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Immune correlates of protection for emerging diseases – lessons from Ebola and COVID-19

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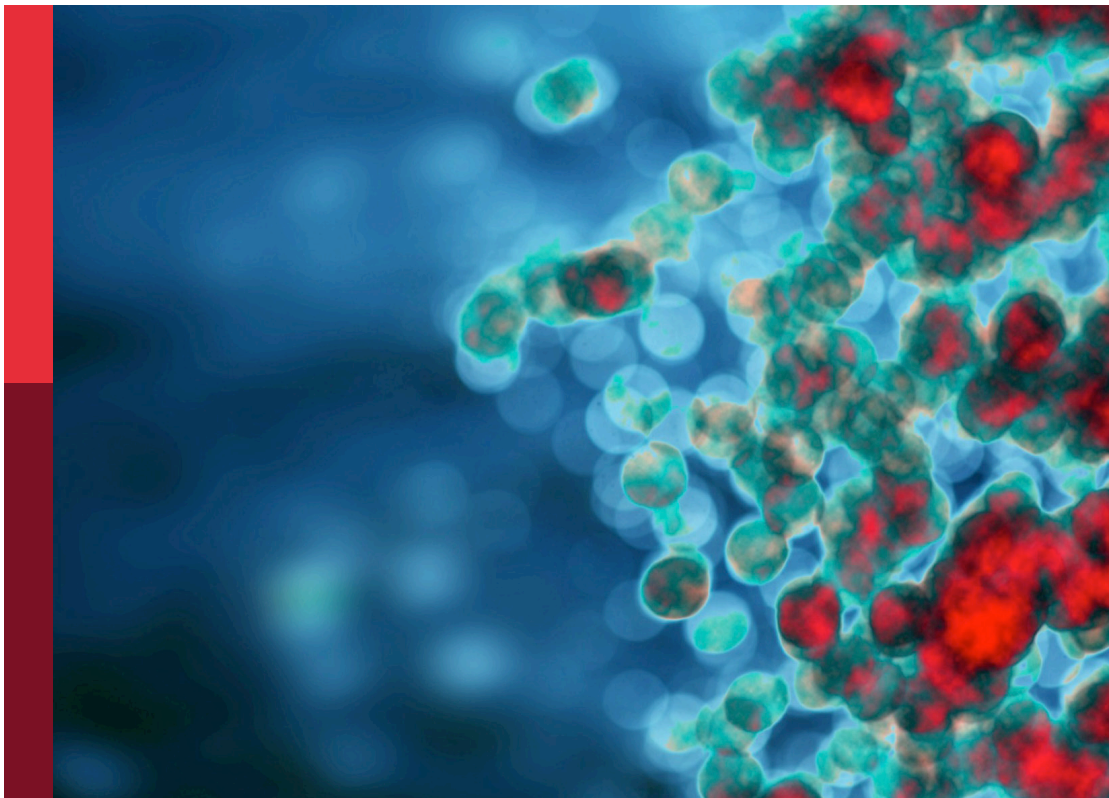

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Immune correlates of protection for emerging diseases – lessons from Ebola and COVID-19

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Kinetics of vaccine-induced neutralizing antibody titers and estimated protective immunity against wild-type SARS-CoV-2 and the Delta variant: A prospective nationwide cohort study comparing three COVID-19 vaccination protocols in South Korea

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Introduction: Despite vaccine development, the COVID-19 pandemic is ongoing due to immunity-escaping variants of concern (VOCs). Estimations of vaccine-induced protective immunity against VOCs are essential for setting proper COVID-19 vaccination policy.

Methods: We performed plaque-reduction neutralizing tests (PRNTs) using sera from healthcare workers (HCWs) collected from baseline to six months after COVID-19 vaccination and from convalescent COVID-19 patients. The 20.2% of the mean PRNT titer of convalescent sera was used as 50% protective value, and the percentage of HCWs with protective immunity for each week (percent-week) was compared among vaccination groups. A correlation equation was deduced between a PRNT 50% neutralizing dose (ND_{50}) against wild type (WT) SARS-CoV-2 and that of the Delta variant.

Results: We conducted PRNTs on 1,287 serum samples from 297 HCWs (99 HCWs who received homologous ChAdOx1 vaccination (ChAd), 99 from HCWs who received homologous BNT162b2 (BNT), and 99 from HCWs who received heterologous ChAd followed by BNT (ChAd-BNT)). Using 365 serum samples from 116 convalescent COVID-19 patients, PRNT ND_{50} of 118.25 was derived as 50% protective value. The 6-month cumulative percentage of HCWs with protective immunity against WT SARS-CoV-2 was highest in the BNT group (2297.0 percent-week), followed by the ChAd-BNT (1576.8) and ChAd (1403.0) groups. In the inter-group comparison, protective percentage of the BNT group (median 96.0%, IQR 91.2–99.2%) was comparable to the ChAd-BNT group (median 85.4%, IQR 15.7–100%; P =0.117) and significantly higher than the ChAd group (median 60.1%, IQR 20.0–87.1%; P <0.001). When Delta PRNT was estimated using the correlation equation, protective immunity at the 6-month waning point was markedly decreased (28.3% for ChAd group, 52.5% for BNT, and 66.7% for ChAd-BNT).

Conclusion: Decreased vaccine-induced protective immunity at the 6-month waning point and lesser response against the Delta variant may explain the Delta-dominated outbreak of late 2021. Follow-up studies for newly-emerging VOCs would also be needed.

KEYWORDS

protective immunity, vaccination, neutralizing antibody, SARS-CoV-2, COVID-19

Introduction

Since its emergence in late 2019, coronavirus disease 2019 (COVID-19) has been a serious threat to humanity. Several vaccines against severe acute respiratory syndrome virus 2 (SARS-CoV-2) have been developed to overcome the ongoing pandemic, and both mRNA vaccines and adenovirus-vectored vaccines were approved in South Korea in 2021. Among them, BNT162b2, an mRNA vaccine developed by Pfizer and BioNTech (BNT), and AZD1222 ChAdOx1, an adenovirus-vectored vaccine developed by Oxford University and AstraZeneca (ChAd), were widely administered to the public (1, 2). Both vaccines were initially designed for administration in two doses at three- (BNT) or four- (ChAd) week intervals (3, 4), but vaccination strategies in South Korea have been amended several times because of serious vaccine-induced adverse effects and vaccine supplements (5–8). Meanwhile, breakthrough infections were observed earlier than expected, mostly due to the rapid spread of the Delta variant (B.1.617.2) (9), which reduces vaccine-induced neutralizing activity by three- to four-fold (9–17). A third dose of vaccine was introduced globally to overcome the Delta variant–predominant outbreak (18), while the newly

emerging Omicron variant (B.1.1.529) with multiple mutations has become a following threat to vaccine-induced immunity.

To establish vaccination strategies for ongoing pandemic and continuously emerging SARS-CoV-2 variants, it is necessary to evaluate the vaccination strategies of the first year of COVID-19 vaccination and estimate the impact of Delta variant predominance during the 2021 outbreak. In particular, an ability to accurately estimate protective immunity based on the kinetics of neutralizing antibody titers is essential for predicting the persistence of the protective effect after a third vaccine dose (19–22). For this purpose, we investigated changes in the serologic response following vaccination using three major strategies implemented in South Korea: two doses of the BNT vaccine at a three-week interval (BNT group), two doses of the ChAd vaccine at a 12-week interval (ChAd group), and a single dose of ChAd followed by heterogeneous boosting with BNT at a 12-week interval (ChAd-BNT group) (8). For the estimation of protective immunity, we utilized the 20.2% of the mean PRNT titer of convalescent sera for 50% protective value as suggested by Khoury DS et al. (23).

Methods

Study population

This nationwide, multicenter, prospective cohort study was initiated under the leadership of the Korean Disease Control and Prevention Agency (KDCA) to evaluate the safety and efficacy of the national COVID-19 vaccination program. An earlier analysis of data from this study was published previously, which compared adverse effect and peak antibody response between the vaccination protocols (8). In the present analysis, we conducted a six-month follow-up analysis of the cohort. Healthcare workers (HCWs) from 10 hospitals in South Korea were recruited. To estimate protective immunity, we used 365 serum samples previously collected from reverse transcription polymerase chain reaction (RT-PCR)-confirmed COVID-19 patients who were infected in 2020 (24–27). Because the proportion of VOC among domestic cases was negligible before March 2021, those infected in 2020 are considered to have been non-VOC infections (28, 29). All participating HCWs and COVID-19 patients provided written informed consent, and the study protocol was approved by the institutional review board of each participating hospital.

HCWs receiving either the BNT or ChAd vaccines were recruited between March and April 2021. According to the early guidelines of the national vaccination program, BNT was assigned to HCWs designated for COVID-19 patient care, and ChAd was prescribed to those involved in non-COVID-19 patient care. The ChAd-BNT group was additionally recruited between May and June 2021. This cohort contained HCWs who experienced any adverse effects after the first dose of the ChAd vaccine in March 2021 and were willing to receive the BNT vaccine as a second dose. HCWs with a history of previous SARS-CoV-2 infection confirmed either by RT-PCR or detectable anti-nucleocapsid protein (NP) antibody at the baseline sampling were excluded from the present analysis.

Data acquisition and sample collection

Data on the baseline characteristics of age, sex, height, body weight, and underlying diseases were collected. Use of acetaminophen (AAP) or non-steroidal anti-inflammatory drugs (NSAIDs) after vaccination was neither recommended nor prohibited. The reactogenicity data after the first and second vaccination were collected for seven days using an electronic diary (eDiary) format, which was developed based on phase III clinical trials of the vaccines (3–5). Side effects of pain, redness, swelling, fever, chill, myalgia, arthralgia, fatigue, headache, vomiting, and diarrhea were investigated, as was the need for AAP or NSAIDs to control side effects. Participants rated each symptom on a scale of 0 to 4 (0 for no symptoms, 1 for mild, 2 for moderate, 3 for severe, and 4 for critical). For AAP/NSAID use, a score of 0 was selected for no need for AAP/NSAIDs, 1 for 1–2 tablets per day, 2 for 3–4 tablets, 3 for 5–6 tablets, and 4 for more than 7 tablets per day (5).

Blood specimens were collected at five points, which varied by group: at week 0 (baseline for the ChAd and BNT groups), week 3 (after the first dose for the ChAd and BNT groups), week 5 (after the second dose for the BNT group), week 11 (before the second dose for the ChAd and ChAd-BNT groups), week 13 (the first waning point for the BNT group), week 14 (after the second dose for the ChAd and ChAd-BNT groups), and week 26 (the first waning point for the ChAd and ChAd-BNT groups and the second waning point for the BNT group).

Laboratory procedures

Anti-SARS-CoV-2 spike protein total antibody assay

To estimate total antibody titers against the receptor binding domain (RBD) of the spike protein, the Elecsys® Anti-SARS-CoV-2 S assay (Roche Diagnostics, Basel, Switzerland) was used. The kit was developed for *in vitro* qualitative and semi-quantitative measurement of anti-SARS-CoV-2 spike protein antibodies and uses an electro-chemiluminescence (ECLIA) method conducted with Cobas e modules (Roche Diagnostics). A recombinant RBD of the spike protein was used with the double-antigen sandwich principle. Although the antigen used in the kit is predominantly captured by IgG, IgA and IgM are also detectable (30). The range of measurement is 0.4–250 U/mL (up to 2,500 U/mL with onboard 1:10 dilution and up to 12,500 U/mL with onboard 1:50 dilution). Values higher than 0.8 U/mL were considered positive.

Anti-SARS-CoV-2 NP antibody assay

To detect anti-SARS-CoV-2 NP antibody induced by past SARS-CoV-2 infection, an Elecsys® Anti-SARS-CoV-2 kit (Roche Diagnostics) was used. The double-antigen sandwich principle was used, and the ECLIA method was applied with Cobas e modules. The detectable isotypes included IgA and IgG, and a cut-off index greater than or equal to 1.0 was considered positive (31).

Plaque-reduction neutralization test (PRNT)

To evaluate the neutralizing activity of sera from vaccinated HCWs, PRNTs were conducted at the KDCA. PRNTs against WT SARS-CoV-2 were performed for 100 HCWs in each vaccination group. Briefly, 12-well plates were seeded with 2.5×10^5 Vero cells (ATCC CCL-81)/mL/well and incubated at 37°C in a 5% CO_2 incubator for 24 hours. Heat-inactivated (56°C for 30 minutes) serum samples in 96-well plates were serially diluted four-fold with Dulbecco's Modified Eagles Medium containing 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The diluted serum was incubated at 37°C in a 5% CO_2 incubator for 1 hour. A dilution of 50 plaque forming unit/well of SARS-CoV-2 (βCoV/Korea/KCDC03/2020 NCCP No.43326) was prepared. Vero cells on a 12-well plate were inoculated with the serum and virus mixtures and incubated at 37°C and 5% CO_2 for 1 hour. After the inoculums were removed, the cells were overlaid with 1 ml of Minimum Essential Medium containing 0.75% agarose and 2% FBS. The plates were incubated at 37°C and 5% CO_2 for three days and then stained with 0.07% crystal violet, 10% formaldehyde, and 5% ethanol. The visualized plaques were counted. The 50% neutralizing dose (ND_{50}) titer was calculated using Karber formula: $\log_{10} ND_{50} = m\text{-}\Delta(\Sigma p\text{-}0.5)$ (32). A 140 serum samples obtained after the second dose of vaccination (40 sera from the ChAd, 50 sera from the BNT, and 50 sera from the ChAd-BNT) were additional tested for both WT SARS-CoV-2 and the Delta variant (hCoV-19/Korea119861/KDCA/2021; NCCP43390). PRNTs of sera from convalescent COVID-19 patients were performed with the Vero E6 cell line (ATCC CRL-1586) using the same laboratory procedures.

Statistical analysis

Descriptive statics are presented as mean ± standard deviation (SD) or median {interquartile range (IQR)}. To compare baseline characteristics and clinical variables, one-way analysis of variance was used for continuous variables, and the chi-square test was used for categorical variables. For comparison of reactogenicity after the first and second doses, a summation of reactogenicity scores was used. To estimate the 50% protective neutralizing titer, 20.2% of the mean PRNT titer of convalescent sera was used based on a previous report that analyzed seven vaccine studies and one convalescent study (23). Convalescent sera from RT-PCR-confirmed COVID-19 patients collected between days 28 and 100 were used for this estimation (23, 33, 34). Subjects with an ND_{50} level equal to or higher than the estimated 50% protective neutralizing titer were considered to have protective immunity against SARS-CoV-2. For a quantitative comparison of protective immunity among the three vaccination strategies, the individual ND_{50} for each week after the first dose was calculated using the slope between sampling points. The percentage of HCWs with protective immunity during each week was compared among vaccination groups. For estimation of protective immunity against the Delta variant, we applied a conversion formula between the ND_{50} against WT SARS-CoV-2 and that against the Delta variant calculated from a linear regression model due to a limited number of samples used in PRNT against the delta variant. All P values were two-tailed, and values < 0.05 were considered to be statistically significant. All statistical analyses were performed using R version 4.1.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Study population

Overall, 822 HCWs were enrolled: 375 for the ChAd group, 347 for the BNT group, and 100 for the ChAd-BNT group. All the enrolled HCWs were tested for binding antibodies, and 100 subjects from each vaccination group were selected for PRNTs by order of enrollment. During follow-up, two HCWs in the ChAd group, three HCWs in the BNT group, and one HCW in the ChAd-BNT group dropped out, hence 816 HCWs were finally evaluated. The timeline for vaccination and blood sampling is illustrated in Figure 1. For the present analysis, the HCWs were followed for 26 weeks after their first vaccination. No cases of breakthrough infection occurred during the study period, which was identified by negative result of NP antibodies of the follow-up specimens. The baseline characteristics are presented in Table 1. The average age of the HCWs was 37.1 years, and the BNT group (average 35.3 years) was younger than the ChAd group (average 38.8 years). The subjects were mostly female (75.4%), and their average body mass index was 22.4 kg/m^2. Only 12.6% of the HCWs had comorbidities, most of which were mild and well controlled.

To estimate protective immunity, we investigated 365 serum samples collected within 100 days of illness from 116 RT-PCR-confirmed patients (Supplementary Figure 1): 111 samples from 76 mild-to-moderate patients and 254 samples from 40 severe-to-critical patients. Fifty-two (44.8%) of the patients were male, and the average age was 44.2 years. According to PRNT ND_{50} titer, seroconversion occurred between the first and second weeks of illness, and the peak PRNT response was observed before day 21. One hundred forty-three serum samples collected after 28 days of illness were used as convalescent sera. The mean PRNT titer of the

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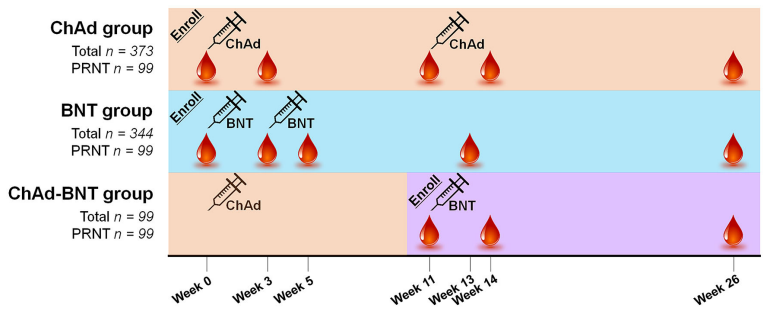

FIGURE 1
Vaccination schedule and sample acquisition timeline for each group. ChAd, AZD1222 ChAdOx1 vaccine; BNT, BNT162b2 vaccine; PRNT, plaque-reduction neutralizing test.

convalescent sera was 585.4 ± 854.6, and the median IQR was 206.8 (45.5–795.8). Patients with severe-to-critical illness (n = 70, median 599.2, IQR 199.5–1,681.0) showed higher titer than those with mild-to-moderate illness (n = 73, median 70.4, IQR 22.5–216.6; $P < 0.001$). Based on a previous estimation (23), we calculated a PRNT ND_{50} of 118.25 as the 50% protective neutralizing titer (20.2% of the mean).

Measured neutralizing and anti-RBD antibody levels

The measured binding and neutralizing antibody levels are presented in Figure 2. The PRNT ND_{50} titer peaked after the second dose in each vaccination group, as previously reported (8), and waned thereafter. The peak PRNT response of the

TABLE 1 Baseline characteristics of the study participants.

Characteristics	Total participants (n = 816)	ChAd group		BNT group		ChAd-BNT group		*P* value	
		Total (n = 373)	PRNT (n = 99)	Total (n = 344)	PRNT (n = 99)	Total (n = 99)	PRNT (n = 99)	Total	PRNT
Age, years	37.1 ± 9.4	38.8 ± 9.4*	39.0 ± 10.0*	35.3 ± 9.3*	34.5 ± 8.7*	37.1 ± 9.2	37.1 ± 9.2	< 0.001	0.003
Gender, female	615 (75.4)	284 (76.1)	69 (69.7)	250 (72.7)	71 (71.7)	81 (81.8)	81 (81.8)	0.159	0.112
BMI, kg/m^2	22.4 ± 3.0	22.4 ± 2.9	22.1 ± 2.5	22.4 ± 3.0	22.3 ± 3.3	22.0 ± 3.1	22.0 ± 3.1	0.416	0.727
Comorbidity, any	103 (12.6)	57 (15.3)	14 (14.1)	34 (9.9)	6 (6.1)	12 (12.6)	12 (12.1)	0.093	0.162
Hypertension	21 (2.6)	12 (3.2)	6 (6.1)	8 (2.3)	1 (1.0)	1 (1.0)	1 (1.0)	0.435	0.040
DM	11 (1.3)	7 (1.9)	1 (1.0)	3 (0.9)	1 (1.0)	1 (1.0)	1 (1.0)	0.483	1.000
Thyroid disease	21 (2.6)	11 (2.9)	2 (2.0)	8 (2.3)	0 (0.0)	2 (2.0)	2 (2.0)	0.813	0.363
Cardiovascular disease	5 (0.6)	2 (0.5)	1 (1.0)	2 (0.6)	0 (0.0)	1 (1.0)	1 (1.0)	0.862	0.604
Pulmonary disease	5 (0.6)	3 (0.8)	0 (0.0)	2 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)	0.657	NA
Gastrointestinal disease	3 (0.4)	1 (0.3)	0 (0.0)	2 (0.6)	1 (1.0)	0 (0.0)	0 (0.0)	0.639	0.367
Liver disease	2 (0.2)	2 (0.5)	1 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.304	0.367
Renal disease	1 (0.1)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.552	NA
Malignancy	8 (1.0)	4 (1.1)	1 (1.0)	2 (0.6)	1 (1.0)	2 (2.0)	2 (2.0)	0.428	0.776
Other	27 (3.3)	15 (4.0)	4 (4.0)	8 (2.3)	3 (3.0)	4 (4.0)	4 (4.0)	0.407	0.910
Reactogenicity, score sum									
After first dose	14.6 ± 17.8	20.1 ± 18.5*	23.1 ± 16.3*	7.6 ± 6.9*	7.4 ± 6.5*	39.9 ± 34.3‡	39.9 ± 34.3‡	< 0.001	< 0.001
After second dose	14.3 ± 15.5	7.5 ± 10.3*†	8.4 ± 9.5*†	19.8 ± 17.1*	22.7 ± 20.8†	20.2 ± 15.5†	20.2 ± 15.6†	< 0.001	< 0.001

Data are expressed as the number (%) of HCWs or mean ± SD.
*Statistically significant differences between ChAd and BNT groups. †Statistically significant differences between ChAd and ChAd-BNT groups. ‡Reactogenicity data of ChAd-BNT group after the first dose of vaccination were available for 49 HCWs and have potential risk for recall bias. Reactogenicity score of ChAd-BNT group after first dose was significantly higher than ChAd and BNT groups.
ChAd, AZD1222 ChAdOx1 vaccine; BNT, Pfizer and BioNTech vaccine; PRNT, plaque-reduction neutralization test; BMI, body mass index; DM, diabetes mellitus; NA, not available.

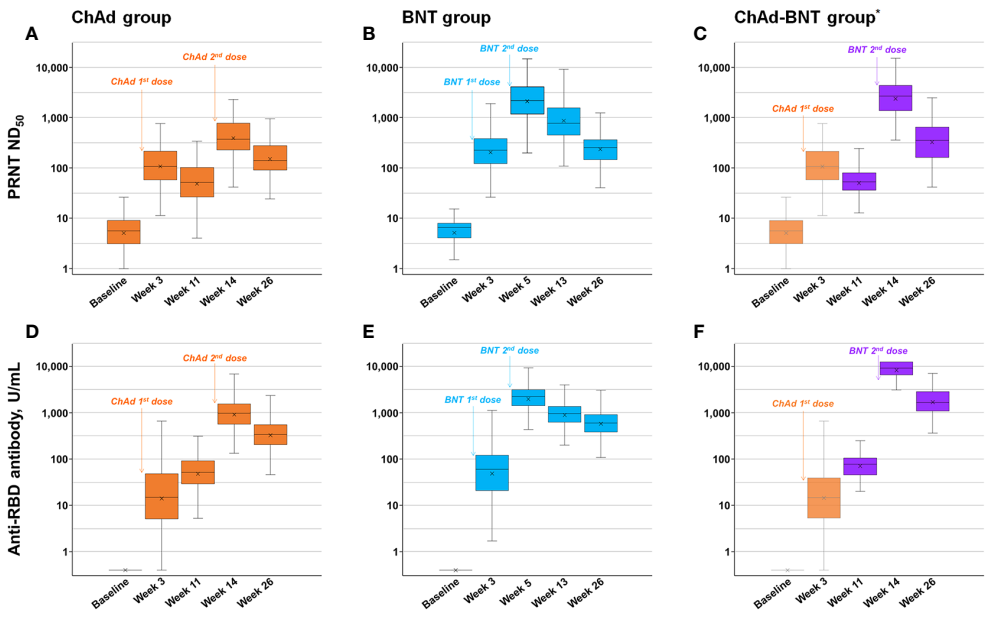

FIGURE 2
Measured neutralizing and anti-RBD antibody levels. Measured neutralizing antibody levels of ChAd **(A)**, BNT **(B)**, and ChAd-BNT **(C)** groups and anti-RBD antibody levels of ChAd **(D)**, BNT **(E)**, and ChAd-BNT **(F)** groups are depicted. *Because the ChAd-BNT group was enrolled later, the baseline and week 3 antibody levels of the ChAd-BNT group were adopted from the ChAd group. ChAd, AZD1222 ChAdOx1 vaccine; BNT, BNT162b2 vaccine; PRNT, plaque-reduction neutralizing test; ND_{50}, 50% neutralizing dose; RBD, receptor binding domain.

ChAd-BNT group (median 2637.0, IQR 1377.0–4261.0) was comparable with that of the BNT group (median 2151.9, IQR 11.65.4–4030.5; P =0.375) and higher than that of the ChAd group (median 374.0, IQR 231.0–783.0; P <0.001). Six months (26 weeks) after the first dose, the ChAd-BNT group (median 355.1, IQR 160.9–649.0) showed a higher PRNT ND_{50} titer than both the ChAd (median 139.6, IQR 90.5–273.6; P <0.001) and BNT groups (median 253.6, IQR 145.8–365.7; P =0.003).

The peak anti-RBD antibody response of the ChAd-BNT group (median 9266.0, IQR 6590.8–12500.0) was significantly higher than that of the BNT group (median 2245.0, IQR 1431.8–3169.3; P <0.001) and ChAd group (median 972.0, IQR 567.0–1549.0; P <0.001). Six months after the first dose, the ChAd-BNT group (median 1663.0, IQR 1058.3–2769.0) showed a higher anti-RBD antibody titer than both the ChAd (median 341.0, IQR 202.8–555.5; P <0.001) and BNT groups (median 597.0, IQR 383.5–908.0; P <0.001).

Estimated protective immunity against WT SARS-CoV-2 and Delta variant

For a quantitative comparison of the estimated protective immunity against WT SARS-CoV-2 among the vaccination groups, the percentage of HCWs with protective immunity (PRNT ND_{50} ≥118.25, Figure 3) during each week was estimated. At each measured time point, 0.0%, 45.5%, 16.2%, 92.9%, and 58.6% of the HCWs in the ChAd group; 0.0%, 77.8%, 99.0%, 98.0%, and 84.8% of the HCWs in the BNT group; and 0.0%, 45.5%, 9.1%, 99.0%, and 83.8% of the HCWs in the ChAd-BNT group had protective immunity. The 26-week cumulative percentage of HCWs with protective immunity was highest in the BNT group (2297.0 percent-week), followed by the ChAd-BNT (1576.8) and ChAd groups (1403.0). When the median values were compared among groups, the BNT group (median 96.0%, IQR 91.2–99.2%) was comparable to the ChAd-BNT group (median 85.4%, IQR 15.7–100%; P =0.117), and they were both significantly higher than the ChAd group (median 60.1%, IQR 20.0–87.1%; P <0.001).

Using the test results from 140 serum samples that underwent simultaneous PRNTs against WT SARS-CoV-2 and the Delta variant, we deduced a correlation equation: Log_{10}(Delta PRNT ND_{50}) = 0.7358*Log_{10}(WT PRNT ND_{50}) + 0.3166 (Figure 4A). There was no statistical difference between the measured and calculated values at each sampling points (Supplementary Table 1). Using that equation, the WT PRNT ND_{50} at each measured time point was converted to the Delta PRNT ND_{50} (Figure 4B). At each measured time point, 0.0%, 20.2%, 2.0%, 69.7%, and 28.3% of the HCWs in the ChAd group; 0.0%, 46.5%, 96.0%, 91.9%, and 52.5% of the HCWs in the BNT group; and 0.0%, 20.2%, 3.0%, 98.0%, and 66.7% of the HCWs in the ChAd-BNT group were estimated to have protective

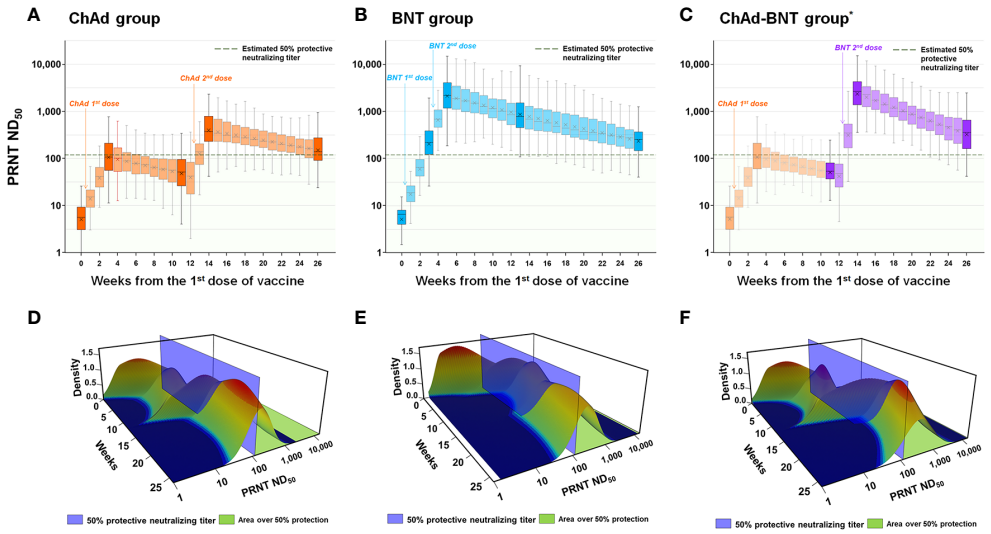

FIGURE 3
Estimation of protective immunity against WT SARS-CoV-2 for a 26-week period after vaccination. Individual PRNT ND_{50} values were calculated for each week after the first vaccine dose (transparent color) using the slope between the measured sampling points (dark color) and plotted for each vaccination group [ChAd **(A)**, BNT **(B)**, and ChAd-BNT **(C)**]. The distributions of PRNT ND_{50} titers for each week in ChAd **(D)**, BNT **(E)**, and ChAd-BNT **(F)** groups are presented using three-dimensional graphs. *Because the ChAd-BNT group was enrolled later, the baseline and week 3 antibody levels of the ChAd-BNT group were adopted from the ChAd group. WT, wild-type; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; PRNT, plaque-reduction neutralizing test; ND_{50}, 50% neutralizing dose; ChAd, AZD1222 ChAdOx1 vaccine; BNT, BNT162b2 vaccine.

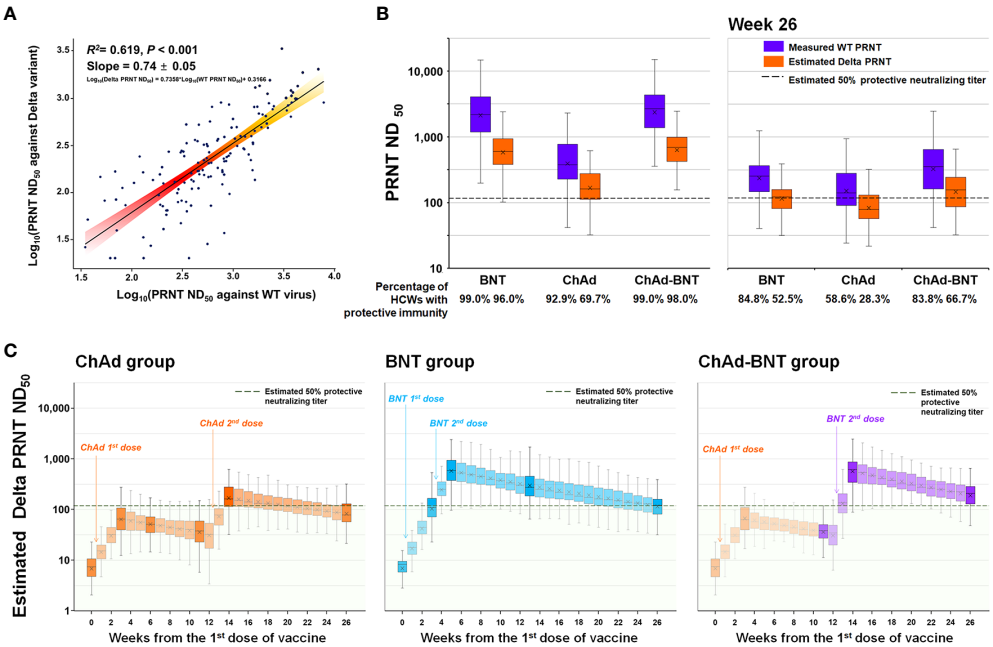

FIGURE 4
Estimated protective immunity against the Delta variant using the PRNT correlation equation. A correlation equation between the PRNT ND_{50} against WT SARS-CoV-2 and that against the Delta variant was deduced using 140 serum samples that underwent PRNT against the two strains simultaneously **(A)**. The PRNT ND_{50} against the Delta variant was calculated for each measured time point **(B)**, and the PRNT ND_{50} for each week was estimated using the slope between measured points **(C)**. WT, wild-type; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; PRNT, plaque-reduction neutralizing test; ND_{50}, 50% neutralizing dose; ChAd, AZD1222 ChAdOx1 vaccine; BNT, BNT162b2 vaccine.

immunity against the Delta variant. The 26-week cumulative percentage of HCWs with protective immunity against the Delta variant was highest in the BNT group (1973.7 percent-week), followed by the ChAd-BNT (1318.2) and ChAd groups (737.4) (Figure 4C). When the median values were compared among groups, the BNT group (median 89.9%, IQR 59.6–95.2%) was comparable to the ChAd-BNT group (median 66.2%, IQR 4.8–98.0%; P =0.280), and they were both significantly higher than the ChAd group (median 24.2%, IQR 7.3–49.7%; P <0.001). For the validation of estimated Delta PRNT ND_{50}, calculated values were compared with measured values of Delta PRNT ND_{50} using identical sera. The peak response and 6-months waning point were compared, and no statistically significant differences were noticed between estimated and measured Delta PRNT ND_{50} (all P > 0.05; Supplementary Figure 2).

Discussion

Despite the rapid development and wide distribution of SARS-CoV-2 vaccines, the COVID-19 pandemic is ongoing after more than two years, mainly because emerging VOCs escape vaccine-induced immunity (8, 10, 12, 13, 15, 29). *In vitro* studies showed that the vaccine-induced neutralizing activity against the Delta variant would decrease by 3–10-fold compared with that against the WT virus (8, 10, 13, 15), but the decrease in vaccine-induced protective immunity over time has not been well evaluated. In this analysis, the PRNT titers against the WT and Delta variant showed a linear correlation with a logarithmic scale, which allowed us to deduce a correlation equation of $\log_{10}$ PRNT ND_{50} against the WT and Delta variant. Using the previously reported estimation method for a 50% protective neutralizing titer (23), we calculated the percentage of HCWs with protective immunity against the WT and Delta variant. The ChAd group showed the lowest WT PRNT titer (8), and the percentage of HCWs in the ChAd group with protective immunity against the WT at the 6-month waning point decreased to 58.6%, whereas more than 80% of the HCWs in the BNT and ChAd-BNT groups maintained their immunity. According to the calculated Delta PRNT titers, the percentage of HCWs with protective immunity decreased to 28.3%, 52.5%, and 66.7% in the ChAd, BNT, and ChAd-BNT groups, respectively. According to a KDCA report, the nationwide overall protection effect of two vaccine doses, regardless of vaccination protocol, was 58.2% during the fifth week of December 2021, the peak of the Delta-predominant outbreak (35). The protective effect of each vaccination protocol has not been precisely calculated, but practitioners frequently reported breakthrough infections in the ChAd group (36). Although accurate validation of our estimation is not possible due to lack of an individual vaccination and SARS-CoV-2 infection database, our results regarding the estimated waning of protective immunity appear to reflect the real-world outbreak situation.

Of note, the cumulative protective percentage for the 6-month estimation was highest in the BNT group (2297.0 percent-week), followed by the ChAd-BNT (1576.8) and ChAd groups (1403.0). The peak PRNT response was achieved first by the BNT group, whereas the titer was highest in the ChAd-BNT group. Other published reports also suggest that heterologous boosting induced higher immunologic responses (37–40), and we further evaluated peak and waning response of antibodies according to the timeline to estimate cumulative protective effect. When the COVID-19 vaccine was introduced, the optimal interval between the first and second vaccine doses was debatable (20, 41). Our estimation suggests that reaching the peak PRNT titer sooner would provide better protective immunity for a 6-month period. However, the actual outbreak phenomenon was more complicated because the Delta-dominant large scale outbreak started 3–6 months after the initiation of wide-scale vaccination. The South Korean healthcare authority implemented a third dose as a booster shot to overcome the Delta-dominated outbreak (35), and that third dose was reported to provide a higher PRNT titer against VOCs, including the Omicron variant (42). Follow-up studies estimating the protective immunity for emerging VOCs need to continue.

Although many studies have reported the vaccine-induced immune response (9, 16, 17, 43–48), few performed the serial neutralizing test because it requires a biosafety level 3 facility, experienced personnel, and enormous time. We could not perform Delta PRNTs for enough serum samples to present the kinetics with measured Delta PRNT titers. Instead, we noticed a linear correlation between the $\log_{10}$ PRNT ND_{50} against the WT and Delta variant and deduced a correlation equation between them. With the calculated Delta PRNT value, we could estimate protective immunity against the Delta variant. However, estimating protective immunity against the Omicron variant would require additional validation. After the third vaccination, a 6–17-fold reduction of PRNT titer against the Omicron variant (compared with the WT) was observed and the correlation between PRNT ND_{50} values against WT and Omicron variant was poor (49). Interpretation of immunoassays measuring binding antibodies needs to be cautious because most assays are produced based on the receptor binding domain of WT SARS-CoV-2. Continuous efforts are needed to establish ways to measure meaningful antibody titers that predict protective immunity, and our data could provide background knowledge for such efforts using the standard vaccination dose.

The present study has several limitations. First, as an observational cohort study, the study population had differences in demographics and underlying diseases. Specifically, the HCWs in the BNT group were younger than those in the other groups. Second, because of the different

intervals between the first and second doses with different vaccines, the follow-up period after the second vaccination differed among the groups. Third, we did not include cell-mediated immunity in the present analysis. However, the present study focused on protective immunity in terms of neutralizing antibody titers in PRNTs, a gold standard test method for measuring neutralizing activity. Fourth, as the estimation of protective immunity in the present study is based from the pooled analysis of various vaccine studies and a correlation equation between WT and Delta PRNT (23), it would not exactly reflect protective immunity of each individual. An elaborate calculation of protective immunity against VOCs would be extremely challenging due to rapidly changing outbreak situations. Despite these limitations, we could compare each vaccine protocols with regard to the actual outbreak situation by utilizing the estimated values of protective immunity.

In conclusion, the percentage of HCWs with protective immunity against WT SARS-CoV-2 at the 6-month waning point maintained over 80% in the BNT and ChAd-BNT groups but only 58.6% in the ChAd group. Further decreases in protective immunity against the Delta variant may explain the Delta-predominated outbreak of late 2021. Follow-up studies need to be conducted for newly emerging VOCs.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Samsung Medical Center and all other included institutions. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: EN, J-HK, K-HS, HJ, S-WK, KK, SK, and KP. Investigation: EN, J-HK, K-HS, EK, KL, YS, YB, JA, JC, YK, YP, WC, SB, S-HK, E-SK, HJ, S-WK, and KK. Laboratory work: J-YC, H-JK, BK, H-YL, K-CK, H-CJ, and SK. Data analysis: EN and J-HK. Writing – original draft: EN and J-HK. Writing – review and editing: HJ, S-WK, KK, SK and KP. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.968105/full#supplementary-material

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Exposed seronegative: Cellular immune responses to SARS-CoV-2 in the absence of seroconversion

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The factors determining whether infection will occur following exposure to SARS-CoV-2 remain elusive. Certain SARS-CoV-2-exposed individuals mount a specific T-cell response but fail to seroconvert, representing a population that may provide further clarity on the nature of infection susceptibility and correlates of protection against SARS-CoV-2. Exposed seronegative individuals have been reported in patients exposed to the blood-borne pathogens Human Immunodeficiency virus and Hepatitis C virus and the sexually transmitted viruses Hepatitis B virus and Herpes Simplex virus. By comparing the quality of seronegative T-cell responses to SARS-CoV-2 with seronegative cellular immunity to these highly divergent viruses, common patterns emerge that offer insights on the role of cellular immunity against infection. For both SARS-CoV-2 and Hepatitis C, T-cell responses in exposed seronegatives are consistently higher than in unexposed individuals, but lower than in infected, seropositive patients. Durability of T-cell responses to Hepatitis C is dependent upon repeated exposure to antigen – single exposures do not generate long-lived memory T-cells. Finally, exposure to SARS-CoV-2 induces varying degrees of immune activation, suggesting that exposed seronegative individuals represent points on a spectrum rather than a discrete group. Together, these findings paint a complex landscape of the nature of infection but provide clues as to what may be protective early on in SARS-CoV-2 disease course. Further research on this phenomenon, particularly through cohort studies, is warranted.

KEYWORDS

SARS-CoV-2, seronegative, T-cells, exposed, hepatitis C

Introduction

Exposure to viral pathogens does not guarantee infection. The clearest examples of this phenomenon are in the failure of test subjects in human challenge studies to consistently become infected (1–3). Variation in host susceptibility has been linked to host genetics, inoculum viral load, and prior exposure to related pathogens (2–6). Among those individuals

who are exposed but fail to become infected, a small but well-documented population generate pathogen-specific T-cell responses in the absence of viraemia or antibodies (7–9). The earliest reports of this phenomenon occurred in the late 1980s concerning apparent Human Immunodeficiency virus (HIV) resistance in at-risk individuals (10–14). These patients, despite exposure to HIV and measurable cellular immunity, failed to develop an antibody response and were therefore classified as "exposed seronegative" (ESN). In the 30 years since initial reports in HIV, the phenomenon has been appreciated to occur following exposure to Hepatitis C virus (HCV), Hepatitis B virus (HBV), and Herpes Simplex virus 2 (HSV-2) (8, 15–18), and recently Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2), a pathogen to which most of the global population has been exposed (7, 19, 20). SARS-CoV-2, a member of the *Coronaviridae* family, is the causative agent of coronavirus disease 2019 (COVID-19). Canonical immunity to SARS-CoV-2 is well characterised despite its recent emergence: a delay in innate immune activation resulting from viral evasion of interferon (IFN) responses enables infection to occur (21). Both humoral and cellular mechanisms are essential for viral control; weak or delayed adaptive responses can lead to severe or fatal COVID-19, with immunopathology and cytokine hyperactivation characteristic of end-stage disease (21).

The causes and consequences of the ESN phenomenon following exposure to SARS-CoV-2, as well as other viruses, are the focus of this review. We outline the circumstances in which seronegative cellular immunity occurs and examine the quality of the T-cell response. In addition, we address the role of viral exposure on response durability, and whether this offers protection against infection. Finally, potential mechanisms are discussed, as well as gaps in current knowledge that future research must fill.

Definitions and terminology

For this review, an ESN is defined as an individual who mounts a cellular immune response following viral exposure without generating detectable virus-specific antibody. Instances where infection has been prevented by the innate immune system alone, as reported elsewhere (22, 23), are out of scope for this review due to not inducing a T-cell response. In the literature, the terminology for ESNs is largely consistent within but not between viruses and include exposed seronegative(s), exposed uninfected, infected seronegative, immune seronegative, and highly exposed persistently seronegative. These terms will be collectively referred to as ESN.

At-risk demographics

The dynamics of exposure, such as inoculum viral load, exposure frequency, and exposure duration, may influence cellular responses in ESNs. To make robust comparisons between SARS-CoV-2 and other viruses, we examine cellular immunity in two demographics at high risk of exposure – close contacts of seropositive individuals, and healthcare workers (HCWs). This enables the analysis of common and differing patterns of immunity to unrelated viruses through similar modes of exposure.

Close and household contacts

Close contacts of SARS-CoV-2-infected individuals represent a population exposed to SARS-CoV-2 over short time periods. Wang et al. (2021) assessed cellular immunity in 90 seropositive individuals and 69 seronegative contacts who had been within 1.5m of a patient for over one hour or in the same household for over 24 hours (20). The authors observed higher $CD8^+$ and $CD4^+$ T-cell activation in both cohorts compared to unexposed controls, as measured by IFNγ production following stimulation with Spike (S), Membrane (M), Nucleocapsid (NP), and Envelope peptides. Gallais et al. (2021) also identified T-cell responses in close contacts of SARS-CoV-2-infected family members (24).

Family members of HCV-infected individuals face viral exposure through sexual and *in-utero* transmission. Kamal et al. (2004) identified 14 seronegative sexual partners of HCV-infected individuals who generated IFNγ responses to HCV, although at lower magnitudes than seropositive resolvers (25). HCV is a positive-sense RNA virus in the *Flaviviridae* family (26). Canonically, adaptive immune responses appear one to two months after infection (27), and both $CD4^+$ and $CD8^+$ T-cells are associated with control (26). In individuals exposed to HCV *via* a family member, Scognamiglio et al. (1999) identified $CD8^+$ T-cells targeting both structural and non-structural proteins (NSPs) (16). There was no correlation between magnitude of response and mode of exposure, suggesting that similar cellular responses are generated following HCV exposure through different routes.

HCWs represent a population where SARS-CoV-2 exposure in clinical settings enables the study of ESNs. da Silva Antunes et al. (2021) recruited 26 PCR+ HCWs, 32 seronegative HCWs at high risk of exposure (treated as ESNs here), and 33 community controls (28). ESNs demonstrated higher T-cell responses than unexposed individuals. Notably, the T-cell activation markers HLA-DR and CD38 were upregulated in PCR+ HCWs, but not in ESNs, leading authors to conclude that cellular responses in ESNs were generated by exposure rather than infection and waning of antibody. Considerable overlap in levels of HLA-DR/CD38 expression was observed between seropositive and ESN HCWs. A study of SARS-CoV-2 ESN HCWs in Swadling et al. (2022) found a significant correlation between the magnitude of the T-cell response and transcript levels of *IFI17*, an IFN-inducible marker of early infection (7). Swadling et al. concluded that transient infection had occurred, but was aborted by early T-cell responses (7). However, ESNs with elevated *IFI17* transcripts only represented 10% of ESNs, and mean *IFI17* expression was lower in ESNs than seropositive HCWs (7). ESNs may therefore represent not one discrete group, but a spectrum of immune engagement, from subclinical exposure to transient infection.

Ogbe et al. (2020) identified SARS-CoV-2-specific T-cell responses in exposed HCWs: three of 10 generated IFNγ to S, M and NP, whilst all generated cellular responses to M and NP by proliferation assay, indicating the potentially higher sensitivity of this assay (19). T-cell responses in ESNs were of greater magnitude than unexposed controls for $CD4^+$ but not $CD8^+$ cells, although both $CD4^+$ and $CD8^+$ cells targeted a greater number of antigens in ESNs compared to controls. The presence of T-cells targeting multiple antigens in ESNs is supported by da Silva Antunes et al. (2021), where cellular immunity targeting S as well as the rest of the proteome was higher in ESNs compared to controls (28).

Kubitschke et al. (2007) identified T-cell immunity in seronegative HCWs exposed to HCV-contaminated needles (29). $CD4^+$ responses occurred in four of 10 individuals within eight weeks of injury but were absent after 2.5 years. Heller et al. (2013) assayed cellular immunity in HCWs exposed to HCV *via* needlestick, cut, or mucosal exposure, and identified HCV-specific proliferation and IFNγ production in 48% (n=30) and 42% (n=26) of individuals, respectively (30). Three-quarters of responses were directed towards NSPs. Responses peaked four to six weeks after exposure, unlike canonical HCV infection where immunity generally peaks at seven to 14 weeks (31). The authors suggested this may reflect boosting of pre-existing cellular memory. However, responses were transient and returned to baseline within months, unlike canonical long-lived memory responses (32). The dynamics of cellular immunity in HCV ESNs appears distinct from seropositive infection in its more rapid induction and reduced longevity.

Finally, Clerici et al. (1994) studied eight ESN HCWs following HIV+ needlestick injury. Four to eight weeks after injury, production of interleukin (IL)-2 by T-cells specific for the *env* glycoprotein was observed in six of eight ESNs, compared to only one of nine unexposed controls (33). However, two ESNs seroconverted at six and 19 months after sampling, reflecting early-stage infection rather than exposure.

Duration and dose

Viruses that have been well-studied for decades provide valuable information on the roles of exposure frequency and response durability in ESNs. Thurairajah et al. (2011) studied seronegative injection drug users exposed to HCV with differing injection behaviours (non-injectors in rehabilitation, infrequent injectors, and continuing injectors) (34). Continuing injectors had stronger and more numerous T-cell responses to HCV compared to non-injectors and healthy controls. Furthermore, individuals who had last injected over 12 months ago had a lower proportion of positive responses than those who had injected in the last six months. These data indicate that ongoing exposure to virus is one factor in the maintenance of T-cell responses in HCV ESNs.

Animal studies also provide insight into the role of antigen exposure for HCV. Shata et al. (2003) demonstrated that two chimpanzees exposed at six month intervals to increasing doses of HCV generated transient T-cell responses (35). 12 months later, both chimpanzees were exposed to a tenfold greater dose of the virus and became infected. The chimpanzee with consistently stronger T-cell responses cleared infection whilst the other developed chronic disease. Interpretation of this result is limited by the small cohort size. Furthermore, macaques exposed to infectious doses of simian immunodeficiency virus seroconverted but generated weak cellular responses, whilst those exposed to sub-infectious doses generated cellular responses only (36). These findings suggest that dose may factor into which arm of adaptive immunity dominates upon viral exposure. Similar challenge studies in primates or humans exposed to differing doses of SARS-CoV-2 would be necessary to make conclusions about the role of dose in SARS-CoV-2 ESNs.

T-cell responses in seronegative household contacts exposed to SARS-CoV-2 suggest that prolonged exposure may not be essential for cellular immunity (7, 19, 28). The durability of these responses is unknown due to the short timescale since virus emergence as well as the confounding influence of vaccination against SARS-CoV-2. Future studies on SARS-CoV-2 ESNs would benefit from sampling high-risk seronegative individuals, but although NP-targeting immunoassays could be used, these studies will be hamstrung by vaccination.

Target antigens

Determining which antigens are targeted in SARS-CoV-2 ESNs provides insight into mechanisms of response. T-cells targeting the replication-transcription complex (RTC) of SARS-CoV-2 were described by Swadling et al. (2022) in ESNs (7). The RTC is comprised of the RNA polymerase NSP12, a co-factor NSP7, and the helicase NSP13 (37). Its expression early in the SARS-CoV-2 replication cycle makes the RTC a target for rapidly-induced T-cell responses (7). The authors identified fivefold-higher RTC-specific T-cell responses in ESNs compared to unexposed controls. Furthermore, cellular immunity in ESNs preferentially targeted the RTC over structural proteins compared to seropositive individuals. However, the authors did not assay cellular responses to other NSPs. In a study of six ESN sexual partners of HSV-2-infected individuals by Posavad et al. (2010), T cell responses in ESNs were skewed towards peptides expressed early in the virus replication cycle, whereas HSV-2 seropositive individuals more frequently generated responses to structural proteins present in virions (8). The authors speculated that this skew in ESNs reflected early T-cell engagement with infected cells before the production of infectious virions. Together, these data support a model whereby rapid T-cell responses targeting early translated NSPs may prevent infection from gaining a foothold.

Cytokine profile

In a cohort of 52 household contacts of SARS-CoV-2-infected individuals, Kundu et al. (2022) identified higher frequencies of IL-2-, but not IFNγ-, secreting T-cells in ESNs compared to individuals that later became infected (38). A similar study of household contacts from Brand et al. (2021) reported no T-cell recognition of SARS-CoV-2 epitopes in seronegative individuals – this was measured by a novel IFNγ assay, which may lack sensitivity for low magnitude responses in ESNs (39). Assays that measure IFNγ production alone may underestimate the prevalence of cellular immune responses in ESNs, highlighting the need for multiple sensitive immunophenotyping methods, such as flow cytometric or proliferation assays, to accurately quantify responses.

T_H1-focussed cytokine production has been described in HBV ESNs (18). Sexual partners of infected individuals generated proliferative T-cell and IFNγ responses to HBV peptides. No ESNs generated TNFα or IL-10 responses, unlike seropositive individuals (40). Finally, IFNγ secretion was described in seronegative individuals exposed to Ebola virus (EBOV) (41). A study of EBOV close contacts (n=42) from Thom et al. (2021) identified two ESNs. However, responses in these ESNs were not present in further samples, potentially reflecting experimental artefacts. This further highlights the need for sensitive immunophenotyping assays to examine the true prevalence of ESNs.

Proposed mechanism

To prevent infection before seroconversion, a rapid cellular response appears critical. Chandran et al. (2021) assayed weekly nasopharyngeal swabs and blood samples from HCWs, and demonstrated that SARS-CoV-2 specific T-cell proliferation can occur before PCR positivity (42). These rapid responses may originate from pre-existing, cross-reactive T-cells specific for human coronaviruses (HCoVs). Cross-recognition of SARS-CoV-2 by HCoV-specific T-cells has been widely described (43–50), and T-cells from COVID-19 convalescents preferentially target conserved epitopes over SARS-CoV-2-specific epitopes (49). HCWs display higher levels of HCoV-specific T-cells than community controls (28), which may contribute to the abundance of ESNs amongst HCWs. The activation of cross-reactive T-cells by related viruses has been termed 'heterologous immunity' (51). This is distinct from autologous viral infection in that neutralising antibody responses to the heterologous virus may be suboptimal, allowing cellular memory to dominate.

The RTC is highly conserved between SARS-CoV-2 and HCoVs (7). Tetramer staining of T-cells with an HCoV-HKU1 homologue of the RTC component NSP7 showed strong responses in SARS-CoV-2 ESNs. Swadling et al. (2022) suggested that prior exposure to HCoV-HKU1 generates cross-reactive T-cells specific for NSP7, enabling rapid abortion of SARS-CoV-2 infection (7). A study of camel workers in Saudi Arabia identified both $CD4^+$ and $CD8^+$ responses to Middle-East Respiratory Syndrome coronavirus in four highly-exposed seronegative individuals, suggesting that the ESN phenomenon may be common to other human-infective coronaviruses (52).

It is unclear whether cross-reactive T-cells contribute to ESN immunity in HCV, HIV, HBV or HSV. Cross-reactivity between HCV and influenza A has been described, with HCV-seronegative individuals generating T-cell responses to a cross-reactive HCV epitope (53). However, human viruses with homology to HIV, HBV or HSV have not been described and are thus unlikely to be the driver of the ESN phenomenon for these viruses.

Correlates of protection from infection

Key to understanding correlates of protection against SARS-CoV-2 infection is deciphering the role of cellular versus humoral immunity. Seropositivity may not always be the most appropriate marker if cellular immunity is protective. This is particularly relevant for assessing vaccine-induced protection against disease where neutralizing antibody titres are a common endpoint, and particularly for SARS-CoV-2 where an arms race between booster vaccination and waning antibody titres has begun.

In a model whereby cellular immunity in ESNs is protective, one would reasonably expect that the magnitude of cellular response in ESNs would be greater than in seropositive individuals, to compensate for the lack of humoral immunity. Cellular immunity is able to clear SARS-CoV-2 infection in isolation; patients with X-linked agammaglobulinemia who cannot produce antibodies eventually clear SARS-CoV-2 infection, and mount higher magnitude $CD8^+$ T-cell responses to SARS-CoV-2 compared to immunocompetent individuals (54). However, in Wang et al. (2021) the magnitude of the SARS-CoV-2-specific $CD4^+$ T-cell response was twice as high in infected individuals compared to ESNs. This casts doubt on their role in protection against infection.

In influenza virus infection, cytotoxic T-cells target conserved non-structural proteins while antibodies target the divergent neuraminidase and hemagglutinin proteins and are thus strain-specific. In 1983, McMichael and colleagues demonstrated that individuals with cross-reactive T-cells targeting influenza A were able to clear infection in the absence of subtype-specific antibody (55). Later studies showed cross-reactive CD4+ and CD8+ T-cells are associated with milder disease in individuals lacking cross-reactive antibody (56, 57). Animal challenge models have shed light on whether cellular immunity following vaccination can confer protection against influenza. Vaccination of mice with a virus-like particle vaccine against influenza A virus promoted cross-reactive CD8+ T-cell-mediated protection against later challenge with a heterosubtypic strain, supporting the idea that cellular immunity in the absence of subtype-specific antibody can confer protection against infection (58). The applicability of cross-reactive T cell responses in influenza virus models to other virus families is unclear. However, the observations in these studies strongly support cellular immunity being considered in estimates of correlates of protection for viral infection.

Discussion

A model for the dynamics of adaptive immunity in infection versus exposure is shown in Figure 1. In canonical infection (Figure 1A), T-cells and antibodies reduce viral load and contribute to disease resolution. In ESNs (Figure 1B), T-cells proliferate alone and at lower levels than canonical infection. Viral load never reaches detectable levels and clinical disease does not occur. This may result from early proliferation of cross-reactive memory T-cells. Rather than being a discrete group, ESNs likely represent points along a spectrum of immune engagement, influenced by viral and host factors (Figure 1C). Discrepancies across studies likely reflect individuals at different points along this spectrum, dependent upon viral dose, existing cross-reactive immunity, and frequency of exposure.

Table 1 displays a summary of findings for SARS-CoV-2 and other viruses. However, significant gaps in our understanding of this phenomenon remain. Specific areas that would provide further clarification include:

1. **Repeated exposure and response durability**. Frequent exposure appears critical in the durability of HCV-specific cellular responses. SARS-CoV-2 challenge studies in primates and humans would clarify the role of dose and exposure frequency in ESNs, durability of responses, and the extent to which cellular immune responses correlate with protection against infection.
2. **Interaction with innate components**. Although not covered here, cross-reactive T-cells likely act in coordination with innate immunity to prevent infection. Natural killer cells have

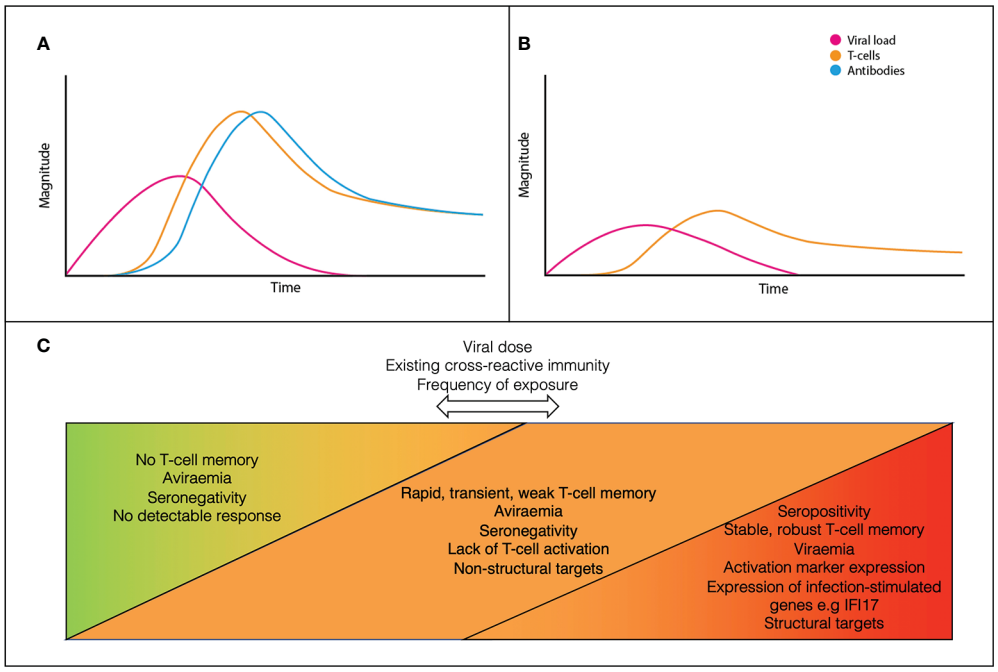

FIGURE 1
A conceptual model for the dynamics of canonical acute infection **(A)** vs ESN infection **(B)**. ESNs (orange) represent points along a spectrum of immune engagement, influenced by viral dose, immune cross-reactivity, and frequency of exposure **(C)**.

TABLE 1 Summary of ESN findings for SARS-CoV-2 and other viruses.

	SARS-CoV-2	Other
Canonical immune response	Both humoral and cellular immunity weeks after infection. Resolution usually within weeks with a small percentage experiencing severe or fatal outcomes.	HCV: Adaptive immunity months after infection. Cellular immunity with some contribution from neutralising antibodies.
Exposure duration and dose	Prolonged exposure not essential. Duration of exposure required is unclear.	HCV: Ongoing or recent exposure important for response maintenance. Potential role for low doses of virus. Responses peak earlier than in seropositive infection.
Durability	Unknown	HCV: Limited, with waning of responses observed within one year
Target antigens	Early translated antigens More non-structural targets than seropositive individuals	HCV: Early life cycle peptides
Cytokine profile	IL-2, IFNγ in some cases but not always	HBV: IFNγ production EBOV: IFNγ production
Potential source of response	Cross-reactivity with HCoVs	MERS: Potential cross-reactivity with HCoVs

been demonstrated to mediate resistance to HCV infection (59–61), and polymorphisms in immune mediator genes such as *IL28B* likely contribute to disease susceptibility (62). Future research into correlates of protection for SARS-CoV-2 should examine both innate and cellular components in seronegative infection, for example with the use of flow cytometric assays that enable precise dissection of immune components.

3. **Other T-cell subsets.** Many of the studies outlined in this review use whole blood samples representing circulating immunity. It is critical for future research to consider mucosal and tissue-resident cells to generate a complete picture wherever possible (63). This would require additional sampling such as nasopharyngeal swabs or respiratory samples.

Our understanding of the ESN phenomenon remains in its infancy yet offers opportunities for development. The remarkable heterogeneity in outcome following SARS-CoV-2 exposure makes understanding infection susceptibility crucial for prevention and treatment. Significant insight can be gained into correlates of protection against SARS-CoV-2 by further investigating this phenomenon and gaining a deeper understanding of the role of cellular immunity in protection against infection.

Author contributions

CJ conceived the idea and wrote the manuscript. JR assisted with finalising the manuscript. LT, PG and PK proofread the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Recent updates on correlates of vaccine-induced protection

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Correlates of protection are key for vaccine development against any pathogen. In this paper we summarize recent information about correlates for vaccines against dengue, Ebola, influenza, pneumococcal, respiratory syncytial virus, rotavirus, shigella, tuberculosis and Zika virus.

KEYWORDS

dengue, Ebola, influenza, pneumococcal, respiratory syncytial virus, rotavirus, shigella, tuberculosis

Introduction

A correlate of protection (CoP) is an immune function that correlates with and may be biologically responsible for vaccine-induced efficacy. The literature on this subject has grown considerably since it was identified as an important issue in vaccinology (1–5). The importance of CoP with regard to vaccines against SARS-2, the coronavirus causing COVID-19, needs no emphasis, and numerous papers have been published on that subject (6). However, not so much has been published recently about vaccines against other diseases. This paper is an effort to summarize recent findings in a number of important examples.

It should be acknowledged that the subject of CoP has become more complex due to increasing knowledge concerning Fc Effector antibody mediated functions and T cell mediated functions (7, 8). However, while it is evident that CoPs are often multiple and synergistic, their utility depends on identifying responses that are major and measurable. The fact that immune responses are often synergistic does not negate the value of identifying the main immune function that correlates with the protection generated by vaccination.

The SARS-2 novel coronavirus has been with us for only the last two years, but much work has been expended on defining a CoP, as recently summarized (6). The chief CoP is clearly neutralizing antibodies, with a gradual increase in efficacy as the titer increases. Although T cell responses and Fc effector antibodies are important in modifying the

results of infection, antibodies play the major role in preventing infection in the first place (9–11). However, antibodies must be specific for the variant virus, as the neutralizing epitopes differ between strains (12).

Dengue viruses

The correlates of protection against the four serotypes of dengue virus remain debatable, despite the development of several vaccines that have demonstrated some degree of efficacy (13–17). That efficacy has been influenced by age of the vaccinated population and serotype of the circulating dengue virus. It appears that efficacy is related to the induction of homotypic antibodies, whereas heterotypic antibody may actually enhance disease caused by other serotypes. The Dengvaxia vaccine is licensed only for those aged 9 years or above, and is most effective against dengue serotype 4, against which it induces homotypic specific antibodies. However, in a trial conducted in subjects aged 9 to 16 years with evidence of prior infection with a single serotype, efficacy was 67%, 67%, 80% and 89% respectively against types 1, 2, 3, and 4. Thus, homotypic neutralizing antibodies was the best correlate of protection against infection, but once an individual had infection by one serotype the Dengvaxia vaccine gave efficacy against other serotypes (18, 19). A live attenuated vaccine developed by Takeda was shown to induce that type of response (20). FC effector antibody functions may have a role in protection (21), and antibodies to NS1 reduce severity of disease (14) although no absolute correlate is available. However, at this point the best correlates of protection appears to be type-specific neutralizing antibodies (19).

Ebola virus

Vectored vaccines against the Ebola filovirus have been highly successful in controlling outbreaks of the disease in Africa. All of the vaccines are able to induce antibodies to the glycoprotein that is present in quantity on the elongated virus particle. However, the CoP is more complex than antibodies alone, and there is strong evidence that CD8+ T cells reacting against Ebola virus are necessary for high efficacy. Thus, Ebola is an example of where both arms of the immune system must respond in order for a vaccine to optimally prevent disease (22–28).

The functions of immune responses induced by successful Ebola vaccines are multiple, involving antibodies to the glycoprotein but also T cell responses (29). The role of the latter in protection appears to depend on the host species, being more important in infections occurring in primates (30). Study of vaccinated subjects also suggests important synergies between antibody and cellular immune functions. Moreover, the selection of adjuvant influences the mechanism of protection (31). For example, a CpG adjuvant stimulating the TLR-9 receptor gave superior survival. In summary, IgG antibody to the viral glycoprotein is the major CoP for Ebola, but is influenced by the type of adjuvant used. In addition, the sheer quantity of glycoprotein on the elongated virus particle may influence the quantity of responses correlated with protection (22, 32–38).

Influenza

The CoP for influenza that is commonly accepted for influenza is a 1/40 hemagglutinin-inhibition titer, which is credited with signifying a 50% protective ability. This is an oversimplification and ignores many other immunological functions that contribute to the efficacy of influenza vaccines (24, 39). Age of the vaccinee and the type of immunogen also influence the CoP. The widely used HAI titer of 1/40 corresponds to about 50% efficacy in young adults who have had immunologic priming by prior influenza infections. However, that titer conveys lower efficacy in older adults. The single radial hemolysis assay of >25 mm corresponds to about 70% efficacy in adults. In children who have not had prior infection or vaccination an HI titer of 110 gives 50% protection. In any case, in adults protection rises with HI titers, but protection is not guaranteed at higher titers nor absent at lower titers (24, 40).

Although neutralization is clearly an important function of antibodies, Fc effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADP) play a role in protection. In addition, influenza vaccines contain neuraminidase, though often unmeasured, which contributes to protection (25, 26). Neuraminidase concentration may vary between strains (41). Finally, cell-mediated immunity has not received enough attention and probably contributes to controlling virus replication (42). In summary, multiple antibody functions contribute to influenza vaccine efficacy (43–46).

Pneumococci

The vaccines against pneumococcal disease are composed of pneumococcal capsular polysaccharides conjugated chemically with proteins to increase immunogenicity, especially in children. For many years an antibody response measured by ELISA with a level of 0.35 micrograms/ml was accepted as a CoP. However, a seminal paper has demonstrated that the protective level is very much dependent on serotype, with type 3 being the most resistant; types 1, 7F, 19A, and 19 F requiring high antibody levels; and types 6A, 6B, 18C and 23F being less resistant (47). Thus, 0.35 mcg gives only a general estimate of a CoP with little precision. For type 3 relatively high levels of antibody are needed, estimated to be 2.83 mcg/ml. This means that the efficacy of conjugated pneumococcal polysaccharide vaccines

will vary with the epidemiology of serotypes, and that vaccines will vary in efficacy depending on the composition of serotypes and the sites of infections. The variability of CoPs for different serotypes was recently confirmed by the results of a study in African toddlers that gave 0.26 mcg/ml as CoP for type 14 but 1.93 mcg/ml as CoP for type 23F (28). The conclusion must be that the CoPs for pneumococcal serotypes are variable and must be determined individually.

Respiratory syncytial virus

Protection against RSV lower respiratory illness is complex: There are two distinct syndromes, one occurring in young infants who have only transplacental neutralizing antibodies to RSV, and a second occurring in the elderly, in whom the pathogenesis of disease is more complex. Antibodies having high neutralizing function are clearly protective in the very young, as shown by the correlation between antibody titer and protection, as well as the prophylactic value of administered monoclonal antibodies (23, 43, 44). A group A RSV inhibitory titer of 1/239 and a group B RSV inhibitory titer of 1/60 were associated with protection against disease (45). Antibodies against the prefusion form of the F protein are those that correlate best with protection. However, Fc effector as well as neutralizing functions of antibody are important, particularly in the lower respiratory tract (23, 34, 46, 47).

On the other hand, pathogenesis of RSV disease is less clear in seropositive elderly adults, in whom administration of antibodies is less effective. In part this may be due to the need to direct antibodies against other antigens of the virus and to elicit functions other than neutralization, particularly T cell functions (35). Antibodies and T cell responses against the small hydrophobic (SH) protein appear to be more important in adult infections. Thus, the problem of RSV vaccine development is less for infants, in whom monoclonal antibodies are protective. The level of neutralizing antibodies in infants predict protection from RSV (45) and thus the problem of vaccine development in infancy could be solved by developing a vaccine based on the prefusion form of the F protein (36, 37). Previously, numerous attempts to develop an RSV vaccine for the elderly have failed to give high levels of protection, despite the use of many strategies including nanoparticle, subunit, live-attenuated and vector-based (36–38, 48). However, the use of prefusion forms of the fusion protein rather than the post-fusion form has recently given encouraging immune responses in adults (49). Although it is uncertain as to whether the efficacy relates to serum or mucosal antibody responses. In addition, cell-mediated immunity may be important for protection of adults (50). However, a monoclonal antibody against prefusion F was successful in preventing RSV disease in children (51).

Rotavirus

Rotavirus vaccination has been spectacularly successful in high-income countries, though less so in poor countries where children are exposed to many pathogens soon after birth. Over the years since introduction of rotavirus vaccines, an intestinal IgA response and its surrogate, serum IgA, has been considered to be the principal CoP (52–54). A level of more than 20U/ml has been proposed as the protective level (55). However, other studies have not found serum IgA to be a convincing CoP, particularly in low-income countries (56, 57). A thorough review by Clarke and Desselburger (57) concluded that VP6 antibodies may be a better correlate. VP6 is part of the capsid of rotaviruses, and although it does not induce neutralizing antibodies, non-neutralizing antibodies to VP6 develop after infection or vaccination and thus may be a good correlate for protection. However, attempts to develop parenteral vaccines against rotavirus have so far failed, and it appears that secretory responses at the level of the intestine are the best correlates. However, serum IgA serves as an indicator of IgA responses in the intestine (52, 58).

Shigella

In a review published in 2007 Levine et al. (59) wrote that "Identification of protection is arguably the most crucial catalyst needed to accelerate the development of effective Shigella vaccines," but added that no clear correlate had been identified. Multiple candidate vaccines against shigella continue to be studied, including those containing the surface O antigen, antibodies to which are one proposed correlate (60, 61). In a detailed analysis Clarkson et al. (62) conclude that there are multiple CoPs, which may differ from one species to another. It appears that both serum and mucosal responses may serve as CoP depending on the challenge situation. This may simply reflect a situation in which the shigella organism must first replicate in the intestine by overcoming mucosal antibodies, but then invade the intestine, where systemic antibodies may be more important. Nevertheless, serum antibodies measured in various ways correlate with efficacy of shigella vaccines (63).

Tuberculosis

Bacille Calmette-Guérin, an attenuated *Mycobacterium bovis*, has been used for many years as a vaccine against TB, but with efficacy largely confined to vaccination at birth. Many attempts have been made to improve on BCG, for which identification of a CoP would be key. Studies in cows confirm that protective immunity correlates with a Th1 bias and induction of interferon gamma producing T lymphocytes.

The presence of central memory T cells also correlates with protection (64). Intravenous BCG given to macaque monkeys also protected against active tuberculosis, which correlated with induction of T cells reacting to tuberculosis antigens (65).

The search for an easily administered and more effective vaccine against human tuberculosis continues. There is agreement that T cells, both CD4+ and CD8+, are key to protection particularly with regard to interferon secretion, but Th17 cells may also play a role. Vaccine delivery by an aerosol route might be preferable (66). A recent review concluded that BCG is only effective in children (67). In any case, it is likely that a T cell function that has not yet been identified will provide the best correlate of protection against tuberculosis (68).

Zika

As Zika virus is transmitted by mosquito bite, it is not surprising that antibodies in the blood stream are protective. In macaques neutralizing antibody titers of about 1/100 induced by inactivated virus vaccines were shown to be highly protective (69, 70). However, cross-reactive antibodies with other flaviviruses raise questions about whether inducing Zika antibodies might enhance their replication (71).

Summary

Knowledge concerning correlates of protection by vaccines is critical to their application and continues to grow (5). In this article we report some recent findings for selected vaccines. Although from a biological point of view vaccines produce a variety of protective functions, some are more important than others, and are useful to predict efficacy. Table 1 lists correlates of protection for some major vaccines.

Current interest in correlates has been raised by the SARS-2 new coronavirus vaccines. As discussed elsewhere (6), the principal correlate of protection is antibodies measured by

TABLE 1 Selected correlates of protection after vaccination.

Vaccine	Immune Function	Protection Level
Anthrax	Toxin Nt Ab, Anti-PA IgG	1/3000, 10 μg/mL
Diphtheria	Toxin Nt Ab	0.01-0.1 IU/mL
***H. influenzae* conjugate**	ELISA Ab	0.15 ng/mL
Hepatitis A	ELISA Ab	20 m1U/mLl
Hepatitis B	ELISA Ab	10 mlU/mL
Influenza, inactivated	HI Ab	1/40 = 50% protection 1/320 in children
	NtAb	1/40 = 50% protection
Lyme	ELISA Ab	1400 U/mL
Measles	ELISA Ab	≥120 miU/mL
Meningococcal	Bactericidal Ab	≥1/4
Pneumococcal, conjugated	ELISA Ab	0.20-0.35 μg/mL
Polio, inactivated	Nt Ab	≥1/8
Rabies	Nt Ab	≥0.5 IU
Tetanus	Toxin Nt Ab	0.01-0.1 IU/mL
Tick-borne encephalitis	Nt Ab	≥1/10
Yellow Fever	Nt Ab	≥0.7 LNI

neutralization or ELISA. However, although there is no threshold value for protection, titers of approximately 1/100 give efficacy against disease better than 50%, whereas titers of 1/1000 or more give efficacy over 90%.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Approaches to demonstrating the effectiveness of filovirus vaccines: Lessons from Ebola and COVID-19

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Zaire ebolavirus (EBOV), Sudan ebolavirus (SUDV) and Marburg virus (MARV), are members of the *Filoviridae* family that can cause severe disease and death in humans and animals. The reemergence of Ebola, Sudan and Marburg virus disease highlight the need for continued availability of safe and effectives vaccines as well as development of new vaccines. While randomized controlled trials using disease endpoints provide the most robust assessment of vaccine effectiveness, challenges to this approach include the unpredictable size, location, occurrence and duration of filovirus disease outbreaks. Thus, other approaches to demonstrating vaccine effectiveness have been considered. These approaches are discussed using examples of preventive vaccines against other infectious diseases. In addition, this article proposes a clinical immunobridging strategy using licensed EBOV vaccines as comparators for demonstrating the effectiveness of filovirus vaccine candidates that are based on the same licensed vaccine platform technology.

KEYWORDS

filovirus, Ebola, Marburg, Sudan, vaccine, effectiveness, correlates of protection immunobridging

Introduction

Viral haemorrhagic fever is a deadly disease in humans and nonhuman primates (NHPs) caused by two genera of the larger virus family of *Filoviridae*. The most commonly known belong to the genera Ebolavirus and Marburgvirus. Of the six known Ebolavirus species, four can cause Ebolavirus disease (EVD) in humans: Zaire Ebolavirus (EBOV), Sudan ebolavirus (SUDV), Tai Forest virus (TAFV) and Bundibugyo virus (BDBV) (1). Marburg Virus disease (MVD) usually appears in sporadic outbreaks throughout Africa and is caused by the Marburg virus (MARV) which is a genetically unique virus of the filovirus family (2). The members of the *Filoviridae* family share a common mechanism of action with regard to tropism, cellular and disease pathology (3–6). Also, the genomic organization of Ebolaviruses and Marburgvirus is highly similar with seven sequentially arranged genes encoding: the

nucleoprotein (NP), the virion protein 35 (VP35), the VP40, the glycoprotein (GP), the VP30, the VP24, and the polymerase (L). The surface of the filovirus virion is coated by spike-like projections of the GP, which is responsible for the viral antigenicity upon entry and is the target of virus neutralizing antibody (7). Although there is no established immune correlate of protection for filoviruses, levels of GP-binding antibody have been linked to protection (8, 9).

Historically, EBOV has caused most filovirus disease outbreaks and cases. The EBOV outbreak of 2014 - 2016 in West Africa caused over 28,000 cases of EVD and more than 11,000 deaths and led to the rapid development of preventive vaccines against EVD (10). In response to the reemergence of large outbreaks of this deadly disease, several EBOV vaccines were rapidly developed. ERVEBO, a live attenuated, replication competent recombinant vesicular stomatitis virus (rVSVΔG-ZEBOV-GP) vaccine expressing the GP antigen of EBOV was licensed in 2019 by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (11, 12). Effectiveness was demonstrated in a Phase 3 cluster-randomized ring vaccination study conducted in affected areas during the 2014 - 2016 outbreak (13). Zabdeno/Mvabea (Ad26-ZEBOV/MVA-BN-Filo), a heterologous prime-boost vaccine consisting of the non-replicating adenovirus serotype 26 expressing the EBOV GP and the Modified Vaccinia Ankara (MVA) encoding glycoproteins from EBOV, SUDV, MARV as well as TAFV nucleoprotein, was licensed by EMA in 2020 under the exceptional circumstances pathway (14). Effectiveness of the vaccine was inferred from challenge/protection studies in NHPs and clinical immunogenicity data. Although the booster dose of this vaccine expresses SUDV, MARV, and TAFV antigens in addition to EBOV, the vaccine is only approved for prevention of disease caused by EBOV. An adenovirus serotype 5 (Ad-5 EBOV) vaccine expressing the EBOV GP was licensed by the Chinese Food and Drug Administration, and a heterologous prime boost vaccine consisting of recombinant VSV and Ad-5 expressing EBOV GP was licensed by the Ministry of Health of the Russian Federation, both for emergency use (15). There is currently no licensed vaccine indicated for the prevention of disease caused by SUDV or MARV.

Repeated outbreaks of EVD such as the one ending in 2020 in the Democratic Republic of Congo (DRC) and reported cases of MARV in Ghana as well as the SUDV outbreak in Uganda in 2022 underscore the need for additional safe and effective vaccines to protect against filovirus disease (16, 17). However, the sporadic nature of these outbreaks, uncertainties in occurrence and duration and geographic location presents challenges to conducting randomized controlled efficacy trials in particular in preventive settings and thus, other approaches to demonstrating vaccine effectiveness are considered to enable licensure of these products. While approaches to establishing vaccine safety to support licensure are well-established, here we describe strategies to demonstrating vaccine effectiveness using examples of licensed preventive vaccines and present considerations for use of clinical immunobridging strategies to support science-based predictions about the effectiveness of new filovirus vaccine candidates (Figure 1).

Approaches to demonstration of effectiveness of preventive vaccines

Clinical disease endpoint efficacy studies and/or use of scientifically well-established marker

Randomized controlled clinical trials using prevention of disease as an endpoint represent the gold standard to demonstrate the efficacy of preventive vaccines. Recent examples include DENGVAXIA for the prevention of dengue disease (18) and COMIRNATY and SPIKEVAX for the prevention of disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (19, 20). For these products, efficacy was demonstrated in pivotal Phase 3 studies using symptomatic virologically confirmed disease as endpoint. Vaccine effectiveness can also be demonstrated using a scientifically well-established and validated marker, e.g, an immune marker, that predicts protection. Examples of such a marker with a defined and validated threshold include anti-Hepatitis B antibody titer (10mIU/

	Immunobridging Strategies			
Clinical efficacy studies	Scientifically well-established biomarker	Biomarker reasonably likely to predict protection	Biomarker for demonstrating effectiveness using animal studies	Biomarker without threshold value
Randomized controlled clinical trials using clinical disease endpoint. Biomarker not required for licensure	Scientifically well established biomarker, e.g., antibody level, predicting protection Clinical trials comparing the biomarker induced by the candidate vaccine to licensed comparator	Biomarker, e.g., antibody level, reasonably likely to predict benefit but not robustly established Clinical trials comparing the biomarker induced by the candidate vaccine to licensed comparator	Biomarker, e.g., antibody level, not robustly established Animal and clinical trials to bridge vaccine-induced immune response in animals to humans using biomarker	Biomarker, e.g. antibody without a serological cut-off but associated with protection and proportional to other protective components of the immune system Clinical trials comparing the biomarker induced by the candidate vaccine to licensed comparator
Examples				
Prevention of symptomatic virologically confirmed dengue cases; Dengvaxia (dengue tetravalent vaccine, live)	Anti-Hepatitis B antibody titer (≥ 10 mIU/ml) Post-marketing studies to confirm benefit not needed	Influenza virus hemagglutination-inhibition (HI) antibody titer (≥ 1 to 40) Post-marketing studies to confirm benefit	Anthrax toxin neutralizing antibody (TNA) level Post-marketing studies to confirm benefit	Anti-SARS Cov-2 neutralizing antibody Post-marketing studies to confirm benefit not needed

FIGURE 1
Approaches to demonstrating the effectiveness of preventive vaccines.

ml). This marker has been used as predictors of vaccine effectiveness and has supported licensure of vaccines against Hepatitis B (21). Using this example, in cases where new Hepatitis B vaccines are being developed, in addition to demonstrating attainment of the validated marker of protection, immunological non-inferiority was also demonstrated against the approved comparator product. This is usually based on demonstrating similar geometric mean antibody titers and/or seroconversion rates based on pre-specified statistical criteria.

Use of immune markers likely to predict protection from disease

Vaccine effectiveness to support licensure has also been inferred based on a surrogate endpoint (e.g, immune marker) thought to be *reasonably likely* to predict clinical benefit even though not robustly established. As in the above section, adequate well-controlled trials comparing the surrogate endpoint in persons administered the candidate vaccine versus the licensed comparator using pre-specified statistical criteria must be conducted. However, as there is uncertainty in regard to ability of the surrogate endpoint to predict effectiveness, post-licensure studies are required to confirm the clinical benefit of the vaccine. In the US, this is referred to as accelerated approval (AA) under 21 CFR 601.40/41 (22). An example of a surrogate endpoints supporting AA include influenza virus hemagglutination-inhibition antibody titer of ≥ 1 to 40 (23). In 2019, FDA convened its Vaccines and Related Biological Advisory Committee (VRBPAC) to discuss data necessary to establish an immunologic marker *reasonably likely* to predict clinical benefit for Chikungunya virus (CHIKV) vaccines (24). Disease outbreaks caused by Chikungunya, like those caused by filoviruses, are irregular and unpredictable making clinical disease endpoint efficacy studies challenging. Furthermore, there is no relevant animal model reflecting clinical CHIKV disease in humans. Data derived from animal models and human epidemiological studies have suggested that CHIKV neutralizing antibody could be used as a surrogate endpoint to support vaccine licensure. Based on these data, FDA and VRBPAC agreed that a CHIKV neutralizing antibody titer *reasonably likely* to predict protection could be established from passive transfer of human antibodies in NHP followed by challenge with wild-type CHIKV. A similar approach could be considered for developing vaccines against SUDV and MARV disease.

Animal challenge/protection studies

If demonstration of effectiveness is not possible based on a clinical disease endpoint efficacy study, and if a scientifically well-established marker and/or a surrogate endpoint *reasonably likely* to predict protection is not identified, it may be necessary to conduct challenge/protection studies in qualified animal models to demonstrate the effectiveness of the candidate vaccine in preventing disease. Some national regulatory authorities have provisions to allow licensure of a vaccine candidate using this approach (25–27). In the US, this pathway is referred to as the "animal rule" (AR) under 21 CFR 601.91 (28). Under the AR there are specific criteria that must be met including that the animal study endpoint is clearly related to the desired benefit in humans, which is generally the enhancement of survival or prevention of major morbidity. Predicting effectiveness using animal/challenge protection studies includes a) determining that the marker being measured in the animal (usually antibody levels) is associated with protection against virus challenge, b) evaluating the marker in humans and using the information accrued, c) bridging of animal and human data on this marker to establish an effective dose in humans. The vaccine dose in humans should elicit levels of the marker comparable to that of animals protected by the vaccine whereby the dose chosen may not be the same. Furthermore, the marker selected for bridging does not need to be causally responsible for protection.

One example of a vaccine approved based on effectiveness data in animals is BioThrax, anthrax vaccine absorbed (AVA) for post-exposure prophylaxis (PEP) (29). Two General Use Prophylaxis (GUP) challenge/protection studies in rabbits and NHPs studies were performed to estimate protective antibody levels measured in a validated anthrax toxin neutralizing antibody (TNA) assay. TNA levels corresponding to 70% survival probability in the animals were determined. Immunogenicity data in animals were then bridged to human immunogenicity data. The proportion of clinical study subjects achieving a TNA response corresponding to 70% survival probability in animals was determined to estimate effectiveness of the vaccine in humans.

Another example, although not U.S. approved under the AR, is the Zabdeno/Mvabea, Ad26.ZEBOV/MVA-BN-Filo EBOV vaccine for which marketing authorization by EMA in 2020 was based on data demonstrating that immunization with this prime-boost vaccine fully protected NHPs against a lethal EBOV exposure (14). Data on immunogenicity and survival outcome were derived from NHP challenge/protection studies using the selected vaccine dose regimen and a 56-day dose interval. To infer effectiveness of the vaccine in humans, immunobridging was performed based on EBOV GP-binding antibodies measured by the validated EBOV GP FANG ELISA assay used for quantitation of both human and NHP anti-GP IgG. A similar approach could be considered for developing vaccines against SUDV and MARV disease; however, differences between the immune responses in NHPs and humans vaccinated with EBOV vaccines raise some uncertainty with this approach to identifying levels of antibody that would predict protection (30).

Inference of effectiveness using clinical immunobridging studies

Clinical immunobridging refers to studies in which the effectiveness of a new vaccine candidate is inferred by comparing the vaccine-induced immune response (e.g, neutralizing antibody titer) to that induced by a comparator vaccine for which efficacy was previously demonstrated. The above include examples where clinical immunobridging studies were conducted to infer effectiveness of the candidate vaccine using either a scientifically well-established immune marker or a marker *reasonably likely* to predict protection at a defined threshold. However, immunobridging can also be used as an important tool in the absence of an agreed upon serological cut-off or threshold value of a selected immune marker. Using this approach,

one key consideration is that the immune response measured (e.g, neutralizing antibody, total binding antibody) is correlated to protection against disease and is also positively correlated with other protective components of the immune response. In addition, the efficacy of the comparator vaccine will inform statistically appropriate criteria (non-inferiority vs. superiority).

Clinical immunobridging studies have been conducted to demonstrate the effectiveness of COVID-19 vaccines against COVID-19 variant of concerns (VOCs) and new COVID-19 vaccines using neutralizing antibody titers as biomarkers (31). Note that in these cases a correlation between neutralizing antibodies and protection has been confirmed across different vaccine modalities or platforms even though an antibody threshold has not been established (32). In the US, these recommendations pertain to modified vaccines generated using the same process and manufacturer as the authorized or approved parental or "prototype" vaccine. Other regulatory authorities, including the Public Health Agency of Canada (PHAC) have accepted immunobridging studies to authorize not only modified versions of COVID prototype vaccines, but also new COVID vaccines (e.g, vaccines produced by a different manufacturing process) despite the lack of an established correlate or surrogate marker of protection (33). WHO has also promulgated a framework for immunobridging of COVID vaccine efficacy, focusing on the ability of viral neutralizing antibody responses to predict other immune mechanisms of protection for any given vaccine, as well as the effectiveness of the comparator (34).

Similar to COVID-19, although there is no established level of SUDV or MARV GP-specific antibody responses predicting protection against EVD or MVD, levels of filovirus GP-binding antibody are associated with protection against disease (8, 9). Thus, using the analogy of COVID-19, clinical immunobridging studies using a licensed comparator vaccine based on the same platform could be considered for filoviruses. For example, the EBOV GP insert in the licensed rVSVΔG-ZEBOV-GP vaccine, ERVEBO, could be replaced by the SUDV or MARV GP, followed by an immunobridging study demonstrating that the level of anti-GP antibody induced in subjects is comparable to that induced by the parental prototype vaccine. This approach would require confidence that anti-GP antibody responses could predict protection at similar levels for different filoviruses. This confidence is enhanced if the efficacy of the original vaccine is high (as indeed, it is for rVSVΔG-ZEBOV-GP vaccine) and if the immunopathogenesis of the diseases are similar, including rates of disease evolution and potential immune evasion mechanisms used by each virus.

Discussion

Some or all of the approaches to demonstrating vaccine effectiveness described in this article may be considered to demonstrate the effectiveness of new filovirus vaccine candidates recognizing that each approach presents with challenges and uncertainties. As stated, the sporadic nature of Filoviral outbreaks may not allow the demonstration of protection against EVD and MVD by way of conducting clinical disease endpoint efficacy studies unless there is timely availability of filovirus vaccine candidates at the time of a large outbreak as was the case during the EBOV outbreak in West Africa in 2014 - 2016. Furthermore, there is no scientifically well-established validated immunologic marker that predicts protection against EVD or MVD disease.

There are some important considerations for demonstrating effectiveness based on a surrogate endpoint *reasonably likely* to predict protection for MARV, EBOV or SUDV vaccines. Notably, one must identify a surrogate endpoint, e.g., neutralizing antibody, binding antibodies or cellular immune markers, *reasonably likely* to predict protection. These immune markers may be derived from naturally infected or exposed and protected humans including those participating in vaccine clinical trials in outbreak areas. They may also be derived from animal challenge protection studies (e.g, NHPs). Furthermore, when evaluating whether a particular immune marker is *reasonably likely* to predict protection against EVD or MVD, the conclusion may be different for vaccine candidates that are based on different platforms. For example, immune responses induced by differing vaccine modalities (e.g, a replication deficient-, a replication-competent-, or inactivated virus, a recombinant protein-based and/or nucleic acid-based product) will likely be different, not only in magnitude, but also in the type and breadth of the immune mediators induced (35). It may also be different for virus species that are either homologous or heterologous to the vaccine targeting antigens. Finally, there is a requirement that the immunologic assays used to demonstrate effectiveness are validated.

Demonstration of filovirus vaccine effectiveness using challenge protection studies in animals can be considered if it cannot be demonstrated by other approaches. For filoviruses, the NHP represents an adequate animal model and the disease presentation between humans and NHPs is similar (36–40). However, comparing disease courses between experimentally infected NHPs and naturally infected humans is difficult as route of exposure and challenge dose selected may not resemble natural exposure. Furthermore, there is currently no established EBOV GP antibody titer threshold value associated with clinical benefit. Moreover, studies have demonstrated that immune responses in animals vaccinated with EBOV vaccines are higher than those induced in humans, resulting in uncertainties regarding level of antibody that would predict protection (41).

Numerous studies have been conducted to characterize both vaccine-induced and naturally acquired immunity to filoviruses in humans and animal models. Data indicate that both humoral and cell-mediated immune responses are critical in protecting from filovirus disease (42–44). In NHPs, although cell-mediated immunity plays a role in protection from disease, vaccine effectiveness was consistently associated with the presence of ELISA IgG (45–48). Monoclonal antibodies isolated from human survivors of EVD in the 2014 - 2016 outbreak in West Africa afforded protection in animal EBOV challenge model (49, 50). Human monoclonal antibodies were licensed by FDA for the treatment of infections caused by EBOV in adult and pediatric populations (51, 52). Grais et al. assessed antibody levels induced by the licensed rVSVΔG-ZEBOV-GP vaccine, ERVEBO, using serology data from participants of three immunogenicity trials conducted in Guinea, Sierra Leone and Liberia during the time of the EBOV outbreak in 2014 – 2016 (41). Their analysis supported the Ebola GP-ELISA as a tool for predicting vaccine effectiveness even though contributing protective effects afforded by cell-mediated immunity could not be excluded. However, it is likely that all filovirus vaccines using the

rVSVΔG platform will induce cellular responses in similar proportion to humoral responses, supporting use of humoral responses to predict overall responses including cellular responses.

Together, even though the underlying immune mechanism affording protection against filovirus disease is not fully elucidated, anti-GP antibodies play a significant role in providing protection against EVD and MVD. Thus, clinical immunobridging studies using anti-GP ELISA-based IgG levels as an endpoint should be considered to infer effectiveness of new filovirus vaccines. For example, demonstration of statistically pre-specified anti-GP antibody titers induced by the licensed rVSVΔG-ZEBOV-GP vaccine (for which efficacy was demonstrated) and vaccine candidates using the same platform and modified to express the SUDV or MARV GP could potentially serve as the basis for vaccine approval much in the same way as modified COVID-19 vaccines are approved to address VOCs. Importantly, because mechanisms of protection may vary by vaccine platform, such a clinical immunobridging strategy is likely only applicable to vaccine candidates that are based on the same or similar platform as that of the licensed comparator vaccine. This strategy was discussed by global regulators at a recent workshop entitled " Realizing the potential of correlates of protection for vaccine development and licensure" sponsored by Wellcome held in London, UK, in September 2022. Regulators considered clinical immunobridging studies to infer effectiveness of filovirus vaccine candidates a useful approach provided supportive data would be available. Such data should consist of challenge/protection studies in NHPs demonstrating protective effectiveness of filovirus vaccine candidates against the respective challenge viruses (e.g, SUDV, MARV). Of note, while data derived from challenge/protection studies in animal models would be supportive of the clinical immunobridging strategy, this approach would not be an approval under the AR as the primary data would be derived from comparison of human clinical immunogenicity. Additional supportive data should provide evidence that the pathogenicity and immune mechanism of protection for the filoviruses are similar and that the immune response (humoral and cell mediated immune response) induced by the filovirus candidate vaccines is comparable to that induced by the licensed comparator. The importance of validated assays to assess the immune response induced by the various filovirus vaccine candidates was stressed.

Regardless of the approach chosen to demonstrate effectiveness of filovirus vaccine candidates, clinical safety studies to support a favorable benefit risk ratio of the vaccine will be essential. In addition, real world effectiveness studies of the vaccine post-licensure in the event of an outbreak should be conducted to confirm clinical effectiveness.

In summary, additional vaccines to protect people from filovirus disease in endemic areas, notably Africa, are critically needed. There are a number of approaches to demonstrating vaccine effectiveness including clinical disease endpoint efficacy trials, use of scientifically well-established immune markers, surrogate endpoints *reasonably likely* to predict protection and challenge/protection studies in adequate animal models. In addition, we propose clinical immunobridging studies comparing filovirus vaccine candidates to licensed filovirus comparator vaccines as an approach to infer vaccine effectiveness. Clinical immunobridging has the advantage of being able to directly bridge to clinical efficacy data by way of the licensed comparator vaccine. This approach would need to be supported by data derived from challenge/protection studies in animal models, data on the pathogenesis and protective immune mechanisms for filoviruses and a characterization of the immune response induced by the vaccines. In all cases, the combined data will need to support reasonable likelihood of clinical benefit and a favorable benefit-risk profile. It is the preponderance and strength of the evidence that will determine the licensure pathway used by regulatory authorities.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

SR is currently an employee of GSK group of companies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

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SR is currently an employee of GSK group of companies, but any views or opinions in this paper are personal and do not necessarily reflect those of his employer.

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Mid-titer human convalescent plasma administration results in suboptimal prophylaxis against SARS-CoV-2 infection in rhesus macaques

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Introduction: SARS-CoV-2 is a respiratory pathogen currently causing a worldwide pandemic, with resulting pathology of differing severity in humans, from mild illness to severe disease and death. The rhesus macaque model of COVID-19 was utilized to evaluate the added benefit of prophylactic administration of human post-SARS-CoV-2 infection convalescent plasma (CP) on disease progression and severity.

Methods: A pharmacokinetic (PK) study using CP in rhesus monkeys preceded the challenge study and revealed the optimal time of tissue distribution for maximal effect. Thereafter, CP was administered prophylactically three days prior to mucosal SARS-CoV-2 viral challenge.

Results: Results show similar viral kinetics in mucosal sites over the course of infection independent of administration of CP or normal plasma, or historic controls with no plasma. No changes were noted upon necropsy via histopathology, although there were differences in levels of vRNA in tissues, with both normal and CP seemingly blunting viral loads.

Discussion: Results indicate that prophylactic administration with mid-titer CP is not effective in reducing disease severity of SARS-CoV-2 infection in the rhesus COVID-19 disease model.

KEYWORDS

SARS-CoV-2, COVID-19, rhesus macaque, convalescent plasma, prophylaxis

Introduction

SARS-CoV-2, a pathogenic beta-coronavirus, is the cause of an ongoing worldwide pandemic. The disease resulting from infection by this virus, COVID-19, while largely presenting as a mild to moderate self-limiting respiratory illness, affects a percentage of individuals much more severely. This has resulted in almost six million deaths worldwide (1), and over one million deaths in the US (2), to date. The virus is highly transmissible as an airborne respiratory pathogen, with a low estimated infectious dose, making it highly successful at inducing large numbers of infections, often moving through populations rapidly. This has resulted in a large effort to produce an effective therapeutic or prophylaxis against infection or severe disease, in addition to the efforts toward production and distribution of vaccines.

Few options for prophylaxis and therapy were available early during the pandemic. One of the investigated options consisted of administration of convalescent plasma (CP) from individuals who recovered from prior infection by SARS-CoV-2. Some initial work indicated a potential for modulation of severe disease (3) and lowering viremia (4). This early promise led to clinical trials, specifically the Mayo Clinic's COVID-19 Convalescent Expanded Access Program (EAP), eventually resulting in emergency use authorization from the FDA for administration to COVID-19 patients (5). Administration of CP has been correlated with lowered positivity by PCR for SARS-CoV-2 (6), and early delivery has been shown to reduce progression of disease (7). Accordingly, in designing this study, a prophylactic approach was selected initially to evaluate protective benefit of this source of CP before subsequent therapeutic assessments were performed.

Despite the early optimism surrounding CP administration, later analyses determined there to be no benefit, though there were no analyses performed on group subsets (8). The clinical trial focusing on emergency department CP treatment (NHLBI C3PO) was discontinued (9), and the RECOVERY trial showed no difference in 28-day mortality with or without treatment with CP (10). Many of these studies focused on administration of CP at a later time point, such as within 72 hours post symptom onset (8). This late time point administration may have a negative effect on the efficacy of CP therapy.

We hypothesize that administration of CP prior to SARS-CoV-2 challenge will maximize protective effect of the prophylactic intervention. Prior work has shown administration within 24 hours has a limited effect on viral shedding and clinical signs of disease (11). We utilize a nonhuman primate model of infection, shown before as susceptible to a mild to moderate disease process (12), to investigate the prophylactic administration of CP.

Materials and methods

Study approval

The Tulane University Institutional Animal Care and Use Committee approved all procedures used during this study. The Tulane National Primate Research Center (TNPRC) is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC no. 000594). The U.S. National Institutes of Health (NIH) Office of Laboratory Animal Welfare number for TNPRC is A3071-01. Tulane University Institutional Biosafety Committee approved all procedures for work in, and removal of samples from, Biosafety Level 3 laboratories.

Virus and cells

Virus used for animal inoculation was strain SARS-CoV-2; 2019-nCoV/USA-WA1/2020 (BEI# NR-52281) prepared on subconfluent VeroE6 cells (ATCC# CRL-1586) and confirmed via sequencing. VeroE6 cells were used for live virus titration of biological samples and were maintained in DMEM (#11965092, Thermo Scientific, USA) with 10% FBS.

Animals and procedures

A total of nine rhesus macaques of Indian origin (*Macaca mulatta*), between 3 and 11 years old, were utilized for this study. All rhesus macaques (RMs) were bred in captivity at TNPRC. For the PK study, three RMs were intravenously infused at standard rates with 4 mL/kg of human convalescent plasma (CP) obtained from prior, recovered SARS-CoV-2 infection or normal plasma (NP). Serum from RMs were monitored for RBD binding as well as neutralizing activity routinely for 68 days to determine pharmacokinetics. The NT_{50} of the CP used for both the PK and challenge studies was 1:1597 by pseudovirus neutralization assay. This plasma met the FDA recommended minimum neutralizing titer of CP to be used in therapy against SARS-CoV-2 of 1:160 (13). We define this as mid-titer plasma due to meeting the FDA recommended limit but falling below that of prior work selecting high titer plasma at levels of 1:3200 or above (7).

For the viral challenge study, four of the RMs were intravenously infused at standard rates with 4 mL/kg CP three days before challenge, with two RMs similarly infused with normal plasma. They were then exposed via intratracheal/intranasal (IT/IN) installation of viral inoculum (1mL intratracheal, 500 uL per nare, total delivery 2e+6 $TCID_{50}$). Four historic controls of the same species and viral challenge dose, variant and route, are utilized for the purposes of comparisons in figures. Animal information, including plasma dosage and type, can be found in Table 1. Historic controls are listed as the final 4 animals.

The animals were monitored twice daily for the duration of the challenge study, with collections of mucosal swabs (nasal, pharyngeal, bronchial brush) as well as fluids (bronchoalveolar lavage) were taken pre-exposure as well as post-exposure days 1, 2, 3, 5 and at necropsy (or 1, 3 and necropsy for bronchial brush and BAL). For the PK study, BAL was performed through day 21 post infusion. Bronchial brushes were performed endoscopically. BAL consisted of instillation of 40mL of saline via feeding tube followed by removal via the same tube. Blood was collected pre-exposure, as well as 1, 2, 3, 5 and at necropsy for the challenge study, or up to day 68 post infusion for the PK study, in order to follow antibody levels. Physical examinations were performed daily after exposure, and necropsy occurred between 7- and 9-days post-exposure. During physical examination, rectal temperature and weight of each animal was performed. No animals met humane euthanasia endpoints during this study. Animals were euthanized at prescribed timepoints based upon experimental design of this evaluation. Animals were first anesthetized using ketamine and

TABLE 1 Study Animal Information.

Animal ID	Species	Age (years)	Source	Sex	Weight (kg)	Viral Dose ($TCID_{50}$)	Plasma Dose (mL/kg)	Plasma Type
II67	*Macaca mulatta*	11	TNPRC	F	8.3	N/A	4	CP
JK23	*Macaca mulatta*	9	TNPRC	M	9.6	N/A	4	CP
L147	*Macaca mulatta*	5	TNPRC	M	9.1	N/A	4	CP
LI78	*Macaca mulatta*	5	TNPRC	M	8.2	2.0 X 10^6	4	NP
LL28	*Macaca mulatta*	4	TNPRC	M	7.7	2.0 X 10^6	4	NP
LC59	*Macaca mulatta*	6	TNPRC	M	7.5	2.0 X 10^6	4	CP
IE32	*Macaca mulatta*	11	TNPRC	F	7.4	2.0 X 10^6	4	CP
JJ76	*Macaca mulatta*	9	TNPRC	F	7.6	2.0 X 10^6	4	CP
LJ15	*Macaca mulatta*	5	TNPRC	M	9.2	2.0 X 10^6	4	CP
LM74	*Macaca mulatta*	4	TNPRC	M	6.2	2.0x106	N/A	N/A
IK92	*Macaca mulatta*	11	TNPRC	M	6.9	2.0 X 10^6	N/A	N/A
KF89	*Macaca mulatta*	8	TNPRC	M	8.2	2.0 X 10^6	N/A	N/A
LM30	*Macaca mulatta*	4	TNPRC	M	8.1	2.0 X 10^6	N/A	N/A

N/A, not applicable.

then administered euthanasia agent (Fatal plus, sodium pentobarbital, Lexington, KY). Death was confirmed by auscultation and absence of heartbeat. During necropsy, tissues were collected in media, fresh frozen, or in fixative for later analysis.

Prior to being assigned to the study, animals underwent the following: physical examination by a veterinarian, assessment of hematology and clinical chemistry, fecal direct and indirect examinations for intestinal parasites, and viral/pathogen screenings (including simian immunodeficiency virus (SIV), simian retrovirus type D (SRV), measles virus (MV), human papilloma virus 2 (HPV2), simian t-lymphotropic virus 1 (STLV1), SARS-CoV-2, *Mycobacterium tuberculosis, Burkholderia* sp., *Shigella* sp., *Salmonella* sp., *Campylobacter sp*, *Escherichia coli*, *Tryapanosoma cruzi*, *Plasmodium* sp., and the study-specific pathogen SARS-CoV-2. Only animals considered healthy and determined to be free of screened pathogens were assigned to the study.

The animals underwent a one-week acclimation period following transfer to the ABSL3 facility prior to challenge for the purpose of allowing physiological and psychological stabilization before experimental manipulation. The TNPRC facilities are accredited by AAALAC International. Housing space requirements set forth by The Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act are used to establish the minimum standard for housing all species at the TNPRC. Nonhuman primate standard caging dimensions are 4.3 ft^2 x 36"H for those animals under 10kg, which included all animals under this study. The temperature set points for holding rooms for all nonhuman primates housed at the TNPRC ranged between 69-72°F, with a relative humidity target of 70%. Light cycle was set at 12:12 h of light:dark. All nonhuman primates were fed Purina LabDiet nonhuman primate diet, which is nutritionally complete. The Purina Mills diet was supplemented with a variety of fruits and vegetables at a minimum of three times each week. Water was provided *ad libitum*. For all procedures, animals were anesthetized per internal SOPs, with pain control occurring as per veterinary discretion.

Quantification of Viral RNA in swab and tissue samples

Viral load in tissues, swabs and BAL cells and supernatant was quantified using RT-qPCR targeting the nucleocapsid (genomic and subgenomic) or envelope gene (subgenomic) of SARS- CoV-2. RNA was isolated from non-tissue samples using a Zymo Quick RNA Viral Kit (#R1035, Zymo, USA) or Zymo Quick RNA Viral Kit (#D7003, Zymo, USA) for BAL cells, per manufacturer's instructions. RNA was eluted in RNAse free water. During isolation, the swab was placed into the spin column to elute the entire contents of the swab in each extraction. BAL supernatant was extracted using 100 µL, and serum was extracted using 500 µL. Viral RNA (vRNA) from tissues was extracted using a RNeasy Mini Kit (#74106, Qiagen, Germany) after homogenization in Trizol and phase separation with chloroform.

Isolated RNA was analyzed in a QuantStudio 6 (Thermo Scientific, USA) using TaqPath master mix (Thermo Scientific, USA) and appropriate primers/probes (14) with the following program: 25°C for 2 minutes, 50°C for 15 minutes, 95°C for 2 minutes followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Signals were compared to a standard curve generated using *in vitro* transcribed RNA of each sequence diluted from 10^8 down to 10 copies. Positive controls consisted of SARS-CoV-2 infected VeroE6 cell lysate. Viral copies per swab were calculated by multiplying mean copies per well by amount in the total swab extract, while viral copies in tissue were calculated per microgram of RNA extracted from each tissue.

Detection of neutralizing antibodies in serum

The ability of antibodies in serum to disrupt the binding of the receptor binding domain (RBD) of SARS-CoV-2 spike protein to Angiotensin Converting Enzyme (ACE_2) was assessed via the Surrogate Virus Neutralization Test (GenScript# L00847) using the

included kit protocol modified per the following: Serum samples were diluted from 1:10 to 1:21, 870 to determine an IC_{50} for RBD/ACE_2 binding. Pseudovirus neutralization testing of matched serum was performed using a SARS-CoV-2 D614G spike-pseudotyped virus in 293/ACE_2 cells, with neutralization assessed via reduction in luciferase activity (15, 16). Pseudovirus assay was utilized for both determination of RM plasma titers over time as well as CP material prior to RM infusion.

Statistical analysis

Comparisons between the area under the curve measurements of vRNA were made using ANOVA with Geisser-Greenhouse correction and Holm-Šídák multiple comparisons test. Time-based comparisons of vRNA were performed using a two-way ANOVA with Geisser-Greenhouse correction and a Tukey multiple comparisons test.

Results

Antibody levels wane rapidly following CP administration

We infused three RMs with convalescent plasma and followed antibody levels long term for pharmacokinetic determination (Figure 1A). Using a surrogate ELISA examining the ability of antibody to disrupt RBD/ACE_2 interaction, we determined antibody levels were higher overall in BAL on day 3 post infusion, but wane in both BAL and serum rapidly (Figures 1B, C). Pseudovirus inhibition assay performed on serum shows a similar pattern, with levels falling to baseline in one individual by day 10 post infusion (Figure 1D). Based on this data, a separate set of RMs were challenged with SARS-CoV-2 three days post infusion with normal plasma (NP) or convalescent plasma (Figure 1A). One day post challenge, ID_{50} levels were between 1:40 and 1:82 (data not shown).

Mucosal viral RNA content shows mild reductions with use of CP

We challenged a cohort of RMs with $2x10^6$ $TCID_{50}$ SARS-CoV-2 and followed viral loads via qPCR using nasal and pharyngeal swabs, bronchial brushes, and BAL cell isolation, as these are the primary sites of infection for this virus. No differences were found between the AUC of viral genomic or subgenomic content of RMs administered normal or convalescent plasma except for BAL cell subgenomic E content (Figure 2). No differences were found between those two groups and historic controls administered no plasma at all (Figure 2). Some sites, including bronchial brush, show a trend toward a reduction in vRNA in individuals administered CP, indicating a potential effect that is not significant due to small sample size.

Viral RNA day-by-day shows an increase in subgenomic N content one day post challenge in the cohort administered no plasma as compared to that administered normal plasma. No other significant differences were seen, though there were patterns indicative of slight effect (Figure 3). Viral RNA content was blunted earlier in the bronchi upon CP administration compared to normal plasma, but did not reach significance, likely again due to a small

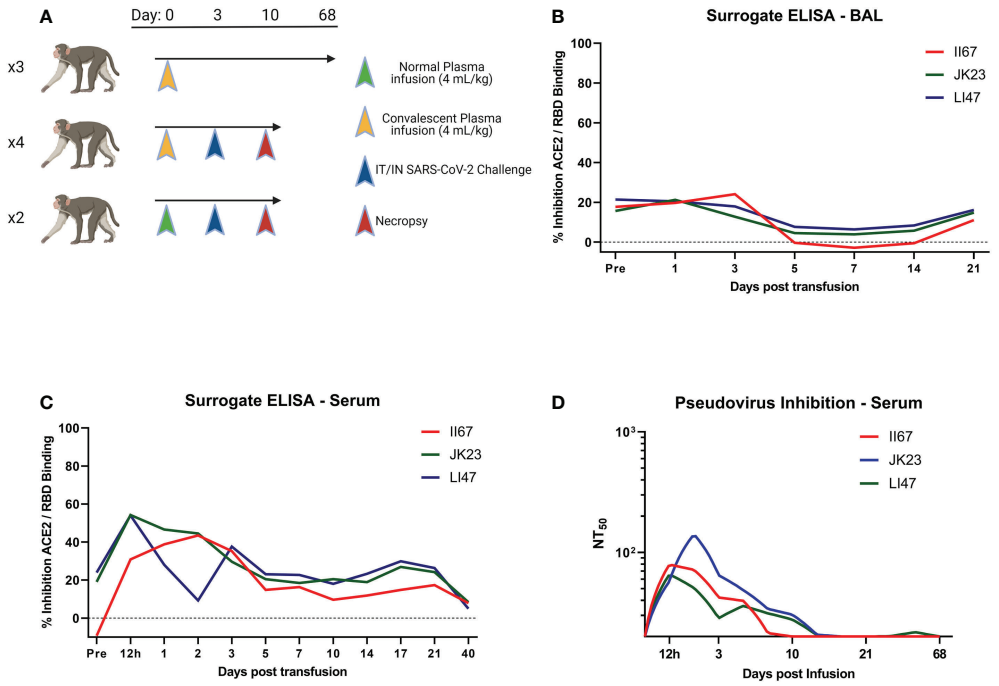

FIGURE 1
Study Design and Antibody Kinetics. **(A)** Three RMs were given 4 mL/kg CP and followed for 68 days to determine pharmacokinetics. Following this, 6 RMs were given either NP or CP and challenged with SARS-CoV-2 three days later. **(B, C)** Surrogate ELISA was used to follow plasma kinetics of BAL and serum, respectively. **(D)** Serum kinetics were followed by pseudovirus inhibition assay. Study design made in Biorender.

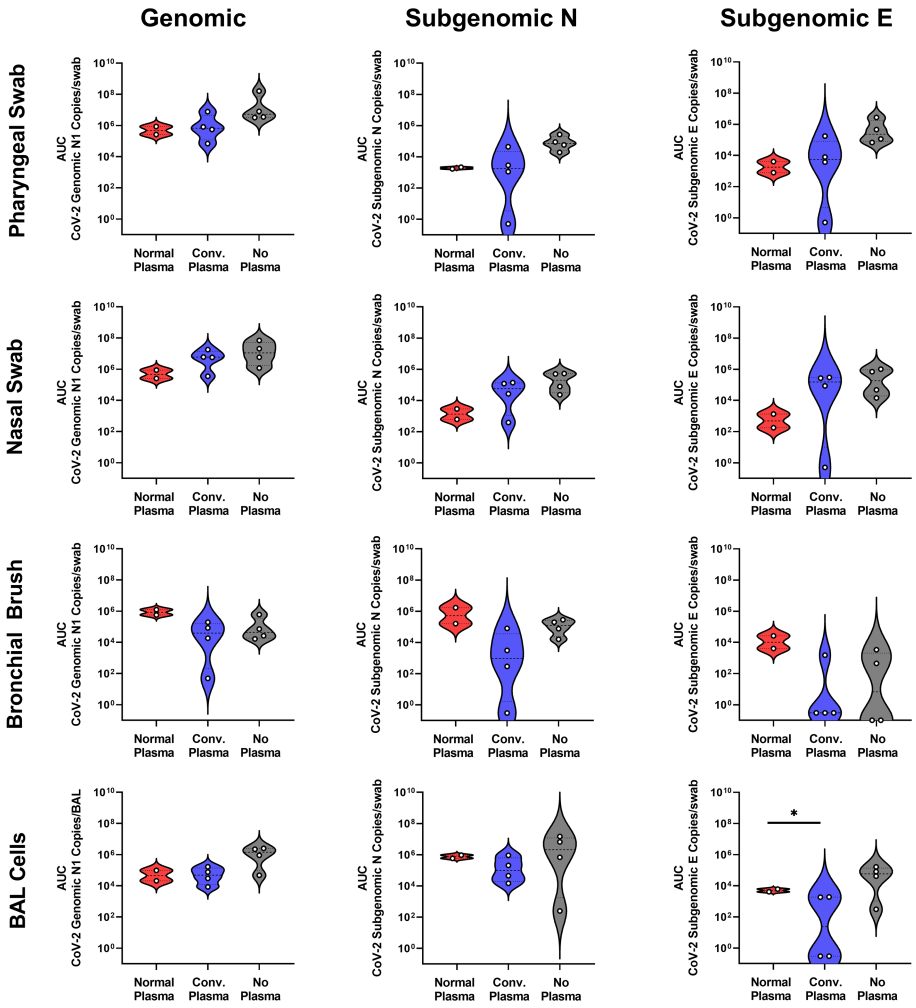

FIGURE 2
Mucosal vRNA Content post SARS-CoV-2 Challenge. Viral loads, measured via genomic N and subgenomic (E and N) vRNA content, were determined in pharyngeal and nasal swabs, as well as bronchial brushes and BAL cells. Data is represented as area under the curve of vRNA content over the course of the study. Groups were compared via ANOVA with Geisser-Greenhouse correction and Holm-Šídák multiple comparisons test (*p<0.05).

sample size (Figure 3). Genomic and subgenomic content persisted longer in the pharyngeal swabs in both the CP and no plasma cohorts (Figure 3).

Tissue viral RNA content at necropsy is similar between normal plasma and CP

After necropsy, vRNA content was examined in respiratory and gastrointestinal (GI) tissues. In respiratory sites, genomic and subgenomic content was generally lower in both NP and CP animals than the no plasma cohort. Gastrointestinal sites showed higher genomic vRNA loads in the CP cohort than the NP cohort, but less than the no plasma cohort. Very little subgenomic N vRNA was found in the GI tract, with the same pattern being displayed. No subgenomic E content was found in either NP or CP cohorts for either respiratory or gastrointestinal sites (Figure 4).

In addition to vRNA content of tissues, administration of CP did not alter the clinical course of SARS-CoV-2 infection. No significant disease was observed in either treated or untreated animals beyond mild respiratory signs that were not significantly different between cohorts.

