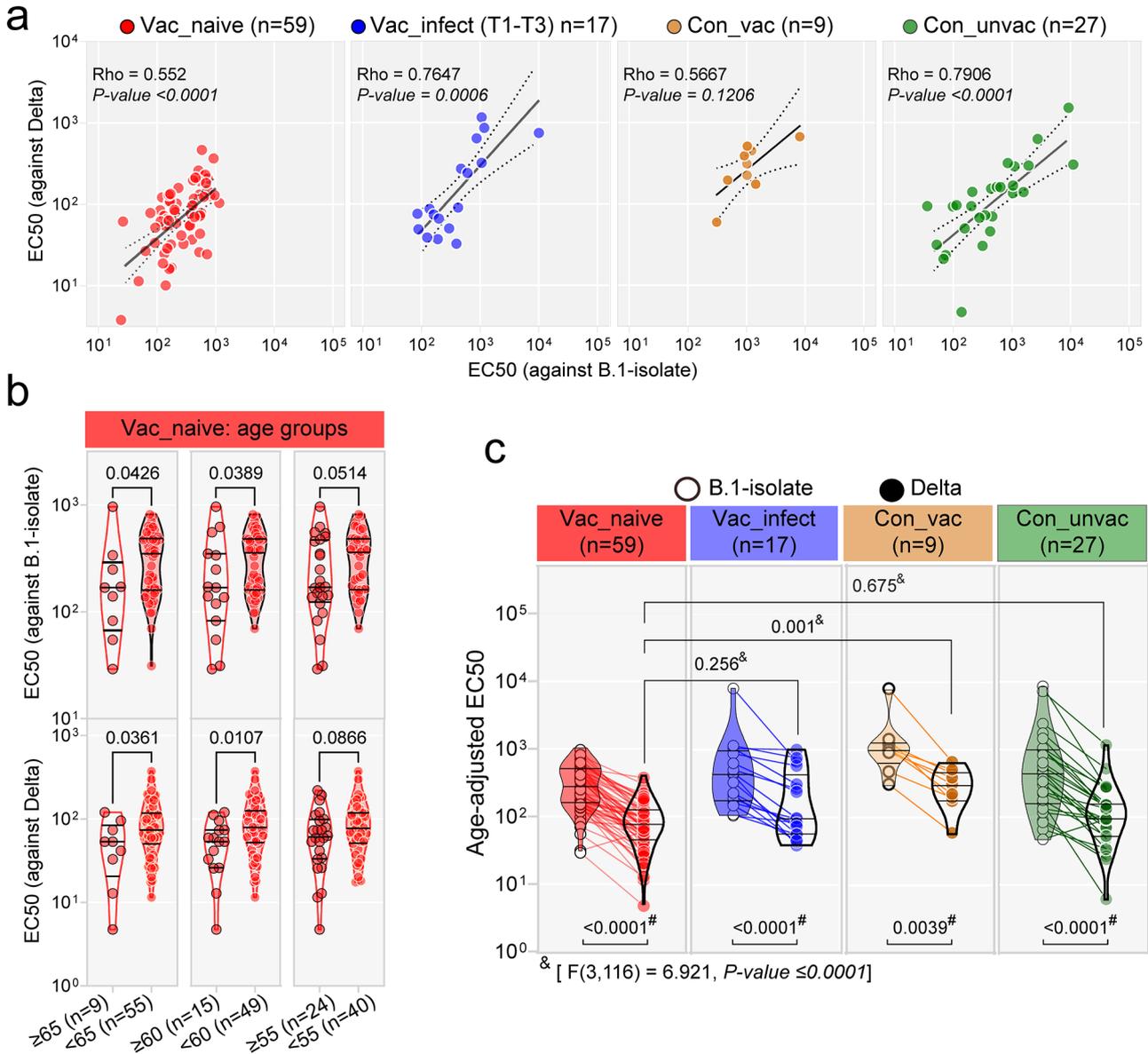
Figure 4. Reduced neutralizing capacity against SARS-CoV-2 Delta variant at 5 months postvaccination and in unvaccinated convalescent individuals. (a) Correlation between neutralization capacities (presented as EC50: half-maximal effective concentration) against B.1-isolate and Delta variant as evidenced by the Spearman correlation coefficient. (b) Neutralization capacities against B.1-isolate (top panels) and Delta variant (bottom panels) in older Vac_naive individuals as compared to younger Vac_naive individuals. Significance was determined using two-sided Mann–Whitney *U* tests. (c) Age-adjusted neutralization capacities against Delta (filled circles) compared to those against B.1-isolate (open circles). #: Significance was determined using the two-sided Kruskal–Wallis test followed by a two-stage-step-up Benjamini, Krieger, and Yekutieli false discovery rate method for multiple comparison correction. &: The rank ANCOVA was used to evaluate neutralization capacities across study groups, while controlling for the confounding effect of age. The rank ANCOVA results were presented at *F*-distribution with *p*-value. The rank ANCOVA was followed by two-sided Dunnett's post hoc tests to correct multiple comparisons.