Discussion

Convalescent plasma therapy has been used for infectious diseases for over a century since von Behring developed the practice in the late 19th century. Early on mostly utilized for bacterial diseases, it has also been used against viral infections spread by the respiratory route including influenza and measles (17–20). Mechanisms thought to be protective include neutralization that mitigates viral burden as well as non-neutralizing Fc-based antibody functions that reduce lung inflammation (21). Due to the novel nature of SARS-CoV-2 at the beginning of the worldwide pandemic, CP was explored as a

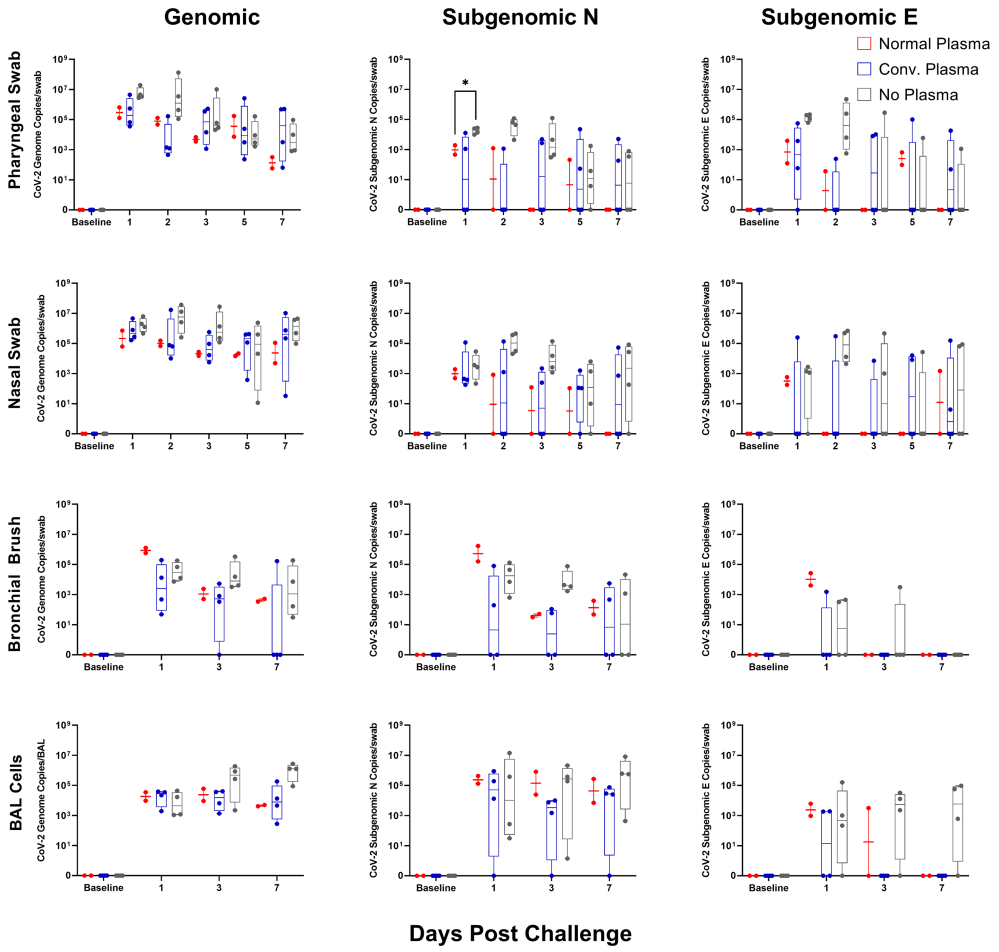

FIGURE 3
Mucosal vRNA Content post SARS-CoV-2 Challenge. Viral loads, measured via genomic N and subgenomic (E and N) vRNA content, were determined in pharyngeal and nasal swabs, as well as bronchial brushes and BAL cells. Data is represented as copies per swab or BAL of vRNA content per collection timepoint. Groups were compared via two-way ANOVA with Geisser-Greenhouse correction and a Tukey multiple comparisons test (*p<0.05).

therapeutic approach in the absence of any available virus-specific antiviral or vaccine. Accordingly, we tested the prophylactic potential of CP administered three days prior to infection with SARS-CoV-2. We demonstrate that CP that is of insufficient titer does not alter most viral kinetics in the host. This data underscores the lack of utility of CP in the prophylaxis of COVID-19.

Antibodies capable of RBD/ACE2 binding inhibition or neutralization were highest in BAL at three days post

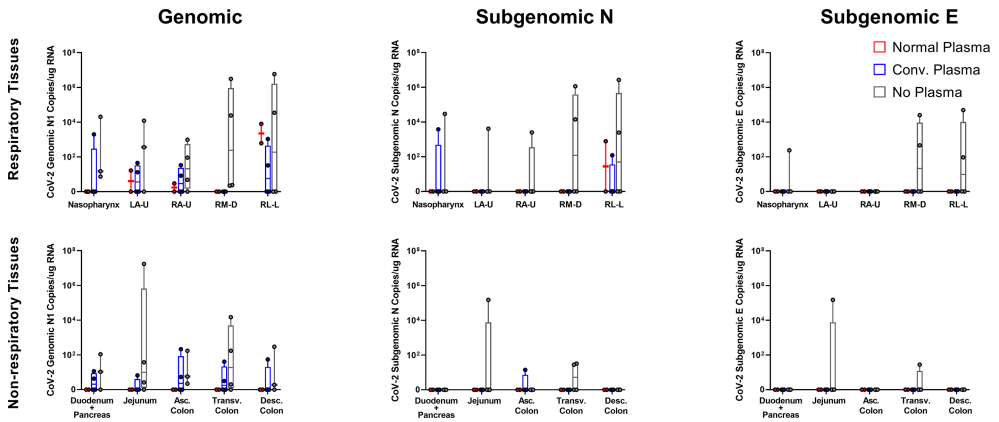

FIGURE 4
Tissue vRNA Content post SARS-CoV-2 Challenge. Viral loads, measured via genomic N and subgenomic (E and N) vRNA content, were determined in respiratory and digestive tissues, as well as bronchial brushes and BAL cells. Data is represented as copies per ug extracted RNA at necropsy.

administration and between 12 hours and three days post administration in serum, though particularly high levels were never achieved, even with the more sensitive pseudovirus inhibition assay. Human antibody kinetics in macaques are skewed toward shorter half-lives, though this 3-day time frame is not of sufficient length for that be a driving factor of efficacy in this study (22). Viral kinetics in mucosal sites were similar regardless of administration of NP or CP. Tissue viral loads showed differences, with NP and CP both seemingly able to lower tissue vRNA content one week post challenge, especially in the subgenomic content. There was nothing significant noted in histopathology at necropsy (data not shown).

This work agrees with earlier work in the nonhuman primate model that focused on CP administration soon after SARS-CoV-2 challenge, also utilizing mid-titer CP (11). Lack of histopathological modification, viral kinetics, or changes in development of later immune responses such as antibody development indicates lack of disease modification capacity whether given early after infection, or even before infection. Treatment early post infection with high titer CP in another nonhuman primate model did produce a potentially clinical benefit, with reduced lung pathology and viral loads seen (23). This indicates a high degree of variability in therapeutic efficacy between pools of CP, leading to less certainty as to the effective nature of any given CP lot.

In contrast, prophylaxis by potent, long half-life, neutralizing monoclonal antibodies has been shown in this model of SARS-CoV-2 infection to reduce viral loads at mucosal sites up to 75 days post administration (14). With multiple lots of a monoclonal antibody (mAb) preparation being much more standardized than a CP preparation (24), this makes therapeutics consisting of mAbs much more appealing. Indeed, trials regarding therapy via CP have been discontinued due to lack of apparent efficacy (9, 10). With the advent of robust vaccines and antiviral medications, CP therapy and prophylaxis are no longer an avenue of significant exploration regarding infection by SARS-CoV-2.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Tulane IACUC.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Pre-clinical models to define correlates of protection for SARS-CoV-2

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A defined immune profile that predicts protection against a pathogen-of-interest, is referred to as a correlate of protection (CoP). A validated SARS-CoV-2 CoP has yet to be defined, however considerable insights have been provided by pre-clinical vaccine and animal rechallenge studies which have fewer associated limitations than equivalent studies in human vaccinees or convalescents, respectively. This literature review focuses on the advantages of the use of animal models for the definition of CoPs, with particular attention on their application in the search for SARS-CoV-2 CoPs. We address the conditions and interventions required for the identification and validation of a CoP, which are often only made possible with the use of appropriate *in vivo* models.

KEYWORDS

animal models, SARS-CoV-2, vaccines, immunity, correlates of protection (CoP)

1 Introduction

The outbreak of a novel coronavirus, subsequently named severe acute respiratory coronavirus 2 (SARS-CoV-2), in Wuhan, China, in early December 2019, rapidly escalated to pandemic status on March 11th 2020 (1). SARS-CoV-2, the causative agent of coronavirus disease, COVID-19, has since claimed over 6 million lives (2). Fortunately, the development, approval and deployment of effective SARS-CoV-2 vaccines has since significantly reduced fatality rates and ameliorated the impact of SARS-CoV-2 infections on billions of lives.

Since the beginning of the pandemic, international efforts have been made to understand SARS-CoV- 2 pathogenicity and host immune responses. However, the immune profile/s associated with protection is/are still unclear. A person's immunity to a pathogen can be inferred by measurement of the component of the "immune response that is responsible for and statistically interrelated with protection," also known as a 'correlate of protection (CoP)', a term coined by Stanley Plotkin (3). Profiling the prevalence of a CoP in community samples of vaccinees and convalescents would efficiently capture rates of immunity against the disease of interest. Hence, a CoP is a

parameter that is invaluable for the fields of vaccinology, infectious disease immunology, epidemiology and health policy.

To define a CoP unequivocally, animal research remains critical - from the early stages of understanding the immunology of a disease, to providing proof-of-concept evidence in support of candidate CoPs. SARS-CoV-2 is no different, with COVID-19 animal models being central to the extraordinary immunology research and vaccine development efforts over the last three years. This review focuses on the mechanisms by which SARS-CoV-2 animal research assists, and in some cases supersedes, human research in the search for SARS-CoV-2 CoPs, with reference to the immunological insights provided by COVID-19 animal models used in challenge and vaccine pre-clinical studies.

2 Correlates of protection

Identifying a CoP that is universally observed and easily measured in an immunised population is of significant value for; a) vaccine development, such that vaccine candidates aim to drive the protective response, b) vaccine licensure, whereby protection likelihood can be inferred upon measurement of a CoP in vaccinees (also known as immunobridging), and c) public health policy-making in the midst of a pandemic, where accurate rates of immunity can be measured to appropriately inform governmental decisions on the application of non-pharmaceutical interventions (NPIs). A prime example of a validated CoP that supports vaccine development is haemagglutinin inhibition (HAI) titres (of at least 1:40) for the approval of seasonal influenza vaccines (4).

For the purpose of this review, it is important to consider precisely what 'protection' means in the context of SARS-CoV-2 infection, particularly as COVID-19 has many variable manifestations in humans, including asymptomatic, mild, moderate, severe or fatal disease, and chronic disease. Successful management of a pandemic relies on reduced transmission rates and/or lower rates of hospitalisation, therefore either low virology scores or minimal pathology could be considered as a 'protected' outcome. This review focuses on animal models where veterinary pathologist assessments, post-cull, engender the scope for precise characterisation of clinical as well as virology outcomes post-challenge. This is particularly valuable as the mechanism of limiting viral load may differ from the mechanism of infection control and resolution.

The terms 'co-correlate' and 'surrogate' describe a collaborative and redundant network of protective immune mechanisms, respectively (3). 'Absolute' and 'relative' correlates capture the weight of involvement of the immune parameter in mediating protection, either by dominating the protective response (the former) or by variably contributing to protection (the latter) (3). In the search for a CoP, knowledge gaps must also be accounted for; a statistically significant CoP may, in actuality, be a by-product of an unknown protective mechanism. We need to recognise the limitations and shortcomings of statistical correlations and that even with supporting 'proof-of-concept' investigations, such as adoptive transfer or depletion experiments, unknown knock-on effects of such immune manipulations may contribute to the observed exacerbation or elimination of infection.

Additionally, given immune system variability with age, sex, genetics, epigenetics and microbiome composition, a CoP in one population may differ to that in another population, particularly with respect to the magnitude of responses and epitope-specific responses. In which case, a universal CoP for SARS-CoV-2 may not exist, with different parameters defining protection in different groups. Hence, it is important to bear in mind which populations are involved in the study and whether data from different cohorts is pooled for analysis in an attempt to extract a universal CoP, or alternatively the data is separated by age, sex or ethnicity to define cohort-specific CoPs. Furthermore, each vaccine platform or formulation may exploit different protective immune mechanisms, thereby pointing to the existence of vaccine-specific CoPs. Such factors convolute the definition of a CoP.

3 COVID-19 animal models

It is essential that CoP research is based on authentic, relevant and reproducible *in vivo* models. Since the emergence of SARS-CoV-2 in December 2019, significant efforts have been made to develop accurate COVID-19 animal models (as extensively reviewed in (5–7) and summarised in Figure 1 and Table 1) to probe and understand COVID-19 pathology and immunology in order to develop effective therapeutics and vaccines. Given how critical an animal model is for research into a novel, pandemic-causing virus, the World Health Organisation (WHO) Research and Development Blueprint Team launched WHO-COM, a group focused on 'COVID-19 *in vivo* modelling' (55), as has the National Institute of Health (NIH) through the 'Accelerating COVID-19 Therapeutic Interventions and Vaccines' (ACTIV) preclinical working group.' The WHO recognises non-human primates (NHPs), ferrets, mice and hamsters, to differing degrees, as models that capture the major physical manifestations of COVID-19, including pathology and/or immune signatures.

The genus of Old World Monkeys (OWMs) (taxonomically known as *Cercopithecidae*), are the gold standard for modelling human disease, given the extensive homology between the closely-related species that diverged twenty-three to twenty-five million years ago. The Rhesus Macaque Genome Sequencing and Analysis Consortium reported that macaques and humans share 93.54% sequence identity, with 97.5% sequence identity in high confidence orthologues (56). Salguero et al. has provided a direct comparison of the key macaque species used in COVID-19 research, rhesus macaques (*Macaca mulatta*) (RhMs) and cynomolgus macaques (*Macaca fascicularis*) (CyMs). Matched viral strains, viral loads and routes of challenge were used to conclude that RhMs and CyMs have a similar capacity to recapitulate a mild-to-moderate human SARS-CoV-2 infection, both pathologically, virologically and immunologically (8).

BALB/c and C57BL6 mice also model COVID-19, with the added advantages of housing- and manipulation-ease. They share 69.1% sequence identity with humans. Murine angiotensin-converting enzyme 2 (ACE2), which shares 82.11% of its gene sequence with humans, does however have negligible affinity to SARS-CoV-2 Spike (57). Thus, the murine SARS-CoV-2 infection

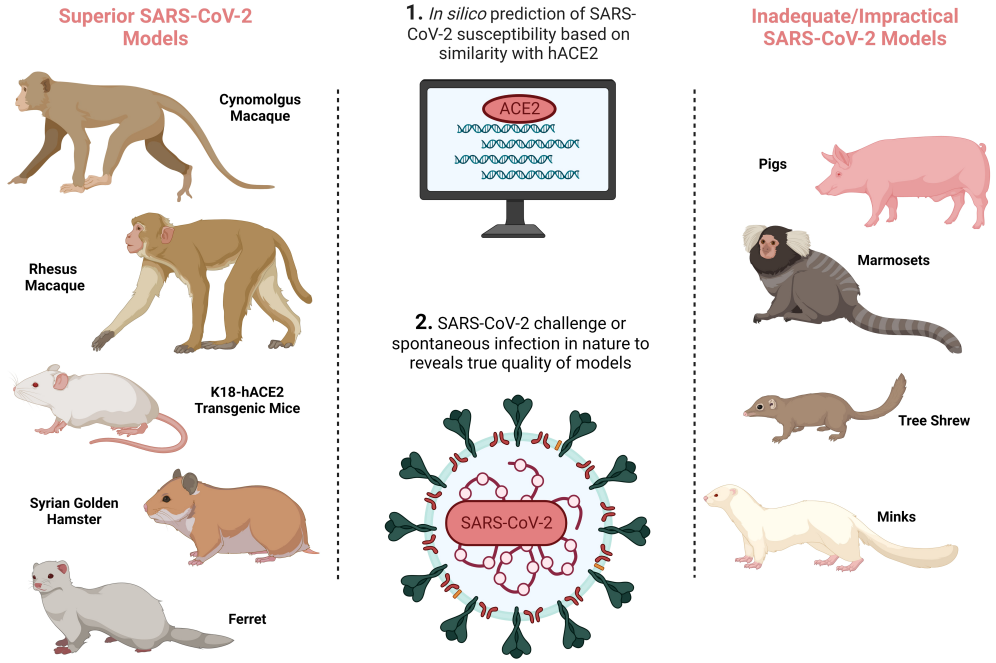


FIGURE 1
SARS-CoV-2 animal model development overview demonstrating the preferred SARS-CoV-2 animal models on the left, and inadequate/impractical COVID-19 models with defined limitations on the right, and the processes by which animal models are identified in the central column. Created with BioRender.com.

model requires either genetic manipulation of the SARS-CoV-2 virus so as to become mouse-adapted, or the breeding of transgenic mice that express human ACE2 (hACE2) e.g. K18-hACE2 C57BL/6J mice (a flaw of which is the variability of hACE2 expression levels) (7). It is important to note that cause of death in mice following SARS-CoV-2 infection is high viral burden in the brain rather than in the lung, implying COVID-19 pathology differs between the species (42). However, these models do capture human COVID-19 clinical symptoms of anosmia, thrombosis, and weight loss (41).

Syrian Hamster ACE2 has affinity to SARS-CoV-2 Spike and hence the species are naturally susceptible to SARS-CoV-2 infection (41). Clinical symptoms consistent with COVID-19 are seen, including weight loss, respiratory distress and inflammation-driven lung pathology (41). For example, a 5% increase in percentage weight loss as a consequence of high dose challenge was reported in Syrian hamsters, with 75% of these animals meeting the humane euthanasia criteria by day seven post-challenge, thus capturing severe COVID-19 (32). Furthermore, this small animal model mirrors the age-bias of COVID-19, with thirty-two to thirty-four-week-old hamsters exhibiting more substantial weight loss during acute infection, as well as persistent inflammation and unresolved lung tissue damage fourteen days post-challenge with SARS-CoV-2, in comparison to the six-week-old hamsters that had recovered by this timepoint (33).

Ferrets are susceptible to SARS-CoV-2 infection as evident from the mild lung pathology and detection of viral shedding in the nose and throat (41, 48). SARS-CoV-2 is detectable in the upper respiratory tract (URT) of ferrets by day two post-challenge and they present with similar URT symptoms to humans, including nasal discharge and sneezing (41).

Inadequate/impractical SARS-CoV-2 infection models include pigs, where despite *in silico* prediction of swine ACE2 and SARS-CoV-2 Spike affinity, supported by their natural susceptibility to SARS-CoV-1, they are poor SARS-CoV-2 hosts. Minks on the other hand are susceptible to SARS-CoV-1 and SARS-CoV-2 with outbreaks of the latter occurring in mink farms (58), however, their aggressive nature restricts their use as a common laboratory model (41).

4 Limitations of human studies for the definition of SARS-CoV-2 CoPs that are met by animal models

Prior to the emergence of effective vaccines, human largescale serology studies reported that SARS-CoV-2-specific immune responses were detectable following primary exposure, which conceptually could provide protection upon re-exposure (59–64). However, the degree of protection in the studied populations was heterogenous [further complicated by variants of concern (VOCs)]. Identifying the role of the humoral and/or the cellular response in mediating protection, as well as the precise characteristics of the humoral and/or cellular response that engender this protection, remains a challenge. *In vivo* models have played a key role in pin-pointing which immune parameters are mediating this protection in humans.

TABLE 1 Summary of the pros and cons associated with each model and the research papers discussed in this review that applied these models for the definition of CoPs.

Animal	Pros	Cons	Is the model in use?	COVID-19 Research References
Rhesus Macaque	* Captures Mild-Moderate COVID-19 lung pathology (8) * Old RhMs Model Severe COVID-19 (8) * 94% Genetic Similarity to Humans	* Shortages (8) * Ethical Issues * Housing requirements are costly	Yes	(8–28)
Cynomolgus Macaque	* Captures Mild-Moderate COVID-19 (8) * Restricted HLA (8)	* Lung Cell ACE2 Expression Patterns Differ to Humans (29) * Ethical Issues * Housing requirements are costly	Yes	(8, 30, 31)
Syrian Golden Hamster	* Small * Models Severe COVID-19 when Challenged with High Doses (32) * Captures age-bias of COVID-19 (33)	* Lack of Hamster Reagents * Lung Cell ACE2 Expression Patterns Differ to Humans (34)	Yes	(12, 13, 32, 33, 35–40)
K18-hACE2 C57BL/6 and hACE2 BALB/c Mice	* Small * Manipulate Genetics * Captures Clinical COVID-19 Symptoms (41)	* Inter-Mouse Variability of hACE2 Expression * High Viral Load in the Brain (42)	Yes	(40, 41, 43–47)
Ferret	* Susceptible to SARS-CoV-2 Infection * Captures Mild Clinical Symptoms (41, 48)	* Predominantly Models URT Infection (41)	Yes	(21, 23, 49)
Mink	* Susceptible to SARS-CoV-2 Infection (41)	* Lab Handling Difficulties	TBD	
Marmoset	* Susceptible to SARS-CoV-2 Infection	* Fail to Mount IgG Response (50)	No	
Tree Shrew	* Susceptible to SARS-CoV-2 Infection	* No SARS-CoV-2 Shedding (51) * Only Clinical Symptom is Body Temperature Changes (51) * Inverse age bias (52)	No	
Pig	* Strong similarities with human anatomy, physiology and immunology	* No Affinity between Swine ACE2 and Spike Protein * Not Susceptible to SARS-CoV-2 Infection (53) (54)	No	

A major limitation of human studies for the identification of CoPs, is the failure to conclude, with confidence, whether the anamnestic response following natural primary infection or vaccination is protective upon re-exposure. Firstly, researchers cannot say with certainty whether or not a convalescent or vaccinated participant has been exposed to the pathogen during the follow-up period, particularly if an individual were to have sterilising immunity against the pathogen and would therefore lack any serological evidence of breakthrough/reinfection (an anti-SARS-CoV-2 Nucleocapsid response would only emerge post-infection). Vaccine efficacy is deduced from the reduced rates of infection seen in vaccinees versus placebo controls in an area where the virus is endemic and in active circulation during the clinical trials. However, vaccinees are not necessarily ever exposed to the aetiological agent during the course of the trial. Government-enforced lockdowns and other NPIs in place during the phase II and III clinical trials would no doubt impact the rate of transmission, which firstly, may disguise the true weight of impact of vaccination while also limiting the number of infections, thus restricting the statistical significance of outcomes. Swadling et al. did attempt to ascertain exposures to SARS-CoV-2, using IFI27 as a blood biomarker of viral exposure at subclinical levels, such that PCR negativity and IFI27 positivity would suggest protection from reinfection (59, 65, 66). However, it is important to note that IFI27 is non-specific for SARS-CoV-2, and is upregulated in response to other respiratory infections including H1N1/09 influenza and respiratory syncytial virus (66). In which case, it must be recognised that only the endpoint of failed protection can be confidently defined in humans, with the eventuality of a positive PCR result post-vaccination or primary infection, while the endpoint of successful protection is largely ambiguous.

The incidence of asymptomatic COVID-19 further limits our ability to characterise successful protection. Rates of asymptomatic SARS-CoV-2 infections are high, with 47% of 165 SARS-CoV-2 positive cases in a ~12,000 frontline worker cohort reported as asymptomatic (64). PCR testing was predominantly encouraged for those presenting with COVID-19 symptoms in large-scale human studies, therefore endpoints of failed protection in the form of asymptomatic COVID-19 often fail to be recorded. The consequence of failing to capture asymptomatic cases is seen in the phase III trial of mRNA-1273. mRNA-1273 was reported to be 94.1% effective in preventing COVID-19 from fourteen days post-boost, however, this trial only identified symptomatic COVID-19 cases, given the limited capacity for routine screening of the large population size (n = ~30,000) (67). The revised efficacy of mRNA-1273 was subsequently found to be 82% when asymptomatic cases were accounted for (68). Failing to accurately capture asymptomatic

cases also limits the drawing of associations between vaccine-induced immune responses and protection against asymptomatic infection, as was unsuccessful in the study by Feng et al. (69).

Inherent human biases have also been shown to skew PCR testing frequency. In a study by Lumley et al., baseline seropositive and seronegative healthcare workers (HCWs) were screened biweekly in an attempt to define an association between the rates of PCR-positivity and anti-Spike IgG titres. The group observed that seronegative HCWs had a much greater attendance to the non-obligatory asymptomatic screenings than seropositive individuals, epitomising a phenomenon known as 'outcome ascertainment bias,' which manifests in convalescent participants with an inherent assumption that reinfection is unlikely. The problem with bias in this particular setting, is that over the course of this investigation, seropositive HCWs that did return PCR-positive results during mandatory testing presented with asymptomatic infections, further increasing the likelihood that there were undetected PCR-positive results amongst the seropositive HCWs (64). Testing of human participants must therefore be frequent and mandatory, irrespective of symptom presentation, if researchers carrying out vaccine efficacy studies wish to detect near 100% of infections.

High variability between human re-exposure events is also inevitable in a real-world setting. This includes variable viral inocula which will have an impact on whether or not COVID-19 disease manifests, and cannot be accounted for when comparing human vaccinees and control groups. Furthermore, given the rapid evolutionary trajectory of a virus upon zoonosis to a new host species, it is possible that human participants are exposed to different viral strains. In fact, reinfection of study participants or cases of vaccine breakthrough have been, and continue to often be, caused by SARS-CoV-2 VOCs that escape the immune responses induced during primary immunisation (e.g (70).). Although many studies sequence the virus from reinfected participants to confirm infection by a VOC, this complicates the interpretation of immunological parameters associated with protection. Specifically, it is difficult to extrapolate whether prior immunisation provided, or would have provided, strain-specific protection versus broad-range protection.

As the timing of viral challenge is also unknown, the viral and immune trajectories at critical timepoints post-challenge cannot be studied in human trials. Strong correlations between the IFI27 biomarker and a subset of memory CD8+ T cells, implied HCoV cross-reactive SARS-CoV-2-reactive memory CD8+ T cells to facilitate 'abortive SARS-CoV-2 infection' (59). However, if reinfection were to have occurred in a controlled manner, many immune parameters could have been traced. This would rule out the possibility that this CD8+ T cell subset is only a consequential biomarker of controlled infection, with a different mechanism actually being responsible for viral control.

Only controlled human challenge studies can overcome the aforementioned limitations of human clinical trials and longitudinal studies. However, human challenge studies are associated with high cost and risk, and in the case of SARS-CoV-2, there is also a paucity of naïve participants due to vaccination or previous challenge (71)). Therefore, though extremely valuable, human challenge studies are infrequently performed. Animal challenge studies not only fill this void, but also overcome the drawbacks of human challenge trials.

Considering the many limitations of COVID-19 vaccine efficacy clinical trials discussed above, there are several obvious benefits of animal models. Animal models overcome biases, lack of attendance to screenings and escape of asymptomatic cases, as frequent mandatory testing is considerably more feasible. In pre-clinical vaccine or rechallenge studies, animals are intranasally- and intratracheally-challenged with matched inoculation doses following vaccination. In which case there is a known, comparable exposure event, and the endpoint of protection can be determined with near certainty. Animal studies also follow identical regimens for vaccine dose administration, challenge and rechallenge, therefore immunologists can map immune landscapes at each critical timepoint. Carrying out challenge and testing under the same, known conditions facilitates direct comparability and the extraction of CoPs with greater confidence.

It is also important to remember that the definition of CoPs relies on a range of outcomes arising post-immunisation to extract immune profiles that differentiate protected from unprotected groups. However, stratification of outcomes was limited in human trials as, a) suboptimal SARS-CoV-2 vaccines did not progress through human clinical trials, b) the inclusion of placebo control groups in SARS-CoV-2 vaccine trials during the pandemic was an ethical dilemma (72) which limited the duration of study of these groups, and c) SARS-CoV-2 vaccine candidate efficacy was high. Pre-clinical studies however involved the testing of a range of vaccines with different efficacies, different dose numbers, dosing intervals and formulations, under the same conditions to yield an array of phenotypes post-challenge that increases the power behind the correlations that are drawn between immune parameters and protection.

Another advantage of using animal models to define CoPs, is that research animals, often contained within closed facilities, have a more definable immunological history in comparison to human participants that have a more diverse and undetermined virome and bacteriome. This is particularly relevant as human studies have identified a CD8+ T cell response against SARS-CoV-2 Nucleocapsid ($N_{105-113}$) epitope, presented by HLA-B*07:02, in 80% of naïve participants and is significantly associated with mild COVID-19 (73–76). This response has been proposed to originate from a seasonal human coronavirus (HCoV) infection, due to the marked homology between this SARS-CoV-2 Nucleocapsid epitope and HCoV-OC43 and HCoV-HKU1 Nucleocapsid (73–76). Similarly, Swadling et al. proposed that pre-existing memory T cells against the highly conserved HCoV proteins NSP7, NSP12, and NSP13 of the replication transcription complex (RTC) facilitates abortive infection in seronegative HCWs (59), as also reported by Kundu et al. (77). Upon a complex immunological background, it is difficult to conclude the source of protection. Animal challenge studies however, can describe more accurately the specific immune signature left by SARS-CoV-2 vaccination and infection, as they are less likely to have been exposed to closely-related HCoVs.

An array of clinical (CT, X-ray), immunological (serology and blood analysis) and viral (PCR from URT and lower respiratory tract (LRT)) assessments can be carried out on human challenge study participants at timepoints throughout immunisation and acute infection to capture the clinical, immune and viral dissemination trajectories. However, the thoroughness of pathological assessment of a human subject will never match that possible in animal subjects (except perhaps in the context of post-mortem analysis of human COVID-19 fatalities (78), which is not as relevant for CoP identification). Performing histopathologic assessment of animal tissue post-cull supports the precise definition and stratification of disease endpoints which can be compiled with immunological data to reach conclusions on CoPs for the disease-of-interest (examples of measurable immunological data are demonstrated in Figure 2).

Candidate CoPs identified from preclinical and/or human phase III vaccine trials, can also be validated in later immunology research studies involving animal models (Figure 3). For example, animal thymectomies, cell depletion or adoptive transfer experiments can be carried out to determine whether the feature identified in human phase III studies directly explains protection outcomes when an animal is rechallenged with SARS-CoV-2. Hence outputs from clinical research can also be complemented by animal research.

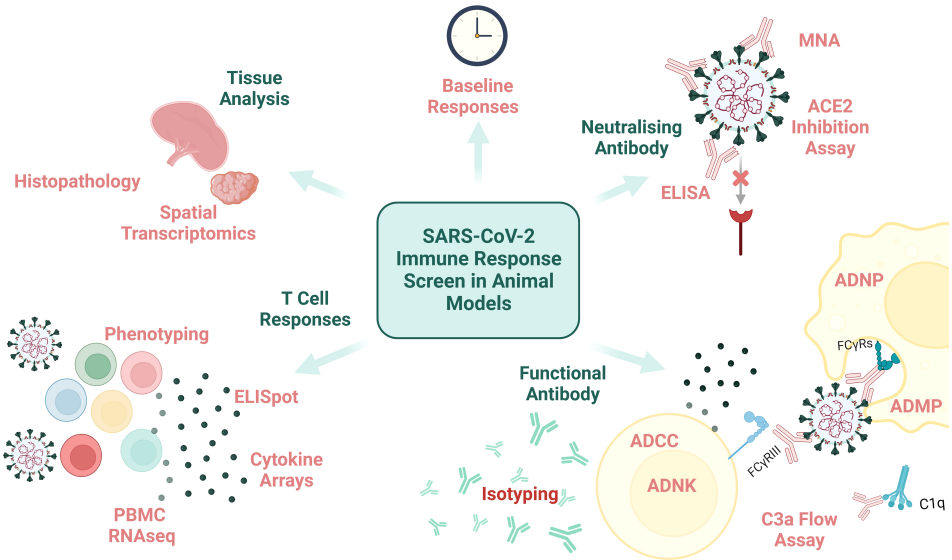


FIGURE 2
An overview of immunological assays and analysis that can be performed on animal samples. MNA, microneutralisation assay; ELISA, enzyme-linked immunosorbent assay; ADNP, antibody-dependent neutrophil phagocytosis; ADMP, antibody-dependent macrophage phagocytosis; FcγRs, fragment crystallisable of antibody receptor; ADCC, antibody-dependent cellular cytotoxicity; ADNK, antibody-dependent NK cell activation; PBMC, peripheral blood mononuclear cells; RNAseq, RNA sequencing. Created with BioRender.com.

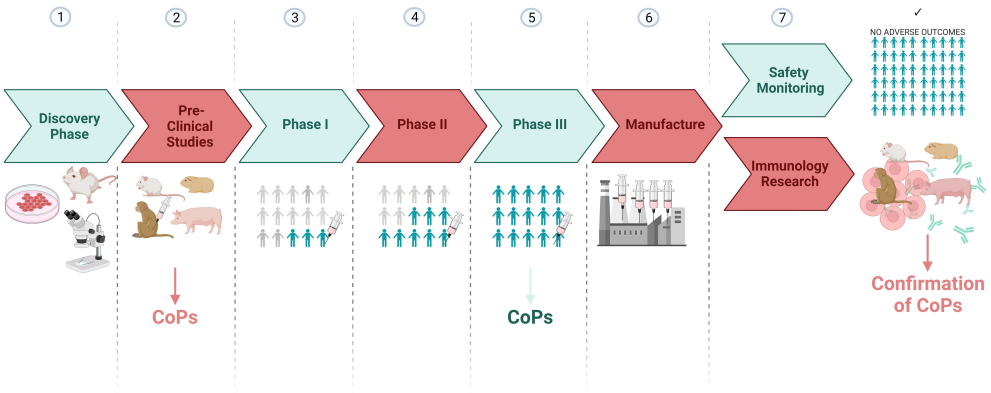


FIGURE 3
A vaccine development pipeline highlighting the stages where candidate CoPs can be identified and confirmed. Created with BioRender.com.

5 Strategies to facilitate CoP identification in COVID-19 animal models

5.1 Adoptive transfer and cell depleted animal models

As mentioned, one of the major advantages of using animal models for the identification of CoPs is the capacity to manipulate the immune response post-immunisation to demonstrate the consequence of introducing or withdrawing immune parameters on outcome post-challenge (described in Figure 4).

The independent role of T cells in protection against SARS-CoV-2, was investigated by McMahan et al. and Hasenkrug et al. in the macaque challenge model for SARS-CoV-2 by performing T cell depletion experiments.

Hasenkrug et al.'s experiment involved anti-CD4 and anti-CD8a monoclonal antibody (mAb)-mediated depletion of CD4+ and CD8+ T cells, prior to primary challenge with ~4 x 10^5 $TCID_{50}$. In the four CD8+ T cell-depleted RhMs, they report amplified CD4+ T cell numbers and responsivity in the cervical lymph nodes (LNs) and spleen during acute infection, likely to compensate for the lack of CD8+ T cell-mediated cellular adaptive immunity. Viral load measurements by qPCR confirms infection clearance in the control group by day fourteen, while in T cell-depleted groups, the infection was resolved by day twenty-one. Therefore, while the delay in viral clearance is likely attributable to the lack of T cell response, eventual clearance is not impacted by impaired T cell activity (28).

In contrast to Hasenkrug et al. that depleted the T cell subsets prior to primary infection, McMahan et al. depleted CD8+ T cells seven weeks post-primary infection, prior to rechallenge with 1 x 10^5 $TCID_{50}$, to investigate the role of memory CD8+ T cells in protection against reinfection. In comparison to the five control sham-mAb treated RhMs, that were successfully protected from reinfection, 100% and 25% of the eight CD8-depleted RhMs had detectable SARS-CoV-2 RNA in nasal swabs and BAL samples, respectively (10). Together, the results of these RhMs studies, implies a role for T cells in controlling viral load.

Despite expansion of receptor-binding domain (RBD)-responsive memory CD4+ and CD8+ T cells post-vaccination of hACE2-C57BL/6 mice with an alum-adjuvanted recombinant RBD vaccine, adoptive transfer of splenic CD4+ and CD8+ T cells from vaccinated-mice did not protect recipient naïve mice upon challenge, but passive transfer of immunised sera was found to be protective (9). Similarly, Matchett et al. reports on the persistence and expansion of Nucleocapsid ($N_{219-227}$)-specific memory CD8+ T cells in the lung and lung draining mediastinal LN post-challenge and post-successful vaccination with a SARS-CoV-2 Nucleocapsid-expressing human adenoviral vector 5 (HAd5)-vectored vaccine. However CD8+ T cell depletion only partially abrogated protection upon challenge of vaccinated K18-ACE2 C57BL/6 mice (40). While these results are most likely due to the mouse less faithfully modelling the human immune system, it is also possible that T

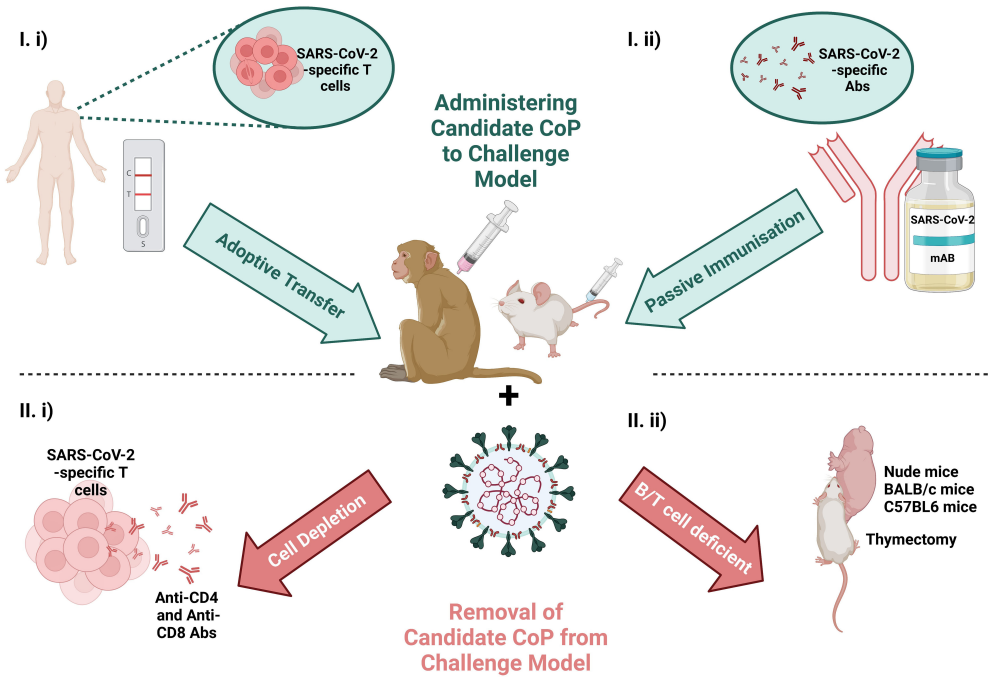


FIGURE 4
Schematic of the scope to manipulate the immunological response by I) administering the candidate CoP to the challenge model (at top in blue) *via* i) adoptive transfer of T cells from a convalescent individual or ii) passive immunisation with convalescent serum or a therapeutic mAb or II), removing candidate CoP from challenge model (at bottom in red) *via* i) T cell depletion or ii) use of an immunodeficient animal model. Challenging the animal with virus and ascertaining the effects of removal/addition of these immune features on virology and pathology, can provide evidence for/against the candidate CoPs. Created with BioRender.com.

cells are only a 'surrogate' of protection, that support the emergence of a protective humoral response.

Understanding the relationship between the cellular and humoral response in the context of COVID-19 can also be thoroughly investigated *via* T cell manipulation experiments in animal models. The aforementioned study by Hasenkug et al., observed significantly attenuated peripheral B cell responses in the CD4+ T cell-depleted RhMs in comparison to control animals, in all but the animal that failed to achieve >90% CD4-depletion, confirming the involvement of CD4+ T cells at this adaptive axis (28). A delayed induction of IgM or isotype switching post-challenge was also observed in 50% of CD4+ T cell-depleted RhMs (28). This rate of dependency on CD4+ T cells for the induction of an antibody response explains the positive correlation between anti-Spike or RBD IgG titres and Spike-specific CD4+ T cell frequency and activity, found in human studies (79). However, the effect of CD4+ T cell-depletion on antibody development had no additional consequence on outcome post-challenge of this RhM cohort (28). Therefore CD8+ T cells may sufficiently mediate protection under these circumstances, as implied by the findings of McMahan et al.

Rydyznski et al. reports that the three arms of the antigen-specific adaptive immune response (antibody, CD4+ and CD8+ T cells) were mounted successfully in 73% of mild human COVID-19 cases, with unsuccessful coordination of such responses occuring in the elderly (greater than sixty-five years old) most prone to severe COVID-19 disease (80). The RhM SARS-CoV-2 model confirms a role for T cells in protection, with evidence thus far suggestive of T cell responses supporting viral control. Further work is necessary to underpin the true weight of the role of T cells in providing protection and to define the precise T cell population responsible for protection.

5.2 Passive transfer of antibody to animal models

The scope to manipulate animal immune responses to identify the parameters crucial for protection continues with passive transfer of antibody. Given the above indications that the role of T cells impacts B cells responsivity and antibody titres, proof-of-concept that antibodies are in fact the key mediators of infection resolution and protection can be achieved *via* the passive transfer of antibody to animals involved in pre-clinical vaccine and rechallenge studies.

Rogers et al. isolated S+ and RBD+ memory B cells (MBCs) from eight SARS-CoV-2 human convalescent donors and neutralising mAbs were passively transferred to Syrian hamsters by intraperitoneal infusion at five different concentrations. Twelve hours later, the animals were intranasally challenged with a dose of 1 x 10^6 PFU of SARS-CoV-2. Using weight loss as the measure of disease magnitude, neutralising antibody (NAb) titres of ~22 ug/ml and 12 ug/ml, confers full protection or a 50% reduction in disease burden, respectively (35). Similarly, passive transfer of 10 mg of mRNA-1273-vaccinated RhM IgG to Syrian Hamsters also provided protection upon SARS-CoV-2 challenge (but 2 mg did not) (81).

McMahan et al. isolated SARS-CoV-2-specific NAbs from nine challenged macaques. Twelve RhMs, divided into four groups, were intravenously infused with concentrations of IgG that differed by an order of magnitude and were subsequently challenged with 1 x 10^5 $TCID_{50}$ of SARS-CoV-2. A dose-dependent effect of SARS-CoV-2-specific NAb titrations on viral load was observed, with the group infused with the highest IgG titres (250 mg/kg) yielding negative PCR results from BAL and nasal swab samples and hence are protected from infection. McMahan et al. was the first to propose NAbs as a CoP for COVID-19, as it is an immune parameter that significantly differentiates protected from non-protected NHPs and correlates with protection (10).

The pre-clinical evaluation of pharmaceutical mAbs for their therapeutic and/or prophylactic effects, represents another setting for experimental passive transfer of NAbs to animals, to assess the role of antibody in protection against SARS-CoV-2 challenge. Two mAbs, tixagevimab and cilgavimab, of AstraZeneca's Evusheld, which potently and collaboratively target the 'open' and 'closed' conformations of the ACE2 RBD, reduce pathology and viral load when tested as a therapeutic intervention and provides protection when administered prophylactically to female hACE2-BALB/c mice (44). Similarly, the REGN-COV pre-clinical trial of the mAb cocktail, casirivamab plus imdevimab, successfully limited pathology and viral load when administered prophylactically and therapeutically in the mild COVID-19 RhM challenge model and severe COVID-19 Syrian golden hamster challenge model with low and high dose SARS-CoV-2 inocula (12).

The functional capacity of the Fc domain of passively transferred antibody must also be considered. McMahan et al. found functional antibody responses including antibody-dependent complement deposition (ADCD), antibody-dependent NK cell activation (ADNKA) and antibody-dependent neutrophil phagocytosis (ADNP) to correlate with protection in their passive transfer experiments (10). Administering genetically-engineered antibody to animals has also accelerated the field's understanding of the role of the Fc domain of antibody in the context of SARS-CoV-2 infection. An Fc-mutated mAb fails to confer clinical, viral and pathological protection when administered therapeutically to both K18-hACE2 transgenic mice and Syrian hamsters, however the functional Fc mAb did successfully protect these animal models in the early days post-challenge (13). This is suggestive of a crucial role for the Fc domain in the control of acute infection in these models. Whether the Fc has a prophylactic role is less clear. Serum levels of intraperitoneally administered anti-RBD NAb, prior to intranasal challenge with 1 x 10^3 PFU of SARS-CoV-2, correlated with clinical protection from COVID-19 and inversely correlated with lung vRNA in the K18-hACE2 transgenic mouse model, irrespective of whether the Fc region was loss-of-function mutated (13). Meanwhile, a non-RBD-based S2 stem helix-targeting neutralising mAb, S2P6, that activates Fc-mediated effector functions antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP), effectively limits lung vRNA when administered prophylactically in the Syrian hamster challenge model (36). These results demonstrate the potential collaborative effect of neutralising and Fc functional SARS-CoV-2-specific antibodies to confer complete protection.

Additional immune manipulation strategies facilitated the interrogation of the potential mechanisms underlying antibody Fc-mediated protection. The investigative strategies adopted were based on the observation that functional Fc mAb-treated mice had reduced counts of TNFa+iNOS+CD80+CD11b+ monocytes and an amplified frequency of activated CD8+ T cells (13). Depletion of monocytes in functional Fc mAb-treated mice, resulted in the loss of clinical protection, yet a sustained ability to reduce viral burden. Depletion of CD8+ T cells in functional Fc mAb-treated mice contributed to the loss of viral control, but not a loss of clinical protection (13). This is in support of the aforementioned T cell depletion experiments that proposed T cells as mediators of viral control. The proposed mechanism of protection of functional Fc mAb-treated mice is that phagocytosis and antigen presentation follows virus-Fab-Fc-FcyR immune complex formation on monocytes, so as SARS-CoV-2-reactive cytotoxic CD8+ T cells can be activated and destroy virally infected cells (13).

5.3 High versus low dose vaccine comparison

The identification of a CoP relies equally on vaccine breakthrough as it does successful immunisation in order to stratify outcomes and define the immune profiles that differentiate protected from unprotected groups. Formulated in lipid nanoparticles (LNPs), Moderna's mRNA vaccine, mRNA-1273, was 94.1% effective at preventing symptomatic disease and 100% effective in preventing severe disease, when two doses were administered twenty-eight days apart in a phase III trial. However, given the efficacy of the vaccine, with only eleven of 15,210 vaccinated participants (0.07%) contracting COVID-19, CoPs could not be identified (67). As in the name, CoP, statistical power and 'correlations' underpin the investigation of candidate CoPs, therefore the low number of vaccine breakthrough cases means this need will not be met by human SARS-CoV-2 vaccine clinical trial data. Hence, pre-clinical vaccine studies that investigate protective and sub-protective vaccine dosing strategies under matched conditions, diversifies challenge outcomes and immune profiles, from which statistically significant correlations with protection can be drawn (summarised in Figure 5).

BALB/cJ, C57BL/6J and B6C3F1/J mice were intramuscularly vaccinated with mRNA-1273 as part of a two-dose regimen with three week intervals (47), as were twelve RhMs at four week intervals (14, 47). A vaccine dose-dependent effect on binding and NAb emergence was observed (14). Challenge of vaccinated BALB/cJ mice with 1 x 10^5 PFU of SARS-CoV-2 at week five or week thirteen post-boost, and vaccinated RhMs challenged with 7.6 x 10^5 PFU at week four post-boost, revealed a vaccine dose-dependent reduction in lung viral load (47), such that NAb responses were negatively correlated with viral load in the nasal turbinates (14).

He et al. found that the lowest dose of Ad26.COV2.S kept SARS-CoV-2 sgRNA levels at a minimum in the LRT of RhMs challenged with 1 x 10^5 $TCID_{50}$ six weeks post-single-dose vaccination. However, a higher vaccine dose also protected against the establishment of a SARS-CoV-2 infection in the URT, in addition to the LRT (15). This was attributed to the trend for poorer anti-RBD IgG and NAb kinetics and response magnitudes, as well as reduced T cell and RBD-specific IgG+ MBC activity in the low dose vaccine groups (15). Specifically, the MBC compartment was amplified in the higher dose groups, which was found to be associated with completely protected groups, in comparison to non-protected or partially protected groups. MBC frequency positively correlated with respective antibody titres and negatively correlated with nasal swab sgRNA levels (15).

Minimal lung pathology and a significant decline in viral load was observed in RhMs vaccinated with a high dose of beta-propiolactone-inactivated SARS-CoV-2 vaccine candidate, PicoVacc. This was observed following intratracheal challenge (1 x 10^6 $TCID_{50}$) one week post-completion of the three-dose regimen (16). Medium-dose vaccinated animals induced lower NAb titres and increased incidence of SARS-CoV-2 detection in the pharynx and lung. These results were replicated in PiCoVacc-immunised BALB/c mice and Wistar rats, providing additional support for NAb as a mediator of viral control (16). No significant difference in CD3+, CD4+ or CD8+ frequency, or inflammatory cytokines, were noted between the vaccinated and control groups (16).

From the above-mentioned vaccine pre-clinical studies, a vaccine dose-dependent effect on antibody titres was observed, that subsequently correlated with viral load. This research highlights the necessity for a range of vaccine doses to be tested in animals in order to draw associations between immune profiles and protection.

5.4 Comparison of matched optimal and sub-optimal vaccine candidates

Many vaccine platforms, such as vector-based vaccines or nucleic acid vaccines, are amenable to the testing of different antigenic components that may differ in their immunogenicity and hence in the level of protection the induced immune response provides. Hence, pre-clinical studies of vaccines, that differ only in their antigen composition, provides another mechanism of promoting challenge outcome and immune profile divergence that favours SARS-CoV-2 CoP identification.

Ad26 vector-based vaccines, incorporating different forms of SARS-CoV-2 Spike (Spike sequences that differ in length, that incorporate the furin cleavage site mutation and/or further stabilising mutations) were first tested in RhMs (17). Due to the range of challenge outcomes yielded post-vaccination with the different Ad26 candidate vaccines, the group concluded that NAb titres were the factor that differentiated protected from unprotected RhMs post-challenge, with ADNKA and ADCP also contributing to the separation of these protection statuses (17). In fact, it was proposed that the collaborative effect of antibody neutralisation and Fc-mediated effector functions had an improved correlation with protection (revealed following logistic regression analyses) (17), supportive of the results from the therapeutic Fc functional mAb pre-clinical trial discussed above (13).

The Ad26 vaccine encoding pre-fusion stabilised full-length Spike (Ad26.COV2.S) generated the most substantial immunological effector functions and viral control responses, with BAL samples from RhMs in this group lacking detectable virus upon challenge with 1 x 10^5 $TCID_{50}$ of SARS-CoV-2 at week six (17). This evidence-based optimal vaccine was also tested against a suboptimal Ad26 SARS-CoV-2 vaccine in fifty Syrian golden hamsters that model more severe COVID-19. An array of outcomes post-intranasal challenge with 5 x 10^5 $TCID_{50}$ of SARS-CoV-2 were elicited, including successful, partial and failed protection which support CoP identification. Clinical and viral outcomes were also found to inversely correlate with anti-RBD and/or NAb responses in this model (32).

Formulated in LNPs, Pfizer/BioNTech's mRNA vaccine candidates, BNT162b1 and BNT162b2, encoded soluble RBD or pre-fusion stabilised full-length Spike, respectively (18). The intranasal and intratracheal challenge of twelve BNT162b1/BNT162b2-immunised and nine control RhMs with 1.05 x 10^6 PFU of SARS-CoV-2, revealed that BNT162b2-vaccinated macaques' BAL PCR results remained negative throughout post-challenge sampling, in comparison to the control and BNT162b1-vaccinated macaques that had a higher incidence of BAL PCR vRNA positivity (18). At challenge, matched neutralising responses were seen in RhMs vaccinated with the BNT162 candidates, hence the aetiology of the improved BNT162b2 efficacy does not fall with enhanced neutralising responses. Augmented levels of circulating CD8+ T cells were detected in mice vaccinated with BNT162b2 versus BNT162b1, likely due to the broader range of T cell epitopes encoded by the BNT162b2 candidate. However, mice were not challenged as part of this study to relate this disparity to protection outcomes (18).

Thirty-five RhMs were intramuscularly vaccinated with one of six DNA vaccine candidates, each encoding different SARS-CoV-2 Spike variants, or the sham vaccine, to induce heterogenous response profiles (19). Subsequent challenge with 1.1 x 10^4 PFU of SARS-CoV-2 *via* intranasal and intratracheal routes, resulted in a ~two-fold reduction in median BAL and nasal viral loads in vaccinated groups in comparison to the sham control group (19). URT and LRT sgRNA levels were found to inversely correlate with NAb titres (with a systems biology approach indicating a potential collaborative effect with Fc-mediated responses of ADCD and ADCP), while ELISpot and ICS results did not correlate, inferring that the humoral and not the cellular compartment mediates this protection (19).

Efficacy of CureVac's LNP-formulated SARS-CoV-2 mRNA vaccine, CVnCoV, was observed in twenty Syrian golden hamsters in the form of reduced lung pathology, minimal URT and undetectable LRT viral loads (37). RhMs that received high dose CVnCoV vaccination also experienced significantly reduced lung lesion severity and undetectable LRT vRNA (20). However a statistically significant difference in URT vRNA copies between high dose CVnCoV recipient versus unvaccinated/low dose CVnCoV-vaccinated RhMs was not observed following intranasal and intratracheal challenge with 5 x 10^6 PFU (20), which may provide an explanation for the poor 48.2% efficacy reported from clinical trials, irrespective of the induction of Spike- and RBD-specific IgG and NAbs (82). The failure of the first generation CureVac vaccine was also attributed to the incidence rate of breakthrough infections caused by VOCs (82).

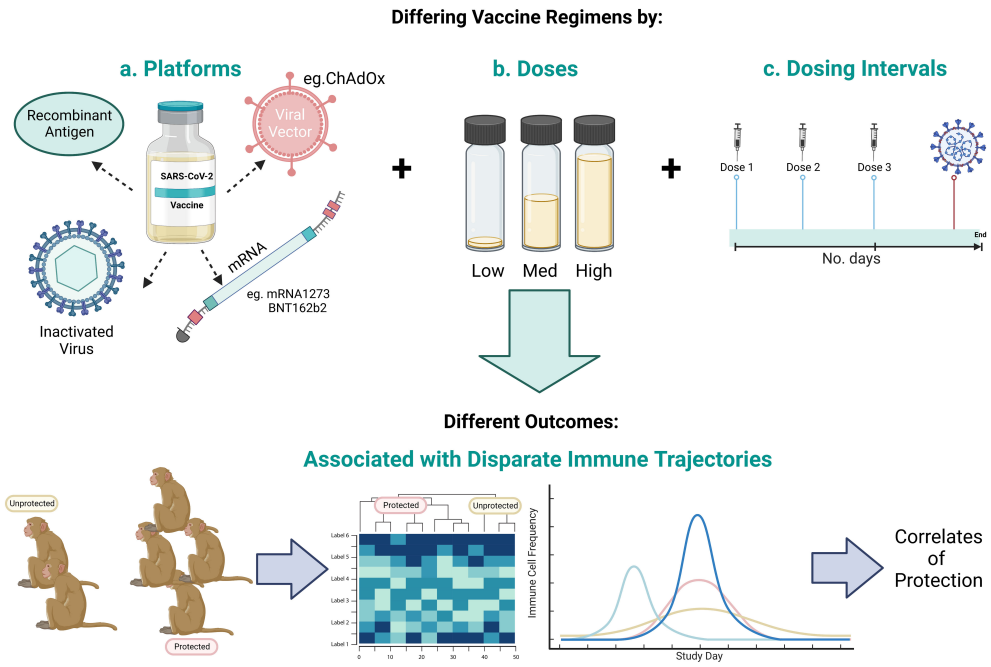


FIGURE 5
Schematic representing the scope to identify CoPs by investigating a range of a) vaccine candidate platforms, b) number of vaccine doses, and c) vaccine dosing regimens to give a range of outcomes in order to identify the immune profile that differentiates protected from unprotected animals. Created with BioRender.

Hence, a second generation CureVac SARS-CoV-2 vaccine (CV2CoV) was developed with enhanced intracellular Spike transcript stability to optimise antigen expression (83). Following positive initial immunogenicity and efficacy results of a high dose prime-boost CV2CoV regimen in Wistar rats (83, 84), a comparison of CVnCoV and CV2CoV vaccines was drawn in eighteen CyMs, inclusive of six control CyMs, which yielded a range of immune profiles and outcomes (30). The higher innate cell, NAb, MBC and T cell responses post-CV2CoV vaccination versus CVnCoV vaccination, coincided with lower sgRNA copies in the URT and LRT post-intranasal and -intratracheal challenge with 1 x 10^5 $TCID_{50}$ eight weeks post-vaccination with CV2CoV (30). In fact, NAb titres at two weeks post-boost were found to inversely correlate with BAL and nasal swab sgRNA (30). This is another example of how side-by-side comparisons of vaccines accelerate CoP identification.

Direct comparison of sub-optimal and optimal vaccines, mediates the partitioning of outcomes and immune profiles to identify correlations between immune parameters and protection. A vaccine developed to interrogate the vaccine-associated enhanced disease (VAED)-potential of SARS-CoV-2 vaccines, a formaldehyde-inactivated viral (FIV) SARS-CoV-2 vaccine with a Th2-skewing adjuvant, alhydrogel, was studied in ferrets and RhMs and further supports SARS-CoV-2 CoP investigations (21). While a single dose of FIV did not provide clinical protection (there were insignificant differences between FIV-vaccinated and sham control RhM CT scores, weights and temperatures), vaccinated RhMs did yield significantly lower mean vRNA concentrations, pathology scores and infected lung area post-intranasal and -intratracheal challenge with 5 x 10^6 PFU of SARS-CoV-2. Deeper analysis of the immune profile associated with this 'sub-optimal' protection, revealed that FIV vaccination only elicited a modest neutralising response in ferrets and RhMs providing the most likely explanation for the lack of protective efficacy (21). This example demonstrates how sub-optimal vaccination highlights deficiencies in the immune response, not seen in optimally-vaccinated animals, that ultimately contribute to the lack of protection, thus increasing our confidence in candidate CoPs.

5.5 One versus two vaccine dose comparison

Pre-clinical efficacy studies often investigate the number of vaccine doses required to achieve optimal protection. As a result of this exploratory research, control unvaccinated, primed-only and prime-boosted groups of animals yield a hierarchy of outcomes, from which parameters that distinguish protected from unprotected animals can be identified to inform CoP research. This approach offers significant advantages as monitoring the protective efficacy post-prime, and then subsequently post-boost, in the same animal, can illustrate the trajectory of protective immune response development which may help to identify predictors of immunity. Additionally, it must be considered that protection may be provided *via* one mechanism post-prime, that evolves to establish a different protective profile post-boost.

The preclinical study of Ad26.COV2.S involving sixty RhMs, found a two-dose regimen to fail to improve protective efficacy as the median number of days with detectable sgRNA in the URT was minimally different between the primed-only and prime-boosted NHPs, despite significantly higher anti-Spike IgG and NAb responses in boosted-RhMs (22). While the boost similarly had a 2.6-2.9-fold amplification effect on NAb titres of Ad26.COV2.S-vaccinated humans, this did not improve protective efficacy, and provides the rationale for Janssen's adoption of a single-dose regimen (85).

Though the Ad26.COV2.S studies illustrated that one dose was optimal, many of the approved SARS-CoV-2 vaccines adopted a two-dose regimen. Whilst two doses were found to improve the immunogenicity, response longevity and efficacy of these vaccines, many did provide considerable protection post-prime also. In which case, what vaccine-induced response/s is/are responsible for the primary protection and the enhanced protection achieved post-boost?

In the multicontinental clinical trial of ChAdOx-1 nCoV-19, the vaccine was 64.1% protective post-prime, not-too-dissimilar to the 70.4% efficacy reported post-boost (86). NAb titres over the twenty-seven days post-prime of RhMs with ChAdOx-1 nCoV-19 were found to increase (23, 24). This is concordant with the natural increase in NAb titres and the frequency of responders eliciting a neutralising response from week four post-Ad26.COV2.S prime which provides sufficient protection (85).

An evolving humoral profile during the interval between prime and boost is also observed with Pfizer/BioNTech's SARS-CoV-2 vaccine, BNT162b2. Thomas et al. reported an increase in protective efficacy from 58.4% post-prime to 91.7% from day eleven post-prime to day twenty-one (the day of boost), in 43,409 human participants vaccinated with BNT162b2 (87). The antibody profile post-BNT162b2 prime, reported by Walsh et al., was predominantly non-neutralising, even in the 50% of vaccine recipients that had detectable neutralising responses (88). Protection may therefore be explained by the evolution of non-neutralising antibody with enhanced Fc-effector functionality post-prime (88, 89). In which case, antibody 'quality' rather than 'quantity' may be responsible for BNT162b2-mediated protection. Alternatively, these results may be explained by *in vitro* neutralisation assay limitations, such as sensitivity (90), and disregard for the contribution of other serum factors, such as complement, in neutralisation, as reported by Mellors et al. for Ebola virus (91).

Therefore, the boost-induced superior protective response is explained by which immune parameters? NAb titres do increase in humans post-boost with ChAdOx-1 nCoV-19, which correlate with viral and clinical protection and likely contributes to the improved efficacy of ChAdOx-1 nCoV-19 to 70.4% post-boost (23, 38). However, an increase in IgG1 and IgG3 titres may also contribute to this improvement (92). A second dose of BNT162b2, which is reported to improve vaccine efficacy supports the emergence of potent and broadly-neutralising antibody and a predominately class-switched IgG+ SARS-CoV-2-specific MBC repertoire in humans (88, 89).

However, in pre-clinical studies of Novavax's vaccine, NVX-CoV2373, largely equivalent NAb titres are induced in the single and double-dosed groups. Multivariate analysis revealed that multi-subclass Spike-specific Ig responses, ADCD and NAbs separate fully protected RhMs (both URT and LRT protection) from partially (LRT protection only) or unprotected RhMs. As partially or unprotected RhMs had a poorer ability to drive Fc-mediated effector functions, and functional antibodies explosively mature post-boost with respect to the less dramatic change in NAb titres post-boost, functional antibody may underpin the enhanced efficacy of a two-dose regimen of NVX-CoV2373 (27). In the phase III NVX-CoV2373 trial involving 14,039 participants that took place during Alpha variant circulation, eight of ten vaccine breakthrough cases were Alpha variant infections (70). This observation can be explained by the discovery that RhM and human antibodies lack the ability to simultaneously bind both the FcR and SARS-CoV-2 variants that harbour the E484K mutation, such as the Alpha variant (27). This real-world scenario provided additional support for the role of the Fc of NVX-CoV2373-induced SARS-CoV-2-specific antibody in mediating protection.

A combination of data collated from animal and human trials has aided understanding of the evolution of the immune response required for optimal vaccine-mediated protection. The combination of animal and human efficacy and immunogenicity data post-prime and -boost has been used to deduce that functional binding antibody and NAbs are strong CoP candidates.

5.6 The use of different adjuvants

Optimising a vaccine's adjuvanticity is a crucial consideration in any vaccine design process. Adjuvanticity describes a vaccine's ability to stimulate innate immune cells (required for the eventual induction of an antigen-specific response by adaptive immune cells) mediated by the 'adjuvant' component of the vaccine formulation. A number of factors influence a vaccinologist's decision to use a particular adjuvant, including safety profiles, vaccine dose-sparing aims and a pathogen's CoP, particularly were protection to be T cell subset-dependent (93). Commonly used adjuvants include water-in-oil emulsions, aluminium-containing adjuvants, pattern recognition receptors and LNPs (94). The use of the Th2-skewing alhydrogel adjuvant for the FIV SARS-CoV-2 vaccine discussed above, for example, highlights the capacity of adjuvants to diversify the post-challenge outcomes for the investigation of CoPs in pre-clinical vaccine studies.

Pre-clinical studies support the optimisation of vaccine immunogenicity *via* the testing of different adjuvants. For example, hACE2-BALB/c mice primed and boosted with NVX-CoV2373, of recombinant Spike plus saponin-based Matrix-M adjuvant, achieved higher frequencies of multifunctional effector memory T cells, T follicular helper (Tfh) cells and germinal centre (GC) B cells, as well as an amplified anti-Spike antibody response, than those administered the vaccine that lacked the Matrix-M adjuvant. This enhanced immunogenicity likely explains the minimal virology and pathology seen in SARS-CoV-2-challenged hACE2-BALB/c mice, CyMs and RhMs vaccinated with NVX-CoV2373, in comparison to groups that received the vaccine lacking the Matrix-M adjuvant (31, 45).

Many adjuvants have been investigated and compared under the same experimental conditions in animal models, in an attempt to optimise SARS-CoV-2 vaccine candidate efficacy. Arunchalam et al. reports that different adjuvants yield an array of COVID-19 outcomes. AS03-adjuvanted RBD-nanoparticle-vaccinated RhMs were found to be the most protected upon intranasal and intratracheal challenge with 3.2 x 10^6 PFU of SARS-CoV-2 at week four, with undetectable vRNA in pharyngeal, nasal and BAL samples. AS03 adjuvant was found to induce the highest NAb titres, with NAbs significantly correlating with protection in this study. This NAb response positively correlated with the CD4+ T cell response, with a balanced Th1-Th2 response, as well as a higher frequency of circulating Tfh cells, being attributable to the adjuvant in use (25). ADNP also differentiated protected from unprotected RhMs following partial least-squares discriminant analysis and negatively correlates with viral load, thus providing further evidence for the role of functional antibody (25).

With adjuvant as the basis of comparison in the aforementioned studies, Lederer et al. investigated the effects of adjuvant on GC reactions in a mouse model. Given the theorised adjuvanticity of the LNP formulation of mRNA vaccines, the GC reactions of SARS-CoV-2 mRNA-vaccinated BALB/c mice were compared with those seen post-vaccination with the less-optimal recombinant RBD vaccine candidate adjuvanted with Addavax, a MF59-like adjuvant (rRBD-AddaVax) (95). In the mRNA-vaccinated mice, the frequency of SARS-CoV-2-specific GC B cells in the inguinal LN and the popliteal draining LN remains elevated at day twenty-eight post-vaccination, reminiscent of prolonged GC reactions (95). On the contrary, rRBD-Addavax-vaccinated mice lack evidence for GC reactions and unsurprisingly, NAbs do not emerge (95). Additionally, in stark contrast to the poor magnitude and kinetics of the IgG1-dominant response seen in rRBD-Addavax-vaccinated mice, Th1-polarisation of Tfh cells in the mRNA-vaccinated mice ensures IgG2a and IgG2b class switching in this group (95). Influencing GC reactions *via* the adoption of different adjuvants in animal models, sheds further light on the cellular and humoral profiles associated with protection outcomes.

5.7 Vaccine recipients with variable immune functionality

Next, we must explore the idea that vaccinees may have aged immune systems, conditions associated with immunodeficiency, or are being treated with immunosuppressive drugs. Immune features that are naturally compromised in vaccinated individuals, can indirectly provide evidence for protective mechanisms. For example, failed protection in participants with immunoglobulin deficiency would provide support for the role of the humoral response in protection. Additionally, the potential for redundancy mechanisms to be at play in these recipients may also point to a 'surrogate of protection'. This is particularly relevant due to the age-bias of COVID-19. The phenomena of 'inflammageing' and thymic involution in the aging population equally heightens the

requirement for immunisation of this population, as it does explain their increased risk of severe infection and the potential for a failed vaccination. Often in early phase I human clinical trials, only healthy participants below the age of fifty-five years are enrolled. Aged and/or immunocompromised individuals are only included in much later trials and studies, and so there is a considerable lag before it is possible to investigate the immune response to vaccination in these populations.

Hence, 'aged' or immunocompromised animal models, can accelerate and further support this research. For example, lower antibody titres were reported in the aged Syrian golden hamster model with respect to the younger cohort. This difference in humoral response magnitude impacted their ability to protect against challenge with 1 x 10^5 PFU of SARS-CoV-2 (33). While young hamsters had undetectable vRNA in the lung by day five and recovered from infection by day fourteen, aged hamsters had sustained high viral loads in the lung and persistent inflammation (33).

Silva-Cayetano et al. compared the immunogenicity of ChAdOx-1 nCoV-19 in three-month-old versus 'aged' twenty-two-month-old C57BL/6 mice. In the twenty-two-month-old 'aged' mouse model, the percentage of GC B cells post-ChAdOx-1 nCoV-19 vaccination was lower, coinciding with the absence of GCs in the spleen, reduced numbers of proliferating Tfh cells, an impaired type I IFN response, as well as lower anti-Spike IgG and NAb titres, as seen in older humans (46). The compromised GC response was rescued by the second ChAdOx-1 nCoV-19 vaccine dose in the aged mouse model, with draining LN plasma cells, GC B cells and Tfh cells being detectable by day nine post-boost, which occurs in parallel with an eight-fold increase in anti-Spike IgG and NAb responses (46). Similarly, human ChAdOx-1 nCoV-19 vaccine recipients over the age of seventy had lower Th1 cell frequencies post-prime, but both Spike-specific CD4+ and CD8+ T cell responses were elevated post-boost to match frequencies seen in the younger cohorts (96). Therefore, the vaccine was 61% effective between one to four weeks post-boost in recipients over the age of sixty-five (97). A boost also appears to be sufficient for the induction of a class-switched Spike-specific MBC response in immunosuppressed kidney transplant patients (98).

Recognising the immunogenicity and efficacy of different vaccination strategies for the more challenging vaccine recipient versus healthier vaccine recipients, can further enhance our understanding of candidate SARS-CoV-2 CoPs.

5.8 Alternative vaccine administration routes

The routes of entry of SARS-CoV-2, as a respiratory pathogen, include the mucosal sites of the respiratory system – the nose, throat and lung. Hence, to achieve 'sterilising immunity,' one may require sufficient SARS-CoV-2 reactivity at these sites. Therefore, induction of a strong SARS-CoV-2-specific mucosal immune response would likely improve vaccine efficacy, which intuitively can be achieved through intranasal or oral vaccination. The field of mucosal immunology has advanced over the last number of years (99). However rigorous research in animals is required prior to human trials of intranasal/oral vaccination, given the adverse events associated with this vaccine administration route, stemming from strong associations between an intranasally-administered influenza vaccine and the development of Bell's Palsy in Switzerland (100). Therefore, animal models provide an opportunity to investigate mucosal vaccination, while also determining the role of mucosal responses in protection against a respiratory pathogen. Vaccination of animals *via* different administration routes will further diversify the immune response and challenge outcomes to deduce CoPs.

A comparison between the intramuscular (IM) and needle-free oral administration routes of an MVA-expressing Spike and Nucleocapsid vaccine, was addressed in RhM studies. Following challenge with 1 x 10^8 PFU of the Delta variant four weeks post-boost, three protection outcomes were recorded; 1) robust protection *via* IM vaccination, 2) moderate protection *via* the buccal route, 3) failed protection *via* the sublingual route. A higher magnitude of serum and mucosal IgG, and functional Ab-dependent cellular activity was observed in the IM-vaccinated RhMs. Nasal anti-RBD IgG and NAbs, as well as serum ADCD, ADCP and ADNKA, were found to inversely correlate with viral load, providing further evidence for the collaborative efforts of neutralising and non-neutralising antibody to protect against SARS-CoV-2 challenge (101). T cell responses were comparable between the IM and buccal administration routes, hence they may contribute to protection also (101).

Adenoviruses are respiratory viruses, with binding affinity to the coxsackievirus and adenovirus receptor (CAR) expressed on respiratory mucosa, and are responsible for seasonal colds. Therefore unsurprisingly, intranasal (IN) administration of ChAdOx-1 nCoV-19 has been explored for its ability to induce lung-specific and systemic immune responses. ChAdOx-1 nCoV-19 IN administration has been shown to be associated with reduced pathology and URT and LRT virology post-challenge, when compared to IM administration in animal models (38, 49). A horizontal transmission experiment, whereby a naïve hamster is exposed to a challenged hamster for four hours (which more realistically mimics SARS-CoV-2 infection than direct intranasal inoculation), revealed that SARS-CoV-2 Nucleocapsid was undetectable in the lung tissue of the SARS-CoV-2-exposed IN-vaccinated hamsters, in comparison to control and IM-vaccinated hamsters where SARS-CoV-2 Nucleocapsid was detectable (39). This is reflective of LRT viral control, perhaps mediated by the six-fold higher titres of serum anti-Spike, anti-RBD and NAbs induced in IN- versus IM-vaccinated hamsters (38). However, whilst ChAdOx-1 nCoV-19 and Ad5-S-nb2 IN-vaccinated ferrets and RhMs were more protected than the IM-vaccinated animals following SARS-CoV-2 challenge, this cannot be explained by immune parameters that are measurable from blood samples (26, 49). In fact, IN-vaccinated animals failed to induce serum SARS-CoV-2-specific IgG titres or cell-mediated immune responses equivalent to those of IM-prime-boosted animals (26, 49).

It is possible and likely that mucosal rather than serum antibody is providing the improved protection observed following IN-vaccination. Upon challenge with 1 x 10^6 $TCID_{50}$ of SARS-CoV-2, IN-ChAdOx-1 nCoV-19-vaccinated RhMs had lower pathology,

viral titre and frequency of virus detectability in the URT and LRT in comparison to controls (however many of these differences were insignificant) (39). Nasosorption sampling facilitated the analysis of the mucosal response to IN-vaccination, with mucosal SARS-CoV-2 IgA being detectable post-prime and amplified post-boost. A booster-effect on mucosal IgG was also observed from BAL sampling (39). Principle component analysis defined protected IN-vaccinated animals by their SARS-CoV-2-specific IgA and IgG responses in BAL and nasal samples, with correlations being drawn between nasal and BAL IgA and IgG samples and nasal and BAL vRNA, respectively (39). Similarly, IN-immunisation of female BALB/c mice with Ad5-S-nb2 induced anti-Spike IgA in the BAL, that was undetectable in the IM-immunised animals (26).

Mao et al. developed a vaccination strategy involving IM-priming with BNT162b2 mRNA vaccine, followed by an IN-boost with unadjuvanted recombinant prefusion-stabilised Spike, coined 'Prime and Spike'. This vaccine strategy, administered to K18-hACE2 transgenic mice, elicited the amplification of nasal, lung and serum IgA and IgG, resident MBCs, long-lived plasma cells (LLPCs), and CD4+ and CD8+ tissue-resident memory (Trm) cells. In comparison to prime-only with a low dose of BNT162b2, known to be unprotective in the K18-hACE2 mouse model, this 'Prime and Spike' regimen significantly minimised lung pathology and reduced viral burden in the URT and LRT upon challenge with 6 x 10^4 PFU. CD8+ Trm cells in the lung and BAL IgA were detected in the 'Prime and Spike' group only, while serum IgA and IgG, and BAL IgG, were matched between animals immunised *via* 'Prime and Spike' or prime-boosted with BNT162b2. As challenge was not performed, associations between these mucosal profiles and protection, could not be drawn, but informs the scope to induce mucosal immune responses by IN-vaccination (102).

In summary, comparison of vaccine administration routes in animal models, alludes to tissue-resident and mucosal immune features as CoPs for a respiratory pathogen such as SARS-CoV-2.

5.9 Tissue examination and manipulation scope

A major advantage of the use of animal models for CoP research is the scope for in-depth pathological analysis to better stratify post-challenge outcomes based on well-defined pathology scoring systems such as that seen in Salguero et al. (8). Additionally, an in-depth analysis of animal tissues such as lung, spleen and thymus, and the immune cell populations at these sites, can accelerate our search for a SARS-CoV-2 CoP.

Shaan Lakshmanappa et al. characterised the GC cell populations of RhMs by digesting LNs obtained at necropsy to generate a single cell suspension for flow cytometric analysis (11). A robust GC Tfh cell population in the mediastinal LN and spleen of RhMs was detectable following challenge with ~1.7 x 10^6 $TCID_{50}$ of SARS-CoV-2 intranasally, intratracheally and intraocularly (11). Bronchial-associated lymphoid tissue has also been observed in both RhMs and CyMs, with similar frequency and semblance, following pathological analysis, indicative of the induction of localised GC reactions upon challenge (8).

Lung and spleen isolated from BALB/c mice IM-vaccinated with an RBD, full-length Spike- or control luciferase-encoding LNP-formulated mRNA vaccine, revealed the emergence of polyfunctional IFNy+CD4+ and IFNy+CD8+ T cells in the spleen, and to a greater extent, in the lung parenchyma, demonstrative of lung homing and extravasation (103). At nine weeks post-vaccination, Spike- and RBD-specific IgG1+ and IgG2a/b+ MBCs in the spleen were detected, as were LLPCs of varying subsets in the bone marrow by flow cytometry and ELISpot analysis, revealing the scope for a durable protective response (103).

These studies capture the invaluable insights we gain from the in-depth analysis of animal tissue post-vaccination and post-challenge, that peripheral blood mononuclear cell (PBMC) samples from humans fail to provide. PBMC phenotypes are not demonstrative of Trms or GC cells in the LNs (98), hence we only capture a fraction of the immune cell landscape in the absence of tissue. Only study of human cadavers that succumbed to COVID-19 infection was carried out amidst the pandemic which highlighted the profiles associated with fatal COVID-19 (78), but could not aid CoP identification.

6 Future of CoP research in animals

6.1 Flaws of animal research in the search for CoPs

On the basis of the animal studies discussed above, a diverse response by the adaptive arm of the immune system is required for resolution and protection against SARS-CoV-2 infection. A downfall of the NHP challenge model for the definition of COVID-19 CoPs, is the high frequency of protection at reinfection (6), particularly frequent due to challenge with matched SARS-CoV-2 strains, the short intervals between vaccination and challenge, and the mild manifestation of this disease in the animals (except in old RhMs/CyMs). In which case, comparing immune parameters that differentiate protected from non-protected animals at rechallenge can be complicated unless precise pathology scoring systems are used.

Difficulties surrounding the breeding, handling and housing of the animals that most accurately recapitulate human COVID-19, i.e. RhMs and CyMs, contribute to the decision to cull animals soon after challenge. Such difficulties also limit the interval length between vaccine doses and between immunisation and challenge. While cull of animals soon after challenge/rechallenge captures the immune landscape during acute infection, this sacrifices the possibility for analysis of immune response durability and long-term immunity months post-immunisation, post-infection or post-reinfection.

Another clear limitation of animal models for the definition of a CoP, is the poor reproducibility of animal results in humans as was the case in the search for a rotavirus and HIV CoP using an NHP model (3). This is due to the lack of conservation of some immune features. For example, disparities between human and macaque NK cells include the high background activity of macaque NK cells (104) and the difference in frequency of cell surface marker

expression (105, 106). This may explain why candidate HIV vaccines that did provide protection against SIV at the pre-clinical stage (which was attributed to ADNKA), were unsuccessful in human clinical trials (107). Contributing to this limitation is the deficiency of species-specific or species-cross-reactive reagents. With that said, murine reagents are widely available and the availability of NHP reagents is improving [NHP-reactive antibody clones are reported on databases such as NIH NHP Reagent Resource (www.nhpreagents.org/_)]. However, the sparser reagent pool and incomplete characterisation of animal model immune components, together limits our ability to yield results that are replicable in human studies.

Furthermore, the scope for genetic manipulation at the NHP level is minimal, particularly in comparison to that of mice, where immunodeficient mice can help us understand the weight of the role of particular immune parameters in mediating protection. Therefore, for NHP-level immune manipulation we rely on immunodepleting with mAbs against specific cellular subsets, or FcγR inhibitors, prior to challenge and rechallenge to define the relevance of cell subsets and Fc effector functions in mediating protection, respectively. However, this approach is not 100% effective, as was observed in CD4+ depletion experiments referenced in (28), which may in some cases be attributable to the limited or less-optimal NHP-reactive reagents. Additionally, for genetic manipulation studies, we must be at a stage to confidently predict candidate CoPs in order to minimise animal sample sizes and unnecessary/wasteful use of research animals.

6.2 Advances in human research to assist in the search for CoPs

A combination of human and animal data often yields the greatest insights into a pathogen's CoP. Furthermore, the best model for human infection is no doubt the human itself. Hence, advances in biotechnology, immunology and human challenge trials must be applied to further improve CoP research.

To delve into immune responses post-vaccination in detail, LN GC reactions are analysed using digestion or microscopic dissection of isolated animal tissue. However, until recently blood biomarkers such as CXCL13 and circulating Tfh cells were relied upon to detect GC reactions in humans (98). The shortcomings of such techniques include the uncertainty of the antigen-specificity of the reactions, and the limited and short traceability of these markers (98). Fine needle aspiration (FNA) has since allowed for the analysis of GC reactions in the ipsilateral axillary draining LNs (IADLN) of 15 humans vaccinated with BNT162b2 or mRNA-1273 (98). Using fluorescently labelled SARS-CoV-2 probes, an amplification of SARS-CoV-2-specific GC B cell, Tfh cell, class-switched MBC and plasma cell frequencies could be observed post-boost in the IADLNs (98). The significance of the development of the FNA technique is exemplified by the fact that circulating Tfh cell populations, that are amplified post-vaccination, did not correlate with IADLN Tfh cells, SARS-CoV-2-specific GC B cells or NAb responses (98). In other words, whilst these peripheral cells are likely indicative of ongoing LN GC reactions, they are not accurate biomarkers of SARS-CoV-2-specific GC B cell, Tfh cells and MBCs in the IADLN and so fail to accurately illustrate GC reactions, thus highlighting a notable place for FNA in human GC research (98). Therefore, the void that FNA will fill in the study of human GC reactions will no doubt contribute to a greater understanding of this node of the immune system and its role in mediating a protective immune response.

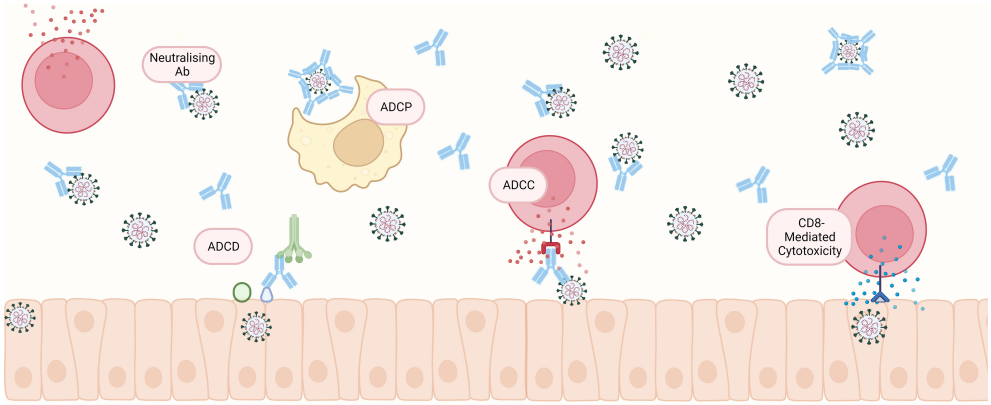
Irrespective of the challenges associated with carrying out a human challenge study as discussed previously, the immune response to a species-specific pathogen is best studied within the species of interest. Hence human COVID-19 challenge trials will be invaluable to the field of immunology research. To date, only provisional findings on viral kinetics have been reported by University College London and Imperial College London. Of the 36 young, naïve and unvaccinated participants, 53% became infected upon challenge with 10 $TCID_{50}$ of SARS-CoV-2, 89% of which experienced mild-to-moderate symptoms and the remaining 11% were asymptomatic cases. Only reports on the induction of Spike IgG and NAbs post-challenge have emerged thus far, with future studies aiming to pinpoint the immune parameters providing protection in the 47% that did not become infected following challenge (71).

Additionally, the development of organoid, 'LN-on-a-chip' technologies will reduce the demand on research animals, thus providing ethical and logistical solutions to the challenges associated with animal research. An organoid developed in the Singh lab, is a gelatin and silicate nanoparticle-based network, that with the addition of appropriate stimuli including integrins, IL-4 and CD40L, has the capacity to direct the differentiation of GC-like B cells at controlled rates within one week (108, 109). 'From one mouse spleen, 500 organoids can be generated, or one human tonsil can mediate the synthesis of 1,000 organoids' (110). This exciting field of research will likely attract extensive interest in coming years and perhaps define the future of immunology.

7 Conclusion

Defining pathogen-specific CoPs is a valuable, yet challenging, endeavour for vaccinologists and immunologists. SARS-CoV-2 is now an endemic CoV and will likely persist as another seasonal human coronavirus infection. Therefore, aged or immunocompromised individuals are likely to receive a seasonal vaccination, as is currently advised for influenza virus. However, the difference between SARS-CoV-2 and influenza virus, is the lack of confidence in the SARS-CoV-2 CoP. The absence of a SARS-CoV-2 CoP minimises the capacity for immunobridging, which would support the approval of yearly variant vaccines, thereby slowing the vaccine approval process and putting pressure on vaccine supply networks.

While proposed SARS-CoV-2 CoPs have successfully facilitated immunobridging for the accelerated approval of SARS-CoV-2 vaccines in subgroups of the population who were not included in original human trials (children and pregnant women), and informed 'boosting' regimens (111), regulatory agencies remain reluctant to approve vaccines in the absence of an accepted



Animal Model	T Cells	Neutralising Antibody	Binding Antibody	Functional Antibody	Mucosal Antibody
a)	(19, 20, 39)	(22, 25-27, 29, 31, 35, 37, 39)	(20, 22, 25-27, 29, 31, 35, 37, 39, 46, 101)	(27, 29, 35, 37, 101)	(46)
b)		(14, 15, 22, 42, 45, 81)	(14, 15, 22, 42, 45, 81)	(23, 43)	
c)	(28)	(49, 52, 95)	(23, 36, 49, 52, 95)	(23)	(36)

FIGURE 6
Graphical summary of CoPs proposed in the referenced literature following computational analysis of preclinical study datasets, involving a) macaques, b) Syrian golden hamsters, and c) mice. ADCP, antibody-dependent cellular phagocytosis; ADCC, antibody-dependent cellular cytotoxicity; ADCD, antibody-dependent complement; Ab, antibody. Created with BioRender.com.

SARS-CoV-2 CoP. Although this is not unheard of when immunogenicity data is directly compared with an approved vaccine, for example, VLA2001 when compared to ChAdOx nCoV-19 (112). Additionally, regulatory agencies recommended approval of the bivalent mRNA-1273.214 (WT/BA.1) based on comparison with approved mRNA-1273 (113), and BNT162b2 Bivalent (WT/BA.4/BA.5) when compared with approved BNT162b2 (114). Acceptance of a SARS-CoV-2 CoP, for which standardised assays have been or can be developed (as is seen with the HAI assay for influenza), will improve the scope for immunobridging, thus accelerating SARS-CoV-2 vaccine approval to meet global demands.

Animal models remain a crucial tool for the identification and confirmation of such CoPs, for reasons outlined in this review. As summarised in Figure 6, the weight of evidence from *in vivo* studies, supported by clinical trials, provides a degree of confidence that a SARS-CoV-2 humoral response CoP will soon be defined and accepted by both the scientific community and regulators. Designing animal studies to further characterise CoPs, *via* the mechanisms discussed in this review, will expedite CoP research and vaccine development for SARS-CoV-2 and future pathogens.

Author contributions

CB & MC were responsible for the concept of the review. CB drafted the manuscript. All co-authors reviewed and contributed to the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Immune correlates of protection for SARS-CoV-2, Ebola and Nipah virus infection

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Correlates of protection (CoP) are biological parameters that predict a certain level of protection against an infectious disease. Well-established correlates of protection facilitate the development and licensing of vaccines by assessing protective efficacy without the need to expose clinical trial participants to the infectious agent against which the vaccine aims to protect. Despite the fact that viruses have many features in common, correlates of protection can vary considerably amongst the same virus family and even amongst a same virus depending on the infection phase that is under consideration. Moreover, the complex interplay between the various immune cell populations that interact during infection and the high degree of genetic variation of certain pathogens, renders the identification of immune correlates of protection difficult. Some emerging and re-emerging viruses of high consequence for public health such as SARS-CoV-2, Nipah virus (NiV) and Ebola virus (EBOV) are especially challenging with regards to the identification of CoP since these pathogens have been shown to dysregulate the immune response during infection. Whereas, virus neutralising antibodies and polyfunctional T-cell responses have been shown to correlate with certain levels of protection against SARS-CoV-2, EBOV and NiV, other effector mechanisms of immunity play important roles in shaping the immune response against these pathogens, which in turn might serve as alternative correlates of protection. This review describes the different components of the adaptive and innate immune system that are activated during SARS-CoV-2, EBOV and NiV infections and that may contribute to protection and virus clearance. Overall, we highlight the immune signatures that are associated with protection against these pathogens in humans and could be used as CoP.

KEYWORDS

immune correlates of protection, emerging viruses, humoral immunity, cell-mediated immunity, SARS-CoV-2, Nipah virus, Ebola virus

1 Introduction

The human immune system responds through multiple interactive mechanisms to any invading pathogen, ultimately controlling the virus infection or clearing it from our system and enabling a more rapid response on subsequent encounters with such a pathogen. Correlates of protection (CoP) may be defined as those immunological parameters, characteristic of a specific immune mechanism, that are associated with protection against infection or disease (1). Understanding these specific immune mechanisms can help to identify specific immune CoP, which can then be used as surrogate measurements of vaccine protective efficacy and to assess the susceptibility of individuals and populations to a specific pathogen. It is important to note that whilst protection against different viral infections can be mediated by similar immune effector mechanisms, CoP are specific for a viral disease or infection (or even a specific manifestation of a disease), for a specific population group (elderly vs children) and even for a specific type of vaccine (CoP for an inactivated Influenza A vaccine may not necessarily coincide with that of an intra-nasal live Influenza vaccine, for example) (2–4).

The immune system is classically divided into two branches: the innate and the adaptive immune response. While innate immunity consists of a rapid but less specific inflammatory response, adaptive immunity develops more slowly, but is long-lasting and highly specific (5). However, this is a non-strict dichotomy since both arms of the immune response are strongly inter-connected. The interplay between the different immune cell populations and the complexity of immune reactions renders the rational design of effective vaccines against a specific pathogen difficult. For instance, while protection mediated through antibodies is prominent during the acute phase of an infection, cell-mediated responses normally play an important role in virus infection clearance and/or during the chronic phase of infection. However, this is not the case for all pathogens (6).

The precise protective role of the different effector mechanisms of the immune system have only been fully characterized for a small number of pathogens. However, this has not prevented the statistical association of specific immune mechanism signatures with protection against a disease manifestation. These statistical correlations are built from data (immunological, virological and clinical readouts), collected from field infections and vaccine clinical trials. Once a statistical association has been made between protection and an immunological biomarker it is difficult to improve, modify or introduce a novel CoP. Therefore, in order to derive reliable CoP, it is necessary first to analyze and review in detail how the immune responses develop in experimental and natural infections. This process would in turn lead to the selection of relevant immunological bio-markers with which to evaluate vaccine efficacy in clinical trials, which ultimately will result in establishing a CoP.

2 Types of protective immune responses

There is a wide range of cell populations and soluble factors involved in the development of a protective immune reponse against a particular pathogen. Each of these immunological parametres is measurable and constitutes the basis from which to define a CoP. Hereinafter, we describe the main players of the immune responses that can lead to pathogen clearance and protection against disease.

2.1 Innate immunity

Innate immunity is the first line of defense against invading pathogens. There are several cell types involved in the innate immune response: monocytes, dendritic cells (DC), macrophages, mast cells, basophils, eosinophils, natural killer (NK) cells and innate lymphoid effector cells. Other than the anatomic and physiologic barriers, innate immune responses comprise endocytic or phagocytic and inflammatory processes as defense mechanisms (7). Both phagocytic and inflammatory immune processes promote the clearance of pathogens and activation of the adaptive immune response (7–9).

For instance, the innate immune response may lead to the activation of the complement cascade which will induce the opsonization of certain pathogens thus rendering them susceptible to phagocytosis, enzymatic degradation or lysis in the case of bacterial pathogens. Another mechanism used during the innate immune response is the production of cytokines and chemokines. This process can lead to an inflammatory state with local activation of cellular responses and recruitment of additional immune cells to sites of infection. The presence of foreign nucleic acid molecules, such as double-stranded RNA (dsRNA) inside the cell, precedes the secretion of interferons (IFNs), the major soluble factors of the innate immune response.

There are several types of IFN but the most notable in virus clearance are type I IFN, which includes IFN-α and IFN-β, IFN type II (IFN-γ) and IFN type III (IFN-λ) (10). The main role of IFN consists in inhibiting viral replication in cells that are already infected but it can also contribute to the protection of neighboring, uninfected cells. Interferon activates signaling pathways that lead to the degradation of the invading pathogen and the activation of some kinase proteins that will shut down the embattled host cell, thus inhibiting viral replication without killing the cell. In some cases, however, the infected cell can also die to prevent viral replication. Hematopoietic cells are the main producers of IFN-α and amongst them, plasmacytoid DCs (pDCs) are the major source (11). On the other hand, most infected cells are capable of producing IFN-β (12). However, the function of innate immune cell populations can be significantly affected during certain infections.

Early innate immune responses do not represent an isolated compartment of the immune system since their activation influences the type of adaptive immune response that develops during the course of the infection (9).

Thus, since soluble factors, molecules and cells involved in the innate immune response influence the development of specific effector mechanisms of the adaptive immune response (antibodies, T cells, immunological memory), which typically define CoP, the measurement of specific innate immune response

signatures could potentially be used during an early phase of a vaccination trial to determine early on the protective capacity of a specific vaccine. In other words, innate immune response signatures can serve as alternative CoP. This is an area that has not been sufficiently exploited so far and deserves further investigation.

2.1.1 Innate effector cells

Innate effector T cells can act without previous exposure to antigens. Examples of innate effector T cells include invariant natural killer T (iNKT) cells and γδ T cell receptor expressing cells. Despite their limited T cell receptor (TCR) diversity and low capacity for proliferation, these cells can rapidly execute effector functions, such as the release of various cytokines, chemokines and growth factors. These mechanisms can initially control infection, interact with the adaptative immune response and even promote the development of effector and memory T cells (13). An example of a robust γδ T cell and effector memory T cell response is that observed after smallpox vaccination (14).

2.2 Adaptive immunity

The adaptive immune system, by virtue of one of its most defining features, immunological memory, enables a fast and effective response against an invading pathogen upon a second exposure to that pathogen and, in many cases, confers long lasting protection. However, for some diseases, it has been shown that protection declines over time. Whilst the level of circulating antibodies is commonly used as a CoP to assess the protective efficacy against many viral diseases, there are instances where protection has been observed in the face of very low virus-specific antibody titers. In these instances, measurements of virus-specific memory B-cell frequency might serve as more realistic CoP than simply measuring serum antibody levels. In other cases, cellular immunity might play a more important protective role than previously recognized. Indeed, during Influenza virus infections it has been clearly demonstrated that virus-specific T-cell responses limit the severity of disease (15).

As a general rule, antibodies tend to prevent cell infection whereas cellular immune responses rather act once replication of the pathogen takes place (16, 17).

2.2.1 Humoral immunity

Humoral immunity results in the production of antibodies that target specific pathogens. There are 5 isotypes or classes of antibodies, IgA, IgD, IgE, IgG and IgM, that have different biological functions. This classification is made according to their heavy chain, namely alpha, delta, epsilon, gamma or mu respectively (7). Additionally, antibodies can be subdivided into several subclasses (IgG1, IgG2, IgG3, IgG4, IgA1, IgA2) that are structurally and functionally different. While the Fab region of an antibody performs mostly a recognition and/or neutralization role, the Fc region is rather used in cell-mediated immune functions (18, 19).

After a first encounter with a pathogen, B cells will differentiate into effector B cells or plasma cells, which will then secrete antibodies specific to the pathogen encountered. A fraction of these cells will then become memory B cells which are long-lived and can respond quickly after a second exposure to the pathogen. IgG antibody production against a first time encountered pathogen can take up to two weeks to develop, however, if re-infection occurs, antibodies are produced after only a day or two thanks to these antigen-specific memory cells (20).

Antibody production is generated by B lymphocytes, however, CD4+ T cells are required in this process. When antigen specific CD4+ helper T cells interact with activated B cells, they produce IL-4 and IL-5 that will then induce B cell proliferation and antibody production. These antibodies can bind to pathogens and prevent their proliferation through different mechanisms such as neutralization, opsonization and complement activation. Neutralization consists of the binding of antibodies to the surface of the pathogen, thus blocking the pathogen's attachment to the cell or interfering with virus uncoating within the cell. Opsonization on the other hand, requires the pathogen to be 'marked' by opsonins (such as IgG antibody, C3b or C1q molecules of the complement) for the subsequent phagocytic removal of the pathogen (21). Complement-dependent cytotoxicity (CDC) takes place when complement protein C1q (in the classical complement pathway), C3b (in the alternative complement pathway) or Mannose binding lectin (MBL), in the Lectin complement pathway, bind to the Fc region of IgG or IgM, coupled to a pathogen antigen expressed on the surface of an infected cell. This activates the complement pathway that will lead to the formation of a membrane attack complex (MAC) that will then cause cell lysis (22, 23).

In some cases, B cells can direct other immune cells to eliminate the pathogen *via* Fc-Fc receptor (FcR) interactions, thus combining the strong antiviral functions of innate immune effector cells with the specificity of the adaptive humoral activity. These mechanisms comprise Antibody Dependent Cellular Cytotoxicity (ADCC) and Antibody Dependent Cellular Phagocytosis (ADCP) (24). During these cell-mediated immune mechanisms, antibodies are produced that will bind the pathogen and these will then be recognized by effector cells that have FcR, namely NK cells, neutrophils, macrophages and dendritic cells. In the case of ADCC, the effector cell will lyse the targeted cell containing the pathogen on the surface coated with IgG1 or IgG3 containing the bound Fc. Pathogen infected cells can be eliminated through the action of cytokines, reactive oxygen species (ROS), perforin and/or granzymes. In contrast, during ADCP, the targeted cell will be engulfed and processed for phagolysosomal degradation. The main leukocytes involved in ADCP include monocytes, macrophages, neutrophils, and eosinophils (25, 26). In addition, B cells can activate and present antigens directly to effector T cells. B cells can directly recognize certain antigens *via* their surface IgG. These specifically bound antigens will be endocytosed, processed and their peptides presented to specific antigen matching T helper cells. As a result of this interaction, B cells express costimulatory molecules that can activate the T helper cells that will then coordinate effector functions.

It is also important to mention the role of immune memory, which during certain infections can be highly correlated with protection. B cell memory is generated by two different cell subsets: memory B cells and long-lived plasma cells or memory plasma cells. Thus, upon a second antigen exposure, memory plasma-cells can rapidly produce antibodies and memory B cells can differentiate faster into plasma cells and start a quick and robust response producing antibodies, isotype switching, effector functions and affinity maturation besides rapid proliferation (27). These processes can play an important role when low, pre-existing antibody levels are present or if the existing antibodies are overcome by the infectious agent (8, 16).

The ability to induce a strong humoral response is the hallmark of an effective host defense against certain infections (7). There are several factors that may affect the efficiency of the antibody response such as the titer, location, subclass of antibody, time of appearance and durability. However, the specific threshold levels of antibody titers conferring protection against many specific pathogens are either currently undetermined or variable amongst pathogens (28). Nevertheless, due to the ease of measuring antigen-specific antibody levels in various clinical specimens and bodily fluids, CoP based on antibody level measurement (e.g. virus neutralization, antibody binding assays, hemagglutination inhibition assays) have been used extensively to assess the immunity of populations against a specific pathogen and to evaluate vaccine efficacy. Furthermore, collection and processing of clinical material for antibody analysis is relatively simple in comparison to collecting, storing and processing PBMC for the assessment of cell-mediated immune responses.

2.2.2 Cell-mediated immunity

Most infectious pathogens are susceptible to the action of antibodies during the extracellular phase of their infection cycle. However, humoral immune responses are not completely effective at clearing pathogens when they are inside cells and cell-mediated immune effector mechanisms are called upon to clear viral infection. These mechanisms are mediated typically by CD8+ cytotoxic T-lymphocytes, which bind in a specific manner *via* their T-cell receptors, to the MHC-I molecules of infected cells that display viral antigen-derived peptides. However, this is not the only cell-mediated effector mechanism of T lymphocytes. Indeed, upon encountering infected cells, T cells secrete pro-inflammatory cytokines, co-stimulatory soluble factors and other regulatory signals. Thus, cell-mediated immunity (CMI) is relevant for intracellular pathogens and this protective mechanism can also synergize with an antibody production strategy in order to achieve a protective response. For instance, it has been shown that certain antibodies can activate Th1 cells through FcR, thus facilitating the rapid processing of antigens (29). Likewise, as alluded to earlier, ADCC and ADCP can also be considered as hybrid effector mechanisms of immunity involving the synergistic action of antibodies and innate immune effector cells.

T cells are considered as the main mediators of cellular adaptive immune responses. They have a crucial role in immunosurveillance since they can discriminate pathogen-derived peptides from native "self" proteins. In order to mount an efficient immune response, after recognition of foreign peptides these cells undergo activation.

During infection, antigen-presenting cells (APCs) recognize and process invading pathogens thus presenting these foreign epitopes to T cells through major histocompatibility complex (MHC) molecules. Such peptides can only be recognized by T cells when they are presented by MHC molecules. There are two types of MHC: class I, which is expressed on the surface of all nucleated cells, and class II which is located on surfaces of specialized APCs. CD8+ and CD4+ T cells will bind MHC I and MHC II respectively.

CD4+ T cells play a central role in the development of the adaptative immune response since they direct downstream effector mechanisms of other immune cells through the secretion of different types of cytokines and chemokines. Through their MHC-II molecules, APCs can present pathogen peptides to naïve CD4+ T cells. If activated, APCs then provide specific co-stimulatory signals resulting in T cell proliferation and differentiation of naïve CD4+ T cells into specific functional T helper (Th) cell subsets, namely: Th1, Th2, Th9, Th17, Th22, T follicular helper (Tfh) and regulatory T (Treg) cells, amongst others (Figure 1). These Th cells contribute to immunoregulation of inflammatory, humoral or CMI responses through the release of effector molecules. A Th1 response for instance, involves the release of tumor necrosis factor-alpha (TNF-α), IFN-γ, and interleukin-2 (IL- 2) amongst others, that will mainly help to clear intracellular pathogens. Th2 responses will release IL-4, IL-5, IL-6 and IL-13, that are mainly involved in the clearance of extracellular pathogens. Th17 effector cells will secrete IL-17, IL-21 and IL-22 and are responsible for the clearance of some extracellular pathogens, however they are also involved in auto immune processes. On the other hand, Treg cells are involved in tolerance and secrete mainly TGF-β and IL-10. It is important to take into consideration that the release of the mentioned cytokines is not exclusive to T cells and that some other immune cells are also an important source of cytokines and chemokines that play crucial roles during infection. In addition, CD4+ T cells also release soluble factors that contribute to the generation and maintenance of CD8+ T cells (30).

As mentioned earlier, cytotoxic CD8+ T lymphocytes (CTL) also play an important role during CMI responses. When activated through MHC I presentation of certain intracellular antigens, these cells release cytotoxic proteins such as perforin, granzyme and cytokines including IFN-γ, TNF-α, IL-2, IL-4, and IL-10, that trigger the killing of specific target cells (8, 31).

Once infection is cleared, antigen-specific effector T cell (CD4+ and CD8+ T) populations decline and a small cellular subset is maintained as antigen-specific effector and long-lived memory T cells (CD4+ and CD8+ T cells) (32). Hence, in a secondary immune response the numbers and the activation status of T cells rapidly increase, the stimulatory antigen requirement to induce a response is reduced and as a consequence, a faster response of the effector functions takes place compared to a first contact with the pathogen (32). There are two main subpopulations of memory cells: effector-memory T cells and central-memory T cells. Effector memory T cells circulate through non-lymphoid tissues and provide an immediate response at pathogen sites of entry, but they have a poor proliferative capacity. Central memory T cells, on the other hand, are located in secondary lymphoid tissues, have a long-life

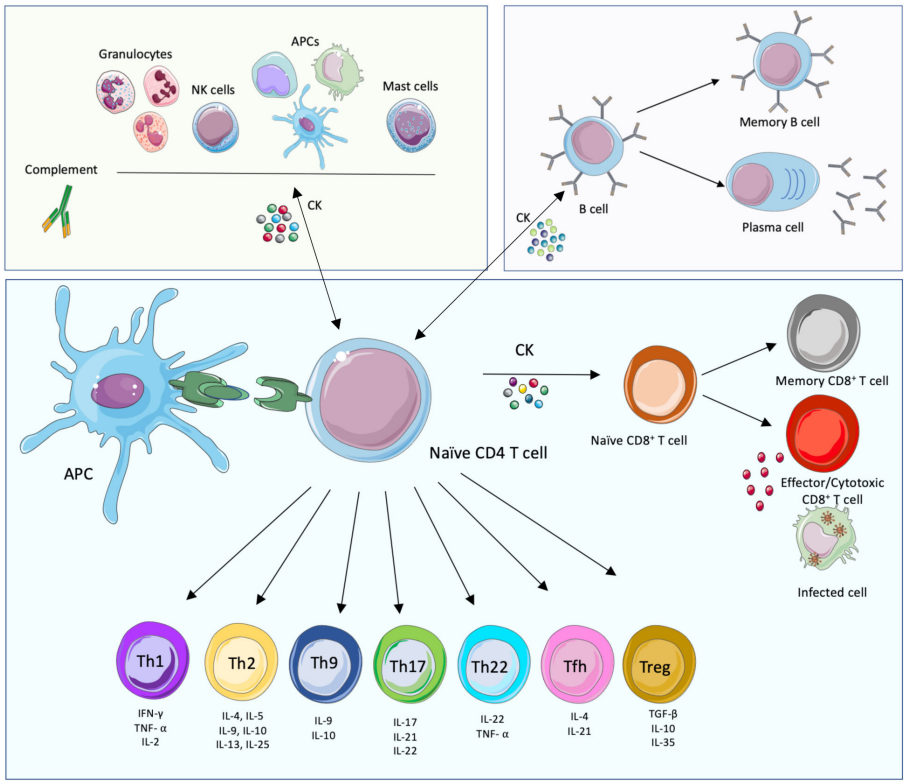

FIGURE 1
Innate, humoral and cellular-mediated immune responses. The main cellular immune players of the cell-mediated innate (green background, top left), humoral (purple, top right) and adaptative cell-mediated (blue, bottom) immune responses and their interconnections are displayed. The components of the innate immune system provide, together with their effector functions and soluble mediators, an immediate response to pathogens. This response triggers in turn the adaptive immune system, mostly T cell-mediated immune responses that lead to the activation of effector T cells and the activation of B cell functions. This branch of immunity provides specific, long-lasting immune responses. The adaptive and innate immune systems are connected; importantly, while soluble mediators are important to link both arms of immunity, the presentation of foreign peptides (in green) by Antigen Presenting Cells (APCs) is also necessary, together with immune mediators such as cytokines (CK). This figure was created with smart.servier.com.

span and a high proliferative capacity. Together, effector and central memory T cells have been shown to protect and reduce infection levels in several vaccine studies (32, 33).

Factors such as T-cell phenotype, which antigen the cells are specific to and their function can influence the potential of the CMI response to be an immune CoP during infection. Thus, T cell proliferation and the specific cytokine profile secreted by immune cells in response to specific antigens could be used as CoP for certain infections.

3 Protective immune responses during SARS-CoV-2, Nipah virus and Ebola virus infection

3.1 SARS-CoV-2

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is an enveloped virus with a positive single-stranded RNA genome that belongs to the *Coronaviridae* family and the ß-Coronavirus genus (34).

SARS-CoV-2 is transmitted mainly *via* respiratory droplets and can cause the syndrome known as coronavirus disease 2019 (COVID-19). While most patients are asymptomatic or mildly symptomatic with flu-like symptoms, 13.9% of patients can experience complications that may lead to acute respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC) or organ-failure, amongst others (35, 36). This, together with its high transmissibility, makes this virus a public health threat for humans.

Upon entry into a host's cells, SARS-CoV-2 causes cell damage and triggers a host immune response. There are several molecular mechanisms by which the human immune system can be hijacked by SARS-CoV-2. In this way, innate immune responses are affected, adaptive immune responses are delayed and as a consequence, viral clearance is inefficient and the virus can spread systemically.

3.1.1 The role of cytokines in SARS-CoV-2 infection

While inflammation is crucial for the development of an efficient and coordinated antiviral immune response, an exacerbated inflammatory response can become detrimental for the host. This is the case for SARS-CoV-2 infection where a

'cytokine storm' signature has been shown to be a common denominator in severe cases of COVID-19 (37–40). These high levels of proinflammatory cytokines have been associated with injury and loss of lung function, increased levels of SARS-CoV-2 load and severe or fatal outcomes (41). Some proinflammatory markers that are elevated during severe COVID-19 include IL-6, IL-8, IL-1β, TNF-α, MCP-3, TGF-β, CXCL10 and IL-17, amongst others (42, 43). Severe cases of SARS-CoV-2 also correlate with the release of ROS. It is believed that ROS, in turn, increases the expression of proinflammatory cytokines, which further contribute to disease severity (44, 45). The elevated expression of multiple chemokines during COVID-19 also leads to high numbers of neutrophils and monocytes, which in severe cases, can infiltrate the alveolar spaces and are believed to contribute to lung injury and increased disease severity (46, 47). Moreover, this cytokine storm also has an impact on the adaptive immune response, since the low expression of HLA-DR induced by high concentrations of IL-6 and TNF-α leads to a pronounced lymphopenia in severe COVID-19 cases (48).

Amongst cytokines, it is also important to mention IFN. Despite its importance in viral clearance, the precise role of IFN during COVID-19 has still not been elucidated; IFN-I production can be partially impaired by SARS-CoV-2 proteins such as the M protein or non-structural proteins nsp1, nsp6, nsp13-15 and orf6 (49–51). The efficacy of IFN during COVID-19 is however controversial, while some studies have shown a potential protective role during SARS-CoV-2 infection (52, 53), others have suggested that it may be detrimental during infection (54). For instance, it has been shown that high levels of IFN-α are associated with high viral loads and severity, thus indicating that in some severe cases, IFN signatures fail to clear the viral load (55). However, this could be explained by a delayed production of IFN when SARS-CoV-2 titers are already too high and thus IFN cannot clear the virus.

On the contrary, it seems that an early production of IFN and an efficient adaptive immune response correlate with control of SARS-CoV-2 infection, whilst both absence or prolonged presence of IFN can lead to cellular hyperactivation with high inflammation levels that may cause a detrimental clinical outcome (56). Some other studies have suggested a link between severe human cases of COVID-19 and defective IFN responses (52, 57, 58). For instance, it has shown that the impairment of IFN responses, caused by insufficient production of IFN or the presence of autoantibodies against interferons in the host, is correlated with COVID-19 severity (58, 59). In agreement with an IFN protective role, several treatments have been shown to alleviate the severity of COVID-19 by accelerating viral clearance and decreasing levels of certain pro-inflammatory cytokines (60–63). Interestingly, a recent study showed that treatment with a single subcutaneous injection of pegylated interferon lambda in (majoritarily vaccinated) patients with acute COVID-19 decreased disease severity by 51% when compared with placebo-treated patients (64). While not all aspects of IFN effects during COVID-19 are clear, it would appear that IFN treatment during COVID-19 is more efficient in the early phases of the disease (65–67).

In addition to the timing of IFN production or its presence or absence during COVID-19, the anatomical location of IFN also seems to be relevant to determine disease severity. Currently, complete data regarding the circulating levels of IFN-I in association with the severity of COVID-19 disease are lacking. Some studies have shown that circulating IFN-α levels were not significantly different between severe and mild cases when measured in plasma (68) and that prolonged IFN production during SARS-CoV-2 infection can be detrimental for the host and cause negative clinical outcomes (54, 69). However, with regards to local IFN lung production, high IFN-III levels in the upper respiratory tract have been seen to be protective during COVID-19 resulting in mild cases, while high IFN-I and IFN-II levels in the lower respiratory tract have been associated with severe cases of COVID-19 (70). Other factors, such as the age of the patients, seem to be relevant for the severity of the disease. This could be related to the fact that IFN production is impaired by age through the decrease in RIG-I signaling efficiency and pDC IFN production capacity (71). For instance, in a recent study on SARS-CoV-2 infected macaques, it was shown that, in aged macaques, there was higher expression of pro-inflammatory cytokines, lower IFN-I and increased lung pathology (72).

Therefore, the role of IFN during COVID-19 may be strongly influenced, in addition to its presence or absence, by its anatomical location, the moment at which it is produced and the pre-existing cytokine host environment and pre-immune status. Thus, depending on the host and disease context, IFN kinetics can result in a protective or detrimental outcome.

3.1.2 The role of T cell immunity in SARS-CoV-2 infection

Adaptive immune responses are essential in controling and clearing SARS-CoV-2 infection, thus cellular and humoral immunity can confer protection during COVID-19. However, adaptative immune responses are highly influenced during infection by multiple factors, including the immune status of the host (genetic and acquired factors), the efficacy of the innate immune response and the initial virus load, amongst others (73–75).

T cell responses seem to correlate with protection during SARS-CoV-2 infection, however, they are partially impaired in severe cases of COVID-19 thus leading to exacerbated activation and lymphopenia (56). Although the mechanisms responsible for this phenomenon are not fully understood; it would appear that lymphocyte hyperactivation, exhaustion and impaired lymphocyte proliferation can contribute to disease, especially in severe COVID-19 cases (76, 77). The type of T cell response seems to also be relevant. While a biased Th1 phenotype seems to be associated with milder COVID-19 cases and good clinical outcome, Th2 and Th17 responses have been shown to be more prominent and detrimental in severe cases (76, 78). However, it is difficult to draw a general conclusion from such studies, as the observations are often based on relatively low numbers of patients.

In terms of cellular immunity, it is important to identify SARS-CoV-2 specific epitopes that elicit efficient responses in humans. The immunodominance and immunoprevalence of a peptide correspond respectively to how strongly and how frequently a given peptide sequence is recognized by T cells. In the context of

SARS-CoV-2, it is possible that an optimized immunodominance and immunoprevalence could improve the efficiency of host immune responses. Therefore, by knowing which specific epitopes can elicit an efficient T cell response, it is possible to modulate the immune responses, thus possibly improving the outcome of the disease by providing immunological memory.

It has previously been shown that convalescent COVID-19 patients harbor an efficient CD4+ T cell response against the SARS-CoV-2 spike (S) glycoprotein. This response also seems to correlate with the presence of specific IgG and IgA titers. Interestingly, several studies showed that some individuals unexposed to SARS-CoV-2 had S specific CD4+ T cells and, at a low level, specific CD8+ T cells (79–81). The presence of these specific responses in non-previously infected individuals could be explained by cross-reactivity responses from previous coronavirus infection. In support of this, an additional study showed that some human samples obtained before the SARS-CoV-2 virus pandemic harbor preexisting memory CD4+ T cells that are cross-reactive to specific SARS-CoV-2 epitopes but also to other common cold coronaviruses (82). Of note, while CD4+ T cells from healthy donors mostly target the C terminal part of the S glycoprotein, CD4+ T cells from COVID-19 patients target almost equally both the N- and C-terminal parts of the SARS-CoV-2 S glycoprotein. This is probably due to the fact that the C-terminal part of the S glycoprotein of many betacoronaviruses has high homology (83). Another difference is that CD4+ T cells from convalescent patients from mild to severe COVID-19 are in an activated state (81). In addition, S glycoprotein-specific T CD4+ cell responses are considered to support antibody generation, thus correlating cellular with humoral immunity in the memory phase (84). Whether S specific T cell responses provide a potential protective role or modulate the severity of the disease in healthy individuals when exposed to SARS-CoV-2 remains to be determined. Another mechanism of protective immunity that has been asasociated with protection against COVID-19 is the presence of resident memory T cells in the lungs, which can last up to 10 months post-infection regardless of the severity of COVID-19 (85, 86).

CD8+ T cells responses seem to be highly heterogeneous between COVID-19 patients. A correlation has been shown between a high expression level of effector molecules by $CD8^+$ T cells and a positive clinical outcome (78). This is supported by non-human primate (NHP) models, where, in SARS-CoV-2 infection in macaques, $CD8^+$ T cell responses have an important role in protection even when neutralizing antibody levels are low (87). The relevance of CD8+ T cells was also supported in recovered COVID-19 patients, where they were shown to harbor not only SARS-CoV-2-specific $CD8^+$ T cells, but also $CD8^+$ T cell memory cells (88, 89). Likewise, clonal expansion of CD8+ T cells has been suggested to be present in mild COVID-19 cases (56). Several studies have identified the importance of respiratory $CD8^+$ T cell responses and the importance of the interaction between cytotoxic CD8+ T cell and epithelial cells in the upper respiratory tract (90).

In conclusion, severe COVID-19 cases correlate with a delayed and excessive adaptive immune response, whilst in milder and convalescent cases, it appears of importance to have an early robust T cell response that leads to SARS-2 clearance. Moreover, T cell responses are probably not redundant thus cellular and humoral responses can be simultaneously considered as CoP.

3.1.3 The role of B cells in SARS-CoV-2 infection

In general, a specific level of circulating anti-viral antibodies is necessary to confer humoral protection against infection. Most patients with COVID-19 develop IgM and IgG within days to weeks after the onset of symptoms (91). However, the relevance of SARS-CoV-2 specific antibodies during infection is not yet clear.

The protective effect of humoral immune responses against SARS-CoV-2 re-infection depends on how long the humoral response lasts and the antigenic characteristics of the re-infecting virus. After natural, SARS-CoV-2 infection, virus-specific T cells, memory B cells and protective neutralizing antibodies can be detected more than 1 year after infection (92–94). In general, memory B cells and specific protective antibodies are still present at 12-18 months post-infection (at least as long as the studies lasted). Indeed, in one study, 20 months after initial SARS-CoV-2 infection, natural antibody and cellular immunity were still shown to confer protection against infection and hospitalization in 95% and 87% of cases respectively compared to patients that presented no immunity (95). In comparison, vaccine-induced immunity decays faster than natural immunity. Thus, after vaccination a hook effect is observed, while protection is very efficient in the first months, it has been shown to decline more rapidly, nearly disappearing five months after the second dose (96, 97). Moreover, after vaccination the immunogenic reaction takes place against the spike S protein only and IgA is minimally elicited.

With regards to the natural humoral response, it was shown that 300 days after natural infection, IgG antibodies against SARS-CoV-2 S and N proteins were present in 68% and 87% of subjects respectively (98). In fact, many studies have shown the presence of SARS-CoV-2 IgG neutralizing antibodies months after contracting COVID-19 (88, 99–103). Importantly, recent studies have detected the presence of neutralizing IgAs on the surface of the upper nasopharyngeal airway mucosa, lasting for several months (104, 105).

When hybrid immunity takes place (natural + vaccination immunity) the data is slightly contradictory. Some studies show that vaccination in recovered COVID-19 patients improves the disease outcome or increases antibody titers (106–110). For instance, in the previous mentioned (95) study, hybrid immunity induced by either one or two doses of a COVID-19 vaccine was associated with an additional risk reduction of SARS-CoV-2 reinfection compared with natural immunity for up to 9 months, although with small absolute differences. An additional study has shown that infection probability after vaccination is significantly lower than the possibility of reinfection after natural infection (111). For instance, it has also been shown that hybrid immunity is 95.3% and 97.4% effective in preventing hospital admission and severe disease respectively at 6 and 12 months, with the first vaccination dose after the most recent infection. With regards to reinfection, hybrid immunity effectiveness after primary vaccination decreased to 46.5% and 41.8% at 6 and 12 months respectively (112). On the contrary, several studies have shown no statistically significant differences between natural or hybrid immunity effectiveness in terms

of increase in neutralizing antibodies, cellular immunity, or specific memory B cells in recovered COVID-19 patients after the second vaccine dose (113–116).

Age is also an important factor with regards to antibody titers against SARS-CoV-2. It has been shown that adaptive immune humoral responses wane with age not only after COVID-19 illness but also after vaccination (117–119).

Overall, there are many studies determining the potential protective role conferred by previous infection and/or vaccination and while there is conflicting evidence, it has been shown that in most cases the presence of high antibody titers decreases the risk of infection by SARS-CoV-2, albeit this risk is not completely eliminated. These studies are summarized in (120). Importantly, when reinfection takes place, previously SARS-CoV-2-exposed patients or recently vaccinated individuals seem to be protected from relevant clinical repercussions and against infection by certain variants (121–123). In these studies, neutralizing antibody titers correlated with the level of protection and thus this parameter is often used as a CoP for COVID-19. In contrast, with regards to the potential protection of SARS-CoV-2 pre-existing humoral immunity towards new molecular variants of the virus, some studies in rhesus macaques have shown that neutralizing antibodies developed during a first SARS-CoV2 infection confer clinical protection against some of the new variants (124–126). Moreover, there are indications that existing humoral cross-reactivity does occur between SARS-CoV-2 and other Beta-coronaviruses. For instance, it has been shown that neutralizing antibodies from the 2003 SARS-1 outbreak can neutralize SARS-CoV-2 (82).

It is also important to consider that one-fifth of SARS-CoV-2 infections result in long-term COVID-19, where despite viral clearance, certain symptoms persist and can lead to post-acute sequelae of COVID-19 (PASC). The dysregulation of the host immune response and virus persistence are believed to account for the development of PASC. Among the immune responses noted in PASC, a distinct humoral immune response was observed, with more avid IgM, weaker Fcγ receptor binding anti-SARS-CoV-2 antibodies and an expanded inflammatory antibody response recognizing the human Betacoronavirus OC43 that can cross-react across SARS-CoV-2 and other coronaviruses. In some cases, CD8+ T cells against Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) reactivation have also been detected (127, 128). The mechanism by which these markers lead to PASC are still not well known and may involve different pathophysiological mechanisms that translate into PASC being an heterogenous syndrome with specific endotypes.

In summary, protection against SARS-CoV-2 depends on a coordinated immune response involving various effector mechanisms of the adaptive immune system. The information acquired to date on SARS-CoV-2 immunity is enabling the development of effective treatments and vaccines to reverse the detrimental immune responses sometimes associated with infection. In conclusion, protection against SARS-CoV-2 depends on: (i) eliciting an early non-exacerbated, innate immune response with limited early IFN production, (ii) inducing a robust cellular response without hyperactivation of T cells, (iii) inducing an effective humoral response with neutralizing antibody production, (iv) generating immunological memory and (v) producing cross-reactive, non-specific innate and adaptive immune responses to generate heterologous protection against COVID-19. The main COVID-19 immune CoP candidates are displayed in Supplementary Table 1.

3.1.4 SARS-CoV-2 variants and their interplay with immune responses

The increasing SARS-CoV-2 genomic diversity poses a potential threat to vaccination efficiency since antigenic changes can lead to the appearance of variants of concern (VOCs) with improved viral fitness that can jeopardize a host's immunity in comparison to previous circulating strains. VOCs sometimes have significant mutations that give them unique properties with a functional impact affecting virus-host interactions and infection capacity, transmission and/or replication, amongst others. As of March 2023, the following major VOCs have been indentified: Alpha (B.1.1.7), Beta (B.1.315), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529. */BA.*) (129).

Interestingly, SARS-CoV-2 variants have been shown to differ in their capacity to bind to the SARS-CoV-2 receptor Angiotensin-converting enzyme 2 (ACE 2), in their antibody escape capacity or in triggering different host immune responses. For instance, while the first SARS-CoV-2 variants tended to induce a stronger innate immune response, SARS-CoV-2 Delta has integrated multiple improved mechanisms to evade an IFN response by suppressing the host innate immune response (130).

SARS-CoV-2 Omicron has recently been shown to include many concerning mutations that affect several viral proteins. SARS-CoV-2 proteins can be classified into three categories: structural, non-structural and accessory proteins. Structural SARS-CoV-2 proteins notably play a role in virion assembly and formation. There are four major SARS-CoV-2 structural proteins: the spike protein S, the envelope protein E, the membrane protein M and the nucleocapsid protein N. The Omicron variant has been shown to contain unique mutations mainly in the receptor-binding domain (RBD) and the N-terminal domain of the S1 spike subunit (131). Omicron spike mutations increase binding to ACE 2 and enable antibody escape, thus adding an increased immune evasion capacity to an already higher transmission and replication fitness. While the most explored mutations are found in the spike protein, further mutations in Omicron have also been detected in the N-terminus region of the structural E protein which is known to interact with NSP3 for ubiquitination and glycosylation (132), and also in the M protein that promotes the assembly of new viral particles, affecting membrane integrity and post-translational modifications (133). Unique mutations are also found in the N-terminal region of N which translates into a more significant inhibition of RNA-induced IFN expression (134). Overall, while the functional effect of these mutations has not fully been studied, it is believed that they can modulate host-virus interactions and thus, increase SARS-CoV-2 Omicron replication, pathogenicity, and fitness (135).

Mutations in non-structural proteins for Omicron may also have a crucial effect on immune regulation, transcriptional regulation and viral pathogenesis. For instance, some mutations

take place in NSP1, that binds to ribosomal subunits to stop host protein translation (136), or in NSP3, NSP4, and NSP6 that are responsible for viral budding by modifying the endoplasmic reticulum (ER) into double-membrane vesicles (137, 138). In addition, mutations in NSP14 can cause post-transcriptional modifications (139). Mutations have also been observed in NSP5, the main viral protease which also harbors the binding site for this enzyme, and that might which affect viral enzymatic processing activity. Additionaly, mutations detected in NSP6 could help virus survival through the avoidance of autophagosome fusion with lysosomes (140).

Accessory proteins normally act as virulence factors mainly through immune evasion mechanisms that increase viral survival in the host. In SARS-CoV-2, there are eleven accessory proteins of which some are known to be potent interferon antagonists. For instance, in the Omicron variant some mutations have appeared in ORF3a and ORF7b that inhibit STAT signaling phosphorylation and ISGs expression (141, 142). Other examples include ORF3b, ORF6, ORF7a, ORF8 and ORF9b, that are also known to have IFN-antagonistic activity (143–145). ORF9b and ORF9c are also known to interact with cellular organelles, reducing antiviral responses (129, 144–146).

Altogether, it has been shown that Omicron can evade the host immune response more efficiently than previous VOCs. This is credited to decreased recognition by neutralizing antibodies but also to new acquired mutations that lead to increased viral fitness, higher transmission rates and better host immune evasion amongst others.

3.2 Nipah virus

Nipah virus (NiV) is an enveloped virus with an 18 kb negative-sense single-stranded RNA genome that belongs to the *Paramyxoviridae* family (147). There are two different strains: NiV Malaysia (NiV-M) and NiV Bangladesh (NiV-B) (148).

NiV outbreaks are reported almost yearly, the most recent occurring in India in 2021, notably with one of the highest fatality rates (92%) observed in the last few years (149). NiV infection in humans is generally associated with an acute respiratory and neurological syndrome resulting in a high fatality rate of between 40% and 92%, depending on the local capacity for epidemiological surveillance and clinical management (150–152).

The NiV reservoir has been identified as fruit bats of the *Pteropus* genus (153). It is known that NiV can also cause severe disease in domestic animals such as pigs, resulting in significant economic losses for farmers (154). Currently, there are no approved treatments or vaccines available for either humans or swine infected with NiV.

Here we aim to summarize the main features of the innate and the adaptive immune response to NiV and discuss the identification of potential immune CoP.

3.2.1 The role of cytokines in NiV infection

NiV infection triggers a robust inflammatory and IFN-I response involving the expression of various IFN-induced antiviral genes. However, to counteract this, NiV expresses several structural and non-structural proteins that can efficiently antagonize a host immune response (155). For instance, the structural matrix M protein and non-structural accessory proteins C, V and W play important roles in preventing IFN-I activation and production at many stages of the signaling pathways involved [summarized in (156)]. In addition, the disproportionate production of pro-inflammatory cytokines at the very early steps of NiV infection in humans considerably contributes to its pathogenicity by causing vasculitis and encephalitis characterized by inflammatory cell infiltration (150).

In this regard, neutrophils are very important during the early steps of the innate immune response since they are involved in several defense mechanisms, including the production of antimicrobial peptides or ROS-induced neutrophil extracellular traps (NETs). However, while in general, NETs can trap and act upon viral particles, in some respiratory virus infections, such as with respiratory syncytial virus (RSV) and influenza A virus (IAV), an exacerbated release of cytokines can lead to high levels of neutrophil activation and excessive NET formation leading to airway occlusion and increased lung inflammation that is detrimental for the host (157). While this has not yet been specifically shown for NiV infection, it is very likely that, similar to what occurs in IAV and RSV infection, a strong release of proinflammatory cytokines leads to hyper-activation of neutrophils which can result in tissue damage. Moreover, it has previously been shown that during NiV infection, while neutrophils do not seem to be infected, they play a prominent role in disseminating NiV (158).

Some of the pro-inflammatory mediators released during NiV infection in humans include TNF-α, CXCL10 and interleukin-1β (IL-1β). The three have been shown to have an important role in disrupting the blood-brain barrier (BBB) and contribute to the neurological symptoms observed in severe cases of NiV disease (NiVD) (150, 157, 159). In addition, other pro-inflammatory mediators such as IL-6, IL-8, MCP-1, GM-CSF and G-CSF have been shown to be released at high levels in severe cases of NiV infection, particularly in the lungs. It has also been shown that this increase in inflammatory chemokines correlates with increased monocyte and T lymphocyte chemotaxis (155).

As a result, it is believed that IFN-I impairment and the release of high levels of pro-inflammatory mediators can contribute to the worsening of clinical symptoms (157). Altogether, data indicates that NiV employs many strategies to counteract the innate immune response and that specific levels of IFN-I and pro-inflammatory cytokines could be used to determine the outcome of the infection. Further study is needed in order to establish these correlations more specifically.

3.2.2 The role of T cell immunity in NiV infection

To date, very little information is available on human cellular immune responses to NiV infection. During the 2018 NiV outbreak in Kerala, India, 18 patients were confirmed to be infected with NiV, of which 2 survived the disease. Cell mediated and humoral immune responses were studied during the acute and convalescent phases of the disease (160). Throughout these

periods, surviving patients presented stable T lymphocyte absolute numbers and CD4+ T cells were not more activated than in healthy individuals. However, this was not the case for CD8+ T cells that were more activated and indicated active proliferation and effector functions during the acute phase of the illness and returned to basal levels during the convalescence phase.

Interestingly, the clearance of NiV from the blood seemed to happen before the humoral response (NiV-specific IgG antibodies) took place and rather coincided with the aforementioned activation of CD8+ T cells. In a study where African green monkeys (AGM) were infected with NiV, analysis of the peripheral immune response also showed high levels and activation of T CD8 effector memory cells in surviving AGMs, correlating with an increased release of cytokines and associated cell-mediated immunity (161). This is interesting since CMI was not shown to be relevant in animals that succumbed to NiV infection, thus suggesting that effector memory cells were only relevant in survivors. Interestingly, the activation and proliferation of CD8+ T cells was also observed in the only two survivors of the NiV outbreak in Kerala (160). In contrast, in another study performed in a porcine model, a reduction of CD4 + T cell populations was shown in individuals with a poor clinical outcome (162).

There are several limitations that make it difficult to draw conclusions about cellular immune responses during NiV infection. In human studies, small sample sizes and the lack of samples from disease victims to compare with survivor samples often limit the robustness of the conclusions drawn. Moreover, a complete overview of the relevance of cellular immune responses during NiV infection is lacking, in part due to an absence of CMI response studies in NiV animal models.

During infection, robust T cell responses would enable the development of a faster transition between innate and adaptative immune responses and thus accelerate the production of antibodies and protective immunity. While more data is required, preliminary studies in humans and non-human primates indicate that cellular immune responses, specifically CD8+ T cell activation, seem to be important for protection and therefore CoP for NiVD can be derived from CD8+ T cell measurements.

3.2.3 The role of B cells in NiV infection

Similarly, humoral immune response studies of NiV infection in humans are very limited. However, in the previously mentioned study on the two Kerala NiV survivors in 2018, both patients showed an increased number of B lymphocytes that correlated with the presence of NiV-specific IgG and IgM antibodies within a week after exposure. Moreover, an increased level of activated B cells and plasmablasts was present in both survivor patients (160). However, the specific NiV antigens targeted by the NiV-specific humoral response are yet to be identified.

The correlation between protection against NiV and the presence of antibodies has also been demonstrated in several animal models of infection. For instance, in NiV-infected swine, neutralizing antibodies were detected a week post-infection, with considerably increased titers observed two weeks post-infection. However, NiV RNA could still be detected several months after the initial infection (163). In an AGM model, B cell numbers decreased at twelve days post-infection, a fact that correlated with disease progression and a detrimental outcome (161). In contrast, the only surviving animal in the study showed robust IgM and IgG responses which correlated with an increase in B cell lymphocytes, suggesting that humoral immune responses are relevant during NiV infection and may afford protection against the virus. Moreover, humoral immunity relevance during NiV infection has also been shown in several models including ferrets, hamsters and again in AGM. In these models, the administration of sera or NiV-specific monoclonal antibodies was shown to protect from NiV challenge (164–168).

With regards to fatal cases, there is almost no data for the acute phases of NiVD in humans, and the existing data derives mostly from histopathological analysis of post-mortem samples. However, it has been shown in AGM that lymphopenia takes place in fatal cases of infection with both NiV-M and NiV-B strains (169).

Despite the limitations in human and animal model studies for NiV infection, humoral immune responses seem to play an active role in protection and it is likely that CoP could be derived from humoral immunity parameters such as numbers of plasmablasts and activated B-cells and specific titers of IgM and IgG antibodies.

The main NiVD immune CoP candidates are summarized in Supplementary Table 1.

3.2.4 NiV interplay with immune responses

NiV has several proteins that modulate the host immune response. For instance, NiV viral proteins P, C, V and W can antagonize the IFN signaling response (170). While NiV-W protein sequesters STAT1 in the nucleus to inhibit subsequent ISG activation, NiV-V protein antagonizes IFN by binding STAT1 and STAT2 thus preventing their dimerization and transport to the nucleus for transcriptional activation of ISG genes. NiV-P protein is also able to bind and sequester STAT-1 in the nucleus (171, 172). NiV-C protein prevents IFN production in the cytoplasm, but the details of this process are still not well known (156, 173). Further IFN antagonistic mechanisms of *P* gene products are produced through interactions with TANK-binding kinase 1 (TBK1), Inhibitor of κB kinase ϵ (IKKϵ) and IRF-3 by the NiV-W protein (174, 175) or through inhibition of STAT2 (176), LGP2, RIG-I (177), and MDA5 (178) by NiV-V, thus preventing downstream signaling.

Besides *P* gene products, NiV matrix protein (NiV-M), can also inhibit IFN-I. When NiV-M interacts with TRIM6, it promotes its degradation and reduces IKKϵ polyubiquitination thus reducing IFN-mediated responses (179). Moreover, NiV nucleoprotein N can either directly prevent STAT nuclear import or hamper STAT-complex formation, thus also reducing STAT nuclear accumulation and inhibiting type I and II IFN responses (180).

3.3 EBOV virus

The genus Ebolavirus contains six virus species, namely *Zaire ebolavirus* (EBOV), *Sudan ebolavirus* (SUDV), *Taï Forest ebolavirus*

(TAFV), *Bundibugyo ebolavirus* (BDBV), *Reston ebolavirus* (RESTV) and *Bombali virus* (BOMV). Out of the six, EBOV is the most prominent member having caused many highly lethal outbreaks in the past. EBOV is a single-negative stranded RNA virus from the *Filoviridae* family (181) and is highly pathogenic for humans and non-human primates. There have been many EBOV outbreaks with high morbidity and mortality since 1976, the 2014 outbreak in West Africa being the deadliest, with more than 28000 recorded cases and 13000 fatalities (182). Due to the multiple transmission mechanisms of EBOV, the broad cellular tropism of the virus and the multiple mechanisms used by EBOV to evade human immune responses, EBOV is considered a highly infectious, category A pathogen. EBOV can cause a highly pathogenic disease, known as Ebola Virus Disease (EVD), with a fatality rate of up to 90% in humans. Cases of EVD are often associated with a septic-shock-like syndrome characterized by an exacerbated inflammatory immune response and coagulopathy, that when combined, lead in many cases to multiple organ failure and death (183).

Whilst many treatments that have been tested in animal models and several vaccines have been shown to induce a very promising immune response against EBOV infection in animal models and humans (some of which are licensed for human use), it is not yet completely clear what are the main protective mechanisms of a successful immune response against EBOV. Although virus neutralizing antibodies and enumeration of polyfunctional T cells (IFN-γ, TNF-α, IL-2) have been associated with protection against EBOV, more data is required to validate these as reliable CoP (184–187). Determining more accurate CoP could facilitate the development of novel, better targeted treatments and vaccines.

3.3.1 The role of cytokines in EBOV infection

EBOV has a broad cellular tropism, with monocytes, dendritic cells and macrophages all being primary cellular targets of the virus. After becoming infected by EBOV, these cells have a pivotal role in the systemic dissemination of the virus through the blood and the lymphatic system. Moreover, their infection also triggers the release of inflammatory mediators such as IL-16, TNF-α, MIP-1α, IL-1β, IL-6, IL-10, amongst others (188, 189). Specifically, high levels of IL-10 and TNF-α are believed to correlate with fatal outcomes from EVD (190, 191).

The virus glycoprotein (GP) and soluble viral proteins such as shed GP are released from infected cells into the extracellular medium, where they have been shown to contribute to the release of proinflammatory cytokines, however, the exact mechanisms responsible for the early cytokine storm are yet to be determined (192, 193).

While monocytes, macrophages and dendritic cells are the main producers of proinflammatory products, other cells such as T cells and endothelial cells are also involved in the release of multiple inflammatory mediators. This results in an immunological disbalance that is believed to, in part, contribute to the severity of EVD.

Overall, the immune disbalance observed during EVD has been shown to be a crucial factor in determining disease severity, since fatal cases often present an exacerbated immune response while survivors, in contrast, mostly display a well-regulated inflammatory response (194).

Other soluble mediators that appear to be extremely relevant during EVD include ROS and IFN I. In the case of IFN I, EBOV VP35 and VP24 proteins act as IFN I transcription and signaling antagonists, respectively (195, 196). Thus, while an early and short-lived production of Type I IFN has been associated with a survival outcome, the absence of IFN is believed to contribute to EBOV dissemination (197). In contrast, an early IFN-γ response followed by lymphopenia is believed to correlate with fatal cases of EVD (188, 190).

ROS has also been shown to have a relevant role in EBOV pathogenesis. For instance, high levels of nitric oxide (NO) are associated with mortality in infected patients (198). Abnormal NO levels are believed to contribute to several pathological disorders such as tissue damage, lymphocyte apoptosis and the disruption of vascular integrity.

There are several coagulopathies associated with EVD such as thrombocytopenia or the presence of high levels of fibrin degradation products. In some cases, this leads to DIC, which frequently contributes to multiorgan failure (199). While not all of the mechanisms responsible for triggering EBOV-related coagulopathy are fully understood, the results of several studies strongly suggest that the exacerbated release of proinflammatory mediators considerably contributes to these characteristic EVD coagulopathies. For instance, the hyperproduction of proinflammatory cytokines activates coagulation factors such as procoagulant protein tissue factor (TF), fibrin fragment E and thrombin, which in turn, upregulate the production of proinflammatory cytokines (190, 200).

It has also been observed that endothelial cells are severely affected in late stages of EVD. Due to exceedingly high levels of proinflammatory cytokines (ROS and TF amongst other soluble mediators) endothelial cells are activated and endothelial leakage occurs (201).

Therefore, upon EBOV infection, a chain reaction initiated by an exacerbated inflammation response leads to a disbalanced immune response, systemic virus spread, vascular damage and coagulopathies that altogether will lead to a septic-shock like syndrome and multiorgan failure.

3.3.2 The role of T cell immunity in EBOV infection

Although EBOV does not infect lymphocytes, it can interact with T cells, affecting the development of immune responses. T-cell mediated immune responses during EVD involve a robust activation of T cells followed by their proliferation in both fatal cases and survivor patients (202). The magnitude and diversity of T-cell mediated immune responses in survivors during EVD are more robust when compared to fatal cases. In fatal cases there is an early T cell activation followed by a T cell population collapse, probably due to T-cell exhaustion (203, 204). Moreover, oligoclonal T-cell responses and higher expression of T cell inhibitory molecules CTLA-4 and PD-1 in CD8+ and CD4+ T cells are believed to contribute to an inefficient T cell response in fatal cases that is

associated with higher viral loads when compared to survivors (205, 206). In this regard, the early cytokine storm observed in fatal cases correlates with high later expression levels of CTLA-4 and PD-1 in T cells (206). In contrast, survivors would appear to develop a very diverse T cell response with low levels of CTLA-4 and PD-1 T cell inhibitors, thus contributing to viral clearance. However, a more recent study has shown that West African EVD survivors from 2013-2016, presented an increase in activation and proliferation markers in CD4+ and CD8+ T cell populations by 30% and 50% respectively, when compared with healthy individuals (202). This increased activation and proliferation suggests that survivor patients can develop a robust immune response. This activation was shown to last more than one month after recovery.

Interestingly, during the EVD convalescent phase, CD4+ and CD8+ T lymphocytes from survivor patients were able to respond to EBOV nucleoprotein (NP) thus indicating that EBOV NP can stimulate virus-specific-T cell responses in humans after resolution of the disease. Similarly, in another study in EVD patients from the 2013-2016 outbreak, it was shown that survivor memory CD8+ T cells can secrete IFN-γ and TNF-α and mainly responded to viral NP and to a lesser degree to VP24, VP40, VP35 and GP. This data would appear to corroborate the immunodominance of the EBOV NP-specific T cell responses described in previous studies (205). Studies in mice, guinea pigs and NHP models have also highlighted the importance of T cell responses during EVD and the involvement of the viral NP in generating T-cell immunity (207–209).

In cases of EVD, lymphocytes are severely affected and undergo apoptosis thus making lymphoid depletion a prominent feature of the disease (208, 210). In fatal cases, there is approximatively one fourth less lymphocytes when compared with levels found in survivors (190). This loss of lymphocytes is believed to be due to several factors, including the combined impairment of DC associated with the previously mentioned abnormal release of inflammatory cytokines, including TNF-related apoptosis-inducing ligand (TRAIL) and Fas death receptor, upon EBOV infection (194, 211). Abnormal levels of NO and direct interactions between EBOV and lymphocytes are also believed to contribute to the loss of bystander lymphocytes during infection (183, 212). In addition, it has also been shown that phosphatidylserine associated with EBOV GP can bind and stimulate CD4+ T cells through T-cell immunoglobulin mucin receptor 1 (TIM-1). These cells then release proinflammatory mediators believed to contribute to the cytokine storm and the lymphopenia observed during EVD (213). Other studies have determined that abortive infection of T lymphocytes causes ER-stress in these cells thus contributing to their own apoptosis (214). Importantly, lymphopenia was shown to correlate with fatal cases during the 2000 Ebola Sudan outbreak in Uganda (198).

To summarize, the proliferation of lymphocytes is observed in both survivors and fatal human cases, however in the latter, T lymphocytes display less immune response diversity and frequently show lymphopenia in the later stages of EVD. While it has been determined that robust T cell mediated immune responses can be a CoP during EVD, it is clear that immune responses are not independent compartments and the appreciating the interplay between innate and adaptive immunity may be crucial in understanding the complexity required to produce an efficient protective immune response during EVD.

3.3.3 The role of B cell immunity in EBOV infection

Even though T cell mediated immune responses are crucial during EVD, humoral immune responses also play a very relevant role in EBOV clearance. It has previously been shown that survivors tend to produce early and sustained levels of IgG, while fatalities have rather an impaired humoral response characterized by the absence of EBOV-specific IgG, low levels of IgM and lymphopenia (215, 216). Upon EBOV infection, IgM can be detected as early as day 2 after symptoms appear and in the case of IgG, antibodies are normally present between days 5 to 18 days after symptom onset (202, 217, 218). After one year of symptom onset, an IgM repertoire against VP40 and GP was observed in survivors despite undetectable virus levels. This however could also be an indicator of hidden viral persistence (194). Interestingly, serological surveys of IgG levels in rural villages in Gabon showed EBOV antibody seroprevalence, suggesting either prior exposure to EBOV or the presence of cross-reactive antibodies (219).

It is however unclear how long the immunity in EBOV survivors lasts; in some cases, it has been shown that EBOV-specific antibodies are present for forty years after symptomatic infection (220). These antibodies have been shown to have pan-neutralizing capacity against EBOV *in vitro* and were associated with protective roles in several animal models such as mice, guinea pigs and ferrets (221–225). However, whether these antibodies have a protective potential against EBOV reinfection in survivors remains undetermined. It should be considered that EBOV neutralization may not always translate into protection in humans and frequently, other antibody functions (complement, opsonization…) have been shown to be important in surviving EVD (226, 227).

In order to assess serological immune profiles of EVD survivors, antibody isotypes were analyzed and showed changes in the antibody repertoire over time. While neutralizing EBOV-specific IgG1 persisted over time, IgG3 decreased in early phases and IgG4 appeared later on. Moreover, IgA with innate immune effector functions and long-lasting IgG/IgM/IgA epitope diversity were described in EVD survivors (228, 229).

Not all of the antibodies detected can recognize EBOV GP. Survivors from the 1976 Yambuku outbreak for example have been shown to harbor antibodies with reactivity to GP, NP and to a lesser extent VP40. However, all identified antibodies with neutralizing capacity were GP-specific in humans and animal models (220, 230, 231). It is for this reason that most vaccines are based on EBOV GP.

Antibodies can target nearly any region on the surface of EBOV GP. Conserved GP regions include the receptor binding site (RBS), the base, the internal fusion loop (IFL) and the heptad repeat 2 (HR2). Other regions such as the glycan cap region and mucin-like domain (MLD) are less conserved (232). While conserved regions are normally targeted by cross-reactive antibodies, most of the antibody responses found in survivors target less conserved regions since they are structurally more exposed. However, these antibodies are frequently non-neutralizing, show weak affinity and are non-

cross-reactive (233). There are nevertheless some antibodies that target the glycan cap and are pan-protective, neutralizing several EBOV species (234) Recently, a conserved site named the MLD cradle that connects the MLD to the glycan cap has been identified as an antibody target region that destabilizes the GP quaternary structure, blocking the receptor binding required for effective EBOV infection (235). This is an important step forward in determining the molecular basis of EBOV neutralization by targeting conserved exposed epitopes and could be used to design universal antibody therapeutics.

There are currently two approved vaccines against EBOV, namely rVSV-ZEBOV (Ervebo) and ChAd3-MVA (Ad26) (236, 237). They have both been shown to induce EBOV-specific humoral and cellular responses, however these immune responses are not identical to the ones observed in survivors. In order to generate efficient vaccines, it is thus important to compare immunogenicity and protection between vaccinees and survivors. For instance, there are serological studies of immune memory responses showing that EVD survivors (2-6 months after infection) from the 2013-2016 EBOV outbreak have higher antibody levels and stronger antibody affinity when compared to ChAd3-MVA vaccinees at 2-12 months. Moreover, while this cohort of vaccinees had a predominant IgM response, survivors displayed a higher level of IgG with a more diverse antibody repertoire than the vaccinees (230, 238). Interestingly, survivor antibodies were shown to preferentially target the fusion peptide and HR2 domains of the viral GP2 protein and provide neutralization (238).

In the case of rVSV-ZEBOV, a serological study comparing survivors versus rVSV-ZEBOV vaccinees showed that survivor IgM and IgG do not bind the same EBOV GP epitopes when compared to rVSV-ZEBOV vaccinees (239). Additionally, another study in a similar cohort showed no significant differences in circulating antibody subclass levels. However, survivor antibodies had a higher neutralization capacity and a higher capacity to induce cellular responses than those from vaccinee samples. Importantly, IgG1 levels in survivors correlated with EBOV neutralization capacity, which was not the case in vaccinees (240).

These studies provide a good overview of the potential differences between survivor and vaccinee immune responses and will surely contribute to the development of more efficient next-generation vaccines. The main EVD immune CoP candidates are shown in Supplementary Table 1.

3.3.4 EBOV interplay with immune responses

EBOV has two main strategies to interfere with the host immune response. First, EBOV blocks IFN signaling and production through VP24 and VP35, respectively. This way, both proteins together ensure that IFN production is hampered and in the case that IFN is produced, the infected cell is unable to respond (241). In the case of VP24, this protein can either directly bind to STAT-1 thus blocking its transport to the nucleus or it can bind to karyopherin α1 and consequently block the IFN antiviral response (242, 243). EBOV VP35 on the other hand, antagonizes IFN mainly through blocking Interferon regulatory factor 3 (IRF-3) phosphorylation and protein kinase R (196, 244).

The second mechanism used by EBOV for immune diversion involves several glycoproteins. The EBOV fourth gene encodes for three different glycoproteins depending on the number of uracyls (Us) added at a so-called editing site. The viral structural surface glycoprotein (EBOV GP) is transcribed when 8 Us are found the editing site (245, 246). When 7Us and 6U/9Us are present, different soluble glycoproteins are produced, namely secreted glycoprotein (sGP) and small soluble GP (ssGP) respectively. Moreover, an additional soluble glycoprotein is generated when a percentage of the EBOV GP expressed on the surface of infected cells is cleaved by proteases releasing it in a soluble form with no transmembrane domain, known as shed GP. Due to its structural similarity to EBOV GP, it has been suggested that shed GP has a role in recruiting new primary targets and also binds antibodies directed to the virus (192). While not all functions have clearly been elucidated, it is suggested that soluble glycoprotein sGP may also play a role in immune evasion by binding antibodies initially directed against the viral surface glycoprotein EBOV GP. However, since not all amino acids are identical to the surface glycoprotein, it is believed that sGP also acts as decoy antigen and reduces specific antibody production against surface GP, possibly resulting in antigenic subversion (247). Importantly, sGP also exhibits anti-inflammatory activities in the endothelium and by reducing the amount of CD16b receptor on human neutrophils thus preventing their activation and consequently stunting an innate immune response (248). Regarding ssGP, while it is believed that it could share some of the functions described for sGP (249), its specific role during EBOV pathogenesis has not yet been clearly elucidated.

4 Comparison of immune profiling in SARS-CoV-2, NiV and EBOV infection

To effectively tackle emerging viruses, it is essential to understand the potential similarities and differences between virus families, the viruses themselves and the immune responses that they elicit upon infection in order to establish realiable CoP. SARS-CoV-2, NiV and EBOV share some immune signatures, yet currently not all of the molecular mechanisms involved in fighting infection with these viruses have been elucidated. These emerging viruses all have in common that they dysregulate host immune responses, including both early and late events, and this dysregulation is associated with viral progression during COVID-19, NiVD and EVD. Below we provide a comparison of the key features of immune responses to SARS-CoV-2, NiV and EBOV infection in humans (Table 1), to better understand differences and the potential pathways to derive CoP (or pathology) for the three diseases.

The exacerbated release of cytokines and chemokines plays a major role in SARS-CoV-2, NiV and EBOV immunopathology, as this can eventually lead to severe complications and in some cases death during all three diseases. Moreover, abnormal inflammation

levels have a big influence in late-stage cellular and humoral immune responses. In this regard, the level of activation, proliferation, phenotype and kinetics of T lymphocyte populations can direct specific T cell mediated and humoral responses and influence the severity of the aforementioned emerging diseases.

The presence of neutralizing IgG in serum is in general used as a CoP, however the titer and the type of antibodies that are needed to reach protection for each disease (or disease outcome) are not clear and need to be defined more precisely and more specifically for each manifestation of infection (protection against infection, protection against death or severe disease, chronic infection, etc…). The kinetics of humoral responses and additional antibody functions such as ADCC may also play a crucial role in protection. Moreover, currently, most vaccines are focused on the glycoproteins of the virus envelope as the immunogen, however, as described above, the viral nucleoproteins and other non-envelope proteins should also be considered in future vaccine designs, and consequently the definition of CoP be updated.

Taken in combination, all of these factors highlight the importance of the different immune compartments and their interactions in achieving viral clearance and highlight the need to further understand innate, cellular and humoral responses and their interplay in order to identify more specific CoP. It is also important to consider the relevance of the potential differences amongst host immune responses during disease progression and to appreciate the role of host diversity in determining the ability to survive infection. The evaluation of common immune signatures that lead to the transition from a mild disease state to a severe one will help in finding novel preventive measures and treatments that could reduce mortality rates.

5 Conclusions

In summary, defining which immune effector mechanisms play a role in protective immunity is crucial for the rational design of vaccines and therapeutics and also for deriving CoP. The latter could be used as surrogates of protection so that evaluation of the protective efficacy of new medical countermeasures can be facilitated. This will in turn guide the pathway for the acceleration of the licensing of these products by regulatory agencies.

For certain pathogens, when survival rates are low, it is not easy to define the underlying immune mechanisms that correlate with protection. The lack of a full understanding of natural immune responses and the potential of certain pathogens to evade them complicates the derivation of CoP. The immune response components described above are an example of the variety of the different and non-exclusive mechanisms that the human immune system uses to evoke the desired protective immunity. Moreover, these mechanisms are not always systemic and for instance, there are often organ-specific mechanisms of immunity (mucosal immunity). More research towards the definition of organ-specific protective mechanisms would help in determining more reliable CoP for certain diseases and vaccines.

The nature and complexity of the interactions of the different cells and soluble effectors of the immune system that are involved in protection is remarkable. Indeed, humoral and cell-mediated immune responses do not act in isolation and the innate immune response strongly influences both T-cell mediated and humoral responses. The best protection against most pathogens is achieved when both arms of the immune system act cooperatively in synergy. While pre-existing antibodies and natural immunity mechanisms may provide the first line of adaptive immune defense, when it is breached, memory T and B cell responses come into play. Thus, an ideal vaccine should offer an integrative approach that triggers both protective antibody levels with robust immunological memory and rapid and efficient effector functions. The high degree of variability of surface antigens in certain pathogens and the complex and dynamic nature of host-pathogen interactions, render the development of vaccines against intracellular infections a challenging process. Notably, such infections often also require cell-mediated immunity.

A limitation of current vaccine development strategies however, is the reductionist approach of measuring vaccine efficacy as a function of measurable antibody responses, which are often used as

TABLE 1 Key features of immune responses to SARS-CoV-2, NiV and EBOV infecCon in humans.

	Mild cases or SURVIVORS							Severe cases or NON-SURVIVORS						
	Cytokine release	IFN	Reactive Oxygen species (ROS)	T cell responses	B cell responses	Lymphopenia	Neutralizing antibodies	Cytokine release	IFN	Reactive Oxygen species (ROS)	T cell responses	B cell responses	Lymphopenia	Neutralizing antibodies
SARS-CoV-2														
NiV														
EBOV														

	Normal levels
	Upregulation
	Downregulation
	No information
	Contradictory information

CoP, mainly for reasons related to ease of detection, quantification and ease of standardization. Whilst this strategy has proven useful for certain vaccines, it has also shown limitations. This is exemplified by the case of using a serum antibody titer of 1/40 in hemagglutination inhibition tests as a CoP for Influenza vaccines. This way of assessing protective immunity is becoming obsolete as further studies have revealed that different population sub-groups (i.e. the elderly and children) require different titers for predicting protection, particularly as new vaccine strategies for flu based on viral vectors (including other antigens in addition to HA) and mucosal delivery routes (which induce different type of immune effector mechanisms) become available. In the case of EBOV, NiV and SARS-CoV-2, virus neutralizing antibodies have been used as CoP, but again as explained above, this parameter has its own limitations as it is clear that other arms of the immune system do play a role in protection that may not necessarily correlate with neutralizing Ab levels. Furthermore, it is important to emphasize that CoP need to be defined for a specific set of conditions that are related to host, population group, specific disease manifestation that the vaccine intends to protect against and dose, amongst other factors. The large variation in immune responses of the host and the heterogeneity in terms of genetics, age, sex, individual variation and environmental factors, including previous infection status, adds to the challenge of obtaining efficient vaccines.

For all the reasons described above, gaining a deeper understanding of the underlaying immune mechanisms and requirements for successful outcomes during infection is essential in order to derive CoP that are accurate and reliable. This would translate to the development of more effective vaccines and provide more confidence in the ways in which these vaccines are assessed. Vaccine efficacy could be dramatically improved by targeting specific immune CoP such as the generation and maintenance of distinct memory T cell subsets, the specific release of cytokines or facilitating the production of neutralizing antibodies.

The increasing focus on characterizing immune responses to viral infections has led to the development of novel approaches to detect common immune features conferring protection. This has resulted in the development of *in silico* prediction targets that ultimately may result in the definition of CoP against prominent current pathogens but also for future emerging ones. From a long-term perspective, understanding immune CoP that are specific for certain pathogens could help to promote long-term immunological health. Hence, at a time when emerging infections seem to be more and more frequent, the speed of the efficient establishment of immune CoP appears to be a critical factor in the fight against present and future health threatening diseases.

Author contributions

Conceptualization was done by BE-P and JC-O; writing—original draft preparation, BE-P, PL and JC-O; writing—review and editing, BE-P, PL and JC-O. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1156758/full#supplementary-material

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Pre-pandemic SARS-CoV-2-specific IFN-γ and antibody responses were low in Ugandan samples and significantly reduced in HIV-positive specimens

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Introduction: We investigated whether prior SARS-CoV-2-specific IFN-γ and antibody responses in Ugandan COVID-19 pre-pandemic specimens aligned to this population's low disease severity.

Methods: We used nucleoprotein (N), spike (S), NTD, RBD, envelope, membrane, SD1/2-directed IFN-γ ELISpots, and an S- and N-IgG antibody ELISA to screen for SARS-CoV-2-specific cross-reactivity.

Results: HCoV-OC43-, HCoV-229E-, and SARS-CoV-2-specific IFN-γ occurred in 23, 15, and 17 of 104 specimens, respectively. Cross-reactive IgG was more common against the nucleoprotein (7/110, 15.5%; p = 0.0016, Fishers' Exact) than the spike (3/110, 2.72%). Specimens lacking anti-HuCoV antibodies had higher rates of pre-epidemic SARS-CoV-2-specific IFN-γ cross-reactivity (p-value = 0.00001, Fishers' exact test), suggesting that exposure to additional factors not examined here might play a role. SARS-CoV-2-specific cross-reactive antibodies were significantly less common in HIV-positive specimens (p=0.017; Fishers' Exact test). Correlations between SARS-CoV-2- and HuCoV-specific IFN-γ responses were consistently weak in both HIV negative and positive specimens.

Discussion: These findings support the existence of pre-epidemic SARS-CoV-2-specific cellular and humoral cross-reactivity in this population. The data do not establish that these virus-specific IFN-γ and antibody responses are entirely specific to SARS-CoV-2. Inability of the antibodies to neutralise SARS-CoV-2

implies that prior exposure did not result in immunity. Correlations between SARS-CoV-2 and HuCoV-specific responses were consistently weak, suggesting that additional variables likely contributed to the pre-epidemic cross-reactivity patterns. The data suggests that surveillance efforts based on the nucleoprotein might overestimate the exposure to SARS-CoV-2 compared to inclusion of additional targets, like the spike protein. This study, while limited in scope, suggests that HIV-positive people are less likely than HIV-negative people to produce protective antibodies against SARS-CoV-2.

KEYWORDS

SARS-CoV-2, Common-cold coronaviruses, sero-crossreactivity, Pre-existing IFN-γ, Sub-Saharan Africa, Disease severity, Ugandan population, Cross protection

Introduction

Since the onset of severe acute respiratory syndrome (SARS-CoV-2) outbreak in Uganda and other Sub-Saharan African countries, COVID-19 disease severity and mortality have been lower than in Europe, Asia, and the Americas (1, 2). Several hypotheses were proposed to explain this discrepancy, including cross-reactive adaptive immune responses to SARS-CoV-2 and antigenically related human coronaviruses (3) that cause common seasonal colds. Studies assessing antibody prevalence to SARS-CoV-2 in pre-pandemic serum specimens observed a significant increase in the prevalence of cross-reactivity among sera in SSA compared to other continents (4). Antigenic cross-reactivity between SARS-CoV-2 and human coronaviruses such as HCoV-229E, HCoV-NL63, HCoV-HKU1 and HCoV-OC43 has been demonstrated in other geographical regions through the detection of cross-reactive humoral and cellular immune responses (5). Different conserved human coronaviruses (HCoVs) cross-reactive B-cell epitopes against SARS-CoV-2 N protein are detected in a significant fraction of individuals not exposed to this pandemic virus (6). While the spike and nucleocapsid are the most immunogenic (7), the nucleocapsid is more conserved across various human coronaviruses; thus, cross-reactivity can be expected. The less conserved spike protein is more susceptible to mutations, especially in the receptor binding domain (RBD); thus, cross-reactivity to it would be a potential protective correlate. The cross-reactive antibodies of interest are IgG, as these are the hallmarks of B-cell memory (8).

Pre-COVID-19 pandemic plasmas from Tanzania and Zambia were shown to have a higher prevalence of serological cross-reactivity to SARS-CoV-2, HCoV-HKU1, HCoV-OC43, HCoV-NL63 and HCoV-229E, compared to specimens from the USA (4). A robust pre-existing humoral immunity against SARS-CoV-2 is thus entirely plausible as a reason for the observed lower disease severity and mortality. Although this can be attributed to the presence of pre-existing cross-reactive binding antibodies, it remains to be seen if, and to what capacity and breadth, such antibodies can neutralise SARS-CoV-2 or have other mechanisms of conferring protection (9, 10).

Cross-reactive immune responses to SARS-CoV-2 have been shown in pre-pandemic cohorts and proposed to contribute to host protection. Pre-existing T-cell-mediated immunity to SARS-CoV-2, from prior exposure to antigenically related seasonal coronaviruses, has been demonstrated in other populations previously unexposed to SARS-CoV-2 (11). Pre-existing T-cell contribution to asymptomatic or mild disease, rapid viral clearance, and differences in seroconversion have been suggested (12). Efforts continue to understand the potential role of T-cell memory induced by prior exposure to seasonal coronaviruses in reducing COVID-19 disease severity, and findings have remained conflicting. Epitopes specific to seasonal coronaviruses and those cross-reactive to SARS-CoV-2 have been defined, and their potential roles in vaccine design and pathogenesis have been postulated (13). Airway-resident SARS-CoV-2- T cell cross-reactivity correlated with the magnitude of human seasonal coronavirus immunity highlighting the potential to harness cross-reactive T cells in vaccine design (14). Higher frequencies of non-spike T cell cross-reactivity in PCR-negative exposed household contacts underscored the importance of cross-reactive T cell memory in protecting SARS-CoV-2-naïve contacts from infection (15). In contrast, a case-control study found no association between baseline human coronavirus cross-reactive antibodies and protection against SARS-CoV-2 infection but suggested that pre-existing human coronavirus immunity might aggravate SARS-CoV-2 infection instead, which is critical in considering emerging variants (16). Detection of a pre-existing cellular and humoral immunity against SARS-CoV-2 and other coronaviruses in each population is significant, mainly because the occurrence is not ubiquitous in all populations (17).

For almost three years since the emergence of COVID-19, Uganda, like many sub-Saharan African countries, has experienced lower COVID-19 and a less severe epidemic than other regions worldwide. Prior cross-reactivity is widely postulated as a possible explanatory feature for the observed differences and is essential for interpreting diagnostic strategies. Consequently, we used pre-COVID-19 Ugandan specimens to examine the presence, prevalence, and magnitude of pre-existing

SARS-CoV-2-specific cross-reactive immune responses to test the hypothesis that pre-pandemic natural immunity acquired by some human populations may have contributed to the epidemic outcome.

Materials and methods

Study design and population

This retrospective study estimated the levels of pre-COVID-19 pandemic cross-reactivity to SARS-COV-2 using 110 specimens collected between January 2009 and February 2015 for future optimisation of immunological assays. Of 110 available specimens, 68 (68.12% were HIV-negative, and 36 (32.72%) were HIV-1 positive. 35 of the 36 HIV-1 positive were on cotrimoxazole prophylaxis due to HIV-related progressed CD4 counts, 6 lacked HIV-1 serostatus information, and 2 lacked ELISpot data. Ten of the 36 HIV positive subjects had CD4 count data available, with a median of 483.0 (IQR 231.58-678.2 CD4 cells/ml).

Correspondingly, 110 plasmas and 104 PBMC specimens were available to screen for pre-epidemic anti-SARS-CoV-2 cross-reactive IgG and IFN-γ responses, respectively. Specimens with no documented HIV status were excluded in analyses by HIV stratification. All participants provided written informed consent for their specimens' storage and future use. Study ethical approval was granted by the Uganda Virus Research Institute (UVRI) Research and Ethics Committee (IRB Ref: GC/127/233) and the Uganda National Council for Science and Technology (Ref: HS1030).

SARS-CoV-2, HCoV-229E and HCoV-OC43 peptide pools

The SARS-CoV-2 and the seasonal human coronavirus OC43 (HCoV-OC43) and 229E (HCoV-229E) peptides arrays were 12-17 mers long, overlapping by ten amino acids, obtained from the BEI Resources Repository, NIAID, USA, selected based on availability. The SARS-CoV-2 array constituted 181 spike peptides (S; GenPept: QHO60594 NR-52402), 59 nucleoprotein peptides (N; GenPept: QHO60601, NR-52404), 31 membrane peptides (M; GenPept: QHO60597, NR-52403) and ten envelope peptides (E; NR-52405). Single peptides were combined based on the SARS-CoV-2 proteome region into fourteen spike (S) pools. The S1 region comprised all SARS-CoV-2 spike peptides spanning amino acid 13-684 (NTD: 13-305, RBD: 331-528, and OTHERS: 1-12, 306-330, 529-684). The S2 region comprised all spike peptides spanning amino acids 685-1211 (18). The S1 region was subdivided into NTD-1, NTD-2, NTD-3, RBD-1, and RBD-2 pools, while all other S1 peptides were arranged in ascending order and divided into OTHERS 1 and OTHERS 2. The S2 region was divided into seven peptide pools, namely S2-1, S2-2, S2-3, S2-4, S2-5, S2-6 and S2-7. The nucleoprotein (N) was divided into two pools (N1: N1-N30 and N2: N31-N59), while membrane (M) and envelope (E) had one pool each, summarised in Tables 1 and 2. Common human coronavirus peptides included 226 S protein peptides combined into one pool (NR-3011, BEI Resources) and 195 individual 229E S protein peptides combined into a single pool (NR-3010, BEI Resources). All peptide pools were reconstituted and used at a final concentration of 2μg/ml per peptide.

Detection of T-Cell responses using an IFN-γ ELISpot assay

The cryopreserved cells were permitted to rest in growth media at a temperature of 37°C overnight. Subsequently, the median percentage (interquartile range) of cell recovery was recorded, with values of 78% (74-84) on day one and 63% (45.75-75) on day two. Moreover, the corresponding percentage viability values were determined, with recorded values of 99% (interquartile range 98.25-99) and 100% (interquartile range 99-100) on days one and two, respectively. These

TABLE 1 Pooling arrangement of peptides from BEI Resources.

	Region	Number of Overlapping peptides to Pool	Peptide Numbers from the List	Website links
SARS-CoV-2 Peptides	M	31	1-31	https://www.beiresources.org/Catalog/BEIPeptidesandPeptideArrays/NR-52403.aspx
	E	10	1-10	https://www.beiresources.org/Catalog/BEIPeptidesandPeptideArrays/NR-52402.aspx
	N1	30	1-30	https://www.beiresources.org/Catalog/BEIPeptidesandPeptideArrays/NR-52404.aspx
	N2	29	31-59	https://www.beiresources.org/Catalog/BEIPeptidesandPeptideArrays/NR-52404.aspx
Common Cold Peptides	OC43	226	1-226	https://www.beiresources.org/Catalog/BEIPeptidesandPeptideArrays/NR-53728.aspx
	229E	195	1-195	https://www.beiresources.org/productinformationsheet/tabid/784/default.aspx?doc=80254.pdf

findings indicate the successful recovery and viability of the cryopreserved cells following the resting period, highlighting their potential suitability for subsequent experimental procedures.

Duplicate wells pre-coated with anti-human IFN-γ capture antibody were seeded with 100,000 PBMCs/well and incubated with respective peptide pools at 37°C for 18 hours in a 5% CO_2-in-air environment. Captured IFN-γ was detected with a biotinylated anti-human IFN-γ detection antibody and horseradish peroxidase-conjugated streptavidin, developed using a Vector Novared kit (Vector Laboratories, SK-4800, CA, USA), as described by the manufacturer. Control wells included donor specimens of known reactivity to SARS-CoV-2, HCoV-229E and HCoV-OC43 spike and phytohemagglutinin (PHA) treated PBMCs. Background reactivity was computed from four wells containing cells with mock (media with DMSO peptide diluent) and four with media only. Spots were counted using an AID ELISpot Reader (GMBH, Germany). IFN-γ secreting T-cells were enumerated as Spot-forming units (SFU), and the overall response as SFU per million PBMC (SFU/10^6 PBMC). The test acceptance criteria were ≥300 spots in PHA wells, ≤ 10 in each of the four wells containing cells plus mock, and ≤ 5 in each of the four media-only wells. Positive responses were deduced after subtracting two times the mean of background wells. Test wells with a net response ≥ 55 SFU/10^6 PBMC were considered positive.

Detection of virus-specific binding antibodies using ELISA

SARS-CoV-2-specific S and N binding IgG optical densities (450nm) and concentrations (ng/ml) were quantified using an in-house optimized conventional ELISA, as described before (19). Briefly, 96-well flat-bottomed membrane-binding plates (Greiner Bio-One) coated with 3 µg/ml recombinant S or N protein in PBS were incubated with serum specimens diluted 1:100 in PBS-T containing 1% Bovine Serum Albumin (BSA). A secondary monoclonal horseradish-peroxidase conjugated goat anti-human IgG (IgG-HRP) antibody (catalogue no. A0170, Sigma) was added for 1 h before washing to remove any excess unbound antibody. Tetra-methyl-benzidine (TMB) substrate solution was added for 3 minutes, and the reaction stopped with 2.5% (0.68M) Hydrochloric acid. Bound IgG was quantified as optical densities (ODs) read on an ELx808 microplate reader (BioTek, Winooski, VT, USA) at 450nm. Net OD values were computed by subtracting the OD values of blank wells. Antibody concentrations were extrapolated from serially diluted standards and calibrated to BAU/ml using WHO international standards.

Preparation of SARS-CoV-2 Pseudoviruses for neutralization assays

SARS-COV-2 pseudoviruses were generated by co-transfection of 293T cells with MLV-CMV-firefly plasmids encoding the firefly luciferase gene, MLV gag-pol and Wuhan-strain SARS-COV-2 spike plasmids, using the PEIMAX (Polysciences) transfection reagent. Culture supernatants containing the pseudovirus particles were clarified of cells using a 0.45µm filter, titrated and stored at -80°C. Working aliquots were quickly thawed at room temperature and diluted to pre-determined working concentrations to give sufficient luciferase signals in infected cells.

TABLE 2 Pooling arrangement SARS-CoV-2 spike peptides from JPT.

	Region	Number of Overlapping peptides pooled	Peptide Numbers from the List	SARS-CoV-2 spike amino acids
SARS-CoV-2 Peptides	NTD 1	24	4-27	13-305
	NTD 2	24	28-51	
	NTD 3	23	52-74	
	RBD 1	24	83-106	331-528
	RBD 2	24	107-130	
	OTHERS 1	21	1, 77-80, 133-148	1-12, 306-330 (SD1/2), 529-684 (SD1/2)
	OTHERS 2	21	149-169	
	S2 1	19	172-190	685-1211
	S2 2	19	191-209	
	S2 3	19	210-228	
	S2 4	19	229-247	
	S2 5	19	248-266	
	S2 6	19	267-285	
	S2 7	15	286-300	

SARS-CoV-2 pseudotyped virus neutralization assay

A pseudovirus neutralization assay was used to determine whether the identified cross-reactive binding antibodies neutralised SARS-COV-2. Prior to testing, sera were heat-inactivated at 56°C for 30 minutes. In order to determine the potential neutralizing effect of the sera, serial dilutions ranging from 1:10 to 1:320 were prepared and incubated with fixed dilutions of the virus for a period of 1 hour. This incubation was carried out at a temperature of 37°C in an environment consisting of 5% CO2 in air. Duplicate wells were utilized to ensure accuracy of the results. Ten thousand 293T cells expressing human ACE2 receptors were added, and the wells were further incubated for 72 hours at 37°C, 5% CO_2. The wells were then incubated with Promega 1X lysis buffer and Bright-Glo Luciferase reagent for 15min. Luciferase expression in infected cells was measured using a Perkin-Elmer victor X3 luminometer. Neutralization titres were computed from linear interpolating plotted virus infectivity curves as 50% inhibitory titres (IC50). Inhibitory titres were reciprocals of serum dilutions that reduced the relative luminescence in test wells by 50% compared to the virus control wells. The lower detection limit of the SARS-CoV-2 neutralisation assay was ten, so virus titres less than ten were deemed undetectable.

Statistical analysis

GraphPad Prism 9.4.1 (GraphPad Software Inc, San Diego, California, USA) and R Version 4.1 were used for the statistical analyses and graphical data representation; p-values ≤ 0.05 were considered statistically significant. A two-tailed Mann-Whitney test was used to compare median IFN-γ responses to different peptide pools and median nucleocapsid and spike-directed antibody levels. P-values ≤ 0.05 were considered statistically significant.

Results

Pre-pandemic IFN-γ cross-reactivity to SARS-CoV-2 and human coronavirus structural proteins was detected at a low frequency, but primarily in HIV-1 negatives

Pre-epidemic IFN-γ cross-reactivity to SARS-CoV-2 was assessed in 104 specimens, 36 of which were HIV-1 positive and 68 were HIV negative. Cross-reactivity with HCoV-OC43, HCoV-229E, and SARS-CoV-2 occurred in 23 (22.1%), 15 (14.4%), and 17 (16.4%) of the 104 specimens, respectively. Cross-reactivity IFN-γ frequencies were greater to the human coronaviruses (29; 27.9%) than to SARS-CoV-2 (17; 16.3%), although the difference was not statistically significant (Fishers' Exact test, p-value = 0.065; Figure 1A). Rates of cross-reactivity did not differ between the two human coronaviruses HCoV-OC43 (15; 14.4%) and HCoV-229E (23; 22.1%). SARS-CoV-2-specific IFN-γ cross-reactivity of was greater in HIV-negative specimens (15/68; 22.06%) than in positives (2/36; 5.56%); Fishers' Exact test, p-value 0.0484, Figure 1B. HuOC43 cross-reactivity was also more prevalent in HIV-negative specimens (15 of 68) than in HIV-positive specimens (0 of 36); p = 0.0011. Cross-reactivity to HuCoV229E did not substantially vary by HIV status (p = 0.08). When examined based on the most often targeted SARS-CoV-2 antigens for populational surveillance, the frequencies of nucleoprotein (8 of 104) and spike-directed (5 of 104) IFN-γ cross reactivity did not vary. Similarly, anti-spike IFN-γ cross-reactivity did not vary between HIV-negative (3 of 68) and HIV-positive (2 of 36) tissues; Fishers' Exact test p-value > 0.05. These findings show that pre-existing immunity to SARS-CoV-2 occurred in Uganda, most likely due to prior exposure to other coronaviruses, and that SARS-CoV-2 antigens were not targeted more frequently than other coronavirus antigens. Cross-reactive IFN-γ responses to SARS-CoV-2 antigens were substantially more prevalent in HIV-negative specimens, than in HIV-positive specimens suggesting that the lower level of pre-existing immunity reported in Ugandans may be partly explained by HIV-positive persons' diminished capacity to generate a significant IFN-γ response to SARS-CoV-2 antigens. Thus, the Ugandan data gives an insightful view into the pre-existing immunity to SARS-CoV-2 in various populations and the variables that might impact it.

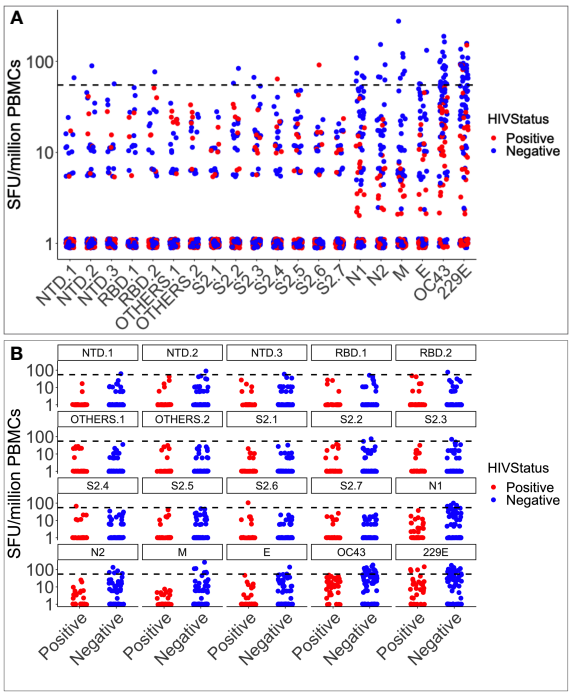

FIGURE 1
Pre-epidemic IFN-γ responses to SAR-CoV-2, HCoV-229E and HCoV-OC43. Illustrates the virus-specific IFN-γ responses to 18 SARS-CoV-2 and two human coronavirus peptide pools (HuCoVOC43 and HuCoV229E). IFN-γ response magnitudes are depicted and quantified as spot-forming units per million PBMC cells. The horizontal dotted red line represents the cut-off threshold for a positive response of 55 SFU/million PBMCs **(A)**. The data represents 104 pre-COVID specimens classified as positive in red and negative in blue based on HIV serostatus **(B)**.

Pre-pandemic anti-SARS-CoV-2 IFN-γ cross-reactivity was uncommon and comparable across the nucleoprotein and spike, but it was broader and more frequent in the HIV-1 negatives

Six of the seven pooled SARS-CoV-2 antigenic regions tested positive for IFN-γ. The evaluated regions comprised the nucleoprotein, spike, NTD, RBD, envelope, membrane, and SD1/2 (categorised here as "OTHERS"). Seventeen of the 104 pre-pandemic specimens tested positive for SARS-CoV-2-specific IFN-γ cross-reactivity; eight specimens (7.69%) targeted the nucleoprotein; eight (7.69%) targeted the spike; six (5.76%) targeted the membrane; two (1.92%) targeted the envelope; and no cross-reactivity occurred to "OTHERS", (Figure 2). In total, 16.3% of the specimens were positive for pre-epidemic IFN-γ cross-reactivity with SARS-CoV-2, with the nucleoprotein and the spike being the most frequently recognized targets. Cross-reactive antigenic pools were more common in HIV-negative specimens (6 of 7 pools) than in HIV-positive specimens (1 of 7 pools); Fishers' Exact test, p-value = 0.029 (Figures 2A, B).

Among the 15 HIV-negative SARS-CoV-2 responders, the number of SARS-CoV-2 cross-reactive pools ranged from 1 to 5, with a median of 1 (IQR; 1-2 pools). Only two of the 36 HIV-positive specimens tested positive for SARS-CoV-2-specific IFN-γ cross-reactivity, and both were against the S2 region of the SARS-CoV-2 spike protein. Using the Wilcoxon test, the median IFN-γ levels in HIV-negative participants were significantly greater than those of HIV-positive participants (p = 0.00038; 15; IQR 5–20 SFU/million PBMCs vs. 10; IQR 6.25–35 SFU/million PBMCs). Taken together, these data demonstrate the presence of pre-pandemic cross-reactive IFN-γ in this population, implying that the immune system had been primed to respond to SARS-CoV-2 antigens prior to the outbreak. These data also show that HIV-positive individuals are less likely to produce cross-reactive IFN-γ against SARS-CoV-2 than those who are HIV-negative. These findings have significant implications for the development of SARS-CoV-2 cross-protective vaccines, which may need to consider HIV infection status when assessing immunogenicity and efficacy.

Cross-reactive IFN-γ responses to pre-pandemic SARS-CoV-2 and human coronaviruses were weakly correlated

Next, correlations between historical human coronavirus IFN-γ cross-reactivity and the pre-epidemic SARS-CoV-2-specific IFN-γ cross-reactivity were explored. Anti-HuCoV IgG-deficient specimens (10/10, or 100%) had a higher frequency of cross-reactive SARS-CoV-2-specific IFN-γ responses than anti-HuCoV IgG-expressing specimens (7/29, or 24.1%); Fishers' exact test, p-value = 0.00001. Specifically, pre-epidemic SARS-CoV-2-specific cross-reactive IFN-γ responses were more common in the absence of anti-HuCoV antibodies, suggesting that exposure to additional variables not assessed here may have contributed to this phenomenon. Pairwise peptide comparisons for the correlation between SARS-CoV-2 and HuCoV-specific IFN-γ responses in both HIV-negative (Figure 3A) and positive (Figure 3B) specimens consistently revealed weak correlations. Regardless of HIV serostatus, robust positive correlations occurred between cross-reactive IFN-γ specific to each of the two human coronaviruses, HCoV-OC43 and HCoV-229E (Figures 3C, D).

The data showed the presence of pre-epidemic SARS-CoV-2-specific T-cell cross-reactivity in this cohort, these could not be fully explained by prior T cell cross-reactivity to human coronaviruses. It implies that most of the epidemic SARS-CoV-2 cross-reactive T cells were specific to SARS-CoV-2, rather than derived from prior coronavirus exposure. The findings suggest that prior coronavirus exposure may have had little to no impact on the pre-epidemic SARS-CoV-2-specific T cell pool, meaning that these T cells detected during the epidemic were likely generated in response to the emergence of the SARS-CoV-2 epidemic itself. Taken together, these findings agree with the existence of pre-existing HuCoV IFN-γ cross-reactivity, but they do not entirely explain the prevalence of SARS-CoV-2 IFN-γ responses before the epidemic.

Pre-epidemic SARS-CoV-2-directed IgG cross-reactivity was more common with the nucleoprotein than the spike, but only in HIV-negative individuals

Cross-reactive IgG against the nucleoprotein was more frequent (17/110, 15.5%) than against the spike (3/110, 2.72%), p = 0.0016,

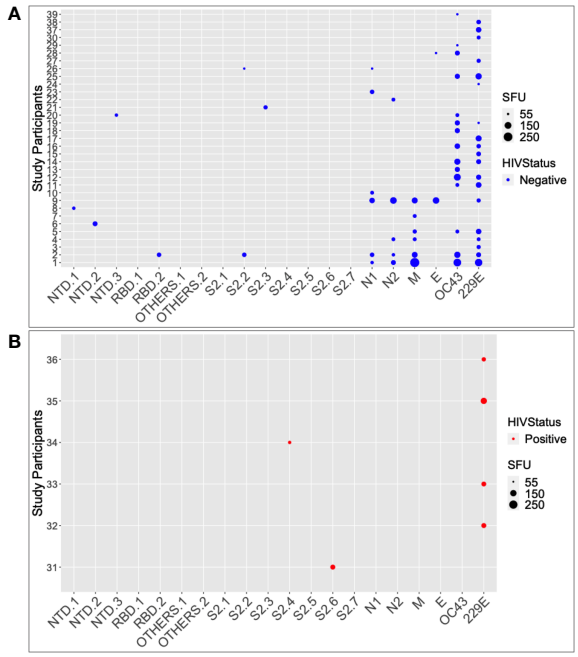

FIGURE 2
The SARS-CoV-2 and HuCoV peptide pools targeted by HIV-1 status. Summarizes the individual responses to each peptide pool for 39 of the participants who responded positively to at least one peptide pool. The data is stratified by HIV serostatus, with HIV negatives in blue **(A)** and HIV positives in red **(B)**. The larger the size of the circle, the greater the response.

Fishers' Exact test (Figure 4A). Cross-reactive IgG was still significantly higher against the nucleoprotein (14/68, 20.59%) than the spike (1/68, 1.47%) in HIV-negative individuals (Fishers' Exact test, p = 0.017, Figure 4B), but not in HIV-positive individuals (Nucleoprotein: 3/36, 8.33% vs. Spike: 2/37, 5.55%), Figure 4C. Nucleoprotein-directed IgG antibody optical densities (0.211; IQR 0.134 - 0.364nm) and concentrations (529.75; IQR 180.78-1154.33 ng/ml equivalent to 265.3; IQR 90.55 – 578.18 BAU/ml) were also significantly higher against the nucleoprotein than the spike (optical density 0.072 nm; IQR 0.048-0.120 and concentration 95; IQR 24.45-483.20 ng/ml equivalent to 37.83; 10.22 – 201.89 BAU/ml), Wilcoxon matched-pairs signed rank test, all p-values of 0.0001). (See Figures 4D, E). Taken together, these findings suggest that the nucleoprotein, rather than the spike protein, is a more important target for developing SARS-CoV-2-directed cross-reactive antibodies in HIV-negative individuals. Antibodies to the nucleoprotein were less specific to SARS-CoV-2 and were much more likely to cross-react with viruses from other families. As a result, surveillance efforts focusing on the nucleoprotein may overestimate SARS-CoV-2 exposure compared to other targets, such as the spike protein, while underestimating exposure, particularly in populations with a higher prevalence of HIV-positive individuals. When calculating and interpreting SARS-CoV-2 exposure for sero-epidemiological surveillance, HIV serostatus is a crucial factor to consider.

Pre-epidemic SARS-CoV-2-directed IgG cross-reactive antibodies were not neutralizing and did not correlate with IFN-γ cross-reactivity

Finally, the functionality of the detected spike-directed IgG cross-reactive antibodies was determined using the pseudotyped virus neutralisation assay.

Based on the SARS-CoV-2 neutralisation assay, the lowest dilution used was ten, rendering virus titres below ten undetectable. All responses obtained from the assay were found to be below the positivity detection limit. Consequently, it can be inferred that the pre-epidemic anti-spike SARS-CoV-2 antibodies were incapable of neutralising the wild-type SARS-CoV-2 pseudovirus *in vitro* (Table 3). Individuals who were infected with other coronaviruses prior to the pandemic, but not with SARS-CoV-2, may not have sufficient immunity to protect against the currently circulating strains of SARS-CoV-2. The inability of pre-pandemic antibodies to neutralize SARS-CoV-2 indicates that prior exposure did not result in SARS-CoV-2 immunity prior to the pandemic; thus, any immunity against the virus at the time of the pandemic was most likely the result of novel immune responses elicited by natural exposure to SARS-CoV-2 or its vaccine. The findings highlight the importance of continuing research into SARS-CoV-2 immunity mechanisms to identify protective

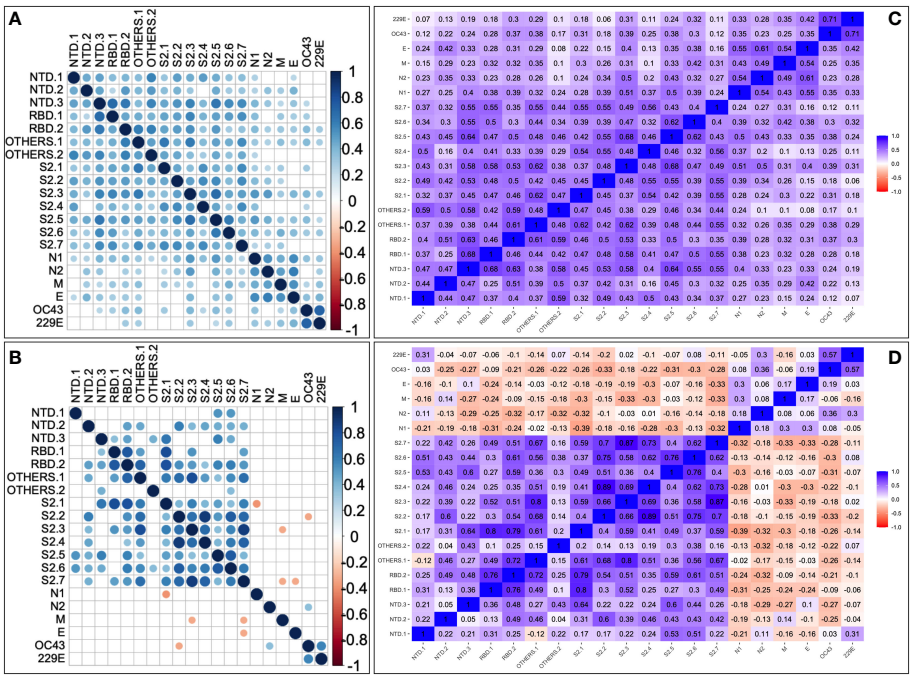

FIGURE 3
Pairwise correlation between SARS-CoV-2 and HuCoV-directed IFN-γ. Depicts correlation plots of all examined SARS-CoV-2 and human coronavirus peptide pools for 68 HIV-negative **(A)** and 36 HIV-positive **(B)** specimens using Spearman's rank correlation test. Blue dots represent a positive correlation, while red dots represent a negative correlation. The darker the circle, the stronger the correlation, and the lighter the circle, the weaker the correlation. The correlation matrices show correlation coefficients between peptides in HIV-positive **(C)** and HIV-negative **(D)** specimens using Spearman's rank correlation. P-values of 0.05 or less are considered significant. Non-significant correlations are indicated by blank cells.

antibodies and develop effective treatments and vaccines. Cross-reactive IFN-γ against SARS-CoV-2 or human coronaviruses was not induced in any of the three specimens with cross-reactive SARS-CoV-2 spike-IgG antibodies (Figure 5A). Despite the presence of cross-reactive SARS-CoV-2 IgG antibodies, most specimens lacked an IFN-γ response. Only one of the 17 specimens with nucleoprotein-directed cross-reactive IgG antibodies produced virus-cross-reactive IFN-γ to SARS-CoV-2, while three produced virus-cross-reactive IFN-γ to either of the two human coronaviruses (Figure 5B). According to these findings, existing spike-directed antibody cross-reactivity may not be specific to SARS-CoV-2. The lack of an IFN-γ response in the presence of SARS-CoV-2 antibodies suggests that SARS-CoV-2-directed IgG antibodies did not elicit a novel IFN-γ response in response to natural SARS-CoV-2 or vaccine exposure. Our understanding of the immune system's ability to respond to SARS-CoV-2 is still limited.

Discussion

This study used pre-pandemic Ugandan specimens to examine the levels of pre-existing cross-reactive cellular and humoral responses to seasonal human coronaviruses and how they fit with Uganda's lower COVID-19 disease severity. We found that pre-pandemic IFN-γ T-cell responses were detectable against the structural proteins of SARS-CoV-2 and HCoV-OC43 and HCoV-229E human coronaviruses. The SARS-CoV-2-specific IFN-γ cross-reactivity targeted the nucleoprotein, spike, membrane, and envelope viral proteins and was broader and stronger in HIV-1-negative specimens than in HIV-1-positive ones. The correlation between pre-pandemic SARS-CoV-2-directed IFN-γ cross-reactivity and human coronavirus-directed IFN-γ cross-reactivity was weak, indicating that cross-reactive T-cells to seasonal human coronaviruses do not fully explain the detection of pre-epidemic

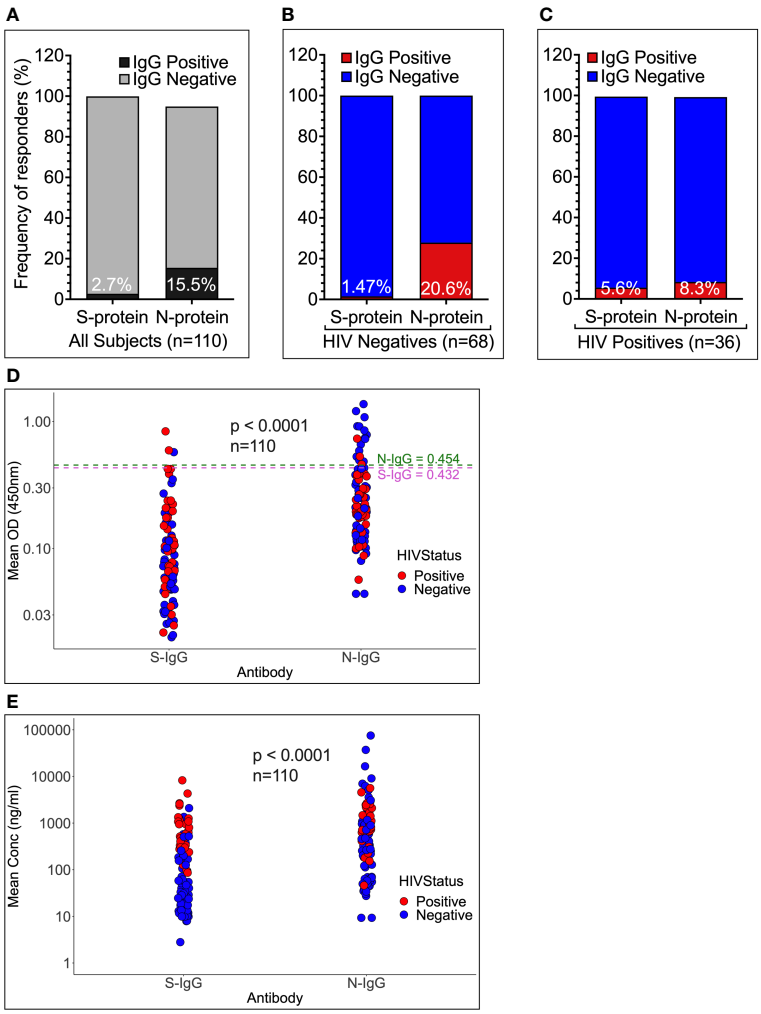

FIGURE 4
Frequencies and magnitudes of SARS-CoV-2 -specific cross-reactive IgG binding antibodies by HIV status. The frequency of pre-epidemic cross-reactive anti-SARS-CoV-2 binding IgG antibodies in Ugandan pre-pandemic sera is depicted in Figure 4. 110 specimens **(A)** were stratified by HIV-1 negative **(B)** and positive serostatus **(C)**, and the proportions of subjects with cross-reactive IgG binding antibodies against the SARS-CoV-2 spike and nucleoprotein illustrated. **(D, E)** summarises the medians of means of duplicate IgG antibody OD values in nm and concentration levels in ng/ml, respectively. The cut-off OD values are shown by the dashed lines.

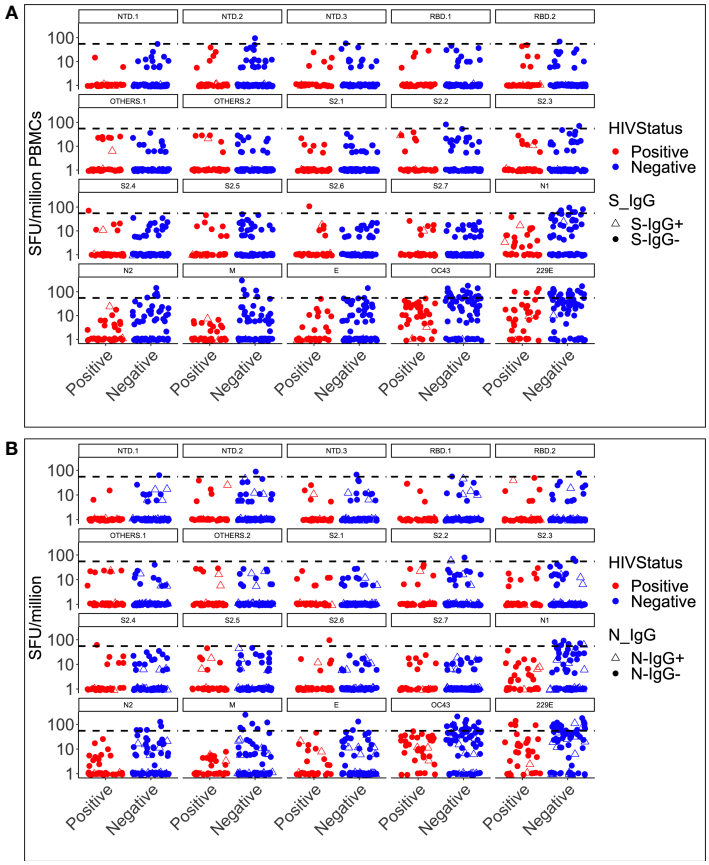

FIGURE 5
IFN-γ cross-reactivity categorised HIV status and spike **(A)** and nucleoprotein **(B)** IgG response. Depicts the simultaneous induction of SARS-CoV-2-specific IgG antibodies and IFN-γ cross-reactive responses in 104 specimens with antibody and T-cell data against the spike **(A)** and the nucleoprotein **(B)**. The data is presented according to HIV serostatus, with HIV-positive individuals in red and HIV-negative individuals in blue. Open triangles represent participants with cross-reactive antibodies and IFN-γ responses

TABLE 3 SARS-CoV-2 directed IgG cross-reactive neutralizing antibody titres. Summarises the responses of nine participants tested for neutralising antibodies against SARSCoV-2.

	LogNT90	NT90	LogNT50	NT50
PARTICIPANTS	1.954	1	1.699	1
	1.954	1	1.699	1
	1.954	1	1.699	1
	1.954	1	1.699	1
	1.954	1	1.699	1
	1.954	1	1.699	1
	1.954	1	1.699	1
	1.954	1	1.699	1
	1.954	1	1.699	1

The highest antibody titre that neutralised 50% and 90% of the virus, NT50 and NT90, respectively, were recorded. The antibody titres were logarithmically scaled to account for minimal differences. Notably, the lower detection limit of the SARS-CoV-2 neutralisation assay was 10, rendering virus titres below this threshold undetectable. Consequently, all responses obtained from the assay fell below the detection limit for positivity. For statistical analysis, NT50 values below det5ection were assumed to be 1.699, while undetectable NT90 values were equated to 1.954.

SARS-CoV-2-specific T-cell cross-reactivity. SARS-CoV-2-specific IgG antibody cross-reactivity was detectable, but it was primarily directed against the nucleocapsid protein. Cross-reactive, spike-directed antibodies did not neutralize SARS-CoV-2 *in vitro*, and no correlation occurred between antibody and IFN-γ responses.

Various hypotheses have been suggested linking the comparatively lower SARS-CoV-2 pathogenesis infection rates in sub-Saharan Africa to possible cross-protection due to prior exposure to seasonal human coronaviruses. The pre-pandemic serological cross-recognition of human coronaviruses was shown to be more prevalent in SSA than in USA specimens (4). Our findings agree with the prevalent serological cross-reactivity to SARS-CoV-2 in pre-epidemic Ugandan specimens reported by others before (20). However, we observed a much lower serological prevalence of 15.5% of 110 specimens (HCoV-OC43 and HCoV-229E combined) compared to the 87.5% reported by Mulabbi et al. (20). Seroprevalence of spike-directed cross-reactivity, the hallmark of antibody protection was even much lower occurring at a frequency of 2.72% of 110 pre-pandemic specimens. Differences in screening methodology could explain the much lower prevalence in the same population. Mulabbi et al.'s study (20) screened with a commercial ELISA kit using wells pre-coated with a nucleoprotein, aa 340-390 peptide; the test

specimens were not diluted, and the cut-off threshold was deduced by adding 0.15 to the mean of negative wells; conditions which in our hands would have resulted in the majority of the specimens being positive. Here, we used a validated in-house ELISA that uses the complete nucleoprotein proteome, with all test specimens first prediluted 100-fold to define a virus-specific response. The assay limits of detection and cut-off thresholds used were established and validated for the Ugandan population (19). The simultaneous ELISpot screening of our specimens using seven SARS CoV-2 antigenic regions to establish a comparable human coronavirus T-cell cross-reactivity prevalence of 27.9% corroborates our antibody prevalence data.

We have established that the cross-reactive binding antibodies detected here are not specific or functional against the prototype SARS-CoV-2 viruses. We have also shown that pre-existing T-cell cross-reactivity is comparable to that reported in South Africa, where a dual colour ELISpot assay detected IFN-γ responses in 29.9% and IL-2 in 39.2% with a combined response rate of 51.6% (21). Higher levels of cross-reactive T-cell frequencies of up to 50% reported in a U.S. study are likely attributable to evaluations of a broader range of seasonal coronavirus strains and a broader range of viral epitopes (11). The broader selection of strains and epitopes likely triggered a higher frequency of cross-reactive T-cells in the U.S. In general, the methodology of these other studies differed slightly from ours in terms of lower cut-off values for positive IFN-γ responsiveness (20 SFU/10 6 PBMCs) and the use of flow cytometry to detect additional T-cell responses (55 SFU/106 PBMCs).Nevertheless, even with these differences in the methodologies, the results of both our study and others showed that some people had some level of pre-existing cross-reactive T cells prior to being exposed to SARS-CoV-2.

We observed a trend toward decreased cross-reactivity in HIV-1-negative individuals of median CD4 counts of 483.0, IQR: 231.5 - 678.2 CD4 cells/µl. HIV-1 seropositive specimens were obtained from patients receiving Cotrimoxazole prophylaxis, which is routinely administered to patients with low CD4+ T-lymphocyte counts in order to prevent potentially fatal bacterial infections. Immunocompromised status may have had a further impact on the frequency of detectable cross-reactivity, as other studies have demonstrated that HIV-1-infected individuals have lower cross-reactive responses to SARS-CoV-2 (4, 22), particularly those with lower CD4 counts (23).

Nevertheless, these results demonstrate that exposure to HCoV-229E and HCoV-O43 has been endemic in Uganda, indicating the potential for the presence of pre-existing cross-reactive immune responses to SARS-CoV-2. Their low prevalence and the lack of correlation between SARS-CoV-2 and human coronavirus-specific immunity suggest that pre-existing cross-reactive immunity may not significantly account for the comparatively mild impact of COVID-19 in Uganda. The data highlight the importance of further studies to understand the role of pre-existing immune responses to HCoV-229E and HCoV-O43 in protecting against SARS-CoV-2 infection in Uganda. Others showed that low comparative T-cell epitope homology between SARS-CoV-2 and common cold coronaviruses was deemed insufficient to offer cross-protective cellular responses in other contexts as well and may therefore not be a critical correlate of low disease severity (24). Here, the cross-reactive IgG antibody response was stronger against the nucleocapsid than the spike, showing that the observed cross-reactive antibody response targets the most conserved regions of coronaviruses (25, 26).

This research had some limitations. While cross-reactive antibodies lacked neutralizing activity, other protective antibody functions not evaluated here are mediated by non-evaluated antibody-dependent effector mechanisms, such as antibody-dependent cellular cytotoxicity and Fc effector functions (4, 18, 27). As a result, we recommend additional research into the diverse effector mechanisms of these cross-reactive antibodies, as they may provide valuable insights into the development of a pan-coronavirus-directed vaccine. By elucidating the molecular and cellular mechanisms through which cross-reactive antibodies function, this research could provide a framework for the development of universal vaccines against coronaviruses, providing an invaluable contribution to the global health landscape. For determining the relationship between prior cross-reactivity and immunity, a longitudinal study design with pre- and post-epidemic specimens is optimal. Such a study design would compare pre-existing levels of cross-reactivity to post-epidemic levels of immunity to determine the extent to which prior cross-reactivity may be responsible for protection against a novel virus. We are conducting a follow-up study to investigate the association between pre-existing cross-reactivity and disease outcome. Further, we recommend investigating the variability in genetic influence on the production of such cross-reactive antibodies, which was not determined in this study, and exploring their epitope specificities to inform vaccine design targets. In addition, due to reagent accessibility constraints, we only tested two human coronavirus strains. To better understand the entire spectrum of prior cross-reactivity, more seasonal coronavirus strains and viral epitopes will need to be evaluated. In addition, it would be instructive to know what the results would have been with a much larger, more heterogeneous, and more representative sample, as this study was limited to only 110 participants, 36 of whom were HIV-seropositive.

Also, only spike-based PNA and nucleocapsid cross-binding antibodies were studied. Functional non-neutralizing antibody responses to SARS-CoV-2 were not evaluated. Because cross-reacting, binding antibodies may have effector mechanisms other than neutralization, evaluating the efficacy of other effector functions, such as N-directed functionality, is critical for understanding the full spectrum of immunological protection that a specific antibody may provide (28, 29). Antibodies, for example, can be evaluated for their ability to activate complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent cell-mediated cytotoxicity (ADCM) (ADCC). Finally, live viral assays shed light on how antibodies interact with viruses and the effector functions that they can perform in addition to spike-based PNA functionality. To fully understand the effector mechanisms of cross-reacting binding antibodies, it is critical to use live viral assays in conjunction with spike-based PNA and nucleocapsid tests.

In conclusion, these results confirm the presence of pre-existing SARS-CoV-2 specific cross-reactivity, but do not demonstrate that these antibodies are entirely specific to SARS-CoV-2. More research

is needed to fully understand the presence and clinical significance of pre-existing SARS-CoV-2 specific cross-reactivity. The findings shed new light on the potential for the nucleoprotein to induce cross-reactive immunity against SARS-CoV-2 and suggest that surveillance efforts targeting the nucleoprotein may overestimate the exposure to SARS-CoV-2 relative to other targets, like the spike protein. Specifically, the data underscores the need for multiple serologic assays targeting a variety of SARS-CoV-2 antigens to more accurately measure exposure. In addition, the data revealed a lower degree of cross-reactivity in HIV-positive individuals compared to healthy individuals, indicating that pre-existing SARS-CoV-2 specific cross-reactive immunity may not be a characteristic shared by all populations. Preexisting cross-reactivity must be considered when developing an accurate serological test for SARS-CoV-2 infection, as this study demonstrates.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved, and ethical approval granted by the Uganda Virus Research Institute Research and Ethics Committee (IRG Ref: GC/127/233) and the Uganda National Council for Science and Technology (Ref: HS1030). The participants provided written informed consent to participate in this study.

Author contributions

Conceptualized the work and drafted the initial manuscript: JSer, HN, VA and JSem. Clinical management and collection of specimens: BU, and FK. Conducted the laboratory assays: HN, JSem, LK, JSK, BG, SM, CB, GO, GKO, IS, AWK and "The COVID-19 Immunoprofiling Team". Data analysis: JSer, VA, HN, JSem, GKO and LK. Scientific Oversight and Supervision: JSer, OJS, SM and PK. All authors wrote and reviewed the manuscript. Reviewed and authorized the final manuscript. JS and PK. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Longitudinal analysis of SARS-CoV-2 infection and vaccination in the LA-SPARTA cohort reveals increased risk of infection in vaccinated Hispanic participants

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Introduction: SARS-CoV-2 is the etiologic agent of coronavirus disease 2019 (COVID-19). Questions remain regarding correlates of risk and immune protection against COVID-19.

Methods: We prospectively enrolled 200 participants with a high risk of SARS-CoV-2 occupational exposure at a U.S. medical center between December 2020 and April 2022. Participant exposure risks, vaccination/infection status, and symptoms were followed longitudinally at 3, 6, and 12 months, with blood and saliva collection. Serological response to the SARS-CoV-2 spike holoprotein (S), receptor binding domain (RBD) and nucleocapsid proteins (NP) were quantified by ELISA assay.

Results: Based on serology, 40 of 200 (20%) participants were infected. Healthcare and non-healthcare occupations had equivalent infection incidence. Only 79.5% of infected participants seroconverted for NP following infection, and 11.5% were unaware they had been infected. The antibody response to S was greater than to RBD. Hispanic ethnicity was associated with 2-fold greater incidence of infection despite vaccination in this cohort.

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Discussion: Overall, our findings demonstrate: 1) variability in the antibody response to SARS-CoV-2 infection despite similar exposure risk; 2) the concentration of binding antibody to the SARS-CoV-2 S or RBD proteins is not directly correlated with protection against infection in vaccinated individuals; and 3) determinants of infection risk include Hispanic ethnicity despite vaccination and similar occupational exposure.

KEYWORDS

SARS-CoV-2, COVID-19, vaccination, infection, serological analysis, high-risk

1 Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) causes Coronavirus-Disease 2019 (COVID-19), a pandemic that emerged in December of 2019. Despite intensive worldwide investigations, specific determinants of risk or protection against SARS-CoV-2 infection and COVID-19 disease remain elusive. Furthermore, as viral variants continue to emerge, correlates of vulnerability and immunity have continued to evolve. Critical to gaining knowledge in this regard are key conceptual distinctions: 1) exposure *vs.* infection *vs.* disease; and 2) immune response *vs.* protective immune response *vs.* determinants of protective immunity.

Among diverse populations, SARS-CoV-2 exposure risks differ relative to several factors, including occupation, healthcare, lifestyle and household structure, among others. Limited information is available regarding SARS-CoV-2 infection, immune response and COVID-19 risk in frontline workers at urban medical centers who have among the greatest chance of exposure. Interestingly, Hancean et al. found that urban medical occupations do not significantly drive viral transmission as compared to non-medical professions (1). The tendency for individuals to associate with those who are similar to themselves (e.g. vaccinated individuals with other vaccinated individuals) has been hypothesized to explain such an observation. However, there is a paucity of information regarding occupation, vaccination or immune response relative to risks of SARS-CoV-2 exposure or infection in frontline healthcare workers.

Humoral immunity has long been correlated with vaccine efficacy and protection against infection, particularly where neutralizing antibody affords immunity against viral pathogens. The humoral response to SARS-CoV-2 vaccination has accordingly been linked to increased protection against infection (2, 3). However, the observed direct correlation between severity of COVID-19 disease and anti-RBD antibody titer (4–6), serious infection despite high vaccine induced anti-RBD antibody titers (7, 8), and broader immune protection arising from natural SARS-CoV-2 infection (9) suggests as yet unknown risks and antibody qualities contributing to outcomes.

During the study period, two SARS-CoV-2 vaccines were FDA-approved for use in the U.S. (Pfizer-BioNTech BNT162b2 or Comirnaty vaccine and Moderna mRNA-1273 or Spikevax vaccine), and one retained its Emergency Use Authorization (EUA) (Johnson & Johnson/Janssen JNJ-78436735 vaccine). However, SARS-CoV-2 continues to cause widespread global morbidity and mortality nearly two years after global vaccination efforts began. Asymptomatic viral carriers contribute to the challenge of infection control measures; it has been estimated that 40-45% of the globally infected individuals are asymptomatic (10, 11). Additionally, the emergence of viral variants has caused confusion to whether vaccine efficacy and knowledge gained based on those who have had natural infection with a previous variant will translate to subsequent variants.

Most serologic assays for SARS-CoV-2 assess either neutralizing or binding antibodies against the spike holoprotein (S), spike receptor binding domain (RBD), or nucleocapsid protein (NP). Serological response to the S protein can be detected in both vaccinated and naturally infected individuals. However, NP is not included in current vaccine formulations; therefore, only those individuals naturally infected by SARS-CoV-2 generate anti-NP responses. This fact allows for the detection of prior infection in individuals who may have been asymptomatic or otherwise unaware of infection.

From its onset, the COVID-19 pandemic has imposed a disproportionate impact on underserved populations in larger urbanized areas (12). Specifically, SARS-CoV-2 disproportionately affects racial and ethnic minority groups in the US (13–15). However, whether such disparities are due to socioeconomic, healthcare utilization, comorbidities, genetic or a combination of these and other factors is not yet clear. In Los Angeles, almost 50% of the infections occur in populations who identify as Hispanic (16). Early studies regarding the COVID-19 pandemic show that Hispanic participants in Los Angeles have a higher prevalence of infection with SARS-CoV-2, but as variants have emerged, there has been little follow up (17, 18). Thus, key questions remain regarding potential correlates of risk or immunity based on race or ethnicity.

To gain new insights and address potential correlates of COVID-19 risk and immunity in real-world context, here we serologically analyzed the Los Angeles cohort of the COVID-19 SeroPrevalence And Respiratory Tract Assessment (LA-SPARTA) study. The current study focused on high-risk individuals according

to their occupation, antibody response to S, RBD, and NP proteins of SARS-CoV-2.

2 Materials and methods

2.1 SPARTA-LA study design

We prospectively enrolled 200 participants with a high risk of SARS-CoV-2 occupational exposure, irrespective of history of SARS-CoV-2 vaccination or natural infection. Participants were enrolled on the campuses of Harbor-UCLA Medical Center and The Lundquist Institute for Biomedical Innovation at Harbor-UCLA in Torrance, CA, between December 2021 and April 2021. Our cohort of participants in Los Angeles, CA is one of eight sites who enrolled participants starting in 2020 as part of PARIS (Protection Associated with Rapid Immunity to SARS-CoV-2)/SPARTA (SeroPrevalence and Respiratory Tract Assessment) for longitudinal analysis of SARS-COV-2 reinfection and correlates of protection. Previously, the cohorts of all PARIS and SPARTA cohorts, including a preliminary analysis of the LA-SPARTA cohort infections and collaborative efforts were reported (19).

2.2 Data collection/storage

Patients were recruited via flyers that were posted around the Harbor-UCLA campus (Supplemental Figure 2). Interested persons contacted a research coordinator and were screened for eligibility via interview. Study inclusion criteria were: age > 18 years of age, able to complete the informed consent process, willing/able to attend and complete scheduled study visits, and who fall within one of the following categories: full-time healthcare workers, work in the inpatient setting, and take care of patients with or at high risk of having COVID-19 infection (such as in the emergency department), or medical center full-time employees who do not have contact with persons with documented or suspected COVID-19 infection, or law enforcement who work full time while having contact with the general public, or paramedics or Emergency Medical Service (EMS) whose duties include full time interaction with patients, or other community members who are able to access the Harbor-UCLA campus. The exclusion criteria were: pregnancy, weight <110 lbs (50 kg), acute non COVID-19 infection, receipt of immunomodulatory or immune suppressive medication (e.g., chemotherapy, systemic steroids), in the prior 12 months, chronic infection (e.g. HIV, Hepatitis C), or conditions with immune dysregulation such as rheumatologic or autoimmune diseases.

Eligible participants gave informed consent and answered surveys to collect data on demographics, medical conditions, employment type, and symptoms of possible COVID infection. Participant height and weight were measured at enrollment and each follow up visit. At baseline, we performed saliva, nasal swab, and blood draws. Over the duration of the study period, participants were surveyed routinely to screen for additional vaccinations/boosters, infections, and health changes via weekly emails. If events were reported, such as vaccination or infection, participants would provide a blood and saliva sample in addition to samples that were collected at 3 months, 6 months, and 12 months after enrollment. Due to funding constraints, participants who had SARS-CoV-2 antibody responses at enrollment were prioritized to be sampled more often. Serological response against the SARS-CoV-2 Spike, RBD, and NP was detected via quantitative ELISA assay and analyzed accordingly. All survey and serological data were stored using the secure, REDCap database management software.

2.3 Quantitative ELISA assay

RBD and Spike Proteins were obtained from the central laboratory of the PARIS/SPARTA collaboration. 100 µL of proteins were coated onto a 96-well microplate at a concentration of 1µg/mL, 2µg/mL, or 0.5µg/mL of SARS-CoV-2 Receptor Binding Domain (RBD), Spike, or Nucleocapsid (NP) proteins, respectively and incubated overnight at 4°C. RBD and Spike proteins from the Wuhan-Hu-1 SARS-CoV-2 isolate were created by Florian Krammer's lab at Mount Sinai (20) and produced and received from the Center for vaccines and immunology CORE Lab at UGA (accession# MT380724.1 and MT380725.1). The NP protein (Sino Biological cat# 40588-V08B) was constructed from the 2019-nCoV SARS-CoV-2 isolate (accession# YP_009724397.2). The concentration of IgG was determined using a previously described method (21, 22). Plates were rinsed three to five times with 0.1% tween 20 in PBS (0.1% PBST) when noted. Plates were blocked with 3% non-fat dry milk in 0.1% PBST for 1h at room temperature (RT). Patient plasma was heat inactivated for 45-60 minutes at 56°C and diluted to 1:120 in 1% non-fat dry milk in 0.1% PBST and added to the plate for 2h at RT or overnight at 4°C after rinsing five times with 0.1% PBST. Next, plates were similarly rinsed using 0.1% PBST and a 1:3000 dilution of goat anti-human IgG-HRP was added to the plates for 1h at RT. Plates were rinsed and developed with SigmaFast OPD tablets in PBS per the manufacturer's instructions for 10 minutes. The reaction was stopped using 3M HCl and scanned at 492 and 700 nm using a BioTek Cytation 5 Microplate reader. The OD700 nm was subtracted from the OD492 nm and the averaged from triplicate runs for each patient sample.

The Standard Curve was run on each plate with patient samples using a concentration gradient of an S1 specific SARS-CoV-2 IgG1 Ab (AbCam cat# ab273073) for the RBD and Spike assays or NP IgG1 Ab (Invivogen cat# covn-mab1) for the NP assay. The log of each average OD492-700nm was logged and graphed and the linear equation was then calculated and applied to the samples to determine the concentration respective of the standard curve, corrected for the dilution factor, and converted from ng/mL to µg/mL.

The positive and negative values for the RBD, Spike, and NP ELISA assays were calculated using a mixtures model to define two distributions in the raw or Log2 transformed ELISA data (i.e., negative and positive) using package "mclust" in RStudio. The mean and standard deviation (SD) of the presumed-negative population

was calculated and the seropositivity cutoff was set as 2 standard deviations above the mean of the presumed-negative population. The cutoff values for the RBD, Spike and NP ELISA assays were 0.383, 0.132, and 2.225 μg/mL, respectively.

2.4 Calculating the time since vaccination

Participants reported their SARS-CoV-2 vaccination dates via surveys at enrollment and were able to notify us through their surveys if they received a vaccination over the course of the study. If participants only reported the date of the first dose, a second dose date of 21 days after the first dose was recorded for Pfizer recipients and 28 days for Moderna recipients. When available, clinical coordinators were able to confirm vaccination dates with participants. In total, 7 participants were missing the date of their second vaccination dose, but had provided the first date. Time since vaccination and booster was calculated as the blood collection date minus the vaccination or booster date divided by 30.

2.5 Definitions

At enrollment, a participant with a positive detected NP or an RBD antibody response without receiving SARS-CoV-2 vaccination was classified at having a prior infection (Supplemental Figure 1). During the study, participants who reported a positive SARS-CoV-2 PCR or antigen test were also categorized as having a reported infection. For those who had a natural infection over the course of the study, we defined a "Breakthrough infection" as a serologically detected infection or reported infection detected by positive SARS-CoV-2 PCR or antigen test in a participant who has been vaccinated with two doses of an mRNA vaccine or single dose of the adenoviral vector-based vaccine. "Infection" was defined as a serologically detected infection or infection detected by positive SARS-CoV-2 PCR or antigen test in an unvaccinated participant. "Serologically detected infection" was defined as NP seroconversion (converting from a negative to positive concentration value) or a ≥4-fold increase in the serological NP concentration, when compared to the previous serological sample tested or RBD seroconversion (converting from a negative to positive concentration value) without a known vaccination event or a ≥4-fold increase in the serological RBD concentration compared to the previous serological sample tested. Re-infection was used to describe participants who had a serologically detected NP antibody response at enrollment (, suggesting a prior infection with SARS-CoV-2, and who were infected over the course of the study (including both reported and serologically detected infections). Serological response was defined as a 4-fold or higher increase in the concentration of antibody compared to the sample prior to when infection was detected or reported. No or minimal response was defined as no change in the antibody concentration or less than 4-fold increase in the antibody concentration in the infection sample compared to the sample prior to when infection was detected or reported. The 4-fold threshold was defined by the PARIS/SPARTA consortium to determine sero-positivity or possible infection (19).

2.6 Statistical analysis

For continuous measures, t-tests were used to compare variables between two groups or time frames. For categorical data, grouped analyses of data pre and post vaccination with prior infection were carried out using Fisher's exact test or a T test was carried out comparing the delta change. Longitudinal comparisons of continuous and categorical variables between groups were carried out using a mixed model analysis. Comparisons of continuous and categorical variables (RBD after vaccination) in a single group were carried out using an ordinary one-way ANOVA with an appropriate multiple comparison's test. For time-to-event data, an interval-censored Cox proportional hazards model was used to estimate relative risks of breakthrough since participants were surveyed periodically.

2.7 Analysis of decay rates

Visual inspection of plots of raw protein levels versus time since vaccination suggested that protein decay could be modeled exponentially. Thus, protein levels were log transformed to facilitate using a random-effects generalized least-squares (GLS) regression model for testing effects among protein type (RBD/Spike) and ethnicity (Hispanic/Non-Hispanic) in initial analyses of decay rates. Models included time-interaction terms and random-subject factors to account for repeated measures within the same individual. We used conventionally derived variance estimators for GLS regression and assumed asymptotic independence and normality of standard errors for statistical inferences.

Deeper inspection of the raw experimental data revealed that protein decay may behave according to a transition over two linear phases – an early and late phase post-vaccination. To estimate early (i.e., left slope)/late (i.e., right slope) decay rates and transition times (i.e., breakpoints), a second analysis was done using a piecewise linear regression model (23) fit to the raw protein levels stratified by ethnicity. Point estimates and their 95% confidence intervals were reported and used for statistical inferences assuming asymptotic approximations due to the lack of proper *a priori* hypotheses.

All statistical analyses were calculated via GraphPad Prism version 9.3.1 or Stata version 17, as needed. P-values were two-sided and judged statistically significant if less than a nominal type-1 error rate of 5%. Due to the study's discovery nature, no additional multiplicity adjustments were made beyond the pairwise ANOVA comparisons listed above and no prediction validations were performed.

2.8 Study approval

The study was approved by Institutional Review Board of the University of California, Los Angeles (IRB#20-001649-AM-00009).

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Written informed consent was received by participants prior to study participation.

3 Results

3.1 Study population, rates of vaccination, and natural infection

The LA-SPARTA study population comprised 29.0% Asian, 57.5% White, 6.5% Black and 7.0% other races (Table 1). In the total participant population, 40.5% identified as Hispanic. The mean age was 40.2 years. The number of female participants was 139 (69.5%) and males were 61 (30.5%) (Table 1). At enrollment, 65.5% of the cohort was vaccinated with at least one dose of a SARS-CoV-2 mRNA or adenoviral vector vaccine. Pfizer-BioNTech (BNT162b2) vaccination predominated the vaccination type received by this cohort (Table 2). A history of prior natural infection in 26.5% of the study participants at enrollment was confirmed serologically via detectable NP or RBD antibody concentration (in participants who had not received a vaccination), (Table 2). The criteria for the initial serological grouping at enrollment are shown in Supplemental Figure 1. A total of 40 infections were detected over the course of the study period, which is described in detail in the next section. 50.7% of unvaccinated participants at enrollment underwent vaccination over the study period (Table 2). At the end of the study period, 83.0% of participants were vaccinated and 35.0% of participants had evidence of a prior infection (Table 2). Pfizer-BioNTech (BNT162b2) was the predominant vaccine received by LA-SPARTA participants, with 84.3% of participants receiving this vaccine by study completion.

3.2 Serological assessment of SARS-CoV-2 infection in participants

Plasma was assessed at enrollment for antibody against S, RBD and NP proteins of the SARS-CoV-2 coronavirus. Participants were further stratified based on antibody reactivity to RBD and NP proteins (Supplemental Figure 1). We observed 6 unvaccinated participants with RBD seroconversion but without NP seroconversion at enrollment (Table 3). This pattern of results suggested these participants had been naturally infected due to evidence of seroconversion to RBD but not NP (Supplemental Figure 1).

TABLE 1 Participant demographic data.

All Participants (n=200)		
Age (Years)		
	Mean (SD)	40.24 ± 12.06
	Min, Max	19, 77
Gender, n (%)		
	Male	61 (30.5%)
	Female	139 (69.5%)
Race, n (%)		
	White	115 (57.5%)
	Asian	58 (29.0%)
	Black or African American	13 (6.5%)
	American Indian/Alaskan Native	1 (0.5%)
	Native Hawaiian or Pacific Islander	4 (2.0%)
	Multiple	8 (4.0%)
	Unknown	1 (0.5%)
Ethnicity, n (%)	Hispanic or Latino	81 (40.5%)
	Non-Hispanic or Latino	118 (59.0%)
	Unknown	1 (0.5%)
Exposure Category, n (%)		
	Medical Center/Healthcare Worker	101 (50.5%)
	Nurse	68 (67.3%)

(Continued)

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TABLE 1 Continued

All Participants (n=200)		
	Physician	11 (10.9%)
	Respiratory Therapist	3 (3.0%)
	Radiology Technician	3 (3.0%)
	First Responder	2 (2.0%)
	Physical/Occupational Therapy	1 (1.0%)
	Other	13 (12.9%)
	Medical Center/Non-Healthcare Worker	44 (22.0%)
	Environmental Services	1 (2.3%)
	Facilities Management	5 (11.4%)
	Laboratory Staff	15 (34.1%)
	Social Worker/Case Manager	3 (6.8%)
	Other	20 (45.5%)
	Local Community Member/Research Staff	55 (54.5%)
	Lundquist Institute Employee	30 (54.5%)
	Person who lives nearby	25 (45.5%)

Data are represented as the number of participants (n) and the percentage of participants in the cohort or group (%), unless otherwise stated.

Accordingly, the criteria for assessing infection throughout the study period included NP seroconversion (defined as converting from a negative to positive concentration value if the value was not already within the positive range) or a ≥4-fold increase in NP concentration compared to the previous serological sample tested in previously positive individuals, or RBD seroconversion without a known vaccination event (converting from a negative to positive concentration value if the value was not already within the positive range) or a ≥4-fold increase in RBD concentration compared to the previous serological sample tested in previously positive individuals.

Over the observation period, 40 total participants either reported a SARS-CoV-2 infection or had a natural infection detected serologically. Out of all infections, 10/40 (25.0%) occurred in unvaccinated participants, while 30/40 (75.0%) infections occurred using the same criteria in vaccinated individuals (termed "breakthrough infection") (Figures 1A, B). It is important to note that the increased number of infections in vaccinated participants is likely because over the course of the study, the study population increased from 65.5% to 83.0% of participants vaccinated. Thus, 20.0% of the study cohort was infected with SARS-CoV-2 during the study period. Among these infections, 8 were unreported in unvaccinated participants, and 15 infections were unreported in vaccinated participants (Figure 1B). Of all participants who either reported a positive polymerase chain reaction (PCR) or antigen test over the course of the study (termed "reported infections") and those who had serological evidence of infection (termed "unreported" or "serologically detected" infection), 20.5% of participants had no evidence of NP seroconversion (Figure 1C). In all infections, including those reported and serologically detected, there were post-infection increases in the concentrations of RBD, S, or NP antibodies in the plasma (Figure 1D). However, not all infections yielded an RBD response of ≥ 4-fold increase in concentration (Table 3). The RBD-, S-, or NP-specific serological responses did not significantly differ between reported infections and those unreported but detected serologically (Figures 1E–G). Many of the reported infections during the study period took place as the Omicron SARS-CoV-2 variant began to circulate in California, according to data from the California department for Health and Human Services (24) (Figure 1H). However, it is unclear if this is also true for the serologically detected infections, as we were unable to estimate the timing of the serologically detected infections due to a lack of positive test date.

3.3 SARS-CoV-2 vaccination response differs between RBD and non-RBD antibodies

Of the unvaccinated participants at enrollment, 50.7% received a SARS-CoV-2 vaccination over the course of the study period (Table 2). At study completion, 83.0% of the study participants had received at least 2 doses of an mRNA-based or a single dose of an adenoviral vector-based SARS-CoV-2 vaccine (Table 2). Among participants who had samples collected both before and after vaccination, the concentration of RBD and S antibodies significantly increased after vaccination, while the concentration of NP antibodies did not significantly change (Figure 2A). To determine the kinetics of the binding antibody response to SARS-CoV-2 vaccination, confounding samples were censored (e.g. those collected after a booster vaccination or infection) and the remaining longitudinal samples were analyzed post-vaccination. This analysis

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TABLE 2 Participant serological status at enrollment and over the study period.

All Participants (n=200)	
At Enrollment	
Vaccinated, n (%)	131 (65.5%)
Moderna (mRNA-1273), n (%)	20 (15.2%)
Pfizer (BNT162b2), n (%)	110 (84.0%)
J&J/Janssen (JNJ-78436735), n (%)	1 (0.8%)
Not Vaccinated, n (%)	69 (34.5%)
Prior Infection, n (%)	53 (26.5%)
No Prior Infection, n (%)	147 (73.5%)
Prior Vaccination and Infection, n (%)	25 (12.5%)
Prior Vaccination without Infection, n (%)	106 (53.0%)
No Vaccination with Infection, n (%)	28 (14.0%)
No Vaccination without Infection, n (%)	41 (20.5%)
Vaccination Over the Study Period	
Unvaccinated to Vaccinated, n (%)	35/69 (50.7%)
Moderna (mRNA-1273), n (%)	3 (8.6%)
Pfizer (BNT162b2), n (%)	30 (85.7%)
J&J/Janssen (JNJ-78436735), n (%)	2 (5.7%)
Received Booster Vaccination, n (%)	67/166 (40.4%)
At Study Completion	
Vaccinated, n (%)	166/200 (83.0%)
Moderna (mRNA-1273), n (%)	23 (13.9%)
Pfizer (BNT162b2), n (%)	140 (84.3%)
J&J/Janssen (JNJ-78436735), n (%)	3 (1.8%)
Not Vaccinated, n (%)	29/200 (14.5%)
Unknown, n (%)	5/200 (2.5%)
Prior Infection, n (%)	70/200 (35.0%)
No Prior Infection, n (%)	120/200 (60.00%)
Unknown, n (%)	10/200 (5.0%)

Data are represented as the number of participants (n) and the percentage of participants in the cohort or group (%), unless otherwise stated. The number of participants who were vaccinated and infected at study completion is representative of each participant while they were in the study.

revealed a response to vaccination that differed between RBD- and S-specific responses (Figures 2B, C). RBD-specific antibodies appeared to wane more rapidly than S-specific responses after vaccination, with RBD antibodies significantly decreasing by 3 months (Figure 2B) post-vaccination as compared to S responses which did not significantly decrease until ≥ 6 months (Figure 2C). There was no significant change in the NP antibody concentration after vaccination (Figure 2D). Accordingly, S antibody responses were higher than RBD antibody responses after vaccination (Figure 2E). The average ratio of RBD to S antibodies was 4.56 ± 7.7 (SD) μg/mL following vaccination (Figure 2F).

To compare the kinetics of RBD and S antibody decay, we first analyzed log-transformed RBD and S antibody levels (i.e., consistent with exponential decay) over time using a Generalized Least Squares regression model (Figure 2G). The constant decay rates (expressed as log ug/mL values) were -0.11 units/month and -0.02 units/month for anti-RBD and anti-S antibody, respectively. These two rates were significantly different (p=0.001). Also, average initial antibody levels (i.e., the estimated intercepts) were higher versus S than RBD, with initial values of 5.5 ug/mL (e^1.71) and 3.1 ug/mL (e^1.14), respectively (p<0.001). Next, we applied piecewise linear regression models separately for each antibody target to compare time estimates of two linear phases of raw decay change, or the "breakpoint". Accordingly, the concentration of RBD antibodies decayed at a rate (left slope) of -2.1 (95% CI: -2.7, -1.4) ug/mL/month for 3.4 months (95% CI: 2.6, 4.1) and then decreased to 0.3 (95% CI: -0.02, 0.5) ug/mL/month as indicated by the slope thereafter (Figure 2H). Notably, after the breakpoint at 3.4 months, an *ad hoc* hypothesis test that decay ceases (i.e., the right slope equals zero) was not rejected at a 5% error rate since the 95% CI lower bound is negative and the upper bound is positive. For S protein, the concentration of antibodies decreased at a rate of -0.7 (95% CI: -1.1, -0.2) ug/mL/month for 6.2 months (95% CI: 2.5, 10.0) and then 0.1 (05% CI: -0.5, 0.7) ug/mL/month thereafter (Figure 2I). Similarly, after the breakpoint at 6.2 months, the *ad hoc* hypothesis that the antibody level was constant was not rejected.

3.4 Vaccination response differs in participants with prior infection

To analyze potential differences in the response to vaccination based on the concentration of RBD specific antibodies, we compared antibody concentrations in various groups of participants following vaccination. Using the same method of eliminating confounding samples described above, we analyzed whether differences were detectable in the response to vaccination based on the concentration of RBD specific antibodies. Antibodies specific to the SARS-CoV-2 RBD protein have been shown to be specific to this virus, as the RBD domain is poorly conserved among other members of the SARS-coronavirus clade and other common coronaviruses (25–27). Thus, we focused on RBD-specific responses, as it is the key target of SARS-CoV-2 vaccines and binding RBD antibodies are associated with strong neutralizing SARS-CoV-2 responses (28, 29). To assess potential demographic correlates (age, ethnicity, and biological sex) that may influence the SARS-CoV-2 vaccination-induced antibody response, we compared the kinetic responses stratified to these variables. No significant differences in the binding antibody response to SARS-CoV-2 vaccination occurred according to age, ethnicity, or sex in this cohort (Figures 3A–C). There was however a significant difference based on whether the participants had a prior infection detected serologically. This difference occurred in the early vaccination response, noted at the time in between the 1st and 2nd vaccination dose (p=0.0003) and after 0-2.99 months post-vaccination (p<0.0001) (Figure 3D). Importantly, this difference in response was short lived, as no significant difference was detected in the response at ≥3 months

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TABLE 3 Serological response to infection based on RBD and NP antibody response.

LA-SPARTA Infections			
Previous Infections at Enrollment, n (%)	53 (26.5%)		
NP Seroconversion, n (%)	47 (88.7%)		
RBD Seroconversion, n (%)	49 (92.5%)		
NP & RBD Seroconversion, n (%)	43 (81.1%)		
Infections During the Study Period			
	Reported	**Unreported**	**Total**
Infection in un-vaccinated person, n (%)	2 (1.0%)	8 (4.0%)	10 (5.0%)
Re-Infection, n	1	4	5
NP response, no/minimal RBD response, n	1	1	2
RBD response, no/minimal NP response, n	0	4	4
Minimal Serological Response, n	1	0	1
RBD & NP response, n	0	3	3
Breakthrough or Infection in Vaccinated Person, n (%)	15 (7.5%)	15 (7.5%)	30 (15.0%)
With serological Data, n	14	15	29
Re-Infection, n	4	6	10
NP response, no/minimal RBD response, n	5	8	13
RBD response, no/minimal NP response, n	0	2	2
Minimal Serological Response, n	2	0	2
RBD & NP response, n	7	5	12

Data are represented as the number of participants (n) and the percentage of participants in the cohort or group (%), unless otherwise stated.

post-vaccination, based on prior infection status (p=0.441 at 3-5.99 months post-vaccination and p=0.7532 at >6 months post-vaccination) (Figure 3D). Using this same dataset, we also analyzed the pre-and post- vaccination response in all participants who were vaccinated over the course of the study, using the first sample post-vaccination, without separating the data by time post vaccination. There was no significant difference in the change in antibody response when comparing participants with *vs.* without prior infection (p=0.9234) (Figure 3E).

3.5 Factors associated with breakthrough SARS-CoV-2 infection

Breakthrough infection occurred in 30 of 166 (18.1%) of the vaccinated participants over the course of the study. Thus, we sought to determine if there were correlates associated with greater prevalence of breakthrough infection. Based on cumulative breakthrough infections in the study cohort, there was a significant increase in the concentration of RBD-, S- and NP-specific antibodies after infection as compared to infection naive (Figure 4A). Interestingly, there was an equivalent incidence of community members (10 participants) and healthcare workers (14 participants) who had breakthrough infection in this cohort (Figure 4B). To compare the variables that may be associated with a higher risk of breakthrough infection, we compared breakthrough participants with control participants who had been vaccinated against SARS-CoV-2 but did not have breakthrough infection over the course of this study. The demographic characteristics of these populations are shown in Table 4. Analysis of these data revealed a trend of increased incidences of infection among participants self-identifying as having Hispanic ethnicity in the breakthrough participant group compared to the control group (p=0.19; Table 4). Therefore, we next examined the possibility that Hispanic participants have a disproportionate risk of breakthrough infection with SARS-CoV-2 relative to time since vaccination. As shown in Figure 4C, we estimated probabilities of breakthrough infection as the time post-vaccination increases using an interval-censored Cox Regression model. This approach revealed that Hispanic participants exhibited twice the relative risk of breakthrough infection compared with non-Hispanic participants (hazard ratio=2.07; $p< 0.05$). We attempted to compare breakthrough risk among non-White participants but we only had sufficient study subject numbers to analyze Asian versus non-Asian participants. Comparison of the incidence of breakthrough infection in Asian versus non-Asian participants there was no significant difference in the breakthrough risk (data not shown).