which we clinically isolated during the first wave of COVID-19 in the United States – March 2020)[26] and a clinical Delta isolate (B.1.617.2) at T3 and T4 for all participants.

We observed positive correlations between the neutralization capacities (as measured by half-maximal effective concentration – EC50) toward B.1-isolate and those against the Delta variant in both vaccinated (regardless of natural infection status) and unvaccinated convalescent individuals (Con_unvac) (Figure 4(a)), indicating the strong cross-reaction of antibodies to the two variants. Furthermore, the strongest correlations were observed in infected individuals that were either vaccinated or non-vaccinated (Figure 4(a) second and last panel).

Of note, recent studies have shown that older participants exhibited lower antibodies following vaccination with BNT162b2.[27,28] Although the effect was modest, we similarly found that neutralization capacities against the B.1-isolate were significantly and negatively correlated to age in the Vac_naive group (S1a Supplemental Figure, top left panel). A more striking effect of age on neutralization capacity was observed for the Delta variant in Con_vac group (S1a Figure penultimate bottom right panel). To further determine thresholds of this effect, we age-categorized individuals based on three age partitions: $<$ or ≥ 65 years of age (yoa), $<$ or ≥ 60 yoa, and $<$ or ≥ 55 yoa. Indeed, within Vac_naive group, we found that older individuals ≥ 60 yoa exhibited

significantly lower neutralization capacities against both B.1-isolate and Delta variant compared to younger (< 60 yoa) participants (Figure 4(b)). Finally, we observed a similar, but not statistically significant trend, when evaluating those ⩾ 55 yoa (Figure 4(b)).

Vaccinated and convalescent individuals exhibited a significant reduction in neutralizing capacity against the SARS-CoV-2 Delta variant compared to the B.1-isolate at 5 months postvaccination

As recently reported,[8,9] we also found that vaccinated individuals, regardless of previous infection status, exhibited a significant reduction in neutralization capacity against the Delta variant as compared to those against the B.1-isolate at ~ 5 months postvaccination (Figure 4(c)). Sera neutralization capacities against Delta were 4.85 (95% CI: 3.85–5.86, *p*-value ⩽ 0.0001), 3.26 (95% CI: 1.92–4.60, *p*-value ⩽ 0.0001), 4.77 (95% CI: 2.17–7.37, *p*-value ⩽ 0.0039), and 6.41 (95% CI: 3.6–9.2, *p*-value ⩽ 0.0001) fold lower than against B.1-isolate for Vac_naive, Vac_infect, Con_vac and Con_unvac groups, respectively (Figure 3(c) and Supplemental Figure S1b). Our and others' data clearly demonstrate the age dependency of neutralization capacities resulting from vaccination with BNT162b2[27,28] (Figure 4(b)). Since the Vac_naive group was significantly older than convalescent group (Supplemental Figure S1c), to control for the confounding effect of age, a rank ANCOVA was used to evaluate the difference in neutralization capacities between study groups. Overall, Vac_naive group exhibited similar neutralization capacities against Delta variant as compared to Vac_infect and Con_vac groups. Intriguingly, sera from vaccinated individuals with a history of previous natural exposure/infection (Con_unvac) significantly outperformed Vac_naive group in neutralizing the Delta variant (Figure 4(c)) after adjusting for age (F [3,116] = 6.921, *p*-value ⩽ 0.0001). Thus, we reasoned that vaccination served as a "booster" in previously convalescent individuals, potentially enhancing protection against emerging variants.[29]