Additionally, we compared the logarithmic-scale decay rate for antibodies against RBD and S proteins between vaccinated Hispanics and non-Hispanics. Although initial RBD antibody levels were statistically equivalent, the RBD decay rates (expressed as log ug/mL values) were -0.05 units/month and -0.14 units/month

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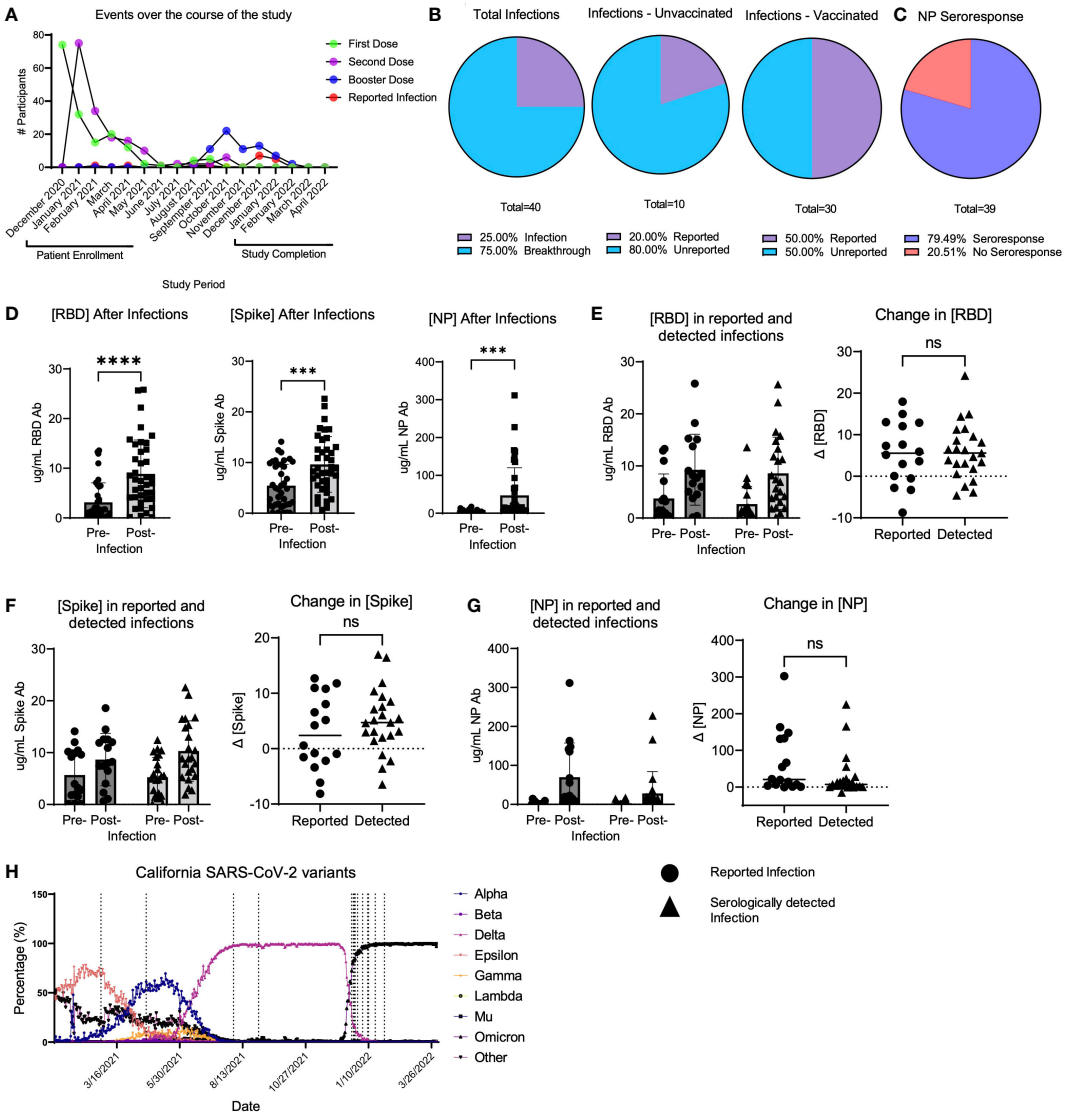

FIGURE 1
LA-SPARTA infections during the study period. **(A)** shows the events that occurred for all LA-SPARTA participants over the course of the study period. **(B)** describes both the reported and serologically detected infections in unvaccinated participants and breakthrough infections in vaccinated participants. **(C)** shows the percentage of infections and breakthrough infections in which the NP concentration increases by ≥4-fold compared to the previous blood sample collected. **(D)** shows the concentration of RBD, Spike, and NP in all serologically detected and reported infections and breakthrough infections. **(E)** shows the concentration of RBD in infections and breakthrough infections, separated by serologically detected versus reported infections. The delta change between the pre-infection sample collected and post-infection samples is also shown. **(F)** shows the concentration of Spike in infections and breakthrough infections, separated by serologically detected versus reported infections. The delta change between the pre-infection sample collected and post-infection samples is also shown. **(G)** shows the concentration of NP in infections and breakthrough infections, separated by serologically detected versus reported infections. The delta change between the pre-infection sample collected and post-infection samples is also shown. **(H)** uses SARS-CoV-2 variant data available from the California department of Health and Human Services from Los Angeles County to illustrate the percentage of each variant circulating at the time of reported positive tests (vertical dashed lines) in the LA-SPARTA cohort. *** designates P >0.001 and **** designates P >0.0001. ns, not significant.

for Hispanic and Non-Hispanic subjects, respectively (Figure 4D). These two rates were significantly different ($p<0.03$). By comparison, while initial S antibody levels were higher among Hispanic participants (7.3 ug/mL *vs.* 4.6 ug/mL, $p<0.03$), their logarithmic decay rates were indistinguishable from one another (data not shown). Given the overall difference in log-RBD antibody decay slopes, we used piecewise linear regression to find times at which the slopes of raw values changed. Here, we found similar temporal patterns and overlapping confidence intervals among Hispanics and non-Hispanics (Figure 4E). Finally, we compared the initial response against SARS-CoV-2 vaccination relative to concentration of anti-RBD, S, and NP antibodies within the first 4 months after vaccination. Results suggested a statistically significant increase in the early concentration of RBD antibodies after vaccination in the breakthrough infection group ($p=0.0493$); however, these individuals also had higher concentrations of anti-NP antibodies ($p=0.0261$) (Figure 4F). These findings suggested that the higher antibody response to vaccination was associated

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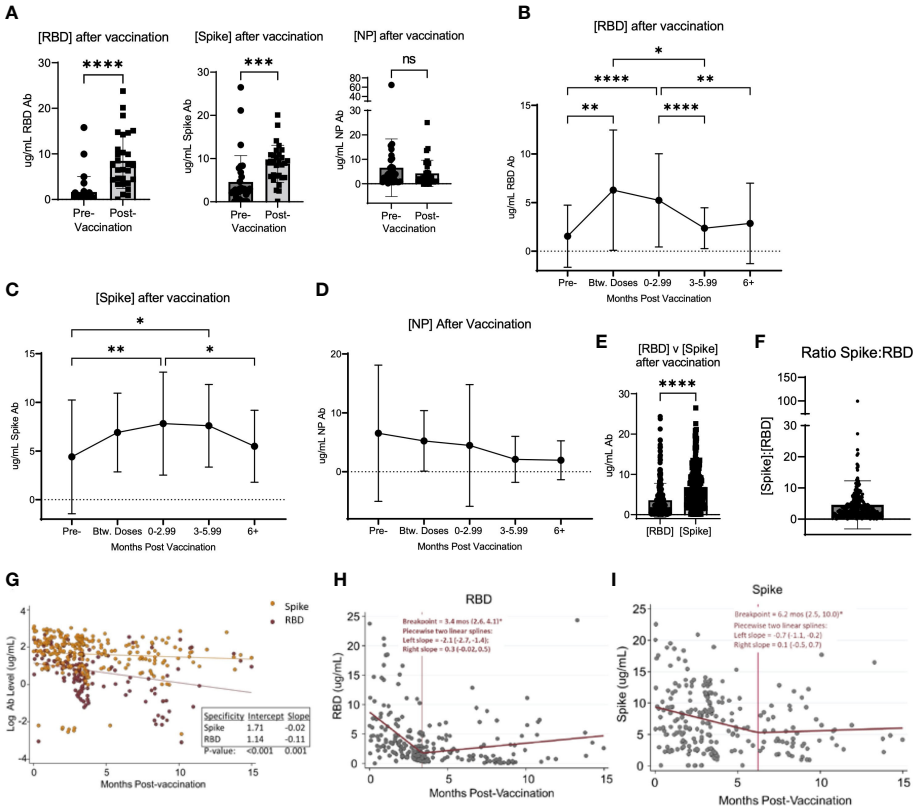

FIGURE 2
Vaccination induced changed in serological response to SARS-CoV-2. **(A)** shows the change in the concentration of RBD, Spike, and NP in participants who were vaccinated over the course of the study. **(B)** shows the change in RBD, Spike **(C)**, and NP **(D)** concentration after vaccination in all participants, regardless of when participants were vaccinated and when their blood was collected after vaccination. **(E)** shows the value of [RBD] and [Spike] in all participants after vaccination. **(F)** shows the ratio of the concentration of RBD to Spike for all participants after vaccination. **(G)** shows the log-transformed RBD (red data points) and Spike (orange data points) concentrations (i.e., to be consistent with exponential decay) over time using a GLS regression model **(H)** The lines represent the decay lines. Panel **(H)** shows the plot generated from piecewise linear regression models for the RBD protein or Spike protein **(I)**. The vertical line represents the "breakpoint", which is the estimate of when the two linear phases of raw decay change. The two decay phases are shown before and after each breakpoint. The breakpoint and decay slopes for the left and right portion are noted in the graphs. * designates P >0.05, ** designates P >0.01, *** designates P >0.001, and **** designates P >0.0001. ns, not significant.

with having SARS-CoV-2 infection prior to vaccination. We analyzed the incidence of previous infection at enrollment when comparing Hispanic (28/53) versus non-Hispanic (25/93) participants and found that there was a relationship between ethnicity and prior infection (p=0.0496). When comparing the population of participants who had breakthrough infection, the early concentration of RBD and NP antibodies after vaccination were not significantly different between Hispanic and non-Hispanic participants (Figure 4G). Taken together, these results suggest that there may not be a direct quantitative link between early antibody response and breakthrough infection, or longer-term antibody kinetics (persistence or decay) and breakthrough infection.

4 Discussion

The results of this study illustrate differential exposure and immune response patterns to SARS-CoV-2 among a diverse population of high-risk individuals in the urban LA-SPARTA cohort from December 2020 – April 2022. This time period encompasses a major shift in SARS-CoV-2 variant emergence from alpha and beta, to delta to omicron lineages. Using a strategic serological approach, studies were designed to detect response to vaccination as well as previous natural SARS-CoV-2 infections. We identified a significant proportion of participants who experienced one or more SARS-CoV-2 infections over the course of the study. Of note, the emergence of the omicron variant was temporally associated with a significant increase in the number of reported SARS-CoV-2 infections. Many of these infections were detected serologically, without symptoms or illness reported by the participant.

Importantly, our results suggest that confirmatory detection of SARS-CoV-2 infection may be best based on both seroresponse against RBD and NP proteins. Others have noted that anti-NP seroconversion does not occur in all naturally-infected individuals (30). Our observation regarding the decreased NP seroresponse compared to RBD agrees with such studies, in which SARS-CoV-2 PCR positive participants may be non-responders to NP protein. To our knowledge, the present study is the first to directly report humoral responsiveness to RBD, but not NP after natural infection (31, 32). Other studies in which all participants achieved seroconversion have hypothesized that a decrease in viral RNA

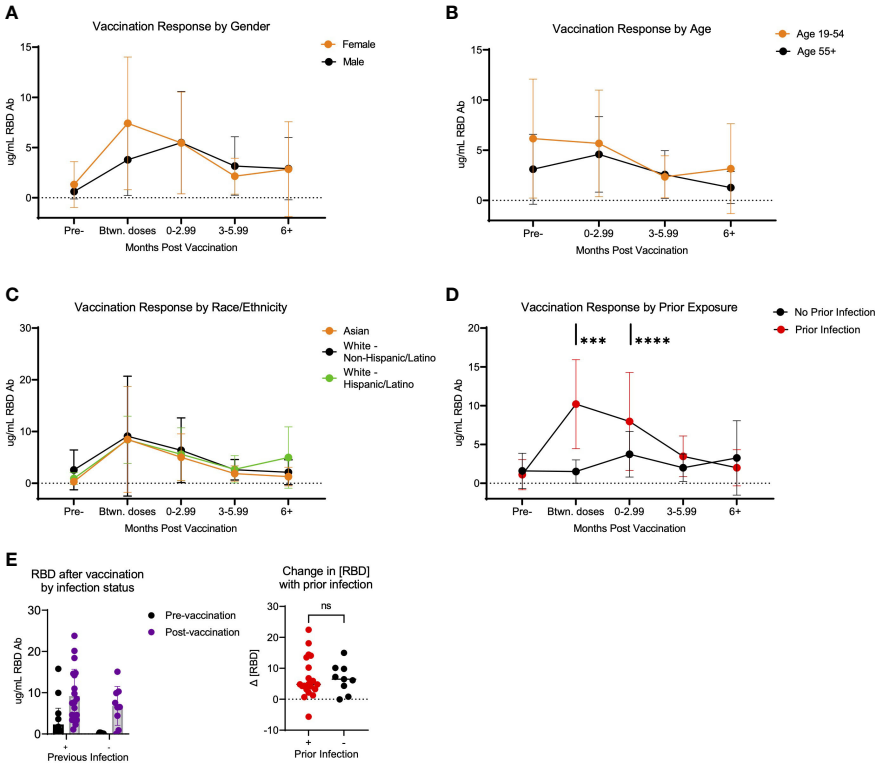

FIGURE 3
Response to SARS-CoV-2 vaccination in LA-SPARTA participants differs with prior infection. **(A)** shows the concentration of RBD over time according to age. Participants over 55 years of age were compared to all participants below 55 years of age. **(B)** shows the concentration of RBD over time according to gender. **(C)** shows the concentration of RBD over time according to race/ethnicity. **(D)** shows the concentration of RBD over time according to prior infection status before vaccination. **(E)** shows only the difference in RBD concentration in participants who were vaccinated over the course of the study, using the first available sample after vaccination (regardless of timing after vaccination) according to whether they had a prior infection at enrollment and prior to vaccination. The change in RBD concentration is also shown between the pre-infection and post-infection blood samples. *** designates P >0.001 and **** designates P >0.0001. ns, not significant.

copy number or magnitude of symptoms are associated with a decreased anti-NP seroconversion (33). Parallel studies have also noted that lower Ct values via SARS-CoV-2 PCR testing are associated with lower seroconversion for S or NP proteins (34).

Collectively, the present results suggest that participants with prior infection had a stronger and more rapid response to the SARS-CoV-2 vaccination as compared to infection-naïve vaccinees. However, this effect did not impact the longer-term RBD-specific antibody response and was not associated with relative risk of exposure based on occupation. Notably, when determining whether there was a significant change in the pre- to post- vaccination antibody concentration respective of infection status, we found no significant correlates over the course of the study. This result may be due to the smaller sample size in those who were vaccinated.

We found that the RBD-specific antibody response to SARS-CoV-2 vaccination was significantly decreased by 3 months post-vaccination regardless of occupation or exposure risk. The antibody-based threshold of protection is unknown and antibody alone is unlikely to afford full protection against SARS-CoV-2 infection or COVID-19 disease. It is reasonable to hypothesize that even though decreased, antibody response can still contribute to protection in vaccinated individuals. Conceivably, quality of antibody in neutralizing virus to prevent or mitigate pathogenesis may be as or perhaps more important than quantity. Supporting this concept are several lines of evidence. In a large cohort study (SIREN) in the United Kingdom, while protection against infection was 72-92% efficacy after ~2 months post-vaccination, protection remained at 22–69% efficacy at 6 months post-vaccination despite a significant drop in antibody titer (35). Additionally, a recent meta-analysis found that protection against infection decreased by 20-30% after 6 months, however, protection against severe COVID-19 disease decreased by only 10% at 6 months post-vaccination (36). However, neither reports showed any corresponding antibody concentrations that correlate with protection from infection. Lastly, patients suffering the greatest frequency of infection or most severe COVID-19 disease can have among the highest titers (4, 5). Thus, qualitative protection may supersede absolute quantity of antibody response.

Of the total population in the current study, 20.0% were infected over the course of follow up, according to both reported infections and serological detection of infected individuals. Of the infections that occurred during the study period, 75% were in vaccinated participants and 25% in unvaccinated participants. However, it is important to note that 65.5% of participants were vaccinated at enrollment and the vaccination frequency increased over the course of the study, such that 83.0% of participants were vaccinated by study completion. Taking this into account, 30 out of

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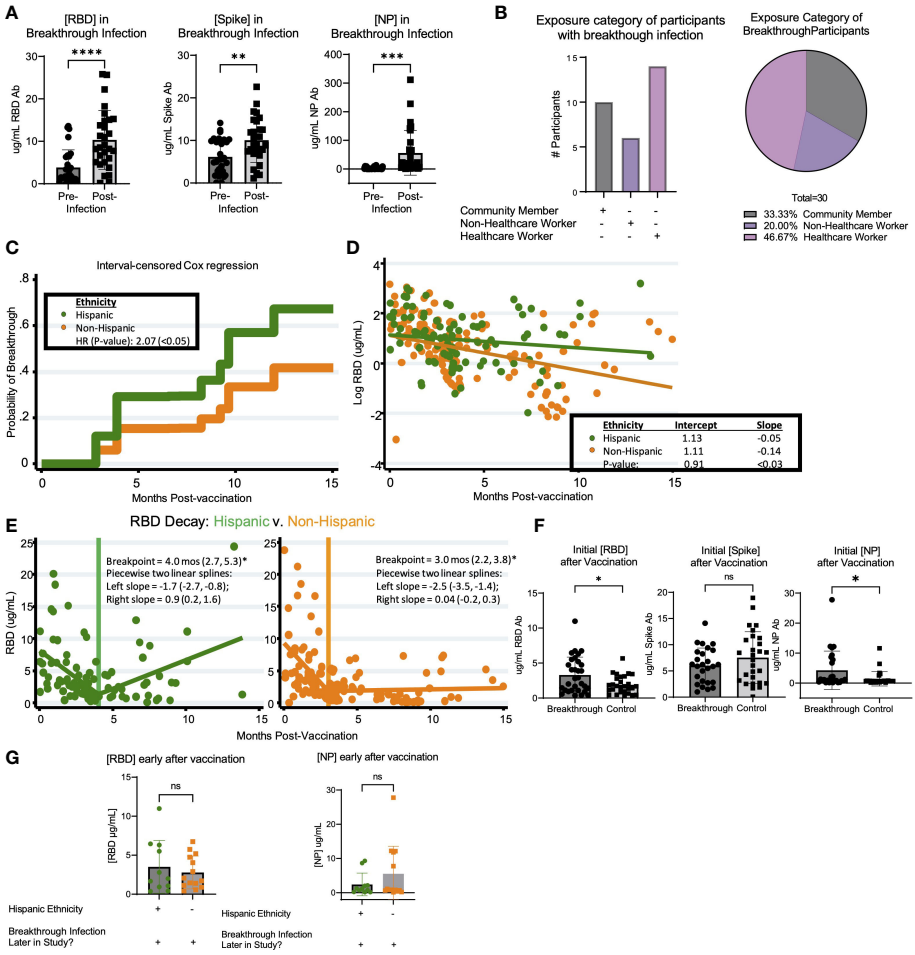

FIGURE 4
Breakthrough infection in vaccinated LA-SPARTA participants is more prevalent in Hispanic participants. **(A)** shows the change in RBD, Spike, and NP concentration between the reported and serologically detected breakthrough infections at the prior blood sample before the breakthrough infection was either reported or detected and after. **(B)** shows the distribution of risk category in the participants with breakthrough infections by number (left) and percentage (right). **(C)** An interval-censored regression model was used to compare the hazard ratios for breakthrough infection between Hispanic and Non-Hispanic participants. The Kaplan Meier curve is shown to represent this analysis. **(D)** shows the decay rate for antibodies against both RBD and Spike proteins on the log scale between Hispanic and non-Hispanic vaccinated participants. Panel **(E)** shows the piecewise linear regression model to find times at which the slopes of raw values change among Hispanic (left plot) and non-Hispanic participants (right plot). This analysis included 65 Hispanic participants, who contributed 95 samples and 93 non-Hispanic participants, who contributed 148 samples to the analysis. **(F)** shows the concentration of RBD, Spike, and NP after roughly 1-4 months after vaccination, in participants who had breakthrough infection later in the study and control participants who did not have breakthrough infection. **(G)** shows the concentration of RBD and NP after roughly 1-4 months after vaccination, in participants who had breakthrough infection later in the study according to ethnicity.* designates P >0.05, ** designates P >0.01, *** designates P >0.001, and **** designates P >0.0001. ns, not significant.

166 (18.0%) of vaccinated participants had breakthrough infection, whereas 10 of 29 (34.5%) unvaccinated participants were infected. Additionally, it is important to note that all infections that occurred during this study were mild and many may have even been asymptomatic based on the absence of reported participant symptom data on their surveys. Additionally, interpretation should be tempered by the fact that efficacy in terms of protection against infection and severe disease differs across studies.

Analysis of the cohort who had breakthrough infection during the study period despite vaccination revealed several interesting observations. First, healthcare workers who are at high risk for occupational exposure to SARS-CoV-2 did not appear to be at a higher risk for breakthrough infection in our cohort. Second, Hispanic participants exhibited twice the relative risk of breakthrough infection compared with non-Hispanic participants. Third, the antibody concentration early after vaccination did not appear to be predictive of breakthrough infection, nor did the concentration of antibodies prior to breakthrough infection. This may suggest other determinates, such as molecular (e.g. interferons) and cellular (e.g. CD8+ T cells) effectors or mucosal immunity may be integral to protective immunity. There may also be other factors such as how different aspects of the immune system interact to mediate protection against different SARS-CoV-2 variants. For example, mucosal IgA generated by WT or earlier SARS-CoV-2 variant infections may be more protective against later variants such as Omicron (37). Thus, additional correlates of protection against breakthrough infection should be studied. Additionally, it is possible that participants were infected with different SARS-CoV-

TABLE 4 Breakthrough and vaccinated control participant demographic comparison.

	Total (n=60)	Breakthrough (n=30)	Control (n=30)	p-value
Age (years), mean ± SD	41 ± 12	41 ± 12	41 ± 12	0.96
Gender, n (%)				1.00
Female	44 (73%)	22 (73%)	22 (73%)	
Male	16 (27%)	8 (28%)	8 (27%)	
Race, n (%)				0.28
Asian	18 (30%)	7 (23%)	11 (37%)	
Black/African American	3 (5%)	2 (7%)	1 (3%)	
White/Caucasian	35 (58%)	19 (63%)	16 (53%)	
Other	4 (7%)	2 (7%)	2 (7%)	
Ethnicity, n (%)				0.19
Hispanic/Latino	24 (40%)	15 (50%)	9 (30%)	
Non-Hispanic/Latino	36 (60%)	15 (50%)	21 (70%)	
BMI, mean ± SD	29 ± 7	30 ± 7	28 ± 8	0.33
Exposure, n (%)				0.65
Medical Center/Healthcare Worker	25 (42%)	14 (47%)	11 (37%)	
Medical Center/Non-Healthcare Worker	15 (25%)	6 (20%)	9 (30%)	
Community Member/Research Staff	20 (33%)	10 (33%)	10 (33%)	

T-test was used to compare continuous variables such as age and BMI. Fisher's Exact test was used for comparing categorical variables such as gender, race, and ethnicity. Data are represented as the number of participants (n) and the percentage of participants in the cohort or group (%), unless otherwise stated.

2 variants, against which the serological response to the RBD and S of the Wuhan-Hu-1 SARS-CoV-2 isolate may not be as protective.

A recent study showed that in a population of adults in Chicago, Hispanic participants were at higher risk for infection with SARS-CoV-2 than non-Hispanic participants (14). Comparatively, our results show that Hispanic participants may be at higher risk of SARS-CoV-2 breakthrough infection. Interestingly, 28 (52.8%) Hispanic and 25 (47.2%) Non-Hispanic participants were infected at enrollment. Recent reports show that COVID-19 has disproportionately affected racial and ethnic minorities in terms of infection, hospitalizations and deaths with Hispanics having worse outcomes than non-Hispanics (16, 38). While the causes of racial and ethnic COVID-19 disparities remain unclear, there may be many reasons for this observed effect including socioeconomic factors, multigenerational households, or immunogenetic differences. We did not collect data on these factors and our cohort size deters us from inferring any possible differences based on these factors. Thus, additional studies with a larger population size are warranted to investigate these differences in SARS-CoV-2 infection and breakthrough infection in this population.

The limitations of our study include the relatively small number of participants, the absence of a positive SARS-CoV-2 PCR test for all infected participants, and the range in time for sample collection after vaccination for vaccinated participants. Thus, our data regarding the difference in SARS-CoV-2 infection in Hispanic participants should be validated in a larger cohort of participants. In addition, we did not require that our participants provide proof of a positive SARS-CoV-2 PCR or antigen test when testing for SARS-CoV-2 infection. We did, however, screen the participants at enrollment for serological evidence of prior infection. Using the criteria in this study it is possible to have missed participants with a prior infection if they had been vaccinated, as the NP response has been shown to decrease faster than that of S (39). Thus, if a participant was vaccinated, we would only be able to base our determination of prior infection on the NP antibody concentration which may be below the positivity threshold, depending on when the infection took place. We were also unable to determine whether there were differences in the kinetic response to vaccination and infection in our cohort because of this missing information. Additionally, the absence of consistent post-vaccination timepoints for all participants likely increased variation in our study data. It is also important to note that we did not measure the antibody neutralizing capacity or cellular immune characteristics in the study cohort. We used the anti-RBD monoclonal antibody CR3022 to establish a standard curve to ascertain the IgG concentrations in participant serum as previously described (20–22). To control for the possibility of reduced sensitivity of the ELISA assay due to potential lower affinity of mAb CR3022, we validated mAb CR3022 to ensure its sensitivity and specificity using stringent washing conditions and a short 2-hour incubation time. Parallel tests were performed using a human IgG reference protein from plasma, which showed comparable results.

In conclusion, our study shows that in a cohort of high risk-individuals, occupational exposure to SARS-CoV-2 and vaccination response varied. However, this variation was not based on occupational exposure risk, as healthcare providers and non-providers exhibited equivalent outcomes. Further, not all infected participants developed a serological response to the NP protein of SARS-CoV-2. As such, a non-

trivial proportion of participants had serological evidence of infection without their knowledge based on reporting symptoms. The humoral response was greater and more stable in response to the full SARS-CoV-2 S protein as compared to the RBD, the latter being the target of existing vaccines. Demographically, Hispanic participants are at a higher risk of breakthrough infection than non-Hispanic participants among vaccinated individuals in this cohort of high-risk frontline workers at an urban medical center community in southern Los Angeles County. Finally, the antibody response to RBD was not predictive of breakthrough infection after vaccination or before infection.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board of the University of California, Los Angeles (IRB#20-001649). The patients/participants provided their written informed consent to participate in this study.

Author contributions

MJ performed the experiments, analyzed the data, and wrote the manuscript. HP contributed data analysis and validated the initial assay used in this study. YZ and DG significantly contributed to statistical analyses. DP and EF are clinical coordinators/phlebotomists who recruited all subjects and directed patient interactions, sample collections, and input the data into RedCap. DK designed the surveys and reviewed of all clinical data collection elements. TR provided necessary proteins for the ELISA assays and reviewed the manuscript. JS, LM, MY, and ER designed the study, significantly contributed to data interpretation and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1139915/full#supplementary-material

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Durability and extent of protection of SARS-CoV-2 antibodies among patients with COVID-19 in Metro Manila, Philippines

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Introduction: Information on the magnitude and durability of humoral immunity against COVID-19 among specific populations can guide policies on vaccination, return from isolation and physical distancing measures. The study determined the durability of SARS-CoV-2 antibodies after an initial infection among Filipinos in Metro Manila, Philippines, and the extent of protection SARS-CoV-2 antibodies confer against reinfection.

Methods: We conducted a cohort study to monitor the antibody levels of patients diagnosed with COVID-19. Receptor-binding domain (RBD)-specific antibodies were measured at Days 21, 90, 180, 270 and 360. Antibody levels were reported as geometric mean titers (GMT) with geometric standard deviation (GSD). Differences in GMT were tested using Friedman test and Kruskal Wallis test, with Bonferroni multiple comparisons procedure. Adjusted hazard ratios on the development of probable reinfection were estimated using Cox proportional models.

Results: There were 307 study participants included in the study, with 13 dropouts. Study participants received SARS-CoV-2 vaccines at varying times, with 278 participants (90.5%) fully vaccinated by the end of study. The GMT of the study cohort increased over time, from 19.7 U/mL (GSD 11) at Day 21; to 284.5 U/mL (GSD 9.6) at Day 90; 1,061 U/mL (GSD 5.3) at Day 180; 2,003 U/mL (GSD 6.7) at Day 270; and 8,403 U/mL (GSD 3.1) at Day 360. The increase was statistically significant from Day 21 to Day 90 ($p<0.0001$), Day 90 to Day 180 ($p=0.0005$), and Day 270 to Day 360 ($p<0.0001$). Participants with more severe initial infection

demonstrated significantly higher antibody levels compared to those with milder infection at Day 21. Sixty-four patients had probable COVID-19 reinfection (incidence of 20.8%, 95% CI 16.4, 25.8%). The GMT of these 64 patients was 411.8 U/mL (GSD 6.9) prior to the occurrence of the probable reinfection. Majority (87.5%) were fully vaccinated. Antibody titers significantly affected the risk of developing reinfection, with adjusted hazard ratio of 0.994, 95% CI 0.992-0.996, p<0.001.

Conclusion: Antibody levels against SARS-CoV-2 increased over a one-year follow-up. Higher antibody levels were observed among those with more severe initial infection and those vaccinated. Higher antibody levels are associated with a lower risk of probable reinfection.

KEYWORDS

antibody, humoral response, SARS-CoV-2, COVID-19, reinfection

1 Introduction

Coronavirus disease 2019 (COVID-19) is a global pandemic that has caused tremendous health and socioeconomic consequences in the Philippines. As of March 2023, the Philippines has recorded over 4.08 million cases and over 66,118 deaths due to COVID-19. The Philippines' poverty incidence rose to 23.7% in 2021, compared to 21.1% in 2018. Millions of Filipinos were unemployed, with the poor and marginalized sectors suffering most from the pandemic (1).

An important factor in controlling the spread of the infection and reinstating normal societal activities is determining what proportion of the population have developed antibodies (seroprevalence of the disease), and understanding whether the development of antibodies translates to immunity against subsequent infection in the long-term.

Several studies that monitored the long-term course of humoral immune response among those naturally infected with SARS-CoV-2 have shown a gradual decrease in receptor-binding and serum neutralizing antibody titers at varying time periods after infection (2–5).

A living review summarized the variation in antibody response to COVID-19 infection by age, sex, race, comorbidities and disease severity. Severe disease was associated with a more robust antibody response, with higher total antibody levels and neutralizing antibody capacity. Severe disease was also associated with a longer duration of detectable antibodies. Studies generally did not find a significant variation in antibody levels by age and sex. Evidence is unclear whether comorbidities are associated with antibody variation. In terms of variation of antibody levels by race or ethnicity, results suggest that non-Caucasians may exhibit higher antibody levels (3).

COVID-19 reinfection is well documented, and occurs when a person who has recovered from a previous SARS-CoV-2 infection becomes infected again. One study in the USA reported an incidence rate of 0.35 cases per 1,000 person-days among healthcare workers (6). Another study in India reported an incidence density of reinfection of 7.26 per 100 person-years (7).

The relationship between antibody levels and SARS-CoV-2 reinfection is a major area of clinical and public health interest. A case-control study done among unvaccinated individuals found that increasing anti-spike levels were associated with reduced risk of reinfection (odds ratio [OR] 0.63, 95% confidence interval [CI] 0.47 to 0.85). Using live virus microneutralization tests, titers >40 were associated with protection against reinfection. For pseudovirus microneutralization, titers>100 were associated with protection against reinfection (8).

In the Philippines, two studies evaluating COVID-19 seroprevalence have been published. One study determined seroprevalence (seropositivity defined as total SARS-CoV-2 immunoglobulin [Ig] ≥1 AU/mL) prior to the national vaccination program. The seroprevalence rates were 11.3% from May to July 2020, 46.8% from August to September 2020, 46% from December 2020 to January 2021, and 44.6% in March 2021 among residents in the city of Manila (9). Another study determined seroprevalence (seropositivity defined as receptor-binding domain [RBD]-Ig ≥0.8 U/mL) from June to December 2021, which coincided with the vaccine roll-out of the country in March 2021. The seroprevalence of the study population, which consisted of faculty, staff and students in a private tertiary university, ranged from 28.8% to 65.1%. The seropositive rate showed an increasing trend during the 7-month study period (10).

There are currently no studies among Filipinos that systematically monitors the quantitative antibody levels over a long-term period. Information on the timing, magnitude, and durability of humoral immunity among Filipinos is essential to guide the deployment of vaccine stocks, and can help guide strategies for returning from isolation and relaxing physical distancing measures. This study aimed to determine the durability of SARS-CoV-2 antibodies over a period of one year and the extent

of protection these antibodies confer against reinfection among patients diagnosed with COVID-19. Specifically, we aimed to describe the pattern of antibody levels according to severity of initial COVID-19 infection, determine the incidence of reinfection among previously diagnosed COVID-19 patients, and determine if SARS-CoV-2 antibodies are protective against future infection

2 Methods

2.1 Study design

We conducted a cohort study to monitor the antibody levels of patients diagnosed with COVID-19. We followed up these patients to determine if there was reinfection within the first year after initial infection.

2.2 Study setting

We identified potential study participants from various COVID-19 hospitals and quarantine facilities in Metro Manila. We also invited potential participants by means of posters disseminated in social media platforms. We conducted the study remotely from the University of the Philippines, Manila from March 6, 2021 to July 12, 2022.

2.3 Study population

Patients who met the following eligibility criteria were enrolled to the study: 1) adult (≥18 years old); 2) diagnosed with COVID-19 through reverse transcription polymerase chain reaction (RT-PCR), including patients with asymptomatic, mild, moderate, severe or critical disease; 3) within 21 days since onset of symptoms (if symptomatic) or since RT-PCR positivity (if asymptomatic); 4) owned a mobile phone; 5) permanent address within Metro Manila; and 6) able to provide informed consent.

Due to anticipated changes in the circulating antibody levels, participants who received or intended to receive convalescent plasma or intravenous immunoglobulin during the follow-up and monitoring period were excluded.

Participants who received COVID-19 vaccine prior to enrollment were excluded from the study. However, due to ethical reasons, study participants who subsequently received the vaccine were still included in the study follow-up and determination of antibody levels.

2.4 Study procedures

Participants were followed up for one year, counting from the first day that they showed symptoms of COVID-19 or the day of RT-PCR positivity for asymptomatic patients.

2.4.1 Remote coordination of study activities during the COVID-19 pandemic

The study researchers underwent training on Good Clinical Practice, study-specific consent process and documenting consent, and study-specific conduct of interviews of study participants prior to the start of study implementation. Study researchers operated from a virtual study hub, interacting with study participants through phone calls. The researchers performed eligibility screening, informed consent process, study data collection (at enrollment and follow-up), scheduling and coordination of study-related diagnostic tests, and tracking of patient location throughout the study. To minimize the risk of infection transmission, face-to-face interactions was limited to healthcare workers wearing the appropriate personal protective equipment and directly in charge of the clinical care of the study participants.

Third-party service providers were tapped to facilitate collection of specimens from the participants at the isolation center, at their respective residences or work place, or at barangay health centers, or at the nearest branch of the designated diagnostic laboratory, depending on the preference of the study participants. All collected blood specimens were transported to the accredited diagnostic laboratory who carried out the tests according to the manufacturer's recommendations.

Healthcare workers were involved in referring potentially eligible participants to the study staff. At the start of the study period, COVID-19 cases in Metro Manila were reaching 2,300 to 3,600 cases per day with variants P.1, P.3, B.1.1.7 and B.1.351 detected in the country (Figure 1) (11, 12). We anticipated that 20% of study participants would develop severe disease and require hospitalization. For this subset of patients, the study staff contacted the healthcare worker in the hospital to coordinate scheduled blood extractions and inquire about the results of diagnostic tests that were done as part of the study participant's clinical care.

2.4.2 Scheduled measurement of SARS-CoV-2 antibodies

We periodically measured the level of SARS-CoV-2 antibodies at Days 21, 90, 180, 270 and 360 (+/- 15 days) from onset of symptoms or date of RT-PCR positive test for asymptomatic patients, with allowable window period of +/-2 days for day 21 and +/- 15 days for the rest of the timepoints. This study used a laboratory-based semi-quantitative test, ECLIA (Elecsys® Anti-SARS-CoV-2 S assay) to measure antibody levels. It detects the RBD-specific total antibody levels (IgG, IgA, IgM). The test is described in detail in Appendix 1. The lower limit of detection of the laboratory test used is 0.40 U/mL, while the upper limit of detection is 250 U/mL. For study participants who had results<0.40 U/mL, the result was recorded as 0.39 U/mL in the database in order to facilitate mathematical computation and data analysis. For study participants with results >250 U/mL, 10-fold dilution was performed to increase the upper limit of detection to 2,500 U/mL (13). Further dilution was performed as necessary to increase the upper limit of detection up to 250,000 U/mL.

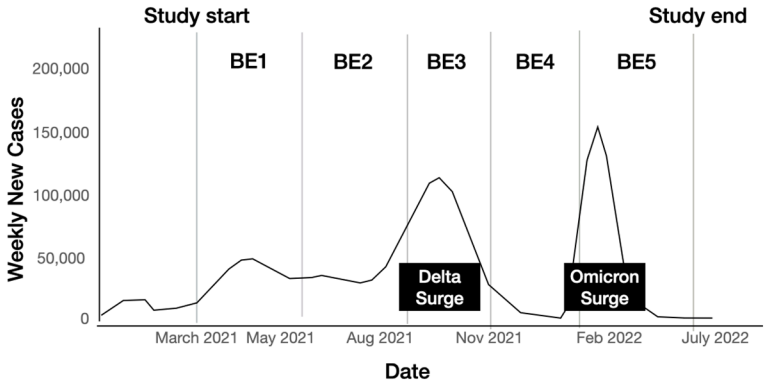

FIGURE 1
Epidemiological context in the Philippines and the timing of study implementation. (Image modified from the https://doh.gov.ph/covid19tracker)[12]. BE1 = first blood extraction at day 21, BE2 = second blood extraction at day 90, BE3 = third blood extraction at day 180, BE4 = fourth blood extraction at day 270, BE5 = fifth blood extraction at day 360.

2.4.3 Monitoring for COVID-19 reinfection

During the one-year follow-up period, remote monitoring of study participants was done every two weeks to inquire the development of symptoms consistent with COVID-19 and result of the RT-PCR test or SARS-CoV-2 antigen test, if done.

An adjudication committee composed of five clinical epidemiologists, three of whom were also infectious disease specialist and one immunology-allergy specialist was formed. The committee classified participants who developed any COVID-19-like symptoms as confirmed, probable, possible or unlikely to have COVID-19 reinfection (defined in Appendix 2) based on the following: demographic information, relevant medical history, date of RT-PCR test indicating COVID-19 infection prior to enrollment, antibody levels before and after symptoms occurred, symptoms, duration of symptoms, history of exposure, type of occupation, RT-PCR test results and cycle threshold values (if available), and vaccination status. These information were collected by the study researchers through phone call to the study participants. The committee members were blinded to the identity of the patients and majority vote was followed.

2.5 Study variables

The following variables were collected at baseline COVID-19 disease severity, age, sex, co-morbidities. On follow-up SARS-CoV-2 antibody levels, incident COVID-19 (based on self-report of symptoms and laboratory results such as SARS-CoV-2 RT-PCR or antigen test, if available), vaccination status were recorded.

2.6 Biologic specimens

At each blood extraction, 10 ml of whole blood was drawn and placed in non-citrated vials for serum separation. One 5-ml vial each was collected for the following 1) laboratory-based antibody test, and 2) biobanking.

Blood specimens were stored for future testing, particularly PRNT once it is available. Serum samples were aliquoted in cryotubes and stored at the University of the Philippines National Institutes of Health (UP-NIH) at -70 to -80 degrees Celsius. We obtained written informed consent from the study participants for the storage of their blood samples for future testing. The blood samples collected in this study will be stored at the UP-NIH for a maximum of 25 years, according to the institution's COVID-19 Samples Storage and Biobanking Policy.

2.7 Data collection and management

We used a secure data management software (Epidata) for study data collection. User access was restricted through user profiles designated according to user roles. Access to the system was given through individual accounts with password protection. A code assigned to each participant was used in the electronic questionnaires, which is only known to the researcher and the study staff. Electronic data was collated centrally and backed-up every day, at the end of the work day.

Data quality control was implemented by using both preventive and corrective actions. The electronic database, which captured the data electronically, was programmed with data quality rules that automatically perform calculations (e.g. age from birthdate), restrict allowable values to a specific range (e.g. a normal range of values for quantitative laboratory tests), use branching logic (e.g. *If yes* questions), and have mandatory items (i.e. empty response not allowed). At the end of the study, and before performing data analysis, frequency distribution of all variables was examined for out of range values and outliers. Data was also counterchecked from other data sources (e.g. medical records), as applicable. Furthermore, the electronic case forms of a random 10% of all the respondents underwent internal audit by an independent staff

member who did not perform data collection to check the accuracy and completeness of the data.

2.8 Data analysis

Study data were processed using MS Excel and analyzed using STATA 17 software. Demographic, laboratory, and clinical data were presented using descriptive statistics. Mean with standard deviation (or median and IQR) was used to describe quantitative data. For qualitative data, frequencies were used. Antibody levels were reported as geometric mean titers (GMT) with geometric standard deviation (GSD) at each period of observation, as these are the recommended measures of location and dispersion for antibody titers (14). Antibody GMTs with GSD were also reported according to initial COVID-19 severity classification and the vaccination status of the participants.

Friedman test was used to compare GMTs across the 5 timepoints. If significant differences was found, pairwise sign test was done at 5% level of significance with adjustments using Bonferroni method ($\alpha = 0.05$ divided by 10 pairwise comparisons). The adjusted alpha used was 0.005 and all p-values were compared with the adjusted alpha. Kruskal Wallis test was used to compare the GMTs based on severity classification. If significant difference was found, Dunn's test was done at Bonferroni adjusted level of significance of 0.005 for severity classification ($\alpha = 0.05$ divided by all 10 pairwise comparisons).

The incidence of reinfection was estimated at 95% confidence level. Unadjusted and adjusted hazard ratios for the effect of antibody levels on the development of probable reinfection were estimated using Cox proportional hazards model. Antibody level was treated as a continuous variable. The antibody level prior to the reinfection was used for those with probable reinfection. For those without probable reinfection, their GMT across the 5 timepoints were used. Hazard ratios were adjusted for possible confounders including age, sex, co-morbidities, and vaccination status.

2.9 Sample size computation

Liu et al. (15) reported a standard deviation of 246 IgG RU/ml for patients with COVID-19 infection on day 14. Using this standard deviation, 244 participants are needed to estimate the mean IgG titer at 99% level and 80% probability of achieving a target width of 88 RU/ml. The level of confidence was adjusted for multiple comparisons by the Bonferroni method since the mean titer will be estimated at 5 periods of observations (alpha=0.05/5 = 0.01).

Taking into consideration a possible dropout rate of 20%, this study targeted to recruit a total of 307 participants. Dropout is defined as a situation where all outcome data of the participant are missing after a certain timepoint. This includes mortality, withdrawal of consent, and loss to follow-up.

2.10 Ethical considerations

This study was conducted following the principles outlined in the Declaration of Helsinki, the WHO International Ethical Guidelines for Health-related Research Involving Humans, and the Philippines' National Ethical Guidelines for Health and Health-Related Research. This research was reviewed and approved by the UP Manila Research Ethics Board. The study protocol was submitted to the UP Manila Institutional Biosafety and Biosecurity Committee for review and clearance. Ethics Review Board (ERB) approval was secured before the start of the study (UPMREB 2020-698-01).

3 Results

3.1 Study participants

From March 6 2021 to June 15 2021, a total of 536 participants were screened. Potential participants came from quarantine facilities (QF) and COVID-19 centers in Metro Manila. We also posted an infographic describing the objectives of the study and inclusion criteria in different social media platforms (i.e. Facebook, Instagram, Twitter and group chats) which included contact details of the research staff for anyone interested to participate. Of the 536 participants screened, 229 were excluded. The reasons for exclusion included no permanent address in Metro Manila (n=53), onset of symptoms beyond 21 days (n=21), receipt of SARS-CoV-2 vaccine or convalescent plasma (n=16), no mobile phone (n=7), no consent to participate (n=124), inability to have the first blood extraction done due to logistical difficulties (n=5) and mortality prior to the first blood extraction (n=3).

Of the 307 participants enrolled, 123 (40.1%) came from QFs and COVID-19 centers, while 184 (59.9%) were identified through social media. The participants were followed up for one year, with the end of follow-up period on July 12, 2022. Over the course of the study, there were four participants who died. Two participants died due to acute respiratory failure after the day 21 blood extraction. One participant developed respiratory failure from hospital-acquired pneumonia and the other one developed respiratory failure due to a mixed connective tissue disease that was diagnosed in 2006. At the time of demise, the SARS-CoV-2 RT-PCR test in the 2 participants taken 30 days and 39 days from the initial positive RT-PCR test, respectively were negative. Two participants died after the fourth (day 270) blood extraction timepoint. One participant had chronic kidney disease stage 5 secondary to chronic glomerulonephritis, requiring maintenance hemodialysis. She was reported to have missed dialysis sessions due to vascular access malfunction. At the time of demise, she had sudden onset of difficulty of breathing, for which she was brought to a hospital where sudden cardiac death was declared as her primary cause of death. Her last blood extraction was three months prior to her demise, with results of 6,527 U/mL. The other participant had no known co-morbid conditions but reportedly had cardiomegaly detected by chest radiograph 6.5 months after enrollment with no

further work-up done. He developed a probable COVID-19 re-infection 7 months after enrollment, 4 months prior to his demise. The participant presented with mild symptoms and recovered after completion of home isolation. His last blood extraction was 2.5 months prior to his demise, with SARS-CoV-2 antibody levels of 6,545 U/mL. He was found dead at home with an unknown cause of death.

There were 9 patients who withdrew from the study. The reasons for withdrawal included refusal to have additional blood extractions done (4 participants), inability to contact the study participants (2 participants), maritime employment (2 participants) and difficulty in scheduling blood extractions due to work (1 participant).

The study participants' flow diagram is shown in Figure 2. All 307 study participants underwent the first blood extraction. There were only 301 study participants who underwent the second blood extraction because 2 participants died, 3 participants withdrew consent, and 1 participant was unable to have the blood extraction done due to logistical difficulties. This one participant who had logistical difficulties for the second blood extraction was still considered to be enrolled in the study with a missing data point for the second blood extraction. Of the 302 study participants in the study cohort after the second blood extraction, 297 underwent the third blood extraction. There were 3 additional participants who withdrew consent, and 2 participants who were unable to have the third blood extraction done due to logistical difficulties. Of the 299 study participants in the study cohort after the third blood extraction, 293 underwent the fourth blood extraction. There were 2 additional participants who withdrew consent, and 2 participants who were unable to have the third blood extraction done due to logistical difficulties, and 2 participants who opted to defer the fourth blood extraction due to medical reasons. One had anemia, while the other participant was on blood thinners and had multiple hematomas. Of the 297 study participants in the study cohort after the fourth blood extraction, 289 underwent the fifth blood extraction. There were 2 participants who died, 1 participant who withdrew consent, and 5 participants who were unable to have the fifth blood extraction done due to logistical difficulties.

The baseline characteristics of the study participants are shown in Table 1. The median age was 36 years old (interquartile range [IQR] 19), with slightly more females (53.4%). Majority of the participants were classified to have mild COVID-19 infection (55.4%).

Of the 307 enrolled participants, 117 (38.1%) had co-morbidities. The most common co-morbidities were hypertension (20.5%), pulmonary diseases (12.4%) and diabetes mellitus (9.8%). Among the respiratory diseases, asthma was the most common (n = 27). Other respiratory diseases reported were interstitial lung

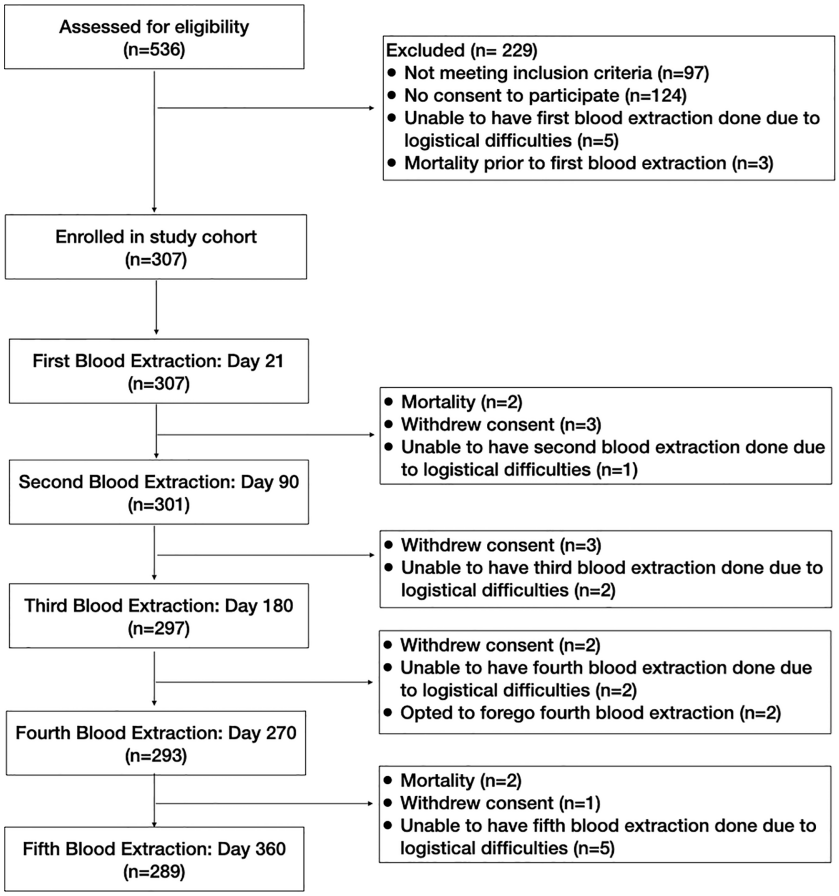

FIGURE 2
Flowchart of screening, enrollment and monitoring procedures.

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TABLE 1 Baseline demographic and clinical characteristics of the study participants, Metro Manila, Philippines (N = 307).

Variable	Frequency (%) N = 307
Age	Median 36 years (IQR 19)
19 to 59 years old	279 (90.9)
60 and above	28 (9.1)
Sex	
Male	143 (46.6)
Female	164 (53.4)
Severity	
Asymptomatic patients	78 (25.4)
Mild disease	170 (55.4)
Moderate disease	24 (7.8)
Severe disease	28 (9.1)
Critical Disease	7 (2.3)
On immunosuppressants	3 (1.0)
Co-morbidities	
Hypertension	63 (20.5)
Pulmonary diseases	38 (12.4)
Diabetes	30 (9.8)
Gastrointestinal disorders	17 (5.5)
Neurologic disorders	16 (5.2)
Dyslipidemia	7 (2.3)
Rheumatologic diseases	7 (2.3)
Oncologic disorders	5 (1.6)
Chronic kidney disease	4 (1.3)
Cardiac diseases	4 (1.3)
Endocrine diseases	4 (1.3)
Psychiatric diseases	3 (1.0)
Hematologic diseases	2 (0.7)

disease, chronic obstructive pulmonary disease and tuberculosis. Other comorbidities were gastrointestinal disorders (e.g., cholelithiasis, ulcer, gastroesophageal reflux disease, fatty liver, hepatitis, Crohn's disease) and neurologic conditions such as cerebrovascular accident, migraine, vertigo and Parkinson's disease, pituitary macroadenoma and history of encephalitis. Chronic cardiac conditions identified were arrhythmia, mitral valve prolapse, and coronary artery disease. Some participants also had chronic kidney conditions (e.g., polycystic kidney, nephrolithiasis), five of whom were undergoing hemodialysis. Cases of neoplastic diseases in the cohort included breast, colorectal, prostate, nasopharyngeal cancer and chronic myelogenous leukemia.

There were no cases of HIV infection among the study participants. Three participants were on immunosuppressants for autoimmune conditions including primary macroadenoma, Sjogren's disease and systemic lupus erythematosus.

3.2 Antibody levels of study participants

The total RBD-specific immunoglobulin levels of the entire study cohort for each of the five blood extraction timepoints are shown in Figure 3. The GMT of the study cohort increased over time. At day 21, the GMT was 19.7 U/mL, with GSD 11 (n=307). At day 90, the GMT significantly increased to 284.5 U/mL (GSD 9.6; n=301), p=<0.0001. At day 180, the GMT was 1,061 U/mL (GSD 5.3, n=297). The increase from day 90 to day 180 was statistically significant (p=0.0005). At day 270, the GMT was 2,003 U/mL (GSD 6.7; n=293), although this increase from day 180 was not statistically significant (p=0.098). At day 360, the GMT significantly increased to 8,403 U/mL (GSD 3.1; n=289) compared to the day 270 GMT (p=<0.0001).

However, it should be noted that at Day 21, 22 study participants had antibody titers below the lower limit of detection (<0.40 U/mL). To facilitate data analysis, these were encoded as 0.39 U/mL. Hence the GMT of 19.7 U/mL at Day 21 is likely an overestimate of the actual value. At Day 90, only 7 study participants had antibody titers<0.40 U/mL. Similarly, the GMT of 284.5 U/mL at Day 90 is likely an overestimate of the actual value. There were no study participants with titers<0.40 U/mL on Days 90 to 360.

3.2.1 SARS COV-2 antibody levels according to initial COVID-19 severity classification

Figure 4 shows the antibody GMT according to initial COVID-19 disease severity classification. It can be observed that regardless of baseline severity classification, the antibody GMTs generally showed an increasing trend from Day 21 until Day 360.

At day 21, study participants with critical disease severity had significantly higher GMTs compared to those with mild disease (p=<0.0001) and asymptomatic disease (p=0.002). Those with severe disease and moderate disease also had higher antibody GMTs compared to those with mild disease (p=<0.001 for severe, p=0.0001 for moderate), and asymptomatic disease (but not statistically significant at p=0.008 for severe vs asymptomatic, p=0.019 for moderate vs asymptomatic). Similarly, although those with critical disease had higher antibody GMTs compared to those with moderate and severe disease, the difference did not reach statistical significance (p=0.072 for critical vs moderate, p=0.088 for critical vs severe).

The antibody GMTs of participants with severe and critical infection remained higher compared to those with asymptomatic, mild, and moderate infection on Day 90 and D180, but the differences were not statistically significant. Similarly, those with critical infection had higher antibody GMTs compared to the rest of the severity groups on Day 270, but the difference was not statistically significant.

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SARS-CoV-2 Antibodies Through Time

Day	D21	D90	D180	D270	D360
Number	307	301	297	293	289
GMT (U/mL)	19.7	284.5	1061	2003	8403
GSD	11	9.6	5.3	6.7	3.1
SARS-CoV-2 Vaccinated (%)	0	19.3	86.2	95.2	96.2
Reinfected (%)	0	0.3	2.0	7.8	11.8

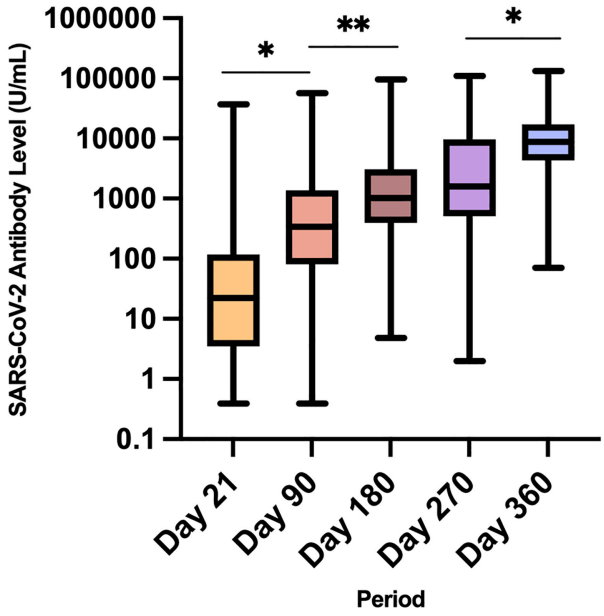

FIGURE 3
Geometric mean titers of SARS-CoV-2 antibodies (U/mL) of all study participants across the 5 timepoints *p=<0.0001, **p=0.0005.

The highest antibody GMTs were observed on Day 360 across all the severity groups. Those with more severe infection had higher GMTs compared to those with milder severity classification.

Among the 22 participants with titers<0.40 U/mL at Day 21, 11 had asymptomatic infection, 9 had mild infection, 1 had moderate infection, and 1 had severe infection. Of the 7 participants with titers<0.40 U/mL at Day 90, 3 had asymptomatic infection and 4 had mild infection.

3.2.2 Subgroup analysis of antibody levels by vaccination status at the end of the study

The study participants received the SARS-CoV-2 vaccine at varying times. At the time of the day 90 blood extraction, 117 participants (38.1% of the entire study cohort) were partially vaccinated and 60 (19.5%) were fully vaccinated and by the end of the follow-up period (Day 360), 278 (90.5%) had been fully vaccinated, with 66 (21.5%) completing only the primary series, 209 (68%) receiving 1 booster dose, and 3 (1%) receiving 2 booster doses.

Figure 5 shows the antibody GMT according to vaccination status at the end of the study. Regardless of baseline severity classification, the antibody GMTs generally showed an increasing trend from Day 21 until Day 360.

From Day 180 to 360, as more study participants received COVID-19 vaccine, the antibody GMTs of the fully vaccinated group and the booster group increased at a greater magnitude compared to those in the unvaccinated group. By the end of the study, when the majority of the participants had already received booster doses, the antibody GMT of the booster group was higher than the group that received only the primary series.

3.3 Reinfection in the study cohort

There were a total of 303 reports of COVID-19-like symptoms during the one year follow-up. Some participants had more than 1 report of COVID-19-like symptoms within the follow-up period. Of

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	D21 (N=307)			D90 (N=301)			D180 (N=297)			D270 (N=293)			D360 (N=289)		
	n	GMT	GSD	n	GMT	GSD	n	GMT	GSD	n	GMT	GSD	n	GMT	GSD
Asymptomatic	78	26	18	76	388	14	75	1006	4.7	74	2644	6.8	72	5293	3.0
Mild	170	11	7.7	170	219	9.5	166	983	5.5	163	1969	6.8	162	8093	2.8
Moderate	24	63	9.6	24	312	5.6	24	707	5.8	24	1118	7.6	24	11937	3.6
Severe	26	66	6.1	26	524	4.9	26	2016	4.9	26	1547	5.4	26	17383	3.5
Critical	7	263	2.7	5	507	2.0	6	5476	5.4	6	3334	5.9	6	17199	4.8

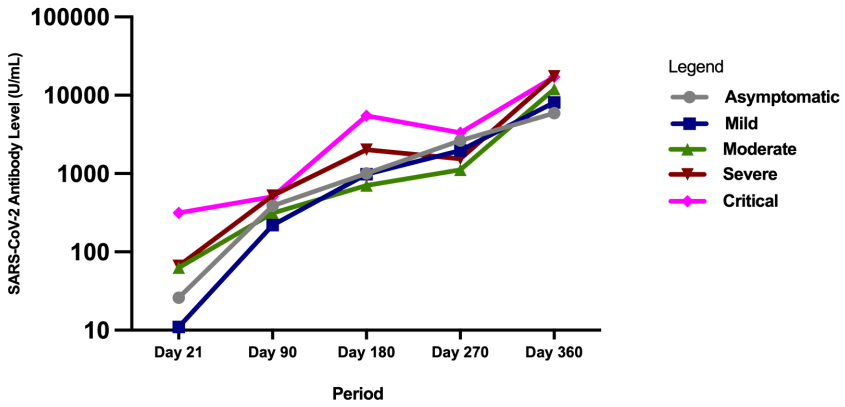

FIGURE 4
Geometric mean titers of SARS-CoV-2 antibodies (U/mL) according to initial COVID-19 disease severity classification.

the 303 reports, 64 (21.1%) were adjudicated to have had probable COVID-19 reinfection, 101 with possible reinfection (33.3%), and 138 were unlikely to have had reinfection (45.5%). There were no confirmed COVID-19 reinfection because genomic testing could not be performed in the respiratory specimens taken from each infection episode. The incidence of probable COVID-19 reinfection in this cohort was 20.8% (95% CI 16.4 to 25.8%).

3.3.1 Probable reinfection

There were 64 cases of probable reinfection occurring in 64 study participants. Six (9.4%) occurred during the Delta variant surge from August to October 2021 while 39 (60.9%) occurred during the Omicron variant surge from January to February 2022. The remaining 19 (29.7%) did not coincide with the observed surges in the Philippines.

	n	D21 GMT (U/mL)	D21 GSD	D90 GMT (U/mL)	D90 GSD	D180 GMT (U/mL)	D180 GSD	D270 GMT (U/mL)	D270 GSD	D360 GMT (U/mL)	D360 GSD
Unvaccinated	19*	122.6	16.0	191.3	12.8	432.2	4.1	909.6	5.7	4499.6	9.9
Fully vaccinated	76**	28.3	12.4	183.3	7.6	1420.7	6.8	2734.8	7.0	6423.2	3.6
Fully vaccinated with booster	212***	14.7	9.3	342.3	10.1	1010.1	4.8	1893.1	6.6	9437.2	2.6

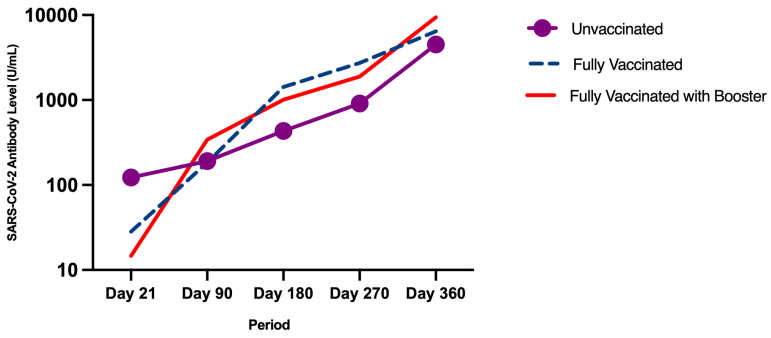

FIGURE 5
Geometric mean titers of SARS-CoV-2 antibody levels (U/mL) according to vaccination status at the end of the study. *Day 90 - 5 dropouts (n=14); Day 180 - 1 dropout and 1 missed blood extraction (n=12); Day 270 - 1 missed blood extraction (n=12); Day 360 - 1 dropout and 1 missed blood extraction (n=11). **Day 90 - 13 partially vaccinated and 13 fully vaccinated; Day 180 - 13 partially vaccinated and 54 fully vaccinated, 2 dropouts and 1 missed blood extraction (n=73); Day 270 – 68 fully vaccinated, 2 dropouts and 3 missed blood extractions (n=69); Day 360 – 66 fully vaccinated, 2 dropouts and 4 missed blood extractions (n=66). ***Day 90 – 104 partially vaccinated and 47 fully vaccinated, 1 missed blood extraction (n=211); Day 180 – 9 partially vaccinated, 202 fully vaccinated (n=212); Day 270 – 1 partially vaccinated, 155 fully vaccinated, 56 with booster; Day 360 – 212 with booster.

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The characteristics of the study participants with probable reinfection are shown in Table 2A. More than half (36 participants, 56.3%) of the probable cases of reinfection occurred among participants who previously had a mild COVID-19 infection. None of the participants who had previous critical infection had a probable reinfection. The most common co-morbidities of those who experienced probable reinfection were hypertension (23.4%) and diabetes mellitus (9.4%).

The antibody GMT of participants with probable reinfection at the first blood extraction timepoint (day 21) was 16.8 U/mL (GSD 9.8, range<0.40 to 1,269). Prior to reinfection, the antibody GMT of the 64 participants with probable reinfection was 422.2 U/mL (GSD 6.3, range 1.98 to 34,570), with an average time interval from the

TABLE 2 Clinical and laboratory characteristics of the study participants with probable, possible and unlikely COVID-19 reinfection, Metro Manila, Philippines (N=262).

Variable	A. Probable reinfection n=64 Frequency (%)	B. Possible reinfection n=88 Frequency (%)	C. Unlikely reinfection n=110 Frequency (%)
Age, *years* [a]	34 (IQR 16)	36 (IQR 14.5)	38 (IQR 16.8)
Sex			
Male	30 (46.9)	42 (47.7)	44 (40%)
Female	34 (53.1)	46 (52.3)	66 (60%)
Initial Severity Classification			
Asymptomatic patients	14 (21.8)	21 (23.9)	25 (22.7)
Mild disease	36 (56.3)	50 (56.8)	57 (51.8)
Moderate disease	9 (14.1)	8 (9.1)	10 (9.1)
Severe disease	5 (7.8)	8 (9.1)	15 (13.6)
Critical Disease	0	1 (1.1)	3 (2.7)
Co-morbidities			
Hypertension	15 (23.4)	13 (14.8)	24 (21.8)
Diabetes mellitus	6 (9.4)	4 (4.5)	6 (5.5)
Asthma	4 (6.3)	9 (10.2)	13 (11.8)
Cardiac conditions	3 (4.7)	1 (1.1)	1 (0.9)
Oncologic conditions	2 (3.2)	2 (2.3)	1 (0.9)
Gastrointestinal esophageal reflux disease	1 (1.6)	3 (3.4)	1 (0.9)
Allergic rhinitis	1 (1.6)	0 (0)	1 (0.9)
Chronic kidney disease	1 (1.6)	3 (3.4)	3 (2.7)
SARS-CoV-2 antibody level (U/mL)[b]			
Day 21	16.8 (9.8)	15.6 (12.6)	21.6 (11.4)
Prior to reinfection	411.8 (6.9)	501.0 (8.9)	197.8 (18.9)
Vaccination status[c]			
Unvaccinated	7 (10.93)	14 (13.9)	49 (35.5)
Partially vaccinated	1 (1.6)	6 (5.9)	14 (10.2)
Fully vaccinated (without booster)[d]			
Sinovac	32 (50)	40 (39.6)	40 (29.0)
Astra-Zeneca	7 (10.9)	4 (4.0)	4 (2.9)
Sputnik	4 (6.3)	0	7 (5.1)
Pfizer	4 (6.3)	4 (4.0)	15 (10.9)

(Continued)

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TABLE 2 Continued

Variable	A. Probable reinfection n=64 Frequency (%)	B. Possible reinfection n=88 Frequency (%)	C. Unlikely reinfection n=110 Frequency (%)
Moderna	4 (6.3)	12 (11.9)	2 (1.4)
J&J	0	1 (0.9)	2 (1.4)
Fully vaccinated with Booster	5 (9.3)	20 (19.8)	5 (3.6)
History of exposure to COVID-19[e]			
Symptomatic close contact	33 (51.5)	20 (19.8)	6 (4.3)
High-risk employment[f]	6 (9.4)	5 (5.0)	4 (2.9)
None	25 (39.1)	76 (75.2)	128 (92.8)

[a]Median, interquartile range (IQR).
[b]Geometric mean titer (GMT), geometric standard deviation (GSD); N=101 cases for possible reinfection, N=138 cases for unlikely reinfection.
[c]At the time of report of COVID-19 symptoms, N=101 cases for possible reinfection, N=138 cases for unlikely reinfection.
[d]Sinovac - inactivated vaccine; Astra-Zeneca, Sputnik, Johnson & Johnson - adenovirus vector vaccine; Pfizer, Moderna - mRNA vaccines.
[e]N=101 cases for possible reinfection, N=138 for unlikely reinfection.
[f]High risk employment refer to frontline workers including healthcare workers and protective service workers (e.g. police).

date of antibody determination to the onset of symptoms of 53.8 days (SD 49.8).

Of the 64 probable reinfection cases, 56 were fully vaccinated, one was partially vaccinated and 7 were unvaccinated at the time of re-infection. Of the 56 fully vaccinated who developed probable reinfection, there were 5 who developed probable reinfection after receiving 1 booster dose. The most common vaccine received was an inactivated SARS-Co-V-2 vaccine, Sinovac (36 participants or 56.3%, of which 32 participants completed the primary series of two doses, while 4 participants received one additional mRNA booster vaccine dose).

A little more than half of the participants with probable reinfection had a history of exposure to a symptomatic close contact (51.5%). Thirty (46.9%) were diagnosed through a positive RT-PCR test, 14 (21.9%) were diagnosed through a positive SARS-CoV-2 antigen test, and 20 (31.2%) were diagnosed based on a spike in their SARS-CoV-2 antibody levels not otherwise explained by vaccination.

TABLE 3 Timing and outcomes of the patients with probable reinfection, Metro Manila, Philippines (n-64).

Variables	Probable reinfection n=64 Frequency (%)	Antibody GMT U/mL (GSD)[a]
Timing of reinfection (from initial		
COVID-19 infection)<3 months	1 (1.6)	0.61
3 to<6 months	6 (9.4)	4.1 (9.7)
6 to<9 months	23 (35.9)	15.9 (6.8)
9 to 12 months	34 (53.1)	24.5 (11.3)
Severity classification of reinfection		
Asymptomatic	0	–
Mild	47 (73.4)	470.8 (9.0)
Moderate	16 (25)	638.0 (2.5)
Severe	1 (1.6)	269.8
Critical	0	–
Outcomes		
Recovered after home isolation	63 (98.4)	422.2 (6.3)
Hospitalized	1 (1.6)	269.8
Mortality	0	–

[a]Geometric mean titer (GMT), geometric standard deviation (GSD); Antibody levels obtained at Day 21 for timing of reinfection; Antibody levels obtained prior to reinfection report for severity classification and outcomes.

The timing of reinfection and outcomes are shown in Table 3. There was only 1 study participant who had a probable reinfection less than 3 months from the initial COVID-19 infection. This participant reported complete resolution of symptoms after the initial mild infection, but there was no documentation of a negative RT-PCR test. The antibody titer at Day 21 was 0.61 U/mL. This study participant presented with symptoms of cough, fever, fatigue, sore throat, and nasal congestion and had a positive repeat RT-PCR test taken 82 days after the initial infection. This participant was unvaccinated at the time of the probable reinfection.

There were 6 participants (9.4%) who developed reinfection 3 to 6 months from the initial infection, of which 2 were unvaccinated, 1 was partially vaccinated, and 3 were fully vaccinated. The antibody GMT of the unvaccinated participants prior to infection was 58 U/mL (GSD 4.2) while the vaccinated participants had a GMT of 103.7 U/mL (GSD 4.1). There were 23 participants (35.9%) who developed probable reinfection 6 to 9 months from the initial infection, all of whom were fully vaccinated. Their antibody GMT prior to infection was 603.6 U/mL (GSD 6.1). Majority of the participants (34 participants or 53.1%) developed probable reinfection 9 to 12 months after the initial infection. Of the 34 participants, 4 were unvaccinated, 25 were fully vaccinated, and 5 had 1 booster dose. The antibody GMT of the unvaccinated participants prior to infection was 416.6 U/mL (GSD 2.1) while the vaccinated participants had a GMT of 522 U/mL (GSD 5.8). The average time to reinfection from initial infection among the 64 participants was 253 days (SD 56).

Majority (73.4%) had mild disease on reinfection. There were 16 participants (25%) with moderate disease and 1 participant (1.6%) with severe disease. The participant who developed severe disease upon reinfection needed hospitalization and oxygen support through nasal cannula. This participant was a 31-year-old female with diabetes mellitus and heart failure. This participant was fully vaccinated with Sinovac 3.5 months prior to reinfection, with no booster dose received. She was treated with remdesivir, dexamethasone, and enoxaparin, with improvement of symptoms and was discharged well after 11 days. Her antibody titers at the first extraction timepoint (Day 21) was 38.3 U/mL, while her antibody titers 45 days prior to the probable reinfection was 269.8 U/mL. In contrast, those with mild reinfection had antibody GMT of 470.8 U/mL (GSD 9.0) extracted on the average 58 days prior to the development of reinfection. Those with moderate reinfection had antibody GMT of 638.0 U/mL (GSD 2.5), extracted on the average 40 days prior to the development of reinfection. Majority of the participants (98.4%) with probable reinfection recovered after home isolation.

3.3.2 Possible reinfection

There were 101 reports of COVID-19 like symptoms adjudicated as possible reinfection occurring in 88 study participants. There were 11 participants with 2 possible reinfection, and 1 participant with 3 possible reinfections over the one-year study period. Of the 101 cases, 15 (14.9%) occurred during the Delta variant surge from August to October 2021 while 53 (52.5%) occurred during the Omicron variant surge from January to February 2022. The remaining 33 (32.7%) did not coincide with the observed surges in the Philippines.

The characteristics of the study participants with possible reinfection are shown in Table 2B. Similar to the patients with probable reinfection, most (56.8%) of the possible cases of reinfection occurred among participants who previously had mild infection. The most common co-morbidities of those who experienced possible reinfection were hypertension (14.8%) and asthma (10.2%).

The antibody GMT of those with possible reinfection at the first blood extraction timepoint (day 21) was 15.6 U/mL (GSD 12.6). Of the 101 reports, 14 were unvaccinated at the time of possible reinfection, 6 were partially vaccinated, 61 were fully vaccinated, and 20 had 1 booster dose. Prior to reinfection, the antibody GMT of the unvaccinated participants was 141.5 U/mL (GSD 10.1), while the GMT of the vaccinated participants was 594.3 U/mL (GSD 8.1). The average time interval from the date of antibody determination to the onset of symptoms of 42.5 days (SD 31.3).

The most common vaccine received was an inactivated COVID-19 vaccine (Sinovac) in 54 cases or 53.5%, of which 40 completed the primary series while 14 received one booster vaccine dose. Of the 20 participants who received a booster dose, 8 participants received Pfizer mRNA vaccine, 7 participants received Moderna mRNA vaccine, 4 participants received an adenoviral vector vaccine (Astra-Zeneca), and 1 participant received an inactivated vaccine (Sinovac).Only 20 cases of possible reinfection (19.8%) had a history of exposure to a symptomatic close contact.

3.3.3 Unlikely reinfection

There were 138 reports of COVID-19-like symptoms adjudicated as unlikely reinfection occurring in 110 study participants. There were 23 participants with 2 COVID-19-like events, 1 participant with 3 events, and 1 participant with 4 events, subsequently adjudicated as unlikely reinfection. Of the 138 reports, 39 (28.3%) occurred during the Delta variant surge from August to October 2021 while 6 (4.3%) occurred during the Omicron variant surge from January to February 2022. The remaining 93 (67.4%) did not coincide with the observed surges in the Philippines.

The characteristics of the study participants with unlikely reinfection are shown in Table 2C. Similar to the probable reinfection, most (51.8%) of the cases occurred among participants with previous mild infection. The most common co-morbidities were hypertension (21.8%) and asthma (11.8%).

The antibody GMT of those adjudicated as unlikely reinfection at the first blood extraction timepoint (day 21) was 15.6 U/mL (GSD 12.6). Prior to reinfection, the antibody GMT was 197.8 U/mL (GSD 18.9), with an average time interval from the date of antibody determination to the onset of symptoms of 36.9 days (SD 41.1).

Of the 138 reports, 49 were unvaccinated, 14 were partially vaccinated, 70 were fully vaccinated, and 5 received 1 booster dose at the time of the report. The most common vaccine received was an inactivated vaccine (Sinovac) (in 43 cases or 31.2%, of which 40 completed the primary series while 3 received one heterologous

booster vaccine dose). There were 49 cases (35.5%) who were unvaccinated, and 14 (10.2%) were partially vaccinated. Of the 5 participants who received a booster dose, 4 participants received mRNA Moderna booster and 1 participant received mRNA Pfizer booster. Only 6 cases adjudicated as unlikely reinfection (4.3%) had a history of exposure to a symptomatic close contact.

3.4 Association of SARS-CoV-2 antibody levels and development of reinfection

To determine the association of antibody titers on the development of probable reinfection, the hazards ratio was estimated using Cox proportional hazards model, with antibody titers taken as a continuous variable. The unadjusted hazard ratio (HR) was 0.994, 95% CI 0.992 to 0.996, p<0.001. Adjusting for age, sex, co-morbidities, use of immunosuppressants and vaccination status, the adjusted HR was similar at 0.994, 95% CI 0.992 to 0.996, p<0.001 (Table 4). In effect, for one unit increase in antibody titer, the risk of symptomatic reinfection decreased by 0.6%.For every 10 units increase in antibody titer, the risk of symptomatic reinfection decreased by 6%.

4 Discussion

4.1 Key findings

The total RBD-specific immunoglobulin levels in this study cohort increased over the one-year follow-up after natural SARS-CoV-2 infection. However, these results must be interpreted with caution since 288 study participants received varying types and doses of SARS-CoV-2 vaccine over the course of the study follow-up. With the high vaccination rate of the study cohort, an increase in the antibody levels is expected, as vaccination induces the production of anti-RBD binding and neutralizing antibodies (16).

We were able to observe the antibody levels of 11 study participants who remained unvaccinated for the entire 1-year follow-up period. In the subgroup of unvaccinated participants, the antibody titers also demonstrated an increase throughout the year. This result differs from the findings of other studies that reported a decline in IgG levels starting 6 months after natural SARS-CoV-2 infection (2, 5).

It is important to consider the epidemiologic context, particularly the timing of community surges of COVID-19 infection, in relation to the timing of the blood extractions. The Philippines experienced a surge of COVID-19 infection from the Delta variant from August to October 2021 and another surge from the Omicron variant from January to February 2022. The Delta variant surge coincided with the blood extraction for the third timepoint (Day 180) while the blood extraction for the fifth timepoint (Day 360) started during the peak of the Omicron variant surge. The timing of blood extractions in relation to the number of cases of COVID-19 in the Philippines is shown in Figure 1.

The 2.3-fold increase in antibody titers among the unvaccinated study participants at 6 months (day 180) compared to 3 months (day 90) may be explained by the surge of infections from the Delta variant in the community. These participants may have developed asymptomatic COVID-19 reinfection, which would cause an increase in the antibody titers. Studies also show that exposure to the SARS-CoV-2 virus may produce a mild increase in antibody titers, as observed among close contacts of COVID-19 patients who were not infected. However, this antibody response was observed to be more short-lived and declined more rapidly compared to those who developed the infection (17). A 4.9-fold increase in antibody titers was observed at 12 months (day 360) of follow-up compared to 9 months (day 270), which may be explained by the Omicron variant surge in the community. Similarly, asymptomatic reinfection or exposure to the SARS-CoV-2 virus may have caused the increase in the antibody titers of the unvaccinated study participants.

Population studies in other countries show different results. In China, the RBD-specific IgG, IgM and IgA antibodies were tested using an indirect electrochemiluminescence immunoassay kits (Kangrun Biotech Co., Ltd). There was a 2.87-fold decrease in RBD-IgG within 3 months (825 to 287 AU/mL), RBD-IgM decreased to negative levels within 3 months, and RBD-IgA became negative at 12 months,{2} In Lithuania, out of 38 study participants with quantitative SARS-CoV-2 S IgG levels measured

TABLE 4 Estimates of hazard ratio derived using the Cox proportional regression analysis.

Factor	Adjusted Hazards Ratio	95% Confidence Interval	P-value
Antibody, U/mL*	0.9939	0.9920 to 0.9958	<0.001
Vaccinated	1.2030	0.4574 to 3.1645	0.708
Sex	1.0321	0.6080 to 1.7520	0.907
Age, years	1.0118	0.9935 to 1.0305	0.209
Comorbidities, number			
1	1.8854	0.8180 to 4.3457	0.137
2 or more	0.9352	0.4981 to 1.7557	0.835

*Antibody level prior to reinfection for those with probable reinfection; antibody GMT for the 5 timepoints for those without probable reinfection. Unadjusted HR: 0.9939, 95% CI 0.9921 to 0.9959.

using quantitative Enzyme Linked Immunosorbent Assay or ELISA (UAB Imunodiagnostika, Lithuania), 17 (44.7%) exhibited a decline in IgG levels from 6 months to 13 months, 14 (36.8%) had stable IgG levels, while 7 (18.5%) had increase in IgG levels (5). In Spain, a gradual decline was observed in S1 protein IgG antibodies detected through ELISA (Euroimmun AG, Lübeck, Germany) from 4 to 7 months (4).

The same laboratory test (Roche Elecsys® Anti-SARS-CoV-2 S assay) was used in a seroprevalence study in South India. Authors reported an overall seroprevalence of 62.7% (95% CI 59.3 to 66.0), using 0.80 U/mL as the cut-off for a positive test. The case-to-undetected-infected ratio (CIR) was 1: 8.65 (95% CI 1:8.1 to 1:9.1) (18). Other studies that use the same laboratory test evaluated humoral responses to vaccination, not natural infection.

The results of this study also demonstrated that participants with more severe COVID-19 infection had significantly higher antibody titers compared to those with milder infection at day 21, consistent with the findings of other studies (2, 3, 5). The antibody titers persistently remained higher until day 180 among those with severe and critical infection, but the difference was no longer statistically significant.

The vaccination rate in this study cohort was high, with 90.5% participants who were fully vaccinated, and 69% receiving at least 1 booster dose. This rate was higher than the national rate of 77.8% for fully vaccinated individuals (as of June 2022) (19). Factors that may have led to a higher vaccine coverage in this cohort include better health seeking behavior as indicated by their willingness to participate in scientific research, and their residence in Metro Manila, which may lead to easier access to vaccine centers, Another possible factor is the frequent follow-up calls by the research team, where several participants would inquire about the safety and effectiveness of the vaccines. These calls provided good opportunities for the participant to express their concerns about the vaccines, and for the research team to clarify common misconceptions regarding vaccination.

All study participants demonstrated an increase in antibody titers regardless of initial disease severity classification and vaccination status. However, the participants who received SARS-CoV-2 vaccines had a greater rise in antibody levels compared to the unvaccinated group. This highlights the importance of vaccination even among previously infected individuals.

Studies that compared the antibody responses of vaccinated and naturally infected individuals report higher levels of anti-RBD or anti-S1 antibodies among those who received the vaccine (4, 20). These studies also reported a faster decline in antibodies among vaccinated individuals compared to those naturally infected, with one study reporting a decline in RBD antibodies within 6 months after vaccination, compared to 8 months for those naturally infected (20). In our study, we did not observe a decline in antibody levels for those unvaccinated and vaccinated individuals during the one-year follow-up.

In this study, there were 64 cases of probable reinfection. Due to the inaccessibility of genomic testing, reinfection could not be documented in this study. Instead, we estimated the prevalence of probable reinfection at 20.8%. Of the 64 participants with probable reinfection, only 1 was severe enough to necessitate hospitalization. This is consistent with the findings of other studies, which reported 90% lower odds of hospitalization or death for reinfections compared to primary infection. This is most likely due to the priming effect of the primary infection on the immune system, which enables a better immune response against the SARS-CoV-2 virus upon reinfection (21).

The 64 study participants with probable reinfection had an antibody GMT of 411.8 U/mL prior to the reinfection. Of the 64 study participants, 56 (87.5%) received the primary vaccine series and were considered fully vaccinated, while 5 of the 56 participants received a booster dose. Thus, these vaccinated participants could be classified as having breakthrough COVID-19 infection as well. Reinfection occurred at 143 days on average (range 13 to 236 days) after completing the primary vaccine series. Among those who remained unvaccinated, reinfection occurred on average 198 days after the initial infection or the positive RT-PCR test result for asymptomatic patients.

In this study, increased antibody levels were found to be significantly associated with reduced symptomatic reinfection rate ($p<0.001$). This is consistent with other studies that reported on the correlation of high levels of anti-RBD IgG with a reduced risk of symptomatic infection (22). Although there is no well-defined cut-off for the antibody level that confers protection against COVID-19 infection, one study involving fully vaccinated participants reported 80% vaccine efficacy against symptomatic infection with the alpha variant of SARS-CoV-2 with anti-RBD immunoglobulin levels of 506 binding antibody unit (BAU)/mL (95% CI 135 to beyond data range) (17). These findings highlight the importance of vaccination, and the potential of properly timed booster doses to enhance the protective immune response.

It can also be observed in this study that the study participant with severe reinfection had lower antibody titers compared to those with mild or moderate reinfection. This finding is consistent with the results of a cohort study in South Korea wherein antibody levels were found to predict the clinical course of patients with delta and omicron variant COVID-19 infection. Those with increased antibody levels had decreased occurrence of fever, hypoxia, CRP elevation, and lymphopenia (23).

4.2 Limitations

The limitations of this study include the focus of the study on determining antibody titers and its correlation with protection against future infection. Recent studies have highlighted the major role of T cells in developing immunity against SARS-CoV-2. T cells have been observed to last for at least 6 months after natural infection. T cells were also observed to increase upon exposure to low-dose SARS-CoV-2 virus, leading to the hypothesis that memory T cells may provide protection against severe reinfection (24).

Another limitation is that the study involved the measurement of binding antibodies (anti-RBD antibodies) and not neutralizing antibodies. Neutralizing antibodies play a critical role in protecting against SARS-CoV-2 by clearing the virus. Neutralizing antibodies interfere with the binding of the virus to its receptor, block the

uptake of virus into host cells, and prevent the uncoating of viral genomes (25). Levels of neutralizing antibodies are highly predictive of immune protection from SARS-CoV-2 infection (26). Measurement of neutralizing antibodies against SARS-CoV-2 utilizing the plaque reduction neutralization test (PRNT) was originally planned since this is the reference standard. The test, which measures the level of neutralizing antibodies, is tedious and takes 4 to 5 days to complete. The procedure typically requires the use of live virus, using a specialized set-up in a biosafety level 3 (BSL3) laboratory (13, 27). At the time of study implementation, there was no certified BSL3 laboratories in the country. However, several studies report that neutralizing and anti-RBD IgG antibody levels are strongly correlated, and that anti-RBD IgG antibody levels can be used for the accurate assessment of immunity following SARS-CoV-2 infection or vaccination (4, 28, 29).

It is also important to note that amino acid substitutions in the RBD of the different COVID-19 variants may affect the binding of antibodies (30). Furthermore, only 1 immunoassay was used in this study. Correlation of antibody titer results with other immunoassays could not be done.

In this study, the laboratory test used had a lower limit of detection of 0.40 U/mL and an upper limit of detection of 250 U/mL. As per manufacturer recommendations, 10-fold dilution was performed to increase the upper limit of detection to 2,500 U/mL. However, several results still exceeded 2,500 U/mL. Further 100-fold and 1,000-fold dilutions were done to increase the upper limit of detection to 250,000 U/mL. The accuracy of the test may have diminished at these higher range of values.

Another limitation in this study is the variation in interval between determination of antibody GMT levels and the development of reinfection. Determination of antibody GMTs was performed at fixed time points based on the time of initial diagnosis of COVID-19 regardless of the time of diagnosis of reinfection or the time of vaccination.

Furthermore, due to limitations in the study funding, testing *via* RT-PCR or antigen test was encouraged but not provided for free for the study participants. Some study participants who developed symptoms consistent with a COVID-19 reinfection refused to undergo testing. We identified 101 reports of possible reinfection in the study cohort that could be true reinfections; however, the lack of supportive tests preclude definite classification. The study was also unable to detect cases of asymptomatic reinfection. Thus, the number of cases of reinfection reported in this study may be underestimated.

4.3 Contribution to knowledge and future research implications

This study observed an increase in antibody levels over the study period among both unvaccinated and vaccinated patients previously infected with COVID-19 infection residing in Metro Manila, Philippines. These data contribute to knowledge on the long-term humoral immune response to COVID-19, which is affected by severity of initial disease, vaccination, COVID-19 reinfection, and exposure to new variants during the Delta and Omicron surges. It also demonstrates that higher antibody levels are associated with a lower risk of symptomatic reinfection. Future research that monitor levels of neutralizing antibodies against specific COVID-19 variants, as well as research on the levels of neutralizing antibody among patients who develop definite asymptomatic and symptomatic re-infection may be done.

5 Conclusion

This cohort study demonstrated an increase in antibody levels against SARS-CoV-2 over one year among those who had COVID-19 infection. Several factors could have led to the steady increase in antibody levels, including COVID-19 vaccination, COVID-19 reinfection, and exposure to new variants during the Delta and Omicron surges. Participants with more severe COVID-19 infection had significantly higher antibody levels compared to those with milder infection at day 21. There were 64 cases of probable reinfection identified in the study cohort, of which 56 (87.5%) were fully vaccinated. Higher antibody levels were associated with a lower risk of symptomatic reinfection. Information on the timing, magnitude, and durability of humoral immunity among Filipinos is essential to guide the deployment of vaccine stocks, and can help guide strategies for returning to the workplace and relaxing social distancing measures.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by University of the Philippines Manila Research Ethics Board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors contributed to conception and design of the study. MD, CT-L organized the database. CT-L, CC performed the statistical analysis. CT-L wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Longitudinal study of humoral immunity against SARS-CoV-2 of health professionals in Brazil: the impact of booster dose and reinfection on antibody dynamics

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Introduction: The pandemic caused by SARS-CoV-2 has had a major impact on health systems. Vaccines have been shown to be effective in improving the clinical outcome of COVID-19, but they are not able to fully prevent infection and reinfection, especially that caused by new variants.

Methods: Here, we tracked for 450 days the humoral immune response and reinfection in 52 healthcare workers from Brazil. Infection and reinfection were confirmed by RT-qPCR, while IgM and IgG antibody levels were monitored by rapid test.

Results: Of the 52 participants, 19 (36%) got reinfected during the follow-up period, all presenting mild symptoms. For all participants, IgM levels dropped sharply, with over 47% of them becoming seronegative by the 60th day. For IgG, 90% of the participants became seropositive within the first 30 days of follow-up.

IgG antibodies also dropped after this period reaching the lowest level on day 270 (68.5 ± 72.3, p<0.0001). Booster dose and reinfection increased the levels of both antibodies, with the interaction between them resulting in an increase in IgG levels of 130.3 arbitrary units.

Conclusions: Overall, our data indicate that acquired humoral immunity declines over time and suggests that IgM and IgG antibody levels are not associated with the prevention of reinfection.

KEYWORDS

antibody, COVID-19, healthcare workers, humoral immunity, reinfection, SARS-COV-2

1 Introduction

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), emerged in China in 2019, and has affected more than 200 countries. By the end of 2022, there had been 647,972,911 confirmed cases of COVID-19, including 6,642,832 deaths, in the world. In Brazil, the first case was diagnosed in February 2020, and by the end of December 2022, the country had had more than 35 million confirmed cases (1). Healthcare workers (HCW) were a group greatly impacted by the pandemic, leading COVID-19 to be recognized as an occupational disease (2).

Due to the significant impact of the pandemic on health systems, a global effort has sought various alternatives to reduce the harm caused by the disease (3). Thus, vaccines have been developed and approved (4). Due to their higher risk of exposure, HCW were the first group to be vaccinated (5). In January 2021, the vaccination schedule began in Brazil. Until December 2022, more than 80% of the population have had a complete vaccination schedule, and almost 50% have had a booster dose (6). Although these vaccines reduce virus levels in the body, and consequently reduce viral transmission, they do not fully prevent new SARS-CoV-2 infections (7–9). Vaccines have proven to be safe, effective, and timely tools to prevent severe outcomes of COVID-19, including hospitalization and death. However, the efficiency of vaccination can change depending on the type of vaccine and other factors, such as the emergence and/or introduction of new viral variants (9–11).

Since the first recorded cases of COVID-19, new variants of SARS-CoV-2 have been identified. Therefore, in order to establish control and monitoring goals for the new variants, the World Health Organization (WHO) has established three classification categories: variants of concern (VOCs), variants of interest (VOIs), and variants under monitoring (VUMs). The four previously circulating VOCs are Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Delta (B.1.617.2), while Omicron (B.1.1.529, including BA.1, BA.2, BA.3, BA.4, BA.5 and their descendent lineages) are currently circulating (1). Each SARS-CoV-2 VOC is associated with a new wave of infection, such as the Gamma variant in December 2020 and the Omicron variant in December 2021, which affect human health worldwide (1, 12). Studies involving these new variants have elucidated fundamental aspects of SARS-CoV-2 biology, including viral transmissibility, disease severity, immune system escape, vaccine efficiency, clinical treatment, and management strategies (3, 13, 14).

It is already known that it is possible to become reinfected with SARS-CoV-2 (12, 15). However, since some people have a recurrence of positive test results for SARS-CoV-2 RNA detection during apparently the same infection (16), in order to be considered a reinfection, the CDC has established that the nucleotide sequences of positive samples must be from different lineages or there must be a difference of 90 or more days between positive results (17). Previously published data on the prevalence of SARS-CoV-2 reinfection have highlighted its low level, ranging from 0.10 to 0.65% in the range of 6-9 months (18–20). However, the reinfection rate in Brazil is still unknown, especially in risk groups such as health professionals. The emergence of new variants could increase the reinfection rate, as they can escape the immune response triggered by existing vaccines, as with VOCs (e.g., Omicron) (14, 21, 22). Identifying the potential for SARS-CoV-2 reinfection is crucial to understanding the long-term dynamics of the pandemic. Previous studies suggest that the presence of IgG antibodies reduce the risk of reinfection (23). According to a study carried out in England, a primary infection reduces the reinfection rate by 84% over the following seven months (24). Therefore, understanding the dynamic behavior of SARS-CoV-2 infection, and assessing reinfection rates, the impact of genetic variants and vaccines on immune memory kinetics, and their application in the global vaccination campaign are some key points that still need to be elucidated (25).

In this study, we investigated, in HCW in the city of Belo Horizonte in Brazil, the dynamics and longevity of the humoral immune response up to 450 days after the initial onset of COVID-19 disease symptoms and laboratory confirmation of SARS-CoV-2 infection. During the study, the kinetics of the humoral response were monitored both before and after the initial vaccination scheme and subsequent booster dose. Our study demonstrated the occurrence of reinfection in 19 study participants, and showed that (i) the humoral immunity of HCW declined over time, and (ii) the booster dose was essential to increase antibody levels, mainly IgG, but not enough to protect against

reinfection with new variants. Robust and constant surveillance is, therefore, essential for responding to future epidemic waves, and provides a basis for recommendations for immunization programs and vaccine updates.

2 Materials and methods

2.1 Ethical approval

This study was conducted in accordance with current legislation including the Declaration of Helsinki and Resolution No. 466/2012 of the Conselho Nacional de Saúde do Brasil. Ethical approval was obtained from the institutional review board of the Instituto René Rachou, Fundação Oswaldo Cruz, CAAE: 31.919520.8.0000.5091, approval numbers: 4177931; 4291836; 4343318; 4624187; 5294423. Written informed consent was obtained from all participants before any study procedure was undertaken.

2.2 Study population and enrollment

The population selected for this study was composed of HCW who worked in at least one of the public hospitals: Hospital das Clínicas (HC) at the Universidade Federal de Minas Gerais, the Unidade de Pronto Atendimento (UPA) Centro-Sul, and the Hospital Metropolitano Dr. Célio de Castro (HMDCC). All three health centers are in the city of Belo Horizonte, the state of Minas Gerais, Brazil. As inclusion criteria, in addition to what has already been mentioned above, all participants had to (i) present with at least one of the following symptoms within the previous seven days: fever (equal to or greater than 37.5°C), cough (dry or productive), fatigue, dyspnea, sore throat, anosmia/hyposmia, and/or ageusia; and (ii) have a positive result for the detection of SARS-CoV-2 RNA by RT-qPCR. Participants were invited by telephone, and those who met the inclusion criteria were included in the study. Individuals who reported volunteering in COVID-19 vaccine clinical trials, prior diagnosis of COVID-19, or reported pregnancy, were excluded. Hospitalization also resulted in loss of follow-up due to inability to perform the tests. The enrollment of participants took place between October 2020 to April 2021. Individuals were followed up for 450 days, with capillary blood samples collected on days 7, 10, 15, 20, 25, 30, 60, 90, 180, 270, and 450 after being enrolled in the study (referred to as D7, D10, D15 and so on). In addition to capillary blood collections, participants were contacted every 30 days after the 60th day (from December 2020 to June 2022) to assess possible SARS-CoV-2 reinfection. Besides, all participants were instructed to contact the research team when they present any symptoms that constitute suspicion of COVID-19. The predetermined symptoms which were monitored as evidence for reinfection were: fever (equal to or greater than 37.5°C), cough (dry or productive), sore throat, fatigue, dyspnea, and diarrhea. Also, any suspicious symptom established by medical criteria was monitored. All suspected cases were tested for the presence of viral RNA by RT-qPCR and serological status. In January 2021, during the study period, public roll-out of the vaccination scheme using the CoronaVac vaccine was started in Brazil. Fifteen days after the second dose, the initial two-dose vaccination scheme is considered "complete". However, in September 2021, a booster dose was rolled-out (6). The workflow for our study is shown in Figure 1.

2.3 Study design

All symptomatic healthcare professionals were tested for SARS-CoV-2 by RT-qPCR using the Charite Institute protocol (26). During the isolation period, kits were sent to participants to perform a self-collection of saliva or nasopharyngeal and oropharyngeal swabs on D1, D3, and D5 after enrollment in the study. Swab or saliva was placed in a tube containing a lysis buffer with guanidine (4 M Guanidine Isothiocyanate, 25 mM Sodium Citrate, pH 7.4) that were stored at 4°C. Samples were collected in participants' houses within a 5-day interval and were transported under refrigeration to the central laboratory for RNA extraction. As demonstrated by Carvalho et al., 2021 (27) this buffer, besides decreasing the virus infectivity, maintains RNA integrity for up to 16 days at room

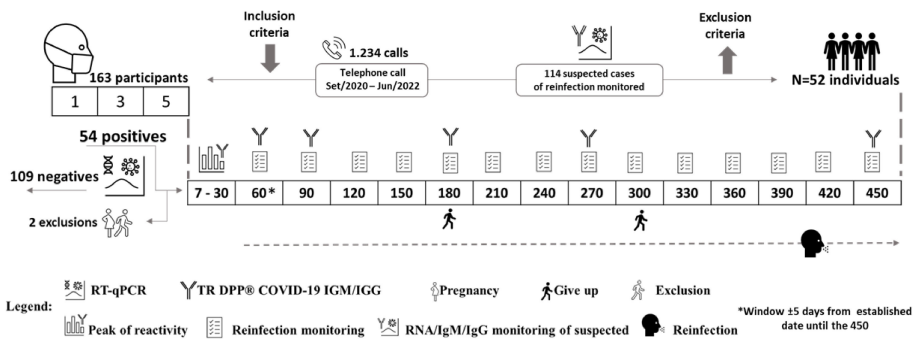

FIGURE 1
Flowchart illustrating our study workflow. The numbers inside the squares represent the days of visits on which patients were followed-up. The icons shown in the legend represent the study design. After 60 days, questionnaires were answered to assess symptoms consistent with suspected SARS-CoV-2 reinfection, if reported by participants, and clinical samples were collected. Fifty-four participants were confirmed positive for COVID-19, of which one participant became pregnant and another was hospitalized, both were subsequently excluded from the study.

temperature. In addition to the material for saliva/swab sample collection, the kits included materials, together with an instruction manual, for performing a rapid test to detect IgM and IgG antibodies against SARS-CoV-2 antigens (TR DPP® COVID-19 IgM/IgG - Bio-Manguinhos). Participants characterized as infected with SARS-CoV-2 must have had at least one positive result for detection of SARS-CoV-2 RNA on D1, D3 and/or D5. Participants characterized as non-infected presented negative results in all three samples analyzed and were not included in the follow-up period of the study.

2.4 RNA extraction

RNA was extracted from the nasopharyngeal and oropharyngeal swabs or saliva samples using the QIAamp® Viral RNA kit (QIAGEN®, USA) according to the manufacturer's instructions. Briefly, 140 µL of sample was added to 560 µL of AVL buffer containing carrier RNA, and after 10 minutes at room temperature, 560 µL ethanol were added. This solution was applied to an RNA affinity column and this column was centrifuged at 6,000 x g for 1 minute. Then, the column was washed with AW1 and AW2 buffer solutions in that order. After the washing process, RNA was eluted using AVE solution and used in the RT-qPCR test.

2.5 RT-qPCR (Quantitative PCR)

The RT-qPCR reactions were performed using the ViiA™ 7 Real-Time PCR System of the communal Real-Time PCR platform at the Instituto René Rachou. The RT-qPCR assays were performed using 5 uL of sample RNA, and the 200 nM GoTaq® Probe 1-Step RT-qPCR System Kit (Promega). This kit uses GoTaq Probe qPCR Master Mix with dUTP (10 uL), GoScript RT Mix for one-step RT-qPCR (0,4 uL), sense and antisense primers (400 nM) and nuclease-free water to a 20.0 uL final volume. The conditions for the amplification were: 45°C for 15 minutes and 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and hybridization at 60°C for 1 minute. The results were analyzed using the Thermo Cloud platform, according to the following criteria: samples with amplification of the E gene (Ct<37) and the RNAse P gene (RP) (Ct<35) were considered positive; samples without E gene amplification or with detection above Ct 37, with RP amplification (Ct<35), were considered negative. Samples with RP amplification above Ct 35 were considered invalid, and the test was performed again using RNA obtained from another extraction of the samples collected.

2.6 TR DPP® COVID-19 IgM/IgG test (Bio-Manguinhos)

In order to detect IgM and IgG antibodies against SARS-CoV-2, the DPP® COVID-19 IgM/IgG kit supplied by Bio-Manguinhos (FIOCRUZ, Brazil) was used according to the manufacturer's instructions. Briefly, a digital puncture was performed, and a blood sample was diluted in the buffer provided in the kit. The sample was then applied to the cassette. After 5 minutes, 9 drops of the buffer were added to the cassette, and the results were read after an additional ten-minute period. The interpretation of the test was performed with the aid of the DPP® Micro Reader, which provides the intensity of the reactive line. The DPP® COVID-19 IgM/IgG system uses an algorithm that includes assay-specific cutoff values to determine test result. Values equal to or greater than 30 for the IgM and IgG antibodies were considered reactive. In order to assess antibody levels, the test was performed on scheduled days and/or when the participant was suspected of reinfection (Figure 1).

2.7 Next-generation sequencing

Positive samples, with cycle threshold (Ct) lower than 36, were sequenced by Next-Generation Sequencing (NGS) on the Illumina MiSeq Platform using the Illumina COVIDSeq Kit for library construction (Illumina, San Diego, USA) generating paired-end reads 150 bp long. The raw reads were trimmed using Trimmomatic version 0.39 (28) with a sliding window of 4 nucleotides with a minimum average Phred score of 20. Trimmed reads smaller than 50 bp were removed. The filtered reads were mapped to the SARS-CoV-2 reference genome (NC_045512) using BWA version 0.7.17 (Li 2009) with the default parameters. The nucleotide variants were identified using iVar version 1.3 (29)., with a minimum frequency of 40% and depth of 30 reads. The consensus sequences generated by iVar were submitted to Pangolin version 4.2 (30) to identify the coronavirus lineage. Sequences that met the GISAID criteria were submitted to the EpiCoV database and are deposited at The European Nucleotide Archive under project number PRJEB49204 (https://www.ebi.ac.uk/ena/browser/view/PRJEB49204).

2.8 Statistical analysis

GraphPad Prism v.8.0.1., Jamovi 2.3.18.0 (https://www.jamovi.org/), and the statistical software R (https://www.r-project.org/) version 4.1.2 (31) were used for data analysis and generation of figures. Data organization and pre-processing of some graphs and figures were done using Excel and PowerPoint (Microsoft 365). The chi-square test or the Fisher's exact test were used to assess the association of categorical demographic variables and the infection status of individuals, as well as the correlation of symptoms reported during the first infection, reinfection and in suspected cases of reinfection. Paired and unpaired t-tests were used to analyze the difference in viral load between infection vs reinfection, and females vs males, respectively.

Generalized Estimating Equations (GEE) (version 1.3.9) were used to evaluate the longitudinal data. Proposed by Lian and Zeger (1986) (32), the proposed model jointly estimates an average effect and intra-individual variations, considering the structure of correlation or dependence between the repeated measures. The outcome of interest was the numeric scale of IgM and IgG levels and the covariates were vaccine status, follow-up time, booster dose and reinfection. In the GEE model, the peak of IgM and IgG levels in the interval from D7 to D30 was considered. A model segmented in time, 180 and 270 for IgM and IgG, respectively, was adjusted to make the average structure more accurate.

In order to assess whether there is an association between antibody levels and reinfection, differences in the levels of IgM and IgG antibodies against SARS-CoV-2 among individuals with a

confirmed case of reinfection and those not reinfected were evaluated. This analysis only considered samples taken during the period of the study when reinfection occurred (i.e., between July 2021 and July 2022), which was also the period when the delta and omicron variants circulated in Belo Horizonte. For this evaluation, the Mann-Whitney U-test was used. For individuals who did not become reinfected, the arithmetic means of all of the values of the antibody levels determined during the period stated above were considered. For individuals who became reinfected, we considered only the values of the antibody levels determined when reinfection was confirmed.

3 Results

3.1 Description of the study cohort

A total of 163 health professionals aged between 19 to 68 years were included in the initial study cohort between October 2020 to April 2021 (Figure 1). Fifty-four (33%) of these individuals subsequently had positive test results by RT-qPCR. Two individuals were excluded from further involvement in the study due to pregnancy or hospitalization, while two other participants withdrew from the study during the follow-up period, but allowed the use of their data and the samples already collected. Therefore, our final study cohort comprised 52 individuals, with 50 of these participants remaining until the end of the 450-day follow-up period. Among the starting 52 participants, four (7.7%) worked at UPA, 16 (30.8%) at HMDCC, and 32 (61.5%) at HC. Overall, the average age of the subjects was 37.38 ± 6.99 years, and 55.8% were female. The final study cohort consisted of 22 (42.3%) physicians, 14 (26.9%) nursing technicians, 10 (19.2%) nurses, and 4 (7,7%) physiotherapists (Table 1).

In January 2021, the vaccination schedule started in Brazil. Thus, 30 (58%) participants were infected with SARS-CoV-2 before starting the vaccination schedule, 11 (21%) during the schedule interval, and 11 (21%) after the complete vaccination, that is 15 days after the second dose of the initial vaccination scheme. During the 450 days of follow-up, 46 (88%) participants had suspected reinfections; of these, 19 (37%) cases were confirmed by RT-qPCR (Figure 2). The proportion of women who became reinfected was higher than that of men (p=0.011). Among the participants who became reinfected 58% had already received the booster vaccine dose when the reinfection was confirmed.

TABLE 1 Demographic characteristics of the final study cohort (n = 52).

Characteristics	Infection	Reinfection	Total	p-value
Health lefts				
HC	20 (38.5%)	12 (23.1%)	32 (61.5%)	1.000
HMDCC	10 (19.2%)	6 (11.5%)	16 (30.8%)	
UPA	3 (5.8%)	1 (1.9%)	4 (7.7%)	
Total	33 (63.5%)	19 (36.5%)	52 (100%)	
Age				
Range 21-30	7 (13.5%)	5 (9.6%)	12 (23.1%)	0.084
Range 31-40	7 (13.5%)	9 (17.3%)	16(30.8%)	
Range 41-60	19 (36.5%)	5 (9.6%)	24(46.2%)	
Total	33 (63.5%)	19 (36.5%)	52(100%)	
Sex				
Female	14 (26.9%)	15 (28.8%)	29 (55.8%)	0.011
Male	19 (36.5%)	4 (7.7%)	23 (44.2%)	
Total	33 (63.5%)	19 (36.5%)	52 (100%)	
Professional Category				
Physicians	15 (28.8%)	7 (13.5%)	22 (42.3%)	0.691
Nurse Technician	7 (13.5%)	7 (13.5%)	14 (26.9%)	
Nurse	6 (11.5%)	4 (7.7%)	10 (19.2%)	
Physiotherapist	3 (5.8%)	1 (1.9%)	4(7.7%)	
[1]Others	2 (3.8%)	0 (0.0%)	2 (3.8%)	
Total	33(63.5%)	19 (36.5%)	52 (100%)	
Complete vaccination scheme (i.e., initial two doses only)				
Yes	5 (9.6%)	19 (36.5%)	11 (21.2%)	<0.001
No	28 (53.8%)	0 (0.0%)	41 (78.8%)	
Total	33(63.5%)	19 (36.5%)	52 (100%)	

[1]Professionals included in the study: social workers, nutritionists, and psychologists.

3.2 IgM and IgG antibodies against SARS-CoV-2

The antibody peak observed between the seventh and thirtieth day (D7-D30) of each participant was chosen to be included in the analysis and represented a reaction average of 72.6 (± 68.0) for IgM (Figure 3A) and 193.7(± 121.2) for IgG (Figure 3B). Nine (17.3%) of the 52 participants did not IgG seroconvert within 30 days, and two (3.8%) individuals did not seroconvert at any time during the study to either of the two monitored immunoglobulins. In addition to these two latter individuals, 12 (23%) did not seroconvert to IgM throughout the study. The reactivity rate for IgM varied from 65%, 36%, 24%, 10%, 15%, and 24%, (Figure 3A) whereas for IgG it was 83%, 85%, 90%, 76% 58%, and 72% (Figure 3B) on days D7-30, D60, D90, D180, D270, and D450, respectively. Immunoglobulin levels decreased over time, reaching their lowest level on day 270 after infection when the mean level for IgM was 13.8 (± 17, p <0.0001) (Figure 3A) and for IgG was 68.5 (± 72.3, p<0.0001) demonstrating a significant reduction in comparison to the values observed at D7-D30. Figures 3C, D show the individual profile for anti-SARS-CoV-

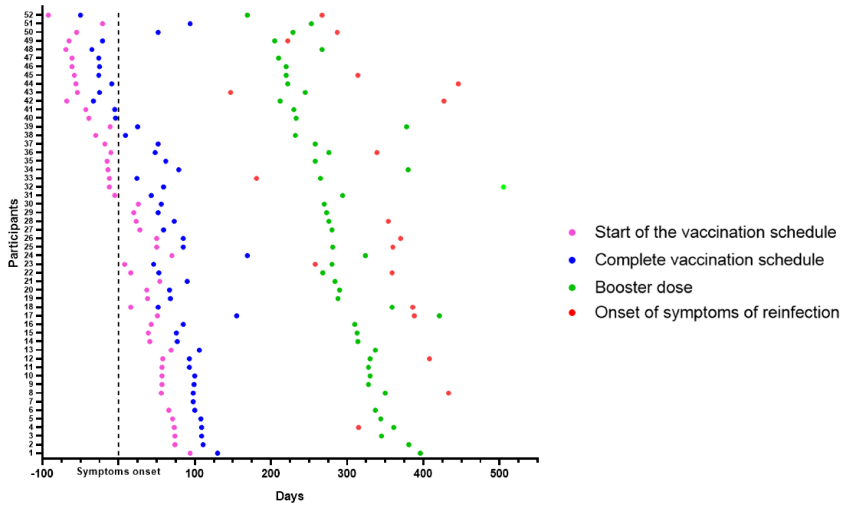

FIGURE 2
Overview of participant cohorts. Longitudinal follow-up of 52 participants who had COVID-19 confirmed by RT-qPCR. The day of symptom onset was called 0 (Black stroke - Symptom onset). The longitudinal timeline shows the days of the start of the vaccination schedule (pink dots), of the complete vaccination schedule, considering 15 days after the second dose (blue dots), and of the booster dose (green dots). Nineteen participants had confirmed SARS-CoV-2 reinfection (red dots).

2 IgM and IgG in the study participants, respectively. Although the mean levels of anti-SARS-CoV-2 IgG antibody did not differ significantly between days 270 and 450, representing the period when the majority of reinfection and vaccination booster doses occurred, a tendency for IgG levels to increase was observed (Figures 3A-F). There was no difference between biological sex in the dynamics of antibodies (Figures 4A, B). However, significant differences in the dynamics of antibody levels were observed, with older individuals presenting the highest levels of IgM and IgG (Figures 4C, D) and a slower decrease in anti-SARS-CoV-2 IgG levels (Figure 4D) compared to IgM (Figure 4C). No significant differences in the Ct value obtained in the RT-qPCR at baseline were observed between the different sex and the age groups (Figures 4E, F).

The dynamics of IgM and IgG levels over time are shown in Figures 3E, F. For IgM and IgG immunoglobulins, there were two behaviors: the drop in the mean antibody levels up to 180 and 270 days, respectively, followed by a slight increase after this time point. Tables 2 and 3 demonstrate the behavior of IgM and IgG estimated by the segmented model. The GEE results indicate that the average IgM and IgG values decreased by 0.606 and 0.645 units each day until days 180 and 270, respectively. After this time, the slope is positive for both IgM (0.363) and IgG (0.224). As shown in Figure 3E, the observed mean IgM levels were below the cutoff despite this upward trend. We can also observe that 60 days after symptom onset, the mean anti-SARS-CoV-2 IgM levels are below the cutoff, while anti-SARS-CoV-2 IgG levels drop, but not below the cutoff, around 270 days after symptom onset (Figures 3E, F).

No significant difference in IgM and IgG antibody levels with regard to the vaccination status of the participants was observed (Tables 2, 3). However, after the booster dose, the anti-SARS-CoV-2 IgM and IgG levels both significantly increased. The IgM levels after the booster dose were 38.388 units higher. In addition, IgM levels were significantly higher after reinfection by 20.378 units. The interaction between these two covariables was not significant. In contrast, when we analyzed the IgG levels, the interaction between the booster dose and reinfection was significant, with the mean IgG levels 130.3 [95%CI: 82.19-178.30] units greater in those that received the booster dose and got reinfected. Before the booster dose, there was no difference in the mean IgG level when comparing reinfected and non-reinfected participants ($p = 0.348$). After the booster dose, the mean level of IgG for reinfected individuals was 87.8 [27.3; 148.0] units, which was higher than for non-reinfected individuals. For non-reinfected individuals, after the booster dose, the mean IgG was 52.22 units higher than before.

Of the 19 participants were reinfected (Figure 5), 13 (68.4%) were after and 6 (31.6%) before the booster dose. In four (21.0%), increased levels of IgM and IgG antibodies were not observed, even after booster dose and re-infection, these individuals being non-reactive to SAR-CoV-2 antigens at day 450 for both immunoglobulin classes (Figures 5F, G, J, O). One individual (Figure 5F) did not seroconverted during the whole period of follow-up. Of the remaining 15 individuals were reinfected, considering IgM dynamics, four of them never seroconverted during the follow-up period (Figures 5B, H, K, S). Four individuals (Figures 5C–E, R) who were negative during visits D7-D30 only had IgM seroconversion after reinfection or the booster dose. For four other individuals (Figures 5L, M, P, Q) that became IgM reactive during period D7-D30, but turned non-reactive during the longitudinal assessment, the booster dose and reinfection were not able to stimulate production of this class of antibody. For the remaining three participants (Figures 5A, I, N), IgM levels declined to non-reactive, but the booster dose or reinfection seroconverted them again. For IgG, the dynamics are different, since all 15 reinfected participants seroconverted at some point during the study. Of these, one only seroconverted after the booster dose (Figure 5K). The other 14

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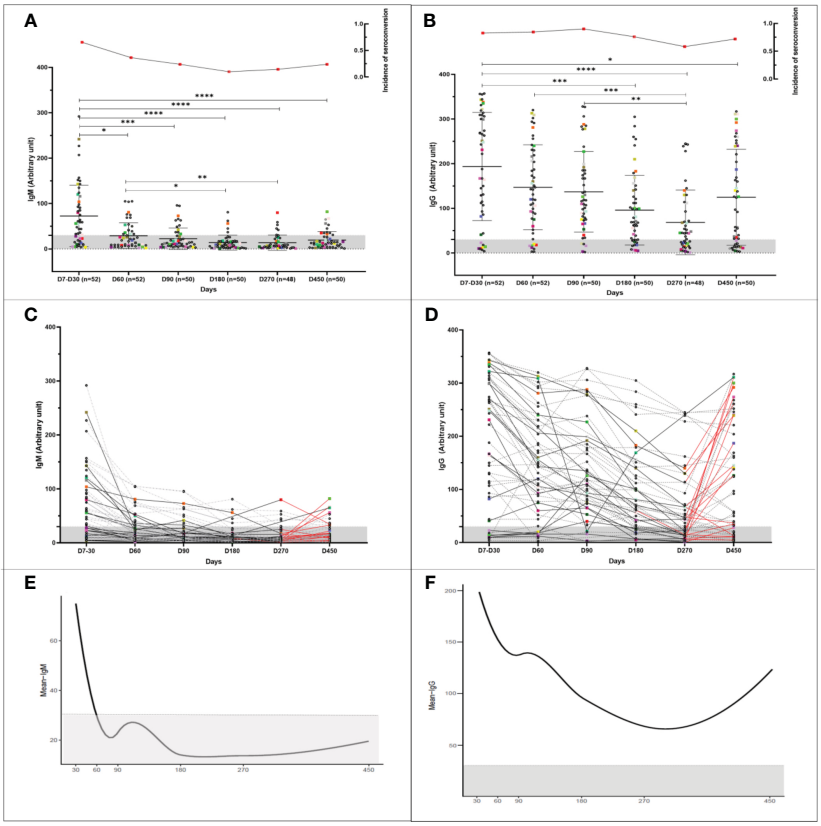

FIGURE 3
Longitudinal humoral immune response in participants infected with COVID-19. Kinetics of the levels of anti-SARS-CoV-2 IgM and IgG antibodies over time. A serum sample was considered positive when the reaction intensity had a value equal to or above 30. Colored dots indicate individuals who became reinfected over the course of the study. **(A, B)** Show the reactivity of IgM and IgG specific for SARS-COv-2 antigens over time. The frequency of seroconversion is shown at the top of the figure. The number of individuals tested (*n*) varies according to the time point evaluated and is indicated on the graph. *p<0.05; **p<0.01; ***p<0.001 ****p <0.0001. Statistical significance was measured using a Kruskal-Wallis test at a significance level of 5%. **(C, D)** Each line represents one participant (*n* = 52). Dashed lines represent participants infected once with COVID-19. Reinfected participants are represented by solid lines. The red lines indicate the interval in which reinfection occurred. **(A-D)** Values below 30 are shown in the gray zone of the graphs. **(E, F)** Mean IgM and IgG immunoglobulin levels over time. For the analysis, the peak antibody level for each participant, observed from the seventh to the thirtieth day, was considered (30). Follow-up evaluation of anti-SARS-CoV-2 IgM and IgG levels was performed at days 60, 90, 180, 270, and 450 after enrollment in the study. A test was considered positive when the detected value was equal to or greater than 30.

participants had reactive IgG antibodies at baseline. For all of them, anti-SARS-CoV-2 IgG antibody levels declined, with seven participants becoming non-reactive to SARS-CoV-2 antigens for this class of antibody. For the remaining seven seroreactive participants, six showed increased IgG levels after reinfection or booster dose (Figures 5H, I, L, M, P, R). For the individuals who became IgG seronegative over the course of the study, the booster dose or reinfection was able to cause secondary IgG seroconversion of all such participants (Figures 5A–E, N, S).

3.3 Reinfection

Every 30 days or when participants presented symptoms that indicated a suspicion of COVID-19 reinfection, saliva or naso-oropharyngeal swab collection was performed to detect viral RNA. At the same time, a capillary blood sample was collected to evaluate immunoglobulins. The interval between symptoms onset and swab/saliva collection or antibody assessment varies from 1 to 15 days, with 75%of the participants being tested until 6 days of symptoms onset (Supplementary Figure S1). Of the 52 participants, 46 (88%) described at least some symptoms compatible with suspected COVID-19, with 9 (17%) reporting it once, 19 (36%) twice, 9 (17%) three times, five (10%) four times, and four (8%) participants reporting five times (Figure 6A). In total, 114 suspected episodes of COVID-19 were recorded, all suspected cases were tested by RT-qPCR and of these only 19 were confirmed positive by RT-qPCR. Of all the symptom episodes/types (n = 185) reported by participants during the study, cough (61.6%) followed by congestion or runny nose (49.2%) and sore throat (41.1%) were the most frequent. The most common symptoms reported during the suspected and confirmed reinfections were congestion or runny nose (57.0% and 74%), cough (40.4% and 63%), and sore throat (45.6% and 47%), respectively. The profile of symptoms presented by reinfected individuals differs from those reported during the first SARS-CoV-2 infection, with statistical significance (p<0.001) for headache, fever and chills, myalgia, anosmia, and ageusia between groups were observed (Table 4). Ageusia and anosmia were not

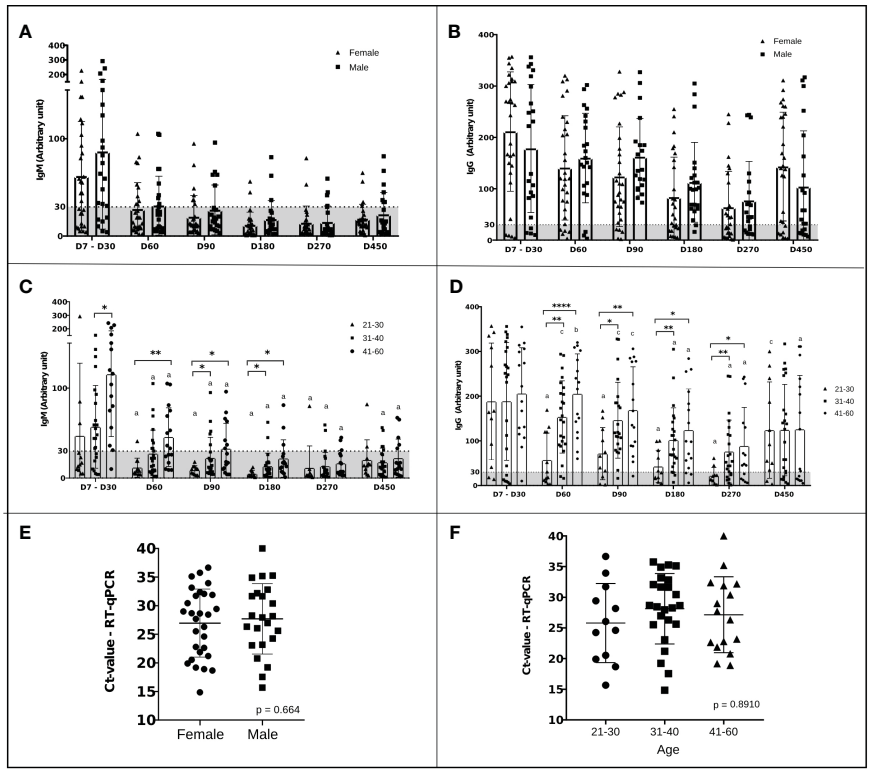

FIGURE 4
Dynamics of IgM and IgG levels against SARS-CoV-2 and Ct values over time stratified by sex and age. **(A-D)** The left and right panels represent the distribution of IgM and IgG levels, respectively. A serum sample was considered positive when the reaction intensity had a value equal to or above 30. Values below 30 are shown in the gray zone of the graphs. P values were determined using a Sidak's multiple comparisons test, ANOVA, or unpaired t-test. **(A, B)** Stratification of the humoral response over time by sex. The triangles represent the antibody level of women and the squares that of men. There was no statistical difference. **(C, D)** Stratification of humoral response over time by age, 21-30 years (triangles), 31-40 years (squares), and 41-60 years (dots). "a" significant difference in comparison with D7-D30 ($p<0.005$); "b" significant differences in comparison with D180 ($p< 0.005$); "c" significant differences in comparison with D270 ($p< 0.005$); *$p<0.05$; **$p<0.01$; ***$p<0.001$ ****$p <0.0001$. **(E)** Ct values of males (squares) and females (dots). Statistical significance was measured using an unpaired t-test at a significance level of 5%. **(F)** Ct values in participants stratified by age: 21-30 years (dots), 31-40 years (squares), and 41-60 years (triangles). Statistical significance ANOVA at a significance level of 5%.

TABLE 2 Parameter estimates according to Generalized Estimating Equations (GEE) analysis of the relative numeric scale of IgM levels.

Covariable	Estimated IgM level in arbitrary units (95%CI)	p-value
Time		
<=180	-0.606 (-0.884; -0.328)	< 0.001
>180	0.363 (0.172; 0.554)	< 0.001
Vaccination status		
Unvaccinated	Reference	
Partially vaccinated	22.151 (-10.597; 54.90)	0.185
Fully vaccinated	9.934 (-8.511; 28.38)	0.291
Booster dose		
Before the booster dose	Reference	
After the booster dose	38.388 (19.091; 57.685)	< 0.001
Reinfection status		
No reinfection	Reference	
Reinfected	20.378 (2.869; 37.888)	0.023

Vaccine status, follow-up time, booster dose, and reinfection were used as covariates.

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TABLE 3 Parameter estimates according to Generalized Estimating Equations (GEE) analysis of the relative numeric scale of IgG levels.

Covariable	Estimated IgG level in arbitrary units (95%CI)	p-value
Time		
<=270	-0.645 (-0.811; -0.479)	< 0.001
>270	0.224 (0.127; 0.321)	< 0.001
Vaccination status		
Unvaccinated	Reference	
Partially vaccinated	15.977 (-15.343; 47.298)	0.317
Fully vaccinated	26.848 (-7.077; 60.773)	0.121
Booster dose		
Before the booster dose	Reference	
After the booster dose	52.221 (15.406; 89.035)	0.005
Reinfection status		
No reinfection	Reference	
Reinfected	9.715 (-10.566; 29.996)	0.348
Interation		
Booster dose - Reinfection	87.8 (27.3; 148.0)	0.001
Reinfection - Booster dose	130.3 (82.19;178.30)	0.001

Vaccine status, follow-up time, booster dose, and reinfection were used as covariates.

reported in confirmed cases of reinfection (p<0.001). Other symptoms reported during the first infection in the suspected and confirmed cases of reinfection can be seen in Table 4.

The first five (26%) cases of reinfection occurred between August and September 2021 (Table 5). At this time, the Delta and Gamma variants were circulating in Belo Horizonte (Figure 7). Between December 2021 and January 2022, 12 cases (63%) of reinfection were confirmed, a period that overlaps with the new wave of transmission caused by the Omicron variant (Figure 7). Of the reinfection samples collected during this period that were sequenced, nine were identified as the Omicron variant. Sequencing samples with low viral load (Ct value > 36) was not

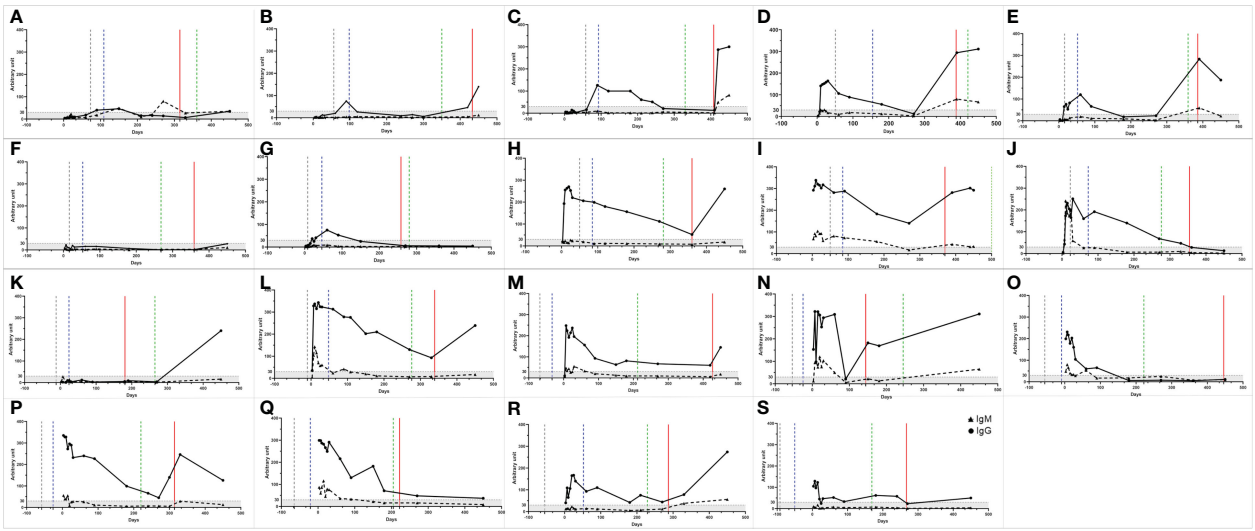

FIGURE 5
IgM and IgG antibody profiles of participants who were reinfected. **(A-S)** The triangles represent the IgM and the dots the IgG values. The gray, blue and green dashed lines represent the beginning date of the vaccination, the complete vaccination scheme, and the booster dose, respectively. The red line represents the reinfection period. A serum sample was considered reactive when the reaction intensity had a value equal to or above 30. Values below 30 are shown in the gray zone of graphs.

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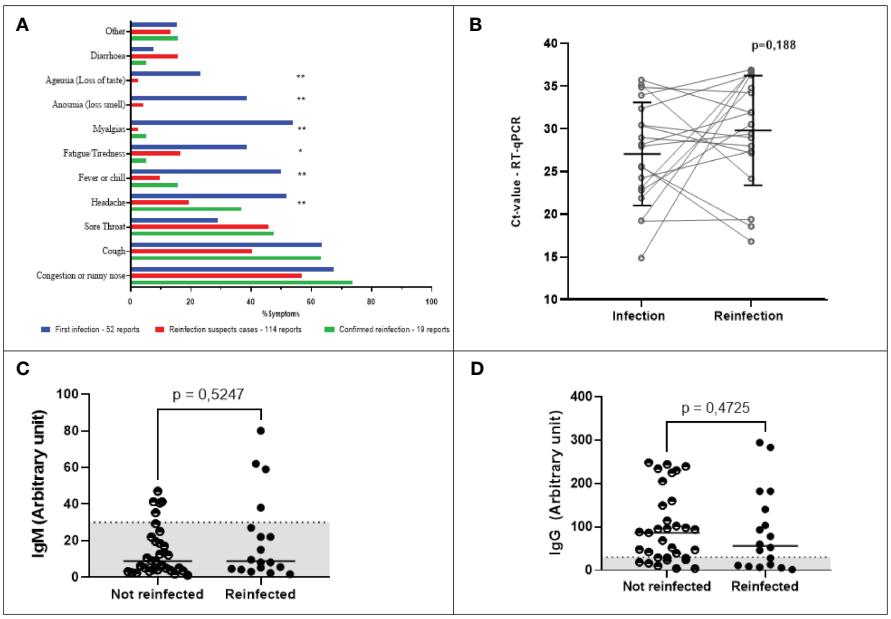

FIGURE 6
Main self-reported symptoms, dynamics of IgM and IgG levels, and Ct- values stratified according to SARS-CoV-2 infection/reinfection status. **(A)** Main self-reported symptoms described by participants. Blue bars indicate the percentage of symptoms reported by the 52 COVID-19 positive participants. Red bars show the symptoms reported in cases of suspected reinfection, but without confirmation by detection of viral RNA. Green bars show the main symptoms of participants with confirmed reinfection by SARS-CoV-2. Statistical significance was measured using either the Chi-square test or Fisher's exact test **p < 0.001, *p=0.002. **(B)** SARS-CoV-2 viral load. Statistical significance was measured using a paired t-test at a significance level of 5%. **(C, D)** Antibody levels of reinfected and non-reinfected participants. For individuals who were not reinfected, the values shown are the arithmetic means of multiple measurements of their circulating antibody levels taken throughout only the period of the study when the waves of the Delta and Omicron variants occurred. For individuals who were reinfected, the antibody levels shown are single point estimates determined only at the time of reinfection confirmation. The left and right panels represent the distribution of IgM **(C)** and IgG **(D)** levels, respectively. A serum sample was considered positive when the reaction intensity had a value equal to or above 30. Values below 30 are shown in the gray zone of graphs. P values were determined using the Mann-Whitney U test at a significance level of 5%.

possible. The mean CT of infected participants was 30.17 (± 6.5) (Table 5). There was no significant difference between Ct values detected during infection and reinfection (Figure 6B). When assessing the humoral response of reinfected and non-reinfected individuals, we did not observe differences in IgM and IgG levels between these groups (Figures 6C, D). The intervals between infection and vaccination and seroconversion status are given in Tables 5, 6.

TABLE 4 Main symptoms reported by the final study cohort.

Symptoms	First infection	Suspected reinfection	Confirmed reinfection	Total	p-value
Congestion or runny nose	35 (67.3%)	65 (57.0%)	14 (73.7%)	114 (61.6%)	0.234
Cough	33 (63.5%)	46 (40.4%)	12 (63.2%)	91 (49.2%)	0.010
Sore throat	15 (28.8%)	52 (45.6%)	9 (47.4%)	76 (41.1%)	0.106
Headache	27 (51.9%)	22 (19.3%)	7 (36.8%)	56 (30.3%)	<0.001
Fever or chill	26 (50.0%)	11 (9.6%)	3 (15.8%)	40 (21.6%)	<0.001
Fatigue/Tiredness	20 (38.5%)	19 (16.7%)	1 (5.3%)	40 (21.6%)	0.002
Myalgias	28 (53.8%)	3 (2.6%)	1 (5.3%)	32 (17.3%)	<0.001
Anosmia (loss smell)	20 (38.5%)	5 (4.4%)	0 (0.0%)	25 (13.5%)	<0.001
Ageusia (loss of taste)	12 (23.1%)	3 (2.6%)	0 (0.0%)	15 (8.1%)	<0.001
Diarrhea	4 (7.7%)	18 (15.8%)	1 (5.3%)	23 (12.4%)	0.287
Other[1]	8 (15.4%)	15 (13.2%)	3 (15.8%)	26 (14.1%)	0.855
Total	52 (28.1%)	114 (61.6%)	19 (10.31%)	185 (100%)	

[1]Other symptoms reported to healthcare professionals included: malaise, throat irritation, pain in the face, abdominal pain, inappetence, asthenia, arthralgia, retro orbital, pain and sweating, nausea or vomiting, and red or irritated eyes.

TABLE 5 Genomic strain, Ct value, and classification of symptoms of confirmed cases of SARS-CoV-2 infection and reinfection.

Date of onset of symptoms	Sample RT-PCR	CT (value)	Symptoms	Genomic Strain	Period between infections (days)	Date of onset of symptoms	Reinfection			
							Sample RT-PCR	CT (value)	Symptoms	Genomic Strain
Nov-20	Saliva	28.17	Mild	Zeta (P.2-like)[&&]	214	Sept-21	Saliva	31.92	Mild	ND
Nov-20	Saliva	33.94	Mild	ND	433	Jan-22	Saliva	36.94	Mild	ND
Nov-20	NS	24.25	Mild	Zeta (P.2-like)[&1]	408	Jan-22	NS	27.37	Mild	Omicron (BA.1.15)[&&]
Dec-20	NS	19.22	Mild	B.1.1.33[&2]	299	Dec-21	NS	36.89	Mild	ND
Jan-21	NS	27.95	Mild	Zeta (P.2-like)[&3]	388	Jan-22	Saliva	29.41	Mild	ND
Jan-21	NS	25.61	Mild	Zeta (P.2-like)[&&]	386	Jan-22	Saliva	16.8	Mild	Omicron (BA.1.1)[&10]
Jan-21	NS	14.85	Mild	B.1.1.28[&4]	359	Sept-21	Saliva	36.45	Mild	ND
Jan-21	Saliva	25.52	Mild	ND	258	Jan-22	Saliva	18.58	Mild	Omicron (BA.1.1)[&11]
Jan-21	Saliva	30.44	Mild	B.1.1.28[&5]	360	Jan-22	NS	28.95	Mild	Omicron (BA.1.1.1)[&&]
Jan-21	NS	23.07	Mild	Zeta (P.2-like)[&6]	370	Jan-22	Saliva	36.73	Mild	ND
Jan-21	Saliva	34.9	Mild	ND	354	Aug-21	Saliva	34.24	Mild	ND
Jan-21	Saliva	35.75	Moderate	B.1.1[&&]	339	Jan-22	NS	31.91	Mild	Omicron (BA.1)[&&]
Mar-21	Saliva	30.38	Mild	ND	181	May-22	NS	27.17	Mild	Omicron (BA.2)[&12]
Mar-21	Saliva	28.98	Mild	B.1.1[&&]	427	Aug-21	NS	34.78	Mild	B.1[&&]
Mar-21	Saliva	35.19	Mild	ND	446	Jun-22	NS	24.16	Mild	Omicron (BA.2.23)[&13]
Mar-21	Saliva	32.38	Moderate	ND	314	Jan-22	Saliva	36.31	Mild	ND
Mar-21	NS	21.88	Mild	Gamma (P.1-like)[&7]	222	Aug-21	Saliva	34.78	Mild	ND
Mar-21	NS	22.79	Mild	Gamma (P.1-like)[&8]	287	Jan-22	Saliva	30.54	Mild	Omicron (BA.1.1.1)[&&]

(Continued)

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TABLE 5 Continued

Date of onset of symptoms	Sample RT-PCR	CT (value)	Symptoms	Genomic Strain	Period between infections (days)	Date of onset of symptoms	Reinfection			
							Sample RT-PCR	CT (value)	Symptoms	Genomic Strain
Apr-21	NS	19.17	Mild	Gamma (P.1-like)[89]	267	Jan-22	Saliva	19.39	Mild	Omicron (BA.1)[&14]

Mild symptoms: no oxygen support but other COVID-19 symptoms (which may include congestion or runny nose, cough, sore throat, fever, chills, shortness of breath, change in taste or smell, headache, nausea, vomiting, diarrhea, fatigue, myalgias, and other symptoms).
Moderate symptoms: supplemental oxygen. Severe symptoms: intensive care unit admission.
NS: nasopharyngeal swabs. ND: Not determined. & Submitted in GISAID. && No coverage for submission in GISAID.
Submission number GISAID &1: EPI_ISL_3031326; &2: EPI_ISL_3031330; &3:EPI_ISL_3031331; &4:EPI_ISL_3031335; &5: EPI_ISL_3031336; &6:EPI_ISL_3031338; &7:EPI_ISL_3031346; &8:EPI_ISL_12042548; &9:EPI_ISL_12042549; &10:EPI_ISL_10706100; &11: EPI_ISL_10706101; &12: EPI_ISL_14675090; &13: EPI_ISL_14675091; &14: EPI_ISL_10706102.

4 Discussion

Elucidating the kinetics of the humoral response to SARS-CoV-2 is crucial for controlling the pandemic, and for designing, planning and implementing the most appropriate vaccine schemes (1). Here, we investigated for 450 days the dynamics and longevity of IgM and IgG antibodies from healthcare professionals. The long follow-up period allowed us to monitor dynamics of the humoral response after vaccination booster doses and reinfection.

Many previous studies have evaluated this profile. However, such studies have a variety of follow-up durations ranging from 50 days (33), 100 days (34–37), 210 days (38), 360 days (39), and 480 days (40). Some of these studies followed specific antibodies against the nucleocapsid (N), the receptor-binding domain (RBD), or the spike (S) protein, using either ELISA assays, immunochromatographic tests, or looking for neutralizing antibodies. In our study, a commercial immunochromatographic test was used to detect both IgM and IgG against viral proteins. It has been shown by some authors that there is a correlation between the detection of total and neutralizing antibodies (36, 40, 41).

Our study reinforces the view, and provides evidence, that antibodies are initially produced, but decline over time. The drop of IgM starts on the 30th day, reaching non-reactive levels by the 60th day after symptom onset, the same profile as previously described (36, 37). For IgG, a less pronounced decline is observed, and around the ninth month, we observe the lowest mean reactivity. The stability of the IgG reactivity for three months has been previously demonstrated (36, 37). Gil-Manso et al. (2020) (42) and Gaebler (2021) (43) observed that the IgG response lasted longer, about six months, and the levels of neutralizing activity were proportional to anti-RBD IgG antibody titers. Some studies demonstrate that the duration of the response depends on the studied target: anti-RBD antibodies remained stable for between six to 12 months, while anti-N antibodies decreased over the same period (39). In contrast, Yang (2022) (40) described a peak of anti-RBD antibodies around 120 days after the onset of symptoms with a subsequent decline, maintaining positivity until day 400 after symptom onset. A recent study describes that for non-vaccinated individuals, IgG antibodies, evaluated by ELISA, persist for one year (44).

The results of longitudinal studies, however, may critically vary according to different conditions and variables. Mioch et al. (2023) (44) report that loosening epidemiological control measures increases the chance of re-exposure to the virus. The sensitivity of the tests and different methods used may vary. In addition, patients with comorbidities may have different antibody kinetics, as demonstrated by the rapid decline of antibodies in diabetic patients (45). Yang et al. (2022) (40) conducted a long follow-up of the humoral response in individuals confirmed to be free of re-exposure and vaccination against SARS-CoV-2. Although their study is significant, it does not portray the global reality since we have more than 60% of the population vaccinated worldwide, while in Brazil more than 80% of people completed the vaccination schedule and almost 50% took a booster dose (1). In our cohort, all healthcare professionals who worked on the front line completed the vaccination schedule with the Coronavac vaccine and took the Pfizer vaccine as a booster dose (6)

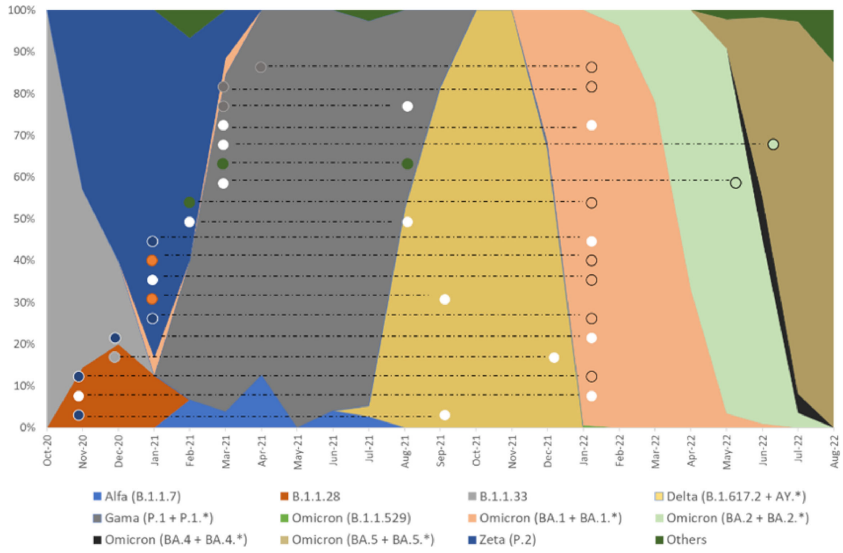

FIGURE 7
Overlap of genomic strains sequenced in this study in relation to strains circulating in Belo Horizonte during the same period. Absolute frequency of SARS-CoV-2 genomic samples sequenced in Belo Horizonte from October 2020 to August 2022 (Stacked area graph). The colors inside the circles indicate the sequence of SARS-CoV-2 strains. White dots represent samples for which the SARS-CoV-2 strain was not determined. The dashed lines connect results obtained in samples from the same participant during their first infection and subsequent reinfection.

For our analyses, we considered the peak of reactivity to be between days 7-30 (D7-D30). Thus, there was no difference in the increase of antibody levels for fully and partially vaccinated and unvaccinated individuals. Based on the dynamics presented, the slight increase observed in the mean antibody levels (D60-D90) may have been induced by the Coronavac vaccination (46). After using the Generalized Estimating Equations (GEE), it is possible to establish that the Pfizer booster dose increased antibody levels for both IgM and IgG. The decline of antibodies after the Coronavac vaccination, even in individuals who became infected, has also been already described. The booster dose is essential to restimulate the humoral response (47–49) and the same profile is also observed for other vaccines (50–53).

Vaccines are essential to reduce the morbidity and mortality from COVID-19. However, they cannot completely prevent new infections and reinfections (50). In our cohort, 36% of participants were reinfected either before (31.6%) or after (68.4%) the booster dose. The reinfections coincided with the spread of new variants in Brazil, such as Delta and Gama in August-September 2021, and Omicron in December 2021. The emergence of new variants has been a matter of great concern, as they can reduce neutralization and even escape vaccination, as demonstrated for Delta (46, 54), Beta and Gamma (47, 55), and Omicron (56–58) variants.

Cases of reinfection have been reported since the beginning of the pandemic (12, 20, 59–61), including in Brazil (62–64). Studies have shown that an acquired immune response can reduce the risk of transmission by up to 90%, with an interval of 6-10 months (65–67). The reinfection rate is relatively low, ranging from 0.1-0.65 (18–20, 68–70), with the highest rates reported in the UK study at 1.9% and 4.5% in India (24). In our study, we reported a high rate of reinfection that might be due to some important factors. Most of the reinfections occurred nine months after the first infection, when antibody levels were already low. Additionally, reinfections coincided with the entry of new variants into Brazil, which, as already mentioned, have a high rate of transmission, and escape from immune responses (14, 21, 25, 67, 71).

Some studies show that men are more likely to test positive and develop severe COVID-19 (72). Petersen et al. (2022) (73) showed a faster decrease in IgG in males. Frauke et al. (2022) (41)observed that the decline of neutralizing antibodies in men was faster than in women but that afterward, there was no difference in response. The same was observed by (74), in which no correlation exists between neutralizing antibodies and biological sex. We also did not notice any difference in the behavior of the humoral response between men and women. Evaluating reinfection cases, our data corroborate the data of Alexander Lawandi et al. (2022) (75), in which we observed a higher rate of reinfection in women, which contrasts with the review made by Sahar (2022) (24), while other studies did not find a relationship between sex and reinfection (76). This heterogeneity of results demonstrates that other factors must be evaluated, such as comorbidity, lifestyle, workplace, biological and immune differences. Understanding sex differences is fundamental to improving disease management, predicting outcomes, and planning specific interventions for men and women.

There is no relationship between age and cases of reinfection in health workers in our study, as demonstrated by Alejandra Svartza (2023) (77). There was also no difference between the general population and healthcare workers who were reinfected. Ren et al. (2022) (22) and Sahar Ghorbani (2022) (19) review that there is a wide age distribution among reinfected patients, ranging from 15 to 99 years. Hansen (2021) (78) described that an age greater than 65 years might influence the increase in the relative risk of reinfection. Healthcare professionals in our cohort who became reinfected were between 24 and 55 years old, and all of them had mild symptoms.

TABLE 6 Seroconversion of IgM and IgG after infection, vaccine doses, and reinfection.

Infection				Vaccination							Reinfection			
Date of onset of symptoms	Symptoms	Seroreactivity after 30 days (IgM)	Seroreactivity after 30 days (IgG)	Date of full vaccination	Vaccination time relative to infection time (days)	Seroreactivity after full vaccination (IgM)	Seroreactivity after full vaccination (IgG)	Booster dose date	Days between infection and booster dose	Period between infections (days)	Date of onset of symptoms	Symptoms	Seroreactivity after reinfection (IgM)	Seroreactivity after reinfection (IgG)
Nov-20	Mild	No	No	Feb-21	109	No	N/A	Nov-21	361	214	Sept-21	Mild	Yes	Yes
Nov-20	Mild	No	No	Mar-21	98	No	N/A	Nov-21	350	433	Jan-22	Mild	No	Yes
Nov-20	Mild	No	No	Feb-21	93	No	Yes	Oct-21	330	408	Jan-22	Mild	Yes	Yes
Dez-20	Mild	Yes	Yes	May-21	155	No	Yes	Feb-22	421	299	Dec-21	Mild	Yes	Yes
Jan-21	Mild	No	Yes	Feb-21	52	No	Yes	Dec-21	359	388	Jan-22	Mild	Yes	Yes
Jan-21	Mild	No	No	Mar-21	53	No	No	Oct-21	268	386	Jan-22	Mild	No	No
Jan-21	Mild	No	Yes	Feb-21	46	No	Yes	Oct-21	280	359	Sept-21	Mild	No	No
Jan-21	Mild	No	Yes	Apr-21	85	No	Yes	Oct-21	281	258	Jan-22	Mild	No	Yes
Jan-21	Mild	Yes	Yes	Apr-21	85	Yes	Yes	Jun-22	504	360	Jan-22	Mild	Yes	Yes
Jan-21	Mild	Yes	Yes	Apr-21	73	No	Yes	Oct-21	276	370	Jan-22	Mild	No	No
jan-21	Mild	No	No	Feb-21	24	No	No	Oct-21	265	354	Aug-21	Mild	No	No
Jan-21	Moderate	Yes	Yes	Mar-21	48	No	Yes	Nov-21	276	339	Jan-22	Mild	No	Yes
Mar-21	Mild	Yes	Yes	Feb-21	-33	Yes	Yes	Oct-21	212	181	May-22	Mild	No	Yes
Mar-21	Mild	Yes	Yes	Feb-21	-25	Yes	Yes	Nov-21	245	427	Aug-21	Mild	No	No
Mar-21	Mild	Yes	Yes	Mar-21	-9	Yes	Yes	Oct-21	222	446	Jun-22	Mild	No	Yes
Mar-21	Moderate	Yes	Yes	Feb-21	-26	Yes	Yes	Oct-21	220	314	Jan-22	Mild	No	Yes
Mar-21	Mild	Yes	Yes	Mar-21	-21	Yes	Yes	Oct-21	205	222	Aug-21	Mild	Yes	Yes
Mar-21	Mild	No	Yes	May-21	-52	No	Yes	Nov-21	229	287	Jan-22	Mild	No	Yes
Apr-21	Mild	No	Yes	Mar/21	-50	No	Yes	Out-21	169	267	Jan-22	Mild	No	Yes

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Even though age was not associated with reinfection, we observed that the dynamics of antibody levels varied over time and behaved differently across age groups. The level of antibodies produced by the younger group was lower than the older groups, as observed by others works (73, 79, 80).

Many studies have sought to understand and relate antibody profiles and Ct values with disease severity and protection against reinfection (34, 40, 81, 82). Omid Dadras et al., 2022 (83), concluded that the relationship between viral load and disease severity is inconclusive. We observed no significant difference in viral load at first infection and reinfection. Except for two participants who required oxygen support for their first infection, all other participants had mild disease. As expected, the main symptoms reported were nasal congestion, coughing, sore throat, and headache. Other symptoms were also reported as described in other studies (22). The reinfection period of most participants in our study overlaps with the spread of the Omicron variant, as described by Menni et al. (2022), Karina Vihta (2022) (84, 85) and Machado Curbelo (2022) (86), who demonstrated that anosmia and ageusia were less frequently associated with the Omicron variant. No reinfected participants reported anosmia or ageusia in our study. Symptoms such as cough, fever, shortness of breath, myalgia, fatigue, and headache were less frequently reported by participants who had suspicion of COVID-19 either confirmed or not by RT-qPCR. In contrast, sore throat was more frequently reported by those participants, although no significant difference in frequency was observed among infection/reinfection status. These clinical conditions corroborate those described by Karina Vithta (2022) (85).

Our study has some limitations. We evaluated total antibodies and did not determine if they were neutralizing, nor the quality of memory B cells necessary to produce antibodies against reinfection. Also, we monitored reinfection in symptomatic participants, but there is a possibility that cases of asymptomatic reinfection also occurred (24).

The humoral response declines after the first infection and vaccination but increases substantially after reinfection and booster doses, especially for the IgG antibody class. There is no association between circulating antibody levels and cases of reinfection. Overall, we demonstrated that even after the booster dose, health professionals can become reinfected with new variants of SARS-CoV-2. Therefore, our study demonstrates that prolonged protection after COVID-19 infection, even after the booster dose, does not prevent reinfection by new variants, which contrasts with the prolonged immune response cited by other studies (73, 87). Studies show that previous infection and booster dose reduce the risk of reinfection, as seen in Switzerland (88), Qatar (68), and United States of America (89). However, studies have shown a significant decrease in the effectiveness of the vaccine against the Omicron variant within a few months after administration (90). Although our study was not designed to assess whether the booster dose would prevent reinfection, the evidence suggests that the booster dose was not effective in preventing reinfection. Those differences might have been impacted by the vaccine scheme and/or type used during the vaccination campaign.

In this context, our data reinforce the importance of robust surveillance in viral genomics and in the immune response of individuals, especially in high-risk individuals, such as the immunocompromised and health care professionals. Investing in these tools is essential for preparing and responding to new variants and future pandemics.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena/browser/view/PRJEB49204, PRJEB49204 (The European Nucleotide Archive).

Ethics statement

The studies involving human participants were reviewed and approved by Institutional review board of the Instituto René Rachou, Fundação Oswaldo Cruz, CAAE: 31.919520.8.0000.5091, approval numbers: 4177931; 4291836; 4343318; 4624187; 5294423. Written informed consent was obtained from all participants before any study procedure was undertaken. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, CF, MW, PA and RS-P; methodology, CF, GF, PA and SG; validation, NF, TS, TGS and WB; formal analysis, AF-L, GF, ES and TS; investigation, NF, TS, TGS and WB; resources, BA, CF, MM, MW and PA; data curation, CF, NF, TGS, RS-P and WB; writing—original draft preparation, AF-L; writing review and editing, CF, GF, MW, NF, PA, RS-P, SG, TS, TCS, TGS and WB; visualization, AF-L, CF and PA; supervision, CF; project administration, CF and WB; funding acquisition, CF. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1220600/full#supplementary-material

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Cellular immunity to SARS-CoV-2 following intrafamilial exposure in seronegative family members

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Introduction: Family studies of antiviral immunity provide an opportunity to assess virus-specific immunity in infected and highly exposed individuals, as well as to examine the dynamics of viral infection within families. Transmission of SARS-CoV-2 between family members represented a major route for viral spread during the early stages of the pandemic, due to the nature of SARS-CoV-2 transmission through close contacts.

Methods: Here, humoral and cellular immunity is explored in 264 SARS-CoV-2 infected, exposed or unexposed individuals from 81 families in the United Kingdom sampled in the winter of 2020 before widespread vaccination and infection.

Results: We describe robust cellular and humoral immunity into COVID-19 convalescence, albeit with marked heterogeneity between families and between individuals. T-cell response magnitude is associated with male sex and older age by multiple linear regression. SARS-CoV-2-specific T-cell responses in seronegative individuals are widespread, particularly in adults and in individuals exposed to SARS-CoV-2 through an infected family member. The magnitude of this response is associated with the number of seropositive family members, with a greater number of seropositive individuals within a family leading to stronger T-cell immunity in seronegative individuals.

Discussion: These results support a model whereby exposure to SARS-CoV-2 promotes T-cell immunity in the absence of an antibody response. The source of these seronegative T-cell responses to SARS-CoV-2 has been suggested as cross-reactive immunity to endemic coronaviruses that is expanded upon SARS-CoV-2 exposure. However, in this study, no association between HCoV-specific immunity and seronegative T-cell immunity to SARS-CoV-2 is identified, suggesting that de novo T-cell immunity may be generated in seronegative SARS-CoV-2 exposed individuals.

KEYWORDS

SARS-CoV-2, COVID-19, exposed seronegative, family, T-cells

Introduction

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) offered an unprecedented opportunity to study immunity to a novel pathogen in an immunologically naïve population. Early in the coronavirus disease 19 (COVID-19) pandemic, ongoing lockdowns provided an opportunity to examine SARS-CoV-2-specific immune responses in family households where transmission between close contacts was common and social mixing outside of households was limited. In 2020, a significant degree of our understanding of SARS-CoV-2 transmission risk arose from small case studies, many of which involved intrafamilial transmission (1–3). As well as querying transmission dynamics, another benefit of family studies of SARS-CoV-2 infection is the opportunity to interrogate age- and sex-related determinants of immunity.

The role of age in disease severity and immune response is well characterized: low-grade inflammation, thymic aging, and reduced cellular functionality in older individuals associated with "immunosenescence" promote worse disease outcomes for many infections in the elderly (4). Children experience low rates of COVID-19 mortality, perhaps owing to their increased numbers of naïve T cells, high degree of exposure to related respiratory viruses, and reduced inflammatory phenotypes (5). Furthermore, sex-specific differences in immune response owing to the location of immune genes on sex chromosomes, the immunomodulatory effects of sex hormones, and differential cytokine profiles in male and female patients generally promote greater adaptive immune responses and stronger autoimmune phenotypes in female patients (6). These established trends raised the possibility that weaker adaptive immune responses may contribute to the worse disease outcomes and higher mortality identified in male patients with COVID-19 (7). Studying SARS-CoV-2-specific immunity in families provided an opportunity to test these hypotheses in male and female patients of diverse ages with differing levels of exposure to SARS-CoV-2.

Additionally, it has been demonstrated that individuals highly exposed to SARS-CoV-2, as well as other viruses such as hepatitis C virus (HCV), can generate pathogen-specific cellular immunity in the absence of specific antibodies (8–10) and, in the case of SARS-CoV-2, that these cellular responses are of higher magnitude than would be expected from cross-reactivity (such as with the endemic human coronaviruses [HCoVs] OC43, HKU1, 229E, and NL63 that circulate widely in the UK, causing common cold symptoms) in unexposed individuals. The source of elevated cellular responses in SARS-CoV-2-exposed individuals is believed to be expansion of pre-existing memory responses to HCoVs that abort SARS-CoV-2 infection before seroconversion can occur (9). However, this seronegative cellular immunity has not been extensively explored. Questions that remain include the following: How much exposure is required to generate this immunity? Are these responses wholly cross-reactive memory responses against HCoVs or does seronegative exposure also generate *de novo* responses to non-conserved T-cell epitopes? Are T-cell responses equally distributed among seronegative adults and children?

Here, a large dataset of SARS-CoV-2-infected and -exposed families was generated through recruitment of families in Oxford, London, and Cardiff during the winter of 2020 and the spring of 2021. As well as assessing transmission dynamics of SARS-CoV-2, the aim of this study was to characterize immunity to primary SARS-CoV-2 infection in family members, with a focus on differences in age and sex. Furthermore, we examine the quality and likely source of specific T-cell immunity in seronegative individuals.

Materials and methods

Ethics

Venous blood samples were donated from October 2020 to March 2021. Eligible participants were individuals aged 6 years or above who either had experienced symptoms of COVID-19 or had a family member who had. Samples from Wales were collected as part of the CROWN study, where index patients from the CROWN study were recruited along with their families. Families from Oxford and London were recruited by word of mouth from different neighborhoods, whereas families in Cardiff were recruited through GP visits. Families were visited and blood was collected

only once from each family. It was generally possible to collect samples from all members of a household, except on some occasions from children whose parents did not consent or from whom sufficient blood samples were difficult to obtain for practical reasons. As this study was carried out before widespread PCR and lateral flow testing and vaccines became available, history of SARS-CoV-2 infection was determined by anti-Spike (S) immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) above a threshold of 10 ELISA units (EU). ELISAs were carried out by collaborators at the Oxford Vaccine Group on 232/264 individuals. Written informed consent was obtained from patients; ethical approval was granted by the Central University Research Ethics Committee (CUREC R71346/RE001) and Brighton and Sussex HRA Research Ethics Committee (IRAS reference 269506).

Sample collection and processing

Whole blood EDTA samples were transported from site of sampling to the laboratory and processed within 6 h. Isolation of peripheral blood mononuclear cells (PBMCs) and plasma was carried out as described elsewhere (11). Briefly, PBMCs were separated by density gradient centrifugation using Lymphoprep (1.077 g/ml, Stem Cell Technologies). PBMCs were washed twice in RPMI 1640 (Sigma, USA) with 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin (Sigma, USA), and 2 mM L-glutamine (Sigma, USA). Plasma was spun at 2,000*g* for 10 min to remove platelets. Cells were resuspended in RPMI and counted using a Muse Cell Analyser (Luminex, USA). Assays were run on fresh PBMCs, or samples were cryopreserved as 0.5-ml aliquots in 90% fetal calf serum with 10% dimethylsulfoxide (DMSO) and stored at −80°C for later use.

Serological assays

As well as ELISA, total IgG targeting SARS-CoV-2 S, receptor-binding domain (RBD), and nucleocapsid (N) was quantified using a Meso Scale Diagnostics (MSD) v-plex immunoassay "Coronavirus panel 3" (MSD, USA) according to the manufacturer's protocol and as described elsewhere (12). Plates were incubated in Blocker A for 30 min at room temperature with 700 rpm shaking. Serum was diluted 1/1,000 and 1/10,000 in Diluent 100. A seven-point standard curve of MSD reference standard starting at 1/10 was prepared in duplicate; three internal controls and one in-house control of COVID-19 convalescent serum was used. Diluent 100 was used as a negative control. Plates were washed three times in MSD Wash buffer and samples and controls were added to the plate. Plates were incubated for 2 h at room temperature with shaking. Plates were washed three times again before addition of detection antibody and incubation for 1 h with shaking. Plates were washed three times, MSD Gold read buffer was added, and plates were immediately read with a MESO QuickPlex SQ 120 (MSD, USA). Data were analyzed in MSD Discovery Workbench. The threshold for S, RBD, and N positivity (S: 1,160 AU/ml, RBD: 1,169 AU/ml, N: 3,874 AU/ml) was taken from analyses of pre-pandemic sera (12).

Total IgG targeting SARS-CoV-2 S2 was quantified using indirect ELISA. S2 antigen (Sino Biological, China) was diluted to 1 µg/ml in PBS and used to coat 535 Nunc-Immuno 96-well plates (Thermo Fisher Scientific, USA) at 4°C overnight. Plates were washed three times in PBS with 0.1% Tween before blocking with Casein Buffer for 1 h at room temperature. Serum was diluted in Casein Buffer 1/500 and plated in duplicate alongside a 10-point standard curve of pooled COVID-19 convalescent sera beginning at 1/25. Casein Buffer was used as a blank. Plates were incubated at room temperature for 2 h and washed six times in PBS with 0.1% Tween. Goat anti-human IgG conjugated to alkaline phosphatase (Sigma, USA) was diluted to 1/1,000 in Casein Buffer and added to plates for 1 h at room temperature. Plates were washed six times in PBS with 0.1% Tween. 4-Nitrophenyl phosphate in diethanolamine buffer (Pierce, UK) was added and plates were incubated for 15 min. Plates were read on an ELx800 microplate reader at 405-nm absorbance (Cole Parmer, UK). Concentrations were calculated by mapping a line of best fit onto the standard curve, then substituting mean absorbance values for each sample into the line equation. This was then multiplied by the dilution factor of 500 to give the final result.

Proliferation assay

T-cell proliferation was quantified using a CellTrace Violet proliferation assay as described elsewhere (11). Frozen PBMCs were thawed in RPMI with 10% fetal calf serum, 1% penicillin/streptomycin, and 2 mM L-glutamine. After washing twice with PBS, cells were stained with CellTrace Violet at 2.5 µM (Life Technologies, USA) for 10 min at room temperature. Cold fetal calf serum was added to quench the stain. Cells were plated in 96-well round-bottom plates at 250,000 cells per well. Pools of 18-mer peptides overlapping by 10 amino acids spanning the whole SARS-CoV-2 genome [S1, S2, membrane (M), N, open reading frame (ORF) 3, ORF8, non-structural protein (NSP) 1 + 2, NSP3A, NSP3B, NSP3C, NSP4, NSP5 + 6, NSP7-11, NSP12A, NSP12B, NSP13, NSP14, and NSP15 + 16]) were added to wells at a final concentration of 1 µg/ml. RPMI was used as a negative control, and phytohemagglutinin L (Sigma, USA) was used as a positive control at a final concentration of 2 µg/ml. Plates were incubated for 7 days at 37°C, 5% CO_2, 95% humidity with a hemimedia change on day 4. Cells were then washed in PBS and stained for CD4, CD8, and CD3 in PBS with fluorochrome-conjugated antibodies (BioLegend, USA). LIVE/DEAD Aqua was used to stain dead cells (Thermo Fisher Scientific, USA). Cells were fixed in 4% PFA (Sigma, USA) at 4°C for 10 min, washed in PBS, and stored at 4°C in the dark. Samples were acquired the next day on a MACSQuant X (Miltenyi, Germany) and analyzed in FlowJo. Cutoff for positive responses was set at 1% proliferation as determined previously (11).

Neutralization assay

Neutralizing antibody (nAb) titers were calculated using a SARS-CoV-2 lentivirus-based pseudovirus assay displaying a

codon-optimized SARS-CoV-2 S protein (National Center for Biotechnology Information [NCBI] reference sequence: YP_009724390.1) as described elsewhere (13). Briefly, HEK293 T/17 cells were cultured in complete medium [Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% L-glutamine] and incubated at 37°C/5% CO_2. Pseudotyped viruses were produced by transfecting HEK293T cells with 1 µg of codon-optimized S, 1 µg of gag/pol, and 1.5 µg of a luciferase reporter in a plasmid-OptiMem solution.

For the microneutralization assay, a transfection mix was prepared using 2,500 ng of ACE2, 250 ng of TMPRSS2, 1 ml of OptiMem, and 9 µl of Fugene (ProMega, USA) as part of a plasmid-media mix. Cells (10 ml) were transfected with the plasmid-media mix 24 h before the assay. Five microliters of serum and 95 µl of complete media were added to columns 1 and 7 in a 96-well white opaque culture plate. Complete media (50 µl) was added to columns 2 to 6 and 8 to 12 and serum was serially diluted across the plate in a 1:2 dilution. Pseudotyped virus (50 µl) was added to wells, mixed, and incubated for 2 h at 37°C. Each plate contained six positive controls wells (no serum) and six negative control wells (no pseudovirus). A total of 10^4 plasmid transfected HEK293 T/17 cells were added to each well and incubated for 48 h at 37°C/5% CO_2. Supernatant was removed using vacuum filtration. Bright Glo (Promega, USA) was diluted 1:1 with sterile PBS. Bright Glo-PBS mixture (50 µl) was added to each well and allowed to lyse for 5 min, after which luciferase activity was measured using a GloMax Luminometer (ProMega, US). Data were analyzed using Microsoft Excel and GraphPad Prism.

Statistical analysis

All statistical comparisons, logistic regression, and multivariable linear regression were performed in GraphPad Prism 9.0. For pairwise comparisons, two-tailed Mann–Whitney tests were used for unpaired data. For multiple comparisons, Kruskal–Wallis tests with Dunn's multiple comparisons test were used. For correlations, Spearman's rank tests were used.

Results

Cohort

Families were eligible for the study if at least one family member had experienced symptoms of COVID-19 between October 2020 and March 2021. Over a period of 6 months of recruitment, a total of 264 individuals belonging to 81 families were recruited from Oxford, London, and Cardiff (Figure 1A). From each study participant, whole-blood EDTA samples were taken, and PBMCs and plasma were isolated and cryopreserved for later use (Figure 1B). Fifty-five percent of individuals were women, and the median age was 40, ranging from 6 to 88 years of age (Figure 1C). The cohort was predominantly white British (92%). Fifty-two percent (n = 136) of individuals were seropositive for SARS-CoV-2 anti-S IgG at the time of enrolment and 94% (n = 248) were unvaccinated; vaccinated individuals were excluded from later analysis. A total of 19 individuals self-reported pre-existing medical conditions, including Graves' disease, asthma, gout, epilepsy, type 2 diabetes, Crohn's disease, atrial fibrillation, depression, hypertension, obesity, and celiac disease. In most families (74%), the index patient recruited to the study was the mother, and in 51% of families, the index patient was a healthcare worker (HCW). Of the 136 individuals seropositive for SARS-CoV-2 at enrolment, 127 self-reported symptoms. All symptomatic individuals experienced mild disease and none were hospitalized. The following symptoms were self-reported: cough, fever, anosmia, gastrointestinal symptoms, sore throat, fatigue, myalgia, and a runny nose. Among the 127 individuals that self-reported symptoms, sampling occurred a mean of 228 days (7.6 months) after symptom onset.

Transmission dynamics

To examine the dynamics of SARS-CoV-2 infection within and between families, families with similar patterns of seropositivity were identified and grouped into seven family types (Supplementary Figure 1). These included the following: all seropositive (18 families), all seronegative (6 families), seropositive father/adult male (3 families), seropositive mother/adult female (17 families), seropositive children only (5 families), serodiscordant parents with at least one seropositive child (6 families), and double-seropositive parents with at least one seropositive child (7 families). Interestingly, the rarest groups, therefore, were seropositive children only and adult seropositive male only. All double-seropositive parents had at least one seropositive child. Overall, the families represented a diverse group, indicating that intrafamilial transmission can occur through multiple routes.

To assess whether the number and proportion of individuals infected within a family increased someone's risk of infection, the following logistic regression was carried out:

$$\text{Infected ?} \sim \text{Intercept} + \text{Proportion infected} + \text{Family size}$$

The model demonstrated that, holding family size constant, the odds of an individual becoming infected increased by 15% (95% CI [1.04 to 1.28]) for every 10% increase in the percentage of family members infected (p = 0.001). There was no increased infection risk associated with family size (p = 0.12).

Immune dynamics within families

To compare the immune dynamics of SARS-CoV-2 infection between family members, first, a representative case study was examined in detail. A fully seropositive family (Family 008), consisting of a 39-year-old mother, a 41-year-old father, two daughters, and two sons, is shown in Figure 2A. All individuals made IgG responses to SARS-CoV-2 S; the response of greatest magnitude was the father (1,011 EU), and the response of lowest

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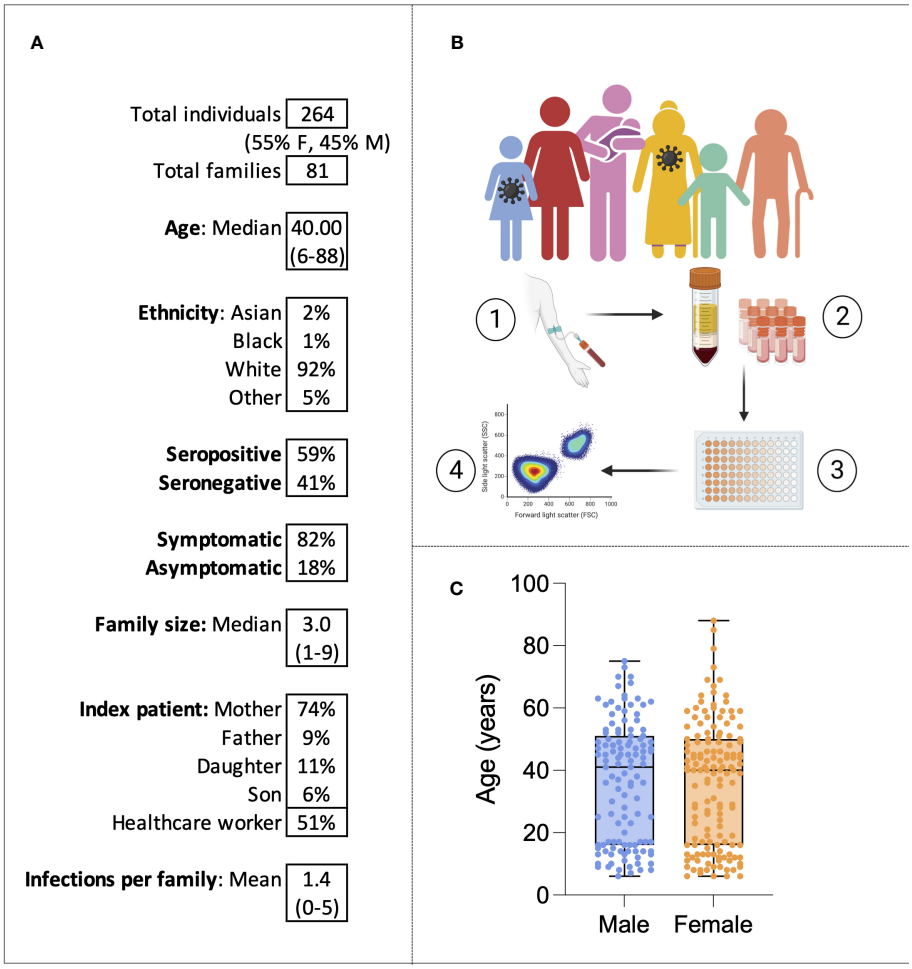

FIGURE 1
The family cohort. Characteristics of the cohort **(A)**. Graphical representation of the study: sampling of infected individuals and their family members, PBMC and serum isolation, assays, and analysis **(B)**. Age distribution of the cohort by sex **(C)**. Virus symbols indicate exemplar seropositive individuals.

magnitude was the mother (18 EU). The greatest nAb response (IC_{50} = 74) and S1-specific CD4+ T-cell response (14% proliferation) also belonged to the father. All individuals made CD4+ T-cell responses to either S1 or S2 pools; CD8+ responses were weaker.

To compare immune responses between all mothers, fathers, daughters, and sons from the "all seropositive" group, and to assess any age- or sex-related differences in immunity, IgG, nAb titer, and S1-specific CD4+ and CD8+ T-cell responses were compared (Figure 2B). Notably, there was no difference in IgG, nAb response, or S1-specific CD4+ or CD8+ T-cell response between any of the family members in this group (IgG: p = 0.65, nAb: p = 0.22; T cell: p = 0.76, Kruskal–Wallis test with Dunn's multiple comparisons).

We next assessed SARS-CoV-2-specific immune dynamics in "all seronegative" families to identify potential cross-reactive immunity from endemic HCoVs. Although these individuals were seronegative, in order to be recruited onto the study, at least one individual self-reported symptoms of COVID-19. A representative case study family was first assessed (Figure 3A). Family 046 consisted of a mother, father, three daughters, and two sons who generated no IgG or nAbs to SARS-CoV-2, as defined (Figure 3A). T-cell responses were absent in most family members, although the 22-year-old daughter generated a weak CD4+ response to S2 (1.3% proliferation), and the 15-year-old daughter generated a weak CD4+ response to N (1.2% proliferation).

To determine if any form of SARS-CoV-2-specific immunity was present in members of the "all seronegative" group, and to uncover any age- and sex-specific trends, IgG, nAbs, and T-cell responses were compared between all mothers, fathers, daughters, and sons in the "all seronegative" group (Figure 3B). Of note, some mothers and fathers generated CD4+ T-cell responses to S1. However, no children generated a CD4+ or CD8+ response to S1, raising the question of whether pre-existing cellular immunity may be found at higher levels in older individuals.

Case study families of mixed-serostatus families were also assessed (Supplementary Figures 2, 3). Cross-reactive cellular immunity appeared widespread in seronegative individuals who

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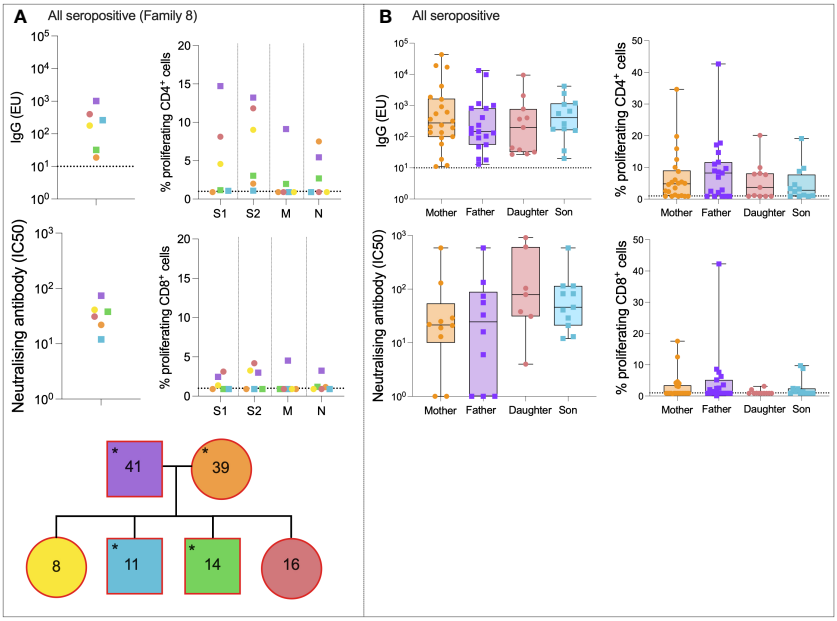

FIGURE 2
All seropositive families. Anti-S IgG, nAb responses, CD4+ T-cell responses, and CD8+ responses in a seropositive family group consisting of mother, father, two sons, and two daughters **(A)**. IgG, nAb, and T-cell responses in all individuals from the "All seropositive" family type **(B)**. Male patients are squares; female patients are circles. Seropositive individuals are outlined in red. Asterisks refer to symptomatic individuals. Proliferation values below 1% were given nominal values of 0.9%. Dotted lines refer to cutoffs as determined previously (11, 12).

had been exposed to SARS-CoV-2 through infected family members. These T-cell responses were present in both seronegative adults and seronegative children.

The cohort consisted of individuals who experienced only mild symptoms; however, these symptoms were self-reported at the time of sampling and therefore individuals could be classed as symptomatic or asymptomatic. Symptomatic individuals had significantly greater magnitude S1-, M-, and N-specific CD4+ T-cell responses (p = 0.002, p = 0.004, and p = 0.009, respectively; Mann–Whitney tests) but anti-S IgG and nAb responses did not differ significantly between symptomatic and asymptomatic individuals.

Age- and sex-specific trends in immunity

To analyze more closely if age and sex had an impact on the magnitude of humoral or cellular immune responses, two multivariable linear regressions were run. Model 1 calculated the effects of age, sex, and days since symptom onset on total IgG response in seropositive individuals. The fitted regression model was:

$$\text{IgG} = 2,744 - 15.78{*}(\text{Age}) - 195.2{*}(\text{Sex[Male]}) - 5.808{*}(\text{Days since symptoms})$$

The overall regression was statistically significant (R^2 = 0.22, F (3, 35) = 3.29, p = 0.03). It was found that neither age (β = −15.78, p = 0.26) nor male sex (β = −195.2, p = 0.67) significantly predicted IgG response. However, days since symptoms (β = −5.808, p = 0.017) was negatively associated with IgG response, indicating some waning of humoral immunity over time.

Model 2 calculated the effects of age, sex, and days since symptom onset on S1-specific CD4+ T-cell responses in seropositive individuals. The fitted regression model was:

$$\text{CD4 + response} = 4.82 + 0.23{*}(\text{Age}) + 8.57{*}(\text{Sex[Male]}) - 0.037{*}(\text{Days since symptoms})$$

The overall regression was statistically significant (R^2 = 0.36, F (3, 35) = 6.69, p = 0.001). It was found that age (β = 0.23, p = 0.008), male sex (β = 8.57, p = 0.004), and days since symptoms (β = −0.037, p = 0.01) were significantly associated with CD4+ response. This indicated that older men generated stronger CD4+ T-cell responses to S1.

Finally, the correlation between anti-S IgG and CD4+ T-cell response was calculated for male and female patients separately. In female patients only, IgG was weakly correlated with S1-specific T cells (r = 0.3, p = 0.006) and S2-specific T cells (r = 0.26, p = 0.02).

Cross-reactive cellular immunity in exposed seronegative individuals

It was hypothesized that that exposure to SARS-CoV-2 through infected family members might expand T-cell immunity to SARS-CoV-2 in seronegative individuals, as has been demonstrated elsewhere (9, 10). To test this hypothesis, individuals were split into three groups—seropositive (n = 121), exposed seronegative (ESN, n = 72) who had at least one seropositive family member, and

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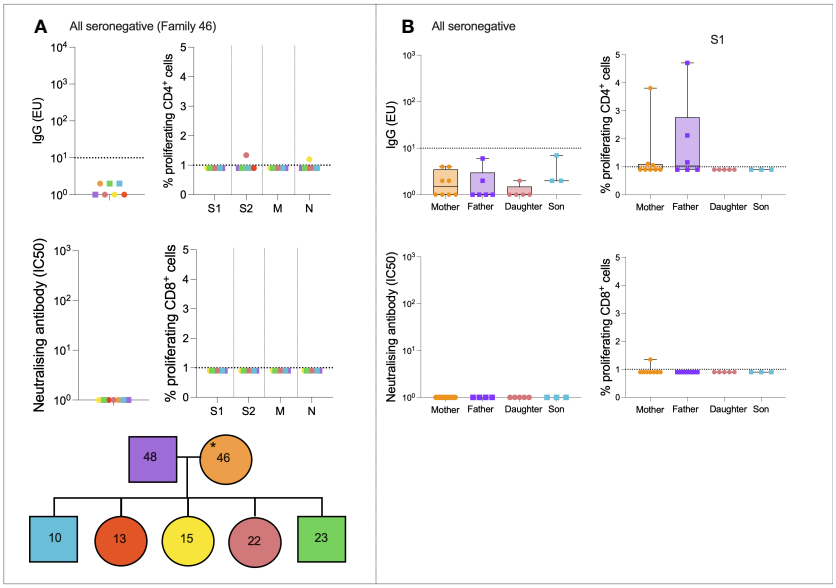

FIGURE 3
All seronegative families. Anti-S IgG, nAb responses, CD4+ T-cell responses, and CD8+ responses in a seronegative family group consisting of mother, father, two sons, and three daughters **(A)**. IgG, nAb, and T-cell responses in all individuals from the "All seronegative" family type **(B)**. Male patients are squares; female patients are circles. Asterisks refer to symptomatic individuals. Proliferation values below 1% were given nominal values of 0.9%. Dotted lines refer to cutoffs as determined previously (11, 12).

unexposed seronegative (USN, $n = 24$) with no history of infection, no seropositive family members, and at least one symptomatic family member.

T-cell responses were then compared between seropositive individuals, ESNs, and USNs (Figure 4). As expected, seropositive individuals generated significantly higher magnitude CD4+ responses compared to ESNs (all $p < 0.0001$, Mann–Whitney tests), and significantly greater CD8+ responses (all $p < 0.0001$, Mann–Whitney tests). Of note, ESNs generated stronger S1-specific CD4+ T-cell responses than USNs ($p = 0.05$, Mann–Whitney test)

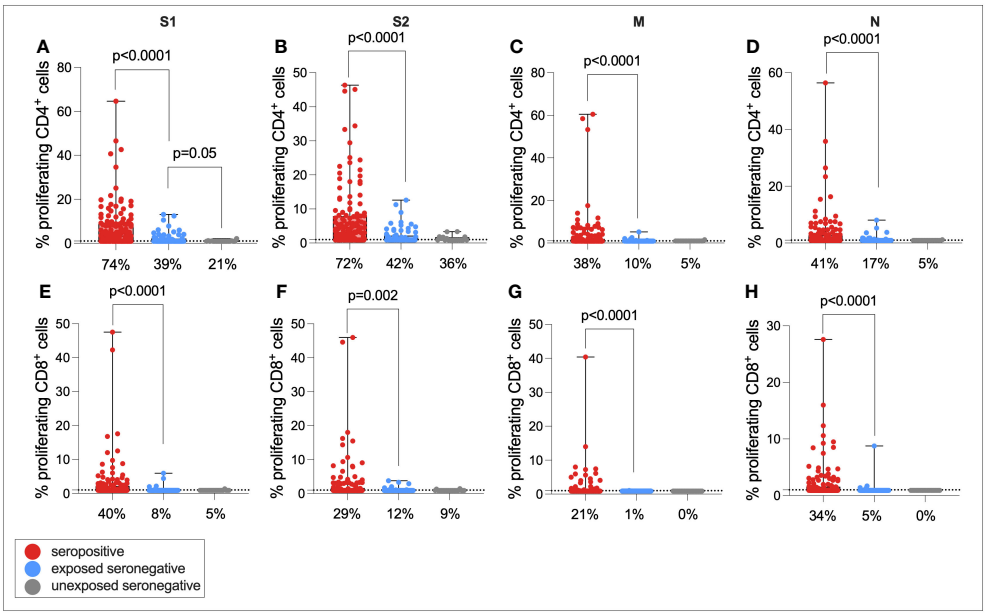

FIGURE 4
Cellular immunity in seropositive, ESN, and USN individuals. CD4+ T-cell responses to SARS-CoV-2 S1 **(A)**, S2 **(B)**, M **(C)**, and N **(D)** as measured by proliferation assay in seropositive individuals (red), ESNs (blue), and USNs (gray). CD8+ T-cell responses to S1 **(E)**, S2 **(F)**, M **(G)**, and N **(H)** as measured by proliferation assay. *p*-values refer to Mann–Whitney test values. Data points below 1% were given nominal values of 0.9%. Dotted lines refer to cutoffs as determined previously (11). Percentages refer to the number of individuals with responses above 1% proliferation.

(Figure 4A), and this trend held for S2, M, and N, although not significantly (S2: $p = 0.6$, M: $p = 0.5$, N: $p = 0.16$, Mann–Whitney tests). There was also a trend of greater CD8+ responses to S1 in ESNs compared to USNs, although again this was not significant ($p = 0.49$, Mann–Whitney test) (Figure 4E).

One possibility is that the same ESNs who generated CD4+ responses to S1 also generated responses to S2, as well as also generating low-level nAb and IgG responses. To test these hypotheses, the correlation between S1-specific CD4+ responses and S2-specific response, nAb response, and IgG response was calculated in ESNs only (Supplementary Figure 4). CD4+ responses to S1 and S2 were correlated ($r = 0.39$, $p = 0.0008$), but responses to S1 were not correlated with nAb or IgG responses, suggesting that ESNs with high magnitude S1-specific T-cell immunity had not simply generated antibody responses that had waned to levels below the limit of detection but remained above zero. Classifying ESN responses in a binary way, irrespective of magnitude, there was a significant relationship between being an S1 responder and being an S2 responder (proliferation>1%) ($p = 0.007$, Fisher's exact test) but not between being an S1 responder and a low-level IgG (EU > 1) or nAb ($IC_{50} > 0$) responder.

It has been demonstrated that a target of T-cell responses in ESNs is the replication–transcription complex (RTC), an early-translated protein that is highly conserved between SARS-CoV-2 and endemic HCoVs (consisting of non-structural protein [NSP] 7, NSP12, and NSP13) (9). To test the hypothesis that ESN individuals generate enhanced responses to the RTC compared to USNs, both CD4+ and CD8+ T-cell responses to pools of overlapping peptides spanning the rest of the SARS-CoV-2 genome (ORF3, ORF8, NSP1 + 2, NSP3A, NSP3B, NSP3C, NSP4, NSP5 + 6, NSP7-11, NSP12A, NSP12B, NSP13, NSP14, and NSP15 + 16) were compared between seropositive, ESN, and USN individuals (Figure 5). Responses were generally stronger in seropositive individuals than ESNs, reaching significance in CD8+ responses to NSP3B ($p = 0.02$, Kruskal–Wallis

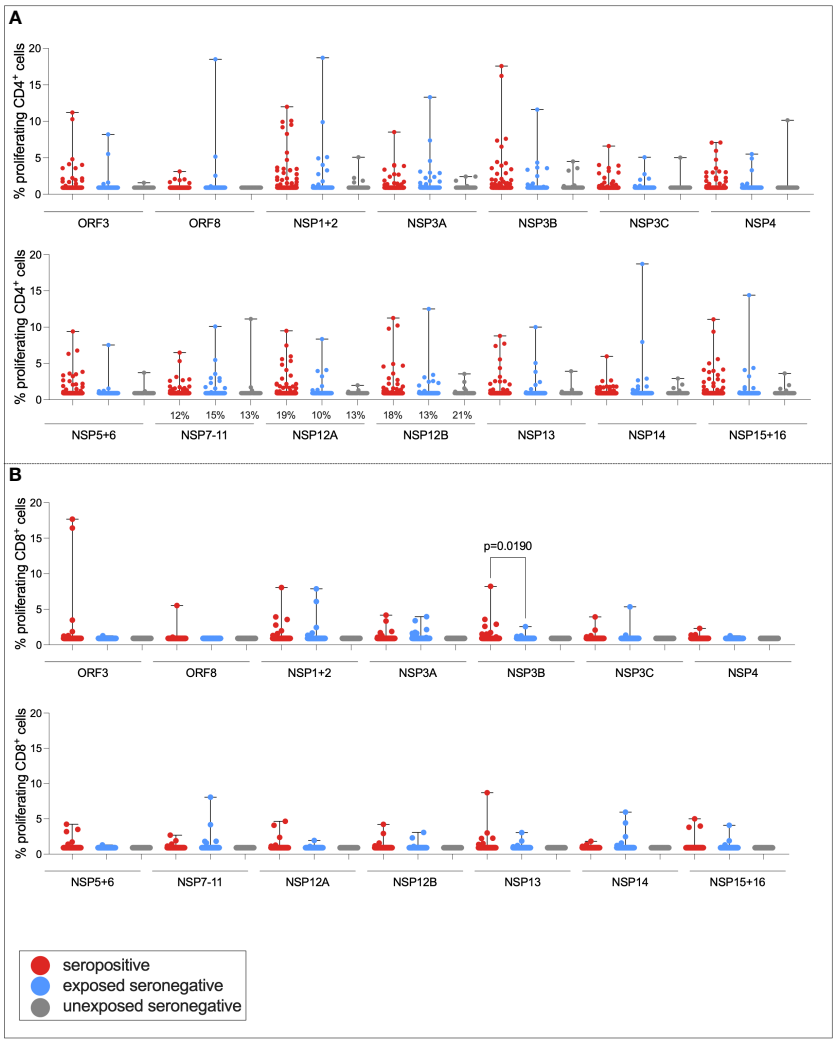

FIGURE 5
T-cell responses to NSPs in ESN individuals. CD4+ T-cell responses targeting SARS-CoV-2 NSPs in seropositive (red), ESN (blue), and USN (gray) individuals **(A)**. CD8+ T-cell responses targeting SARS-CoV-2 NSPs in seropositive, ESN and USN individuals **(B)**. Percentages refer to the number of individuals with responses above 1% proliferation, specifically for the RTC region of the genome. *p*-values refer to Kruskal–Wallis test values with Dunn's multiple comparisons. Data points below 1% were given nominal values of 0.9%.

test with Dunn's multiple comparisons). However, there was no significant difference in responses to the RTC region between ESN and USN individuals, and no obvious trend of greater responses in ESNs. To test for the presence of a T-cell response rather than magnitude, Fisher's exact test identified no association between presence of a T-cell response to NSP7-11, NSP12A, or NSP12B and belonging to ESN vs. USN groups ($p > 0.99$, $p = 0.7$, and $p = 0.3$, respectively).

To determine whether cross-reactive immunity is found at higher levels in seronegative adults compared to children, and whether exposure to SARS-CoV-2 induces T-cell immunity in otherwise T-cell negative children, Spearman rank correlations were calculated between age in years and S1- or S2-specific CD4+ T-cell response for ESNs, USNs, and seropositive individuals (Figure 6). There was a positive correlation between age and S1-specific T cells in USNs ($r = 0.42$, $p = 0.05$) (Figure 6A) and ESNs

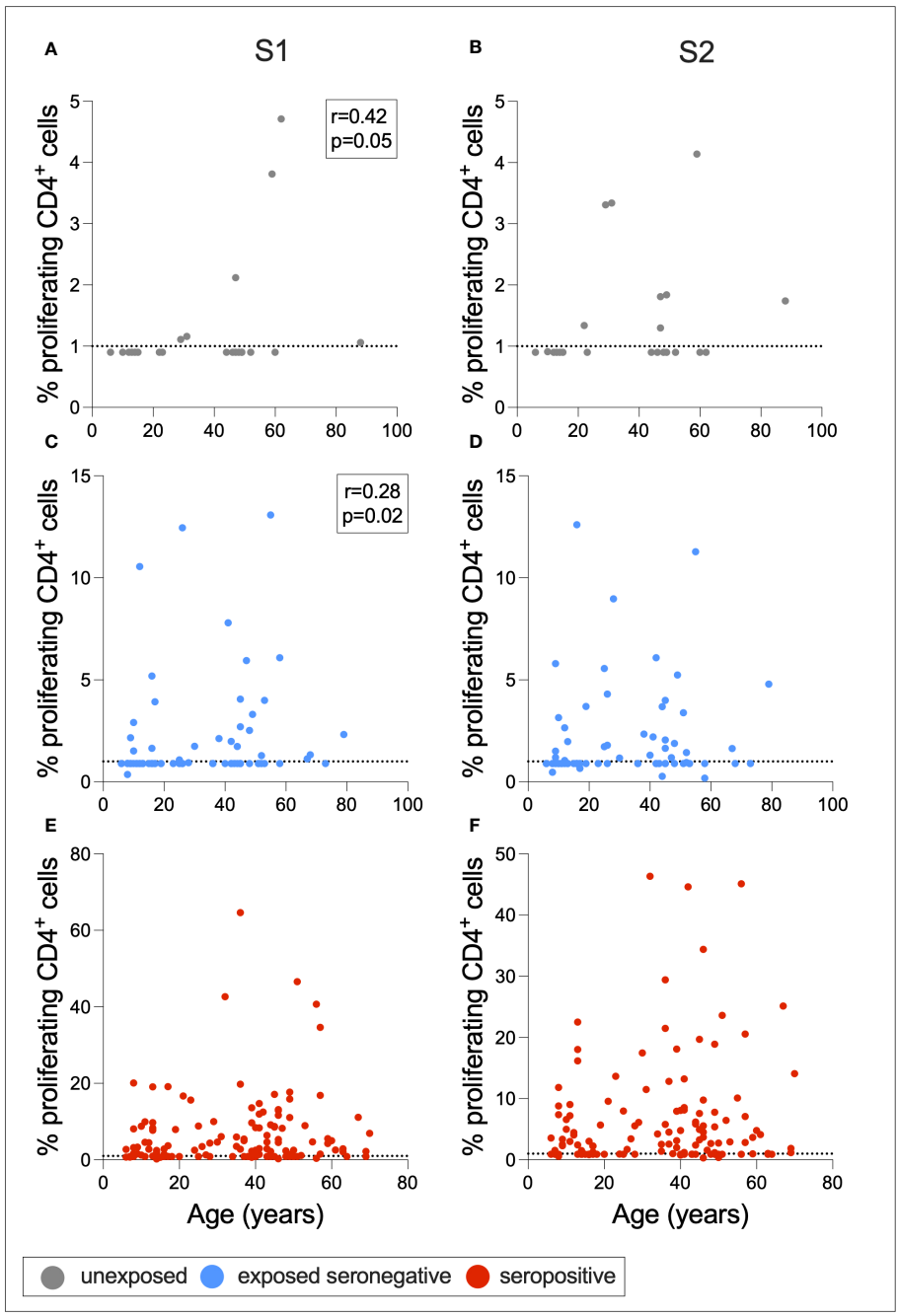

FIGURE 6
Associations between age and T-cell response. Correlations between age and S1- or S2-specific CD4+ T-cell response in USNs (**A, B** gray), ESNs (**C, D** blue), and seropositive individuals (**E, F** red). *R*- and *p*-values refer to Spearman rank test values. Data points below 1% were given nominal values of 0.9%. Dotted lines refer to cutoffs as determined previously (11).

(r= 0.28, p = 0.02) (Figure 6C) (Spearman rank tests), although responses in USNs were of lower magnitude than ESNs and seropositive individuals. There were no T-cell positive USNs under the age of 20, but several T-cell positive ESNs under the age of 20, suggesting that exposure to SARS-CoV-2 may generate T-cell immunity in children and adolescents who otherwise do not display cross-reactive cellular immunity.

As described above, a greater proportion of seropositive individuals in a household was associated with an increased risk of infection. This also raised the question of whether a greater number of seropositive family members would be associated with stronger T-cell immunity in ESNs. To test this hypothesis, CD4+ and CD8+ T-cell responses targeting S1 were compared between seropositive individuals, USNs, and ESNs with different numbers of seropositive family members (Figure 7).CD4+ T-cell responses in seropositive individuals with different numbers of family members were significantly different (p = 0.01, Kruskal–Wallis test) with a trend of increased T-cell immunity in seropositive individuals with fewer infected family members, although no pairwise comparison was significant by Dunn's multiple comparison test (Figures 7A, B). However, S1-specific CD4+ responses in ESNs with two infected family members were significantly higher than in USNs (p = 0.0001, Kruskal–Wallis test with Dunn's multiple comparisons) (Figure 7C). There was no significant difference in T-cell response between individuals with no (n = 22) and one (n = 43) infected family member (p = 0.5, Mann–Whitney test). Of note, ESNs with two infected family members (n = 45) made significantly stronger T-cell responses to S1 than ESNs with one infected family member (p = 0.004, Kruskal–Wallis test with Dunn's multiple comparisons) (Figure 7C), providing a direct link between the intensity of viral exposure and the strength of cellular immunity in seronegative individuals. Responses appeared lower in ESNs with 3+ members infected, although this is likely an artifact of the small numbers of individuals in this group (n = 6).

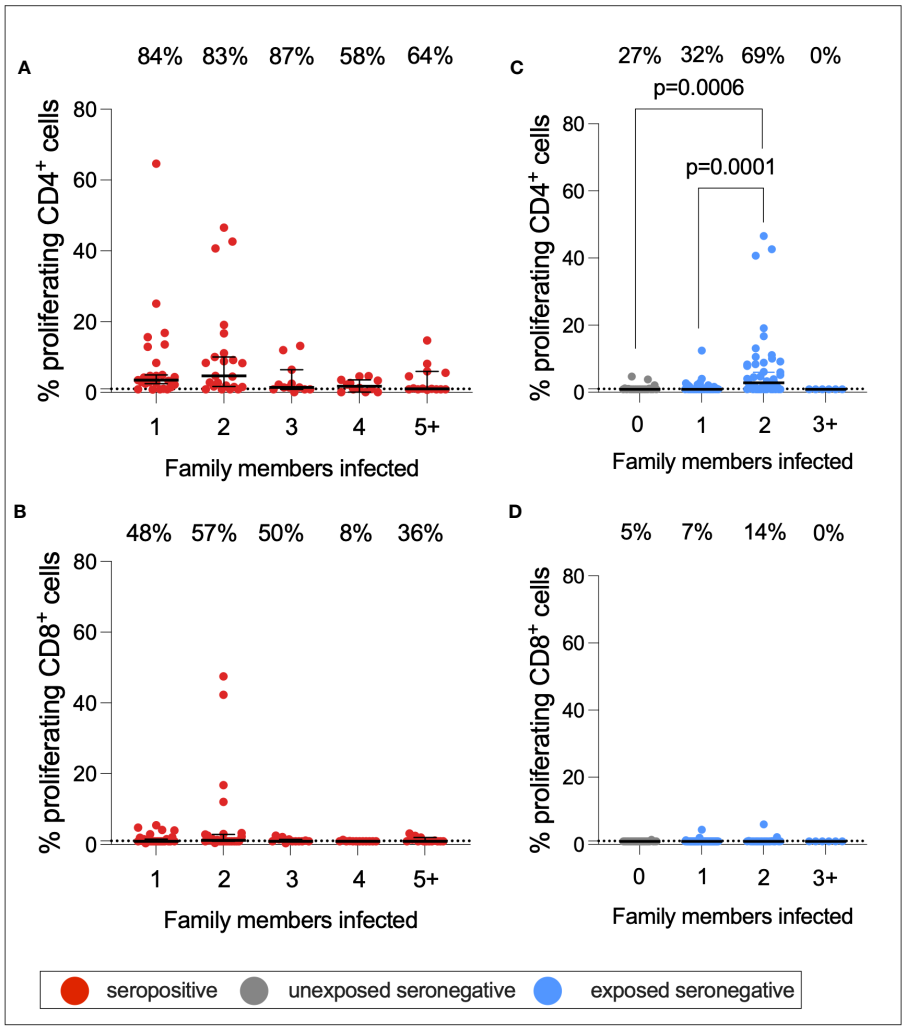

FIGURE 7
S1-specific T-cell responses by number of family members infected. CD4+ **(A)** and CD8+ **(B)** T-cell responses in seropositive (red) individuals with 1–5+ family members infected. CD4+ **(C)** and CD8+ **(D)** T-cell responses in USN (gray) and ESN (blue) individuals with 0–5+ family members infected. p-values refer to Mann–Whitney test values. Values below 1% were given nominal values of 0.9%. Dotted lines refer to cutoffs as determined previously (11). Percentages refer to the number of individuals with responses above 1% proliferation.

To further evaluate the nature of the T-cell response in ESNs, and to assess whether T-cell responses in ESNs target more structural antigens than USNs, the ratio between T cells targeting SARS-CoV-2 structural proteins (SPs) to NSPs was calculated for seropositive individuals, ESNs, and USNs, for both CD4+ and CD8+ T cells (Supplementary Figure 5A) Seropositive individuals had significantly higher SP : NSP ratios than ESNs for both CD4+ (1.8 vs. 0.6, $p < 0.0001$) and CD8+ (0.97 vs. 0.33, $p < 0.0001$) T cells (Mann–Whitney tests). There was no significant different in SP : NSP ratio between ESNs and USNs. To assess the levels of circulating HCoV-specific T cells in these individuals, proliferation assays were carried out on a subset of individuals ($n = 35$) using pools of peptides spanning the S2 region of S from HCoV-OC43 and HCoV-HKU1 (Supplementary Figures 5B, C). Responses to these antigens were small, and there was no significant difference in T-cell responses to HCoV-OC43 or HCoV-HKU1 between seropositive individuals, ESNs or USNs, although there was a trend of greater CD4+ responses in seropositive individuals.

To test the hypothesis that elevated T-cell immunity in ESNs may be due to the expansion of pre-existing, cross-reactive T cells targeting conserved regions of SARS-CoV-2 S, T-cell responses to a pool of 63 peptides highly conserved between SARS-CoV-2 and endemic HCoVs, as defined by Mateus et al. (2020) (14), were compared between seropositive individuals, ESNs, and USNs (Supplementary Figure 5D) (14). Furthermore, to distinguish between the expansion of total S responses versus expansion of conserved responses specifically, the ratio of responses to conserved peptides to S1 response was calculated and compared between groups (Supplementary Figure 5E). There was no significant difference in the magnitude of responses to the conserved pool between seropositive individuals, ESNs, and USNs, although there was a trend towards stronger responses in seropositive individuals. Furthermore, there was no significant difference in conserved S:S1 ratio between seropositive individuals, ESNs, and USNs. Although these comparisons are between small numbers of individuals, this does not support a model of cross-reactive immunity to endemic HCoVs expanding upon SARS-CoV-2 exposure, instead suggesting that *de novo* responses may also play a part.

Humoral immunity in exposed seronegative individuals

Finally, to assess whether there was any difference in humoral immune responses between USNs and ESNs, total IgG targeting SARS-CoV-2 S RBD, S2, and N, as well as nAb titers, were compared between seropositive, ESN, and USN individuals (Figure 8). By definition, responses to S, RBD, S2, and N were significantly higher in seropositive individuals compared to ESN individuals (S: 7,092 vs. 49, $p < 0.0001$; RBD: 2,288 vs. 116, $p < 0.0001$; S2: 24 vs. 19, $p = 0.003$; N: 4,043 vs. 124, $p < 0.001$; Mann–Whitney tests). nAb responses were also significantly higher in seropositive individuals compared to ESNs (38 vs. 1, $p < 0.0001$, Mann–Whitney test). While responses to S2 were higher in ESNs (18 vs. 14, $p = 0.003$, Mann–Whitney test) and IgG responses to total S were not significantly different between ESN and USN individuals, unexpectedly, responses to RBD were higher in USNs (173 vs. 116, $p = 0.03$, Mann–Whitney test). However, taking correction for multiple tests into account, this difference in antibody responses to RBD loses statistical significance. This may also reflect experimental noise as the difference is below the threshold for a positive response. An alternative explanation is that the expansion of cross-reactive S2-specific antibodies in ESNs leads to a reduction in antibody response to less conserved regions such as the RBD through competition for resources between B cells. However, it is perhaps more likely that this finding is artifactual.

Discussion

Here, we describe transmission dynamics and immune responses in family groups sampled during the early stage of the pandemic, where SARS-CoV-2 infections were occurring in immunologically naïve hosts through ongoing household transmission. Notably, we describe elevated T-cell immunity in exposed seronegative individuals that is positively associated with the number of seropositive individuals in a household.

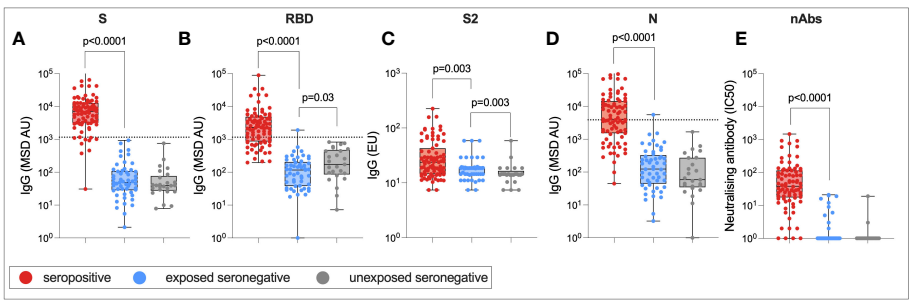

FIGURE 8
Humoral immune responses in ESNs. IgG targeting S **(A)**, RBD **(B)**, S2 **(C)**, and N **(D)**, and nAb titers **(E)** in seropositive (red), ESN (blue), and USN (gray) individuals. *p*-values refer to Mann–Whitney test values. Dotted lines represent seropositivity cutoffs as previously determined (12).

Over half of the cohort was seropositive, suggesting that transmission between family members was consistently occurring. Correlates of protection for SARS-CoV-2 are still uncertain, and it is unclear whether total IgG or nAb response is a better marker for protective immunity. However, the significantly elevated nAb response in seropositive individuals demonstrates that total IgG and nAb responses are well associated following natural infection, as shown previously (15), even many months into convalescence. nAb titers following infection have been shown to be durable and detectable after 8 months as supported here, a mean of 7 months post-infection (16). CD4+ T-cell responses were identified in most seropositive individuals as well as some seronegative individuals in this cohort. In seropositive individuals, responses primarily targeted S1 and S2 regions of S, with some individuals generating responses to M and N as well as NSP3B. Seronegative individuals also targeted these NSPs, although the majority of the response was specific for S1 and S2. Confirming previous findings by Ogbe et al. (12), few seronegative individuals generated T-cell responses to M and N (11). Antigenic targets of T-cell responses in unexposed individuals were well characterized in a study by Mateus et al. (14), which also identified S as the primary target of T-cell responses in pre-pandemic samples (54% of the total positive T-cell response). Eleven percent of this response targeted the RBD region of S, while 44% targeted non-RBD (14).

The findings from multiple linear regression that T-cell immunity was associated with older age and male sex are of note. Takahashi et al. (2020) identified higher T-cell activation in female than in male patients, although poor T-cell immunity was associated with worse COVID-19 outcomes in male patients only, and T-cell response was negatively correlated with age in male patients only (17). The discrepancy between these findings and those published previously may lie in the different cellular assays used, or the fact that these samples were taken months into convalescence. The cohorts in Takahashi et al. (2020) were sampled following a positive PCR test approximately 1 week after symptom onset, were more diverse in terms of ethnicity, and were all adults. Furthermore, the authors employed T-cell surface and intracellular staining rather than proliferation assays to investigate cellular immunity (17). Male patients may generate more proliferative memory responses to SARS-CoV-2 while female patients might express higher levels of activation markers.

It was also identified that S1-specific CD4+ T-cell immunity was positively correlated with age in both USNs and ESNs. This is of note, particularly as cellular immune responses to SARS-CoV-2 in infected children have been demonstrated to be of similar magnitude and functionality compared to adults (18). In this study, the correlation was lost following SARS-CoV-2 infection. It could therefore be inferred that this trend reflects the accumulation of cross-reactive T cells targeting endemic betacoronaviruses over an individual's lifetime, which are less frequently observed in children. However, following SARS-CoV-2 infection, cross-reactive immunity plays a smaller role and therefore the correlation between age and S1-specific T cells is lost. An alternative explanation is that this cross-reactivity is an artifact of using synthetic peptides.

Case studies of individual families provide an opportunity to assess immunity within families in more detail. In families all seropositive for SARS-CoV-2, humoral and cellular responses were varied. Previous studies have identified lower respiratory cycle threshold (Ct) values and higher plasma IgG in "high transmission" families where all individuals become infected compared to "low transmission" families where individuals were mixed serostatus (19). Nasopharyngeal sampling of this cohort during acute infection would have enabled this analysis and further provided an opportunity to assess what makes these "all seropositive" families all become infected, and whether this derives from virus-associated factors such as viral load.

In families all seronegative for SARS-CoV-2, cellular immunity was observed, but only in a few individual parents. In contrast, in mixed serostatus families where parents were either both seropositive or serodiscordant, cellular immunity was also present in seronegative children. This raised the question of whether intrafamilial exposure to SARS-CoV-2 could expand cellular immunity to SARS-CoV-2. This has been demonstrated previously in the context of HCWs (9, 11, 20) and in a small cohort of serodiscordant couples (10). However, this cohort is one of the largest to assess immune responses in ESN family members. In this cohort, elevated CD4+ T-cell responses in ESNs targeted S1, with a trend towards greater responses in S2, M, and N. This is in accordance with previous findings that demonstrate enhanced S-, M-, and N-specific immunity in ESN HCWs (11). However, elevated T-cell immunity to non-S regions of the SARS-CoV-2 proteome has also been identified in ESNs (20). In the data mentioned herein, there was a trend towards stronger T-cell immunity in ESNs for some NSPs such as ORF3, ORF8, NSP3A, and NSP3C, compared to USNs. However, T-cell responses targeting the RTC (NSP7, NSP12, and NSP13) were not significantly elevated in ESNs compared to USNs, in contrast to published findings (9).

Although both CD4+ and CD8+ responses were significantly more structurally targeted in seropositive individuals, there was no difference in the SP : NSP ratio between ESNs and USNs in this cohort. This suggested that ESNs generate a T-cell response more reminiscent of USNs than of seropositive individuals. A skew towards T cells targeting NSPs during ZIKV infection in individuals previously exposed to DENV has been reported; this is due to high homology between flavivirus NSPs (21). Here, though responses to structural proteins such as S1 are significantly higher in ESNs than in USNs, the overall SP : NSP ratio does not differ significantly. There was also no evidence of increased HCoV-specific immunity, or immunity to conserved regions of S, in ESNs compared to USNs. This casts doubt on whether cross-reactivity from endemic HCoVs is the sole source of T-cell responses in ESNs. Another explanation could be a combination of pre-existing cellular immunity combined with low-level *de novo* responses to novel SARS-CoV-2 epitopes upon low-dose viral exposure. It is unclear to what extent this immunity is protective; cross-reactive T cells have been associated with protection against infection with SARS-CoV-2 (22), but further investigation through SARS-CoV-2 challenge is required to confirm these findings.

The role of exposure intensity in ESNs has not been studied for SARS-CoV-2. Here, we describe increased T-cell responses in seronegative individuals with two seropositive family members compared to those with only one seropositive family member, indicating that enhanced exposure intensity is associated with stronger cellular immunity. This may be due to an increased viral dose. The role of dose in ESNs has been studied for HCV and simian immunodeficiency virus (SIV) in non-human primates: transient T-cell responses were demonstrated when two chimpanzees were exposed to increasing doses of HCV at 6-month intervals. Twelve months later, when both chimpanzees were exposed to a 10-fold greater dose of virus, the chimpanzee with consistently stronger T-cell responses cleared infection while the other developed chronic disease (23). Furthermore, macaques exposed to infectious doses of SIV seroconverted but generated weak cellular responses, while those exposed to sub-infectious doses generated cellular responses only (24). These findings suggest that dose may factor into which arm of adaptive immunity dominates upon viral exposure. Similar challenge studies in primates or humans exposed to different doses of SARS-CoV-2 would be necessary to make conclusions about the role of dose in SARS-CoV-2 ESNs. However, the findings described herein suggest that increased dose may promote enhanced cellular immunity in ESNs, while perhaps pushing individuals towards a dose threshold, the surpassing of which leads to infection and seroconversion. An alternative explanation is that an increased number of seropositive individuals within a family increases the duration, rather than the dose, of viral exposure in ESNs. An increased duration of HCV exposure is associated with stronger T-cell responses in ESN injection drug users and is also associated with an increased durability of response (25). ESNs likely represent a spectrum between USN and seropositive individuals, with their position upon the spectrum determined by prior exposure to HCoVs, viral dose, and exposure intensity (Figure 9).

The study has several limitations. The collection of samples in convalescence adds the potential confounding element of response waning over time. An attempt to correct for this using multiple linear regression was carried out, but sampling during acute disease or at a fixed time after infection would have enabled more robust comparisons between individuals. Similarly, sampling during acute disease and nasopharyngeal sampling would have allowed for PCR confirmation of infection rather than using serological data as a marker of prior infection. This is a significant limitation of the study, as ESNs could potentially reflect once-seropositive individuals whose antibodies have waned below the cutoff for seropositivity. However, using nAb response as a determinant of seropositivity instead did not change the results of the analysis, and there was no correlation between T-cell response and humoral response in ESNs, suggesting that ESN individuals with large S1-specific CD4+ responses are not simply convalescent individuals whose humoral immunity has waned below the threshold for seropositivity. Furthermore, although some individuals classed as seropositive by ELISA were seronegative by MSD, classifying seropositivity using the results of the MSD assay or the pseudovirus neutralization assay rather than the ELISA assay did not significantly impact the finding that ESNs generate greater responses to S1 and S2 than USNs, supporting the accuracy of ELISA data to determine seropositivity (Supplementary Figure 6).

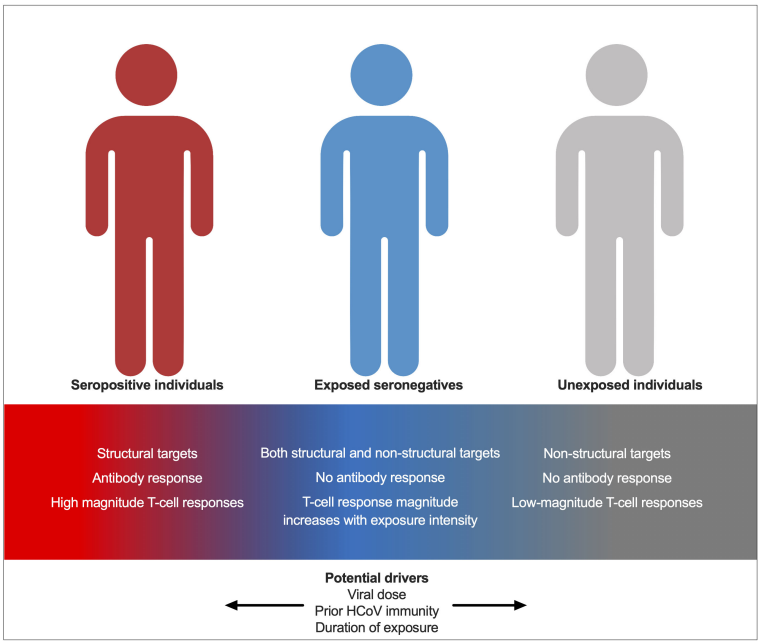

FIGURE 9
A model for immunity in seropositive individuals, ESNs, and USNs. Individuals represent points along a spectrum from USN to seropositive, modulated by viral dose, prior immunity to endemic HCoVs, and duration of exposure.

Finally, sampling of individuals at later time points would also have facilitated an assessment of the potential protective capacity of cellular immunity, as well as its impact on vaccine response.

Overall, this study demonstrates intrafamilial transmission of SARS-CoV-2 as a major route of infection early in the pandemic when individuals were predominantly SARS-CoV-2-naïve. We show an increased risk of infection, and an increased T-cell response in seronegative family members, when more family members become infected. Sex- and age-related differences in immune response appear minimal, although regression analysis identifies an association between older age, male sex, and increased T-cell immunity. Finally, T-cell immunity in ESNs does not appear to originate solely from cross-reactive responses to endemic HCoVs but may also be generated *de novo* upon exposure to SARS-CoV-2. These findings have implications for defining SARS-CoV-2 correlates of protection in the future, as T-cell immunity may be protective when vaccine-induced humoral immunity has waned.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by University of Oxford Central University Research Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

CJ: Conceptualization, Formal Analysis, Investigation, Writing—Original Draft Preparation, Review & Editing, and Visualization. EA: Investigation, Data Curation, Writing—Review & Editing, and Project Administration. AC: Investigation, Data Curation, Writing—Review & Editing, and Project Administration. CD: Investigation, Data Curation, and Writing—Review & Editing. ME: Investigation, Data Curation, and Writing—Review & Editing. C-PH: Investigation, Data Curation, and Writing—Review & Editing. AJ: Resources and Writing—Review & Editing. NL: Investigation and Writing—Review & Editing. SL: Formal Analysis, Investigation, and Writing—Review & Editing. AO: Investigation and Writing—Review & Editing. OS: Investigation and Writing—Review & Editing. DS: Conceptualization and Writing—Review & Editing. OBS: Resources and Writing—Review & Editing. LS: Resources, Methodology, and Writing—Review & Editing. CT: Resources, Writing—Review & Editing, and Methodology. LT: Supervision and Writing—Review & Editing. EB: Conceptualization and Writing—Review & Editing. SD: Supervision and Writing—Review & Editing. MC: Supervision, Writing—Review & Editing, and Methodology. PK: Supervision, Writing—Review & Editing, Conceptualization, and Methodology. CC: Conceptualization, Methodology, and Writing—Review & Editing. PG: Conceptualization, Methodology, Resources, Writing—Review & Editing, Supervision, Project Administration, and Funding Acquisition. LJ: Conceptualization, Methodology, Resources, Project Administration, Funding Acquisition, and Writing—Review & Editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1248658/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Family types. Family ID numbers are shown below each pedigree chart. Example families where all individuals became infected **(A)**. Families where all remained seronegative **(B)**. Families where only the father or an adult male were infected **(C)**. Families where only the mother was infected **(D)**. Families where only children were infected **(E)**. Families where one parent and a child/children were infected **(F)**. Families where both parents and a child/children were infected **(G)**. Squares are male patients; circles are female patients. Seropositive individuals are shown in red; seronegative individuals are in gray.

SUPPLEMENTARY FIGURE 2
Family types C, D, and E. Anti-S IgG, nAb responses, CD4+ T-cell responses and CD8+ responses in a household group consisting of a seropositive male, a seronegative male, and two seronegative female patients **(A)**. IgG, nAb, and T-cell responses in a household group consisting of a seropositive mother and seronegative father, daughter, and son **(B)**. IgG, nAb, and T-cell responses in a household group consisting of seronegative parents, two seropositive daughters, and two seronegative daughters **(C)**. Seropositive family members are outlined in red. Male patients are squares; female patients are circles. Asterisks refer to symptomatic individuals. Proliferation values below 1% were given nominal values of 0.9%.

SUPPLEMENTARY FIGURE 3
Family types F and G. Anti-S IgG, nAb responses, CD4+ T-cell responses, and CD8 + responses in a family group consisting of a seropositive father, a seronegative mother, two seronegative sons, one seronegative daughter, and a seropositive son **(A)**. IgG, nAb, and T-cell responses in a family group consisting of seropositive parents, two seropositive sons, two seropositive daughters, and a seronegative daughter **(B)**. Male patients are squares; female patients are circles. Seropositive family members are outlined in red. Asterisks refer to symptomatic individuals. Proliferation values below 1% were given nominal values of 0.9%.

SUPPLEMENTARY FIGURE 4
Correlations between S1-specific CD4+ response and other immune parameters. Correlation between S1-specific CD4+ T cells and S2-specific CD4+ T cells **(A)**, nAbs **(B)**, and anti-S (IgG) **(C)** in ESNs. *R*- and *p*-values refer to Spearman rank correlation values. Proliferation values below 1% were given nominal values of 0.9%. Dotted lines refer to the cutoff for T-cell positivity of 1%.

SUPPLEMENTARY FIGURE 5
T-cell responses in ESNs are not associated with enhanced HCoV-specific immunity. Ratio of CD4+ and CD8+ SP to NSP responses **(A)**, T cells targeting HCoV-OC43 S2 **(B)**, T cells targeting HCoV-HKU1 S2 **(C)**, T cells targeting a pool of 63 conserved peptides **(D)**, and ratio of response to the conserved pool against total S1 response **(E)** in seropositive (red), ESN (blue), and USN (white) individuals. *P*-values refer to Mann–Whitney test values. Proliferation values below 1% were given nominal values of 0.9%.

SUPPLEMENTARY FIGURE 6
T-cell responses in seropositive, ESN, and USN individuals as defined by MSD or nAb serostatus. % proliferating CD4+ T cells targeting S1 and S2 in seropositive (red), ESN (blue), and USN (gray) individuals, as defined by MSD serostatus **(A)** with an AU > 1,160, or nAb serostatus **(B)** with an IC_{50} > 0. *p*-values refer to Mann–Whitney test values. Proliferation values below 1% were given nominal values of 0.9%.

SUPPLEMENTARY FIGURE 7
Gating strategy for proliferation assay.

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Persistence of immunological memory as a potential correlate of long-term, vaccine-induced protection against Ebola virus disease in humans

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Introduction: In the absence of clinical efficacy data, vaccine protective effect can be extrapolated from animals to humans, using an immunological biomarker in humans that correlates with protection in animals, in a statistical approach called immunobridging. Such an immunobridging approach was previously used to infer the likely protective effect of the heterologous two-dose Ad26.ZEBOV, MVA-BN-Filo Ebola vaccine regimen. However, this immunobridging model does not provide information on how the persistence of the vaccine-induced immune response relates to durability of protection in humans.

Methods and results: In both humans and non-human primates, vaccine-induced circulating antibody levels appear to be very stable after an initial phase of contraction and are maintained for at least 3.8 years in humans (and at least 1.3 years in non-human primates). Immunological memory was also maintained over this period, as shown by the kinetics and magnitude of the anamnestic response following re-exposure to the Ebola virus glycoprotein antigen via booster vaccination with Ad26.ZEBOV in humans. In non-human primates, immunological memory was also formed as shown by an anamnestic response after high-dose, intramuscular injection with Ebola virus, but was not sufficient for protection against Ebola virus disease at later timepoints due to a decline in circulating antibodies and the fast kinetics of disease in the non-human primates model. Booster vaccination within three days of subsequent Ebola virus challenge in non-human primates resulted in protection from Ebola virus disease, i.e. before the anamnestic response was fully developed.

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Discussion: Humans infected with Ebola virus may benefit from the anamnestic response to prevent disease progression, as the incubation time is longer and progression of Ebola virus disease is slower as compared to non-human primates. Therefore, the persistence of vaccine-induced immune memory could be considered as a potential correlate of long-term protection against Ebola virus disease in humans, without the need for a booster.

KEYWORDS

Ebola, vaccine, immunological memory, persistence, correlate, protection

Introduction

Since Ebola virus disease (EVD) was discovered in 1976, outbreaks have continued to occur with increasing frequency in sub-Saharan Africa (1). The 2014–2016 EVD epidemic in Guinea, Liberia, and Sierra Leone remains the largest outbreak to date, with over 11,000 deaths and more than 28,000 confirmed cases. The second largest outbreak occurred only 2 years later in 2018 in the Democratic Republic of Congo and Uganda, underscoring how easily EVD outbreaks can escalate to become epidemics (1).

Clinical trials have shown that the Ad26.ZEBOV, MVA-BN-Filo regimen, which is licensed for use in the European Union and six African countries, is safe and immunogenic and induces both antibody and T-cell responses in adults and children (2–15). Thus far, it has not been feasible to collect classical clinical efficacy data for Ad26.ZEBOV, MVA-BN-Filo in humans. However, the likelihood of clinical benefit was established via an immunobridging approach.

Immunobridging is a statistical analysis that translates human immunogenicity data into the likelihood of protection. This is performed by first establishing how the immune response in non-human primates (NHPs) is associated with the likelihood of protection against lethal Ebola virus (EBOV) challenge and then comparing human vaccine-induced immune responses with the NHP vaccine-induced immune response to infer the likelihood of protection in humans. The intramuscular EBOV Kikwit NHP challenge model is considered stringent, as it is 100% lethal, compared to an average human case fatality rate of 50% during Ebola outbreaks, as reported by the WHO (16). NHPs also have both a shorter incubation time (average of 5.4 days compared to 6.2–9.7 days in humans) and an extremely rapid disease progression with a shorter time to death (after symptom onset, mean survival time in NHPs is 1.4 days relative to 5.8–14.4 days to death for lethal human cases) (16, 17).

It was observed that EBOV glycoprotein (GP) binding antibody levels strongly correlated with vaccine-induced protection against EBOV in NHPs (17). Therefore, a logistic regression model was built using survival outcome as the dependent variable and the EBOV GP-binding antibody levels at 21 days post-dose 2 as the independent variable, using NHP data (n = 66) from four independent challenge studies. Survival probabilities were then estimated based on human Phase 2/3 immunogenicity data assessed at 21 days post-dose 2, using the same EBOV GP-binding antibody ELISA that was validated for both human and NHP serum testing at Q^2 Solutions (18). To evaluate whether the vaccine regimen was likely to provide a protective benefit in humans, the lower limit of the confidence interval (CI) of the mean predicted survival probability was compared with a pre-specified success criterion of 20%. The immunobridging analysis demonstrated a mean predicted survival probability of 53.4% with a lower limit of the pre-planned 98.7% CI of 33.8%, thereby passing the pre-defined success criterion of 20% (18) and indicating that the regimen is likely to confer a protective effect in humans.

The immunobridging model is based only on levels of circulating binding antibodies at 21 days post-dose 2 of the primary vaccine regimen. After completion of the primary vaccine regimen, a vaccine-induced immune memory response is established over time. This is evidenced by a sharp increase in EBOV GP-binding antibody levels within 7 days of re-exposure to the EBOV GP antigen via a booster dose of Ad26.ZEBOV, which indicates that a strong and rapid anamnestic response is activated upon re-exposure to the EBOV GP antigen (6, 14, 15). The disease course of EVD in the NHP model is expected to limit the contribution of an anamnestic response to protection. In contrast, an anamnestic response is expected to contribute to protection in humans because of the longer incubation time and slower disease progression of EVD. This also implies that the high-dose, intramuscular EBOV challenge in NHPs (compared to the primary route of mucosal exposure in humans) may not be a good model for the durability of protection, as protection will likely be underestimated and may no longer correlate with circulating antibody levels. Thus, immunobridging based on NHP studies can inform on the likelihood of a vaccine protective effect in humans, although there is no straightforward approach to derive the extent and the duration of the conferred benefit.

In the current manuscript, we explore whether the persistence of immunological memory could be considered as a correlate of long-term, vaccine-induced protection against EVD in humans. We analyze the persistence of the primary immune response and the persistence of immunological memory in both humans and NHPs, as demonstrated via an anamnestic response. However, alternative assessments of immunological memory, such as B-memory cell

ELISpot, multi-color flow cytometry, or single-cell transcriptomics, could potentially be used in a real-life setting. We show that the onset of an anamnestic response following re-exposure to the EBOV GP antigen via booster vaccination in NHPs provides protection against EVD within 3 days. Based on the longer incubation time and slower disease progression after symptom onset in humans as compared to NHPs, the persistence of immunological memory in humans could be considered as a putative correlate of long-term protection against EVD (16, 17).

Materials and methods

Ethics

All clinical study protocols were conducted following the Declaration of Helsinki and International Council for Harmonisation Good Clinical Practice Guidelines (ICH-GCP) and were approved by both local and national independent ethics committees, as well as institutional review boards (IRBs) (6–11, 14, 15).

All adult participants supplied written informed consent before enrolment. For pediatric participants, parents or guardians provided written informed consent for their child to join the trial. Older children (age varied by country) also gave written assent.

Clinical sample immunogenicity evaluations

Seven late-development studies were selected for inclusion in this analysis: EBL2001, EBL2002, Partnership for Research on Ebola VACcination (PREVAC; hereafter referred to as EBL2004), EBL2011, EBL3001 (Stage 1 was open label, while Stage 2 was randomized and active controlled), EBL3002, and EBL3003 (6–11, 14, 15). In each study, participants received an intramuscular injection of Ad26.ZEBOV on day 1 (5×10^{10} viral particles [vp]; dose 1) followed by an intramuscular injection of MVA-BN-Filo on day 57 (1×10^{8} infectious units [InfU]; dose 2).

With the exception of EBL2004, the EBOV GP-binding antibody concentration at day 21 post-dose 2 (MVA-BN-Filo) and 12 months or more post-dose 1 (Ad26.ZEBOV) was assessed. In the EBL2004 study, EBOV GP-binding antibody response was assessed at day 28 post-dose 2 and 12 months post-dose 1.

The clinical EBOV GP-binding antibody data reported here are from samples analyzed using the same validated Filovirus Animal Nonclinical Group (FANG) ELISA assay, performed at a single analytical laboratory (Q^2 Solutions, San Juan Capistrano, CA, USA). This allows immune responses to be compared more reliably across different studies. All data are from participants who received the primary, heterologous, two-dose Ad26.ZEBOV, MVA-BN-Filo vaccine regimen with dosing on days 1 and 57. Participants in a subset of the studies additionally received a booster dose of Ad26.ZEBOV, at varying time points relative to dose 1 (Ad26.ZEBOV) of the primary regimen.

Results of the current analysis are presented by study, and results from adults and pediatric participants are presented separately. Within the studies that included pediatric participants, results are further stratified by age group. In EBL2002 and EBL3001 Stage 2 (9, 10), the EBOV GP-binding antibody response was assessed in adolescents aged 12–17 years and older children aged 4–11 years. In EBL3001 Stage 2 only, EBOV GP-binding antibody response was additionally assessed in younger children aged 1–3 years. Study EBL2004 assessed EBOV GP-binding antibody response in adolescents aged 12–17 years, older children aged 5–11 years, and younger children aged 1–4 years (11).

Statistics

In each study, the analysis set for immunogenicity was the per-protocol set and included all vaccinated participants who had no major protocol deviations that could have influenced the immune response, had received both vaccinations within the protocol-defined window, and had at least one evaluable post-vaccination immunogenicity sample.

Antibody concentrations, in EU/mL, for the Ad26.ZEBOV, MVA-BN-Filo-vaccinated and matched control groups were summarized at each time point as geometric mean concentrations (GMCs) with corresponding 95% CIs. Responder rates were also reported for each time point post-baseline. Data from the matched control groups are not discussed in this manuscript but can be found in the original publications (6–11, 14).

For all studies, a responder was defined as a participant with an EBOV GP-binding antibody concentration >2.5-fold increased from baseline if the baseline sample was positive, or >2.5 times the lower limit of quantification (LLOQ) of 36.11 ELISA units (EU)/mL if the baseline sample was negative. For all applicable studies, outcomes for adults and pediatric participants were assessed separately.

Additional details on statistical methods, as well as baseline demographic characteristics, can be found in the original publications for each study (6–11, 14, 15).

Non-human primate studies

All NHP studies described utilized a cynomolgus macaque (*Macaca fascicularis*) animal model. Animals were between ~3 years and 5 years of age, weighing between ~3 kg and 6 kg with an approximate 1:1 ratio between males and females. Depending on the study, the vaccination phase took place at Alpha Genesis (Yemassee, SC, USA), Bioqual (Rockville, MD, USA), or Charles River (Reno, NV, USA). For all studies, the EBOV challenge occurred at the Texas Biomedical Research Institute (TBRI, San Antonio, TX, USA). Approval of each institute's Institutional Animal Care and Use Committee (IACUC) was obtained prior to the commencement of each study. All facilities involved were accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and all animal experiments performed complied with the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act regulations.

While animals originated from different vendors and had different origins, this did not appear to affect vaccination or challenge outcomes.

NHPs received specialized, commercially available primate chow on a daily basis, and drinking water was available *ad libitum*. During the vaccination phase of the long-term studies, animals were socially housed. Throughout the studies, animals were provided with cage enrichment in the form of food and non-food items. In all EBOV challenge studies, humane endpoints were predefined to limit potential discomfort.

Study to assess immune memory activation with Ad26.ZEBOV booster vaccination

The vaccination phase of this study was performed at Alpha Genesis. Cynomolgus macaques (n = 12, Chinese origin) were obtained from the breeding colony at Alpha Genesis, except for two animals that were imported from Guangxi Grandforest Scientific Primate Company, Ltd. (Guangxi, China). Prior to the study start, animals tested negative for *Mycobacterium tuberculosis*, Simian immunodeficiency virus, Simian T-lymphotropic virus, Simian retrovirus, and herpes B virus. NHPs received an intramuscular (IM) vaccination with 5×10^{10} vp of Ad26.ZEBOV at study day 0, followed by an IM immunization with 1×10^{8} InfU of MVA-BN-Filo at study day 56 (8 weeks later). NHPs were subsequently divided into two groups of n = 6, with one group receiving no further injections and the other group receiving a booster vaccination with Ad26.ZEBOV at study day 196 (0.5 years). Both groups were observed for an additional 50 weeks to assess the activation and persistence of the memory response by booster vaccination.

Study to assess immune memory activation after late EBOV challenge

The group described above that did not receive the booster vaccination of Ad26.ZEBOV was transferred to TBRI for EBOV challenge 548 days (1.5 years) after the first immunization. A negative control group was included, consisting of animals originating from Alpha Genesis (n = 2, mock vaccinated with Tris-buffered saline) and animals (Vietnamese origin, n = 6, unvaccinated) obtained from Covance Research Products (Alice, TX, USA). Animals were observed for 21 days after the challenge to assess the long-term protective efficacy of the clinical regimen and activation of the memory response following the late EBOV challenge.

Study to assess immune memory activation after early EBOV challenge

The vaccination phase of this study was performed at Bioqual. Cynomolgus macaques (n = 4, Mauritian origin) were obtained from PrimGen (Hines, IL, USA) and acclimatized for 6 weeks before the study started. All animals tested seronegative for *M. tuberculosis*, Simian immunodeficiency virus, Simian T-lymphotropic virus, Simian retrovirus, and herpes B virus. NHPs were vaccinated IM with 5×10^{10} vp of Ad26.ZEBOV at study day 0, followed by an IM immunization with 1×10^{8} InfU of MVA-BN-Filo at study day 56 (8 weeks later). For the EBOV challenge at TBRI on study day 84 (3 months), the group that had received IM injections with empty Ad26 and MVA vectors was included as the negative control (n = 2). Animals were observed for 28 days after the challenge to evaluate the activation of the memory response after early EBOV infection.

Study to assess protection after booster vaccination with Ad26.ZEBOV

The vaccination phase of this study was performed at Charles River Laboratories. Cynomolgus macaques (n = 15, Mauritian origin) were obtained from Bioqual. Prior to the study's start, animals were negative for *M. tuberculosis* and Simian retrovirus. Animals were divided into three groups of n = 5, and on study day 0, animals received either an IM vaccination with 4×10^{10} vp of Ad26.ZEBOV (Group 1) or 1.2×10^{11} vp of Ad26.Filo (Groups 2 and 3). On study day 56, all groups were vaccinated IM with 5×10^{8} InfU MVA-BN-Filo. After transfer to TBRI and prior to the EBOV challenge, animals received an IM booster vaccination with 4×10^{10} vp of Ad26.ZEBOV either 7 days (Groups 1 and 2) or 3 days (Group 3) before the challenge at study day 592 (1.62 years). The negative control group (n = 2) consisted of animals that received mock immunizations with 0.9% NaCl at study days 42 and 56, as well as 7 days prior to the challenge. After the EBOV challenge, animals were observed for 21 days to monitor the protective efficacy of an anamnestic response conferred by the booster vaccinations. One animal from both Groups 1 and 3 had to be taken out of the study due to health issues unrelated to vaccination or EBOV challenge and were therefore not included in the dataset.

EBOV challenge

After arrival at TBRI, animals were acclimatized to BSL-4 laboratory conditions for at least 5 days prior to EBOV challenge. For all studies, animals were exposed to a target dose of 100 plaque-forming units (PFU) (actual dose range 75.5–96 PFU) of the FANG-approved EBOV Kikwit-9510621 strain via intramuscular injection in the deltoid muscle of the right arm. For each study, animals were exposed in order of their TBRI identifier number. After the challenge, animals were observed twice daily, 7 days a week, for their health status and clinical signs of EBOV infection, with observation frequency increasing as clinical signs became apparent. Clinical manifestations were scored via an in-house scoring system, assessing general appearance, condition of skin and fur, nose/mouth/eyes/head, respiration, feces and urine, food intake, petechiae, temperature, and locomotor activity. Animals reaching a clinical score ≥15 or an otherwise moribund state were euthanized after veterinary approval. All TBRI staff were blinded to animal vaccination.

NHP vaccines and challenge material

Ad26.ZEBOV (Janssen Vaccines and Prevention, Leiden, the Netherlands) is a recombinant, replication-incompetent, Ad26-vectored vaccine encoding the EBOV Mayinga GP. Ad26.Filo (Janssen Vaccines and Prevention, Leiden, the Netherlands) is a

1:1:1 mixture of three Ad26-vectored vaccines encoding the EBOV Mayinga variant GP, the Sudan Gulu GP, or the Marburg Angola GP. MVA-BN-Filo (Bavarian Nordic, Hellerup, Denmark) is a recombinant, modified vaccinia Ankara-vectored vaccine, non-replicating in human cells, encoding the EBOV Mayinga, Sudan Gulu, Marburg Musoke GPs, and the nucleoprotein of the Tai Forest virus. All vaccine preparations were tested for sterility and the presence of endotoxins.

EBOV strain Kikwit-9510621, supplied by TBRI, was used for all challenges. A second-cell culture passage (P2) of EBOV Kikwit was obtained from Dr. Tom Ksiazek (at the National Institute of Allergy and Infectious Diseases (NIAID) World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch (UTMB) Health Galveston National Laboratory) in 2012 and propagated at TBRI. The stock virus was passaged for a third time in Vero E6 cells to generate the challenge stock. The challenge stock was confirmed to be wild-type Ebola virus by deep sequencing and determined to be sterile and free of mycoplasma and endotoxins.

NHP blood collection and processing

Animals were bled for serum collection at predefined time points. Blood was collected in clotting tubes, processed for isolation of serum, and subsequently aliquoted and stored at −80°C on the day of collection. Post-challenge sera were transferred to the University of Texas Medical Branch for inactivation of EBOV via a validated gamma irradiation procedure before shipment and analysis of EBOV GP binding antibodies.

EBOV plaque assay using NHP serum

Serum viral load was determined via the FANG-optimized plaque assay for EBOV (19). In brief, frozen serum aliquots were thawed, serially diluted, and added to pre-seeded Vero E6 cells. After approximately 1 hour at 37°C, an agarose overlay medium was added to the wells and allowed to solidify. Plates were incubated for another 7 days before staining with a secondary overlay medium supplemented with neutral red to visualize the plaques. Plaques were counted 24–48 hours after staining. Serum EBOV concentration was calculated as plaque-forming units per milliliter serum (PFU/mL). Serum samples with countable plaques below the lower limit of quantification (LLOQ = 15 PFU/mL) were set at the LLOQ. Samples with 0 PFU/mL were set at 1 to enable graphing on a logarithmic scale. Data are presented in Supplementary Figure 1.

Anti-EBOV GP ELISA using NHP serum

The concentration of EBOV GP binding antibodies in NHP serum was determined via the EBOV GP FANG ELISA at Battelle Biomedical Research Centre (OH, USA). The method was described previously by Rudge et al. (20). Binding antibody concentration was calculated as ELISA units per milliliter (EU/mL) serum based on a reference sample. For all data points, a median EU/mL was generated based on a minimum of two independent analyses that passed all acceptance criteria. Values below the limit of detection (LOD) were set at the LOD of each assay before log10 transformation and graphing.

Results

Persistence of humoral immune response following Ad26.ZEBOV, MVA-BN-Filo vaccination in adults

Data from six Phase 2/3 clinical studies, all analyzed with the same validated FANG ELISA assay performed in the same laboratory, are available to support the persistence of immune response to vaccination in adults, with the majority of studies including a time point of 1 year post-dose 1 (Table 1). Circulating EBOV GP-binding antibody GMCs decline between 21 days post-dose 2 and 6 months post-dose 2 (8 months post-dose 1), at which point a plateau is reached, with some variation in binding antibody GMCs by geographic location (Figure 1). At 1 year post-dose 1, circulating binding antibody GMCs persisted from 259 EU/mL in the randomized, active controlled Stage 2 of EBL3001 in Sierra Leone to 1,205 EU/mL in the UK and France in EBL2001. Responder rates at 1 year ranged from 49% in EBL3001 to 100% in several studies. Study EBL3001 also included a 2-year post-dose 1 time point, where circulating binding antibody GMCs were 279 EU/mL and 255 EU/mL in Stage 1 (open-label) and Stage 2 (randomized, active controlled), respectively. This is comparable to the GMCs observed at 1 year and 1.5 years post-dose 1 within this study, indicating that further decay is slow once the plateau phase is reached.