Breakthrough COVID-19 cases with Delta variant (BC19) among fully vaccinated male individuals ⩾ 65 yoa who exhibited low neutralization capacities from 5 to 8 months postvaccination

During the Delta surge (~5–8 months postvaccination),[2] 16 breakthrough symptomatic COVID-19 cases (BC19) were identified among 105 vaccinated participants. Infection with SARS-CoV-2 was confirmed by RT-PCR in CLIA-approved facilities. Breakthrough with the Delta variant was confirmed through a combination of RNA sequencing (Supplemental Figure S2a), genotyping assay (Supplemental Figure S2b), and epidemiologic data (see Supplementary for details). For comparison, an acutely ill individual (MI), who was found to be Delta positive via RNA sequencing, was used as a reference control for the disease cases (Supplemental Figure S2a, b). Also, all BC19 cases experienced mild-to-moderate upper respiratory tract symptoms for which hospitalization was not required indicating the high efficacy of the BNT162b2 vaccine against severe COVID-19. Interestingly, while all BC19 cases were

found in fully vaccinated individuals without history of previous SARS-CoV-2 infection (*n* = 90), none were identified in Con_vac individuals (*n* = 15) (Figure 2).

We next sought to evaluate potential risk factors for the occurrence of breakthrough COVID-19 cases among fully vaccinated individuals without history of previous SARS-CoV-2 infection. By comparing the neutralization capacities against Delta variant before infection of these 16 BC19 cases with those of the Vac_naive group, we found that BC19 cases exhibited significantly lower viral neutralization capacities as compared to Vac_naive group (prior to infection) (Figure 5(a)). Of note, neutralizing antibody responses were age-dependent (Figure 4(b)), and the risk of COVID-19 was multifactorial. This suggests that other factors, including age, confound the association between viral neutralization capacities and the occurrence of breakthrough COVID-19. Indeed, we found that male participants were at 5.74 times greater risk of developing breakthrough COVID-19 (odds ratio = 5.74, 95% CI [1.62–21.79], *p*-value = 0.0102) (Figure 5(b) and Supplemental Table S1). Likewise, older individuals (⩾ 65 yoa) were at 4.33-fold greater risk of developing BC19 (odds ratio = 4.33, 95% CI [1.16–13.99], *p*-value = 0.0236) (Figure 5(b) and Supplemental Table S1). Intriguingly, individuals with noticeable reactions to COVID-19 vaccine were 4.95 times more likely to experience breakthrough COVID-19 (odds ratio = 4.95, 95% CI [1.38–17.05], *p*-value = 0.0159). There was no significant association between underlying conditions/race/body mass index and the occurrence of breakthrough COVID-19 cases (Figure 5(b) and Supplemental Table S1). Our findings demonstrated history of side effects of COVID-19 vaccine (including sore arm, pain at infection site, chills, headache, nausea, fever, fatigue, and myalgia), gender, and age dependency in breakthrough COVID-19 cases, adding to the growing evidence of risk factors associated with breakthrough disease.[27,28,30–32] Thus, we further assessed the association between viral neutralization capacities and BC19 using multivariable logistic regression with history of reactions to the COVID-19 vaccine, age, and sex as covariants. We found observed that a five-fold increase in EC50 was associated with a 4.15 times lower risk of breakthrough COVID-19 (odds ratio = 0.241, 95% CI [0.06–0.969], *p*-value = 0.045) (Figure 5(b)*).