Persistence of humoral immune response following Ad26.ZEBOV, MVA-BN-Filo vaccination in children and adolescents

Data from four Phase 2/3 clinical studies in pediatric participants show similar results as compared to adults (Table 2). Immune response as measured by EBOV GP-binding antibody GMCs decline between 21 days post-dose 2 and 6 months post-dose 2 (8 months post-dose 1). After this point, circulating binding antibody levels reach a plateau (Figure 2). At 1 year post-dose 1, circulating binding antibody GMCs ranged from 386 EU/mL in adolescents in Sierra Leone in EBL3001 to 1139 EU/mL in children 1–4 years old in Guinea, Liberia, Mali, and Sierra Leone in EBL2004. Responder rates at 1 year post-dose 1 ranged from 70% to 100%, and responses tend to be higher at 1 year post-dose 1 in younger individuals, both compared to adults and between the age group stratifications for pediatric participants. This plateau of circulating binding antibody levels is maintained up to at least 3.1 years in children aged 1–3 years at the time of dose 1 vaccination (934 EU/mL [568–1,534]; 96%) and at least up to 3.8 years in children aged 4–11 years at the time of dose 1

TABLE 1 Persistence of Ebola virus glycoprotein circulating binding antibody response in adults.

	Study (age strata)	Persistence time point analyzed	N	GMC (EU/mL) (95% CI)	% Persisting response
Phase 2 (Q^2 Solutions)	**EBL2001 (FRA, UK)** ***Healthy adults***	1 year post-dose 1 (Day 365)	50	**1,205** (971; 1,497)	100%
	EBL2002 (BFA, CIV, KEN, UGA) ***Healthy adults***	1 year post-dose 1 (Day 365)	133	**342** (291; 401)	78%
	EBL2002 (BFA, CIV, KEN, UGA) ***HIV-infected adults***	1 year post-dose 1 (Day 365)	59	**338** (253; 450)	88%
	EBL2004 (GNA, LIB, MAL, SL) ***Healthy adults***	1 year post-dose 1 (Day 365)	254	**437** (352; 542)	80%
Phase 3 (Q^2 Solutions)	**EBL3001, Stage 1 (SL)** ***Healthy adults***	1 year post-dose 1 (Day 360)	31	**325** (238; 445)	77%
	EBL3001, Stage 2 (SL) ***Healthy adults***	1 year post-dose 1 (Day 360)	168	**259** (223; 301)	49%
	EBL3001, Stage 1 (SL) ***Healthy adults***	2 years post-dose 1 (Day 720)	31	**279** (201; 386)	68%
	EBL3001, Stage 2 (SL) ***Healthy adults***	2 years post-dose 1 (Day 720)	158	**255** (212; 306)	50%
	EBL3002 (USA) ***Healthy adults***	8 months post-dose 1 (Day 237)	131	**1,263** (1,100; 1,450)	99%
	EBL3003 (USA) ***Healthy adults***	8 months post-dose 1 (Day 237)	244	**1,151** (~950; ~1400)	98%

GMC, geometric mean concentration; EU/mL, enzyme-linked immunosorbent assay units per milliliter; CI, confidence interval; FRA, France; UK, United Kingdom; BFA, Burkina Faso; CIV, Côte d'Ivoire; KEN, Kenya; UGA, Uganda; GNA, Guinea; LIB, Liberia; MAL, Mali; SL, Sierra Leone; USA, United States of America.

vaccination (418 EU/mL [287–608]; 77%). These results indicate that circulating binding antibodies persist, with minimal additional decline, for at least 3.8 years post-dose 1.

Persistence of immune memory following Ad26.ZEBOV, MVA-BN-Filo primary regimen vaccination and Ad26.ZEBOV booster vaccination in adult and pediatric participants

Three Phase 2/3 clinical studies included the administration of an Ad26.ZEBOV booster dose at various time points after completion of the primary Ad26.ZEBOV, MVA-BN-Filo vaccine regimen. These studies were conducted in adults (EBL2002, EBL3001) and pediatric participants (EBL2011). In each study, the Ad26.ZEBOV booster was administered at a different time points, ranging from 1 year to 3.8 years, after dose 1 of the primary regimen (Table 3). Regardless of when the Ad26.ZEBOV booster dose was administered, a strong anamnestic immune memory response was observed, as indicated by a sharp increase in EBOV GP-binding antibody GMCs within 7 days (Figure 3). The fold increase in EBOV GP-binding antibody GMCs from pre-booster to 7 days post-booster ranged from approximately 33-fold when the booster was administered at 3.1 years post-dose 1 in 1–3-year-old pediatric participants in EBL2011 to 63-fold in 4–11-year-old pediatric participants in EBL2011 when the booster was administered at 3.8 years post-dose 1 (Table 4).

GMCs continued to rise when assessed at 21 days post-booster relative to day 7, followed by a decline when assessed at 1 year post-booster. At 21-days post-booster, the fold changes from pre-booster increased to between 76-fold when the booster was administered at 3.1 years post-dose 1 in 1–3-year-old pediatric participants in EBL2011 and to 138-fold from pre-booster when the booster was administered at 3.8 years post-dose 1 in 4–11-year-old pediatric participants in EBL2011 (Table 4). When the anamnestic response at 21 days post-booster was compared to the immune response observed at 21 days post-dose 2 of the primary regimen, a 3.2-fold increase was observed if the booster was administered at 3.1 years post-dose 1 in pediatric participants, and this was increased to 5.6-fold if the booster was administered at 3.8 years post-dose 1 in pediatric participants (Table 4).

In terms of persistence of the anamnestic immune response post-booster in adults, circulating binding antibodies were still detectable at 1 year post-booster in studies EBL2002 (booster administered at 1 year post-dose 1) and EBL3001 (booster administered at 2 years post-dose 1). Responder rates in these two studies were 97% at 1 year post-booster in study EBL2002 and 100% at 1 year post-booster in study EBL3001. Additionally,

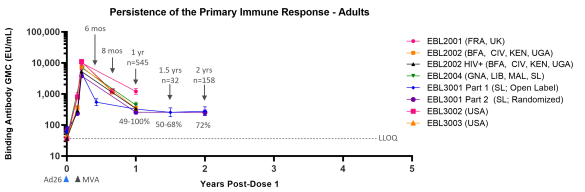

FIGURE 1
Persistence of the primary immune response in adults after vaccination with the Ad26.ZEBOV, MVA-BN-Filo vaccine regimen. EBOV GP-binding antibody GMCs in EU/mL at various time points, with accompanying 95% CIs, are depicted. Samples were analyzed following standard operating procedure at Q^2 Solutions using the FANG ELISA, and a single reportable value for each sample at each time point was uploaded for statistical analysis. The horizontal dashed line indicates the FANG ELISA lower limit of quantification of 36.11 EU/mL. The blue arrowheads below the x-axis indicate the timing of administration of the Ad26.ZEBOV vaccine, and the black arrowheads indicate timing of administration of the MVA-BN-Filo vaccine dose. CI, confidence interval; EBOV GP, Ebola virus glycoprotein; ELISA, enzyme-linked immunosorbent assay; EU, ELISA unit; mL, milliliter; FANG, Filovirus Animal Nonclinical Group; GMC, geometric mean concentration; HIV+, human immunodeficiency virus positive; Ad26, Ad26.ZEBOV; MVA, MVA-BN-Filo; Mos, months; Yr, year; Yrs, years; FRA, France; UK, United Kingdom; BFA, Burkina Faso; CIV, Côte d'Ivoire; KEN, Kenya; UGA, Uganda; GNA, Guinea; LIB, Liberia; MAL, Mali; SL, Sierra Leone; USA, United States of America. N is the total number of participants, from all studies, with data at the indicated time point. Percentages indicate the range of percent responders observed across studies at the indicated time point.

circulating binding antibody GMCs at 1 year post-booster were approximately 12 times higher than the GMCs observed at 1 year post-dose 1 (Table 5).

Persistence of humoral immune responses and immune memory following Ad26.ZEBOV, MVA-BN-Filo primary regimen in NHPs

The kinetics of humoral immune responses in NHPs were similar to those observed in humans. The immune response, as measured by circulating EBOV GP-binding antibody levels, declined between 21 days post-dose 2 and 6 months post-dose 2 (8 months post-dose 1) (Figure 4A), after which a stable plateau phase was reached that persisted for at least 17 months (~1.4 years). A booster dose of Ad26.ZEBOV (5×10^{10} vp) administered 4 months after the two-dose primary regimen elicited an anamnestic immune memory response, indicated by an approximately 40-fold increase in EBOV GP-binding antibody levels by day 7 post-booster. Antibody levels again declined from day 21 post-booster onward and reached a plateau level approximately fourfold higher than after the two-dose primary regimen. Thus, it is clear that immunological memory was also maintained in NHPs, which

TABLE 2 Persistence of Ebola virus glycoprotein circulating binding antibody response in children and adolescents.

	Study (age strata)	Persistence time point analyzed	N	GMC (EU/mL) (95% CI)	% Persisting response
Phase 2 (Q^2 Solutions)	**EBL2002 (BFA, CIV, KEN, UGA)** ***4–11 years***	1 year post-dose 1 (Day 365)	53	**638** (529; 767)	98%
	EBL2002 (BFA, CIV, KEN, UGA) ***12–17 years***	1 year post-dose 1 (Day 365)	54	**541** (433; 678)	90%
	EBL2004 (GNA, LIB, MAL, SL) ***1–4 years***	1 year post-dose 1 (Day 365)	105	**1139** (905; 1432)	100%
	EBL2004 (GNA, LIB, MAL, SL) ***5–11 years***	1 year post-dose 1 (Day 365)	109	**739** (585; 933)	94%
	EBL2004 (GNA, LIB, MAL, SL) ***12–17 years***	1 year post-dose 1 (Day 365)	127	**731** (589; 907)	77%
	EBL2011 (SL) ***1–3 years***	3.2 years post-dose 1 (Day 1168)	27	**934** (568; 1534)	96%
	EBL2011 (SL) ***4–11 years***	3.2 years post-dose 1 (Day 1168)	23	**418** (287; 608)	77%
Phase 3 (Q^2 Solutions)	**EBL3001 (SL)** ***1–3 years***	1 year post-dose 1 (Day 360)	120	**750** (629; 894)	96%
	EBL3001 (SL) ***4–11 years***	1 year post-dose 1 (Day 360)	123	**436** (375; 506)	71%
	EBL3001 (SL) ***12–17 years***	1 year post-dose 1 (Day 360)	132	**386 (326; 457)**	70%

GMC, geometric mean concentration; EU/mL, enzyme-linked immunosorbent assay units per milliliter; CI, confidence interval; BFA, Burkina Faso; CIV, Côte d'Ivoire; KEN, Kenya; UGA, Uganda; GNA, Guinea; LIB, Liberia; MAL, Mali; SL, Sierra Leone; USA, United States of America.

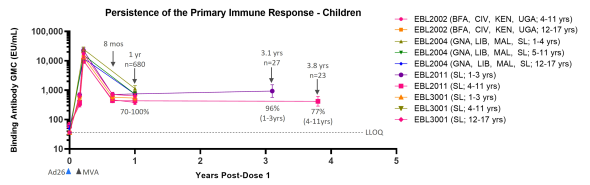

FIGURE 2
Persistence of the primary immune response in children and adolescents after vaccination with the Ad26.ZEBOV, MVA-BN-Filo vaccine regimen. EBOV GP-binding antibody GMCs in EU/mL at various time points, with accompanying 95% CIs, are depicted. Samples were analyzed following standard operating procedure at Q^2 Solutions using the FANG ELISA, and a single reportable value for each sample at each time point was uploaded for statistical analysis. The horizontal dashed line indicates the FANG ELISA lower limit of quantification of 36.11 EU/mL. The blue arrowheads below the x-axis indicate the timing of administration of the Ad26.ZEBOV vaccine, and the black arrowheads indicate timing of administration of the MVA-BN-Filo vaccine dose. CI, confidence interval; EBOV GP, Ebola virus glycoprotein; ELISA, enzyme-linked immunosorbent assay; EU, ELISA unit; mL, milliliter; FANG, Filovirus Animal Nonclinical Group; GMC, geometric mean concentration; Ad26, Ad26.ZEBOV; MVA, MVA-BN-Filo; Mos, months; Yr, year; Yrs, years; BFA, Burkina Faso; CIV, Côte d'Ivoire; KEN, Kenya; UGA, Uganda; GNA, Guinea; LIB, Liberia; MAL, Mali; SL, Sierra Leone. N is the total number of participants, from all studies, with data at the indicated time point. Percentages indicate the range of percent responders observed across studies at the indicated time point.

could rapidly be re-activated by exposure to the EBOV GP vaccine antigen.

Exposure to EBOV activates an anamnestic response, but disease progress in NHPs is too rapid for protection

Based on the kinetics of the anamnestic response and the speed of disease progression in NHPs, it appeared unlikely that the anamnestic response would outcompete disease progression in the NHP model (100 pfu IM infection leads to lethality in approximately 7 days). Indeed, when a cohort of six NHPs was challenged IM with EBOV approximately 1.5 years after the first immunization, all animals succumbed to infection (Supplementary Figure 1A). When EBOV GP-binding antibodies were analyzed post-challenge, an anamnestic response was not observed (Figure 4B), indicating that the persistent level of circulating antibodies at this time point was not sufficient, and the NHPs succumbed before an effective memory response could be mounted. Indeed, starting at day 6 post-challenge, antibody titers declined in all animals, reaching undetectable levels in two animals before they succumbed to EVD. Circulating antibodies were depleted by excess GP production due to viral replication and did not confer sufficient protection in the absence of an anamnestic response.

Contrastingly, when NHPs vaccinated with Ad26.ZEBOV, MVA-BN-Filo were infected with EBOV early after vaccination (4 weeks after dose 2) when levels of persistent circulating binding antibodies are higher, a substantial increase in EBOV GP-binding antibody levels was observed in three out of four NHPs from day 14 post-challenge (Figure 4C). This proves that EBOV exposure can elicit an anamnestic response in NHPs when disease progression is delayed. While some animals displayed clinical signs, none became viremic, and all survived until the study end (Supplementary Figure 1B), suggesting that circulating binding antibodies were able to delay disease progression long enough for the anamnestic response to contribute to protection.

Pre-activation of the anamnestic response elicits rapid protection in NHPs

To investigate whether an anamnestic response to the EBOV GP antigen could contribute to protection in the NHP model, a cohort of immunized NHPs received a booster vaccination 7 days or 3 days prior to the challenge to simulate immune memory activation. A cohort of NHPs were immunized with either Ad26.ZEBOV as dose 1 or Ad26.Filo as dose 1. Ad26.Filo is a 1:1:1 mixture of three Ad26-vectored vaccines encoding the EBOV GP, the Sudan virus GP, or the Marburg virus GP. Thus, Ad26.Filo contains the same EBOV GP antigen as Ad26.ZEBOV. All animals, regardless of whether Ad26.ZEBOV or Ad26.Filo was administered as dose 1, received MVA-BN-Filo as dose 2 in a 56-day interval.

TABLE 3 Overview of clinical studies which administered a booster dose of Ad26.ZEBOV.

Study phase	Study number	Location	Population	Method	Booster dose administration
Phase 2	**EBL2002**	Burkina Faso, Côte D'Ivoire, Kenya Uganda	• Healthy adults • HIV-infected adults • 4–11 years • 12–17 years	Randomized, placebo-controlled, observer-blind	• 1 year
	EBL2011	Sierra Leone	• 1–3 years • 4–11 years	Open label	• 3.1 years • 3.8 years
Phase 3	**EBL3001**	Sierra Leone	• Healthy adults	Staged study with an open-label, uncontrolled Stage 1 followed by a randomized, controlled, double-blind Stage 2	• 2 years

HIV, human immunodeficiency virus.

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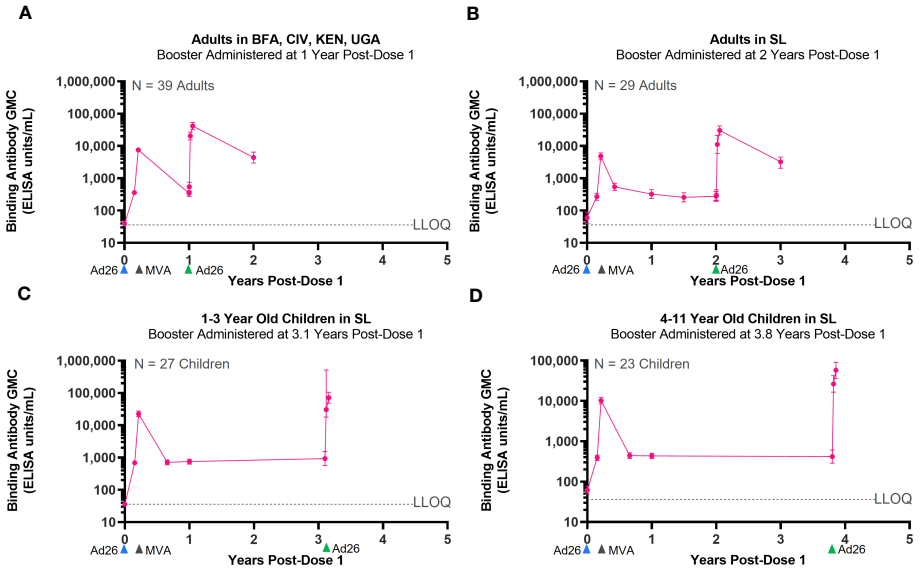

FIGURE 3
Persistence of the primary immune response after vaccination with the Ad26.ZEBOV, MVA-BN-Filo vaccine regimen and activation of an immune memory response after administration of an Ad26.ZEBOV booster dose. EBOV GP-binding antibody GMCs in EU/mL at various time points, with accompanying 95% CIs, are depicted. Ad26.ZEBOV booster dose administered between 1 year and 3.8 years post-dose 1 in adults **(A, B)** and children **(C, D)**. Samples were analyzed following standard operating procedure at Q^2 Solutions using the FANG ELISA, and a single reportable value for each sample at each time point was uploaded for statistical analysis. The horizontal dashed lines indicate the FANG ELISA lower limit of quantification of 36.11 EU/mL. The blue arrowheads below the x-axis indicate the timing of administration of the Ad26.ZEBOV vaccine doses, the black arrowheads indicate timing of administration of the MVA-BN-Filo vaccine dose, and the green arrowheads indicate timing of administration of the Ad26.ZEBOV booster dose. CI, confidence interval; EBOV GP, Ebola virus glycoprotein; ELISA, enzyme-linked immunosorbent assay; EU, ELISA unit; mL, milliliter; FANG, Filovirus Animal Nonclinical Group; GMC, geometric mean concentration; Ad26, Ad26.ZEBOV; MVA, MVA-BN-Filo; Yrs, years; BFA, Burkina Faso; CIV, Côte d'Ivoire; KEN, Kenya; UGA, Uganda; GNA, Guinea; LIB, Liberia; MAL, Mali; SL, Sierra Leone. N is the number of participants with data at pre-booster baseline.

This cohort received a booster (Ad26.ZEBOV) approximately 1.6 years after dose 2 and was challenged with EBOV either 7 days or 3 days later. Irrespective of the preceding regimen, all NHPs that were re-exposed to the EBOV GP antigen by way of an Ad26.ZEBOV booster 7 days or even 3 days prior to EBOV infection survived the challenge, with minimal morbidity and absence of viremia (Supplementary Figure 1C). In agreement with previous data (Figure 4A), a booster immunization 7 days prior to the challenge resulted in a fully developed, protective anamnestic response at the time of challenge (Figure 4D). A booster immunization 3 days prior to the challenge did not result in a detectable increase in EBOV GP-binding antibody levels by the time of challenge, but by day 3 post-

TABLE 4 Activation of immune memory response in humans after administration of an Ad26.ZEBOV booster dose.

		21 days post-dose 2	Pre-booster	7 days post-booster		21 days post-booster		
Column		**A**	**B**	**C**	**C:B**	**D**	**D:B**	**D:A**
Study (age strata)	**Timing of booster administration relative to dose 1 of the primary regimen**	**GMC* (EU/mL)**	**GMC**	**GMC**	**Fold increase**	**GMC**	**Fold increase**	**Fold increase: 21 days post-booster vs. 21 days post-dose 2**
EBL2002	1 year	**7,518**	**366**	**20,416**	**55.8**	**41,643**	**113.8**	**5.5**
EBL3001 Stage 1	2 years	**4,784**	**274**	**11,166**	**40.8**	**30,411**	**111**	**6.4**
EBL3001/ EBL2011 (1–3 years)	3.1 years (1–3 years)	**22,568**	**934**	**30,463**	**32.6**	**71,143**	**76.2**	**3.2**
EBL3001/ EBL2011 (4–11 years)	3.8 years (4–11 years)	**10,212**	**418**	**26,478**	**63.3**	**57,564**	**137.7**	**5.6**

GMC, geometric mean concentration; EU/mL, enzyme-linked immunosorbent assay per milliliter.
*GMC of all participants at 21 days post-dose 2 in the indicated study or parent study of the indicated study as applicable.

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TABLE 5 Persistence of immune memory response in humans after administration of an Ad26.ZEBOV booster dose.

		1 year post-dose 1	Pre-booster	1 year post-booster		
Column		**A**	**B**	**C**	**C:B**	**C:A**
Study (age strata)	**Timing of booster administration relative to dose 1 of the primary regimen**	**GMC* (EU/mL)**	**GMC (EU/mL)**	**GMC (EU/mL)**	**Fold increase**	**Fold increase: 1 year post-booster vs. 1 year post-dose 1**
EBL2002	1 year	**342**	**366**	**4,383**	**12**	**12.8**
EBL3001 Stage 1	2 years	**279**	**274**	**3,237**	**11.8**	**11.6**
EBL3001/EBL2011 (1–3 years)	3.1 years	**750**	**934**	**NA**	**NA**	**NA**
EBL3001/EBL2011 (4–11 years)	3.8 years	**436**	**418**	**NA**	**NA**	**NA**

GMC, geometric mean concentration; EU/mL, enzyme-linked immunosorbent assay per milliliter.
*GMC of all participants at 1 year post-dose 1 in the indicated study or parent study of the indicated study (as applicable).

challenge, a protective anamnestic response had developed. Thus, in NHPs, re-exposure to the EBOV GP antigen via a booster immunization provides protection within 3 days.

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Discussion

Vaccination with Ad26.ZEBOV, MVA-BN-Filo administered in a 56-day interval in humans induces strong EBOV GP-binding antibody responses that persist for at least 3.8 years post-dose 1. This agrees with modeling data, which suggest that antibody concentrations could persist, with minimal decline, up to 5 years after initial vaccination (21). Immunological memory is also maintained up to at least 3.8 years post-dose 1 and can be activated within 7 days by a booster immunization with Ad26.ZEBOV to levels greater than the highest levels observed after dose 2 of the primary vaccine regimen. After booster immunization, antibody levels sharply increase before declining and appear to be stable at 1 year post-booster at levels 12-fold higher than the plateau that persisted after the primary two-dose regimen. The kinetics of Ad26.ZEBOV, MVA-BN-Filo-induced EBOV GP-binding antibody responses are very similar when comparing humans with NHPs. After an initial peak, antibody levels in NHPs decline and reach a plateau phase approximately 6 months after dose 1 that persists for at least 1.3 years post-dose 1. An immunological memory response can be rapidly activated by a booster immunization with Ad26.ZEBOV, with GP-binding antibody levels exceeding levels reached at 21 days post-dose 2 of the primary regimen already observed within 7 days after the booster. Infection with EBOV also increased GP-binding antibody levels in animals infected shortly after vaccination, indicating that infection can activate an anamnestic response when at least partial protection is provided by the primary response. However, due to the rapid progression of EVD in the NHP model, there was no sufficient time for this anamnestic response to confer protection after a late EBOV challenge. An anamnestic response triggered via a booster immunization provided an onset of protection within 3 days, which is prior to a strong increase in circulating EBOV GP-binding antibodies. We will now first discuss the role of circulating EBOV GP-binding antibodies in protection against EVD before turning to the potential contribution of the anamnestic response to protection.

Early studies implicated $CD8^+$ T-cell responses in EVD protection mediated by an adenovirus type 5 (Ad5)-based vaccine (22), and innate immune responses were implicated in early protection by rVSV (23). However, a remarkably consistent picture emerges, across a wide range of vaccine platforms, that circulating EBOV GP-binding antibodies correlate with protection against EVD. This was indeed observed for vaccines based on vesicular stomatitis virus (VSV) (24), Ad5 (22), chimpanzee adenovirus type 3 (ChAd3) (25), virus-like particles (VLPs) (26), Ad26 and MVA (17), parainfluenza virus (PIV), and Newcastle disease virus (NDV) vectored vaccines (27). At least in some cases, antibody functionality appeared to be more closely associated with protection when compared to EBOV GP-binding antibody level per se (27). Circulating EBOV GP-binding antibody levels are therefore potentially a surrogate of an underlying mechanism of protection, such as Fc-receptor binding and neutrophil phagocytosis, which were associated with persistent protection after rVSVΔG-ZEBOV (28). In our studies, the level of circulating EBOV GP-binding antibodies after vaccination with Ad26.ZEBOV, MVA-BN-Filo was strongly correlated with the level of EBOV-neutralizing antibodies and had a similar discriminatory capacity for predicting challenge outcomes in NHPs, while cellular responses were independently correlated with protection (17). In addition, Ad26.ZEBOV, MVA-BN-Filo vaccination also induced antibody effector functions in humans, as shown by antibody-dependent NK-cell activation (29). As yet, it is unclear to what extent different antibody-mediated effector mechanisms are involved in protection against EVD by Ad26.ZEBOV, MVA-BN-Filo, though the long-term presence of Fc-gamma receptor binding antibodies in Sudan virus (SUDV)

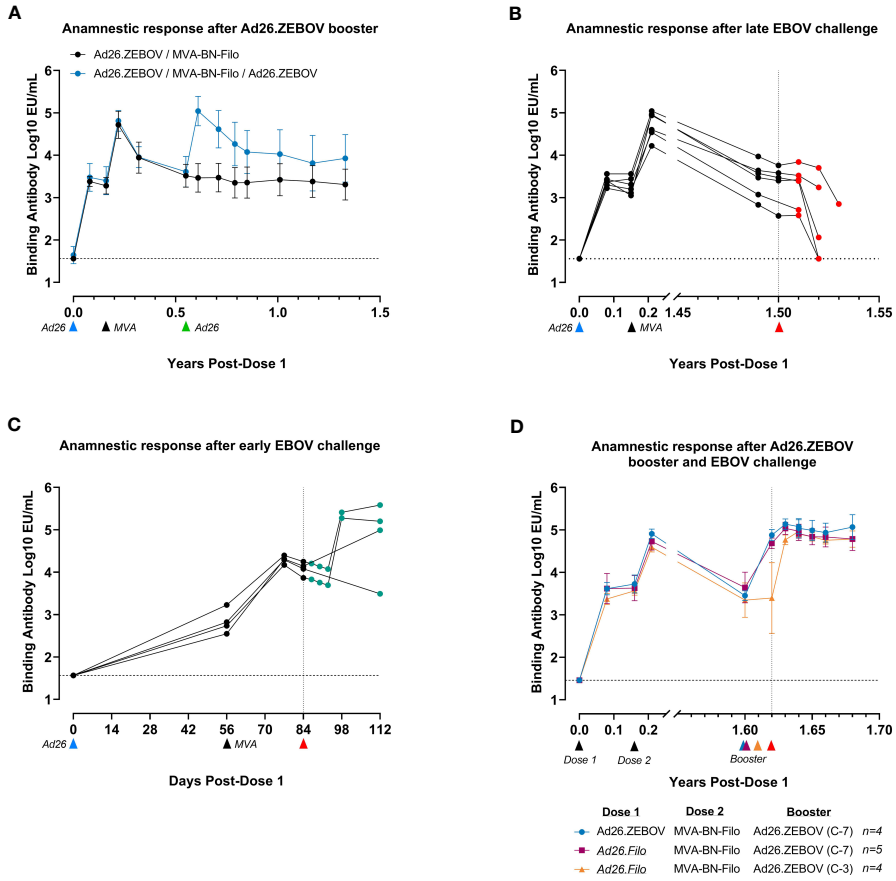

FIGURE 4
Anti-EBOV GP responses in serum of NHPs after vaccination and EBOV challenge as a measure of vaccine immunogenicity and the anamnestic response after challenge. EBOV GP-binding antibody levels at various time points in log10 EU/mL at various time points. **(A)** Comparison of the antibody response over time after vaccination with the clinical regimen with or without Ad26.ZEBOV boost at 0.5 years (day 196). Data are shown as group mean with standard deviation, n = 6/group. **(B)** Serum antibody concentration after vaccination followed by EBOV challenge 1.6 years (548 days) after the first dose. Individual NHP response profiles are shown. **(C)** Serum antibody concentrations after vaccination with Ad26.ZEBOV and MVA-BN-Filo, followed by short-term EBOV challenge 84 days after the first dose. Individual NHP response profiles are shown. The x-axis for this panel is reported in days, rather than years, due to the short time course of the experiment. **(D)** Serum antibody concentrations after vaccination with either Ad26.ZEBOV-MVA-BN-Filo or Ad26.Filo-MVA-BN-Filo, followed by Ad26.ZEBOV booster either 7 days or 3 days prior to EBOV challenge, 1.6 years (592 days) post-first dose. Data are displayed as group mean with standard deviation; group sizes as indicated in the figure legend. C-7, booster administration 7 days prior to EBOV challenge; C-3, booster administration 3 days prior to challenge. In **(A–C)**, the blue arrows below the x-axis indicate the timing of administration of the Ad26.ZEBOV vaccine doses, and the black arrows indicate timing of administration of the MVA-BN-Filo vaccine dose. In all panels, red arrows indicate intramuscular challenge with EBOV. Data points shaded green represent animals surviving EBOV challenge, while data points shaded red represent animals succumbing to the challenge before the study end. Dashed horizontal lines indicate lower limit of detection for each data set. For data in **(C)** the LOD for post-challenge data was set at the LOD of the pre-challenge data (1.46 vs. 1.56 log10 EU/mL). A continuous x-axis is used for panels **(A, C)** and an interrupted x-axis is used for **(B, D)** EU/mL, ELISA units per milliliter; GP, glycoprotein; NHPs, non-human primates; LOD, limit of detection.

survivors suggests that these types of antibodies could be important for protection against infection (30). Similarly, it was observed that EBOV survivors have high levels of antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis as compared to rVSV vaccinated individuals (31). In conclusion, circulating EBOV GP-binding antibodies likely contribute to protection elicited by virtually all GP-based vaccines, though they are also correlated with other mechanisms of protection.

The fact that the EBOV GP-binding antibody level is not necessarily a mechanistic correlate for protection imposes restrictions on when it can be used to infer protection. For instance, it is not a given that the correlation between circulating EBOV GP-binding antibody levels and survival is quantitatively similar at all time points after vaccination, even within a single vaccine platform. This is clearly illustrated by an elegant experiment that explored the onset of protection against MARV, using a ChAd3-based vaccine (32). Though there was a strong correlation between MARV GP-binding antibody levels 4 weeks after vaccination and protection 5 weeks after vaccination, the vaccine provided an onset of protection within 1 week in the absence of detectable MARV GP-binding antibody levels. In our own studies, protection was observed within 3 days after a booster vaccination in NHPs (Figure 4D), while circulating EBOV GP-binding antibodies were below levels that are associated with protection early after vaccination (17). This suggests that the initiation of the anamnestic response, rather than the level of EBOV GP-binding antibodies, could be considered as a potential correlate of protection in NHPs at later time points after vaccination.

In terms of the potential contribution of the anamnestic response to protection at later time points after vaccination, the triggering of the anamnestic response in NHPs needed to be supported by a booster vaccination with Ad26.ZEBOV given just before the challenge. However, the slower progression of EVD in humans could permit enough time for an anamnestic response to be triggered by the GP produced upon EBOV infection. Thus, the vaccine protective effect could evolve over time, being strongly correlated with circulating EBOV GP-binding antibody levels early after vaccination, while the durability of the protective effect would rely on persistent immunological memory (18). A similar situation exists for smallpox, where the vaccine take, as identified by a skin reaction, is considered the best predictor of vaccine efficacy, also highlighting a role for a protective memory response (33). In addition, in the case of hepatitis B, a full course of vaccination confers complete protection against acute clinical disease and chronic hepatitis B infection for long periods of time based on persisting memory responses, even after circulating antibody responses have become undetectable (34). Data from clinical studies with Ad26.ZEBOV, MVA-BN-Filo show that circulating EBOV GP-binding antibody concentrations in humans plateau approximately 6 months post-dose 2, and this plateau is maintained for at least 3.8 years. Although the tentative protective threshold for EBOV GP-binding antibodies of 200 EU/mL was identified for a different vaccine platform, which may have a different correlate of protection, it is interesting to note that the levels of circulating EBOV GP-binding antibodies persisting at 3.8 years after dose 1 of the Ad26.ZEBOV, MVA-BN-Filo vaccine regimen (418 EU/mL) were higher than this threshold. Importantly, administration of a booster dose of Ad26.ZEBOV resulted in a strong and rapid immune memory response, within 7 days of booster administration. This immune memory response was persistent over time and could be re-activated even when the booster dose was administered 3.8 years after dose 1 of the primary vaccine regimen.

The only filovirus vaccine for which a putative correlate of human protection has been identified is the rVSVΔG-ZEBOV vaccine (35). Both circulating EBOV GP-binding and neutralizing antibodies were correlated with protection, with EBOV GP-binding antibodies providing a better differentiation between protected and non-protected individuals. An EBOV GP-binding antibody level of 200 EU/mL was tentatively identified as a protective threshold in humans. This remarkably low level of EBOV GP-binding antibodies would not likely give sterilizing immunity on its own, providing further support for the notion that in humans, a vaccine anamnestic response contributes to protection against EVD, similar to the smallpox vaccine mentioned above. If this is indeed the case for EBOV infection, it may eventually be possible to establish persistent memory as a correlate of protection, irrespective of the vaccine platform, as most vaccine-mediated protection is based on the Ebola GP antigen. Thus, vaccines that have independently established protective efficacy in the NHP model could be evaluated for persistent immunological memory in humans while acknowledging potentially divergent correlates of protection (36). EBOV infection also triggered an anamnestic response in NHPs, albeit with apparently slower kinetics. The slower kinetics after EBOV infection versus an Ad26.ZEBOV booster may be due to a combination of immune modulation by EBOV (37), the time needed for viral replication to reach EBOV GP levels capable of triggering the anamnestic response, and soluble GP produced by EBOV reducing the amount of measurable circulating GP-binding antibodies. Taking into consideration the incubation time and slower disease progression in humans versus NHPs, it is likely that a protective anamnestic response could be mounted upon natural exposure to EVD, even several years after primary vaccination. Therefore, the ability to activate such an immune memory response could be considered as a potential independent correlate of long-term protection against EVD in humans.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Janssen has an agreement with the Yale Open Data Access (YODA) Project to serve as the independent review panel for the evaluation of requests for clinical study reports and participant-level data from investigators and physicians for scientific research that will advance medical knowledge and public health. Data will be made available following publication and approval by YODA of any formal requests with a defined analysis plan. For more information on this process or to make a request, please visit the Yoda Project site at http://yoda.yale.edu. The data sharing policy of Janssen Pharmaceutical Companies of Johnson & Johnson is available at https://www.janssen.com/clinical-trials/transparency.

Ethics statement

The studies involving humans were approved by The French national Ethics Committee (CPP Ile de France III; 3287), the French Medicine Agency (150646A-61), the UK Medicines and Healthcare Products Regulatory Agency (MHRA), and the UK National Research Ethics Service (South Central, Oxford; A 15/SC/0211) (EBL2001); the Burkina Faso Central Ethics Committee, the Comite National D'Ethique de La Recherche (Guinea), the Kenyatta National Hospital/University of Nairobi Ethical Review Committee (Kenya), the Uganda Virus Research Institute Research Ethics Committee, and the Makerere University School of Public Health Research and Ethics Committee (Uganda) (EBL2002); Comité d'Evaluation Ethique de l'Inserm, the London School of Hygiene & Tropical Medicine Ethics Committee the Comité National d'Ethique pour la Recherche en Santé (Guinea), National Health Science Research Ethics Committee (Liberia), the University of Mali Faculty Med Pharmacy & Dentistry (Mali); and the Sierra Leone Ethics and Scientific Review Committee (Sierra Leone) (EBL2004); The Sierra Leone Ethics and Scientific Review Committee, the Pharmacy Board of Sierra Leone, and the London School of Hygiene & Tropical Medicine Ethics Committee (EBL2011); the Sierra Leone Ethics and Scientific Review Committee, the Pharmacy Board of Sierra Leone, and the London School of Hygiene and Tropical Medicine Ethics Committee

(EBL3001); MaGil Institutional Review Board (EBL3002); MaGil Institutional Review Board (EBL3003). For pediatric participants, parents or guardians provided written informed consent for their child to join the trial. Older children (age varied by country) also gave written assent. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Alphagenesis, Bioqual, Charles River, or the Texas Biomedical Research Institute, depending on where the study was performed. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was not obtained from the owners for the participation of their animals in this study because the animals were purchased from commercial suppliers by Janssen. Therefore, Janssen was both the animal owner and study executor.

Author contributions

CM, KD, and RR wrote the manuscript and were involved in the design of experiments, as well as in data acquisition, analysis, and interpretation. AG, BK, MK, MD, CR, RZ, KL, and JH were involved in the design of the experiments, as well as in data acquisition, analysis, and interpretation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

CM, KD, AG, BK, MK, MD, CR, LS, DC-C, LD, MP, BC, JS, HS, RZ, KL, JH, and RR are employees of Janssen Vaccines and Prevention, B.V., and may hold shares of Johnson & Johnson. YW and AV are employees of Bavarian Nordic and may hold shares in the company.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1215302/full#supplementary-material

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Predictive values of immune indicators on respiratory failure in the early phase of COVID-19 due to Delta and precedent variants

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Background: Immune response indicators in the early phase of COVID-19, including interferon and neutralizing responses against SARS-CoV-2, which predict hypoxemia remains unclear.

Methods: This prospective observational study recruited patients hospitalized with COVID-19 (before emergence of omicron variant). As the immune indicators, we assessed the serum levels of IFN-I/III, IL-6, CXCL10 and VEGF, using an ELISA at within 5 days after the onset of symptoms, and serum neutralizing responses using a pseudovirus assay. We also assessed SARS-CoV-2 viral load by qPCR using nasal-swab specimens and serum, to assess the association of indicators and viral distribution.

Results: The study enrolled 117 patients with COVID-19, of which 28 patients developed hypoxemia. None received vaccine before admission. Serum IFN-I levels (IFN-α and IFN-β), IL-6, CXCL10, LDH and CRP were significantly higher in patients who developed hypoxemia. A significant association with nasopharyngeal viral load was observed only for IFN-I. The serum levels of IFN-α, IL-6, CXCL10 were significantly associated with the presence of RNAemia. Multivariable analysis showed higher odds ratio of IFN-α, with cut-off value of 107 pg/ml, in regard to hypoxemia (Odds ratio [OR]=17.5; 95% confidence interval [CI], 4.7-85; p<0.001), compared to those of IL-6, >17.9 pg/ml (OR=10.5; 95% CI, 2.9-46; p<0.001).

Conclusions: This study demonstrated that serum IFN-α levels in the early phase of SARS-CoV-2 infection strongly predict hypoxemic respiratory failure in a manner different from that of the other indicators including IL-6 or humoral immune response, and instead sensitively reflect innate immune response against SARS-CoV-2 invasion.

KEYWORDS

COVID-19, type I interferon, pneumonia, hypoxemia, interleukin-6, CXCL-10, humoral immune response

Introduction

Coronavirus disease 2019 (COVID-19) is a highly transmissible infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); the disease presentation and symptomology of COVID-19 ranges from asymptomatic to severe respiratory failure (1, 2). As increasing medical experience was acquired during the course of the pandemic, it has been recognized that severe COVID-19 is induced predominantly by complex immune regulation, rather than by cytokine storm syndrome (3, 4).

A recent meta-analysis conducted by Qin et al. (5), which evaluated 145 studies examining the association between immune-related indicators and COVID-19 prognosis, suggested that a combination of immunological, hematological, and biochemical parameters might be more sensitive in predicting disease severity and mortality following SARS-CoV-2 infection. As reported in previous studies, interleukin-6 (IL-6), the neutrophil-to-lymphocyte ratio (NLR), C-reactive protein (CRP) levels, and lactate dehydrogenase (LDH) levels have been determined to be the most representative parameters of SARS-CoV-2 infection; these findings have been repeatedly demonstrated in studies conducted in COVID-19 patients (6–8). We note that IL-6 is widely recognized as a pivotal cytokine in immune dysregulation, and also correlates with several important biomarkers (including C-X-C motif chemokine ligand 10 [CXCL10] and CRP) (9, 10).

To date, type I interferons (IFNs), which mainly consist of IFN-α and IFN-β, have emerged as crucial contributors to the innate immune response against SARS-CoV-2 infection (11, 12); interferons act as inhibitors of viral replication in infected cells and play a defensive role in uninfected cells. Although impairment of IFN-α and increased autoantibodies against IFN-α have been recognized as important contributors to the disease severity (11), the association between serum IFN-I levels and patient prognoses following SARS-CoV-2 infection remains unclear. Type III interferons (IFN-III), IL-29/IFN-λ1 and IL-28B/IFN-λ3, and which have received considerable attention as the predominant antiviral cytokines present at the mucosal barriers in the upper respiratory tract of SARS-CoV-2 infected patients, might potentiate the accurate prediction of coronavirus disease prognosis (13, 14).

Aside from those indicators that are representative of the innate immune response, humoral immune responses against SARS-CoV-2 infection have been considered the most robust form of immunity; humoral immune responses are induced by variable antibodies generated shortly after the onset of infection (15). Several studies have investigated the role of the humoral immune response in the early phase of SARS-CoV-2 infection in unvaccinated patients by measuring SARS-CoV-2 anti- receptor-binding domain titers or plasma/serum neutralizing responses using various methodologies (16–19). However, it remains unclear whether neutralizing activities against SARS-CoV-2 in the early phase of infection can predict favorable outcomes.

Herein, we sought to assess the potential of emerging immune indicators of SARS-CoV-2 as predictors of hypoxemia, in comparison with the biomarkers that are already in clinical use. In this study, we focused on the predictive value of immune indicators in the patients who had not received any vaccine against SARS-CoV-2 and infected by the Delta or precedent variants. The primary outcome of this study was to evaluate the association between indicators in the early phase of SARS-CoV-2 infection and the later development of hypoxemic respiratory failure. The secondary outcome of our investigation was to elucidate the pathophysiological implications of these indicators in relation to SARS-CoV-2 distribution.

Materials and methods

Study design

This study was conducted as part of the Toyama University COVID-19 Cohort Study, an investigator-initiated, prospective, single-center study, which was approved by the Ethical Review Board of the University of Toyama (R2019167).

The study period was between December 2020 and October 2021, which consisted of three major waves of the pandemic (before emergence of omicron) in Japan: the third wave (December to January 2021), the fourth wave (April to June 2021, which mainly occurred due to the Alpha variant), and the fifth wave (July to October 2021, which mainly occurred due to the Delta variant). Nasal specimens for reverse transcription quantitative polymerase chain reaction (RT-qPCR) were collected and chest CT was performed at admission; moreover, serum samples were stored

frozen at -80°C after each laboratory examination. Written informed consent was obtained from all patients.

Study participants and study protocol

The inclusion criteria were as follows: 1) men or women aged 18 years or older, who were diagnosed with COVID-19 based on the findings of RT-qPCR assays. 2) patients hospitalized at Toyama University Hospital (Toyama, Japan) between December 2020 and October 2021, and 3) patients with a first blood sample collected within five days after symptom onset.

Since the large population with COVID-19 had not received vaccine against SAR-CoV-2 during the study period, we excluded the following from the present study; patients who had received a vaccine or SARS-CoV-2 antibody treatment, or those who participated in another clinical trial.

Clinical data on patient were collected from patients' medical charts. COVID-19 pneumonia was confirmed by trained pulmonary radiologists (KN and YY), when a newly developed inflammatory lesion was detected by chest CT performed on admission, according to the previous reports (20, 21). Patients without inflammatory lesions were confirmed to be negative for COVID-19 pneumonia.

Hypoxemia requiring oxygen therapy was defined according to a blood oxygen saturation (SpO_2) level of ≤93% at rest on room air. This is a universally accepted criterion for the initiation of oxygen therapy for COVID-19 (22). Patients were excluded from further analysis, if had received oxygen therapy for hypoxemia (SpO_2 ≤93% at rest) or had received any anti-viral or immunomodulate therapy at the time of admission.

Blood samples

The stored blood serum samples of the patients were used for cytokine and RNAemia measurements as described below. Only serum collected within 5 days after symptom onset was used for the analysis.

Cytokine measurement

Serum cytokines and chemokines (IFN-α, IFN-β, IFN-λ1 [IL-29], IFN-λ3 [IL-28-B], IL-6, CXCL10 and vascular endothelial growth factor [VEGF]) were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturers' instructions (see Supplemental Data; Table S1). VEGF, which was also known as potential predictor for prognosis of COVID-19 (23), was included as a comparator indicator. If an analyte signal was below the background signal, it was set to 0; if the signal was detectable but below the manufacturer's lower limit of quantification, it was set to the lower limit of detection. Each lower limit of detection was as follows; IFN-α for 0.43 pg/mL, IFN-β for 1.2 pg/mL, IFN-λ1 for 2.0 pg/mL, IFN-λ3 for 2.1 pg/mL, IL-6 for 1.2 pg/mL, CXCL10 for 13.4 pg/mL.

RT-qPCR

RT-qPCR (for detecting SARS-CoV-2) was performed as previously described in a study conducted at our hospital (24). The detection limit was approximately 0.4 copies/μL (2 copies/5 μL). RNAemia was determined when SARS-CoV-2 was detectable in blood serum specimens.

The presence of mutation on SARS-CoV-2 was examined with the screening PCR tests, which was conducted as administrative tests at Toyama institution of health (Toyama, Japan), during the fourth and fifth wave. The presence of N501Y mutation (suspected as alpha variant, if positive) was examined with patients in the fourth wave, using Primer/Probe N501Y (Takara Bio Inc., Shiga, Japan). The presence of L452R (suspected as delta variant, if positive) was examined with patients in the fifth wave, using Primer/Probe L452R Ver.2 (Takara Bio Inc.).

Pseudo-virus neutralization assay

The neutralizing activity of human serum against pseudo-viruses was measured using the high-throughput chemiluminescent reduction neutralizing test (htCRNT), as previously described (25). The values of samples without the pseudo-virus and those with the pseudo-virus but without serum were defined as 0% and 100% infection (100% and 0% inhibition), respectively.

In order to measure neutralizing activity (NT) against the infected variant of each pandemic wave, we used three pseudo-viruses with expression plasmids for the truncated S protein of SARS-CoV-2; pCAG-SARS-CoV-2 S (Wuhan; wild-type [WT]), pCAGG-pm3-SARS2-Shu-d19-B1.1.7 (Alpha-derived variant), and pCAGG-pm3-SARS2-Shu-d19-B1.617.2 (Delta-derived variant). For all patients, NT was evaluated against the WT strain using a pseudo-virus with the truncated S protein of the WT strain. In addition, NT against the infected variant was measured for the fourth and fifth pandemic waves; NT against alpha variant for the fourth wave, and NT against delta variant for the fifth wave. We defined the NT against each SARS-CoV-2 variant of that pandemic wave as the "adjusted NT."

Statistical analysis

A summary of the participants' medical and demographic characteristics was expressed using medians (interquartile ranges) or numbers (percentages). Differences between the two groups were tested using the Mann–Whitney test or Fisher's exact test. The Mann–Whitney U test with Bonferroni correction was used to compare nominal variables among three groups.

Receiver operating characteristic (ROC) curves and the respective areas under the ROC curve (AUC) were generated using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). The cut-off value for the prediction of hypoxemia was determined using the nearest point relative to the left corner of each ROC curve.

The association between each pair of biomarkers and nasopharyngeal viral load was determined using Spearman's rho correlation coefficient.

The association of each biomarker with hypoxemic respiratory failure was estimated as an adjusted odds ratio (OR) calculated via a logistic regression model adjusting for potential confounders that were determined based on clinical considerations (age, sex, body mass index [BMI], and patients' present history of hypertension/diabetes mellitus). These variables were dichotomized as above or below the cut-off value calculated using ROC curve analysis. The size of the tests was set to 0.05 and statistical significance was set to $p < 0.05$. R statistical software (v.4.1.018; The R Project for Statistical Computing, Vienna, Austria) and GraphPad Prism software (v.9.0) were used for the statistical analyses.

Results

Study participants

The clinical characteristics, microbiological findings, treatments, and outcomes of the 117 patients included in this study are summarized in Table 1. We note that a portion of the study population was included in previous experimental reports investigating the clinical implication of IFN-I (n = 50) (26), and the effect of monoclonal antibody treatment (n = 28) (27). A total of 28 patients in the entire cohort developed hypoxemia, while 89 did not. Age, underlying disease (hypertension, diabetes mellitus), and BMI were significantly different between patients with COVID-19 who did or did not develop hypoxemia. All enrolled patients survived COVID-19 for at least 30 days after symptom onset.

Serum biomarkers and the development of hypoxemic respiratory failure

The results of the biomarker-level analyses are summarized in Figures 1A–J. Because the analyte signals for CXCL10 were higher than the detectable range in four patients (too strong analyte signal to be detected), we set the highest value of CXCL10 to 2,000 pg/mL in further analyses. We found that IFN-α, IFN-β, IL-6, CXCL10, LDH, and CRP levels were significantly higher in patients who later developed hypoxemia than in those who did not.

TABLE 1 Clinical features, microbiological findings of patients in the study.

	Total (n=117)	Hypoxemia required oxygen therapy		P value
		Positive (n=28)	Negative (n=89)	
Age, years	46 [31-54]	57 [51-64]	38 [26-51]	<0.001
Sex; male/female	71/46	23/5	48/41	0.008
Pandemic period				
Third wave	39 (33%)	1 (3%)	38 (43%)	—
Fourth wave	50 (43%)	17 (61%)	33 (37%)	—
Fifth wave	28 (24%)	10 (36%)	18 (20%)	—
Underlying disease				
None	63 (54%)	9 (32%)	54 (61%)	0.010
Hypertension	21 (18%)	12 (43%)	9 (10%)	<0.001
Diabetes mellitus	8 (7%)	5 (18%)	3 (3%)	0.019
Body mass index	22.8 [20.8-25.5]	25.3 [23.4-27.6]	21.6 [20.5-24.4]	<0.001
Initial nasopharyngeal-viral load (log copies/μL)	4.8 [3.8-5.5]	5.2 [4.0-5.6]	4.7 [3.8-5.4]	0.185
RNAemia	31 (26%)	16 (57%)	15 (17%)	<0.001
Treatment				
Remdesivir	29 (25%)	28 (100%)	1 (1%)	—
Dexamethasone	31 (26%)	28 (100%)	3 (3%)	—
Heparin	31 (26%)	28 (100%)	3 (3%)	—
Nasal High Flow	4 (3%)	4 (14%)	—	—
IPPV	2 (2%)	2 (7%)	—	—
30 days-mortality	0 (0%)	0 (0%)	0 (0%)	—

Continuous variables are reported as median [interquartile range (IQR) 25-75]. Categorical variables are reported as number (percentages).
'—' indicates that the data were not applicable for evaluation nor comparison.

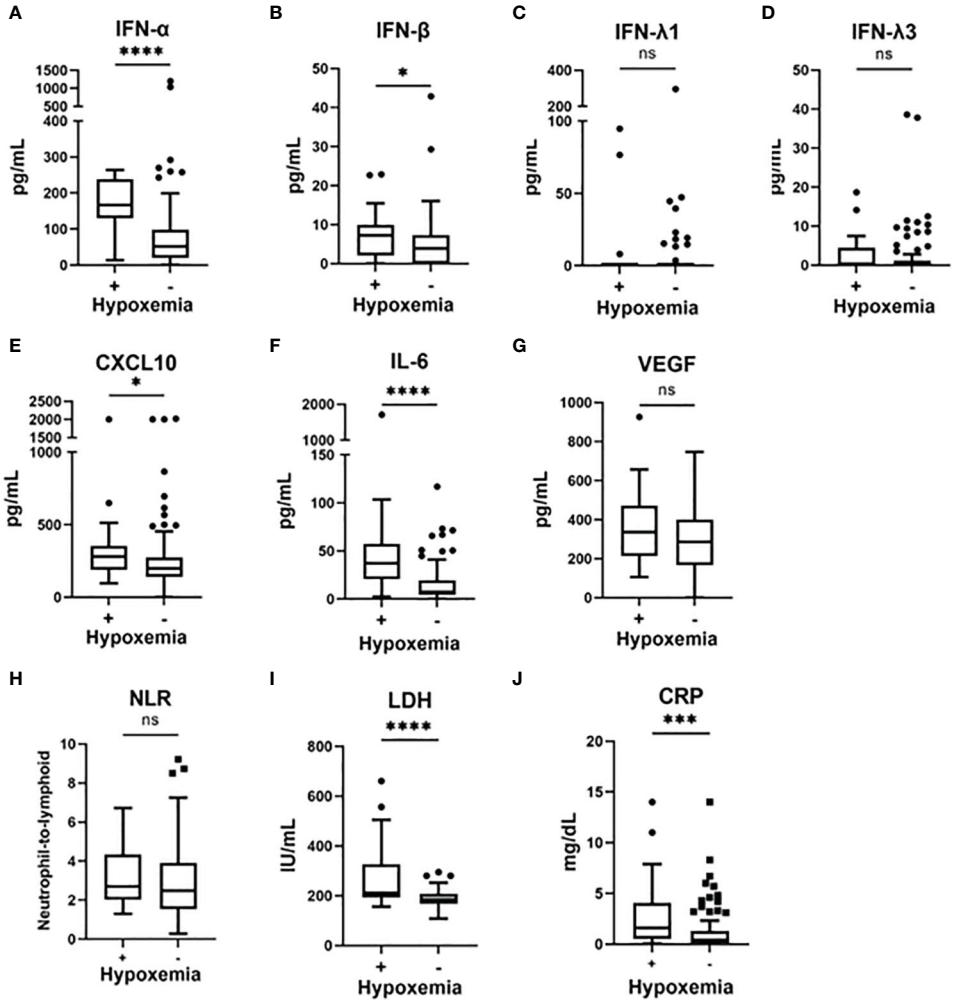

FIGURE 1
Serum biomarker levels in the early phase of SARS-CoV-2 infection and associations with the development of hypoxemic respiratory failure. Serum biomarker levels in the early phase of SARS-CoV-2 infection and associations with the development of hypoxemic respiratory failure: **(A)** IFN-α, **(B)** IFN-β, **(C)** IFN-λ1, **(D)** IFN-λ3, **(E)** CXCL10, **(F)** IL-6, **(G)** VEGF, **(H)** NLR, **(I)** LDH, and **(J)** CRP. Each biomarker level was evaluated at hospital admission (within five days after symptom onset), without hypoxemic respiratory failure being present at that time. Data are presented using Tukey box-plots as well as using individual values. *p <0.05; ***p <0.001; ****p <0.0001; ns, not significant.

Serum neutralizing activities against SARS-CoV-2 strains

Our results in regard to NT against SARS-CoV-2 strains are summarized in Figure 2. With patients in the third, fourth, and fifth wave, NT against the WT strain were not significantly different between those who developed hypoxemia and those who did not (Figure 2A). Similarly, the adjusted NT were not significantly different between patients who developed hypoxemia and those who did not develop hypoxemia (Figures 2B–D).

The presence of N501Y mutation was confirmed in 94% of the fourth wave, and the presence of L452R was confirmed in 82% of the fifth wave. Whereas, neither mutation was unknown in several cases; 3 out of 50 patients in the fourth wave (none developed hypoxemia), and 5 out of 28 the fifth wave (3 developed hypoxemia).

NT values against the WT strain in the whole cohort were significantly lower than the findings for adjusted NT (Figure 2E).

In the measurement of NT against each variant, we simultaneously examined time-dependent changes in NT in several patients to confirm whether NT values could consistently reflect the neutralizing response against SARS-CoV-2 infection. As shown in Figure S1, NT against each strain increased until two weeks after symptom onset, which was confirmed in 14/14 patients with available serum.

Biomarker levels and SARS-CoV-2 viral load

To examine the association between microbiological findings and biomarker levels, we measured the viral load in nasal-swab

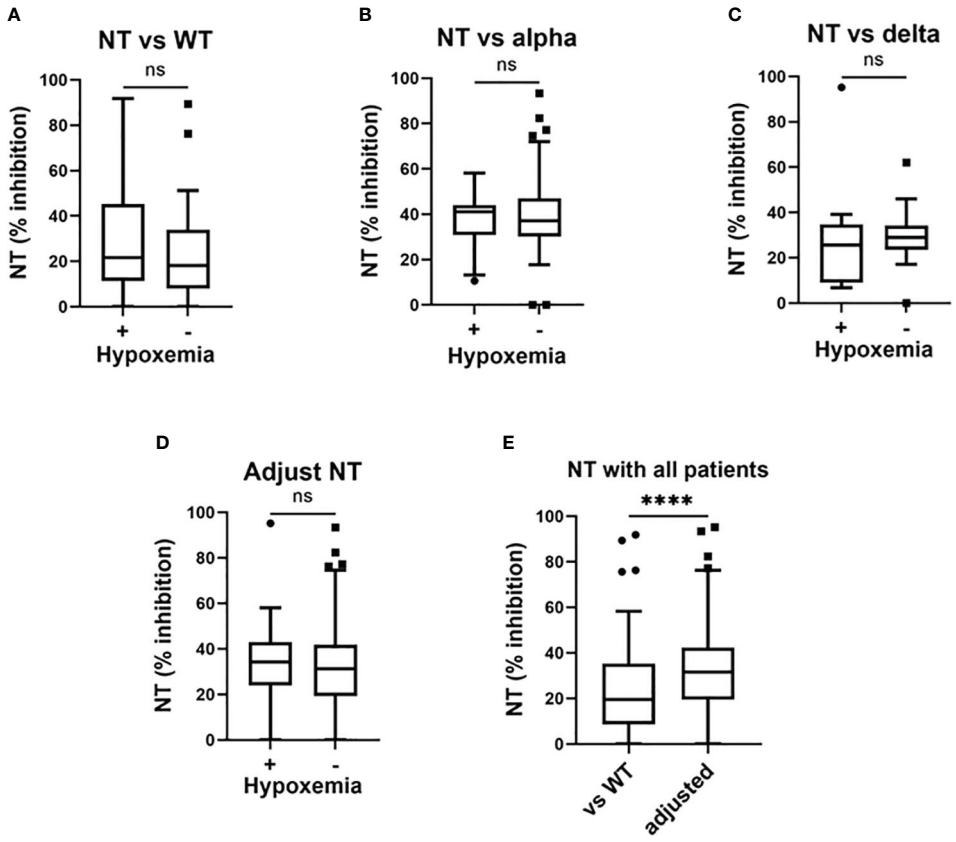

FIGURE 2
Serum neutralizing activities in the early phase of SARS-CoV-2 infection and associations with the development of hypoxemic respiratory failure. Serum neutralizing activities (NT; % inhibition) in the early phase of SARS-CoV-2 infection (i.e., within five days after symptom onset) and associations with the development of hypoxemic respiratory failure: **(A)** NT against the wild-type (WT) strain in patients enrolled in the third, fourth, and fifth pandemic waves (n = 117); **(B)** NT against the Alpha variant in patients enrolled in the fourth pandemic wave (n = 50); **(C)** NT against the Delta variant in patients enrolled in the fifth pandemic wave (n = 28); **(D)** adjusted NT values in patients enrolled in the third, fourth, and fifth pandemic waves (n = 117); **(E)** NT against the WT strain and adjusted NT values in patients enrolled in the third, fourth, and fifth study waves. Each level was evaluated at hospital admission (within five days after symptom onset), without hypoxemic respiratory failure being present at that time. Adjusted NT values were significantly higher than those of NT against the WT strain. ****p <0.0001; ns, not significant.

specimens and in serum. We could not assess the nasal viral load in five patients (all did not develop hypoxemia), because we could not collect the nasal-swab specimen for this observational study. As shown in Figure S2, the levels of IFN-α and IFN-β, as well as evaluations NT against the WT strain, significantly correlated with the SARS-CoV-2 viral load in nasal-swab specimens. A stronger correlation was observed with IFN-β than with IFN-α. Moreover, as shown in Figure 3, IFN-α, IFN-λ1, CXCL10, and IL-6 levels were significantly higher in patients with RNAemia than in those without RNAemia.

Association between biomarker levels and the presence of pneumonia

We analyzed the association between various biomarkers and the presence of pneumonia. As shown in Figure S3, in addition to the biomarkers that were associated with the development of hypoxemia (IFN-α, IL-6, CXCL10, LDH, and CRP), VEGF, NLR, and adjusted NT levels were significantly higher in patients with pneumonia than in those without pneumonia.

Predictors of hypoxemic respiratory failure due to SARS-CoV-2 infection

To determine the predictors of hypoxemic respiratory failure occurring due to SARS-CoV-2 infection and its respective cut-off values, we conducted an ROC analysis for each biomarker (Figures 4A–C; Figure S4). AUCs were higher than >0.8 for IFN-α and IL-6. Multivariate regression analysis showed that serum IFN-α levels higher than the cut-off value of 107 pg/mL showed the highest OR for hypoxemic respiratory failure, demonstrating a greater than two-fold stronger association as compared with IL-6 and CXCL10 (Figure 4D). Among the evaluated biomarkers, IFN-β, CXCL-10, IL-6, LDH, and CRP levels significantly and directly correlated with serum IFN-α levels (Figure 5). In contrast, IL-6 and CXCL10 levels correlated more strongly and significantly correlated

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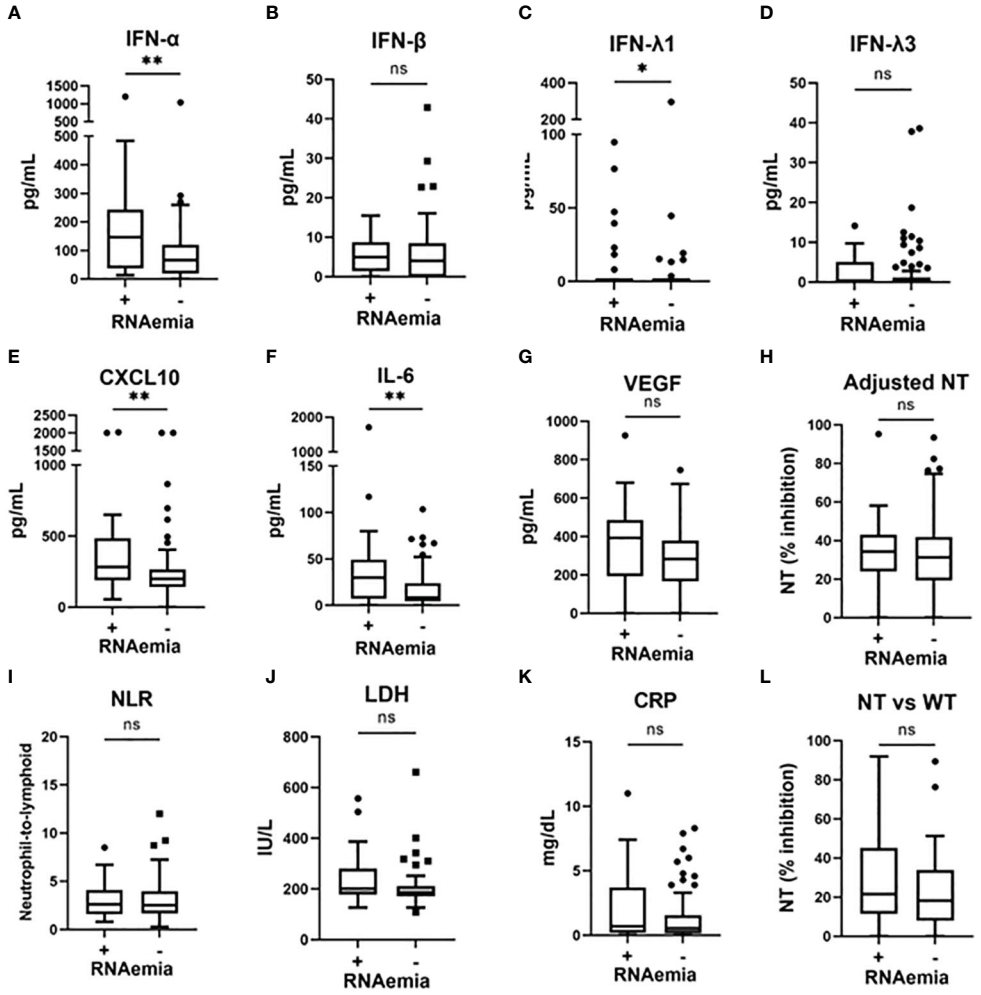


FIGURE 3
Serum biomarker levels in the early phase of SARS-CoV-2 infection and associations with the presence of RNAemia. Serum biomarker levels in the early phase of SARS-CoV-2 infection and associations with the presence of RNAemia: **(A)** IFN-α, **(B)** IFN-β, **(C)** IFN-λ1, **(D)** IFN-λ3, **(E)** CXCL10, **(F)** IL-6, **(G)** VEGF, **(H)** adjusted NT, **(I)** NLR, **(J)** LDH, **(K)** CRP, and **(L)** NT vs WT. Each level was evaluated at a time point at hospital admission (within five days after symptom onset). Data are presented using Tukey box-plots and individual values. *p <0.05, **p <0.005; ns, not significant. NT, neutralizing activities (% inhibition); NT vs WT, neutralizing activities against the wild-type strain.

with LDH and CRP levels. Notably, IFN-β levels significantly correlated with adjusted NT values and with IFN-α levels.

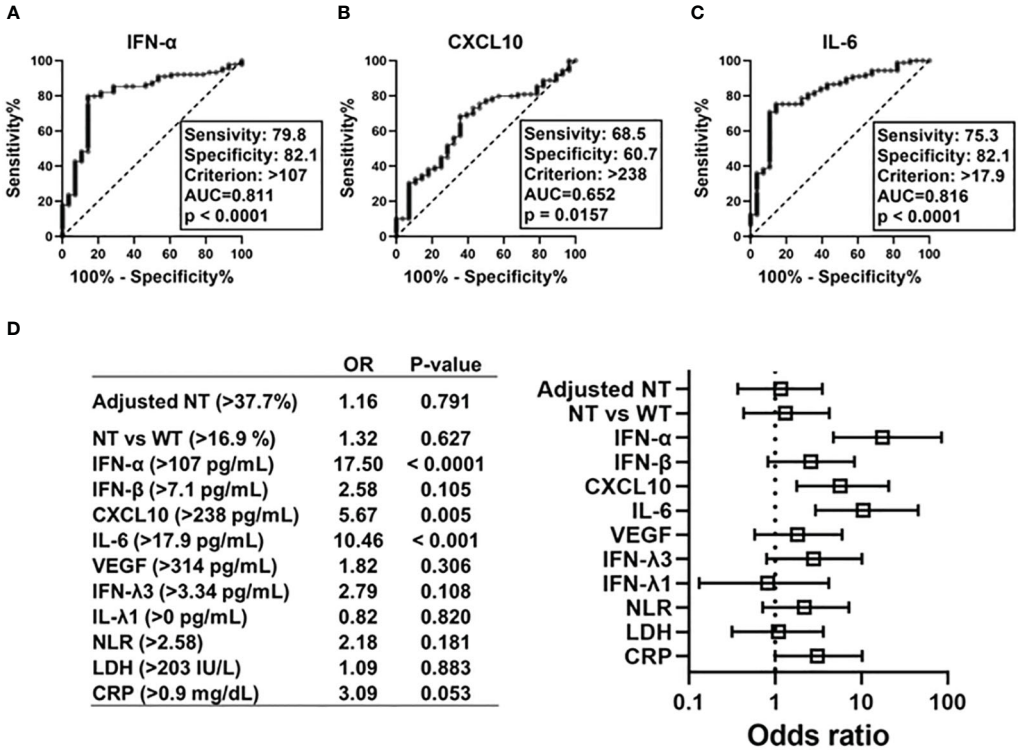
Discussion

This study demonstrated that serum IFN-α levels present a higher OR in regard to the development of hypoxemia in the early phase of COVID-19 than those of other biomarkers, including IL-6. The distinct features of IFN-α in relation to other IFNs and cytokines, the distribution of the virus, and findings in regard to neutralization activity support our preliminary conclusion (26) that IFN-α could be a unique and strong predictor of hypoxemia occurring due to SARS-CoV-2 infection. Also, these strongly support that IFN-α play critical role in the early phase of COVID-19 due to Delta and the precedent variants.

In our study, IL-6 was one of the most significant predictors of hypoxemia due to SARS-CoV-2 infection; hypoxemia was also closely related to CXCL10, LDH, and CRP levels. The mean level of IL-6 in patients with hypoxemia enrolled in our study, 37.3 pg/mL [20.8-55.5], was similar to that reported in a recent study (10).

Among the indicators measured in this study, IFN-I, IFN-α and IFN-β, significantly correlated with nasopharyngeal viral load in the early phase of SARS-CoV-2 infection, indicating that IFN-I sensitively interacts with viral replication rather than with other biomarkers. Moreover, we confirmed that IFN-α was significantly associated with RNAemia, consistent with the findings of our previous study (26). The strong association between IFN-α and RNAemia detected herein may explain why IFN-α was the most significant predictor of hypoxemia in the current study.

Previous studies have suggested that an impaired IFN-I response and lower serum IFN-α levels could be hallmarks of severe COVID-19 (11, 12). To date, a few studies have investigated IFN-α levels in the early phase of SARS-CoV-2 infection (i.e., within five days after symptom onset), and these studies detected a range of serum IFN-α levels (28, 29). In the current study, we confirmed that serum IFN-α



	OR	P-value
Adjusted NT (>37.7%)	1.16	0.791
NT vs WT (>16.9 %)	1.32	0.627
IFN-α (>107 pg/mL)	17.50	< 0.0001
IFN-β (>7.1 pg/mL)	2.58	0.105
CXCL10 (>238 pg/mL)	5.67	0.005
IL-6 (>17.9 pg/mL)	10.46	< 0.001
VEGF (>314 pg/mL)	1.82	0.306
IFN-λ3 (>3.34 pg/mL)	2.79	0.108
IL-λ1 (>0 pg/mL)	0.82	0.820
NLR (>2.58)	2.18	0.181
LDH (>203 IU/L)	1.09	0.883
CRP (>0.9 mg/dL)	3.09	0.053

FIGURE 4
Predictive value of each immune indicators on development of hypoxemic respiratory failure in the early phase of SARS-CoV-2 infection. ROC curves and AUCs for biomarker levels in patients with SARS-CoV-2 infections in regard to diagnostic values indicating respiratory failure: **(A)** IFN-α, **(B)** CXCL10, and **(C)** IL-6. Forest plots representing odds ratios for each biomarker in regard to the development of hypoxemia at an early phase of SARS-CoV-2 infection (within five days after symptom onset) **(D)**. Each variables were dichotomized as above or below the cut-off value calculated using ROC curve analysis. Each odds ratio (OR) is adjusted for age, sex, body mass index, and history of hypertension/diabetes mellitus. AUC, area under ROC curve; NT, neutralizing activities (% inhibition); NT vs WT, neutralizing activities against the wild-type strain; ROC, receiver operating characteristic.

levels with a cut-off level of 107 pg/mL were significantly associated with the development of hypoxemia in patients enrolled during different pandemic periods. In contrast, we also found that a few of our enrolled patients developed hypoxemia with low IFN-α levels (<100 pg/mL), namely 1/17 patients (5.9%) who developed hypoxemia in the fourth wave and 2/10 patients (20.0%) who developed hypoxemia in the fifth wave. These incidents were similar to those of sub-phenotypes with IFN-I autoantibodies (˜20%) (30). With COVID-19 in the early pandemic period (March-May 2020), several studies demonstrated the association between lower IFN-α and fatal COVID-19 (31, 32). In the present study, six patients required mechanical ventilation or nasal high flow, and one of those presented lower IFN-α levels (19.5 pg/mL). This supports the possibility that sub-phenotype may exist, which follow fatal course with lower IFN-α levels at the early phase of COVID-19. The caution might be necessary in regard to specifying the sub-phenotype, in the application of serum IFN-α levels as a clinical predictor of hypoxemia due to SARS-CoV-2 infection.

In this study, we measured serum neutralizing responses against the SARS-CoV-2 WT strain and against each coronavirus variant using the pseudo-virus assay, and explored the relationship between biomarkers and prognosis following SARS-CoV-2 infection. As shown in Figure S1, we confirmed that all neutralizing ht-CRNT values were elevated over 90% until two weeks after symptom onset, which supports the speculation that the lower neutralizing value in the early phase of SARS-CoV-2 infection consistently reflects lower humoral immune responses against SARS-CoV-2. Notably, the assessment of serum neutralizing activities in the early phase of SARS-CoV-2 infection revealed that the adjusted neutralizing value was significantly associated with the presence of pneumonia (Figure S3), but not with the development of hypoxemia or the presence of RNAemia. This was partly consistent with a previous study conducted by Park et al., which found a significant correlation between neutralizing titers and chest radiography scores in 40 patients with COVID-19 (18).

It is possible that a passive immune response could be induced earlier in patients who develop pneumonia than in those who do not. Among the indicators measured in this study, IFN-β and CRP levels significantly correlated with adjusted NT, which strongly suggests interactions between IFN-β, CRP, and the activation of the humoral immunity response against SARS-CoV-2. Additional studies are necessary to investigate the detailed pathophysiology of neutralization as well as associated biomarkers. According to the results of this study, we suggest that the neutralizing value in the early phase of SARS-CoV-2 infection might be poorly associated with the prediction of respiratory failure.

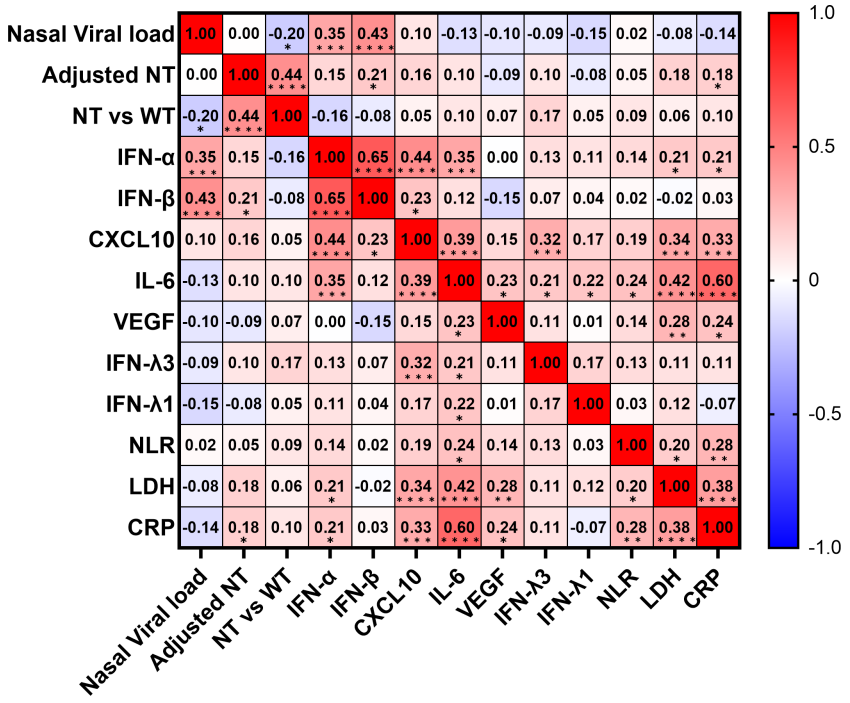


FIGURE 5
Correlation matrix of biomarkers in patients with SARS-CoV-2 infection in the early phase. Correlation matrix of biomarkers in patients with SARS-CoV-2 infection in the early phase (within five days after symptom onset). Results are presented as a correlation matrix. Spearman correlation coefficients are plotted. Cells were colored according to the strength and trend of correlations (shades of red = positive correlations, shades of blue = negative correlations). *p <0.05; **p <0.005; ***p<0.001; ****p<0.0001. NT, neutralizing activities (% inhibition); NT vs WT, neutralizing activities against the wild-type strain.

The present study has several limitations. First, the single-center observational study design of the present study, along with a modest sample size, may have resulted in selection bias. Second, we validated IFN and cytokine levels using serum samples that were not strictly stored immediately after drawing (these samples were instead immediately stored in a 4°C freezer and were then transferred to a -80°C deep freezer). Third, we could not identify the causative strain in the third pandemic wave and a part of the fourth and fifth wave, because genetic identification of the epidemic strain was not available. Therefore, our results in regard to adjusted NT did not completely reflect NT against the infected strain. Fourth, we did not assess the predictive value of immune indicators in patients with COVID-19 by Omicron variant, in this study. The Omicron variant rapidly outcompeted Delta variant by late 2021, and has dominated the pandemic until today (33). In order to promote IFN-α as the predictive biomarker of COVID-19 in clinical use, further assessment in patients with COVID-19 by Omicron variant would be necessary. However, considering our consistent results and the detected associations between IFN, the major cytokine evaluated herein, and NT, we believe that these limitations were not likely to have meaningfully affected our findings, which elucidated the critical part of immune dynamics in COVID-19 without vaccination, due to the precedent virulent variants other than Omicron variant.

In conclusion, we demonstrated that serum IFN-α levels strongly predict hypoxemic respiratory failure in the early phases of COVID-19 by the Delta and the precedent variants, as do IL-6 levels. We suggest that early elevation of serum IFN-α levels may reflect an innate immune response against SARS-CoV-2 systemic invasion, which could be a novel indicator of hypoxemic respiratory failure. These findings highlight the most important feature of immune indicators in COVID-19 during the pandemic period before emergence of Omicron variant, not only as a highly potential predictive factor of hypoxemic respiratory failure but also as a clue to understanding the pathophysiology of COVID-19 due to the current and future variants.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by The Ethical Review Board of the University of Toyama. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, KN; Methodology, KN, HK, HT; Software, KN; Validation, KN, HK, YMo; Formal Analysis, KN; Statistical Analyses, KN, SM; Investigation, KN and HK; Resources, KN; Data Curation, KN, HK, YMu, MK, KK, HN, YMo; Writing – Original Draft Preparation, KN; Writing – Review & Editing, KN, YMo, and YY; Visualization, KN; Supervision, YY; Project Administration, KN and YY; Funding Acquisition, YY. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1197436/full#supplementary-material

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Immunological correlates of protection afforded by PHV02 live, attenuated recombinant vesicular stomatitis virus vector vaccine against Nipah virus disease

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Introduction: Immune correlates of protection afforded by PHV02, a recombinant vesicular stomatitis (rVSV) vector vaccine against Nipah virus (NiV) disease, were investigated in the African green monkey (AGM) model. Neutralizing antibody to NiV has been proposed as the principal mediator of protection against future NiV infection.

Methods: Two approaches were used to determine the correlation between neutralizing antibody levels and outcomes following a severe (1,000 median lethal doses) intranasal/intratracheal (IN/IT) challenge with NiV (Bangladesh): (1) reduction in vaccine dose given 28 days before challenge and (2) challenge during the early phase of the antibody response to the vaccine.

Results: Reduction in vaccine dose to very low levels led to primary vaccine failure rather than a sub-protective level of antibody. All AGMs vaccinated with the nominal clinical dose (2×10^7 pfu) at 21, 14, or 7 days before challenge survived. AGMs vaccinated at 21 days before challenge had neutralizing antibodies (geometric mean titer, 71.3). AGMs vaccinated at 7 or 14 days before challenge had either undetectable or low neutralizing antibody titers pre-challenge but had a rapid rise in titers after challenge that abrogated the NiV infection. A simple logistic regression model of the combined studies was used, in which the sole explanatory variable was pre-challenge neutralizing antibody titers. For a pre-challenge titer of 1:5, the predicted survival probability is 100%.

The majority of animals with pre-challenge neutralizing titer of ≥1:20 were protected against pulmonary infiltrates on thoracic radiograms, and a majority of those with titers ≥1:40 were protected against clinical signs of illness and against a ≥fourfold antibody increase following challenge (indicating sterile immunity). Controls receiving rVSV-Ebola vaccine rapidly succumbed to NiV challenge, eliminating the innate immunity stimulated by the rVSV vector as a contributor to survival in monkeys challenged as early as 7 days after vaccination.

Discussion and conclusion: It was concluded that PHV02 vaccine elicited a rapid onset of protection and that any detectable level of neutralizing antibody was a functional immune correlate of survival.

KEYWORDS

Nipah virus, vaccine, recombinant VSV, immune correlate, neutralizing antibody

Introduction

A central concept in vaccinology is the definition of the immune responses provoked by a vaccine and the role of these responses in protecting against the target (infectious) disease (1). Ideally, the immune response can serve as a surrogate for randomized controlled trials (RCTs) since the former generally requires a much smaller sample size, does not require a population of subjects affected by the disease, can be applied to special populations (e.g., the elderly, infants, and diverse ethnic groups), and can answer important questions—in particular, the durability of protection. The majority of existing vaccines appear to protect against future exposure *via* antibodies—in most cases, mechanistically functional antibodies (2). However, from a regulatory perspective, an immune surrogate does not need to be functional and can be a representative predictor or biomarker, signaling that an underlying functional response that is responsible for clinical benefit to the subject has occurred. The latter concept is embodied in the FDA's "Accelerated Approval" pathway which allows marketing authorization of a vaccine for prevention of a serious condition or for an unmet medical need based on a surrogate endpoint (immunological biomarker) that predicts clinical benefit. The sponsor is required to confirm that there is a meaningful clinical benefit in phase IV efficacy or effectiveness trial post-marketing (3).

Examples of vaccines that have been approved in the US or elsewhere based on an immune surrogate include those for COVID-19, influenza, pneumococcal and meningococcal disease, smallpox, rabies, yellow fever, and Japanese encephalitis, but in most cases, it has been possible to compare the immune response to a pre-existing vaccine with established efficacy or effectiveness. The use of a non-inferiority design is not feasible for vaccines against new target indications without a pre-existing accepted vaccine or when an immunologic correlate has not been defined. In such cases, immune responses in animal disease models where protection can be assessed by experimental challenge are bridged to responses in human vaccine trials. As an example, immune responses to the Ad26 vector prime-MVA-BN-Filo boost vaccine against Ebola virus disease were bridged to human immune responses in clinical trials, showing a close correlation between protection in nonhuman primates and IgG-binding antibody levels (4). Inferences may also be drawn from a comparison of vaccine responses to natural infection immunity (5). Ideally, a level of protective immunity, e.g., an antibody titer, is defined, providing quantitative, statistical means to determine protection based on the surrogate.

NiV disease is a relatively rare but highly lethal bat-borne zoonosis in south and southeast Asia caused by a single-strand, negative-sense RNA virus in the Henipavirus genus, family *Paramyxoviridae*. The disease is characterized by acute, severe pneumonia and encephalitis and has a 40%–75% (or higher) case fatality rate (6). Recrudescent, late encephalitis is described (7), a feature which has implications for vaccine development. The development of NiV vaccines is a high priority for the World Health Organization (WHO) (8) and the Coalition for Epidemic Preparedness Innovations (CEPI) since the virus is transmissible from person to person by the respiratory route and has a pandemic potential. Multiple NiV vaccines are in development, and three have entered phase 1 clinical trials [a subunit protein vaccine (NCT04199169), an mRNA vaccine (NCT05398796), and a recombinant vesicular stomatitis (rVSV)-vectored live, attenuated vaccine (NCT05178901), which is the vaccine candidate described here].

All vaccines against NiV face the same problem for regulatory approval, namely, that (at least in the face of the current

epidemiological situation of unpredictable, intermittent small outbreaks) RCTs for efficacy are likely infeasible. The largest outbreak to date occurred in Malaysia (1998–1999), with 265 cases and 105 deaths (9), and subsequent outbreaks have involved a few to tens of cases. This leaves two potential pathways to regulatory authorization: accelerated approval and the Animal Rule (AR). While both pathways require animal data on immune correlates that are then bridged to human vaccine responses, the AR is acknowledged to be difficult and requires a highly stringent understanding of pathogenesis in the animal model. The time required for the latter may not be consistent with current objectives of rapid vaccine development against emerging public health threats (10). The question, therefore, is whether an immune surrogate for protective immunity against NiV that would allow emergency use authorization and, eventually, full marketing approval can be defined.

At this stage of development of NiV vaccines, there are encouraging indications of the feasibility of identifying an immune surrogate of protection. These indications are based on animal model data since there are few asymptomatic human infections and few survivors of the natural disease for comparison of natural vs. artificial (vaccine-induced) immune responses. As for many other vaccines, antibodies are the principal mediator of protection against NiV (11, 12). Protection against challenge in animal models can be passively transferred by serum polyclonal antibodies (13) and by human monoclonal antibodies with neutralizing activity (14), which have been characterized at the epitope level (15–17). Levels of neutralizing antibody required for protection in a mouse model employing NiV pseudovirus challenge have been defined based on both passive transfer of antibody and active immunization (18). The body of evidence indicates that the classical viral neutralization test result may serve as a surrogate for protection.

In this paper, we describe studies of the rVSV-vectored vaccine in an established nonhuman primate model of NiV disease, the African green monkey (AGM) (19), with a principal objective of elucidating an immune correlate of protection. The vaccine is a live, attenuated recombinant vesicular stomatitis (Indiana) virus (rVSV) developed by reverse genetics in which the glycoprotein (G) gene of VSV (the principal neurovirulence gene) has been deleted and replaced with the corresponding envelope glycoprotein genes of both Ebola virus (Kikwit) (EBOV GP) and Nipah virus (Bangladesh) (NiV G). The EBOV GP is required for fusion and cell entry, which are not mediated by the NiV G protein. The G protein responsible for attachment to cell receptors, principally ephrin B, and antibodies prevent the attachment of and infection by wild-type NiV. The EBOV GP is irrelevant in the context of a NiV vaccine. Previous studies demonstrated that a single intramuscular (IM) dose of rVSV expressing the NiV (Malaysia genotype) attachment glycoprotein (G) is highly attenuated and protected AGMs against lethal intratracheal challenge with NiV (Malaysia) virus (20). Our rVSV vaccine candidate was modified to express the NiV (Bangladesh) genotype, plaque-purified, manufactured to quality specifications, and studied in AGMs challenged with the more virulent (21) Bangladesh virus strain. This vaccine, code named PHV02, is now in clinical development (NCT05178901).

Results

Nipah virus challenge in control AGMs

All animals that received rVSV-EBOV succumbed to intranasal/intratracheal NiV challenge or met euthanasia criteria by day 7 to day 8 and had active NiV infections, viremia, clinical illness, and hematological and biochemical abnormalities. NiV viremia and shedding in oral swabs was evident by day 3 or 7 after challenge at titers of 3–6 log10 copies/mL. Lung radiograph abnormalities were seen by day 3, and radiograph scores increased by day 7. All AGMs in the control vaccine group developed typical signs of NiV disease in the last several days before death, including muddy or cyanotic mucous membranes, decreasing blood pressure, elevated respiratory rate, dehydration and/or vibrations felt over the chest wall, tachypnea, tachycardia, hypoxemia, and bloody/crusty nasal discharge. On day 7, most animals had increases in neutrophil and monocyte counts, mild thrombocytopenia, and increased hemoglobin and hematocrit. Increased serum transminases, creatinine and blood urea nitrogen, serum electrolyte abnormalities, and decreased total protein and albumin were observed variably. The lung pathology in the VSV-EBOV-vaccinated control animals (necropsied at day 7 to 8 after NiV-B infection) showed interstitial pneumonia with pulmonary edema, alveolar fibrin accumulation, leukocyte exudate within alveolar spaces, and epithelial or endothelial cell syncytia formation. Splenic lymphocytolysis was a common feature. At euthanasia, all rVSV-EBOV-vaccinated control animals displayed high genome copies (4–9 log10 copies/g) and infectious titers (4–5 log10 pfu/g) in respiratory and other selected tissues at euthanasia characteristic of a fulminant NiV-B infection (Supplementary Figure S5).

Only one of six AGMs in the group vaccinated on day 7 before challenge had detectable viremia and oral and nasal swabs following NiV challenge (low copy numbers 1–2 log 10 copies/mL). No other vaccinated animals had detectable viremia or shedding.

Survival in vaccinated AGMs is dose-related and associated with an "all-or-nothing" immune response

In a first study (Figure 1), groups of four adult (>3 kg) male and female AGMs were given a single 2-mL IM (1 mL/caudal thigh) inoculation of graded doses of PHV02 (high: 1.7×10^6, mid: 1.8×10^4, or low: $<6.6 \times 10^2$) 50% tissue culture infectious doses ($TCID_{50}$) based on back-titration of the diluted virus used for inoculation. The low dose is unknown and is based on the sensitivity (lower limit of quantitation, LLOQ) of the assay since no virus was observed in the back-titration of the material used for vaccination (see "Materials and methods"). The controls received rVSV-EBOV (2×10^7 $TCID_{50}$/mL in 2 mL). The AGMs were challenged on day 28 post-vaccination with approximately 1,000 LD_{50} (2×10^5 $TCID_{50}$) of NiV (Bangladesh) by the combined intranasal and intratracheal (IN/IT) routes. In total, 100% of the AGMs in the high and mid PHV02 dose groups survived and had no clinical signs, with no detectable viremia after challenge, whereas two

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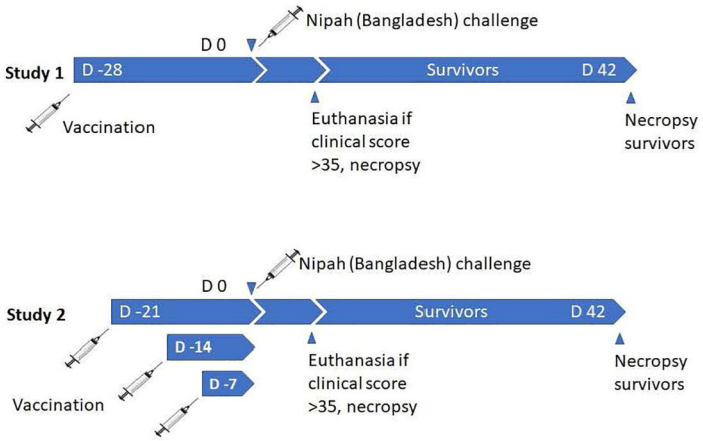

Study	Group	N	Vaccination				Challenge			
			Vaccine	Day*	Inoculum	Route	Virus	Day	Dose	Route
1	1	4	PHV02 (rVSV-EBOV GP NiV G)	-28	High: 1.7 x 10^6 $TCID_{50}$ in 2 mL	IM	Nipah (Bangladesh)	0	2 x 10^5 $TCID_{50}$	Intranasal and intratracheal
	2	4			Mid: 1.4 x 10^4 $TCID_{50}$ in 2 mL	IM				
	3	4			Low: <6.6 x 10^2 $TCID_{50}$ in 2 mL	IM				
	4	4	rVSV-EBOV		2.0x 10^7 $TCID_{50}$ in 2 mL	IM				
2	1	6	PHV02 (rVSV-EBOV GP NiV G)	-21	2.0x 10^7 pfu in 2 mL	IM	Nipah (Bangladesh)	0	2 x 10^5 $TCID_{50}$	Intranasal and intratracheal
	2	6		-14	2.0x 10^7 pfu/ in 2 mL	IM				
	3	6		-7	2.0x 10^7 pfu in 2 mL	IM				
	4	4	rVSV-EBOV	-7	2.0 x 10^7 $TCID_{50}$ in 2 mL	IM				

FIGURE 1
Experimental design of two studies in African green monkeys (AGMs) designed to test the protective efficacy of PHV02 and to define the immunological correlates of protection. D, Day.

animals in the low dose group (50%) and all four rVSV-EBOV control animals died or were euthanized because of severe clinical illness by day 7 (Figure 2A). Survival in the high and mid dose groups was significantly higher than in the rVSV-EBOV controls (p = 0.0047, log rank test), but there was no statistical difference between the PHV02 low dose and control group (p = 0.1573). All surviving animals were also protected against the clinical signs of illness and had low clinical scores and thoracic radiograph scores (Supplementary Tables S1–S3).

A second study was aimed at determining the time to onset of protective immunity. In this study (Figure 1), groups of six AGMs were given a single IM inoculation of 2 × 10^7 pfu (the highest dose used in the phase 1 clinical trial) of PHV02 at 21, 14, or 7 days before IN/IT challenge with NiV (Bangladesh). The control animals (n = 4) were inoculated with rVSV-EBOV at 7 days before challenge. Day "0" was designated as the day of IN/IT challenge with NiV. All AGMs vaccinated with PHV02 on day -28 survived, whereas the control animals succumbed on day 8 after challenge (Figure 2B). The AGMs vaccinated on day -21 before challenge were also protected against viremia, clinical signs, and radiographic changes (Supplementary Tables S2–S4). All AGMs vaccinated on day -14 before challenge were protected against viremia, and only one of six animals vaccinated on day -7 before challenge had low-level viremia. Some animals vaccinated with PHV02 on day -14 or -7 had signs of clinical illness, but not as severe as in the controls, and all recovered).

When vaccination was given 28 days (study 1) or 21 days (study 2) before challenge, all survivors mounted a neutralizing antibody response before challenge (Figure 3; Supplementary Tables S1, S4). In contrast, the two AGMs in study 1 in the PHV02 low dose group that succumbed to infection (Figure 2A) failed to develop detectable neutralizing antibodies before challenge (Figure 4—shown as "X" and Figure 3—shown as open circles; Supplementary Tables S1, S4). Excluding from analysis the two non-responders (low dose group) in study 1, there were no differences in geometric mean neutralizing antibody titers between the dose groups (two-way ANOVA for dose effect p = 0.4645), as shown also by overlapping 95% confidence intervals in Supplementary Figure S1. It was concluded that the effect of lowering dose was principally on seroresponse rather than antibody kinetics or titer, i.e., at very low dose levels, animals either responded or failed to respond, and among those that responded, dose level had no effect on antibody titer or kinetics of the response. Thus, down-dosing led to primary vaccine failure in two of four animals but did not elicit a sub-protective level of antibody.

Protection is associated with low levels of neutralizing antibody

In study 2, vaccination performed 7 or 14 days before challenge afforded the opportunity to determine protection during the early

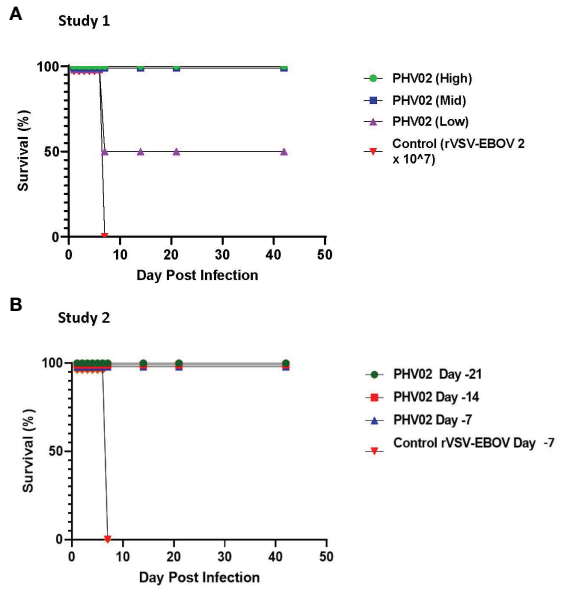

FIGURE 2
(A) Survival ratios, African green monkeys (AGMs) (N= 4 per group) vaccinated with graded doses of PHV02 vaccine 28 days before intranasal/intratracheal (IN/IT) challenge with 2 x 10^5 TCID50 of Nipah (Bangladesh). Controls (n=4) received rVSV-EBOV.
(B) Survival ratios, AGMs (N= 6 per group) vaccinated with PHV02 2 x 10^7 pfu 21, 14 or 7 days before IN/IT challenge with 1 x 10^5 TCID50 of Nipah (Bangladesh). Controls (n=2) received rVSV-EBOV 14 or 7 days before challenge.

phase of the adaptive immune response. The neutralization test was modified to test serial twofold dilutions of serum starting at 1:2.5 (serum dilution when mixed with virus = 1:5), allowing detection at a low (≥1:5) concentration of antibody and a more precise correlation with clinical and virological parameters.

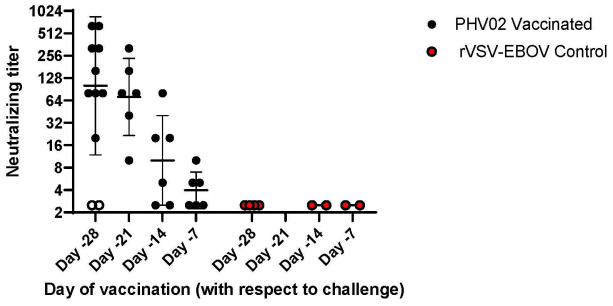

FIGURE 3
Pre-challenge (Day -1) Nipah virus neutralizing antibody titers, AGMs vaccinated with graded doses of PHV02 or with rVSV-EBOV (Controls) 28, 21, 14, or 7 days before IN/IT challenge (Day +1) with 2 x 10^5 TCID50 of Nipah (Bangladesh). Animals in all dose groups in Study 1 are combined since there were no statistical differences between groups, (see text and Supplemental Figure 1). Day -1 neutralizing antibody titers are displayed for animals in study 2 that were vaccinated 21, 14 or 7 days before challenge. Individual animal titers and geometric mean (horizontal bar) and geometric mean standard deviation (GSD) are shown. The two animals in study 1 vaccinated with the low dose on day -28 that did not seroconvert and died are shown in open circles (o).

Survival

In study 2, all monkeys vaccinated with PHV02 at 21, 14, or 7 days before challenge survived, whereas the rVSV-EBOV control animals vaccinated at 7 days before challenge succumbed on day 8 after challenge (Figure 1B).

All six AGMs in study 2 vaccinated on day -21 developed neutralizing antibodies pre-challenge (day -1), with a geometric mean titer (GMT) [± geometric mean standard deviation (GSD)] of 71.3 (± 3.3) (Figure 3; Supplementary Table S4). In the group vaccinated on day -14, four (67%) of six AGMs had a pre-challenge titer of ≥1:5 [GMT (± GSD) 10 (± 4.0)] (Figure 3; Supplementary Table S4). In the group vaccinated on day -7, two (33%) of six AGMs had detectable antibody pre-challenge [GMT (± GSD) 4 (± 1.8)]. It is possible that animals with no detectable antibody would be positive if sera were tested without dilution. Minimal protection, if any, was afforded by non-specific (innate) immunity since all control animals given the rVSV-EBOV vector on day -7 died or had similar survival times (day +8); the survival time was similar or up to 24 h longer than in study 1 where vaccination was 28 days before challenge.

Logistic regression analysis was performed to assess the relationship between pre-challenge (day -1) neutralizing antibodies titers and survival in studies 1 and 2 combined. For regression analysis, vaccinated AGMs with neutralizing titers below the lower level of quantitation (LLOQ) were assigned a value of LLOQ/2 (1:2.5), while control AGMs were arbitrarily assigned a value of 1.25 (LLOQ/4).

A simple logistic regression model was used, in which the sole explanatory variable was pre-challenge log2-transformed neutralizing antibody titers. For a pre-challenge titer of 1:5, the predicted survival probability is 100%, but the 95% CI cannot be estimated (Figure 5A).

A second regression analysis was performed in which random noise (± 1.0 log2) was added to the pre-challenge antibody titers to assess the impact of measurement error on predicted survival. Figure 5B shows the predicted survival curve and its 95% CI. For a pre-challenge titer of 1:5, the predicted survival probability (95% CI) is 88.2% (45.2%, 100%).

The data indicate that any detectable neutralizing response before exposure to NiV was predictive of survival, but the absence of a response when vaccination occurs shortly (e.g., 7–14 days) before exposure does not predict lack of survival. As pointed out below, the survivors without pre-challenge antibody rapidly developed antibodies following challenge. The two AGMs in the low dose group that failed to mount an antibody response in study 1 had been vaccinated 28 days before challenge and represent true vaccine failures.

Clinical scores

Clinical scores were determined to assess whether AGMs without detectable antibody or with low pre-challenge titers in study 2 had evidence for active sub-lethal infections. All animals were scored daily by study staff experienced in signs of illness in the AGM model using a semi-quantitative grading scale (see "Materials and methods"). All rVSV-EBOV control animals had high clinical

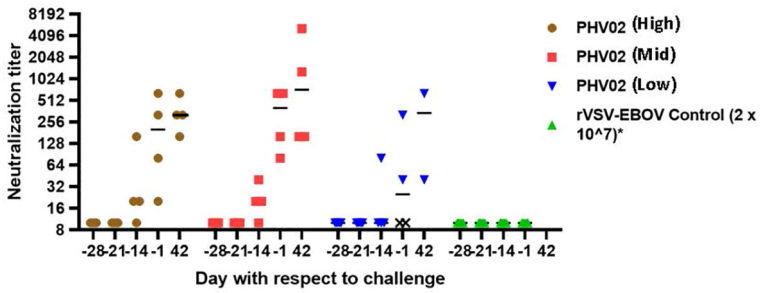

FIGURE 4
Nipah virus neutralizing antibody titers, AGMs vaccinated with graded doses of PH VO2 or with rVSV-EBOV(Controls) 28 days before IN/IT challenge (Day +1) with 2 x 10^5 TCID50 of Nipah (Bangladesh), Study 1. Individual animal titers and geometric mean (horizontal bar) are shown by day with respect to challenge. The two monkeys in the low dose group that failed to develop neutralizing antibodies (designated X) died 7 days after challenge.

scores after NiV challenge (Figure 6; Supplementary Table S4). The AGMs vaccinated on day -21 were protected against illness and had pre-challenge (day -1) neutralizing antibodies (GMT 71, range 10–320). In contrast, four (67%) of six survivor AGMs vaccinated on day -14 before challenge and five (83%) of six survivor AGMs vaccinated on day -7 before challenge had signs of illness as indicated by moderate peak clinical scores. However, these clinical scores were significantly lower than those of the rVSV-EBOV controls (Supplementary Figure S2). The nonhuman primates manifesting illness either had no pre-challenge antibodies or had low neutralization titers (1:5-1:20). The mean clinical scores increased as the time between vaccination and challenge was reduced, but the differences were not statistically different as shown by overlapping 95% CI values (Supplementary Figure S2).

We performed a linear regression analysis of the maximum clinical scores in both studies as they relate to pre-challenge neutralizing antibody titer. The control AGMs were arbitrarily assigned a neutralizing titer of 1.25, while the vaccinated AGMs with titers below the LLOQ were assigned a neutralizing titer of 2.5. For the combined studies, the Pearson correlation coefficient was equal to −0.74 for the association between $\log_2$-transformed pre-challenge neutralizing titers and the maximum clinical score, indicating a moderate inverse correlation. Figure 6A plots pre-challenge titers without and with noise by maximum clinical score for study 1. Figure 6B plots pre-challenge titers without and with noise by maximum clinical score for the time-to-protection study 2. Based on these analyses, a pre-challenge titer of ≥1:40 was predictive of protection against the clinical signs of illness, i.e., maximum clinical scores 0–5.

Thoracic radiographic scores

Thoracic radiographs were taken at scheduled intervals after challenge (Supplementary Table S3), including at the time of euthanasia/death. Pulmonary changes progressed rapidly. In study 2, no or minimal infiltrates were present 1 day before euthanasia,

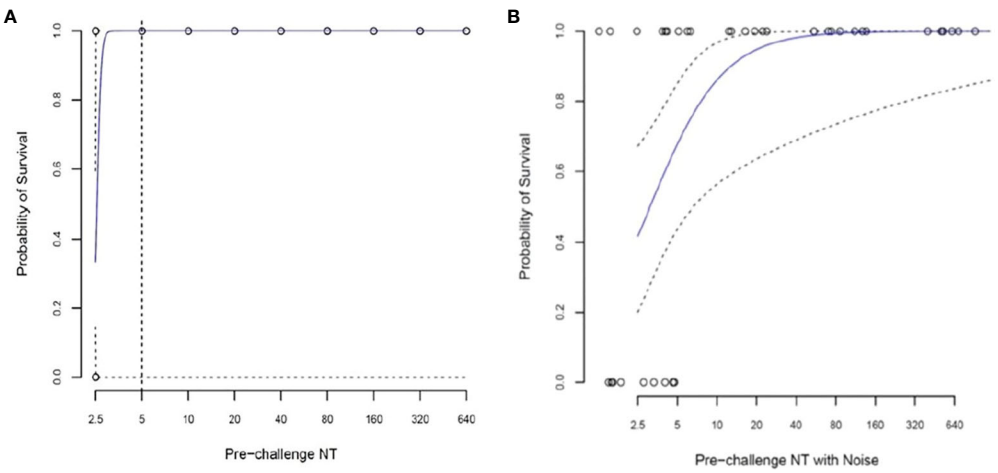

FIGURE 5
Logistic regression analysis to assess the relationship between pre-challenge (Day -1) log2 neutralizing antibody titer (NT) and survival in 38 African green monkeys (Studies 1 and 2 combined). **(A)** Simple logistic regression model with the sole explanatory variable pre-challenge log2 transformed NT. For a pre-challenge titer of 1:5, the predicted survival probability is 100%, but the confidence interval cannot be estimated. **(B)** Random noise (±1.0 log2) added to assess the impact of measurement error on predicted survival. For a pre-challenge titer of 1:5, the predicted survival probability (95% CI) is 73.2% (47.7, 89.1%).

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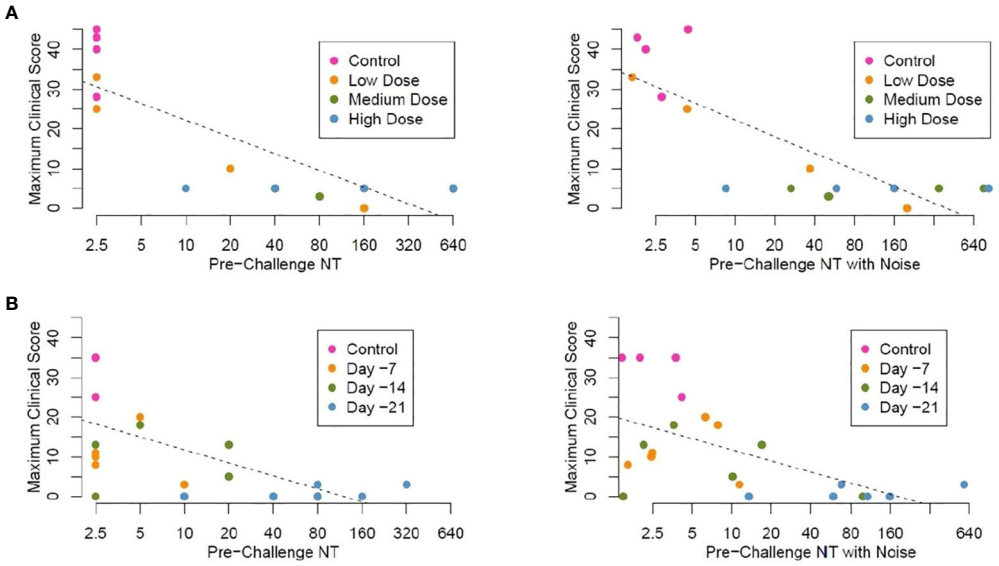

FIGURE 6
Linear regression analysis, maximum clinical scores by pre-challenge (Day -2) neutralizing antibody titers (NT) **(A)**. Study 1 Maximum clinical score by neutralizing titer pre-challenge (Day -1), animals vaccinated IM with graded doses of PHV02 28 days before IN/IT challenge with Nipah (Bangladesh). Regression analysis without (left panel) and with random noise [± 1.0log2) added to the pre-challenge NTs to assess the impact of measurement error] (right panel) **(B)**. Study 2 Maximum clinical score by neutralizing titer pre-challenge (Day -1), animals vaccinated IM with PHV02 ($2x10^7$ pfu) 21, 14 or 7 days before or with rVSV-EBOV 14 or 7 days before IN/IT challenge with Nipah (Bangladesh) without (left panel) and with random noise (right panel). Individual data points are color coded by days from vaccination at the time of challenge. Note, in these plots in which noise has been added to pre-challenge titers unmask data points which are identical for different AGMs. For example, 3 control AGMs in Study 2 had the same maximum clinical score of 35. The estimated linear regression lines are superimposed on each plot.

and only two of four animals had radiographic evidence of pulmonary infiltrates at the time of euthanasia.

Regression analyses were performed for the association between log2-transformed day -1 pre-challenge neutralizing antibody titers and maximum thoracic radiographic score. For the combined studies, the Pearson correlation coefficient is equal to -0.51. In study 1, pre-challenge titers explain 67% of the variability in maximum thoracic radiograph score ($p < 0.001$). However, in study 2, pre-challenge titers only explain 13% of the variability ($p = 0.087$), principally because two of the rVSV-EBOV control monkeys which had severe clinical scores requiring euthanasia had not developed evidence of significant pulmonary infiltrates (Supplementary Tables S3, S4).

Supplementary Figure S3A plots the pre-challenge titers without and with noise by maximum thoracic radiograph score for study 1. Individual data points are color-coded by dose of vaccine received. Supplementary Figure S3B plots the pre-challenge titers without and with noise by maximum thoracic radiograph score for study 2.

In study 1, a pre-challenge titer of ≥20 was predictive of protection against pulmonary infiltrates caused by NiV challenge infection. In study 2, two (50%) of four AGMs in the control group did not develop pulmonary infiltrates, making the correlation with antibody level less clear.

Post-challenge antibody

In AGMs in study 1 and study 2, a pre-challenge neutralizing titer of ≥1:40 predicted the absence of a post-challenge an approximately fourfold rise in titer in the majority of the AGMs (Supplementary Figure S4). There were only two (7%) of 28 AGMs [one in study 1 (vaccinated on day -28 before challenge) and one in study 2 (vaccinated on day -21 before challenge)] with pre-challenge (day -1) titers ≥1:40 that had an antibody rise after challenge. A titer of ≥1:40 was thus sufficient to an anamnestic immune response in most vaccinated AGMs. The lack of an antibody response following virus challenge may be ascribed to the neutralization of incoming virus, preventing replication ("sterilizing immunity"). The AGMs in the days -14 and -7 treatment groups had low pre-challenge titers (Supplementary Table 4), and the antibody response following challenge is indicative of a response to both the infection and vaccination since antibodies elicited by the latter are still evolving. However, a neutralizing titer of ≥1:40 before a severe respiratory NiV challenge appeared to abrogate virus replication and prevent an antibody response. The probability of having an approximately fourfold rise in antibody was significantly reduced at a pre-challenge titer of ≥1:40 ($p = 0.0001$, Fisher's exact test, two-sided).

Tissue RNA levels at necropsy

Necropsies were performed on vaccinated survivors on day +41 or +42 after challenge on day 0 and on low dose PHV02 non-survivors and rVSV-controls at time of death/euthanasia (7 or 8 days after challenge). In study 1, all controls and the two non-survivors in the PHV02 low dose group had high levels of RNA (5–10 $\log_{10}$ copies/g) and infectious virus (3–6 $\log_{10}$ $TCID_{50}$) in multiple tissues including the lung, spleen, and brain on necropsy on day +7 (data not shown). In contrast, the necropsy of survivors

on day +41 revealed only one animal (NiV 117 in the low dose group, Supplementary Table 1) with low levels of detectable genomic RNA and no detectable infectious virus in any tissue (RNA was found in the right middle and lower lobes of the lung, 2.7–3.3 $\log_{10}$ copies/g). Interestingly, this AGM with a low viral load in the lung had a low level of antibody (1:40) both pre-challenge and on day 41 and did not seroconvert after challenge, possibly indicating less robust viral clearance.

In study 2, all rVSV-EBOV control animals had high levels of genomic RNA (4.8–9.5 $\log_{10}$ copies/g) in multiple tissues on necropsy on day 8 after challenge, especially in lung segments and conducting portions of the lower respiratory tract, brain, spleen, and urinary bladder. In vaccinated AGMs, substantially lower levels of detectable genomic RNA (3–4 $\log_{10}$ copies/g) were present in one or more tissue samples, with no differences across the day -21, -14, and -7 vaccine groups (Supplementary Figure S5).

Protection by vaccination shortly before challenge may depend on the post-challenge immune response

As described above, the AGMs in study 2 vaccinated at a short interval (days -14 or -7) before NiV challenge had no detectable (<1:5) or had low titers (1:5–20) of neutralizing antibodies on day -1 before challenge. To determine whether the immune response during the challenge virus incubation period abrogated the infection and resulted in survival, we determined neutralizing titers on days +1, +3, and +7 days after challenge. All AGMs in both the day -14 and -7 treatment groups developed neutralizing antibodies or showed an increase in antibody titer during the 7 days following challenge, whereas the rVSV-EBOV control animals that died on day 8 had no detectable response to Nipah challenge (Figure 7). The largest increase in GMT was seen in the animals vaccinated on day -7 before challenge. The post-challenge response in the day -14 and day -7 groups reflects both the evolution of the response to vaccination and the booster effect of challenge, the latter being most evident in the day -7 vaccine group. The response attributable to the vaccine alone can be estimated from the antibody kinetics without challenge when compared to the observed response after challenge (Supplementary Figure S6). While the post-challenge response was greater than the response expected for the same intervals following vaccination without challenge, the differences were not statistically significant.

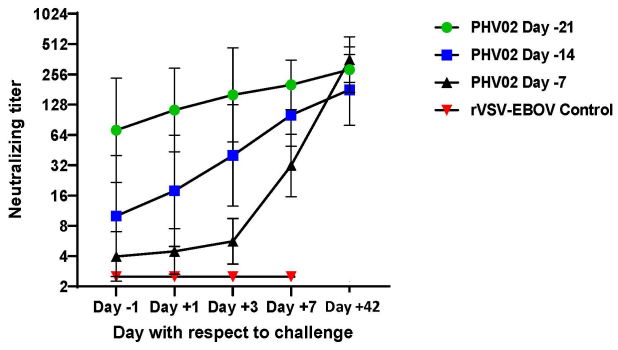

FIGURE 7
Geometric mean (±GSD) Nipah virus neutralizing antibody titers following challenge (Day +1) with 2 x 10^5 TCID50 of Nipah (Bangladesh). AGMs were vaccinated with graded doses of PHV02 or with rVSV-EBOV (Controls) 21, 14, or 7 days before IN/IT challenge.

These results indicated that vaccination given within 7–14 days of a severe virus challenge protected against death and that survival was associated with the appearance of low levels of neutralizing antibodies before infection or shortly after infection, which increased during the incubation period of the challenge infection.

Discussion

In order to assess the relationship between immune response and protection and the potential translation to humans, it is necessary to consider the severity and time course of infection in the challenge model. The AGM is widely accepted as the preferred nonhuman primate model of NiV disease since other species, in particular macaques, do not develop consistently fatal illness (22). The course of NiV infection in the AGM model is more active and lethal than in humans. The 100% mortality ratio in the AGM model is higher than reported in humans (in recent outbreaks caused by the Bangladesh genotype, 73%–89%) (23). The IN/IT route of challenge and high challenge dose (1,000 LD50) likely promote a rapidly progressive pulmonary infection. Survival time in AGMs (7 to 8 days) is shorter than in humans, in which the median incubation period is approximately 10 days and the average duration of hospital stay is 1–9 days (24, 25). The IN/IT route in AGMs may be a model of respiratory exposure and inter-human transmission (26), although human NiV infections also occur following oral ingestion of contaminated date palm sap and probably other modes of contact spread. The outcome of infection and pathogenesis with respect to the onset of pulmonary and neurological infection is dependent on the challenge dose and route (27, 28),. It may be concluded that the AGM model, high challenge dose, and IT route of infection provide a severe test of immunity afforded by a vaccine. If human immune responses can be shown to be similar to those associated with protection in this lethal model, they would likely indicate that the vaccine would provide clinical benefit.

A limitation of the studies reported here is that only humoral immunity (IgG binding and neutralizing antibodies) was investigated, whereas T cells undoubtedly contribute to protective immunity and recovery. Viral clearance mediated by cytotoxic T cells may be necessary to prevent persistent and recurring NiV virus infections (9). In a previous study of the rVSV vaccine expressing NiV G (Malaysia), Prescott et al. found significantly increased CD8+ T cells expressing granzyme B or interferon-γ in vaccinated vs. control animals at 3 and 7 days post-challenge, respectively (20). Prasad and colleagues concluded that both antibodies and T cells contributed to survival in cynomolgus macaques following NiV challenge (22). Antibodies play a major role in pre-exposure protection as shown by passive transfer studies (10, 11), and neutralization is believed to be the principal functional activity

(8). Other Fc-mediated functions of antibody, such as cellular phagocytosis, were also not measured in our studies and could potentially contribute to protection. We did, however, measure IgG-binding antibodies by ELISA; these analyses did not suggest that non-neutralizing antibodies were a better marker of protection than neutralization. Indeed IgG ELISA and neutralizing titers were closely correlated (Supplementary Figure S7), and low levels of neutralizing antibodies were detected without a detectable binding antibody, which is likely due to the lower dilution of serum in the neutralization test (1:5) than in ELISA (1:100).

In the first (dose–response) study, PHV02 vaccine was administered 28 days before NiV challenge. The lowest pre-challenge neutralizing titer was 1:20, and that animal was fully protected against illness, death, and residual viral RNA in tissues collected at necropsy 42 days after challenge. There were two animals in the lowest dose group ($<6.6 \times 10^2$ pfu) that did not seroconvert and developed fatal illness with survival time equivalent to the control animals. The results indicated a very low dose of vaccine which may result in a protective response or in primary vaccine failure. This "all-or-nothing" response is not surprising for a replication-competent viral vaccine that expands its antigenic mass after inoculation. In the case of the replication-competent rVSV-EBOV vaccine, reduction in dose by 10-fold increments to low levels resulted in increasing rates of primary vaccine failure (29).

In the second study, AGMs were challenged 21, 14, or 7 days after vaccination, during the early phase of the adaptive immune response, with the objective of determining a level of antibody predictive of survival. All animals vaccinated at those intervals before challenge were protected against death. All AGMs survived whether or not they had detectable neutralizing antibodies the day before challenge. In those animals with pre-challenge titers <1:5, antibodies appeared rapidly after challenge (Figure 7) and likely abrogated the infection during the first few days after challenge. Nine (75%) of 12 AGMs vaccinated on day -14 or -7 developed signs of illness, from which they recovered, and the clinical scores were substantially lower than in the control animals (Figure 6B; Supplementary Table S4). Of the three AGMs that developed no signs of illness, two had detectable neutralizing antibodies before challenge.