Humoral immune responses against the Delta variant significantly waned from 5 to 8 months postvaccination but are remarkably enhanced following the BNT162b2 booster

As the prevalence of Delta in the state of Louisiana increased from 81.3% (July 10, 2021) to 99% (14 August 2021),[2] we focused on neutralization capacities against the Delta variant at T4 (September 2021). We found that viral neutralization capacities against the Delta variant were reduced 4.31 fold (mean = 4.31, 95% CI [3.289–5.329], *p*-value < 0.0001) from 5 to 8 months postvaccination (Figure 6(a)). These data strongly support the need for a vaccine booster to enhance protection against Delta and other emerging variants (i.e. Omicron variant).

Indeed, a single booster dose of BNT162b2 after ~5 months postvaccination significantly enhanced viral neutralization
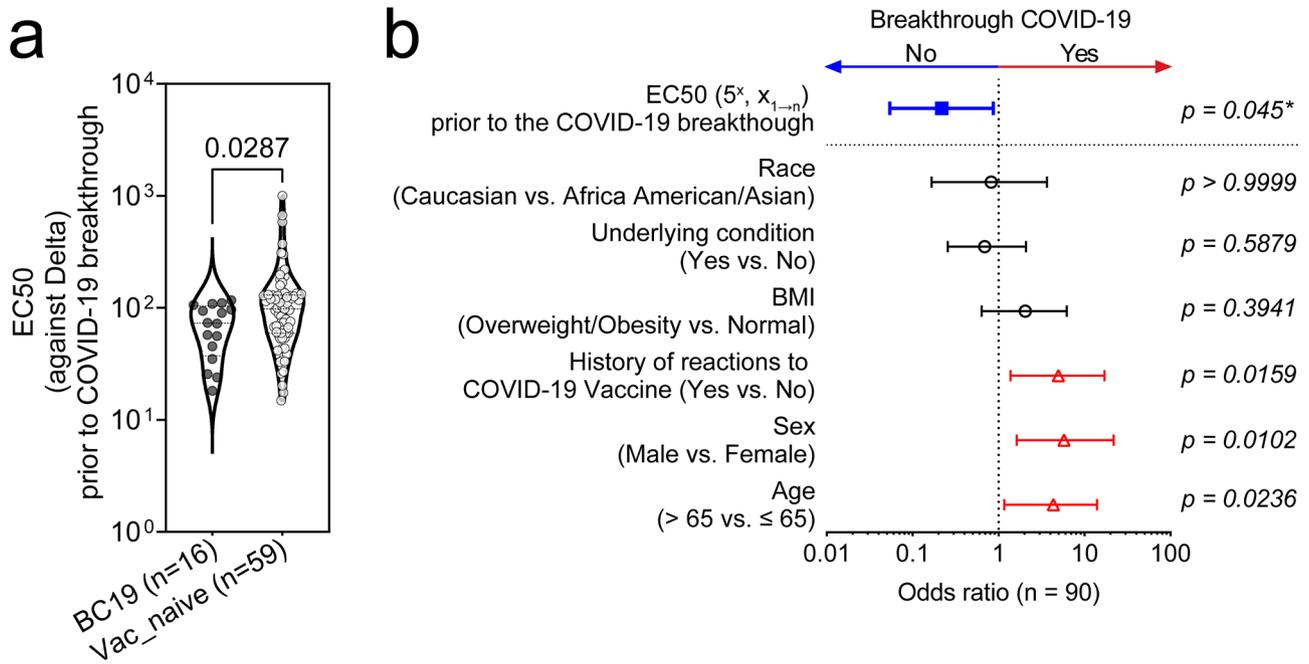
Figure 5. Significantly lower neutralization capacities prior to infection in breakthrough COVID-19 cases. (a) Neutralization capacities against Delta variant (EC50) prior to the occurrence of COVID-19 breakthrough between breakthrough COVID-19 cases and Vac_naive individuals. Significance was determined using the Mann–Whitney U test. (b) Plot of risk factors for the occurrence of breakthrough COVID-19 among, using two-sided Fisher's exact test; *The association of neutralization capacities against Delta variant (EC50) and the risk of the occurrence of breakthrough COVID-19 among vaccinated naïve individuals, including 16 BC19 cases and 74 non-COVID-19 vaccinated individuals: 59 cases of vac_naive group, 10 cases of part_vac_infect group, five breakthrough infection cases – 4 at T3 and 1 at T4 (referred Figure 2), was evaluated using multivariable logistic regression with History of reaction to COVID-19 vaccine, sex, and age ($\geq$65 versus $<$65) as covariants. The vertical line represents an odds ratio of 1. Odds ratio with 95% confidence interval.
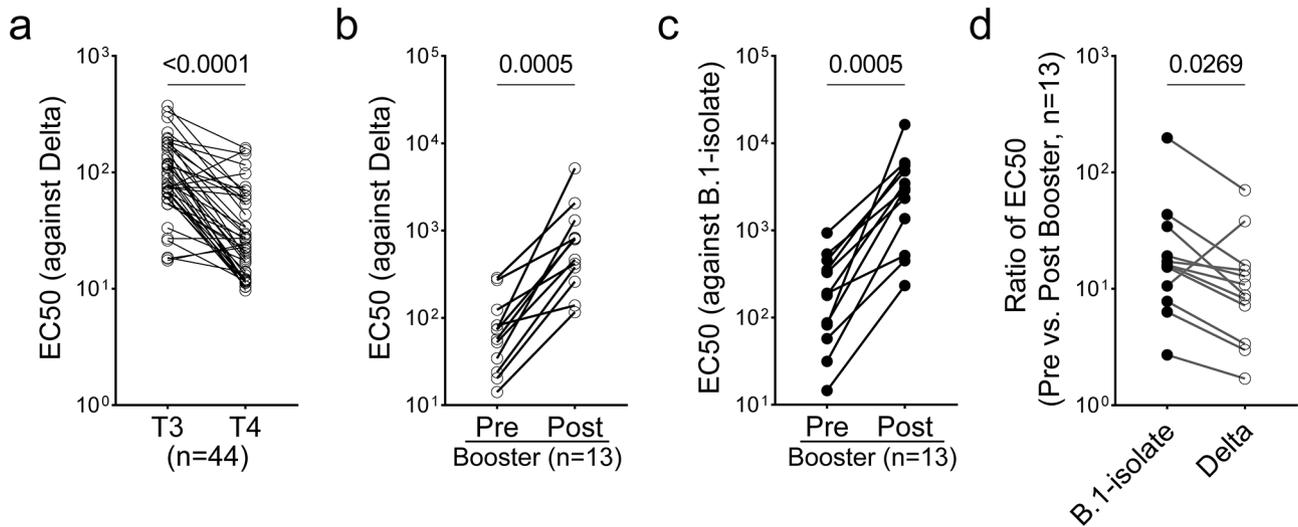