Overall, based on results in a severe challenge model, it may be concluded that any detectable antibody level is predictive of survival against challenge performed up to 28 days after vaccination, that vaccination within as few as 7 days of exposure may protect against severe illness and death, and that the immediate post-challenge immune response protects even where pre-challenge antibodies are low or undetectable. The results are consistent with the observation in a phase 3 clinical trial of the rVSV-EBOV vaccine, in which no one who had been vaccinated within the previous 10 days developed Ebola virus disease (30).

rVSV vectors are known to activate multiple antiviral genes (31). To control for innate immunity in our studies, the animals were given rVSV-EBOV concurrent with PHV02 28 days (study 1) or 7 and 14 days (study 2) before challenge. AGMs given rVSV-EBOV 7 days before challenge had a similar survival time as those challenged at longer intervals, suggesting that, at an interval of ≥7 days, residual innate immunity did not provide protection. None of the control AGMs mounted a neutralizing antibody response to the NiV challenge before death. In contrast to our study, a rVSV vaccine against Marburg virus (MARV) disease given 7 or even 3 days before MARV challenge was shown to protect nonhuman primates; in that experiment, the day -3 survivors were characterized by strong antiviral gene activation, and protection was attributed to innate rather than adaptive immunity (32). Peri-exposure vaccination of nonhuman primates with rVSV-EBOV given 1 or 24 h after challenge protected the animals against both EBOV and the heterologous MARV, again indicating a role for innate immunity in early protection (33). The same conclusion was reached in the case of peri-exposure vaccination of hamsters with the rVSV-Nipah vaccine (34). In our study, we did not evaluate protection by vaccination given after challenge or as early at 3 days before challenge. However, the protection seen in animals vaccinated with PHV02 7 days or more before challenge was attributable to the adaptive immune response which was detected either before or shortly after challenge. Although T cells were not assessed and may have contributed to protection, the neutralizing antibody response provided an adequate explanation for pre-exposure protection.

In our studies, neutralizing antibody induced by PHV02 at ≥1:5 was established as a marker of survival. More work will be required to establish a quantitative level of protection, although the data presented here suggest that a titer of ≥1:40 before exposure was associated with the absence of clinical signs (Figure 6) and protected against an increase in antibody levels post-challenge, which is indicative of sterilizing immunity (Supplementary Figure S3). Using a different model system (Balb/c mice immunized with an HIV pseudovirus expressing NiV G protein and challenge followed by *in vivo* bioluminescence imaging), Nie et al. found a neutralizing antibody level of 170 or greater to be protective (16).

NiV challenge in control AGMs caused a rapidly fatal illness and necropsy tissues, including lung segments and brain that had high levels of viral RNA. In AGMs vaccinated with PHV02 21 or 28 days before challenge, solid immunity was established and protected against death, clinical illness, and infection of vital organs. Nearly all AGMs vaccinated 28 days before challenge had cleared viral RNA by the time of necropsy on day +42. In animals vaccinated at shorter intervals (days -21, -14, or -7) before challenge, the immune response was sufficient to prevent death, but there was residual RNA at low levels in the lung and brain at day 42 (Supplementary Figure S5). This finding indicated that the vaccine given shortly before exposure had abrogated but not prevented infection and that the abortive infection with NiV had resulted in neuroinvasion. Since humans surviving a natural infection with NiV can occasionally develop late-onset or relapsing encephalitis (7) it cannot be excluded that persons vaccinated and subsequently exposed to NiV could be protected against the acute disease but have persistent infection of immune-privileged sites in the brain. Liu et al. also reported subclinical encephalitis with persisting genomic RNA in neurons and glial cells in the brains of nonhuman primates that had survived the acute phase of NiV disease following IT virus challenge (35). Based on the AGM model, it would appear that this phenomenon is more likely to occur in the setting of vaccination

shortly before exposure during the development of the early immune response.

In these studies, vaccination with PHV02 predicts a positive outcome—survival and abrogation of disease with neutralizing antibody seroresponse as a putative biomarker of protection. PHV02 vaccine is a promising candidate for the development of a human vaccine against Nipah disease.

Ethical statement

The use of study animals was approved by the Institutional Animal Care and Use Committee (IACUC) of the Rocky Mountain Laboratories, and the experiments were performed in an AAALAC International-accredited facility following institutional guidelines for animal use, the guidelines and basic principles in the NIH Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, USDA, and the USPHS Policy on Humane Care and Use of Laboratory Animals. Humane endpoint criteria, specified and approved by the IACUC, were applied to determine when animals should be humanely euthanized.

Materials and methods

Vaccines and challenge virus

PHV02 (rVSVΔG-EBOV GP-NiV G) is a plaque-purified rVSV (Indiana) virus with the VSV envelope glycoprotein (G) deleted and replaced with the genes for the surface glycoprotein (GP) of Ebola Zaire (EBOV), which mediates membrane fusion between the virus and the host cell, and the NiV G protein) which binds to ephrin-B2 and ephrin-B3 cell receptors and against which NiV-protective antibodies are raised. The control in animal studies is rVSVΔG-EBOV GP [rVSV-EBOV (Kikwit 1995)], similar to the approved Ebola vaccine. rVSV-EBOV is a similar construct to PHV02 but lacks the expression of NiV G protein. Both viruses are replication competent and highly attenuated in various animal models compared to the parental viruses (i.e., VSV, EBOV, NiV) (36). Both vaccine viruses were produced in Vero cell cultures. The NiV challenge virus is a human isolate, Nipah (Bangladesh, 200401066, GenBank AY988601), passaged three times in Vero cells and frozen in Dulbecco's Minimum Essential Medium (DMEM)–10% fetal bovine serum.

The doses used in the studies deserve further description. In study 1, AGMs were inoculated with a range of doses targeting 2×10^7, 2×10^6, and 2×10^5 pfu (in 2 mL given IM) based on the release titer of the virus by the manufacturer using a qualified potency assay. The inocula were back-titrated by cytopathic effects assay in Vero cells at the facility where the animal study was performed (NIAID). The back-titration showed that virus doses were lower than expected (1.7×10^6, 1.8×10^4, or $<6.6 \times 10^2$ $TCID_{50}$, which are the high, mid, and low doses described in the "Results" section, respectively. The low dose could not be determined since it is below the LLOQ of the assay. The virus content of the inocula on back-titration was confirmed by the manufacturer, which then conducted an investigation showing that the diluent (0.9% saline for injection) used by the NIAID to prepare the dosing materials have low pH (6-6.2), which resulted in a loss of infectivity. For study 2, the diluent used for preparation of the dosing material was changed to DMEM, which resulted in no loss of titer.

Animal procedures

Commercially available monkey chow, treats, and fruits/vegetables were provided twice daily. Water was available *ad libitum*. Environmental enrichment consisted of a variety of human interactions, manipulanda, movies, and music. The AGMs were acclimatized to the study room for a minimum of 5 days. The animals were identified by cage cards and tattoos numbers. The animals were randomly divided into treatment groups and allowed to acclimate to the study room for a minimum of 5 days. All study activities were conducted with the animals under ketamine or Telazol anesthesia. The animals were inoculated IM in the caudal thigh with study vaccines and held for varying intervals before NiV challenge. The monkeys were monitored at least twice daily for any adverse effects to vaccination or signs of disease upon challenge. At protocol-specified intervals, thoracic radiographs, blood, and swabs (nasal, oral, and in some animals, rectal) were collected for clinical laboratory, virology, and immunological tests. Blood was drawn from the femoral, saphenous, or cephalic vein on exam days using a 21–26-gauge needle. The exact volume of blood collected did not exceed 15% of the total circulating blood volume in any 2-week period.

Nipah challenge

Nonhuman primates were given a total dose of 2×10^5 TCID50 NiV (Bangladesh) divided equally over two routes: intratracheal (IT; 4 mL) inoculation was accomplished under anesthesia using a feeding tube advanced down an orally placed endotracheal tube, and intranasal (IN; 0.5 mL into each nostril) inoculation was performed by dripping the inoculum into each nostril using a micropipette.

Clinical endpoints and euthanasia

Experienced staff observed the AGMs and recorded clinical signs using a semiquantitative scoring sheet. The AGMs in severe respiratory distress (open mouth breathing with lack of activity and cyanosis) or with evidence of bloody or purulent discharge from the respiratory tract or severe neurological signs [seizure activity, neurologic signs that interfere with the ability to ambulate or ingest food or water, unconsciousness or moribundity (no or little response to human presence and prompting)] or with body temperature <35°C were euthanized. Any AGM with a clinical score >35 was euthanized.

Thoracic radiographs

Ventro-dorsal and lateral thoracic radiographs were done while the AGMs are under anesthesia on specified examination days and at euthanasia/death. Radiographs were evaluated and scored for the presence of pulmonary infiltrates by two board-certified laboratory animal veterinarians according to a standard scoring system (37).

Briefly, each lung lobe (upper left, middle left, lower left, upper right, middle right, lower right) was scored individually based on the following criteria: 0 = normal examination; 1 = mild interstitial pulmonary infiltrates; 2 = moderate interstitial pulmonary infiltrates, perhaps with partial cardiac border effacement and small areas of pulmonary consolidation (alveolar patterns and air bronchograms); and 3 = pulmonary consolidation.

Thoracic radiograph findings are reported as a single radiograph score for each AGM on each exam day. To obtain this score, the scores assigned to each of the six lung lobes were added together and recorded as the radiograph score for each animal on each exam day. Scores may range from 0 to 18 for each animal on each exam day.

Neutralization

Serial twofold dilutions of heat-inactivated test sera were made in DMEM with 2% heat-inactivated fetal bovine 1 mM L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin, mixed with 100 $TCID_{50}$ of NiV (Bangladesh) virus, incubated (37° C, 60 min), and inoculated onto Vero E6 cells grown in monolayer cultures in 96-well plates. The cells were incubated for 5 to 6 days. Virus neutralization titer is the highest dilution of serum for which no cytopathic effects were observed.

IgG ELISA

Nunc MaxiSorp™ flat-bottom microplates were coated with gamma-irradiated NiV (Bangladesh) in phosphate-buffered saline overnight at 2–8°C. After the removal of coating, antigen plates were blocked 15 min at room temperature with 5% skim milk in 1X DPBS with 0.05% Tween 20. The plates were washed three times with 1x DPBS containing 0.5% Tween 20. Sera were added beginning at 1:100 through 1:6,400 using serial fourfold dilutions and incubated for 1 h at room temperature. The plates were washed three times with 1x DPBS containing 0.5% Tween 20, and secondary antibody (Southern Biotech 6200-005) was applied at 1:1,000 dilution in blocking buffer and incubated for 1 h at room temperature. After the removal of detecting antibody, the plates were washed six times with 1x DPBS containing 0.5% Tween 20. ABTS substrate was added and incubated for 15 min at room temperature and then stopped with 5% SDS in H_2O. Absorbance at 405 nm was read within 30 min of stopping the reaction. ELISA titers were calculated by taking both the average and standard deviation of negative control results. The standard deviation ×3 + the average of the control wells set the negative upper threshold. Only samples with ODs greater than the standard deviation x3 above the average negative results were considered to be positive.

Quantitative PCR

RNA was extracted from swab samples in DMEM and from EDTA blood samples using the QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's instructions. RNA was extracted from tissues using the RNeasy kit (Qiagen); tissues (30 mg) were homogenized in RLT buffer (Qiagen), and RNA was extracted according to the manufacturer's instructions. Moreover, 5 µL RNA was used in a one-step real-time RT-PCR targeting the N gene of NiV using the Rotor-Gene probe kit (Qiagen) according to the instructions of the manufacturer. In each run, dilutions of PCR standards with known copy numbers were run in parallel to calculate copy numbers in the samples. qRT-PCR assays and standards specific for NiV (Bangladesh) or VSV were used.

Statistical methods

Regression analyses were performed with the open-source program R version 4.2.0 (https://www.R-project.org/). GMT, GSD, 95% CI, and group comparison analyses were determined using GraphPad Prism 9, with the test method specified in the text.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by NIAID Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

TM authored the manuscript; TM and RN designed the studies; FF, AG, EH, JC, KM-W, AO, JL, PH, CC, and CS carried out the experimental procedures and virological, clinical, radiographic and pathological tests; WR performed statistical analyses; and JF provided guidance and served as principal investigator on the grant from CEPI that supported the work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

TM is employed by Crozet Biopharma LLC. RN and JF are employed by Public Health Vaccines Inc. WR is an independent consultant.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1216225/full#supplementary-material

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Correlation between pseudotyped virus and authentic virus neutralisation assays, a systematic review and meta-analysis of the literature

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Background: The virus neutralization assay is a principal method to assess the efficacy of antibodies in blocking viral entry. Due to biosafety handling requirements of viruses classified as hazard group 3 or 4, pseudotyped viruses can be used as a safer alternative. However, it is often queried how well the results derived from pseudotyped viruses correlate with authentic virus. This systematic review and meta-analysis was designed to comprehensively evaluate the correlation between the two assays.

Methods: Using PubMed and Google Scholar, reports that incorporated neutralisation assays with both pseudotyped virus, authentic virus, and the application of a mathematical formula to assess the relationship between the results, were selected for review. Our searches identified 67 reports, of which 22 underwent a three-level meta-analysis.

Results: The three-level meta-analysis revealed a high level of correlation between pseudotyped viruses and authentic viruses when used in an neutralisation assay. Reports that were not included in the meta-analysis also showed a high degree of correlation, with the exception of lentiviral-based pseudotyped Ebola viruses.

Conclusion: Pseudotyped viruses identified in this report can be used as a surrogate for authentic virus, though care must be taken in considering which pseudotype core to use when generating new uncharacterised pseudotyped viruses.

KEYWORDS

pseudotype, neutralisation, correlation, virus, live virus correlates of protection

1 Introduction

Serological assays are an invaluable tool in detecting exposure of pathogens in organisms and understanding the immune system's response. The level of insight gained from these assays during a disease outbreak is crucial for the initial medical response, and subsequently understanding the dynamics, strength and longevity of the immune response (1–3). An important protective response requires antibody interaction with the pathogen. Upon infection, the humoral response produces antibodies that bind to the antigens displayed by the pathogen, including those that prevent interaction with the receptors necessary for entry into host cells. Assays for antibody analysis have proved effective during recent viral outbreaks, such as those caused by Ebola virus (4, 5) and Severe Acute Respiratory Coronavirus 2 virus (SARS-CoV-2) (6–8), as they allow for detection and monitoring of viral spread in a population. Such assays are similarly applied to animals, which can also identify intermediary hosts or potential reservoirs and provide information about the potential for zoonotic spillover (9, 10), as well as inform on vaccines and treatment efficacy in preclinical studies.

Some serological assays, such as enzyme-linked immunosorbance assays (ELISA), can identify the presence of antigen-binding antibodies within a day of receiving a human or animal blood sample (11, 12). When considering antibodies targeting a viral glycoprotein, typically a proportion of the binding antibodies to a viral glycoprotein successfully impair the virus entry, whilst other antibodies bind to non neutralising epitopes, enabling other antibody-mediated immune functions (13). This highlights a shortcoming of binding assays such as ELISAs which lack the functional component of measuring virus entry into cells. Owing to this, in order to measure functional activity, specifically the ability of antibodies in preventing entry, a neutralisation assay is required. These assays are considered the gold standard for measuring the presence and magnitude of neutralising antibodies and typically require the use of authentic virus (14). As a result, these assays often take several days to allow the virus to grow and are subject to biosafety containment requirements depending on the virus under investigation. This restricts the study of viruses classified as hazard group 3 or 4, such as SARS-CoV-2 or Ebola virus and Nipah virus, due to the paucity of facilities that possess such high level of biocontainment. An approach to circumvent these requirements is to use a pseudotyped virus (PV), which can be handled at containment level 2 or below (Figure 1). These are comparatively easier to produce, typically by plasmid transfections, and, under optimized conditions, can be produced within 3 to 5 days. Many reviews have been published regarding pseudotype production, core composition, and their uses (15–20). These chimeric viruses commonly use a retroviral or VSV nucleocapsid core are surrounded by a lipid envelope bearing viral glycoproteins of a heterologous virus of interest on their surface. Often, PVs do not contain the virus genomic material required for replication. Instead, the modified genome is replaced by a transgene, for example a reporter gene such as green fluorescent protein (GFP) or luciferase enzyme (16). Upon successful entry into target cells, transgene expression allows for quantification of infected cells. Primarily due to their replication deficiency, PVs can be handled in a containment level 2 laboratories, which are common facilities in biological research laboratories (18). Many viruses of high consequence have been pseudotyped successfully and rapidly during the onset of an outbreak, as authentic viruses typically require isolation and stock amplification, whereas PVs require a published sequence of the viral glycoprotein to be cloned into an expression plasmid. Due to their external mimicry of the virus of interest, with reduced risk of acquiring mutations during production in mammalian tissue culture as seen with authentic viruses, PVs are an effective tool to use in neutralisation assays (18, 19). The COVID-19 global pandemic, caused by SARS-CoV-2, caused a significant rise in the use of pseudotype assays for both serology and molecular virology studies (17, 21). When PVs are used in a multi-well plate assay setting they are often referred to as pseudotype virus microneutralisation assays (pMNA). For the purposes of this systematic review, the alternative authentic virus microneutralisation assay will be referred to as vMNA.

Given that neutralising antibodies are one of the principle components measured to determine correlates or surrogates of protection against disease or infection (22–24), the neutralisation test remains a critical assay. An important aspect when determining a correlate or surrogate of protection is to be able to draw comparisons between data and bridge between studies. By calibrating assays to a common reference reagent, often a pooled sera sample, assay readouts can be standardised across laboratories worldwide as these relative results are reported in a standard unitage (25–27). It is important that such common reagent is used correctly to calibrate in house standards, but in some cases, this is still not enough and the reduction of inter-laboratory variation can only be achieved by sharing common protocols and critical reagents similar to the approach used by the CEPI Centralised Laboratories network. Such reference reagents have been produced for several viruses, including many of high consequence which are applicable to pseudotyping (28–30). Whilst reporting results relative to a reference reagent reduces inter-laboratory variations and allows comparisons between assays, it is fundamentally important to investigate whether surrogate assays, designed to mimic and replace vMNAs which employ highly pathogenic viruses, correlate. If there is a correlation between a pMNA and a vMNA, then the results from either assay could be applied within clinical trials and investigations aimed at identifying the correlates for protection against a virus.

However, it is commonly queried how well the results from a pMNA correlate with those from a vMNA. The question is particularly relevant with the increasing uptake of pMNAs as a consequence of the recent COVID-19 pandemic and their increasing application to clinical trials as focus turns to vaccine development for other high consequence pathogens (31, 32). The studies to-date use a mixture of correlation formulae, most of which are Pearson's R and/or Spearman's Rho (33, 34). Other studies have instead fitted linear regressions to understand the relationship between the two variables, with the R^2 value providing an equivalent measure to the square of Pearson's R in the case of a positive relationship (35). Several reviews on PVs or neutralisation assays have included some of these studies which sought to correlate

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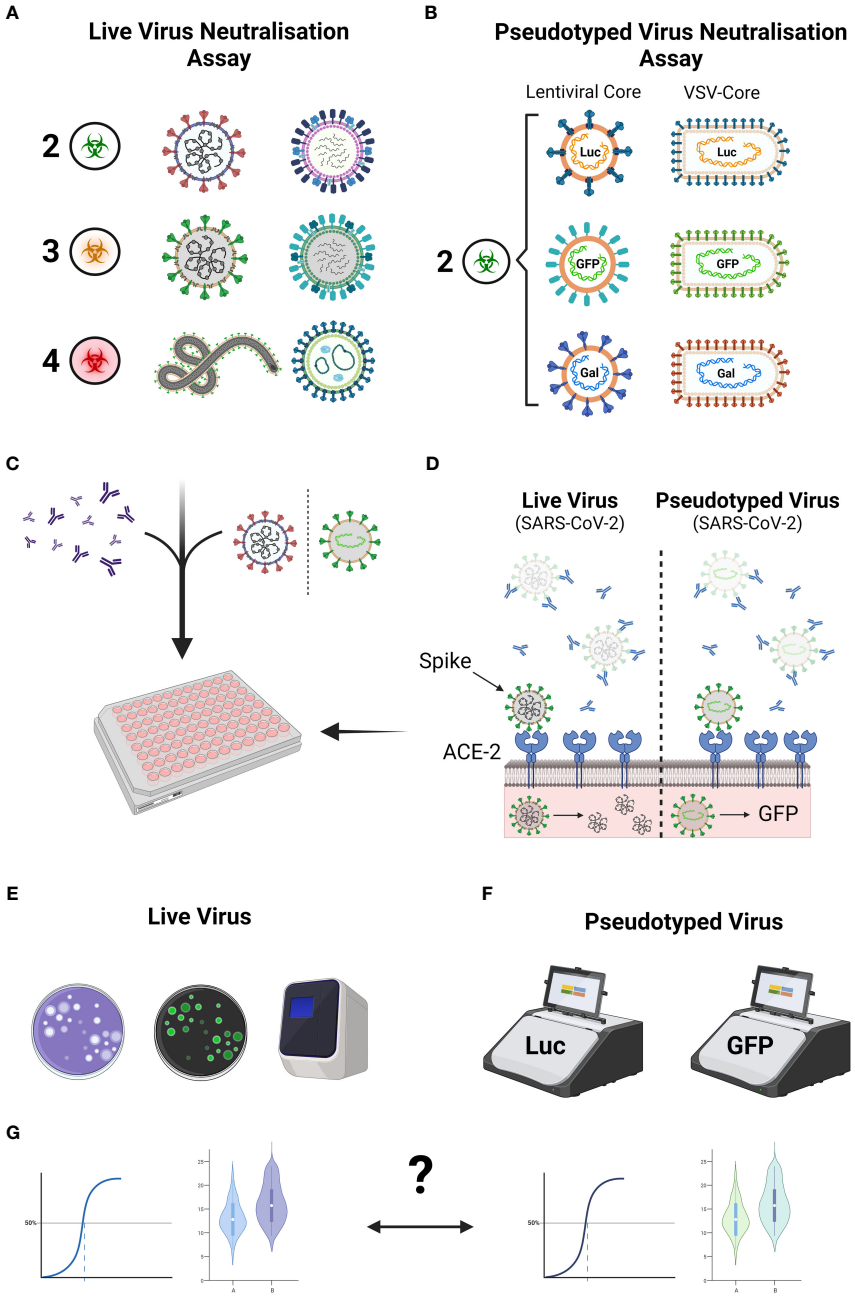

FIGURE 1
Comparison between live virus neutralisation assay and pseudotyped neutralisation assays. Live viruses are commonly used in neutralisation assays though their practicality may depend on the biohazard containment regulations **(A)**. Pseudotyped viruses, despite displaying glycoproteins of highly pathogenic viruses, are designated as a level 2 pathogen **(B)**. The live virus neutralisation assay and the pseudotyped virus neutralisation assay are designed in a similar fashion whereby antibodies are incubated in the presence of virus, followed by addition of a cell line that is susceptible to virus infection **(C)**. In the context of a SARS-CoV-2 neutralisation assay **(D)**, neutralising antibodies bind to the Spike protein of the virus, preventing the virus to bind to the required entry receptor ACE2. Live viruses that enter begin to replicate, whereas pseudotyped viruses only express the desired reporter gene. Plaque assays, fluorescent staining of viral proteins or qPCR are often used to measure neutralisation levels in live virus assays **(E)**, whereas pseudotyped assays typically rely on measuring the intensities of luciferase or fluorescent protein expression **(F)**. The pertinent question of whether the results derived from either assay correlate still remain **(G)**. Figure created with Biorender.com.

results from both assays, yet only a handful are cited (17–19). Despite several studies directly comparing PV and authentic virus neutralization assays, correlation information tends to be buried in the mass of data or supplementary material in these reports. It is likely that for these reasons, the question as to whether the two assays correlate is still frequently posed.

To the best of our knowledge, there is no systematic review nor meta-analysis that has condensed the literature that has correlated pMNA and vMNA. Therefore, the purpose of this systematic review and meta-analysis is to collect the available information on the comparison between the two tests, analyse the strength of correlations, and present the results in a clear and coherent

manner. Overall, we aim to inform the wider community whether pseudotyped viruses can be used as surrogates for authentic virus for the purposes of a neutralisation assay and subsequently to determine the correlates of protection against a virus. Despite the findings within this report, it remains critical that PV-based assays continue to be assayed and correlated with authentic virus wherever possible, particularly if a new PV has been designed for use. Given that correlation coefficient values have different classifications of strength based on the field of study, we included a table based on the definitions that are often cited in the field of medicine (34, 36, 37) (Table 1).

2 Methods

2.1 Search strategy and selection criteria

Google Scholar and PubMed were used to identify published research articles which reported data on correlation between pMNA and vMNAs. The following Boolean search terms were employed to filter studies indexed in Google Scholar and PubMed: "pseudotype|pseudotyped|pseudoparticle" "correlate|correlated|correlation" "live" "virus" "neutralisation|neutralization".

The criteria for inclusion were reports that contained neutralisation assays with both pseudotype virus and authentic virus, as well as application of a mathematical formula to assess the relationship between the results, either by linear regression, Pearson's correlation, Spearman's rank, or a combination of the three. Studies that did not present any form of analysis of correlation were excluded.

2.2 Data collection

We extracted the following data from reports that satisfied our selection criteria: report author name and year, virus used, pseudotype core used, neutralisation assay readout (both for pMNA and vMNA), correlation method, p value of the correlation coefficients, number of samples, and sample types. In total, we identified 67 reports that satisfied our selection criteria and were used for comparative data analysis.

2.3 Statistical analysis

For our meta-analysis, we considered data for the relationships between SARS-CoV-2 PVs and authentic virus. There was insufficient data to consider other viruses in separate meta-analyses and we decided not to analyse the results from multiple viruses together. We instead present the data for other viruses in a table in the supplementary materials (Suppl. Table 1). For the studies reporting a linear regression (R^2), we opted to convert the value by its square-root, so that it may be combined with the Pearson's R values derived from other studies and therefore included in the analysis. We checked that all regressions reported only included the PVs and authentic virus and that the relationships were all positive. We did not have sufficient Spearman's Rho values to analyse and these cannot be directly combined with the Pearson's R values, as they do not measure the same characteristic. Therefore, we did not attempt to carry out a meta-analysis of Spearman's Rho coefficients. These values are reported in the supplementary materials (Suppl. Table 1). We therefore used a dataset of 50 Pearson's R coefficients from 22 papers. Since studies on SARS-CoV-2 used different PV cores (HIV and VSV), PV assays (eGFP, GFP, Luciferase, PRNT and SEAP) and sample types (hamster sera, human mAbs, human plasma and human sera), we checked for differences in the Pearson's correlations between studies using t-tests with a null hypothesis of no difference in the mean Pearson's correlations between the groups containing at least 10 results (Suppl. Figure 1). Since we failed to reject the null hypothesis for any comparison, we decided to carry out our meta-analysis on the full dataset. We had only very limited results reported for different SARS-CoV-2 variants, so that investigating differences in results for each variant alone is left for future work. The analysed datasets used identical variants for PV and authentic viruses.

We conducted a three-level meta-analysis of Fisher's z-transformed Pearson's correlations, using the inverse-variance method, accounting for the dependence between multiple results from the same study (38, 39). We assigned data to "clusters" based on their dependence on other data. All coefficients calculated using the same dataset were considered dependent and were assigned to the same cluster, resulting in 26 clusters in total. Taking the example of Wang et al, 2020 (40), a correlation coefficient was calculated for each of two independent datasets, so that these two coefficients were assigned to separate clusters, while Sholukh et al, 2021 (41) presented four correlation coefficients that were calculated using the same datasets, so that these coefficients were all assigned to the same cluster. Clusters with higher estimated sampling variance of their correlation coefficients, e.g., due to lower sample sizes, are given lower weights in the calculation of the pooled correlation, while clusters are given higher weights if there is less dependence among their correlation coefficients (39). The heterogeneity variance, τ^2, was calculated using the restricted maximum likelihood estimator, with confidence interval estimates calculated using the profile likelihood method. We assessed heterogeneity using the I^2 and H statistics (42) and we calculated prediction intervals (using the t-distribution) for the pooled correlation estimate. While confidence intervals provide measures of uncertainty around the true mean values of correlation, the prediction interval provides a measure of uncertainty around the likely values of correlation to be seen in future studies (38). We checked for influential outliers by removing correlations in turn and recalculating all estimates. We plotted Fisher's z-transformed

TABLE 1 Guide for interpreting correlation coefficients in the medical field of study.

Correlation Coefficient value	Strength of Relationship
>0.8	Very strong
0.6 - 0.79	Moderately strong
0.3 - 0.59	Fair
<0.3	Poor

correlation against standard error (a "funnel plot") to assess possible publication bias. All calculations were carried out in R version 4.3.1 (R Core Team, 2022) using the packages meta (43), metafor (44) and dmetar (45).

3 Results

3.1 Results of literature search

Our search terms returned a total of 33 reports in PubMed and 5,880 reports in Google Scholar. After manually screening abstracts and titles, we identified 80 studies that met our selection criteria and ultimately included 67 reports in this systematic review (Suppl. Table 1). The primary reason for exclusion were reports that either did not include both pMNA and vMNA, or reported neutralisation titres in both the pMNA and vMNA, but did not carry out a correlation analysis between the two methods. Briefly, the total number of reports found for each virus were; SARS-CoV-2 (n=32) (40, 41, 46–75), SARS-CoV-1 (n=2) (76, 77), Canine distemper virus (CDV, n=1) (78), Chikungunya virus (CHIKV, n=1) (79), European bat lyssavirus 1 (EBLV-1, n=1) (80), EBLV-2 (n=1) (80), Ebola virus (EBOV, n=3) (81–83), Hepatitis C virus (HCV, n=3) (84–86), Human immunodeficiency virus (HIV, n=1) (87), Hantaan orthohantavirus (HTNV, n=2) (88, 89), Influenza A virus H5N1 (IAV H5N1, n=5) (90–94), IAV H7N9 (n=1) (95), Japanese encephalitis virus (JEV, n=1) (96), Lagos bat virus (LBV, n=1) (97), Middle East respiratory syndrome virus (MERS, n=4) (98–101), Newcastle disease virus (NDV, n=1) (102), Nipah virus (NIV, n=1) (103), Peste des petite ruminants virus (PPRV, n=1) (104), Puumala

TABLE 2 Summary of reported correlation coefficients. The bounds represent the minimum and maximum point values across the studies.

Virus	No. of Reports	Correlation Range (Linear R^2)	Correlation Range (Pearson's)	Correlation Range (Spearman's)	Correlation Range (Intra-Class)
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)	31	0.385 - 0.993	0.641 - 0.939	0.54 - 1	0.872 - 0.872
Severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1)	2	–	0.69 - 0.78	–	–
Canine distemper Virus (CDV)	1	–	–	0.65 - 0.91	–
Chikungunya virus (CHIKV)	1	0.78 - 0.98		–	–
European bat 1 lyssavirus (EBLV-1)	1	–	0.79 - 0.79	–	–
European bat 2 lyssavirus (EBLV-2)	1	–	0.9 - 0.9	–	–
Ebola virus (EBOV)	3	–	0.96 - 0.96	0.54 - 0.86	–
Hepatitis C virus (HCV)	3	–	0.893 - 0.893	0.7 - 0.93	–
Human immunodeficiency virus (HIV)	1	0.903 - 0.903	–	–	–
Hantaan virus (HTNV)	1	0.91 - 0.91	–	–	–
Influenza A virus H5N1 (IAV H5N1)	5	0.524 - 0980	0.734 - 0.78	0.79 - 0.79	–
Influenza A virus H7N9 (IAV H7N9)	1	–	0.82 - 0.82	–	–
Japanese encephalitis virus (JEV)	1	0.915 - 0.915	–	–	–
Lagos bat lyssavirus (LBV)	1	–	0.83 - 0.83	–	–
Middle East respiratory syndrome virus (MERS)	4	0.96 - 0.96	0.88 - 0.934	0.97 - 0.97	–
Newcastle disease virus (NDV)	1	0.92 - 0.92	–	–	–
Nipah virus (NIV)	1	–	–	–	–
Peste des petits ruminants virus (PPRV)	1	–	–	0.89 - 0.89	–
Puumala virus (PUUV)	1	–	–	0.82 - 0.82	–
Rift Valley fever virus (RVF)	1	–	–	0.77 - 0.77	–
Rabies virus (RABV)	3	0.946 - 0.946	0.915 - 0.918	–	–
Seoul orthohantavirus (SEOV)	1	0.82 - 0.845	–	–	–

Some types of correlation coefficient were not reported in any studies of some viruses and this is indicated by entries containing only "-".

virus (PUUV, n=1) (105), Rift Valley fever virus (RVF, n=1) (106), Rabies virus (RABV, n=2) (107, 108), and Seoul orthohantavirus (SEOV, n=2) (88, 89). A summary of the findings from these reports can be viewed in Table 2, whereas a more detailed breakdown for each report can be viewed in the supplementary file (Suppl. Table 1).

Aside from SARS-CoV-2 which will be analysed in the following sections of this study, we found that in general, most of the pseudotypes correlated well with the vMNA, irrespective of pseudotype cores and the readout techniques used to measure the assay results (Suppl. Table 1.). We found some studies that did not clarify the correlation test used, and were therefore omitted from Table 2, though relevant information including the r value is still included in the Supplementary Table 1. Interestingly, a study analysing the EBOV PVs reported that the choice of the PV core had a substantial impact on correlation with authentic virus (82, 83). When the negative control samples were omitted from the neutralisation assays, the correlation coefficients dropped from 0.68, 0.77 to -0.03 and 0.18, effectively showing no correlation, whereas the samples assayed with the VSV core PVs retained correlation coefficients of 0.84 and 0.96 (Suppl. Table 1.). This study highlights the need to consistently verify whether cores of pseudotypes can affect correlations with vMNAs.

3.2 Three-level meta-analysis results

From 22 SARS-CoV-2 studies we analysed 50 Pearson's correlation coefficients, which were derived from a combined total of 1238 data points by pMNA and vMNA (Figure 2). As stated in the methods, we verified that there were no significant differences in the mean Pearson's correlation values between studies that used different PV cores, neutralising reagents and assay readout types (Suppl. Figure 1). We calculated a pooled correlation of 0.86 (95% CI; 0.82-0.89, $p < 0.01$). These results suggest that there is a strong correlation between the results derived by pMNA and vMNA.

The results indicated the presence of low to moderate between-cluster heterogeneity [I^2 = 37.1% (CI: 11.2%-55.5%); H=1.26 (CI: 1.06 to 1.50); τ^2 = 0.05 (CI: 0.02-0.12)]. This means that there is some weak evidence of differences in the true effect sizes in the study. A 95% prediction interval (PI) for the pooled correlation is 0.69-0.94, which means that it is highly likely that the true correlation between pMNA and vMNA in a future study will lie between 0.69 and 0.94. Since this is entirely greater than 0.5, this provides us with evidence of a positive relationship between pMNA and vMNA for SARS-CoV-2, appropriately accounting for the distribution of effects amongst the studies. Removing results in turn did not lead to substantial reductions in heterogeneity. Our "funnel plot" (Suppl. Figure 2) shows that most points lie within the funnel shape in a symmetrical pattern, providing no evidence of publication bias.

Our "forest plot" (Figure 3) shows the calculated interval estimates for each study. We note that the majority of the interval estimates include our pooled estimate and that all studies except Mykytyn et al. (61), which has very small reported sample sizes, have entirely positive interval estimates.

3.3 Agreement between pMNA and vMNA by Bland-Altman method

Since Pearson's or Spearman's correlation coefficients are used for understanding correlation between two variables, they may not determine whether different assays are strictly in agreement with each other. The Bland-Altman method (109) is a frequently applied analysis which is often used to determine agreement between two methods that aim to measure the same variable, in this case, antibody neutralising capability. Within our literature search, several studies have used the Bland-Altman method of analysis. Therefore, we also refined the literature search used for this study by adding the search terms; "Bland-Altman". All four resulting papers identified were already included from the main literature search. Due to the power of this statistical method, we opted to present the results by the Bland-Altman method within the reports in a separate table (Table 3). All studies that reported results from the Bland-Altman method showed high levels of agreement between pMNA and vMNA.

4 Discussion

Given the interest in the results derived by pMNA compared to vMNA, our systematic review and meta-analysis sought to consolidate the data to inform the wider community on whether there is a correlation and subsequently, agreement between the two assays. The results of the meta-analysis would confirm that for SARS-CoV-2 there is a strong degree of correlation between pMNA and vMNA. Despite the limited number of studies, the Bland-Altman results presented in this manuscript also indicate a high level of agreement between the two assays. This data support the use of pMNA as a surrogate to the vMNA, though more correlation studies by Bland-Altman would be very valuable to perform in future reports.

Moreover, since multiple viral cores can be used for pseudotyping, it is important to assess whether this could impact the pMNA vs vMNA correlation. It would appear that in the case of the Ebola virus, there is a lower concordance, if a lentiviral core is used in the pMNA compared with a VSV core (82, 83). Whilst the precise reason for influence of the core remains unknown, though speculated to be due to the morphological difference between a VSV capsid and a filamentous EBOV particle (82) or the target cells, which is the same for the authentic virus and EBOV-VSV but differ for the lenti-based pMNA. It will be important to determine whether these differences exist in the case of other filoviruses and indeed other viruses, as there may be a high risk of reporting erroneous results. Therefore, it is important to optimize all aspects of the pMNA and different pseudotype cores combined with identical envelope glycoproteins should always be assessed in parallel with the authentic virus in neutralization tests, if possible. Critically, the two EBOV studies observed the reduced correlation of the lentiviral cores when negative control sera were excluded from their analyses. Therefore, we advise future correlation studies to consider not only including negative control samples within their

analyses, but also consider deriving correlations with and without the negative control samples, especially if the number of samples is low and multiple cores are under assessment.

Interestingly, multiple studies have mentioned that one of the benefits of using PVs is that they are more sensitive in discriminating samples containing weaker or a low concentration of neutralising antibodies (92, 100, 104). In fact, one report provided evidence of the vMN assay reporting false negative results on samples that contained neutralising antibodies, successfully detected by the pMN (102). Whilst this would highlight the benefits of using PVs for detecting positive samples within a human or animal population, it is may also bring into question whether the results derived from the weaker samples could protect the individual or animal from subsequent infection, given that the authentic virus was not neutralised. However, it is essential to consider that lower limits of detections can change based on assay design, virus species, the titre of the virus used, and the volume of serum sample used. This highlights reporting of results relative to a reference reagent can add value by enabling comparisons between data produced by different methods. Whilst use of a reference material will not ultimately improve assay performance, it helps to highlight differences. In any case, having a more sensitive assay such as the pMNA would prove to be very useful for epidemiological studies that are aiming to determine whether a virus exists or existed in a particular human or animal population, as opposed to correlating neutralising titres towards disease severity or protection.

Lastly, it is very important to distinguish the type of interpretation derived from either Pearson's R or Spearman's rank correlation analyses and the Bland-Altman plot. Neither the Pearson's R, which is a measure of the linear relationship between two variables, nor the Spearman's rank, that informs on correlation from measurements taken on an ordinal scale, provide information on the agreement between two different assays. In this case, the Bland-Altman method is required (109). Our literature search has shown for multiple viruses that the pMNA and vMNA have high agreement for multiple viruses in several families.

The main limitation of our systematic review is that it was biased towards SARS-CoV-2, due to the sheer number of publications dedicated to this virus in the past three years, providing enough correlation values that allowed for the meta-analysis. Whilst it would have been useful to carry out the same analysis for other viruses, unfortunately there were not enough correlation values. We did not use the Spearman's Rho coefficients in our analyses, but the strong positive values of these, for both SARS-CoV-2 and other viruses (Suppl. Table 1) do not disagree with our main conclusions that PVs and authentic virus showed strong positive relationships. Some of the studies used very small

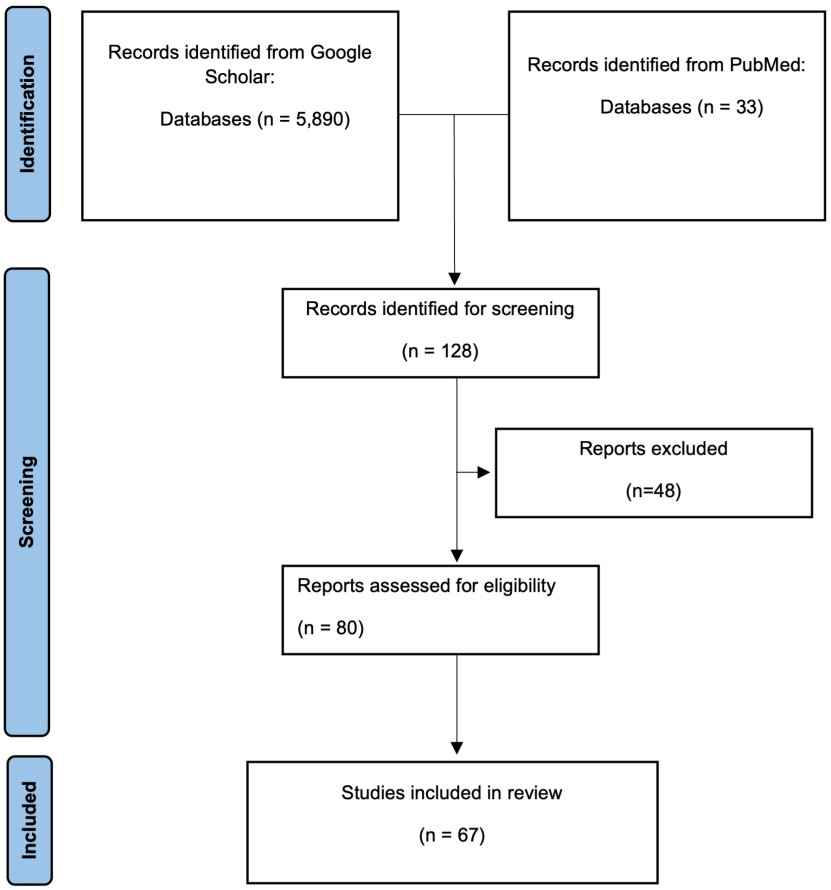

FIGURE 2
Flow diagram of the study identification and selection process.

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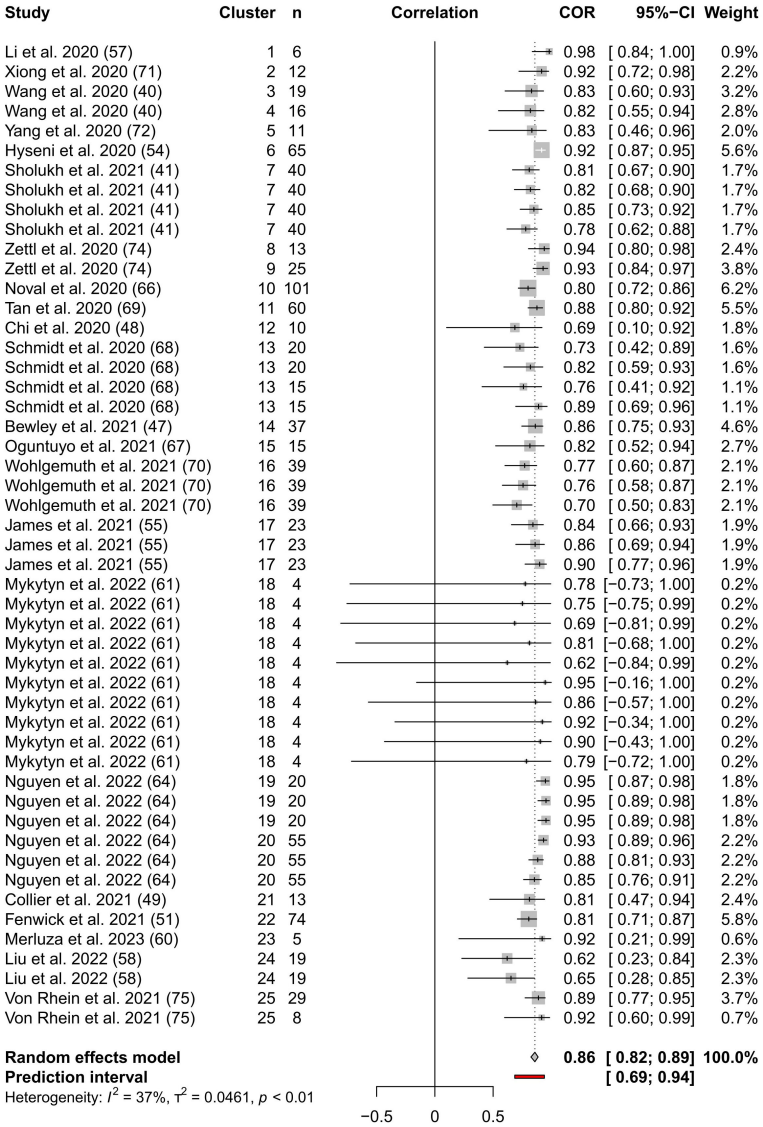

FIGURE 3
Forest Plot of the three-level meta-analysis results. The endpoints of the black or white horizontal lines represent the endpoints of the 95% CIs for the Pearson's correlation coefficients for each study. The grey boxes represent the sample sizes of each study. The vertical dotted line represents the pooled Pearson's correlation coefficient estimate and the grey diamond represents the 95% CI for the pooled Pearson's correlation coefficient estimate. The 95% prediction interval is shown by the red line. The table columns are, respectively, study name, cluster indicator, sample size (n) from which Pearson's correlation coefficient was calculated, correlation as described above, Pearson's correlation coefficients, 95% CI of Pearson's correlation coefficients, and weighting assigned to each coefficient.

sample sizes, which was accounted for through giving lower weights to these studies. We opted to include studies that used PVs that are non-replicative, single cycle of infection, therefore excluding studies that used replicon infection systems, despite some of these reports showing high correlation and high level of agreement between single-round replicons and authentic virus in a neutralisation assay (110, 111). Lastly, new virus and cell-free assays have now been developed for SARS-CoV-2 that measure the capability of

TABLE 3 Reported Bland-Altmann results.

Study	Virus	Samples	Conclusions
Hyseni *et al.*, 2020 (54)	SARS-CoV-2	65	64/65 samples within 95% Limit of Agreement
Lester *et al.*, 2019 (100)	MERS	52	High level of agreement
Nie *et al.*, 2017 (107)	RABV	320	All samples within Limit of Agreement
Buchy *et al.*, 2010 (91)	IAV H5N1	41	High level of agreement

antibodies blocking the spike protein from interacting with its receptor ACE-2, effectively becoming a surrogate neutralisation assay, have shown to have strong correlations with both pMNAs and vMNAs (51, 69, 112–114). Whilst these assays do not fit the scope of this study, we believe it is worth mentioning and monitoring for follow up meta-analyses.

In summary, our systematic review and meta-analysis shows that the pMNA designed for use towards SARS-CoV-2 serological studies demonstrated a high degree of correlation with assays performed using the authentic virus. In addition, many other viruses that have been pseudotyped also show a high degree of correlation. We recommend, where possible, that future studies on methods agreement should continue to investigate the use of multiple PV cores, to determine whether there could be differences in neutralisation titres, such as that exemplified with Ebola virus PVs. It is also essential that future studies incorporate the Bland-Altman analysis to determine the agreement between the two assays as well as this is substantially more informative, especially when both assay results are to be applied to clinical trials and assessed for determining correlates of protection. Ultimately, we would encourage laboratories to calibrate assays to reference materials, if one is available and relevant for the isolate under study, which will support these future comparisons and critically provide traceability to a correlate of protection once derived.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

DC and NT conceptualised the study. DC, CW, EB, MM-N, EW, SS, SR, JC-O, JH, GM assisted in the literature search and proof-reading of the manuscript. CW and SR carried out the statistical analysis. GM, JC-O, JH and NT provided critical evaluation of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

JH is a founder and a shareholder in DIOSynVax Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1184362/full#supplementary-material

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Age- and sex-specific differences in immune responses to BNT162b2 COVID-19 and live-attenuated influenza vaccines in UK adolescents

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Introduction: The key to understanding the COVID-19 correlates of protection is assessing vaccine-induced immunity in different demographic groups. Young people are at a lower risk of COVID-19 mortality, females are at a lower risk than males, and females often generate stronger immune responses to vaccination.

Methods: We studied immune responses to two doses of BNT162b2 Pfizer COVID-19 vaccine in an adolescent cohort (n = 34, ages 12–16), an age group previously shown to elicit significantly greater immune responses to the same vaccine than young adults. Adolescents were studied with the aim of comparing their response to BNT162b2 to that of adults; and to assess the impacts of other factors such as sex, ongoing SARS–CoV–2 infection in schools, and prior exposure to endemic coronaviruses that circulate at high levels in young people. At the same time, we were able to evaluate immune responses to the co-administered live attenuated influenza vaccine. Blood samples from 34 adolescents taken before and after vaccination with COVID-19 and influenza vaccines were assayed for SARS–CoV–2-specific IgG and neutralising antibodies and cellular immunity specific for SARS–CoV–2 and endemic betacoronaviruses. The IgG targeting influenza lineages contained in the influenza vaccine were also assessed.

Results: Robust neutralising responses were identified in previously infected adolescents after one dose, and two doses were required in infection-naïve adolescents. As previously demonstrated, total IgG responses to SARS–CoV-2 Spike were significantly higher among vaccinated adolescents than among adults (aged 32–52) who received the BNT162b2 vaccine (comparing infection-naïve, 49,696 vs. 33,339; p = 0.03; comparing SARS-CoV–2 previously infected, 743,691

vs. 269,985; p <0.0001) by the MSD v-plex assay. There was no evidence of a stronger vaccine-induced immunity in females compared than in males.

Discussion: These findings may result from the introduction of novel mRNA vaccination platforms, generating patterns of immunity divergent from established trends and providing new insights into what might be protective following COVID-19 vaccination.

KEYWORDS

SARS-CoV-2, vaccine, COVID-19, adolescents, immunity

Introduction

The BNT162b2 Pfizer-BioNTech COVID-19 vaccine was authorised for 12–15 year olds in June 2021 in the United Kingdom by the Medicines and Healthcare Products Regulatory Agency (1), with an initial 30 µg dose administered in the winter of 2021 and a second dose administered in early 2022 (2). In the United Kingdom, the first dose of BNT162b2 was administered to adolescents alongside the AstraZeneca intranasal seasonal live-attenuated influenza vaccine (LAIV) FluenzTetra, presenting a unique opportunity to study vaccine-induced immunity in this age group.

Older age is a primary risk factor for severe COVID-19, perhaps due to reduced immune capacity with age, driven by persistent inflammation and cellular dysfunction (3). The overall death rate of COVID-19 was 0.66%, increasing to 7.8% in the 80s (4). The majority of young people experience mild COVID-19; severe disease and multisystem inflammatory syndrome only occur in a minority of paediatric patients (5). Adolescents and children display rapid and adaptable immune responses that may contribute to improved resolution of infections, such as abundant IgM memory B cells, broad and rapidly produced natural antibodies, and lower inflammatory cytokine responses (6, 7). Differential COVID-19 outcomes between adults and children may also be influenced by pre-existing immune responses to endemic coronaviruses that circulate at higher levels in children (6). Notably, adolescents between 12 and 15 years of age generate 1.76-fold higher nAb responses to BNT162b2 than those aged 16–25 years, indicating either potential age-related changes in immune response even during adolescence or an increase in cross-reactivity with endemic coronaviruses that enhance vaccine responsiveness and decline with age (8). However, humoral responses to HCoVs have been associated with worse COVID-19 outcomes through the inhibition of novel responses to SARS-CoV-2 as a result of immune imprinting or 'original antigenic sin' (9). Children have been reported to display higher immunity to endemic HCoVs than adults (10), perhaps because of the high circulation of viruses in schools. Finally, older individuals are more likely to have immunodeficiencies or chronic diseases, which increases their risk of severe COVID-19.

In addition to age, understanding the role of sex in the vaccine response is crucial for the development of more effective vaccines. Adult females aged 18-49 have been shown to generate two-fold greater antibody responses to trivalent influenza vaccines (11), and adult females are more at risk for serious adverse events (SAEs) after vaccination, including after the ChAdOx1 Oxford-AstraZeneca COVID-19 vaccine (12–14). In one study, females administered a half-dose influenza vaccine produced marginally stronger antibody responses than age-matched males who received full-dose vaccine (11). Female children under five also display stronger antibody responses following vaccination against measles (15), diphtheria (16), and hepatitis A (17), although the literature on the subject is often variable, with some evidence of greater immune responses to vaccines, such as measles in males (18), or no significant difference between the sexes (19). Nevertheless, vaccine-induced immune responses in females could potentially facilitate reduced dosing regimens, which may minimise the incidence of SAEs, improve vaccine uptake, and improve vaccine supply. However, young males experience more vaccine-induced myocarditis after BNT162b2 treatment, suggesting that immune responses to mRNA vaccines may be differentially influenced by sex (20, 21). Adolescents undergoing puberty face significant changes in the levels of sex hormones such as testosterone and oestrogen, which are known to modulate immunity to SARS-CoV-2 and influenza (22, 23).

To explore sex- and age-specific differences in humoral and cellular immunity to BNT162b2, we studied adolescent and adult cohorts in the United Kingdom that received this vaccine. Data collected from adolescents in this study were compared to the Protective Immunity from T cells in the Healthcare Workers (PITCH) cohort of vaccinated healthcare workers (HCWs) aged 32–52 years, who received two doses of BNT162b2 and also represented a mixture of previously infected and infection-naïve individuals (24). We explored age-specific effects on immunity within the adolescent cohort as well as between adolescents and adults. Furthermore, we examined whether sex-specific immune effects were evident. As not all adolescents also received LAIV, we were also able to assess whether co-administration of LAIV appeared to influence the magnitude of the response to BNT162b2. Furthermore, many studies on adolescent responses

to BNT162b2 have used prior SARS-CoV-2 infection as an exclusion criterion (8, 25). Here, we enrolled both SARS-CoV-2 infection-naïve and previously infected adolescents to understand the role of prior or ongoing infection in the vaccine response.

Results

Cohort description

In November and December 2021, 34 adolescents aged between 12 and 16 years were recruited into the study through their enrolment at schools in Oxford, UK (Figure 1A). All 34 individuals received the BNT162b2 vaccine; 26 (76%) received LAIV on the same day as the first dose of BNT162b2. Approximately 47% of the individuals (n = 16) were female, and the median age was 14.1 years (12.2–16) (Figure 1B). A total of 33 individuals were Caucasian and one was Asian. None of the individuals were taking any regular medication. All 34 individuals were sampled before the first dose (pre-Vx1) and after the first dose (post-Vx1), 23 individuals were sampled pre-Vx2 and 14 were sampled post-Vx2, giving a dropout rate of 41% over the course of the study.

The adult cohort to which adolescent data were compared was the PITCH cohort of vaccinated HCWs (24, 26). This cohort consisted of 589 adults aged 32–52 years who had received two doses of BNT162b2 28 days apart. IgG data from 79 adults and neutralising antibody (nAb) data from 10 adults were used for comparison with data from adolescents. These samples were randomly selected from the PITCH dataset to include roughly equal numbers of infection-naïve and previously infected samples. Only 10 individuals were included in the nAb data, as they were all available at the time.

Humoral immune responses to BNT162b2 vaccination

To evaluate the immunogenicity of the BNT162b2 vaccine among adolescents, we first characterised humoral responses using MSD-platform immunoassays to quantitatively measure the total immunoglobulin G (IgG) response to the SARS-CoV-2 Spike (S), the receptor-binding domain (RBD) of S, and SARS-CoV-2-N (Figure 2A). Both infection-naïve and previously infected adolescents showed significantly greater IgG responses to S post-Vx1 than to pre-Vx1 (median: 61 vs. 49,696, ×803, p = 0.0005 and 13,409 vs. 788,568, ×55, p <0.0001, respectively, Wilcoxon signed-rank test) and greater anti-RBD IgG responses (263 vs. 16,861, ×64, p = 0.0005 and 6,556 vs. 351,068 ×53, p <0.0001, respectively, Wilcoxon signed-rank test) (Figure 2A) (Supplementary Figures 1A, B). Anti-S and RBD IgG responses increased post-Vx2 in all groups, but only anti-RBD IgG increased significantly and only in previously infected individuals (90,067 vs. 318,687, ×3.5, p = 0.008, Wilcoxon signed-rank test), although this is likely because only four infection-naïve individuals were included post-Vx2 due to the high drop-out rates over the course of the study. Notably, two doses of BNT162b2 in infected individuals gave similar levels of IgG to one dose of the vaccine in previously infected individuals. Recognising the multitude of comparisons made in this section and the risk of committing a type 1 error, a Bonferroni correction for multiple comparisons for the 12 tests conducted for S and RBD demonstrated that six associations remained significant at an alpha value of p = 0.004. These are the increased IgG responses to S and RBD post-Vx1 in both groups, and the waning of response between post-Vx1 and pre-Vx2 time points in previously infected individuals only.

Since nAbs as well as total IgG are reported to be a correlate of protective immunity against symptomatic COVID-19 (27), we next

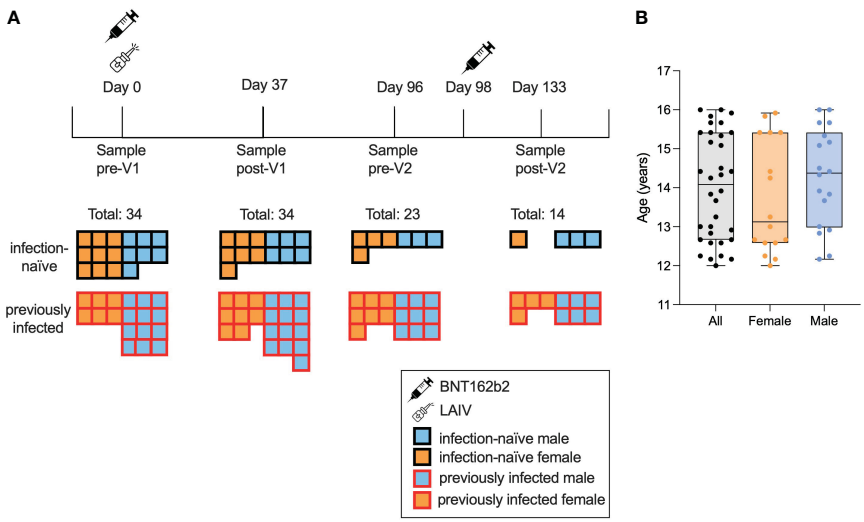

FIGURE 1
Characteristics of the study cohort. A total of 34 adolescents were enrolled and provided consent, of whom 18 were seropositive for S or N pre-Vx1. Samples were taken pre-Vx1 on the day of vaccination, a mean of 37 days post-Vx1, 2 days pre-Vx2, and 35 days post-VX2 **(A)**. The median age was 13 years 1 month for females (orange) and 14 years 5 months for males (blue) **(B)**.

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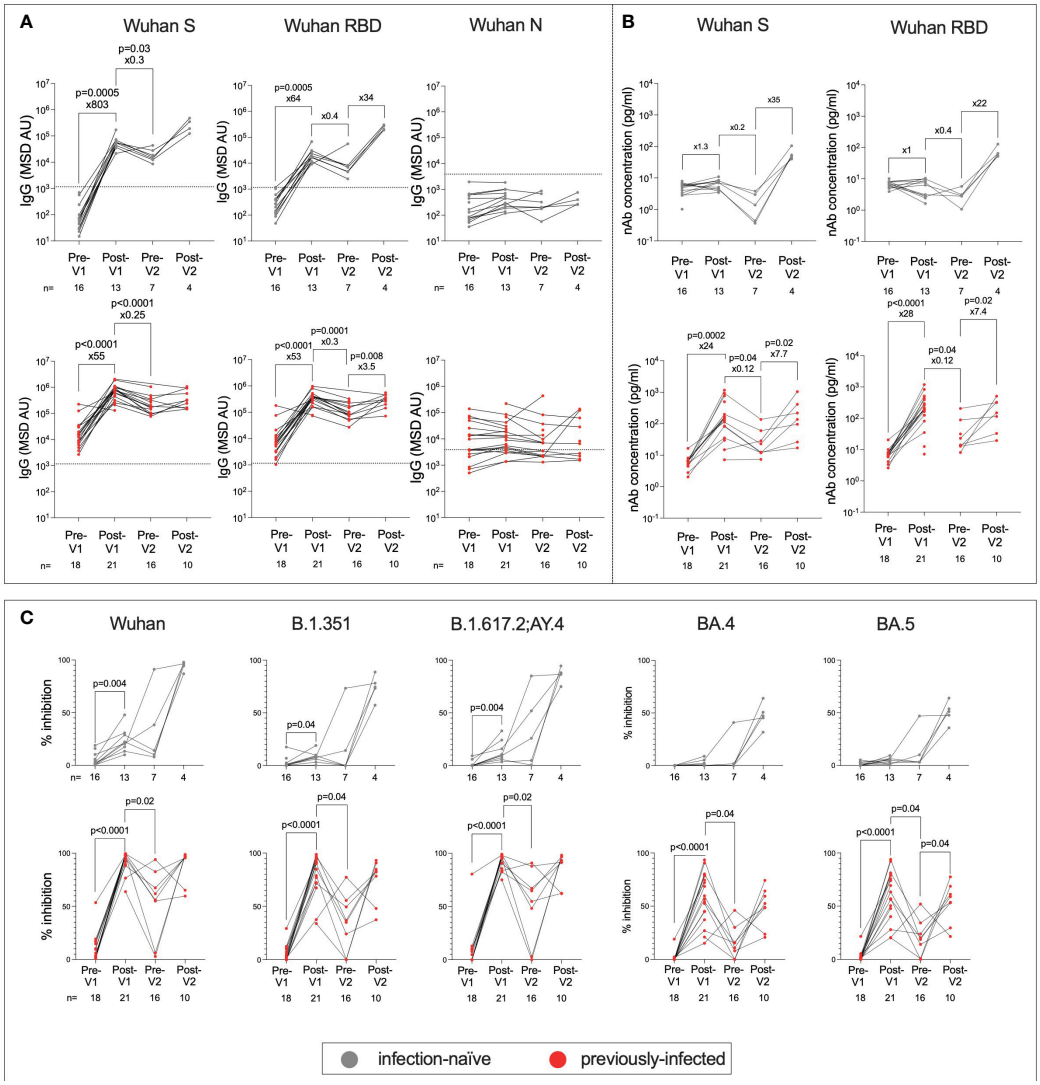

FIGURE 2
Humoral responses following first and second doses of BNT162b2 in previously infected and infection-naive adolescents. Anti-S, RBD and N IgG in infection-naive (grey) and previously infected (red) adolescents **(A)**. The thresholds for IgG positivity were obtained from previous studies (26). nAbs targeting S and RBD in infection-naïve (grey) and previously infected (red) adolescents using MSD ACE2-Spike binding inhibition assays **(B)**. Percent inhibition of SARS-CoV-2 S-ACE2 binding as measured by MSD ACE2 inhibition assay in infection-naive (grey) and previously infected (red) adolescents targeting common SARS-CoV-2 lineages: Wuhan, B.1.351(Beta), B.1.617.2/AY.4 (Delta), BA.4 and BA.5 (Omicron) **(C)**. P-values from Wilcoxon signed-rank tests. Fold-change refers to the difference between the total group medians.

assessed a surrogate of nAb activity using the MSD-platform ACE2 inhibition assay (28), which is well correlated with live virus neutralisation assays (24, 26, 29, 30). In contrast to IgG responses, only previously-infected individuals generated increased nAb responses following the first dose of vaccine (6 vs. 149, ×24, p = 0.0002, Wilcoxon signed-rank test) (Figure 2B), and fold change in nAb response to S and RBD was higher in previously-infected individuals post-Vx1 compared to infection-naïve individuals (S: 24 vs. 1.3, p <0.0001 and RBD: 28 vs. 1, p = 0.0002, respectively, Mann–Whitney tests) (Supplementary Figures 1C, D). After two doses of BNT162b2, infection-naïve individuals reached nAb titres similar to those of previously infected individuals after one dose, supporting the idea that two doses of vaccine are required for a robust neutralising response in infection-naïve individuals. Again, to account for the six tests undertaken under this hypothesis, the Bonferroni correction with an alpha value of p=0.008 revealed that only post-Vx1 nAb responses in previously infected individuals remained significantly increased. The reduction in nAb response pre-Vx2 to levels lower than pre-Vx1 is surprising and may be a result of batch effects in the analysis. Alternatively, higher nAb levels pre-Vx1 may be a result of cross-reactivity with endemic HCoVs following a recent infection.

To determine how the breadth of the nAb response to SARS-CoV-2 variants is impacted by vaccination and prior infection, the MSD-platform ACE2 inhibition assay was carried out against the common variants of SARS-CoV-2 in both infected and previously infected individuals (Figure 2C; Supplementary Figure 2). Notably, previously infected individuals showed broad nAb responses

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against all the studied variants following the first dose, whereas high-titre nAb responses against these variants were only observed following the second dose in infection-naive individuals. Using Bonferroni correction with an alpha value of p = 0.002, for the 30 comparisons made under this hypothesis, % inhibition of ACE2-S binding remained significantly elevated post-Vx1 in previously infected individuals only. In previously infected individuals, median responses were highest post-Vx1 and post-Vx2 for the Wuhan, B.1.351, and B.1.617.2;AY.4 strains, whereas responses to BA.4 and BA.5 were more varied. This may be because some adolescents were infected with the former strains before the study was carried out, whilst some adolescents who became infected during the study were likely infected with BA.4 and BA.5. Therefore, a range of nAb responses to BA.4 and BA.5 were expected in this group. In contrast, in the infection-naïve cohort, all individuals showed weaker responses to BA.4, BA.5 post-Vx2, and post-Vx2 than they do to the first three VOCs. This is likely because none of these individuals were infected with BA.4 and BA.5.

Cellular immune responses to BNT162b2 vaccination

Next, we characterised the cellular immune response in adolescents following the first and second doses of BNT162b2 using a CellTrace Violet (CTV) proliferation assay (Figure 3; Supplementary Figures 3, 4; Supplementary Table 1). The proliferation assay was chosen as it has been used previously to quantify SARS-CoV-2 specific T cells and has been shown to be sensitive to low-magnitude responses, perhaps due to the long incubation period (31). Infection-naïve individuals showed significantly increased responses to S1 post-Vx1 (p = 0.02, Wilcoxon test). However, the small number of individuals included in this analysis makes the interpretation difficult. In contrast to humoral responses to BNT162b2, SARS-CoV-2-specific CD4+ T-cell proliferative responses were similar in infection-naïve individuals compared to previously infected individuals after a single dose of vaccine. The T-cell response in previously infected individuals also increased following one dose of the vaccine, albeit not significantly. For CD4+ responses to S1 in particular, there was a general trend of an increased magnitude of response following vaccination. CD8+ responses were of lower magnitude, and no clear trend of increasing immunity following vaccination could be observed, although responses did increase following Vx1 in some individuals.

Although T-cell responses to HCoV-OC43 S2 and HCoV-HKU1 S2 were identified in several individuals, particularly in previously infected individuals, no significant impact of BNT162b2 vaccination on the magnitude of T-cell responses was observed. Responses appeared to peak pre-Vx2, which may be the result of batch effects within the assay or a delayed response following Vx1. Responses to M and N were also measured (Supplementary Figure 4); these did not increase significantly post-Vx1 or Vx2 treatment, as expected. Some individuals showed increased responses to M and N over the course of the study. As only S is included in BNT162b2, this may reflect reinfection with SARS-CoV-2 in the case of the previously infected group or infection with endemic HCoVs in the infection-naïve group.

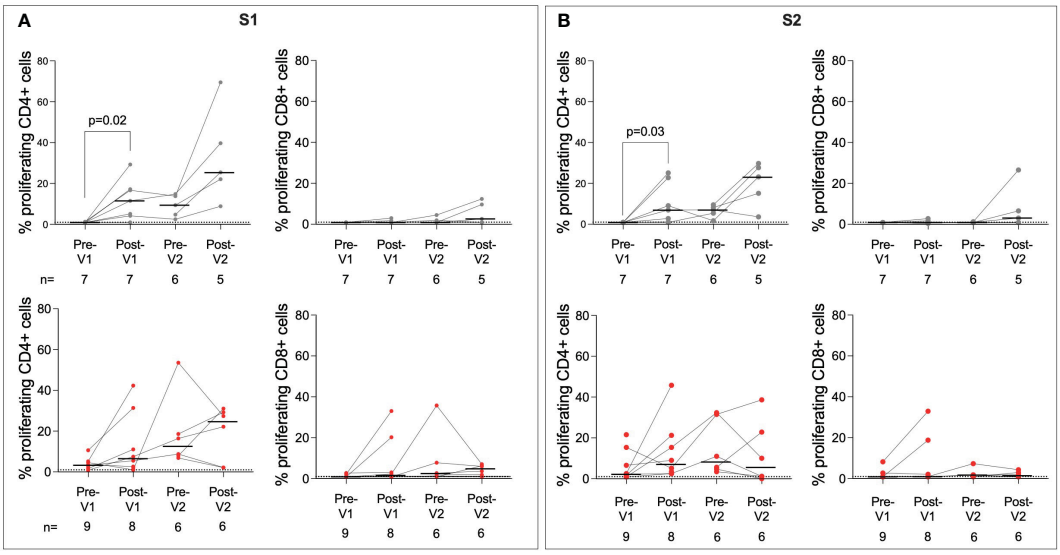

FIGURE 3
T-cell responses to SARS-CoV-2 S are boosted post-Vx1 and post-Vx2. CellTrace Violet stains were used to assess proliferating CD4+ and CD8+ T cells targeting the S1 region of S **(A)** and the S2 region of S **(B)** in infected (grey) and previously infected (red) individuals. Data show proliferating cells as a percentage of the parent populations with subtracted DMSO background values. Thresholds for positivity were set at 1, as determined in previous studies (31). P-values from Wilcoxon signed-rank tests. Fold-change refers to the difference between the total group medians. Values below 1% were given nominal values of 0.9%.

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Higher magnitude antibody responses to BNT162b2 in adolescents versus adults

The role of age in the immune response to vaccination was of particular interest in this study. To determine whether the responses observed in adolescents to the BNT162b2 vaccine were stronger than those observed in adults, as previously shown (8), we compared the adolescent data to humoral responses in adults (32–52 years) from the PITCH cohort 28 days after the first dose of BNT162b2 (Figure 4) (26, 32). PITCH is a consortium of universities and the UK Health Security Agency (UK HSA) with the aim of characterising infection-acquired and vaccine-induced immunity to SARS-CoV-2 in HCWs. Here, as reported for adolescents receiving two vaccines (8, 25), post-Vx1, infection-naive adolescents generated higher magnitude anti-S IgG responses than infection-naive adults (49,696 vs. 33,339, ×1.5, p = 0.03, Mann–Whitney test) and previously infected adolescents generated greater anti-S IgG responses than previously-infected adults (743,691 vs. 269,985, ×2.9, p <0.0001, Mann–Whitney test) (Figure 4A). Post-Vx1 nAb responses did not differ significantly between adolescents and adults, although the small number of previously infected adults (n = 4) included in this analysis limited this conclusion (Figure 4B). Infection-naïve adolescents appeared to have higher levels of pre-Vx1 nAbs than adults; the reasons behind this are unclear but may related to pre-existing cross-reactive immunity to endemic HCoVs. Alternatively, this may be an artefact of the noise at the lower limit of detection of the assay. Despite this higher baseline, nAb titres did not change significantly post-Vx1 in the infection-naïve adolescents. To account for the six comparisons undertaken under this hypothesis and to reduce the possibility of committing a type 1 error, Bonferroni correction was employed. With a new alpha value of 0.008, all but one comparison (infection-naïve adults vs. adolescents) remained significant. It is also possible that the difference in group size (n = 34 adolescents, n = 79 adults) influenced the results of this analysis.

To investigate why infection-naive adolescents did not generate significantly increased nAb responses post-Vx1 despite a strong total IgG response, we sought to address the hypothesis that cross-reactive antibody responses to endemic HCoVs might be present at

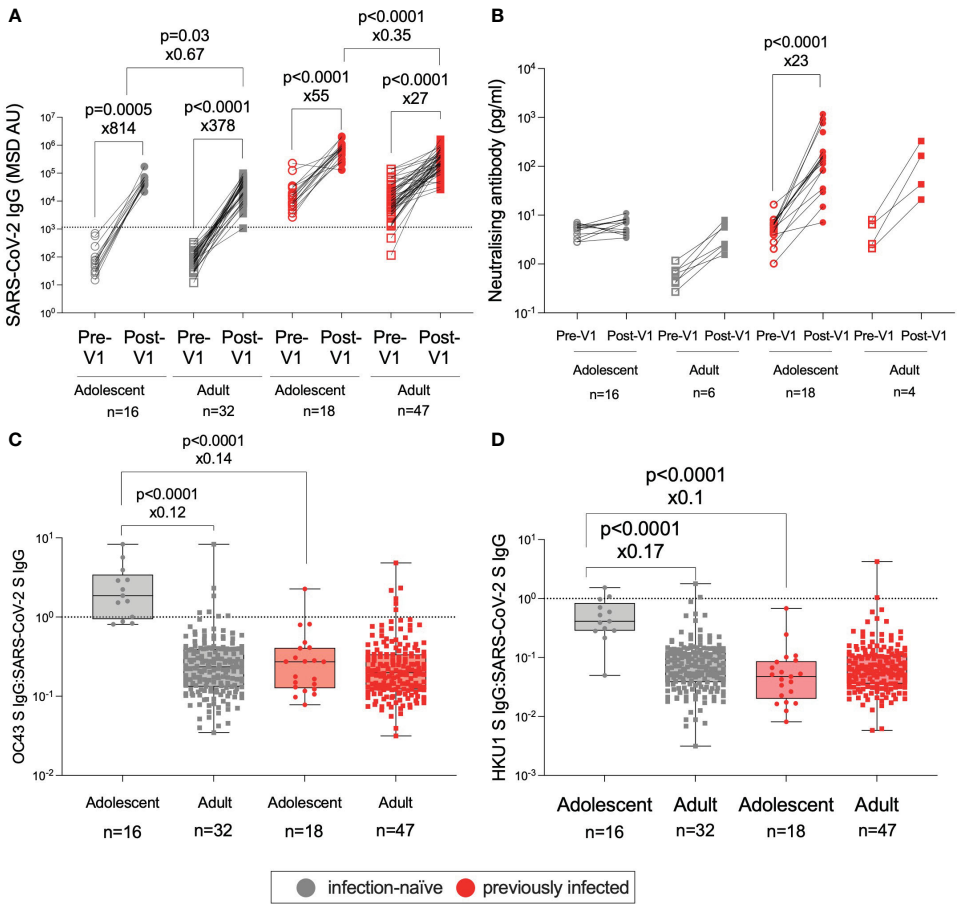

FIGURE 4
Age-specific effects on the humoral response to BNT162b2. IgG targeting S in infection-naïve adolescents (grey circles), infection-naïve adults (32–52 years) (grey squares), previously infected adolescents (red circles), and previously infected adults (red squares), pre-Vx1 (unfilled shapes) and post-Vx1 (filled shapes) as measured by an MSD v-plex immunoassay **(A)**. nAb concentration targeting S in infection-naïve and previously infected adolescents and adults as measured by an MSD ACE2-Spike binding immunoassay **(B)**. The ratio of IgG targeting HCoV-OC43 S to SARS-CoV-2 S **(C)** and the ratio of IgG targeting HCoV-HKU1 S to SARS-CoV-2 S **(D)** in infection-naive adolescents (grey circles), infection-naive adults (grey squares), previously infected adolescents (red circles), and previously infected adults (red squares) post-Vx1. P-values represent Mann–Whitney test values for unpaired data and Wilcoxon signed-rank test values for paired data. The fold change was calculated as the ratio of population medians.

higher levels in infection-naïve adolescents, thereby interfering with the generation of novel SARS-CoV-2-specific responses to BNT162b2, as has been suggested previously (9, 33).

Our data supported the hypothesis that cross-reactive antibody responses to HCoVs are associated with weaker vaccine-induced neutralising responses: in this study, the ratio of IgG targeting betacoronaviruses HCoV-OC43 and HCoV-HKU1 S to IgG targeting SARS-CoV-2 S was significantly higher in infection-naive adolescents versus infection-naive adults (1.9 vs. 0.2, ×8.3, p <0.0001; 0.4 vs. 0.06, ×5.9, p <0.0001, Mann–Whitney tests) and versus previously-infected adolescents (1.9 vs. 0.3, ×7.1, p <0.0001; 0.4 vs. 0.04, ×10, p <0.0001, Mann–Whitney test) post-Vx1 (Figures 4C, D). There was no significant difference in the ratio between infection-naïve adolescents and previously infected adults, perhaps due to several previously-infected adults had a high HCoV:SARS-CoV-2 IgG ratio. Furthermore, the ratio of IgG targeting HCoV-OC43 and HCoV-HKU1 S to IgG targeting SARS-CoV-2 S was significantly negatively correlated with the nAb response (OC43: r = −0.84, p<0.0001; HKU1: r= − 0.75, p <0.0001) in all adolescents, although this significance was lost when adolescents were divided into infection-naïve and previously infected groups (Supplementary Figure 5). Again, considering the eight comparisons made for this hypothesis, utilising the Bonferroni correction revealed a new alpha value of p = 0.006. All four significant differences remained significant after correction.

Sex differences in response to BNT162b2 vaccination

Females typically elicit stronger IgG responses than males following vaccination (12, 13, 23, 34), including after influenza vaccines (11, 23). Surprisingly, infection-naïve males generated significantly higher post-Vx1 IgG targeting both SARS-CoV-2 S and RBD than females (62,270 vs. 36,951, ×2, p = 0.008; 23,860 vs. 11,443, ×2, p = 0.02, respectively; Mann–Whitney tests) (Figures 5A, B). There was no significant difference in IgG response between the sexes of previously infected individuals (Figures 5C, D). Furthermore, there was a trend towards a stronger RBD and S nAb response post-Vx1 in infection-naive males compared to infection-naive females, although this was not significant (p = 0.07 and p = 0.15, respectively, Mann–Whitney tests) (Figures 5E, F), and there was no significant difference in nAb response between previously infected males and females (Figures 5G, H). There was no significant difference in baseline IgG responses between males and females. Furthermore, using a Bonferroni correction to consider the 24 comparisons made under this hypothesis, no comparisons remained significant with a new alpha value of p = 0.002. In addition, the small number of individuals in each group likely affects the statistical analysis undertaken. Furthermore, there was no significant difference in

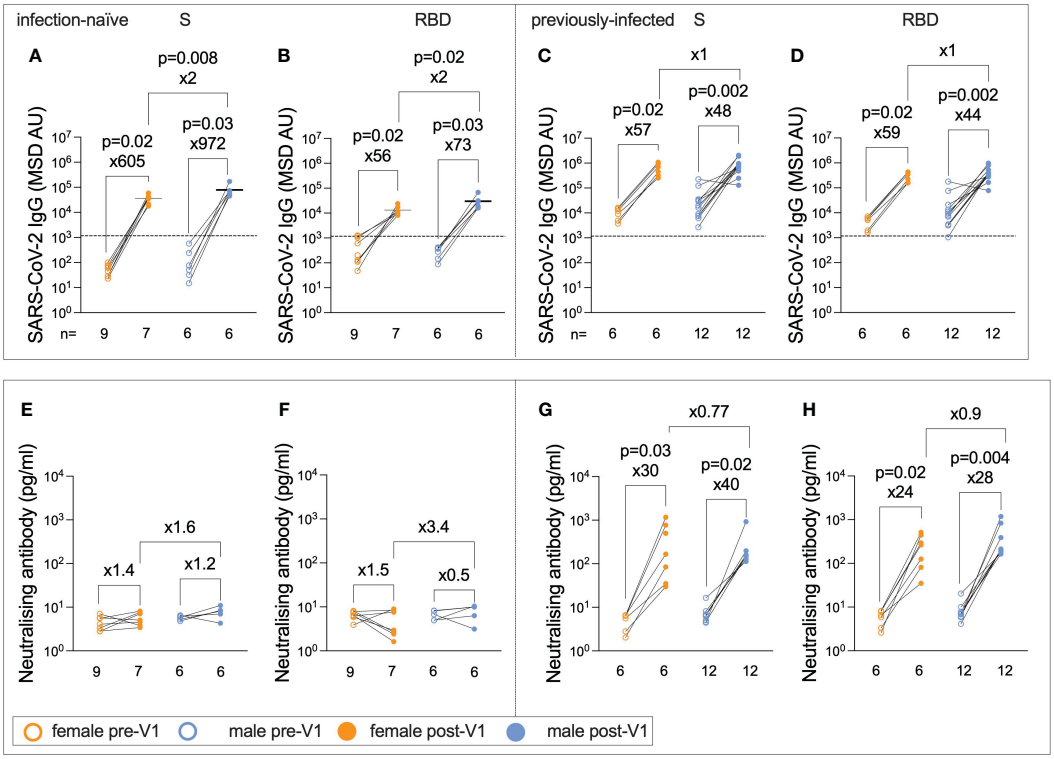

FIGURE 5
Infection-naïve male adolescents generate greater post-Vx1 IgG responses than do female adolescents. IgG targeting S **(A)** and RBD **(B)** in infection-naive adolescents pre-Vx1 (unfilled circles) and post-Vx1 (filled circles) in females (orange circles) and males (blue circles), as measured using an MSD v-plex immunoassay. IgG targeting S **(C)** and RBD **(D)** in previously infected adolescents pre-Vx1 and post-Vx1 in females and males. Concentration of nAbs targeting S **(E)** and RBD **(F)** in infection-naive adolescents measured by an MSD ACE2-Spike binding inhibition assay. Concentration of nAbs targeting the S **(G)** and RBD **(H)** in previously infected adolescents. P-values represent Wilcoxon test values for paired data and Mann–Whitney test values for unpaired data. The fold change was calculated as the ratio of population medians.

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IgG-targeting endemic HCoVs between males and females in this cohort, which may have been a potential confounder in this analysis. There were no reported sex differences in the humoral response to BNT162b2 in adults in previous studies (24, 26).

Humoral responses to LAIV administration

In addition to the immune response to BNT162b2, the co-administration of LAIV enabled the characterisation of immunity against influenza following vaccination. To determine the effect of LAIV on lineage-specific anti-haemagglutinin (HA) IgG titres, enzyme-linked immunosorbent assays (ELISAs) were performed on pre- and post-Vx1 samples from the 26 individuals who received LAIV (Figure 6). As expected, IgG titres were significantly higher post-Vx1 for A/Cambodia (H3N2), A/Victoria (H1N1), and B/Phuket (Yamagata) (9.3 vs. 13.9, ×1.5, p <0.0001; 11 vs. 13.4, ×1.2, p = 0.0002; 7 vs. 10.2, ×1.5, p <0.0001; respectively, Wilcoxon signed-rank tests) (Figure 6A). Employing a Bonferroni correction to account for the four comparisons under this hypothesis resulted in an alpha value of 0.01. All three differences remained significant at this alpha value. Surprisingly, post-Vx1 anti-HA IgG responses towards the B/Washington (Victoria) lineage were not significantly increased compared to pre-Vx1. A possible explanation is that

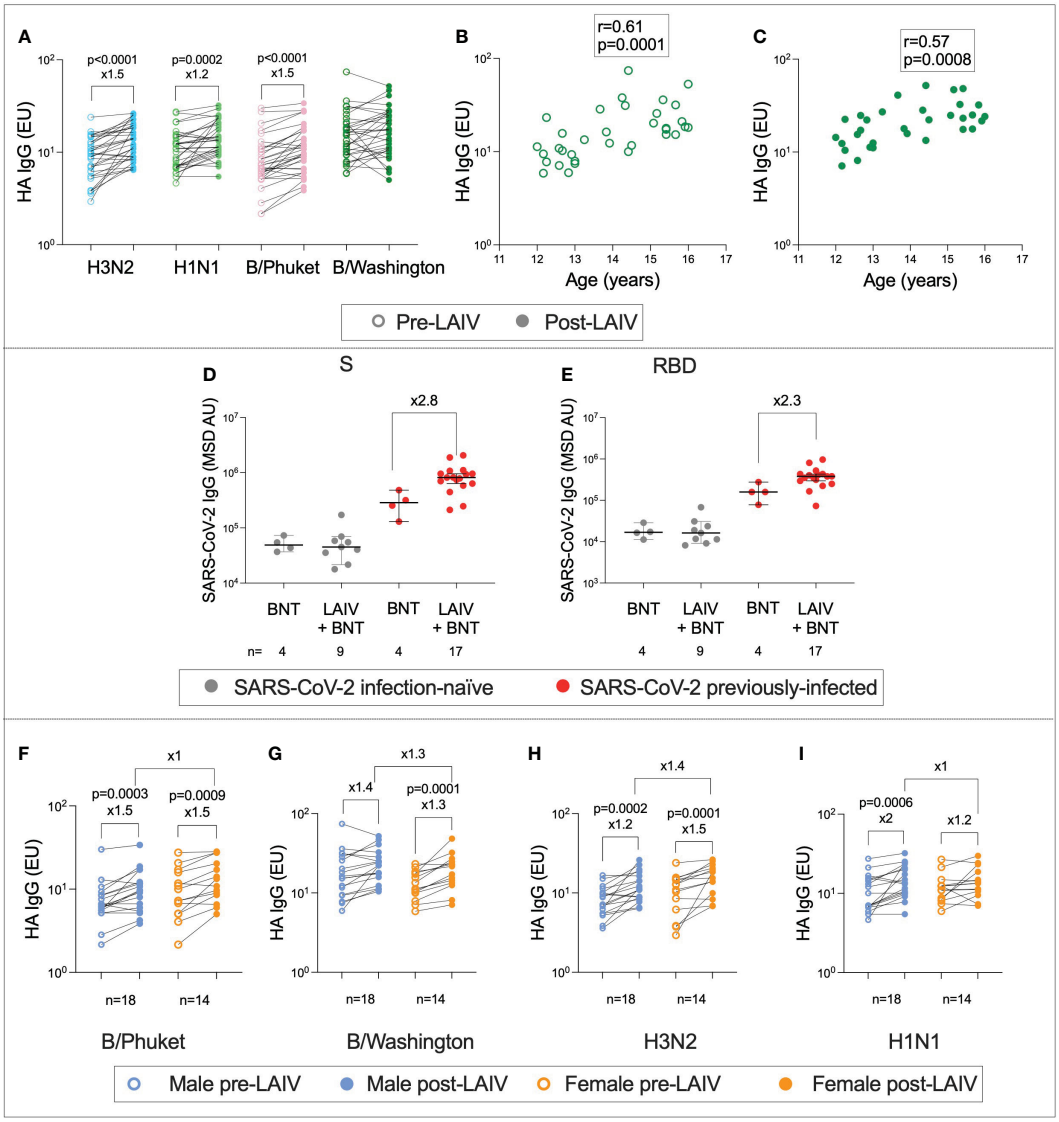

FIGURE 6
Age- and sex-specific immunity to influenza following LAIV administration. IgG targeting haemagglutinin (HA) pre- (unfilled circles) and post- (filled circles) LAIV administration for the four influenza lineages **(A)** (P-values from Wilcoxon tests). Correlation between age and IgG targeting HA for the B/Washington lineage pre-Vx1 **(B)** and post-Vx1 **(C)** (Spearman rank r- and p-values). IgG targeting SARS-CoV-2 S **(D)** and RBD **(E)** in infection-naïve (grey) and previously infected (red) adolescents who received the BNT162b2 vaccine alone (BNT) or co-administered with the LAIV (LAIV + BNT) (Mann–Whitney p-values). IgG targeting HA pre- (unfilled circles) and post- (filled circles) LAIV administration in males (blue) and females (orange) for the four influenza lineages **(F–I)** (Wilcoxon p-values).

responses to B/Washington (Victoria) were strongly correlated with age at both pre- and post-vaccine time points (r = 0.61, p = 0.0001, r = 0.57, p = 0.0008, respectively, Spearman rank test) (Figures 6B, C). In contrast, there was no correlation between age and post-Vx1 IgG for A/Cambodia (H3N2) or B/Phuket (Yamagata), and for A/Victoria (H1N1), pre-Vx1 IgG levels only weakly correlated with age (r = 0.39, p = 0.02, Spearman rank test). This pattern suggests that natural exposure to B/Washington (Victoria) is so frequent in this cohort that vaccination against this strain of influenza does not significantly add to the natural immunity that accumulates during adolescence. Pre-existing immunity to influenza has been widely described from prior infection and vaccination, in support of this finding (35, 36).

Unexpectedly, and in contrast to previous studies (37), adolescents previously infected with SARS-CoV-2 who received both BNT162b2 and LAIV appeared to generate over two-fold-higher higher post-Vx1 IgG targeting both S and RBD compared to adolescents previously infected with SARS-CoV-2 who received BNT162b2 alone. However, as this analysis involved a very small number of individuals, the statistical analysis was not appropriate (Figures 6D, E). Additionally, using Bonferroni correction to account for multiple testing, with a new alpha value of 0.012, these differences were no longer significant. In terms of demographics, the four BNT-alone infection-naïve individuals were two males and two females, aged 12 years 6 months to 16. The nine BNT+LAIV infection-naïve individuals included four males and five females, aged 12 years 3 months to 15 years 11 months. The four BNT-alone previously infected individuals were two males and two females, aged 13 years 8 months to 14 years 5 months. The 17 BNT + LAIV previously infected individuals were six females and 11 males aged 12–16 years. We did not find a sex difference in the IgG response to LAIV (Figures 6F–I).

To assess whether individuals who generated strong vaccine responses to BNT162b2 also generated higher magnitude LAIV responses, the correlation between anti-S IgG post-Vx1 and anti-HA IgG targeting the four influenza lineages was calculated. There was no correlation between anti-S IgG and anti-HA IgG in B/Phuket, B/Washington, or A/Cambodia (H3N2) (B/Phuket: r = −0.1, p = 0.6; B/Washington: r = 0.1, p = 0.62; A/Cambodia (H3N2): r = 0.18, p = 0.37), although there was a moderate negative correlation with A/Victoria (H1N1) (r = −0.43, p = 0.03). However, to correct for multiple comparisons using the Bonferroni correction, with a new alpha value of 0.01, this was no longer significant.

Discussion

Understanding the quantitative markers of vaccine immunogenicity, as well as confounding patient demographic factors, will help to better define the correlates of protection against SARS-CoV-2 and improve the interpretability of future vaccine trials. In this study, by assessing humoral and cellular immunity to SARS-CoV-2, influenza virus, and endemic HCoVs in adolescents receiving BNT162b2, we identified several intriguing patterns that shed light on the immunogenicity of BNT162b2 in this age group. These include the role of prior SARS-CoV-2 infection in promoting a quicker and more neutralising vaccine response, the appearance of stronger humoral responses in adolescents than in adults, and a lack of sex difference following both BNT162b2 and the LAIV.

Due to the discrepancy between IgG and nAb responses in infection-naive adolescents, these data support the use of nAb titre as well as total IgG when assessing vaccine immunogenicity (38, 39). Other studies have established that BNT162b2 and CoronaVac inactivated virus vaccine elicit robust nAb responses post-Vx2 in infection-naive adolescents (8, 40). The totality of the data described herein suggests that a robust nAb response is prompted in infection-naive adolescents after two doses, but previously infected adolescents only require one dose. Previous studies in adults have differed in their evaluation of vaccine-induced versus infection-induced humoral immunity, but these data show that, at least in adolescents, a similar IgG response is elicited after natural infection and one vaccine dose compared to two vaccine doses alone (41). The longevity of these responses is uncertain due to the lack of an extended follow-up in this cohort but should be the focus of future studies.

Other research has shown that two doses of BNT162b2 elicit robust T_H1 T-cell responses in adults, with widespread interferon-gamma (IFNγ) production (26, 42). S-specific T-cell responses following vaccination with BNT162b2 were generated post-Vx2, but not post-Vx1 in another cohort of infection-naive adolescents (25). This contrasts with the data described herein, where one dose of BNT162b2 was sufficient to induce an increase in S-specific CD4+ T cells in infection-naive adolescents. Similar to the IgG response, SARS-CoV-2-specific T-cell responses post-Vx1 in previously infected individuals reached similar frequencies to post-Vx2 in infection-naive individuals. However, further studies on cellular immunity following BNT162b2 are required to supplement the small number of individuals studied here.

BNT162b2 has been shown to promote greater IgG production in adolescents compared to adults post-Vx1 (8, 25). Similarly, both infection-naive and previously infected adolescents generated stronger IgG responses than adults, although this only remained significant in previously infected adolescents when correcting for multiple comparisons. However, only previously infected adolescents generated a strong and broad nAb response targeting multiple variants, and infection-naïve adolescents appeared to generate more cross-reactive antibodies following their first dose of BNT162b2 compared to both infection-naïve adults and previously infected adolescents, as indicated by the higher HCoV: SARS-CoV-2 IgG ratios in infection-naïve adolescents. One interpretation for these patterns is immune imprinting, wherein prior exposure to circulating endemic coronaviruses negatively affects vaccine-induced immunity. Higher levels of cross-reactive IgG have been described in children than in adults (10, 43, 44), which may result in a stronger memory B-cell response, although with no significant boosting of nAb titres, following the first dose of BNT162b2 in infection-naive adolescents. In previously infected adolescents, exposure to SARS-CoV-2 may overcome immune imprinting and enable a robust nAb response. This is supported

by the strong negative correlation between the HCoV:SARS-CoV-2 IgG ratio and nAb response.

Immune responses to many adult and childhood vaccines, as well as responses to natural infection with viral pathogens, are consistently higher in females and associated with increased inflammation and autoimmunity as well as CD4+-skewed T-cell responses and greater B cell activation and IgG production (12, 13, 23, 34). Female IgG responses to influenza vaccines, such as the trivalent inactivated influenza vaccine, have been shown to be twice the magnitude of male IgG responses, and females also report more frequent SAEs to viral vaccines (13, 34). One exception to this trend is COVID-19 mRNA vaccines, for which vaccine-induced myocarditis is more frequent in young males (20, 21, 45). Lower peak anti-S IgG levels were identified in males following two doses of BNT162b2 (46). However, although geometric mean nAb titres to BNT162b2 were slightly higher in females following two doses of BNT162b2 in 12–15- and 16–25-year-olds, this difference was not significant in the US Food and Drug Administration open-label extension report for BNT162b2 (47). Notably, in this cohort, we observed increased post-Vx1 anti-S and anti-RBD IgG responses in infection-naive males compared to infection-naive females, in contrast to expectations based on other vaccines such as inactivated influenza vaccines (11, 13). However, this significance was lost when multiple comparisons were corrected. In addition, no sex differences in immune response have been reported for the adult cohort in previous studies (24, 26). We did not observe a significant sex difference in anti-HA IgG titres following LAIV, which is surprising in the context of established literature (12, 13) but may be obscured by the very small increase in anti-HA IgG post-Vx1 in this cohort, the effect of a live-attenuated rather than inactivated influenza vaccine, the use of different serological assays, or the result of co-administration with BNT162b2 (48). Furthermore, there was no sex difference in anti-SARS-CoV-2 IgG levels for either infected or previously infected adults from the PITCH dataset.

Finally, the correlation between B/Washington influenza IgG responses with age in 12–16-year-olds, as well as the lack of anti-B/Washington HA IgG boosting following LAIV, suggests recent exposure to the B/Washington strain of influenza in this cohort. Our findings that co-administration of BNT162b2 with the LAIV improves IgG response in previously-infected individuals is in contrast with findings for NV×-COV12373, where co-administration with inactivated quadrivalent influenza vaccines reduced SARS-CoV-2-specific IgG titres (37). However, studies of the co-administration of COVID-19 mRNA vaccines with quadrivalent influenza vaccines in adults have reported no reduction in antibody response compared to the administration of mRNA vaccines alone (49, 50). A potential explanation for the improved anti-S IgG responses following co-administration may be the increased innate immune activation due to intranasal LAIV administration, particularly in the nasal mucosa, leading to greater SARS-CoV-2-specific local T-helper cell activation. However, analysis with a greater number of adolescents receiving either BNT162b2 alone or BNT162b2 alongside the LAIV is required to make further conclusions on this hypothesis.

This study had several limitations. The small number of adolescents assayed in this cohort makes broad conclusions difficult, particularly when making comparisons between small subgroups such as co-administered LAIV/BNT162b2 and BNT162b2-alone individuals. No mucosal samples were collected, and mucosal immunity was not assessed in this cohort. Neutralisation responses to SARS-CoV-2 were estimated using an MSD-ACE2 inhibition assay. This has been shown to correlate with live virus assays (24, 26, 29), but live virus neutralisation is likely to be a more accurate measure of nAbs. In addition, no neutralisation assays were performed for influenza lineages, which would have shed further light on the functionality of humoral immunity against influenza. Owing to cell availability, T-cell proliferation assays were only performed on a limited number of individuals in the cohort, reducing confidence in the generalisability of the results. The adolescent cohort was compared with the PITCH cohort of adults. Although this cohort was also divided into infection-naïve and previously infected individuals and received the same vaccine, there are other potential confounders that limit this comparison, such as the sampling of adults 28 days post-vaccination rather than 35 days, as well as comorbidities that may be more prevalent in adults versus adolescents. Furthermore, the lack of an extended follow-up in this study makes assessments of immune durability impossible but should be the focus of future studies.

Another potential confounder for this study is that adolescents of this age group are likely to be at different stages of puberty and therefore have diverse levels of testosterone, oestrogen, and progesterone. Furthermore, males experience puberty at older ages than females; therefore, the sex difference identified herein may result from the confounding effects of puberty. If many male adolescents did not go through puberty at the time of sampling, the increased humoral responses to vaccination in males may result from the absence of immunosuppressive effects of androgens. To ensure that males in this cohort had entered puberty, steroid hormones, including testosterone, dihydrotestosterone (DHT), and progesterone, were measured by tandem mass spectrometry; these data are the focus of a future publication. All but the two youngest males (12 years, 2 months and 12 years, 10 months) demonstrated pubertal androgen levels. Testosterone levels correlated with age in males only ($r = 0.47$, $p = 0.05$). This promotes confidence in the results of the comparisons between sexes, as most males had undergone puberty at the time of sampling. In addition, in this study, the median age of males was approximately one year older than females, which may have affected the results of the study.

Taken together, these data paint a complex picture of vaccine-induced immunity in adolescents, with a potential role for sex and age differences in determining antibody responses to vaccination. These findings have important implications for paediatric vaccination regimens, such as the potential benefit of co-administration with influenza vaccines, and the necessity to consider sex and age when studying vaccine-induced immunity.

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Materials and methods

Ethics

This longitudinal cohort study was conducted between November 2021 and February 2022. Eligible participants were healthy adolescents aged 12–16 years who either had no history of SARS-CoV-2 infection or had experienced mild disease prior to enrolment. Eligible participants were identified by their participation in school-based vaccination. Written informed consent was obtained from all patients and ethical approval was obtained from the Central University Research Ethics Committee (reference: CUREC R71346/RE001). Healthy HCWs aged 32–52 years were recruited as part of the PITCH consortium of HCWs under the GI Biobank Study 16/YH/0247, approved by the Research Ethics Committee (REC) of Yorkshire and The Humber—Sheffield Research Ethics Committee on 29 July 2016, which was amended for this purpose on 8 June 2020.

Sample collection and processing

For the BNT162b2 vaccination (dose 1 (Vx1) and dose 2 (Vx2)), patients received 30 μg of the vaccine intramuscularly. LAIV was administered immediately after Vx1 only; patients received 0.1mL intranasally in both nostrils. Whole blood samples from all 34 individuals were collected immediately before Vx1 (sample pre-Vx1). Samples from all 34 individuals were taken at a mean of 37 days after Vx1 (33-39]) (sample post-Vx1), from 23 individuals 2 days before Vx2 (0–8) and 96 days after Vx1 (81-114) (sample pre-Vx2), and from 14 individuals 35 days after Vx2 (30–40) (sample post-Vx2). All whole blood samples were processed on the same day as collection, as described in the *Materials and methods* section. All serum samples were tested for anti-Spike (S) and anti-nucleocapsid (N) IgG and were classified as seropositive if their anti-N IgG titre was above the previously determined MSD immunoassay cut-off at any point in the study or if their anti-spike (S) IgG titre was above the cut-off pre-Vx1 (26). Only individuals who became infected were included in the seropositive group at the time of seropositivity. The percentage of seropositive patients increased from 52% (n = 18 of 34) to (10 of 14) over the course of the study.

Whole blood samples were transported from the collection site to an academic laboratory and were processed on the same day. PBMCs and plasma were isolated as previously described (31). Briefly, PBMCs were isolated using Lymphoprep (1.077 g/ml, Stem Cell Technologies) via density gradient centrifugation. Plasma and PBMCs were collected, and plasma was centrifuged at 2,000×*g* for 10 min to remove platelets. PBMCs were washed twice with RPMI 1640 (Sigma) containing 10% heat-inactivated foetal calf serum, 2 mM L-glutamine and 1 mM penicillin/streptomycin (Sigma). An estimated 10 million cells were resuspended in the media and counted using a Muse Cell Analyser (Luminex Corporation, USA). Plasma and PBMCs were frozen and stored at −80°C for later use. Steroid hormone concentrations were quantified using tandem mass spectrometry by collaborators at the Imperial College London.

MSD serological assays

IgG responses to SARS-CoV-2 S, N and RBD as well as the S proteins of HCoV-OC43, HCoV-NL63, HCoV-229E, HCoV-HKU1, SARS-CoV-1 and MERS-CoV were measured using a Meso Scale Diagnostics (MSD) V-plex immunoassay 'Coronavirus panel 3' (MSD, USA) according to the manufacturer's protocol. The plates were incubated in Blocker A solution for 30 min at room temperature (RT) with shaking at 700 rpm. Plasma or serum was diluted 1:1,000 and 1:10,000 in Diluent 100, and a seven-point standard curve of the MSD reference standard beginning at 1:10 was prepared in duplicate. Three internal controls and an in-house control of convalescent serum were used, with Diluent 100 used as a blank. Plates were washed three times with MSD Wash Buffer, and samples and standards were added to the plate before incubation at RT for 2 h with shaking at 700 rpm. The plates were washed three times with MSD Wash Buffer, and the detection antibody solution was added. The plates were then incubated for 1 h at RT with shaking at 700 rpm. Plates were washed three times with MSD Wash Buffer. Neat MSD Gold Read Buffer was added, and the plates were read immediately on a MESO QuickPlex SQ 120 (MSD, USA). Data were analysed using MSD Discovery Workbench software. Thresholds for seropositivity were taken from analyses of pre-pandemic sera, as published elsewhere (26), and were defined as 1,160 AU/ml for SARS-CoV-2 S, 1,169 for RBD, and 3,874 for N.

nAb titres were quantified using Meso Scale Diagnostics ACE2 inhibition assays, 'Panel 27,' (analytes: SARS-CoV-2 S, SARS-CoV-2 S (B.1.351), SARS-CoV-2 S (B.1.617.2; AY.4), SARS-CoV-2 S (BA.2), SARS-CoV-2 S (BA.2.12.1), SARS-CoV-2 S (BA.2+L452M), SARS-CoV-2 S (BA.2+L452R), SARS-CoV-2 S (BA.3), SARS-CoV-2 S (BA.4), SARS-CoV-2 S (BA.5)) according to the manufacturer's instructions. The plates were incubated in Blocker A solution for 30 min at RT with shaking at 700 rpm. Serum was diluted at 1:10 and 1:100, and a seven-point standard curve of MSD calibration reagent was prepared with 4-fold serial dilutions. Plates were washed three times with MSD Wash Buffer, and samples and calibrators were added to the plate. The plates were incubated at RT for 1 h, with shaking at 700 rpm. Sulfo-tagged ACE2 protein was added to the plate and incubated at RT for 1 h with shaking at 700 rpm. The plates were washed three times with MSD Wash Buffer and MSD Gold Read Buffer was added. The plates were read immediately on a MESO QuickPlex SQ 120 (MSD, USA). Data were analysed using MSD Discovery Workbench software. Results for VOCs were reported as % inhibition rather than pg/ml, as the standard included in the assay is specific for the Wuhan strain of SARS-CoV-2 only.

Influenza ELISA assay

IgG responses to influenza A/Victoria (H1N1), B/Washington (Victoria), A/Cambodia (H3N2), and B/Phuket (Yamagata) HA

antigens were measured using an indirect ELISA. HA antigens (The Native Antigen Company, Oxford) were diluted to 1 ug/ml in PBS and used to coat 535 Nunc-Immuno 96-well plates (Thermo Fisher Scientific, USA) overnight at 4°C (A/Victoria/2570/2019 (H1N1) pdm09-like virus (NCBI Accession Number: EPI1799581), amino acids 1–528 and C-terminal His-tag; Cambodia/e0826360/2020 (H3N2)-like virus (NCBI Accession Number: EPI1799580), amino acids 46–469 and C-terminal His-tag; B/Washington/02/2019 (B/Victoria lineage)-like virus (NCBI Accession Number: EPI1846769), amino acids 31–469 and C-terminal His-tag, B/Phuket/3073/2013 (B/Yamagata Lineage)-Like virus] (NCBI Accession Number: EPI1799823), amino acids 44–466 and C-terminal His-tag) Plates were washed three times in 0.1% PBS-Tween, before blocking with Casein-PBS Buffer for 1 h at RT. Plasma was diluted 1:200 in Casein-PBS Buffer and added to plates in duplicate. A ten-point standard curve of pooled highly reactive sera, beginning at 1:25, was prepared in duplicate and added to the plates. Casein-PBS Buffer was used as a negative control. The plates were incubated for 2 h at RT and washed six times with 0.1% PBS-Tween. The secondary antibody–goat anti-human IgG conjugated to alkaline phosphatase (Sigma, USA) was diluted 1:1,000 in Casein-PBS Buffer and added to the plates. The plates were incubated for 1 h at RT before washing six times with 0.1% PBS-Tween. 4-nitrophenyl phosphate in diethanolamine buffer (Pierce, Loughborough, UK) was added as a substrate and the plates were incubated for 15 min. The absorbance (405 nm) was measured using an ELx800 microplate reader (Cole Parmer, London, UK).

Proliferation assay

T-cell responses were assayed using the CellTrace Violet Proliferation assay, as described elsewhere (31). Not all individuals were included in this assay because of cell availability: pre-Vx1, n = 16; post-Vx1, n = 15; pre-Vx2, n = 12; post-Vx2, n = 11. Cryopreserved PBMCs were thawed in 30 mL RPMI containing 10% human AB serum (Sigma), 2 mM L-Glut and 1 mM Pen-Strep. Cells were washed twice with PBS and stained with CellTrace Violet (Life Technologies) at 2.5 uM for 10 min at RT. Cold FCS was added to quench the reaction. Cells were plated at 250,000 cells per well in a 96-well round-bottom plate. Peptide pools covering SARS-CoV-2 S1, S2, M, and N, as well as HCoV-OC43 and HCoV-HKU1 S, were added to stimulate cells at a final concentration of 1 ug/ml (Mimotopes, USA) (Supplementary Table 1). Media containing 0.1% DMSO (Sigma) was used as a negative control. Phytohaemagglutinin L (Sigma) was used as the positive control at a final concentration of 2 ug/ml. Plates were incubated at 37°C, 5% CO2, and 95% humidity for 7 days, with a hemimedia change on day 4. On day 7, the cells were washed in PBS and stained with fluorochrome-conjugated antibodies against CD4, CD8, and CD3 in PBS. LIVE/DEAD Fixable Aqua was used as a viability marker (Thermo Fisher Scientific). Cells were fixed in 4% paraformaldehyde (Sigma) for 10 min at 4°C and washed in PBS before being stored at 4°C in the dark before being run on MACSquant X (Miltenyi). The gating strategy is illustrated in Supplementary Figure 6.

Statistical analysis

All analyses were performed using the GraphPad Prism 9.0. For pairwise comparisons, two-tailed Mann–Whitney tests were used for unpaired data and Wilcoxon signed-rank tests for paired data. Spearman rank tests were used for correlations.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by University of Oxford Central University Research Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

CJ: Conceptualisation, Formal analysis, Investigation, Writing—Original Draft Preparation, Review & Editing, Visualisation. EA: Investigation, Data Curation, Writing—Review & Editing, Project administration. AC: Investigation, Data Curation, Writing—Review & Editing, Project administration. NL: Investigation, Writing—Review & Editing. SL: Formal analysis, Investigation, Writing—Review & Editing. AO: Investigation, Writing—Review & Editing. JR: Writing—Review & Editing. OS: Investigation, Writing—Review & Editing. PITCH Consortium: Investigation, Formal analysis, Data Curation. CT: Resources, Writing—Review & Editing, Methodology. EB: Conceptualisation, Writing—Review & Editing. SD: Supervision, Writing—Review & Editing. LT: Supervision. PK: Supervision, Writing—Review & Editing, Conceptualisation, Methodology. MC: Supervision, Writing—Review & Editing, Methodology. PG: Conceptualisation, Methodology, Resources, Writing—Review & Editing, Supervision, Project administration, Funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1248630/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Fold change in nAb and IgG titre in adolescents post-Vx1 and post-Vx2. Fold change in nAbs targeting S **(A)** and RBD **(B)** in infection-naive adolescents (grey circles), and previously-infected adolescents (red circles) post-Vx1 and post-Vx2 as measured by an MSD ACE2-S binding immunoassay. Fold change in IgG targeting S **(D)** and RBD **(D)** in infection-naive and previously-infected adolescents as measured by an MSD v-plex immunoassay. P-values represent Mann-Whitney test values.

SUPPLEMENTARY FIGURE 2
nAb responses to SARS-CoV-2 variants. Percent inhibition of ACE2-S binding for common variants in infection-naïve (grey) and previously-infected (red) adolescents. P-values from Wilcoxon tests.

SUPPLEMENTARY FIGURE 3
Cellular responses to endemic HCoVs. Proliferating CD4+ and CD8+ T-cells targeting HCoV-OC43 S2 **(A)** and HCoV-HKU1 S2 **(B)** in infection-naïve (grey) individuals. % proliferating CD4+ and CD8+ T-cells targeting HCoV-OC43 S2 **(C)** and HCoV-HKU1 S2 **(D)** in previously-infected individuals (red). Values below 1% were given nominal values of 0.9%.

SUPPLEMENTARY FIGURE 4
Cellular responses to M and N antigens. Proliferating CD4+ and CD8+ T-cells targeting M **(A)** and N **(B)** in infection-naïve (grey) and previously-infected (red) adolescents. Values below 1% were given nominal values of 0.9%.

SUPPLEMENTARY FIGURE 5
IgG responses to endemic HCoVs. IgG targeting SARS-CoV-1, MERS-CoV, HCoV-HKU1, HCoV-OC43, HCoV-NL63 and HCoV-229E in infection-naïve (grey) and previously-infected (red) individuals **(A)**. Correlation between HKU1 **(C)** and OC43 **(D)** to SARS-CoV-2 IgG ratio and nAb response in infection-naïve (grey) and previously-infected (red) individuals.

SUPPLEMENTARY FIGURE 6
Gating strategy for T-cell proliferation assay. Gates were drawn on lymphocytes (SSC-A × FSC-A), single cells (FSC-H × FSC-A), live cells (CD3 × Live/Dead stain), and CD4+ and CD8+ cells (CD4 × CD8). Proliferating CD4+ cells were gated as compared to a negative control (CD4 × CTV). Proliferating CD8+ cells were gated as compared to a negative control (CD8 × CTV).

SUPPLEMENTARY TABLE 1
List of peptides used for T-cell assays.

SUPPLEMENTARY TABLE 2
Full author list for PITCH Consortium.

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Distinct anti-NP, anti-RBD and anti-Spike antibody profiles discriminate death from survival in COVID-19

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Introduction: Infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) induces rapid production of IgM, IgA, and IgG antibodies directed to multiple viral antigens that may have impact diverse clinical outcomes.

Methods: We evaluated IgM, IgA, and IgG antibodies directed to the nucleocapsid (NP), IgA and IgG to the Spike protein and to the receptor-binding domain (RBD), and the presence of neutralizing antibodies (nAb), in a cohort of unvaccinated SARS-CoV-2 infected individuals, in the first 30 days of post-symptom onset (PSO) (T1).

Results: This study included 193 coronavirus disease 2019 (COVID-19) participants classified as mild, moderate, severe, critical, and fatal and 27 uninfected controls. In T1, we identified differential antibody profiles associated with distinct clinical presentation. The mild group presented lower levels of anti-NP IgG, and IgA (vs moderate and severe), anti-NP IgM (vs severe, critical and fatal), anti-Spike IgA (vs severe and fatal), and anti-RBD IgG (vs severe). The moderate group presented higher levels of anti-RBD IgA, comparing with severe group. The severe group presented higher levels of anti-NP IgA (vs mild and fatal) and anti-RBD IgG (vs mild and moderate). The fatal group presented higher levels of anti-NP IgM and anti-Spike IgA (vs mild), but lower levels of anti-NP IgA (vs severe). The levels of nAb was lower just in mild group compared to severe, critical, and fatal groups, moreover, no difference was observed among the more severe groups. In addition, we studied 82 convalescent individuals, between 31 days to 6 months (T2) or more than 6 months (T3), PSO, those: 12 mild, 26 moderate, and 46 severe plus critical. The longitudinal analyzes, for the severe plus critical group showed lower levels of anti-NP IgG, IgA and IgM, anti-Spike IgA in relation T3. The follow-up in the fatal group, reveals that the levels of anti-spike IgG increased, while anti-NP IgM levels was decreased along the time in severe/critical and fatal as well as anti-NP IgG and IgA in several/critical groups.

Discussion: In summary, the anti-NP IgA and IgG lower levels and the higher levels of anti-RBD and anti-Spike IgA in fatal compared to survival group of individuals admitted to the intensive care unit (ICU). Collectively, our data discriminate death from survival, suggesting that anti-RBD IgA and anti-Spike IgA may play some deleterious effect, in contrast with the potentially protective effect of anti-NP IgA and IgG in the survival group.

KEYWORDS

COVID-19, SARS-CoV-2, antibodies, receptor binding domain (RBD), spike protein (S), nucleocapsid protein (N), clinical severity

Introduction

The new coronavirus SARS-CoV-2, the etiological agent of COVID-19, is one of the main pathogens that especially targets the human respiratory system (1). COVID-19 has become a public health problem due to high rates of morbidity and mortality, causing millions of deaths and a long-term health burden (2).

The SARS-CoV-2 particle has four structural proteins: spike (S), envelope (E), membrane glycoprotein (M), and nucleocapsid protein (N). To exert its pathogenic mechanism, SARS-CoV-2 binds to host cells through a trimeric glycoprotein that recognizes the angiotensin converting enzyme 2 (ACE2), the S protein, which is cleaved into two domains S1 and S2 (3). The S1 domain contains the receptor binding domain (RBD), which is essential for viral binding to receptor human ACE2 (hACE2) and the establishment of cellular infection (4–6), considered a target for neutralizing antibodies (nAbs). Antibodies that bind to the spike protein, specifically to the RBD and N-terminal domains, inhibit the binding of viruses to cells by neutralizing viral particles (7).

Different profiles of anti-SARS-CoV-2 production and antibody levels and dynamics have been associated with distinct mild or severe clinical outcomes over time (8, 9). However, the underlying mechanisms contributing to better or worse outcomes are still being studied.

Generally, in the early stages of SARS-CoV-2 infection, IgM is the main antibody, IgA- and IgG-mediated protection prevents pathogens from binding and invading the host cells, and IgG is the antibody that has a longer duration in the blood (10, 11). It has been suggested that high levels of IgM and IgG antibodies against the S1 and N proteins, in the first 15 days post-symptom onset (PSO), is considered a risk factor for a more severe clinical outcomes, since these antibodies were detected at higher levels in COVID-19

patients admitted to the intensive care unit (ICU) and in those who died (12–14). High titers of anti-Spike IgM have been reported around 10 to 12 days after symptoms began, with a significant reduction after the 18th day (15). Anti-spike IgA antibodies show induction in the first week of infection and peaking levels around day 20, concomitantly with an increased number of IgA-anti-SARS-CoV-2 secreting plasmablasts (16). There are still several contradictory and unknown issues regarding the levels of anti-SARS-Cov-2 antibodies and the severity of clinical outcomes.

Neutralizing antibodies have mostly been observed to persist up to 180 days after the onset of symptoms (17) and play a critical role in blocking viral entrance into cells. The neutralizing capacity of anti-SARS-CoV-2 antibodies has been reported to be predominantly mediated by IgA, early in infection, and they are seven times more effective than IgG (16). Also, IgM, IgG1 and IgA1 showed neutralizing activity against Spike and RBD proteins early after infection (18). Longitudinal analysis of antibody dynamics in COVID-19 convalescents revealed neutralizing responses up to 16 months after infection (19). In addition, anti-RBD and anti-spike IgG antibodies in hospitalized COVID-19 patients have also been shown to display important participation in complement deposition but a lower capacity in phagocytosis promotion, in comparison to non-hospitalized individuals (20).

In this work, we investigated the IgM, IgA and IgG antibody profiles directed to SARS-CoV-2 antigens, as well as antibody neutralizing capacity, in COVID-19 individuals with different disease outcomes, aiming to determine whether specific profiles were associated with COVID-19 severity or recovery, suggesting potential beneficial versus deleterious antibody functions in COVID-19. We found that higher levels of anti-RBD and anti-Spike IgA distinguished fatal from survival in individuals admitted to the ICU, suggesting that these antibodies may play some deleterious effect in the long run, in contrast with the potentially protective effect of anti-NP IgA and IgG that were higher in survival individuals.

Materials and methods

Ethics approval and consent to participate

Participants, family members, or legal guardians have provided oral recorded informed consent, in accordance with the regulations of the Human Ethical Committee from Hospital das Clínicas, Faculdade de Medicina of Universidade Federal de Goiás (UFG), Goiânia, Goiás, Brazil, and the research protocol was approved by Ethical Appreciation (CAAE: 30804220.2.0000.5078). The sample size was determined by the convenience of sampling, availability at partner hospitals, agreement to participate, and pandemic conditions within the local community.

Study cohort

Sample collection was conducted from June 2020 to June 2021. COVID-19 positive individuals (n = 193), with positive diagnosis of SARS-CoV-2 infection by real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) from nasopharyngeal swabs and/or rapid test assays to detect IgM/IgG (Eco Diagnostics) were enrolled in the study, all before the initial vaccination. Blood samples were obtained in the first 30 days PSO for all positive individuals and a follow-up was conducted in a subset of individuals (n=82) with sample collection up to T1: up to 30 days PSO, T2: 1–6 months PSO, and T3: more than 6 months PSO. The samples were collected at COVID-19 wards and the Intensive Care Unit (ICU) at Hospital das Clínicas, Universidade Federal de Goiás, Goiânia. Brazil. For individuals who were not hospitalized and recovered, blood samples were collected at Laboratório Profª Margarida Dobler Komma, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, Brazil. Additionally, 27 individuals, negative for SARS-CoV-2 by RT-qPCR in nasopharyngeal swabs and pre-vaccination, were enrolled as controls. SARS-CoV-2 positive participants were categorized according to National Institute of Health (NIH), USA, classification for COVID-19 (21, 22) as: mild disease (individuals who had any of the various signs/symptoms but did not have shortness of breath, dyspnea, or abnormal chest imaging, can be managed in an ambulatory or at home), moderate disease (radiologically confirmed pneumonitis, hospitalization and oxygen therapy), severe disease (dyspnea, respiratory frequency ≥30 breaths/min, oxygen saturation [SpO2] ≤93%, and/or lung infiltrates >50% within 24 - 48 hours), and critical disease (treatment in ICU, or complications by other organ failure and/or mechanical ventilation). The fatal group included all participants who required ICU and died. Peripheral blood samples from all participants were collected, and serum was separated and stored at −80°C.