Figure 6. Enhancement of humoral immune responses against Delta after the BNT162b2 booster. (a) Neutralization capacities against Delta variant at T3 compared to those at T4. (b, c) Neutralization capacities pre-and post booster (2–4 weeks post booster) against Delta variant (b) and B.1-isolate (c). (d) Ratio of neutralization capacities pre- over post booster for Delta variant (black dots) and B.1-isolate (solid circles) at T4. Significance was determined using two-sided Wilcoxon matched-pairs signed-rank test.

capacities against the B1 isolate by 32.25-folds (95% CI [1.725–66.22], p-value = 0.0005) and the Delta variant by 16.22-folds (95% CI [3.815–28.63], p-value = 0.0005) (Figure 6(b) and (c)). The growing data demonstrate that BNT162b2 vaccine boosters reduced the risk of SARS-CoV-2 infection and severe COVID-19.[33–35] However, the durability of this protection remains unclear. Because the BNT162b2 booster targets the original form of SARS-CoV-2, we anticipated that the magnitude of protection pre- versus post booster would be different between the original lineage and other variants. Indeed, we found that the BNT162b2 booster enhanced neutralization capacities against the B.1-isolate at a significantly greater magnitude than those against the Delta variant (Figure 6(d)).

BC19 cases exhibited robust neutralizing antibody responses but absent or delayed and muted antibody responses to the viral N protein

BC19 cases exhibited robust neutralizing antibody responses against the Delta variant (Figure 7(a)). It was evident that the difference in viral neutralization capacities (i.e. B.1-isolate over Delta variant) prior to the infection was significantly reduced following breakthrough symptomatic COVID-19 (Figure 7(b)), as viral neutralization capacities against the Delta variant increased (Figure 7(a)). Taken together, these data indicate a robust and more specific neutralizing antibody response to Delta infection. Thus, we expected to observe similar responses to SARS-CoV-2 N protein – the most abundant and highly immunogenic viral protein.[36] It has been shown that antibody responses to SARS-CoV-2 appear 3–5 days post onset of symptoms (dpo) or 1 week postinfection.[37,38] Curiously, there were no detectable antibodies to viral N protein from 10 to 99 dpo in most BC19 cases (13/16 or 81.25%). Antibodies to SARS-CoV-2 N protein were observed in only three BC19 patients (P38, P54, and P94; and were observed much later in the infection: 18, 33, and 64 dpo, respectively (Figure 7(c), left panel).

In contrast, MI, the Delta-infected control, exhibited a typical antibody response with antibodies to N being detectable at 9 dpo. Interestingly, relatively high levels of antibodies to N protein were detected in individuals, who were infected prior to being fully vaccinated and in convalescent individuals (Figure 7(c), second to fourth panels from the left, respectively). The data demonstrated mute/delayed antibody responses to the most abundant viral protein – N protein in fully vaccinated individuals that become infected.

Discussion

Our study, for the first time, demonstrated the lack of antibody responses to SARS-CoV-2 N protein in COVID-19 vaccine breakthrough cases (BC19; i.e. fully vaccinated individuals who get infected) (Figure 7(c)). BC19 cases mounted a robust neutralizing antibody response to the live Delta virus (Figure 7(a) and (b)); however, we unexpectedly observed an absent or delayed and muted antibody response to the viral N protein (Figure 7(c)). While the reason for this is unsubstantiated, we propose that the vaccine limited viral replication and dissemination (as expected) and that this reduced the expression of viral N proteins and subsequent presentation of N antigens and anti-N antibody production. This is supported by our data and others,[39,40] which indicate reduced viral replication in breakthrough symptomatic COVID-19 (Figure S3) and other work which demonstrates that high levels of neutralizing antibodies effectively prevent viral infection and dissemination.[41–43] The repercussion(s) of the lack of anti-N antibodies is unclear, but has recently been suggested to be important for IL6 production and possibly cytokine storm induction.[44] Investigating these possibilities was beyond the scope of our current study but warrants further investigation. Since antibodies against N protein are often used to confirm past infection, our current finding has important implications on the utility of existing serological diagnostic and surveillance for SARS-CoV-2 prevalence in breakthrough infections.[45–47]

Therefore, we strongly encourage further investigation to elucidate the mechanism.

It has been shown that neutralization capacities against SARS-CoV-2 using pseudovirus-based assays are more likely overestimated.[12,13] Therefore, in contrast to previous studies[8,9,48,49] which used pseudovirus-based, we employed a live-virus-based microneutralization assay to more accurately assess viral neutralization capacities against SARS-CoV-2 variants.

Like previous studies,[7–13,50] our study demonstrated that the Pfizer BNT162b2 vaccine remains effective against severe disease and hospitalization. Our data further suggest that humoral immune responses to SARS-CoV-2 wane significantly from 5 to 8 months postvaccination and support the need of vaccine boosters to regain neutralization capacities against emerging variants after this time and thus prevent breakthrough severe COVID-19 (Figure 6).

While we have learned much from these studies,[7–13,50] there is a significant lack of data evaluating risk factors for the occurrence of breakthrough COVID-19. Distinct from previous studies,[7–13,31–33,50] our data demonstrated that male sex, age ($\geqslant$65 yoa), history of side effects to the COVID-19 vaccine, and low viral neutralization capacities were significant risk factors for breakthrough COVID-19 (Figure 5(a) and (b)); and strongly suggest an association of immune responses to the occurrence of breakthrough COVID-19. Since no data were available on the association between viral neutralization capacity or history of side effects of COVID-19 vaccine and the occurrence of breakthrough COVID-19 before our study, we recommend larger studies to evaluate and confirm our observation.

In addition, our data clearly demonstrated that the viral neutralization capacity was age-dependent. Individuals, 60 yoa or older had significantly less neutralizing capacities (Figure 4(b)), strongly supporting the need for vaccine boosters, especially those $\geqslant$60, at 5 months postvaccination. Our data align with observational data from Israel, where boosters of BNT162b2 in the $\geqslant$60 age group reduced symptomatic cases and presumably transmission (as judged by overall numbers of patients in that age group).[34,35]

Since antibody responses to the COVID-19 vaccine were age-dependent, the analysis of neutralization capacities against the viruses without controlling for age as a covariant is potentially misleading. Therefore, in our study, the comparison of neutralization capacities against variants between different studied groups was controlled for age, which was not considered or performed in previous studies.[50] With such an approach, we demonstrated that natural and breakthrough SARS-CoV-2 infection significantly enhanced neutralizing antibody responses against SARS-CoV-2 variants (Figure 4(c)).

Our study is not without limitations. The local study site – Baton Rouge, Louisiana – experienced significant healthcare stress during the surge of the Delta variant in July–September 2021 and a natural disaster (Hurricane Ida) in August 2021. For these reasons, samples were not available for all participants across all time points, though sufficient numbers enabled meaningful comparisons. However, our data clearly suggest the potential utility of viral neutralization capacities in evaluating the association between immunity and
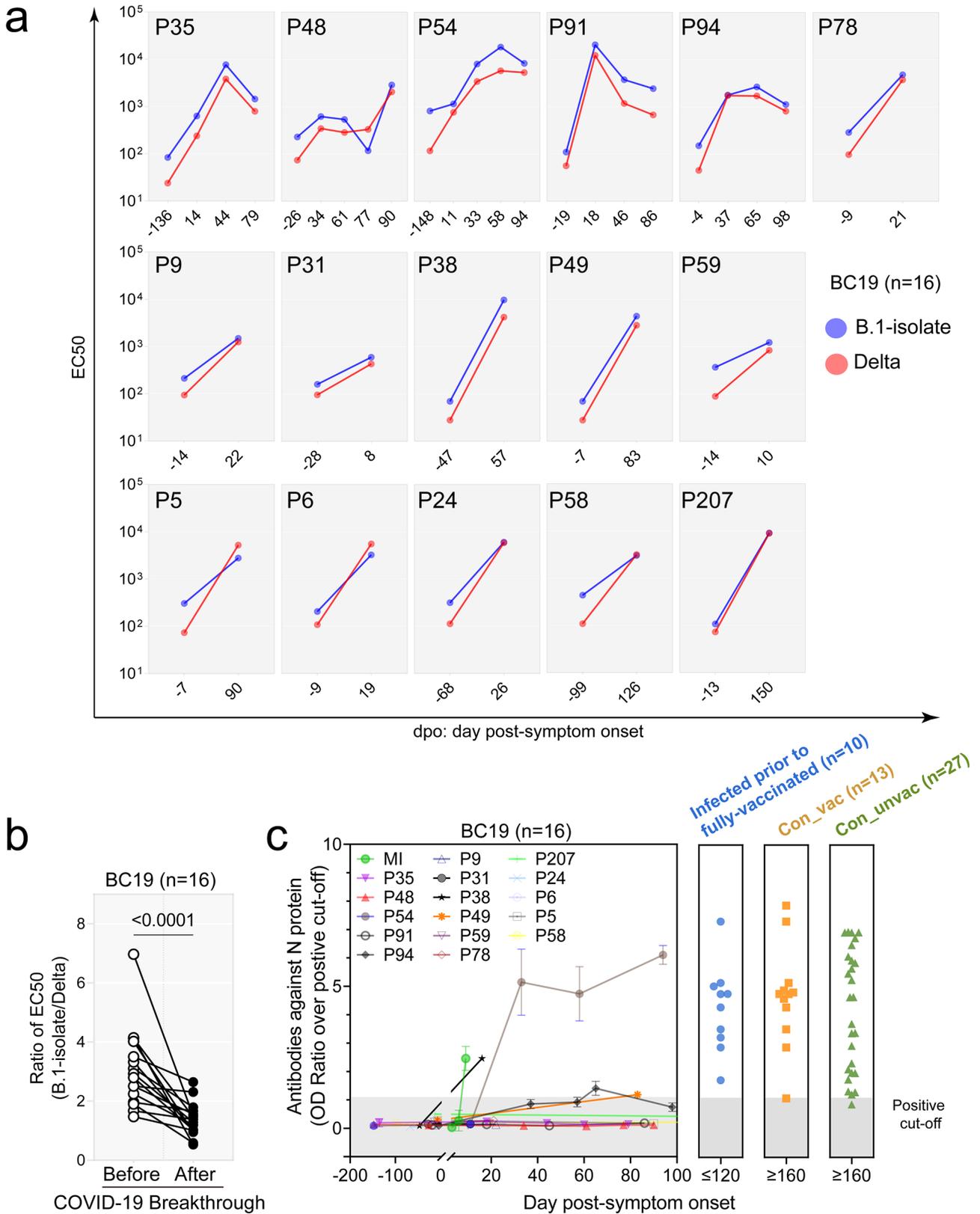
Figure 7. Absent or delayed and muted antibody responses to the viral N protein among BC19 cases. (a) Scatter plots of neutralization capacities pre-and postinfection at different days postsymptom onset (dpo). (b) Ratio of neutralization capacities against B.1-isolate over Delta variant postinfection as compared to the most recent time prior to the infection. Significance was determined using the Mann–Whitney *U* test. (c) Kinetics of antibodies to N protein at days pre-and postsymptom onset; the gray area represents the positive cut-off. Samples were analyzed as triplicates or duplicates from at least three independent experiments.

protection against the virus. Also, the responses of virus-specific B and T cells, which are beyond the scope of our current study, remain to be elucidated and warrant further investigation.

In summary, our study for the first time demonstrated the lack of antibody responses to SARS-CoV-2 N protein in COVID-19 vaccine breakthrough cases. We urge further studies to mechanistically investigate such phenomenal observation. Like previous studies, our study demonstrated that the Pfizer BNT162b2 vaccine remains effective against severe disease and hospitalization. We uniquely demonstrate increased risk of breakthrough COVID-19 for those men ⩾65 yoa, who experienced side effects of COVID-19 vaccine, and who exhibited low viral neutralization capacities. Our data further support prioritizing vaccine boosters for these more susceptible groups.

AUTHORS' CONTRIBUTIONS

LDV, SW, ATQP, SP, ET, SLL, AS, RCC, RR, DJN, KEH, BO, and SAC collected data, performed experiments, analyzed data, provided intellectual input, and contributed to manuscript preparation; LDV, BO, and SAC conceptualized the study; LDV, RCC, BO, and SAC drafted the manuscript. BO and SAC directed the study. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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DECLARATION OF CONFLICTING INTERESTS

ETHICAL APPROVAL

The study protocol was reviewed and approved by Woman's Hospital Foundation Institutional Review Board protocol# RP-20-029, approved on 22 January 2021 with reliance from Louisiana State University's Institutional Review Board and was conducted in accordance with relevant guidelines and institutional policies

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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