SARS-CoV-2 RNA extraction and RT-qPCR

Ribonucleic acid (RNA) extraction was performed using the commercial QIAamp® Viral RNA Mini Kit (Qiagen, Germany), following the manufacturer's protocol. After RNA extraction, samples were submitted to real-time polymerase chain reaction post reverse transcription (RT-qPCR) using the Promega GoTaq® Probe 1-Step RT-qPCR System, according to the manufacturer's protocol (23). Primers and probes targeted two regions of the N gene (N1 and N2) from SARS-CoV-2 and the human RNAse P (RP) gene, and IDT (Integrated DNA Technologies, Iowa, USA). All samples that presented a cycle threshold (Ct) lower than 40 (for N1, N2, and RP targets) were positive for SARS-CoV-2 RNA. Viral loads in genomic copies (GC) per mL/g of clinical specimens were estimated based on a standard curve of serial dilutions (10^6 to 10^0 GC/μL) of the synthetic positive control nCoVPC (severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome, GenBank: NC_045512.2) from Integrated DNA Technologies (24).

Neutralizing antibody assay

A cytopathic effect-based virus neutralization test (CPE-VNT) was performed using 96-well plates, as previously described by Botosso (25). Briefly, serum samples were initially inactivated for 30

minutes at 56°C and subsequently diluted in DMEM containing 2.5% fetal bovine serum from 1/20 to 1/2560. The sera were then mixed vol/vol with 100 tissue culture infectious doses, 50% endpoint (TCID50) of the virus (SARS-CoV-2 wild-type variant B.1.1.28 - MT126808) and pre-incubated at 37° C for 1 h for neutralization. The serum/virus mixture was transferred onto the confluent VERO ATCC CCL-81.4 cell monolayer and incubated for 3 days at 37°C with 5% CO2. After incubation, the plates were analyzed by light microscopy for the presence or absence of SARS-CoV-2 CPE. For confirmation, plates were fixed and stained with amido black (0.1% amido black solution [w/w] with 5.4% acetic acid, 0.7% sodium acetate) for 30 minutes and analyzed to determine the titer. nAb titer (VTN100) is considered the highest serum dilution neutralizing virus growth. Internal positive and negative controls were added to each run. All CPE-VNT procedures were performed in a Biosafety Level 3 at the Institute of Biomedical Science, University of São Paulo, laboratory following the World Health Organization recommendations. nAb titers were transformed in logarithm (log) for normalization.

ELISA to detect SARS-CoV-2 antibodies

Enzyme-linked immunosorbent assay (ELISA) was performed using 96-well high-binding polystyrene COSTAR microplates (Corning, NY, USA, #3590) coated overnight at 4°C with 2.0 µg/mL recombinant Spike protein (26), 1.0 µg/mL NP (27) or 1.5 µg/mL RBD expressed according to Amanat et al. (9) diluted in 0.1 M sodium carbonate-sodium bicarbonate buffer, pH 9.6. Briefly, unbound proteins were removed, followed by blocking with 1% albumin bovine serum (BSA, Sigma) and 5% nonfat dry milk in phosphate buffered saline containing 0.02% Tween 20 (PBST) for 2 or 3 hours at 37°C. After washing three times with PBST, plates were incubated for 45 min at 37°C with 50 µL heat inactivated serum samples (56°C for 30 minutes) diluted to 1:50 for IgA and 1:100 for IgG, in PBST with 0.25% BSA and 5% nonfat dry milk. Each sample was assayed in duplicate. After another series of washing, the plates were incubated for 30 min at 37°C with peroxidase-conjugated goat anti-human IgA (Sigma A0295, 1:2500), IgG (Sigma A0170, 1:4000), or IgM (Sigma A6907, 1:3000) secondary antibodies. After washing, 50 µL of 3,3',5,5'-Tetramethylbenzidine (Life Technologies, Cat. no. 002023) were added to each well and incubated for 10 minutes at room temperature. The reaction was stopped by adding 25µL of 2 N of sulfuric acid. Optical density (OD) was measured at 450 nm using a microplate reader (Labsystems Multiskan, Thermo Scientific, USA). Values were determined as OD minus blank, and the cutoff (CO) was determined as the average OD of samples pre-pandemics or negative ± 2× standard deviation. Each plate we included positive serum for control obtained by SARS-CoV-2 confirmed by RT-PCR. The results were normalized across experiments and transformed as the ratio of sample/cutoff (S/CO). The frequency distribution of antibody detection was calculated as positive when S/CO was higher than or equal to 1.2, and negative detection when S/CO was less than 1.2 (28).

Statistical analysis

All analyses were conducted using GraphPad Prism version 9 for Windows (GraphPad Software, La Jolla California USA). Receiver-operating characteristic (ROC) analyses were performed using MetaboAnalyst. For the correlograms, based on the Spearman correlation, were generated with the package *corrplot* for R studio software (version)". The distribution patterns of the variables were checked using the Kolmogorov–Smirnov and Shapiro–Wilk tests. For frequency calculations, we used the Fisher exact test. For comparisons between paired groups, we used the non-parametric Wilcoxon Matched–Pairs signed-rank test. Unpaired groups were analyzed using the non-parametric Mann–Whitney U test. Multiple group comparisons were analyzed by running a non-parametric Kruskal–Wallis statistical test and were corrected using Dunn's and Dunnett's methods. Spearman correlation coefficients and nonlinear regression analysis were used to assess significance. For all tests, a p value < 0.05 was considered significant.

Results

Clinical and demographic characteristics of COVID-19 individuals and controls

To determine the profile of the SARS-CoV-2 specific humoral immune response, we recruited 27 healthy individuals (control group: negative for SARS-CoV-2 RT-qPCR from nasopharyngeal swabs) and 193 individuals with COVID-19 between June 2020 and February 2021. Part of the samples of cohort was sequenced and the predominant circulating strain was classified as B.1.1.33 lineage of SARS-CoV-2 in that period, as described in our other work (23). All participants were unvaccinated to COVID-19. For our initial analysis, the cohort was first stratified based on disease severity. SARS-CoV-2 positive participants were categorized as: mild (n = 37, from these 26 were not hospitalized participants), moderate (n = 43), severe (n = 63), critical (n = 14), death (n = 36) and recovered (n = 84), according to the NIH classification for COVID-19 (21, 22). It is worth to mention that some patients with mild disease were hospitalized due to decompensation of the underlying disease.

Among all COVID-19 participants, the median age was 58 years (interquartile range [IQR] = 45-71) and was not different from the control group (median=56 years, IQR=50-60). In the COVID-19 group, the median age for mild disease was 33 years, 55 years for moderate disease, 58 years for severe disease, 48 years for critical disease, and 63 years for fatal individuals (Table 1).

The most common comorbidities in COVID-19 participants were hypertension (n = 64, 33.2%), diabetes mellitus (n = 46, 23.8%), and obesity (n = 31, 16.1%). The most common symptoms were cough (n = 102, 52.8%), dyspnea (n=91, 47.1%), fever (n = 81, 42.0%), myalgia (n = 57, 29.5%), headache (n = 49, 25.4%), asthenia (n = 45, 23.3%), diarrhea (n = 29, 15.0%), anosmia (n = 18, 9.3%), and chest pain (n = 15, 7.8%) (Table 1). In our cohort, 86.5% (n=167) were hospitalized for

TABLE 1 Associations between clinical and demographic data of participants and hospital care and interventions (n=220).

Baseline Variable	Control N=27	All patients N=193	Mild N=37	Moderate N=43	Severe N=63	Critical N=14	Fatal N=36	*p* value
Demographic characteristics								
Age, median (IQR)	56 (50-60)	58 (45-71)	33 (25-46)	55 (44-74)	58 (46-71)	48 (34-63)	63 (56-71)	a,f,g,i
Sex, no. (%)								
Male	9 (33)	95 (49)	12 (32)	22 (51)	36 (57)	4 (29)	21 (58)	g,i
Female	18 (67)	98 (51)	25 (68)	21 (49)	27 (43)	10 (71)	15 (42)	
Days post symptom onset collection, median (IQR)	–	14 (10-18)	9 (6-11)	14 (11-18)	14 (12-18)	15 (10-23)	17 (15-20)	f,g,i,h
Comorbidities and risk factors, no. (%)								
Hypertension	7 (29.2)	64 (33.2)	5 (13.5)	15 (34.9)	24 (38.1)	5 (35.7)	15 (42.0)	f,g,i
Diabetes mellitus	1 (3.7)	46 (23.8)	3 (8.1)	13 (30.2)	16 (25.4)	4 (29.5)	10 (28.0)	b,c,d,e,f,g,i
Obesity	6 (22.2)	31 (16.1)	–	7 (16.3)	15 (23.8)	4 (29.5)	5 (14.0)	ns
Heart disease	3 (11.1)	18 (9.3)	2 (5.4)	10 (23.3)	16 (25.4)	-	7 (19.4)	f,g
Pregnancy	–	28 (28.6)	9 (36.0)	6 (29.0)	7 (23.0)	2 (2.0)	4 (27.0)	ns
Symptoms, no. (%)								
Cough	-	102 (52.8)	15 (40.5)	27 (63.0)	43 (68.3)	8 (57.1)	24 (66.7)	g,i
Dyspnea	-	91 (47.1)	12 (32.4)	19 (44.2)	43 (68.3)	8 (57.1)	27 (75.0)	g,i,j,l
Fever	-	81 (42.0)	13 (35.1)	23 (53.5)	29 (46.0)	8 (57.1)	28 (77.8)	i,l,n
Myalgia/Arthralgia	-	57 (29.5)	11 (29.7)	13 (30.2)	28 (44.4)	2 (14.3)	12 (33.3)	ns
Headache	-	49 (25.4)	11 (29.7)	14 (32.5)	19 (30.2)	2 (14.3)	11 (30.6)	ns
Asthenia	-	45 (23.3)	8 (21.6)	9 (20.9)	19 (30.2)	4 (28.5)	14 (38.9)	ns
Diarrhea	-	29 (15.0)	8 (21.6)	2 (4.6)	17 (27.0)	–	3 (8.3)	f,j,n
Anosmia	-	18 (9.3)	6 (16.2)	6 (14.0)	4 (6.4)	1 (7.1)	1 (2.8)	ns
Chest pain	-	15 (7.8)	2 (5.4)	1 (2.3)	9 (14.3)	–	4 (11.1)	j
Hospital support, no. (%)								
Infirmary	-	94 (48.7)	11 (29.7)	43 (100)	40 (63.5)	–	-	f, j
ICU	-	73 (37.8)	-	-	23 (36.5)	14 (100.0)	36 (100.0)	m,n

(Continued)

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TABLE 1 Continued

Baseline Variable	Control N=27	All patients N=193	Mild N=37	Moderate N=43	Severe N=63	Critical N=14	Fatal N=36	*p* value
Respiratory support received, no. (%)								
Nasal Catheter	-	100 (51.8)	11 (29.7)	42 (97.7)	47 (74.6)	-	-	j
Non-rebreathing mask	-	22 (11.4)	–	1 (2.3)	16 (25.4)	–	5 (14.0)	j
Invasive mechanical ventilation	-	45 (23.3)	-	-		14 (100.0)	31 (86.0)	ns

no., number, or values; ICU, Intensive Care Unit; s, second; IQR, Interquartile range; ns, not significant. Comparison of the control (healthy participants) with all patients. Categorical variables represented as number (percentage) and compared using Fisher's exact test. Continuous variables represented as median (interquartile range) and compared using one-way analysis of variance (ANOVA) and Kruskal-Wallis test. *p<0.05. Significance comparing control versus mild = a, control versus moderate = b, control versus severe = c, control versus critical = d, control versus fatal = e; mild versus moderate = f, mild versus severe = g, mild versus critical = h, mild versus fatal = i; moderate versus severe = j, moderate versus critical = k, moderate versus fatal = l; severe versus critical = m, severe versus fatal = n; critical versus fatal = o.

COVID-19; 37.8% (n=73) required intensive care unit (ICU) and only 13.5% (n=26) were not hospitalized. Of the 73 patients in the ICU, 45 (23.3%) required mechanical ventilation for cardiovascular stabilization (Table 1).

The hematological and biochemistry parameters are presented in Table 2. The data showed marked lymphopenia in the severe, critical, and fatal groups compared to control group and in the fatal group compared to mild and control groups ($p<0.05$) and a neutrophilia in critical and fatal groups compared to controls, and to the fatal group compared to the mild, moderate and severe groups and in critical compared to severe groups ($p<0.05$).

Levels of anti-SARS-CoV-2 antibodies in the first month post-infection are associated with distinct COVID-19 outcomes

First, we assessed the levels and frequency of seropositivity to SARS-CoV-2-specific antibodies during the first 30 days PSO. Moderate, severe, critical and fatal groups of COVID-19 participants showed higher levels of antibody anti-SARS-CoV-2 proteins in comparison to controls (Figure 1), except for anti-NP IgM (control *vs.* moderate, p=0.1236) (Figure 1C).

The serum levels of anti-NP IgG antibodies were higher in the moderate (p=0.0002) and severe (p<0.0001) groups than in the mild group (Figure 1A) and higher frequency of seropositivity (95% for moderate, 96% for severe) (Supplementary Figure 1A).

Anti-NP IgA levels were higher in the severe (p<0.0001) and moderate (p=0.0008) groups than in the mild group, while the fatal group showed lower levels (p=0.0038) than the severe group (Figure 1B), with frequency of seropositivity of 100% for severe and critical, 93% for moderate and 86% for fatal group (Supplementary Figure 1B). Anti-NP IgM was higher in the critical (p=0.0406) and fatal (p=0.0301) groups compared to the mild group (Figure 1C), with frequency of seropositivity 64% for critical and 73% for fatal group (Supplementary Figure 1C).

However, regarding the anti-spike specific antibodies, IgG levels showed no significant differences among the COVID-19 groups (p>0.05) (Figure 1D) but the frequency of seropositivity was higher in the severe (96%) and critical (100%) groups than in the mild (75%) group (Supplementary Figure 1D). Anti-spike IgA levels were higher in the fatal (p<0.0001) and severe groups (p=0.0184) than in the mild group (Figure 1E) and higher frequency of seropositivity (85% for fatal, 96% for severe) (Supplementary Figure 1E).

The levels of anti-RBD IgG were higher in the severe group than in the mild (p<0.0001) and moderate (p=0.0263) groups (Figure 1F) with of 95% frequency of seropositive in severe group (Supplementary Figure 1F). In contrast, the severe group presented lower anti-RBD IgA levels than the moderate group (p=0.0342), and the fatal group presented higher anti-RBD IgA levels than the mild (p=0.0347) and severe groups (p=0.0004) (Figure 1G), with of 91% frequency of seropositive in the moderate and 95% for fatal group (Supplementary Figure 1G). Considering the antibodies detected to the three proteins, all participants produced at least one antibody type to at least one

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TABLE 2 Blood biochemical and hematological parameters of participants in the study (n=220).

Baseline Variable median (IQR)	Control N=27	All patients N=193	Mild N=37	Moderate N=43	Severe N=63	Critical N=14	Fatal N=36	*p* value
Blood cell, median (IQR)								
Erythrocytes (10^6/μL)	4.8 (4.5-5.0)	4.2 (3.4-4.8)	4.5 (3.7-5.2)	4.2 (3.7-4.7)	4.4 (4.0-4.9)	3.8 (3.4-4.1)	3.1 (2.8-4.3)	d,e,i,l,n
Hemoglobin (g/dL)	14.3 (13.5-14.8)	12.5 (10.5-14.2)	13.4 (11.7-15.2)	12.5 (11.0-14.3)	13.1 (11.9-14.6)	10.9 (10.2-12.7)	9.3 (8.3-12.7)	d,e,l,n
Leucocyte counts (μL)	6.6 (5.4-7.5)	8.5 (6.0-12.1)	7.3 (5.2-8.6)	8.0 (6.1-11.9)	7.5 (4.8-10.3)	12.7 (8.3-17.8)	12.1 (8.5-16.1)	d,e,m,n
Neutrophil counts (μL)	3.9 (2.9-4.6)	6.1 (3.7-9.2)	3.7 (2.4-6.4)	5.5 (3.,7-8.0)	5.5 (3.4-8.1)	9.8 (5.8-14.4)	9.3 (6.7-12.8)	d,e,i,m,l,n
Lymphocyte counts (μL)	2.2 (1.6-2.8)	1.3 (0.8-1.9)	1.9 (1.5-2.2)	1.7 (0.9-2.2)	1.3 (0.8-1.7)	1.3 (0.7-1.8)	0.7 (0.5-1.4)	c,d,e,i,l
Monocyte counts (μL)	339.0 (189.0-553.5)	413.0 (272.8-703.0)	288.0 (226.5-438.5)	446.0 (281.3-729.0)	399.0 (284.5-618.8)	633.5 (235.5-954.8)	504.0 (281.0-762.0)	ns
Platelet counts (μL)	231.5 (195.3-285.8)	227.0 (177.1-291.4)	213.8 (152.5-253.7)	227.8 (180.5-285.4)	263.1 (195.0-327.1)	247.6 (188.1-301.5)	175.8 (102.7-233.2)	n
Blood biochemistry, median (IQR)								
D-dimer (mg/L)	-	398.0 (72.4-849.0)	328.0 (149.0-503.8)	369.0 (72.2-653.0)	308.5 (757.3-2783.0)	1107.0 (54.9-4526.0)	571.0 (266.3-1142.0)	ns
C-reactive protein (mg/dL)	0.2 (0.1-0.6)	7.0 (1.7-19.6)	1.7 (0.7-4.4)	7.4 (1.8-20.9)	5.6 (0.9-10.9)	9.3 (5.8-34.3)	11.8 (7.3-25.6)	a,b,c,d,e,m
Ferritin (ng/mL)	150.0 (94.1-208.0)	671.0 (285.9-1291)	159.2 (42.8-454.4)	522.6 (303.6-1302.0)	522.9 (205.6-831.6)	901.1 (272.1-1474.0)	1180.0 (711.5-2794)	b,c,d,e,f,i,n
Albumin (mg/dL)	4.3 (4.2-4.4)	3.0 (2.7-3.4)	3.2 (2.8-3.8)	3.3 (3.0-3.7)	3.2 (2.9-3.5)	2.6 (2.4-2.8)	2.6 (2.3-3.1)	h,k,l,m,n
Total bilirubin (mg/dL)	0.6 (0.4-0.8)	0.5 (0.3-0.7)	0.3 (0.3-0.5)	0.5 (0.3-0.6)	0.5 (0.3-0.6)	0.4 (0.3-0.7)	0.5 (0.3-0.8)	a,b,c,d,e,k,l,m,n
Alanine aminotransferase (UI/L)	18 (14-26)	41 (23-65)	15 (10-52)	33 (17-70)	41 (24-62)	47 (29-89)	47 (23-70)	b,c,d,e
Aspartate aminotransferase (UI/L)	22 (19-27)	36 (23-54)	18 (13-30)	32 (20-47)	34 (22-47)	49 (26-98)	49 (33-70)	d,e,i
Creatinine (mg/dL)	0.8 (0.7-1.0)	0.9 (0.7-1.3)	0.6 (0.5-0.7)	0.9 (0.8-1.1)	0.9 (0.7-1.2)	0.8 (0.7-1.5)	1.4 (1.0-2.8)	e,f,g,i,l,n

IQR, Interquartile range; ns, not significant. Comparison of the control (healthy participants) with all patients. Categorical variables represented as number (percentage) and compared using Fisher's exact test. Continuous variables represented as median (interquartile range) and compared using one-way analysis of variance (ANOVA) and Kruskal-Wallis test. *p<0.05. Significance comparing control versus mild = a, control versus moderate = b, control versus severe = c, control versus critical = d, control versus fatal = e; mild versus moderate = f, mild versus severe = g, mild versus critical = h, mild versus fatal = i; moderate versus severe = j, moderate versus critical = k, moderate versus fatal = l; severe versus critical = m, severe versus fatal = n; critical versus fatal = o.

SARS-CoV-2 antigen, except for one participant in the mild group (data not shown).

In order to investigate whether there was a correlation between the SARS-CoV-2 viral copy and antibody isotypes and disease severity, we performed a correlation analysis between Ct values and antibody levels. We considered a correlation when the r is higher than 0.3. The levels of anti-RBD IgG from all COVID-19 patients were positively correlated with SARS-CoV-2 Ct values, which means lower viral load (r=0.3766 p=0.0004) (Supplementary Figure 2A). Nonetheless there was no correlation between Ct value and anti-RBD IgA (Supplementary Figure 2A), anti-Spike IgG and IgA (Supplementary Figure 2B) and anti-NP IgG, IgM, and IgA (r < 0.3) levels (Supplementary Figure 2C). Interestingly, regarding severity, in the mild group, it was observed positive correlation between anti-Spike IgG levels and SARS-CoV-2 Ct values (r=0.4368, p=0.0342) (Supplementary Figure 3A), although there was no correlation with anti-Spike IgA, and anti-NP IgG, IgA and IgM, anti-RBD IgG and IgA levels with SARS-CoV-2 Ct values (Supplementary Figures 3A–C). Moreover, it was observed positive correlation between anti-RBD IgG levels and SARS-CoV-2 Ct value, in the moderate group (r=0.4620, p=0.0265) (Supplementary Figure 4A), even though there was no correlation with anti-RBD IgA, anti-NP IgG, IgA and IgM and anti-spike IgG and IgA levels (Supplementary Figures 4A–C). Moreover, there was a positive correlation between SARS-CoV-2 Ct value and anti-NP IgM and anti-RBD IgG levels in the severe plus critical groups (r=0.5189 p=0.0039) (Supplementary Figures 5A, B), but not with anti-NP IgG and IgA, anti-RBD IgA and anti-Spike IgG and IgA

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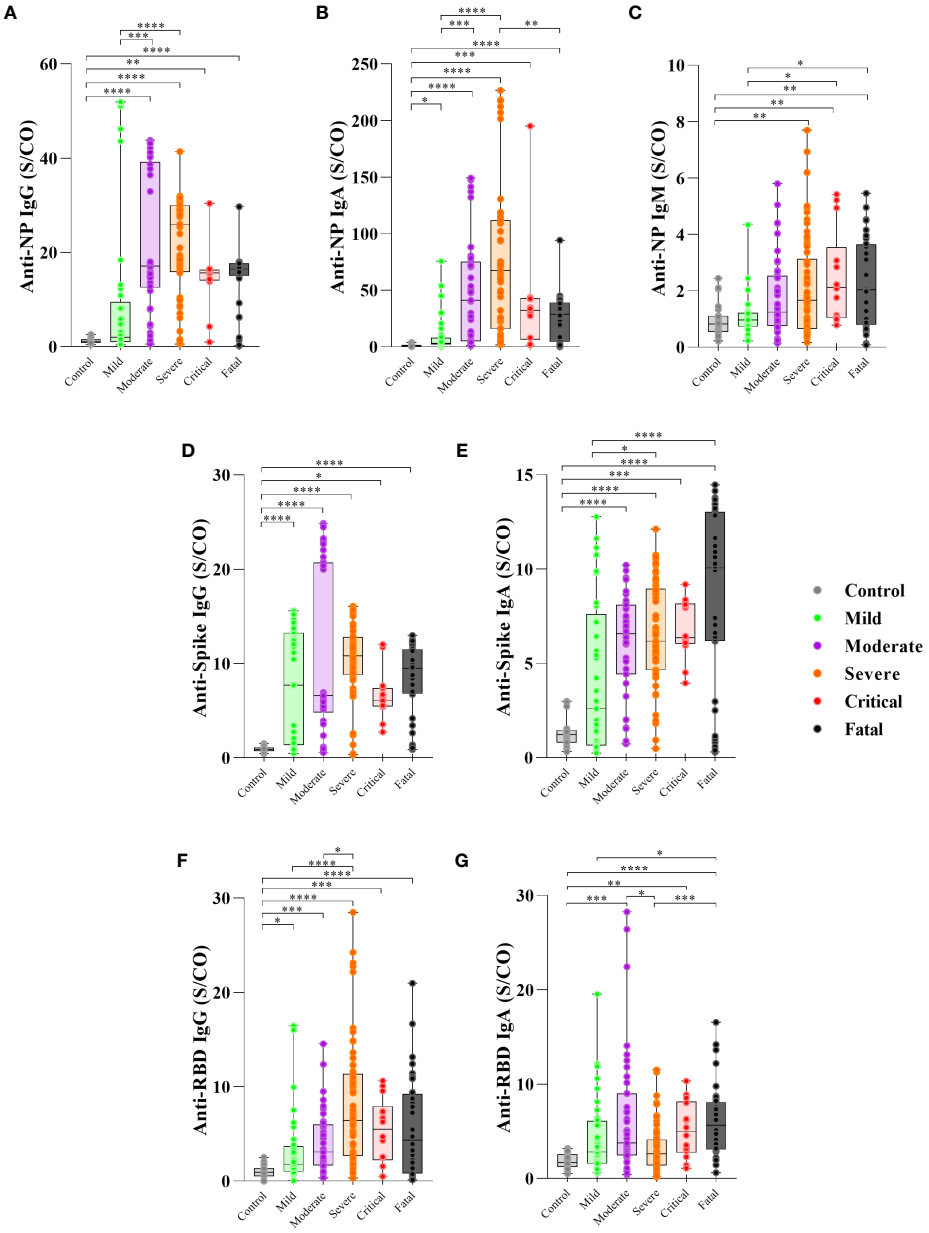

FIGURE 1
Levels of antibody to SARS-CoV-2 proteins in the first 30 days PSO and disease severity. Serum of patients with different clinical status of COVID-19, (uninfected controls, n = 27; mild, n = 37; moderate, n = 43; severe, n = 63; critical n = 14, fatal, n = 36) was analyzed for the presence of anti-nucleoprotein (NP) IgG **(A)**, IgA **(B)** and IgM **(C)**, anti-Spike IgG **(D)** and IgA **(E)** protein and anti-RBD IgG **(F)** and IgA **(G)** antibodies measured by enzyme-linked immunosorbent assay (ELISA). Results are expressed as the index calculated as the ratio of sample/cutoff OD (S/CO) as described in Methods. Boxes represent the 25th to 75th percentiles. Each dot represents a single individual, with distribution in maximum and minimum values. The line inside the box indicates median values. Kruskal-Wallis test was used for comparison of antibody response between groups. *p< 0.05, **p < 0.01, ***p < 0.001 and ****p <0.0001.

(Supplementary Figures 5A–C). Nevertheless, we found no correlation between viral load and anti-SARS-CoV-2 antibodies detected in the fatal group (p>0.05) (Supplementary Figure 6).

Additionally, we have detected several cytokines in the plasma of COVID-19 patients in in different outcomes in our previous study with the same cohort (29). We then analyzed if there was a correlation between the levels of antibodies against proteins of SARS-CoV-2 and the cytokine production (Supplementary Figures 7–10). Taken all COVID-19 patients in the acute phase, we detected a positive correlation between IL-6 and anti-Spike IgA levels (r=0.3517, p=0.0004) (Supplementary Figure 7A), and anti-RBD IgA levels (r=0.3247, p=0.0010) (Supplementary Figure 7B), although there was no correlation between IL-6 and anti-Spike IgG, anti-RBD IgG and anti-NP IgG, IgA, IgM levels (Supplementary Figures 7A–C). In contrast, regarding severity, there was a negative correlation between IL-6 and anti-NP IgG (r=-0.4523, p=0.0232) and IgA (r=-0.4626, p=0.0228) levels in the severe plus critical group (Supplementary Figure 8A), nevertheless, there was no correlation

between IL-6 anti-NP IgM, anti-Spike and anti-RBD IgG, IgA (Supplementary Figures 8A–C). Additionally, the analysis of IL-2 (Supplementary Figure 9) showed a positive correlation with anti-NP IgG levels (r=0.3182, p=0.0014) (Supplementary Figure 9A) in all the COVID-19 patients, however, there was no correlation between IL-2 and anti-NP IgA, IgM, anti-Spike and anti-RBD IgG, IgA levels (Supplementary Figures 9A–C). In the fatal group, we found a negative correlation between IL-2 and anti-Spike IgA levels (r=-0.4253, p=0.0383) (Supplementary Figure 10A). In contrast, there was no correlation between IL-2 and anti-Spike IgG, anti-NP IgG, IgA, IgM and anti-RBD IgG, IgA levels (Supplementary Figures 10A–C). The analysis of other cytokines such as IL-10, IL-4, IFN-γ, and TNF-α did not show any significant correlation with antibody levels against SARS-CoV- 2 proteins (data not shown).

The neutralizing antibody responses to SARS-CoV-2 across the clinical spectrum of COVID-19

We determined the nAb levels in the serum of SARS-CoV-2 infected individuals throughout the clinical course of the infection. We first assessed the levels and frequency of nAb in the first 30 days of PSO (time point T1). We found nAb seropositivity of 81% (data not shown) among COVID-19 individuals. The frequency of nAb detection was significantly higher in participants who developed moderate, severe, critical, and fatal COVID-19 than in those who developed mild disease (Figure 2A). Moreover, higher levels of nAb were observed in individuals who progressed to severe and critical disease and in the fatal group compared to the mild group (*vs.* severe p<0.0001, critical p=0.0075, fatal p=0.0009), no differences among severe, critical, and fatal groups compared to the moderate group (*vs.* severe p=0.1258, critical p=0.8771, fatal p=0.4778) (Figure 2B). Additionally, COVID-19 participants who were hospitalized had higher nAb titers compared to those who were not (*vs.* infirmary p=0.04, ICU p=0.002) (Figure 2C). However, no significant difference in nAb levels was observed between ICU and infirmary admitted participants (p=0.1654) (Figure 2C). Longitudinal follow-up of a subset of individuals in each group of the clinical outcome, between 1 and 6 months PSO (time point 2) and over 6 months PSO (time point 3) did not show significant differences in nAb levels between T1, T2, and T3 time points in any of the COVID-19 groups: mild (Figure 2D), moderate (Figure 2E), and severe plus critical (p>0.05) (Figure 2F). Most of the critical individuals died, thereby, the number of people in this group recruited as recollects was very limited. Thus, due to the scarcity of this group, for follow-up analyses, samples of individuals classified as severe and critical were analyzed together in a single group. The levels of nAbs in the fatal group were not different at the 2 time points analyzed (Figure 2G).

Dynamics of circulating antibodies to SARS-CoV-2 proteins: a longitudinal analysis

Thereafter, we assessed the dynamics of antibody levels longitudinally in a subset of 82 patients at three PSO time points: T1: up to 30 days PSO, T2: 1–6 months PSO, and T3: more than 6 months PSO (only 35 participants for T3).

In the mild group, the levels of anti-NP IgG antibodies increased in T2 (*vs.* T1, p=0.004), but the levels of anti-NP IgA and IgM, anti-spike and anti-RBD IgG and IgA showed no significant differences (*vs.* T2, T3, p>0.050) over time (Figure 3A), suggesting maintenance of the levels over the time.

In the moderate group, anti-NP IgG antibody levels decreased in T3 (*vs.* T1, p=0.004) and T2 anti-NP IgA (*vs.* T1, p=0.020). In contrast, anti-RBD IgG levels increased in T2 (*vs.* T1, p=0.001), while the levels of anti-NP IgM, anti-spike, and anti-RBD IgA did not show significant differences (*vs.* T2, T3, p>0.050) over time (Figure 3B).

For the severe and critical groups analyzed together, we observed a decrease in anti-NP IgG levels in T3 (*vs.* T1, p=0.0289, and *vs.* T2, p=0.0391), IgA levels were lower in T2 (*vs.* T1, p<0.0001) and T3 (*vs.* T1, p=0.0002) and similarly observed for IgM were lower in T2 (*vs.* T1, p<0.0001) and T3 (*vs.* T1, p=0.0001). Moreover, anti-spike IgA was lower in T2 (*vs.* T1, p=0.0063) and T3 (*vs.* T1, p=0.0033 and *vs.* T2, p= 0.0078). The anti-RBD IgG was higher in T2 (*vs.* T1, p=0.0105) and T3 (*vs.* T1, p= 0.0361). The levels of anti-spike IgG and anti-RBD IgA showed no significant differences over time in the severe + critical group (*vs.* T2, T3, p>0.050) (Figure 3C).

The dynamics at follow-up (T1: ≥30 days PSO to T2: ≥60 days PSO) in the fatal group showed that anti-spike IgG antibody levels increased (p=0.0273), while anti-NP IgM levels decreased (p=0.0078) between T1 to T2. Regarding the follow-up of individuals displaying fatal outcomes, the levels of anti-NP IgG and IgA, anti-spike IgA and anti-RBD IgG, and IgA showed no significant difference between the T1 and T2 time points (p=0.7344) (Figure 3D).

Lower levels of IgG and IgA anti-NP and higher IgA anti-spike and anti-RBD discriminate survival *versus* fatal in COVID-19

Considering only individuals admitted to the ICU, we classified them into survivors and fatal individuals and compared the frequency and levels of SARS-CoV-2 antibody production at the first 30 days PSO. The fatal group presented lower levels of anti-NP IgG (p=0.0139) (Figure 4A), lower levels of anti-NP IgA antibodies (p=0.0014) (Figure 4B), but no differences of anti-NP IgM (p=0.7439) (Figure 4C) compared to the survival group. Regarding the antibody levels to spike protein, there was no difference related to IgG (p=0.5016) (Figure 4D), but interestingly the fatal group presented significant higher levels of IgA antibody levels (p=0.0131) compared to survival (Figure 4E). Moreover, there were also no different levels of anti-RBD IgG (p=0.2803) (Figure 4F), but remarkably there was a significant increase of anti-RBD IgA antibodies, in the fatal group compared to the survival (p=0.0052) (Figure 4G).

Concerning the frequency of individuals with positive antibody response to NP protein there was no difference of IgG (p=0.3505) (Supplementary Figure 11A), but it was observed a significant

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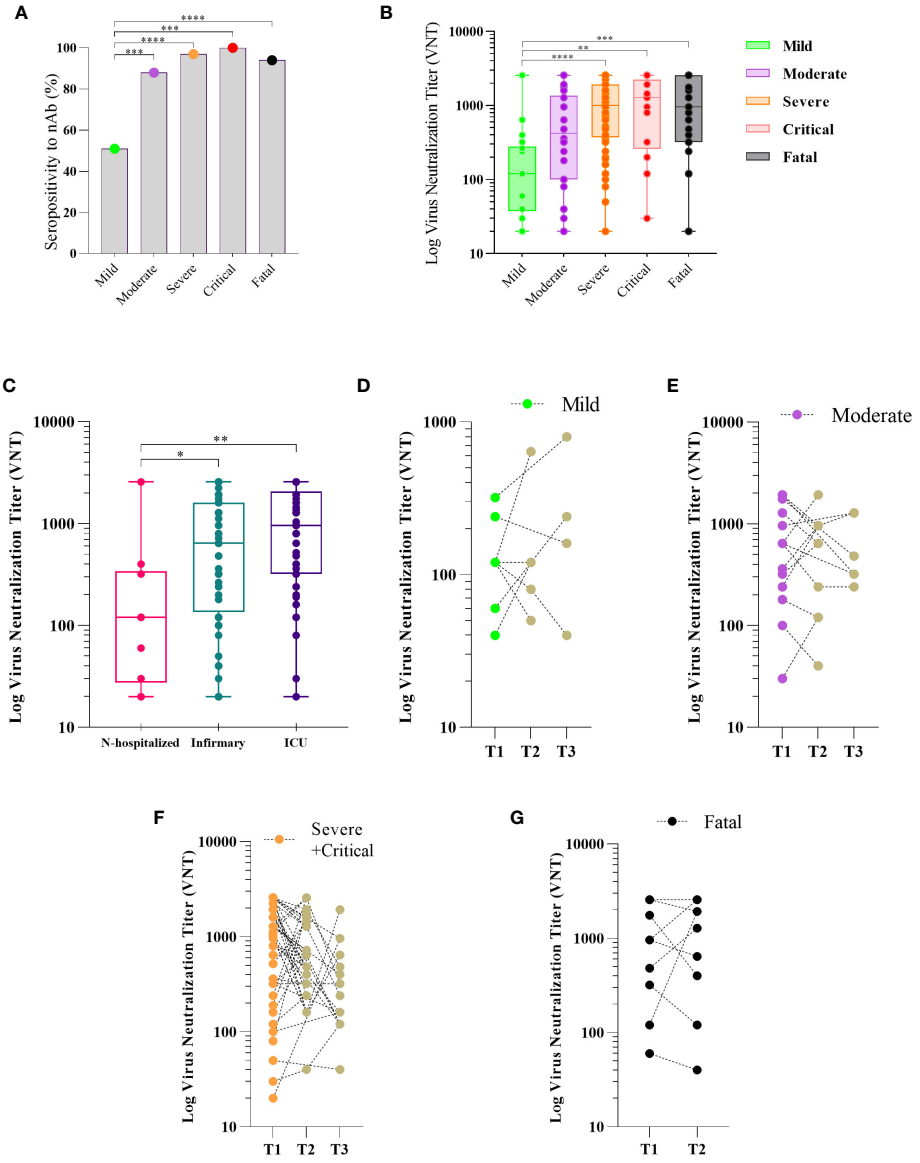

FIGURE 2
Neutralizing antibody response to SARS-CoV-2 and disease severity COVID-19. Sera of patients with different clinical status of COVID-19, (mild, n = 18; moderate, n = 34; severe, n = 60; critical n = 13, fatal, n = 30) were analyzed for the presence of neutralizing antibody (nAb). Maximum neutralization titer was measured by Virus Neutralizing Titers (VNT) and results are expressed as log of VNT as described in Methods. Frequency of positivity to nAb **(A)** and VNT levels **(B)** in the first 30 days after the onset of symptoms (PSO) in the different clinical status of the disease. Boxes represent the 25th to 75th percentiles and each dot represents a single individual, with distribution in maximum and minimum values. The line inside the box indicates median values. nAb response according to hospital care in COVID-19 patients **(C)**. Kinetics of Nab response to SARS-Cov-2 in three periods of time PSO: T1 (≤ 30 days), T2 (>30 and <180 days) and T3 (≥ 180 days) for patients classified as mild **(D)**, moderate **(E)**, severe plus critical **(F)**, and two timepoints (T1 (≤ 30 days) and T2 (>30 and < 60 days) for those who fatal **(G)**. Each dot represents the antibody response of a single individual in different periods of time linked by the dotted line. Fisher's exact test was used for comparison of frequency of nAb response **(A)**, Kruskal-Wallis test was used for comparison of nAb level response **(B, C)**. Wilcoxon matched-pairs test was used for comparison of antibody response among groups **(D–F)**. *p< 0.05, **p < 0.01, ***p < 0.001 and ****p <0.0001.

increase of IgA (p=0.0232) (Supplementary Figure 11B) in the survival compared to the fatal group. In addition, there was no difference in the frequency of anti-NP IgM (p=0.3342) between the groups (Supplementary Figure 11C). A lower frequency of individuals with anti-spike IgG in the fatal group (p=0.0251) (Supplementary Figure 11D), although there was no difference of IgA (p>0.9999) between the groups (Supplementary Figure 11E). In relation to RBD, there was no difference in the frequencies of individuals producing IgG (p=0.0956) and IgA (p=0.0996) (Supplementary Figures 11F, G).

To determine whether the observed differences discriminate individuals who died from those who survived COVID-19, we used anti-NP IgG and IgA, anti-Spike and anti-RBD IgA measurements as input in Receiver Operating Characteristic (ROC). The ROC analysis resulted in an Area Under the Curve (AUC) = 0.901, demonstrating a strong ability of these antibody features to predict

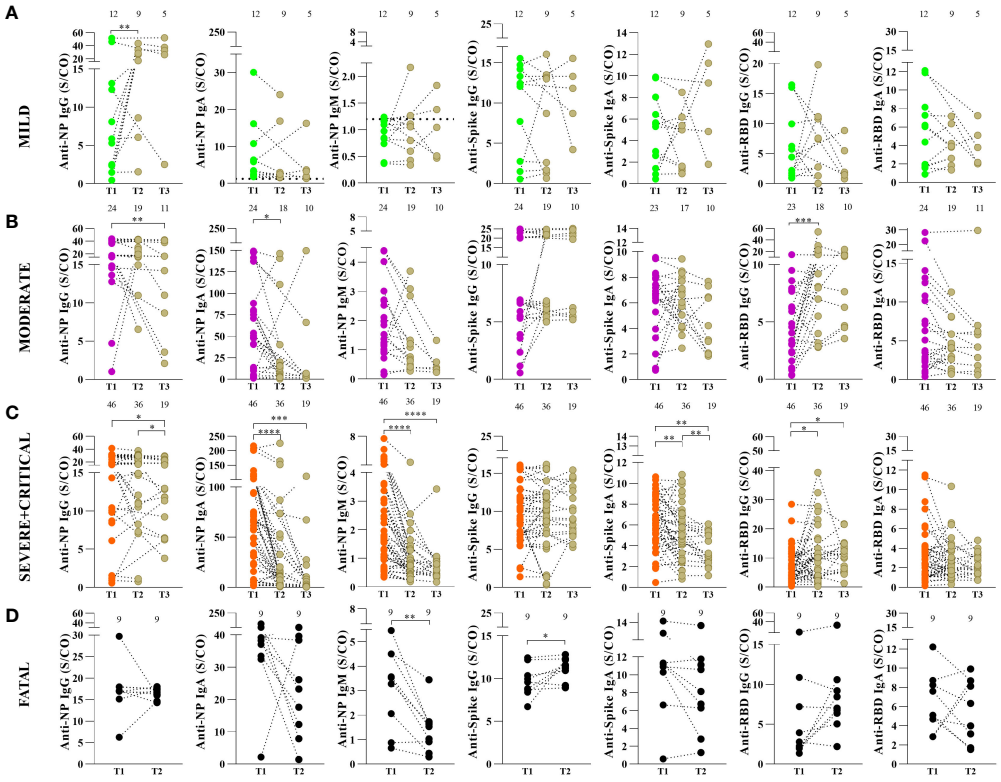

FIGURE 3
Kinetics of antibody response to SARS-CoV-2 proteins in COVID-19 patients according to disease severity. Anti-nucleoprotein (NP) IgG, IgA and IgM, anti-spike IgG and IgA protein and IgG, anti-RBD IgA and IgM antibodies in mild **(A)**, moderate **(B)** and severe plus critical patients **(C)** were measured by enzyme-linked immunosorbent assay (ELISA) as described in Methods. Serum samples were collected in three periods of time after the onset of symptoms: T1 (≤ 30 days), T2 (>30 and <180 days) and T3 (≥ 180 days). Fatal patients **(D)** were analyzed in two timepoints after the onset of symptoms: T1 (≤ 30 days) and T2 (>30 and < 60 days). Results are expressed as sample/cutoff OD (S/CO). The values above the graph are the numbers of patients in each time point. Each dot represents the antibody response of a single individual in the three periods of time linked by the dotted line. Wilcoxon matched-pairs test was used for comparison of antibody response among the different periods of time. *p< 0.05, **p < 0.01, ***p < 0.001 and ****p <0.0001.

fatal outcomes of COVID-19 (Figure 4H). Average importance is given as anti-NP IgA (lower) > anti-NP IgG (lower) > anti-Spike IgA (higher) > anti-RBD IgA (higher) in fatal outcome (Figure 4I).

Moreover, we observed no difference, in time point 2 (T2: >30 and < 60 days) in the levels of anti-NP IgG (p=0.7051) (Supplementary Figure 12A), IgA (p=0.6098) (Supplementary Figure 12B) and IgM (p=0.4634) (Supplementary Figure 12C). The levels were higher of anti-Spike IgG (p=0.0427) (Supplementary Figure 12D), although there was no difference about the anti-Spike IgA (p=0.2538) compared to the survivors (Supplementary Figure 12E). Also, no difference about the levels of anti-RBD IgG (p=0.8307) (Supplementary Figure 12F), but levels were higher of anti-RBD IgA (p=0.0044) (Supplementary Figure 12G). We also compared the levels of antibodies in fatal and survivors in two age groups: 40 to 59 years and ≥60 years. We noted that the levels of anti-NP IgM (p=0.0039), anti-Spike (p=0.0221) and anti-RBD IgA (p=0.0006) were higher in fatal participants with 40–59, although no difference about the levels of anti-NP IgG (p=0.3863) and IgA (p=0.1469), anti-spike IgG (p=0.6518o) and anti-RBD IgG (p=0.9263) (Supplementary Figure 13A). However, the levels of anti-NP IgG (p=0.0321) and IgA (p=0.0204) were lower in participants with ≥60 years of age (Supplementary Figure 13B) who died but was no difference about the levels of anti-NP IgM (p=0.1976), anti-Spike IgG (p=0.1750) and IgA (p=0.0959), and anti-RBD IgG (p=0.1446) and IgA (p=0.4339).

Correlation of antibody levels of SARS-CoV-2 and blood parameters

To determine whether there were correlations between routine blood and biochemical data with antibody levels, we performed analysis using a correlogram, including all COVID-19 participants (Supplementary Figure 14): mild (Supplementary Figure 15A), moderate (Supplementary Figure 15B), severe (Supplementary Figure 15C), critical (Supplementary Figure 15D), and fatal groups (Figure 5A).

In the fatal group, we observed positive correlations between positive anti-NP IgG *vs* IgA, IgM and anti-RBD IgG, anti-NP IgA *vs* IgM and IgG RBD, anti-Spike IgG *vs* IgA, platelets *vs* nAb, erythrocytes *vs* hemoglobin, leukocytes *vs* lymphocytes and monocytes, neutrophils *vs* monocytes and platelets. Additionally, we noted a negative correlation between Ferritin vs anti-Spike IgG and IgA (Figure 5A).

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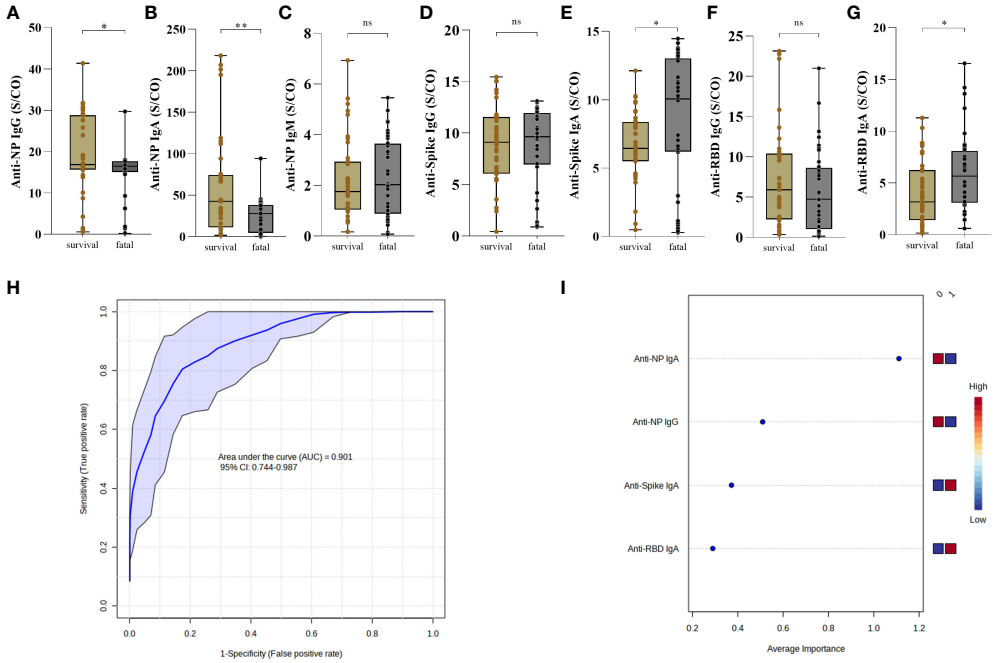

FIGURE 4
Antibody response to SARS-CoV-2 proteins distinguish survival and fatal patients with COVID-19. The presence of anti-NP IgG **(A)** IgA **(B)** and IgM **(C)**, anti-Spike IgG **(D)** and IgA **(E)** and anti-RBD IgG **(F)** and IgA **(G)** were analyzed in the first 30 days post-symptoms onset. Antibodies in the serum of patients who survived (n = 37) and those who died (n = 36) was measured by enzyme-linked immunosorbent assay (ELISA). Results are expressed as sample/cutoff OD (S/CO) as described in Methods. Boxes represent the 25th to 75th percentiles. Each dot represents a single individual, with distribution in maximum and minimum values. The line inside the box indicates median values. Mann-Whitney test was used for comparison of antibody response between groups. Statistical significances are shown as *p < 0.05, **p < 0.01. Receiver Operating Characteristic curve showing sensitivity versus specificity for discrimination of survival and fatal individuals were derived for each combination of the high performing for anti-NP IgG and IgA, anti-Spike and anti-RBD IgA (ROC AUC=0.879, blue line), is presented with 95% confidence intervals (shown in the blue regions) **(H)**. The most discriminating antibody are shown in descending order of their coefficient scores. The color boxes indicate whether antibody concentration is increased (red) or decreased (blue) in [0] survival vs [1] fatal **(I)**.

Among the correlations, we investigated exclusively in the fatal group, the positive correlations between the levels of anti-NP IgA and IgM ($p = 0.0001$, $r^2 = 0.5783$) and between anti-NP IgA and anti-RBD IgG levels ($p = 0.0012$, $r^2 = 0.5006$) (Figures 5B, C). In addition, we investigated potential correlations between antibody levels and those of inflammatory markers, such as C-reactive protein, D-dimer, and ferritin. Ferritin showed higher expression in the fatal group (*vs.* severe, mild, p = >0.05) and a negative correlation ($p = 0.0388$, $r^2 = -0.4772$) with anti-Spike IgA (Figure 5D).

No correlation was observed between other laboratory data and the antibody levels (Supplementary Figure 14). Regarding peripheral blood cell count, we found positive correlations between erythrocytes, hemoglobin, leukocytes, neutrophils, and monocytes in the moderate, severe, and critical groups (Supplementary Figures 15B–D). Concerning biochemical laboratory parameters, D-dimer showed a positive correlation with the levels of anti-NP antibodies, C-reactive protein, and anti-RBD IgA in the mild group (Supplementary Figure 15A).

Discussion

In this study, we investigated the dynamics of the development of SARS-CoV-2-specific antibodies and nAb activity to the wild type of virus in a cohort of unvaccinated COVID-19 patients in Brazil, up to 13 months post-infection and the association with clinical outcomes. The results demonstrated that in general COVID-19 groups showed higher levels of antibodies to SARS-CoV-2 antigens compared to the control group, and the antibody levels increase in the spectrum of more severe disease. confirming that SARS-CoV-2 infection induces a robust humoral immune response according to disease severity. Furthermore, we observed higher levels of nAb in the severe, critical, and fatal COVID-19 groups compared to mild group.

Analyzing the SARS-CoV-2-specific antibodies levels, our results showed that the decreased levels of antibodies and lower frequency of seropositivity in the mild group could be related to the short time of infection, lower viral load, and lower inflammatory response, as well as lower antigen exposure compared to the severe and critical groups (30, 31). Considering the severity of COVID-19, anti-NP IgG and IgA and anti-RBD IgG levels were higher in severe group, anti-NP IgM and anti-spike IgA were higher in the fatal groups. Studies have also reported that higher antibody levels, such as anti-NP IgA (32), anti-Spike, and anti-RBD IgG, were associated with illness progression of COVID-19 between 14 to 42 days PSO (33–35). Our results showed a humoral response mediated by a diversity of antibody isotypes to distinct proteins. The hyperinflammatory state in COVID-19, with exacerbated

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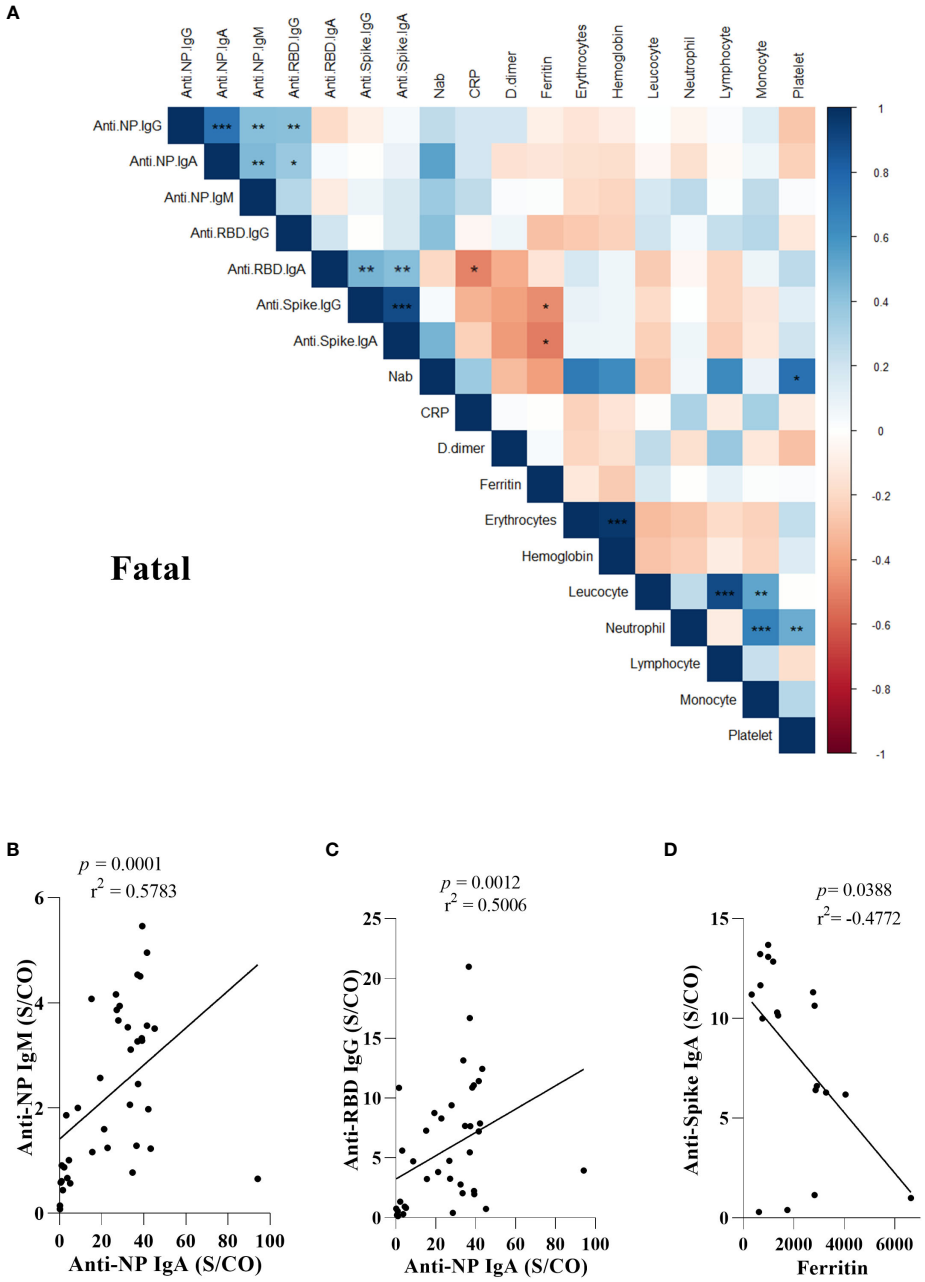

FIGURE 5
Antibody response to SARS-CoV-2 proteins and mortality in COVID-19. Correlations among lymphocyte, monocytes, neutrophil, platelet, erythrocyte, hemoglobin, D-dimer, PCR, Ferritin, and antibodies to SARS-CoV-2 proteins **(A)**. Correlation matrix plot of all variables to identify potential inter-variables correlations. Spearman R values are shown from red (–1.0) to blue (1.0). Only boxes showing a significant correlation and r > 0.5 have been highlighted. Blank fields indicate lack of signal. Hb., Hemoglobin; Neutro., Neutrophil; Lympho. Lymphocyte; Mono., Monocyte; PSO., post-symptoms onset; TAP., partial thromboplastin time; TTPA., activated partial thromboplastin time; CRP., C-reactive protein; ALT., Alanine aminotransferase; AST., Aspartate aminotransferase; nAb. Neutralizing antibody. Spearman correlation between each subclass of specific antibody response to nucleoprotein (NP), Spike and RBD SARS-Cov-2 proteins was analyzed by non-linear regression and those with significant *p* values are shown **(B, C)** and the correlation between anti-Spike IgA and ferritin **(D)**. The presence of antibodies in the serum of patients who died was measured by enzyme-linked immunosorbent assay (ELISA). Statistical significances are shown as *p< 0.05, **p < 0.01, ***p < 0.001.

production of cytokines promotes the class-switching of memory B cells to IgG via IFN-γ or IgA via TGF-β, resulting in a variety of antibodies with distinct kinetics (36) which may be an explanation for the production of different anti-SARS-CoV-2 isotypes. In patients with COVID-19, anti-Spike and anti-RBD IgA levels were positively correlated with the inflammatory cytokine IL-6. This cytokine has been shown to favor an isotype class switching of mucosal B cells to IgA (37, 38). In this context, the high production of IL-6 in severe patients could promote the increase of IgA, which can corroborate with the inflammatory profile and may contribute to the role of IgA in the pathogenesis in severe cases. In contrast, anti-NP IgG levels were positively correlated with the IL-2 levels, which may promote IgG isotype switching (39, 40). Considering, the severe plus critical group, IL-6 was negatively correlated with

anti-NP IgG and IgA levels, and in the fatal group IL-2 was negatively correlated with anti-Spike IgA. Taken together, the results suggest that cytokine production could influence the anti-SARS-CoV-2 antibody isotype switching and in the clinical outcomes, although further studies are necessary to clarify this point.

We observed increase of anti-Spike and anti-RBD IgA levels in fatal group compared to survival group. The association of IgA with disease severity has been shown in the literature (41). For instance, anti-S1 IgA has also been associated with worse clinical evolution (36, 41), suggesting that SARS-CoV-2 infection may be at least in part an IgA-mediated disease since IgA in serum can deposit on tissues, causing damage in several organs, which is a common event in severe cases of COVID-19 (42). Although mucosal IgA was not measured, we do not discard its role in the severity of the disease, as described by Ruiz et al. (43), who showed the persistence of anti-S1 and anti-RBD IgA and the presence of immune complexes in bronchoalveolar lavage in individuals who died, reinforcing the contribution of IgA immune complexes to the immunopathogenesis of SARS-CoV-2 infection (43). Moreover, the crosslink of IgA bound to pathogen antigen with FcRα enhances the signaling cascades, culminating in the increase of inflammation and potentiation of the immune responses that can be protective or detrimental (44). Furthermore, the presence of serum IgA has been suggested as a biomarker for severe COVID-19 (45). Several explanations can rise for the possible pathogenic role of IgA (43, 44, 46). Since IgA are produced against different proteins and may be associated with distinct stages of diseases, our data cannot allow us to link IgA isotype as a deleterious role. Further studies, including antibody subclass function, are need clarify this role of IgA in COVID-19 (47–49).

In the virus neutralization assay, the gold standard assay for the detection of nAbs, a marked presence of nAbs was observed in all groups, but only the mild and moderate groups showed lower levels of nAbs when compared to other groups. Different from our data, Lucas et al. (2021) observed in the first 14 days of PSO that patients who did not present nAb levels progressed to death compared to the other groups according to severity disease, reinforcing that the early production of nAb are associated with survival outcome (50). Our results do not allow us to infer whether the early production of nAbs guided the clinical outcome, since we usually had blood sampling collection at different days PSO. The nAb detected in the individuals in our study might promote virus neutralization in different stages of COVID-19. The overall differential antibody profile found in the fatal group consisted of higher levels and frequency of seropositivity of IgM anti-NP and IgA anti-spike, but lower levels and frequency of detection of IgA anti-NP antibodies and lower frequency of detection of IgG anti-RBD compared to the severe group. Similar to all other clinical groups, the fatal group presented a higher frequency and titers of nAbs, only compared to the mild COVID-19 group. Moreover, we showed a positive correlation of nAb with anti-RBD IgG antibody levels only in the severe and critical groups. In contrast, we showed a significance positive correlation between nAb levels and anti-Spike IgG antibody just in the moderate group. The longitudinal dynamics of nAbs in our cohort showed no significant changes in the different groups over time, suggesting a long-term maintenance of the nAbs for a long time. In relation to this, it was shown that nAbs can persist up to 18 months in patients who had mild COVID-19 (51). In addition, it has been shown that neutralizing activity may decrease after four months (52) or present lower potency in severely ill patients (33). Even though, there are severe, critical and fatal individuals in our cohort, the nAbs levels were present in those patients.

When we investigated the persistence of humoral immunity, in the follow-up analysis of 6 months or more, we observed that anti-spike IgA levels were decreased in the severe plus critical groups compared to the first ≤30 days PSO (T1). These findings are in agreement with those observed by Fedele et al., who demonstrated that there was no decline in IgG levels but that anti-spike IgA decreased after 6 months of infection in mild/moderate and severe groups (53). In fact, humoral response kinetics revealed maintenance of levels in the mild group, with gradual reduction in moderate and severe plus critical groups, revealing loss of this maintenance of anti-NP antibodies, but with stability in the production of anti-spike and anti-RBD IgG antibodies. In the longitudinal antibody levels in the severe + critical group, we must consider that this robust result be related to the number of individuals recruited for another collection of blood in the recovery phase.

Regarding the outcome of SARS-CoV-2 infection, ≤30 days PSO (T1), patients with COVID-19 admitted to the ICU, our analyses revealed a distinct pattern of antibody production, with higher levels of anti-NP IgG and IgA in the survival group, but anti-Spike and anti-RBD IgA had higher levels in the fatal group. In this context, it has been shown that increased levels of antibody in severe individuals may be related to the higher viral load and longer exposure to the virus in severe patients, suggesting that a higher viral load may induce greater antibody production (54–56). Considering that viremia decreases exponentially with the linear increase in Ct values obtained by RT-PCR, when we evaluated the Ct for SARS-CoV-2 and the antibody levels, we saw that the lower is the viremia, the higher are the anti-RBD IgG levels, the same occurring in the moderate and severe plus critical groups. A possible explanation for the control of viremia is the neutralization capacity of anti-RBD IgG and other antibody effector functions via Fc receptors described previously (57). A recent study performed with COVID-19 patients demonstrated that increased anti-spike IgG antibody levels were associated with the worst disease, suggesting that this profile can be explained by antibody-mediated immunopathology (50). The mechanism underling the association of high levels of anti-Spike and anti-RBD with severity and death should be further investigated.

Antibody levels, according to age, showed that individuals with 40-59 years produced higher levels of anti-NP IgM, anti-RBD and anti-spike IgA and individuals with 60 years or more showed less anti-NP IgG and IgA in the fatal group. Age is associated with an increase in the number of people who become seriously ill or die from COVID-19 since older people start to have complications from COVID-19 (53). Immunosenescence is associated with a reduced immune response capacity, either by dysfunction of the innate immune response, increase in inflammatory cytokines, or deficiency in the production of B cells; the repertoire of T-cell

receptors are limited, and regulatory T-cells (Treg) are further efficient, among other changes in the distribution of immune cells (58–61).

The longitudinal analysis of the fatal group in the period between 30 and 60 days PSO showed a decrease in the levels of anti-NP IgM and an increase in anti-spike IgG levels. In general, the increase in the IgG isotype and the reduction in IgM is due to the function of B lymphocytes after interaction with the virus, and later, the exchange of the IgM isotype for IgG, with a consequent production of low-binding plasma cell antibodies (62), which is a common event in infections, especially in the acute phase. A correlation test was performed on inflammatory markers, such as D-dimer, CRP, and ferritin, and we found a negative correlation between anti-spike IgA and ferritin, one of the parameters associated with worsening clinical progression. Increased ferritin synthesis is regulated by the production of pro-inflammatory cytokines or by the extravasation of intracellular ferritin caused by cellular damage (63). Studies reported existence the ferritin positive correlations with anti-Spike IgG, and their increased ratio in severe cases (50, 64), further investigation is necessary to clarify the role of serum ferritin levels with antibodies in the pathogenesis of COVID-19.

A positive correlation of anti-NP IgA with anti-NP IgM and anti-NP IgA with anti-RBD IgG in the blood of fatal patients with COVID-19 was detected. Interestingly, comparison to mild disease presentation, though in the fatal group, levels of anti-NP IgA significantly decreased in comparison to severe COVID-19, suggesting that this could be a relevant indication of disease aggravation and evolution to death. In this context, it has been shown that the antibody anti-NP is more sensitive, conserved, and stable and appears in the first days after the onset of symptoms. Antibodies against NP protein, one of the four structural proteins and main sign for the virus infection, have been used to detect early infection (65). Furthermore, the presence of anti-NP antibodies in the sera of patients has been associated with disease severity (65–68). A possible mechanism that explains this association is that the anti-NP antibody via the Fc-receptor induces the production of the main pro-inflammatory cytokine, IL-6, in lung alveoli infected with SARS-CoV-2 due to the cytokine storm observed in COVID-19, potentiating the disease severity (67, 69).

Our study has some limitations that may explain the differences observed in relation to other studies, as we do not have samples from the first 10 to 15 days after the onset of symptoms from all patients as a definition of the viral shedding duration. The collection of blood samples was late, most donors had specimens collected 14 days after symptom onset, since these patients were mainly treated at the primary health care level and moved to secondary care hospital where the samples were collected. Another point is related to the low number of critical patients and recovered individual. Even though there those limitations, it was possible to study the humoral immune response in acute phase and longitudinally in distinct groups of patients.

In conclusion, we found that higher levels of anti-NP IgM, IgA, and IgG antibodies, as well as anti-spike IgA and anti-RBD IgG, were associated with worse clinical outcome compared the mild disease, suggesting a potentially deleterious effect when these antibodies are found in excess. On the other hand, we found that higher levels of anti-Spike and anti-RBD IgA, and lower levels of anti-NP IgG and IgA characterize fatal outcomes, suggesting these antibody features as predictors of death from COVID-19.

Future studies should be carried out to determine the ability and the mechanism by which antibodies act against the circulating virus and its role in viral infection and/or replication, providing information about antibodies that protect against reinfection or induce clinical worsening.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving humans were approved by Ethical Appreciation (CAAE: 30804220.2.0000.5078). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

Conceived and supervised the study: SGF, CPS, MS-F. Recruited participants, collected samples, epidemiological and clinical data: CPS, LCM, AOG, MASBB, JMMS, ARG-J, MHAL, BGNM, SMS, LEX. Processed samples: CPS, LCM, JMMS, ARG-J. Performed and supervised RT–qPCR analysis: DCCA, FSF, MS. Performed and supervised Elisa: CPS, LCM, MS-F, SGF. Performed and supervised Neutralizing antibody assay: VFB, SACJ, ELD, DCAO. Production of RBD protein: JVB-C, AMM. Performed data analysis and generated figures and tables: CPS, MS-F, SGF. Original draft: CPS, SGF, MS-F. Critical discussion, review and editing of the manuscript: SGF, CPS, VC, MS-F, LCM, DCCA, FSF, MS, AOG, MASBB, SACJ, VFB, PRTR, JVB-C, AMM, ALB, IAHP, NLC, VCRF, LGG. Funding acquisition: SGF. All authors reviewed and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1206979/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Seropositivity to SARS-CoV-2 proteins in COVID-19 patients according to disease severity. Patients with different clinical status were analyzed in the first 30 days post-symptoms onset (mild, n = 37; moderate, n = 43; severe, n = 63; critical n = 13, fatal, n = 36). Detection of anti-nucleoprotein (NP) IgG **(A)**, IgA **(B)** and IgM **(C)**, anti-Spike IgG **(D)** and IgA **(E)** protein and anti-RBD IgG **(F)** and IgA **(G)** antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described in Methods. Results are expressed as frequency of positive response considered with index > 1.2 calculated as the ratio of sample/cutoff OD (S/CO). Fisher's exact test was used for comparison of antibody response between groups. *p< 0.05, **p < 0.01, ***p < 0.001 and ****p <0.0001.

SUPPLEMENTARY FIGURE 2
Ct values reflect and antibodies levels to SARS-CoV-2 proteins. Spearman correlation and non-linear regression (line) were performed between anti-RBD IgG and IgA **(A),** anti-Spike IgG and IgA **(B),** and anti-nucleoprotein (NP) IgG, IgA and IgM **(C),** and with Ct value, performed by RT-PCR. The presence of antibodies in the patients' (each point) serum was measured by enzyme-linked immunosorbent assay (ELISA). Spearman R and p values are included each one graphic.

SUPPLEMENTARY FIGURE 3
Ct values reflect and antibodies levels to SARS-CoV-2 proteins in the mild group. Spearman correlation and non-linear regression (line) were performed between anti-Spike IgG and IgA **(A),** anti-nucleoprotein (NP) IgG, IgA and IgM **(B),** and anti-RBD IgG and IgA **(C)** with Ct value, performed by RT-PCR, in the mild group. The presence of antibodies in the patients' (each point) serum was measured by enzyme-linked immunosorbent assay (ELISA). Spearman R and p values are included each one graphic.

SUPPLEMENTARY FIGURE 4
Ct values reflect and antibodies levels to SARS-CoV-2 proteins in the moderate group. Spearman correlation and non-linear regression (line) were performed between anti-RBD IgG and IgA **(A),** anti-nucleoprotein (NP) IgG, IgA and IgM **(B),** and anti-Spike IgG and IgA **(C)** with Ct value, performed by RT-PCR, in the moderate group. The presence of antibodies in the patients' (each point) serum was measured by enzyme-linked immunosorbent assay (ELISA). Spearman R and p values are included each one graphic.

SUPPLEMENTARY FIGURE 5
Ct values reflect and antibodies levels to SARS-CoV-2 proteins in the severe plus critical group. Spearman correlation and non-linear regression (line) were performed between anti-nucleoprotein (NP) IgG, IgA and IgM **(A),** anti-RBD IgG and IgA **(B),** and anti-Spike IgG and IgA **(C)** with Ct value, performed by RT-PCR, in the severe plus critical group. The presence of antibodies in the patients' (each point) serum was measured by enzyme-linked immunosorbent assay (ELISA). Spearman R and p values are included each one graphic.

SUPPLEMENTARY FIGURE 6
Ct values reflect and antibodies levels to SARS-CoV-2 proteins in the fatal group. Spearman correlation and non-linear regression (line) were performed between anti-nucleoprotein (NP) IgG, IgA and IgM **(A),** anti-Spike IgG and IgA **(B),** and anti-RBD IgG and IgA **(C)** with Ct value, performed by RT-PCR, in the fatal group. The presence of antibodies in the patients' (each point) serum was measured by enzyme-linked immunosorbent assay (ELISA). Spearman R and p values are indicated for each isotype.

SUPPLEMENTARY FIGURE 7
Correlation of IL-6 with antibodies levels to SARS-CoV-2 proteins. Spearman correlation and non-linear regression (line) were performed between anti-Spike IgG and IgA **(A)**, anti-RBD IgG and IgA **(B)**, and anti-nucleoprotein (NP) IgG, IgA and IgM **(C)** antibodies and IL-6 MFI level values (log scale), in the several plus critical group. Concentration of IL-6 was detected by CBA (28). The presence of antibodies in the serum of patients was measured by enzyme-linked immunosorbent assay (ELISA). Spearman R and p values indicated for each isotype. MFI indicates average of median fluorescence intensity units.

SUPPLEMENTARY FIGURE 8
Correlation of IL-6 with antibodies levels to SARS-CoV-2 proteins in the severe plus critical group. Spearman correlation and non-linear regression (line) were performed between anti-nucleoprotein (NP) IgG, IgA and IgM **(A)**, anti-Spike IgG and IgA **(B)**, and anti-RBD IgG and IgA **(C)** antibodies and IL-6 MFI level values (log scale), in the several plus critical group. Concentration of IL-6 was detected by CBA (28). The presence of antibodies in the serum of patients was measured by enzyme-linked immunosorbent assay (ELISA). Spearman R and p values indicated for each isotype. MFI indicates average of median fluorescence intensity units.

SUPPLEMENTARY FIGURE 9
Correlation of IL-2 with antibodies levels to SARS-CoV-2 proteins. Spearman correlation and non-linear regression (line) were performed between anti-nucleoprotein (NP) IgG, IgA and IgM **(A)**, anti-Spike IgG and IgA **(B)**, and anti-RBD IgG and IgA **(C)** antibodies and IL-2 MFI level values, in the several plus critical group. Concentration of IL-2 was detected by CBA (28). The presence of antibodies in the serum of patients was measured by enzyme-linked immunosorbent assay (ELISA). Spearman R and p values indicated for each isotype. MFI indicates average of median fluorescence intensity units.

SUPPLEMENTARY FIGURE 10
Correlation of IL-2 with antibodies levels to SARS-CoV-2 proteins in the fatal group. Spearman correlation and non-linear regression (line) were performed between anti-Spike IgG and IgA **(A)**, anti-nucleoprotein (NP) IgG, IgA and IgM anti-Spike IgG and IgA **(B)**, and anti-RBD IgG and IgA **(C)** with IL-2 of the MFI level values in the fatal group. Concentrations of the IL-2 was detected by CBA (28). The presence of antibodies in the serum of patients was measured by enzyme-linked immunosorbent assay (ELISA). Spearman R and p values indicated for each isotype. MFI indicates average of median fluorescence intensity units.

SUPPLEMENTARY FIGURE 11
Frequency antibody response to SARS-CoV-2 proteins distinguish survival and fatal patients with COVID-19. Frequency of antibody response in COVID-19 according to fatal (n=36) and survival (n=41). Detection of anti-nucleoprotein (NP), IgG **(A)**, IgA **(B)** and IgM **(C)**, anti-spike IgG **(D)** and IgA **(E)**, anti-RBD IgG **(F)** and IgA **(G)** protein antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described in Methods. Fisher's exact test was used for comparison of antibody response between groups; horizontal bar indicates the percentage of frequency with antibodies levels. The line in the middle of box indicates median values. Mann-Whitney test was used for comparison of antibody response between groups. Statistical significances are shown as *p< 0.05, **p < 0.01.

SUPPLEMENTARY FIGURE 12
Antibody response to SARS-CoV-2 proteins distinguish survival and fatal patients with COVID-19 were analyzed between >30 and ≤60 days after the onset of symptoms. The presence of anti-nucleoprotein (NP) IgG **(A)**, IgM **(B)** and IgA **(C)**, anti-Spike IgG **(D)** and IgA **(E)** and anti-RBD IgG **(F)** and IgA **(G)** protein antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described in Methods. Results are expressed as sample/cutoff OD (S/CO) as described in Methods. Boxes represent the 25th to 75th percentiles. Each dot represents a single individual, with distribution in maximum and minimum values. The line in the middle of the box indicates median values. Mann-Whitney test was used for comparison of antibody response between groups. Statistical significances are shown as *p< 0.05, **p < 0.01.

SUPPLEMENTARY FIGURE 13
Antibody response to SARS-CoV-2 proteins distinguish survival and fatal patients with COVID-19 according to the age. Serum of patients with age 40-59 years was analyzed for the presence of anti-NP IgG, IgA and IgM, anti-Spike, and anti-RBD IgG and IgA **(A)**. Serum of patients with age ≥60 years was analyzed for the presence of IgG, IgA and IgM antibodies to NP, IgG and IgA antibodies to Spike and to RBD **(B)**. Antibodies in the serum of patients who survived and those who died was measured by enzyme-linked immunosorbent assay (ELISA). Results are expressed as sample/cutoff OD (S/CO) as described in Methods. Boxes represent the 25th to 75th percentiles. Each dot represents a single individual, with distribution in maximum and minimum values. The line in the middle of box indicates median values. Mann-Whitney test was used for comparison of antibody response between groups. Statistical significances are shown as *p< 0.05, **p < 0.01.

SUPPLEMENTARY FIGURE 14
Correlations among age, days PSO, blood count, biochemical parameters, and antibodies to SARS-CoV-2 proteins in total cohort of COVID-19 patients. Correlation matrix plot of all variables to identify potential inter-variables correlations. The color represents the direction of the correlation. Spearman R values are shown from red (–1.0) to blue (1.0). Only boxes showing a significant correlation and r > 0.5 have been highlighted. Blank fields indicate lack of signal. Abbreviations: Hb., Hemoglobin; Neutro., Neutrophil; Lympho. Lymphocyte; Mono., Monocyte; PSO., post-symptoms onset; TAP., partial thromboplastin time; TTPA., activated partial thromboplastin time; CRP., C-reactive protein; ALT., Alanine aminotransferase; AST., Aspartate aminotransferase; nAb. Neutralizing antibody. Statistical significances are shown as *p< 0.05, **p < 0.01, ***p < 0.001.

SUPPLEMENTARY FIGURE 15
Correlations among lymphocyte, monocytes, neutrophil, platelet, erythrocyte, hemoglobin, D-dimer, PCR, Ferritin, and antibodies to SARS-CoV-2 proteins, with mild **(A)**, moderate **(B)**, severe **(C)**, critical **(D)** COVID-19 patients. Correlation matrix plot of all variables to identify potential inter-variables correlations. Spearman R values are shown from red (–1.0) to blue (1.0). Only boxes showing a significant correlation and r > 0.5 have been highlighted. Blank fields indicate lack of signal. Abbreviations: Hb., Hemoglobin; Neutro., Neutrophil; Lympho. Lymphocyte; Mono., Monocyte; PSO., post-symptoms onset; TAP., partial thromboplastin time; TTPA., activated partial thromboplastin time; CRP., C-reactive protein; ALT., Alanine aminotransferase; AST., Aspartate aminotransferase; nAb. Neutralizing antibody. Statistical significances are shown as *p< 0.05, **p < 0.01, ***p < 0.001.

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Protection from infection and reinfection due to the Omicron BA.1 variant in care homes

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Introduction: Following the emergence of SARS-CoV-2 in 2020, care homes were disproportionately impacted by high mortality and morbidity of vulnerable elderly residents. Non-pharmaceutical interventions (NPIs) and improved infection control measures together with vaccination campaigns have since improved outcomes of infection. We studied the utility of past infection status, recent vaccination and anti-S antibody titres as possible correlates of protection against a newly emergent Omicron variant infection.

Methods: Prospective longitudinal surveillance of nine sentinel London care homes from April 2020 onwards found that all experienced COVID-19 outbreaks due to Omicron (BA.1) during December 2021 and January 2022, despite extensive prior SARS-CoV-2 exposure and high COVID-19 vaccination rates, including booster vaccines (>70% residents, >40% staff).

Results: Detailed investigation showed that 46% (133/288) of Omicron BA.1 infections were SARS-CoV-2 reinfections. Two and three COVID-19 vaccine doses were protective against Omicron infection within 2-9 weeks of vaccination, though protection waned from 10 weeks post-vaccination. Prior infection provided additional protection in vaccinated individuals, approximately halving the risk of SARS-CoV-2 infection.

Discussion: Anti-S antibody titre showed a dose-dependent protective effect but did not fully account for the protection provided by vaccination or past infection, indicating that other mechanisms of protection are also involved.

KEYWORDS

COVID-19, SARS-CoV-2, Omicron (BA1), care homes, outbreaks, correlate of protection, vaccine

1 Introduction

Since the start of the pandemic, United Kingdom Health Security Agency (UKHSA) (formerly Public Health England) has been monitoring a cohort of care homes as a longitudinal cohort study to understand SARS-CoV-2 exposures and transmission within this high-risk setting. By November 2021, as the Omicron (BA.1) variant emerged and spread rapidly across England, staff and residents in these care homes had already been heavily exposed to the original, alpha and delta variants that swept across the country in the previous months (1). Despite this, and high rates of three-dose vaccination (70.8% of residents and 29.4% of staff in England by 23 November 2021) (2), large outbreaks in care homes were observed in late December 2021 (3), although hospitalisation rates remained low (4).

Here, we examine the utility of three possible correlates of protection - past natural SARS-CoV-2 infection, COVID-19 vaccination, and anti-S antibody titres against three clinical outcome measures (a) infection with the Omicron variant, (b) hospitalisation and (c) infectiousness in this highly vulnerable cohort.

2 Methods

2.1 Cohort description

Since May 2020, UKHSA has conducted SARS-CoV-2 surveillance in sentinel care homes across London, England. The care homes selected for study have been previously described (5) comprising a mix of adult residential, nursing, and specialist dementia long term care facilities (LTCFs). Virologic surveillance in nine care homes, involved regular PCR and lateral flow device (LFD) testing for residents and staff in line with national guidelines, as well as periodic serology testing of staff and residents for SARS-CoV-2 spike (S) and nucleocapsid (N) antibodies (5), coupled with detailed genomic analysis of infecting virus strains. The mean age of resident at end of study = 86.3, median = 87.8, and staff average age at end of study – mean = 50.9, median = 51.1.

Omicron outbreaks were recorded in all nine care homes during the surveillance period. A case was defined as an individual with a positive LFD/PCR result in December 2021 or January 2022. All residents and staff with at least one PCR or LFD test in December 2021 or January 2022 (n=1,099, 78% female,) were included in the analysis of the impact of the Omicron variant. Individuals were only included if an LFD/PCR result was available from October or November 2021, to confirm that a resident or staff member was present in the care home during the surveillance period. Prior infection status was determined using PCR, LFD, and anti N and S serology results from the start of the pandemic to the start of the surveillance period (Figure 1).

2.2 Laboratory methods

Nose and throat swabs from residents and staff were sent by courier to the UKHSA Colindale Reference Laboratory for RT-PCR testing using a SARS-CoV-2 assay with E and Orf1ab gene targets as previously described (5). Some PCR testing of staff was also performed via community testing programmes in government Lighthouse laboratories. UKHSA samples with a cycle threshold (Ct) value less than 35 underwent whole genome sequencing. Viral amplicons were sequenced using Illumina library preparation kits (Nextera) and sequenced on Illumina shortread sequencing machines (Nextseq or Hiseq). The bioinformatics protocol to generate consensus sequences utilised Trimmomatic, BWA (mapping), and an in-house variant caller (quasibam) to align against a SARS-CoV-2 reference genome (NC_045512.2). Consensus sequences were generated using a depth cut-off of 20 reads. Genome lineages were allocated where the coverage of the reference genomes was 80% or more. Serological testing was performed using Roche Elecsys® Anti-SARS-CoV-2 S and N antibody testing according to manufacturers instructions.

2.3 Data linkage and analysis

PCR and LFD test results were extracted from the UKHSA Colindale laboratory information management system (LIMS) as well as the relevant national datasets: the Unified Sample Dataset (USD) and the Episode Level Line List. Serological data were also extracted from the UKHSA LIMS. Vaccination and demographic data, including date of death, were retrieved from the national COVID-19 vaccination database, National Immunisation Management System (NIMS). Prior infection status of an individual was allocated if there was evidence of prior infection confirmed by PCR detection or serological testing of blood samples taken prior to the start of the study. Anti N antibody detection and/or presence of anti S antibodies prior to the introduction of vaccination were taken as evidence of prior infection. Hospitalisations were identified using hospital attendance records from the national dataset [Hospital Episode Statistics, HES (6)] and NHS or Lighthouse laboratory COVID-19 test results from the UKHSA USD. Deaths were identified from the NHS Spine, which is included in the NIMS database. A&E attendances without hospitalization and elective admissions for non-COVID-19 conditions and were excluded. All hospitalisations and deaths were confirmed with the appropriate care home manager. To exclude nosocomial infections, cases were only included if any positive result was within 28 days before or after 5 days following hospital discharge, or 28 days before death. This was to ensure that the analysis for correlates of protection was focused on the impact of infection occurring in the community, rather than any nosocomial events. Data were linked together using R (v.4.2.2) in R studio (v.2022.12.0 + 353) by matching of the NHS number, names and dates of birth. Poisson regression modelling of vaccine effectiveness was undertaken in Stata (v.15) and adjusted for period (week), sex, ethnicity, care home, past infection, staff of any age/resident<70yrs/ resident 70-79yrs/resident 80+yrs. Logistic regression was used to investigate the relationship between the most recent S- antibody titre taken prior to the study period and the odds of infection. Titres were grouped at <100,100-999,1000-9999,10000+. Adjustment was made for age on December 1st 2021 and week of the test, and in an exploratory analysis, past infection status was added to see whether the protection from this was mediated through S-antibodies. Ct value analysis was conducted in R using the Kruskal-Wallis test.

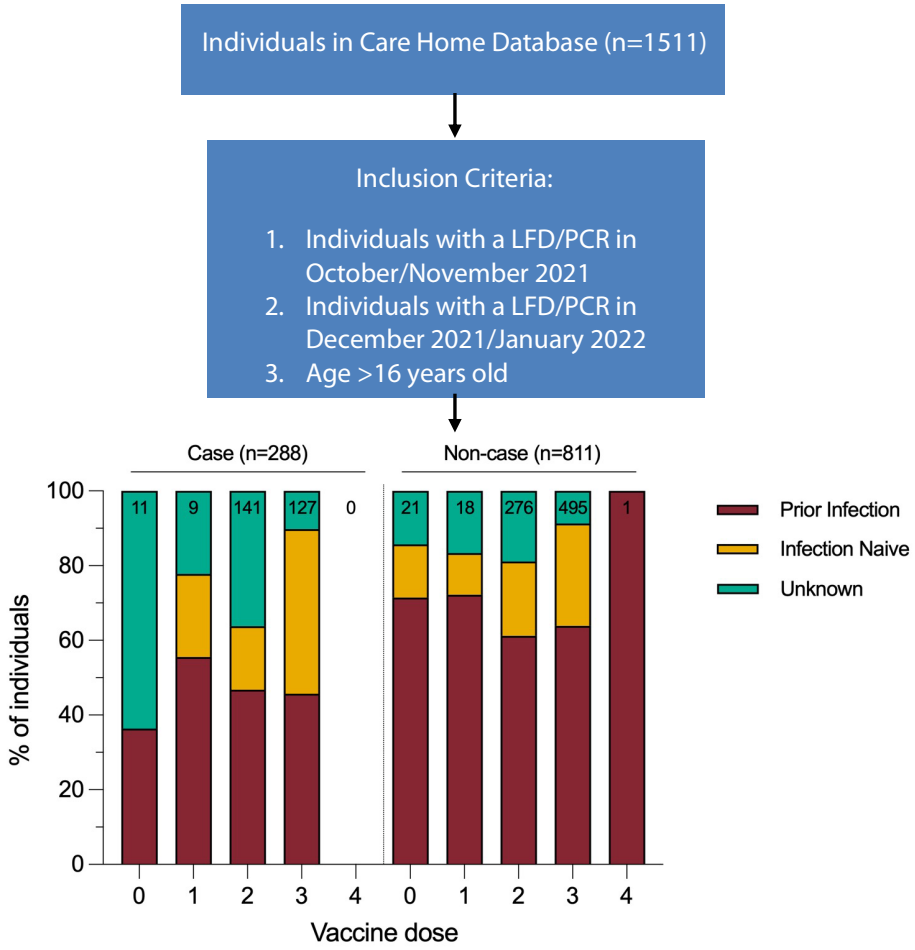

FIGURE 1
Infection status calculated for start of study period (1st December 2021). Vaccination dose for case defined as number of doses received 14 days prior to positive test result. Vaccination dose for non-cases defined as number of doses received 14 days prior to start of study period. Data for this figure was taken from Supplementary Table 2.

2.4 Ethics

The investigation protocol was reviewed and approved by the UKHSA Research Ethics and Governance Group (REGG) (Reference NR0204). Verbal consent for testing was obtained by care home managers from staff members and residents or their next of kin as appropriate. UK Health Security Agency (UKHSA, formerly Public Health England) has legal permission, provided by Section 3 of the Health Service (Control of Patient Information) Regulation 2002, to process patient confidential information for national surveillance of communicable diseases.

3 Results

In total, 112 residents and 176 staff were infected by Omicron variant during the 2-month surveillance period, giving a cumulative infection rate of 26% (288/1099). Staff had approximately twice as many PCR test results available compared to residents. 29% of staff (176/603, median age 50) and 23% of residents (85/375, median age 87) were infected (Figure 2). Case rates were highest in younger age-groups. Approximately half the cases (n=133, 46%) were reinfections and reinfection rates were higher in staff (52%) than residents (38%) (Table 1). 61% (177/288) of positive samples yielded full whole genome sequence, with 99% identified as BA.1 and no other Omicron sub-lineages identified (Supplementary Figure 1).

Vaccination rates were high among residents, with 17% receiving two and 77% receiving three doses compared to 52% and 43%, respectively, among staff. A small proportion of individuals were unvaccinated: 21/496 (4.2%) residents and 11/603 (1.8%) staff were unvaccinated. BNT162b2 (Comirnaty, Pfizer-BioNTech) was the most common vaccine used, representing 52%, 53%, and 90% of first, second and third doses, respectively. ChAdOx1 (Vaxzevria, AstraZeneca) made up the remainder of the primary doses, except for eight (0.7%) individuals receiving Moderna (mRNA-1273) as their primary dose and 72 (11.6%) as their third dose.

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TABLE 1 Cohort infection and reinfection by age and role.

		Individuals	Cases	Case Rate (%)	Reinfection Rate (%)
Age	**≤40**	140	55	39	45
	>40 - ≤60	355	101	28	53
	>60 - ≤80	229	47	21	49
	>80	375	85	23	36
Role	**Staff**	603	176	29	52
	Residents	496	112	23	38
Total		**1099**	**288**	**26**	**46**

3.1 Protection against infection

3.1.1 Protection afforded by vaccination and past infection

Analysis of relative vaccine effectiveness using Poisson regression shows that protection was significantly higher following the 3rd vaccine booster compared to greater than 10 weeks after the second COVID-19 vaccine dose: 43% (95% CI, 17-61%) at 2-9 weeks after 3rd vaccination and 29% (95% CI, 2-48%) at 10+ weeks post-dose 3 (Table 2). The second vaccine dose was also protective (49%, 95% CI, 7-73%) during the first 2-9 weeks compared to 10+ weeks post-dose 2. These findings demonstrate waning of protection against Omicron infection after both the second and third doses. Previous SARS-CoV-2 infection approximately halved the risk of reinfection, irrespective of vaccination status (Table 2). Regular serological assessment increased case ascertainment of symptomatic and asymptomatic infections in our cohort early during the pandemic when PCR

TABLE 2 Cohort vaccination status and vaccine effectiveness (VE) estimates.

Variable	Level	PCR positive events	Person year of follow-up	Rate per person year	Crude relative incidence vs baseline	Adjusted relative incidence vs baseline* (95% CI)	Relative vaccine effectiveness to 2 doses 10+ weeks ago (95% CI)
Vaccination Status	**Unvaccinated**	9	5.4	1.66	0.84	0.81 (0.40-1.64)	
	Dose1: <2 wks ago	2	0.2	8.42	4.24	2.73 (0.61-12.19)	
	Dose1: 2-9 wks ago	0	1.1	0.00	0.00	n/a	
	Dose 1: 10+ wks ago	7	2.7	2.57	1.29	0.95 (0.42-2.12)	
	Dose2: <2 wks ago	0	0.7	0.00	0.00	n/a	
	Dose2: 2-9 wks ago	12	8.8	1.37	0.69	0.51 (0.27-0.93)	49% (7-73)
	Dose2: 10+ wks ago	101	50.9	1.99	baseline	baseline	baseline
	Dose3: <2 wks ago	13	7.7	1.68	0.85	0.79 (0.44-1.41)	
	Dose3: 2-9 wks ago	41	56.3	0.73	0.37	0.57 (0.39-0.83)	43% (17-61)
	Dose 3: 10+ wks ago	103	87.7	1.17	0.59	0.71 (0.52-0.98)	29% (2-48)
Past Infection	**No**	82	55.6	1.47	baseline	baseline	
	Yes	133	133.9	0.99	0.67	0.48 (0.36-0.65)	
	Unknown	73	32.0	2.28	1.54	1.11 (0.78-1.56)	

*Model included period (week), sex, ethnicity, care home, past infection, staff of any age/resident<70yrs/resident 70-79yrs/resident 80+yrs.

testing was more limited. Serological testing was voluntary and only 30% of the cohort underwent serological sampling between October 2021 and February 2022. Despite this, comparison of infection rates by LFD/PCR and seropositivity/seroconversion rates in our cohort during the period indicated that <5% of infections were missed, likely because of already high immunity levels and regular PCR/LFD testing among staff and residents (7).

3.1.2 Protection afforded by anti-S antibodies

Anti-S antibody titres from serum collected in the 90 day period (1 Sept – 1 Dec 2022) immediately before the Omicron variant emerged were a strong predictor of past infection regardless of vaccination status. Those individuals who had previously been infected with an earlier variant had significantly higher anti S antibody titres (median 4170 vs 910, $p < 0.0001$) (Figure 3). The assessment of recent S-antibody on the odds of infection showed reductions in the odds of infection of 49%, 74% and 69% for those with titres of 100-999, 1000-9999, 10000+ compared to titres of <100 respectively (odds ratios 0.51 95% CI: (0.21-1.19), 0.26 (012-0.59), 0.31(0.12-0.76)). This indicates evidence of increased protection up to titres above 1000 and then a plateau, based on samples taken in the month prior to the study period. These antibodies will have been generated by vaccination and/or infection but likely do not mediate all the protective effects. If prior infection status is included in the model this still shows evidence of protection on subsequent infection (odds ratio 0.60, 95% CI (0.35-1.01)). It was not possible to add vaccination status to the model due to its high level of association with S-antibody titres.

3.2 Protection against severe disease`

Clinical outcome data is provided in Table 3. All hospitalizations and fatalities occurred in residents. Hospitalisation rates were much higher among unvaccinated individuals than those who had received two or more vaccine doses (37% vs 3%), as was the case fatality rate (36% vs 5%). Exploration of the relationship between antibody titres, past infection and severe disease was limited by the small numbers of individuals with severe disease.

FIGURE 2
Primary Infection or reinfection during study period stratified by age and role.

3.3 Protection against infectiousness

Cycle threshold (Ct) value obtained during RT PCR testing from samples taken early in infection gives an indication of the magnitude of viral shedding. Several studies have used this as a proxy for infectiousness during primary infection (8). In this study, Ct values were significantly lower (indicating higher viral load) in infection-naive compared with previously infected individuals, (median 24.9 vs 28.4, $p = 0.004$) and did not correlate with role (staff vs resident) (Supplementary Figure 2). In all cases who underwent PCR testing, no significant difference in Ct value distributions was noted by vaccination status (two versus three doses). There were insufficient samples to compare Ct values among unvaccinated cases or those who had only received one dose.

4 Discussion

Whilst Omicron's increased transmissibility with high infection and reinfection rates in the community is well-described (9), less is documented about transmission of this variant in high-risk, highly vaccinated settings. While reducing the force of infection, high rates of prior infection and vaccination did not prevent considerable transmission occurring in all sentinel care homes, despite longstanding implementation of stringent infection control measures and social distancing in this setting. The 46% reinfection rate with Omicron was substantially higher compared to the 3% for the Alpha variant in the same cohort in January 2021 (5), a likely consequence of antibody waning over time since primary infection, combined with immune evasion by the Omicron variant.

Modelled estimates of reinfection with Omicron in the community are similar to the rates that we observed, higher than the estimate of 9.5% derived from national operational testing data (4, 10, 11). Reinfection rates were higher in staff versus residents, likely explained by lower third booster vaccination rates, higher exposure risk in the community likely arising as a result of increased connectivity and increased testing improving ascertainment. Of the 133 reinfections, 67% had primary infection in the pre-Alpha period and 17% in the Alpha and Delta periods, respectively. Notably, 6/133 reinfections were third infections, first with pre-Alpha or Alpha, followed by Delta and then Omicron.

Three possible indicators of protection showed varying levels of correlation against selected clinical outcome measures. Our finding of higher protection with increasing doses of COVID-19 vaccines is consistent with other reports of protection against Omicron infection through vaccination (10) and reassuring about the value of booster vaccine doses. Given the high rates of prior infection in our cohort, our finding that the combination of prior infection and

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TABLE 3 Clinical outcomes within cohort by role, age, number of vaccine doses and past infection status.

		Hospitalised	Died	Hospitalisation Rate (%)	Case Fatality Rate (%)
Role	**Staff (n=176)**	0	0	0.0	0.0
	Resident (n=112)	12	18	10.7	16.1
Age	**60 – 80 (n=47)**	3	3	6.4	6.4
	>80 (n=85)	9	15	10.6	17.6
Vaccination Status	**Unvaccinated (n=11)**	3	4	27.3	36.4
	1 Dose (n=9)	0	1	0.0	11.1
	2 Doses (n=141)	2	5	1.4	3.5
	3 Doses (n=127)	7	8	5.5	6.3
Past Infection	**No (n=82)**	2	4	2.4	4.9
	Yes (n=133)	4	4	3.0	3.0
Total (n=288)		**12**	**18**	**4.2**	**6.3**

vaccination may provide improved protection against Omicron infection than vaccination alone is also noteworthy. Outside the care home setting, prior infection has been estimated to provide 60% protection against reinfection with Omicron compared to Alpha, Beta or Delta variants (12, 13), with vaccination providing additional protection in previously-infected individuals (11).

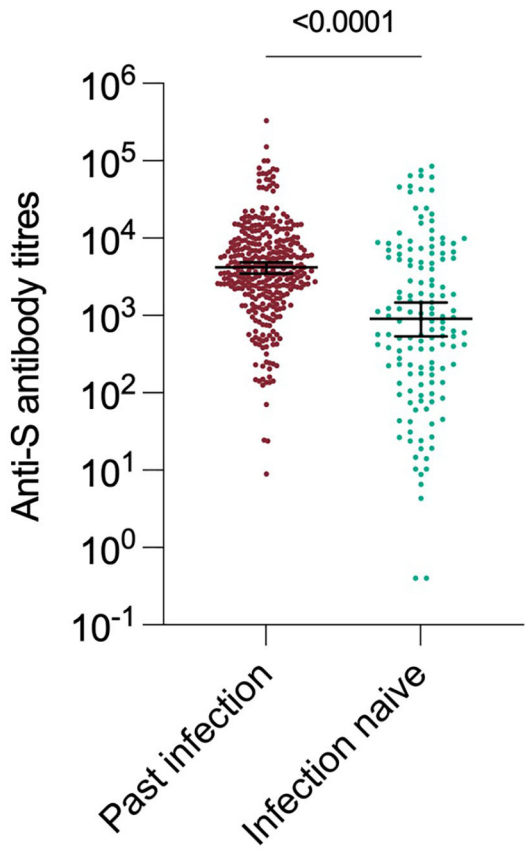

FIGURE 3
Distribution of Antibody titres.

We did not find a correlation between peak viral shedding (for which Ct value is a proxy) and vaccination status. This is consistent with other studies that have shown vaccination reduces the duration and overall amount of viral shedding rather than the peak of viral shedding (14, 15). Our finding of higher Ct values suggestive of lower viral shedding in previously infected individuals is consistent with findings in other community studies outside the care home setting (16). The duration of infectiousness does not reliably correlate with the magnitude of the peak of viral shedding (15, 17).

In this study, we have measured anti-S antibodies as a proxy for neutralizing antibody (Figure 3). Anti-S antibody titre was a dose-dependent correlate of protection from infection. This is consistent with observational studies during the first waves of the pandemic, where levels of neutralising antibody titres were suggested as possible correlate of protection (18, 19). Prior work in this cohort has also shown antibody titres to be correlated with protection against antigenically similar variants (5). The S protein of Omicron variant has a high number of changes and demonstrates significant antigenic distance from earlier variants (20).This work shows that high antibody titres remain protective even in the context of exposure to an antigenically-distant variant.

However, antibody titre was not sufficient to fully explain the protective effect of vaccination or past natural infection and therefore we hypothesise some protective effects must be exerted through pre-existing adaptive or innate immunity mechanisms, including cellular response. Unmeasured contributors to protection may also include innate control mechanisms and mucosal antibodies These require further study. The relative contribution between different classes of strain specific antibodies to viral S protein Receptor Binding Domain (RBD) or N terminal domain vs cross reactive neutralizing antibodies requires detailed analysis of antibody repertoire following primary and secondary infection and the impact of vaccination (21). Examination of the profile of antibody repertoire following boosting by vaccination or re-infection should yield further insights into the nature of antibody correlates of protection. The role or contribution of mucosal antibody in protection from infection has not been considered in this study.

The very low overall hospitalisation rate (<5%) in our cohort during large Omicron outbreaks represents a significant improvement from the pre-vaccine period (1). Emerging data continues to demonstrate decoupling between viral infection or reinfection and severity of disease as measured by hospitalisation during the Omicron and related variants wave. Our study confirming this finding in high-risk settings is reassuring and consistent with others (1, 22) and highlights the importance of ensuring high vaccine uptake and repeated regular boosters to ensure continued protection in this vulnerable cohort (23). Establishing the intrinsic severity of future newly emerging variants will increasingly rely upon animal or *in vitro* models as a result of high degree of population exposure, residual immunity and impact of vaccination.

4.1 Strengths and limitations

Our assessment of Omicron infection risk by prior infection status, vaccination status and time since vaccination was only possible because of the longitudinal nature of our study since the start of the pandemic. There are, however, some limitations. The data included here was censored on 31 January. Therefore, while this paper includes the most intense outbreak period and the majority (>90% of the cases), outbreaks continued at a low level into March 2022. Some outbreaks had not completely terminated at the end of the study period, likely underestimating by a small margin the overall case and reinfection rates.

The higher rate of PCR testing of staff could potentially lead to improved ascertainment of infection and identification of re-infection. Overall, the rates of serological testing were similar between staff and residents, and therefore any bias towards improved detection in staff would be based on the probability of detecting infection by more frequent PCR testing, rather than assessment of past infection status due to serological investigations. This higher ascertainment in staff will not bias estimates of vaccine effectiveness or the effect of past infection since it will apply equally irrespective of vaccination status or past infection status and adjustment is made in the analysis for being staff/resident.

Hospitalisations and deaths were attributed to COVID-19 by temporal correlation, and therefore we do not distinguish 'deaths with COVID-19' from 'deaths from COVID-19'. This may overestimate the number of severe outcomes from COVID-19 infection experienced by the cohort. We were only able to assess protection against the Omicron BA.1 subvariant and, therefore, our findings are not considered for the subsequently dominant BA.4/BA.5 subvariants.

Data availability statement

Genomic data is available from GISAID. The other datasets presented in this article are not readily available because they contain person identifiable information collected and collated as part of an outbreak response under Section 3 of the UK Health Service (Control of Patient Information) Regulations 2002. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by PHE Research and Ethics Governance Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

NI, JC, SL AJ-S and MZ conceived and designed the study. SC also contributed to study design. TR, KM, ER and FA were responsible for data acquisition. SC, TR, KM, NA and MZ conducted data analysis and interpretation, ER, FA and AJS also contributed to data analysis. SC, TR, MZ and KM wrote the manuscript. All authors critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1186134/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Genomic analysis of an Omicron outbreak within a single care home indicating several different introductions, and transmission of the same variant between staff and residents with varying degrees of vaccination.

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Vaccine-induced SARS-CoV-2 antibody response: the comparability of S1-specific binding assays depends on epitope and isotype discrimination

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Background: Quantification of the SARS-CoV-2-specific immune response by serological immunoassays is critical for the management of the COVID-19 pandemic. In particular, neutralizing antibody titers to the viral spike (S) protein have been proposed as a correlate of protection (CoP). The WHO established the First International Standard (WHO IS) for anti-SARS-CoV-2 immunoglobulin (Ig) (NIBSC 20/136) to harmonize binding assays with the same antigen specificity by assigning the same unitage in binding antibody units (BAU)/ml.

Method: In this study, we analyzed the S1-specific antibody response in a cohort of healthcare workers in Germany (n = 76) during a three-dose vaccination course over 8.5 months. Subjects received either heterologous or homologous prime-boost vaccination with ChAdOx1 nCoV-19 (AstraZeneca) and BNT162b2 (Pfizer-BioNTech) or three doses of BNT162b2. Antibodies were quantified using three anti-S1 binding assays (ELISA, ECLIA, and PETIA) harmonized to the WHO IS. Serum levels of neutralizing antibodies were determined using a surrogate virus neutralization test (sVNT). Binding assays were compared using Spearman's rank correlation and Passing–Bablok regression.

Findings: All assays showed good correlation and similar antibody kinetics correlating with neutralizing potential. However, the assays show large proportional differences in BAU/ml. ECLIA and PETIA, which detect total antibodies against the receptor- binding domain (RBD) within the S1 subunit, interact similarly with the convalescent plasma-derived WHO IS but differently with vaccine serum, indicating a high sensitivity to the IgG/IgM/IgA ratio.

Conclusion: All three binding assays allow monitoring of the antibody response in COVID-19-vaccinated individuals. However, the assay-specific differences

hinder the definition of a common protective threshold in BAU/ml. Our results highlight the need for the thoughtful use of conversion factors and consideration of method-specific differences. To improve the management of future pandemics and harmonize total antibody assays, we should strive for reference material with a well-characterized Ig isotype composition.

KEYWORDS

SARS-CoV-2 antibody, spike protein, serological testing, COVID-19 vaccines, humoral immune response, neutralizing antibodies, WHO standard, correlate of protection

1 Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a global health challenge. Since the first case was reported in December 2019, the virus has spread rapidly to become a global pandemic, with more than 760 million confirmed cases and more than 6.9 million deaths worldwide as of May 2023 (1–3). Several countermeasures have been implemented, including the development of COVID-19 vaccines (4, 5).

The SARS-CoV-2 spike (S) protein is a primary target of neutralizing antibodies (nAbs), which are essential for protective immunity against viral infection (6–9). The receptor-binding domain (RBD), located in the S1 subunit of the trimeric S protein, mediates viral attachment by binding to the host cell receptor angiotensin-converting enzyme 2 (ACE2). The interaction between RBD and ACE2 plays a critical role in viral entry, making the SARS-CoV-2 S1 subunit a primary target for vaccine development (10–12).

The mRNA vaccine BNT162b2 (Pfizer BioNTech; hereafter referred to as BNT) and the vector vaccine ChAdOx1 nCoV-19 (Oxford-AstraZeneca; hereafter referred to as ChAd), were among the first COVID-19 vaccines authorized by the European Medicines Agency (EMA) between December 2020 and April 2021 (13, 14). Both vaccines, which encode the full-length S protein of SARS-CoV-2, have demonstrated high vaccine efficacy (VE) in clinical trials (11, 15–17).

Concerns about the durability of immunity and the ability of emerging SARS-CoV-2 variants of concern (VOCs) to evade immune protection have led to ongoing efforts to improve vaccination strategies. In December 2021, the World Health Organization (WHO) and EMA recommended the use of heterologous 'prime-booster vaccination' using different types of COVID-19 vaccines for the first and second doses; also known as the "mix-and-match" approach (18–20).

This decision was based on interim results from several clinical trials suggesting that heterologous vaccination results in a stronger and longer-lasting immune response. In particular, the combination of vector and mRNA vaccines appeared to induce higher levels of neutralizing antibodies than homologous vaccination with the same type of vaccine (21–26). In July 2022, the ECDC and EMA updated their public health recommendation, suggesting a second booster dose at least 4 months after the first (27). Those who received two doses of vector vaccine could receive a third dose of mRNA vaccine. Others received a homologous triple vaccination with three doses of mRNA vaccine, resulting in a heterogeneous vaccinated population.

Serological and cell-based assays are two common approaches used to quantify immune response and immune protection following vaccination (28, 29). While cell-based assays measure cellular immune responses such as T-cell proliferation or cytokine production, serological assays allow for the rapid and cost-effective quantification of SARS-CoV-2-specific antibodies in human serum. Therefore, serological assays are more suitable for routine diagnostics and high-throughput analysis in clinical laboratories. Serological tests can provide valuable information on VE and the durability of antibody protection, helping to identify individuals with suboptimal immune responses who may benefit from alternative vaccination strategies (30).

Neutralizing antibodies against the SARS-CoV-2 S protein are particularly important for assessing VE and predicting immune protection in individuals (31). High nAb titers have been associated with a lower risk of SARS-CoV-2 infection and severe COVID-19 disease. Several studies have shown that individuals with higher levels of neutralizing antibodies are less likely to develop symptomatic COVID-19 following natural infection or vaccination (32–36).

Neutralizing antibody titers have therefore been proposed as a correlate of protection (CoP) from SARS-CoV-2 (37). In a systematic review, Perry et al. found a robust correlation between vaccine-induced antibody levels and VE, despite the profound heterogeneity in vaccination regimens, serological assays, VE endpoints, and populations. The authors conclude that humoral immunity is an integral part of protection against COVID-19 and propose anti-S or neutralizing antibody levels as the most likely immune marker for a SARS-CoV-2 CoP (38).

In 2020, the WHO established the First International Standard (IS) for anti- SARS-CoV-2 immunoglobulin (NIBSC Code: 20/136) to harmonize serological test results worldwide (39–41). Reference standards are intended to improve the accuracy, reliability, and reproducibility of serological tests and facilitate the intercomparison of measurements obtained with different assays and detection methods in different laboratory settings worldwide (42). Lack of standardization can lead to the inaccurate interpretation of serological results, hampering effective disease surveillance and vaccine development (43).

In this study, we compared three SARS-CoV-2 S1-specific routine immunoassays for their ability to monitor humoral immune response and immune protection in a heterogeneous vaccination cohort. Subjects received different homologous and heterologous three-dose vaccination regimens over a period of 8.5 months. The serological tests, which differ in assay method (ELISA, ECLIA, and PETIA), antigens (full S1 subunit vs. RBD only), and isotype specificity (IgG vs. total Ig) were compared using Spearman's rank correlation and Passing–Bablok regression. To define an universal cut-off for immune protection, suitable for real-world settings, we correlated anti-S1/RBD antibody titers (in BAU/ml) with neutralization potential (percentage inhibition of RBD-ACE2 interaction) as assessed by a surrogate virus neutralization test (sVNT).

2 Materials and methods

2.1 Study design and population

In this longitudinal observational study, we monitored the SARS-CoV-2 S1-specific antibody response in a cohort of healthcare workers in Germany who received three COVID-19 vaccinations (n = 76; median age, 50 years; interquartile range, 29–44 years; range, 23–68 years; female/male ratio, 6/1). Blood samples were collected at 11 fixed time points between February 2021 and January 2022. All participants were employed at the Medizinisches Versorgungszentrum für Labormedizin und Mikrobiologie Ruhr GmbH (mvzlm Ruhr) (Essen, Germany). Of the 80 subjects enrolled in this study, four participants were excluded from further analysis due to confirmed COVID-19 diagnosis (n = 2), pregnancy (n = 1), or allergic reaction (n = 1), resulting in a final study population of 76 eligible participants. This study was conducted in accordance with the World Medical Association's Declaration of Helsinki and approved by the local ethics committee (Ärztekammer Nordrhein, No. 2021281). Participants gave written informed consent to participate in this study (44).

The majority of subjects received a homologous prime-boost vaccination with the vector vaccine ChAdOx1 nCoV-19 (Oxford-AstraZeneca; ChAd) as the first and second doses and the mRNA vaccine BNT162b (Pfizer BioNTech; BNT) as the third dose (63/76; 83%; ChAd-ChAd-BNT). The remaining subjects received either a heterologous prime-boost vaccination with ChAd as the first dose and BNT as the second and third doses (8/76; 11%; ChAd-BNT-BNT) or received a homologous vaccination with three doses of BNT (5/76; 7%; BNT-BNT-BNT).

Venous blood samples were collected at the following time points (TP): Before vaccination (TP1; -3/+ 0 days), 12 days (TP2; +/- 1 day) and 28 days after the first dose (TP3; +/- 2 days), the day of the second vaccination (TP4; -3/+0 days; administered 2.5 months after the first dose), 12 days (TP5; +/- 1 day), 28 days (TP6; +/- 2 days), 3 months (TP7; +/- 2 days) and 4 months after the second dose (TP8; +/- 2 days), the day of the third vaccination (TP9; +/- 2 days, administered 5 months after the second dose), and 12 days (TP10; +/- 1 day) and 28 days after the third dose (TP11; +/- 2 days).

Serum aliquots from collected blood samples were stored at −20°C until measurement. For unbiased comparison, an aliquot of each sample was thawed at room temperature and all serum samples for each time point were analyzed on all platforms on the same day, according to the manufacturer's instructions.

2.2 Assays and instruments

2.2.1 Anti-S1 immunoassays

Three different quantitative immunoassays were used to determine the serotiter of anti-SARS-CoV-2 antibodies specific for different proportions of the same S1-antigen (different epitope spectrum) (Supplementary Table S1). The Anti-SARS-CoV-2-QuantiVac IgG (Euroimmun, Lübeck, Germany) is an indirect enzyme-linked immunosorbent assay (ELISA) for the quantification of IgG antibodies against the complete S1 subunit of the SARS-CoV-2 S protein. Anti-SARS-CoV-2-QuantiVac IgG was performed on a fully automated Euroimmun Analyzer I (Euroimmun, Lübeck, Germany). The Elecsys® Anti-SARS-CoV-2-S (Roche Diagnostics, Mannheim, Germany) is an electrochemiluminescence immunoassay (ECLIA) for the quantification of total antibodies (IgG, IgM, and IgA) against the RBD (located in the S1 subunit) in human serum and plasma. Elecsys® Anti-SARS-CoV-2-S was performed on a fully automated e801 Cobas® 8000 analyzer (Roche Diagnostics, Mannheim, Germany). The SARS-CoV-2 UTAB FS (Diasys Diagnostic Systems, Holzheim, Germany) is a particle-enhanced turbidimetric immunoassay (PETIA) for the quantification of total antibodies (IgG, IgA, and IgM) against the RBD in human serum and plasma. SARS-CoV-2 UTAB FS was performed on a fully automated c502 Cobas® 8000 analyzer (Roche Diagnostics, Mannheim, Germany). Samples that exceeded linearity were measured in dilutions: SARS-CoV-2 UATB FS (range: 3.4–250 BAU/ml, dilutions: 1:20, 1:50, and 1:100); Elecsys® Anti-SARS-CoV-2-S (range: 0.4–250 BAU/ml, dilutions: 1:20, 1:50, and 1:100); and Anti-SARS-CoV-2-QuantiVac IgG (range: 3.2–384 BAU/ml, dilutions: 1:10 and 1:100).

2.2.2 Neutralization assay

The SARS-CoV-2 NeutraLISA (Euroimmun, Lübeck, Germany) is a semiquantitative competitive ELISA used as a surrogate virus neutralization test (sVNT; Supplementary Table S1). Neutralizing antibodies in the sample compete with the biotinylated ACE2 receptor in the sample buffer for binding to the precoated RBD. Bound ACE2 is detected by peroxidase-labeled streptavidin, which catalyzes a color reaction. The intensity of absorbance is inversely proportional to the concentration of neutralizing antibodies in the sample. Results are expressed as percentage inhibition (IH%) according to the following formula: IH% = 1 − (absorbance of sample/absorbance of blank) × 100. The negative cut-off is <20 IH % and the positive cut-off is ≥35 IH%. The SARS-CoV-2 NeutraLISA assay was performed on a fully automated Euroimmun-Analyzer I (Euroimmun, Lübeck, Germany).

2.2.3 Harmonization to the WHO IS

All quantitative immunoassays have been harmonized using the First WHO International Standard (IS) for SARS-CoV-2

immunoglobulin (NIBSC code: 20/136) with an assigned unit of 250 International Units (IU) per vial for neutralizing activity. The final concentration after reconstitution is 1,000 IU/ml. Dilutions were 1:256, 1:128, 1:64, 1:32, 1:16, 1:8, and 1:4 (39, 41, 45). For ECLIA (1.0; Roche U/ml = BAU/ml) and ELISA (3.2; Euroimmun RU/ml x 3.2 = BAU/ml), conversion factors provided by the manufacturer were used. The conversion factor for PETIA (1.0; Diasys AU/ml = BAU/ml) was determined through calibration to the WHO IS (Supplementary Figure S1; Supplementary Table S2).

2.3 Statistical analysis

Spearman's rank correlation and Passing–Bablok regression analysis (46, 47) were performed using MedCalc® version 22.006 (MedCalc Software Ltd., Ostend, Belgium) according to the principles of CLSI Guideline C24 (48).

3 Results

3.1 Monitoring of vaccine-induced antibody response by anti-S1 binding assays

Blood samples were collected at 11 different time points (TP1–TP11) during a three-dose COVID-19 vaccination course over 8.5 months. The cohort was vaccinated with ChAd-BNT-BNT, ChAd-ChAd-BNT, or BNT-BNT-BNT. S1-specific antibody serotiters were quantified using three routine binding assays and converted to BAU/ml: ELISA (Anti-SARS-CoV-2-QuantiVac IgG assay), ECLIA (Elecsys® Anti-SARS-CoV-2-S assay), and PETIA (SARS-CoV-2 UTAB FS assay) (Figure 1).

The cohort displayed a heterogeneous antibody response with high interpatient variation in antibody titers (Supplementary Figure S2). Mean antibody levels increased rapidly after each vaccination, peaking at TP5 and TP10 for all three assays (Figure 1). The highest increases were observed 12 days after the second vaccination (from TP4 to TP5), ranging from 43-fold (ELISA) to 297-fold (PETIA), and 12 days after the third vaccination (from TP9 to TP10), ranging from 8-fold (ELISA) to 15-fold (PETIA) (Table 1; Supplementary Figure S3; Supplementary Tables S3-S5).

Antibody levels began to decline as early as 28 days after the second vaccination (from TP5 to TP6) and 28 days after the third vaccination (from TP10 to TP11). Within 5 months after the first booster (from T5 to TP9), mean antibody titers had decreased to 30% (ELISA), 11% (ECLIA), and 7% (PETIA) of the peak concentration at TP5 (Table 1; Supplementary Figure S4).

Despite the similar kinetic profile, the mean BAU/ml values varied widely between the immunoassays, ranging from 959.7 BAU/ml (ELISA) to 12,704.4 BAU/ml (ECLIA) for TP5, and from 2,601.9 BAU/ml (ELISA) to 18,564.4 BAU/ml (ECLIA) for TP10 (Table 1). In general, the Elecsys® Anti-SARS-CoV-2-S (ECLIA) assay measured consistently higher than the Anti-SARS-CoV-2-QuantiVac IgG (ELISA) or SARS-CoV-2 UTAB FS (PETIA) assays. The most considerable differences were observed in samples with the highest antibody titers. In these samples, the mean BAU/ml values for ECLIA were 13-fold (TP5/TP6) and 7-fold (TP10/TP11) higher than those for ELISA (Table 1; Supplementary Figure S2).

3.2 Comparison of anti-S1 binding assays

To further investigate these proportional differences, especially at high antibody titers, we compared all three assays through

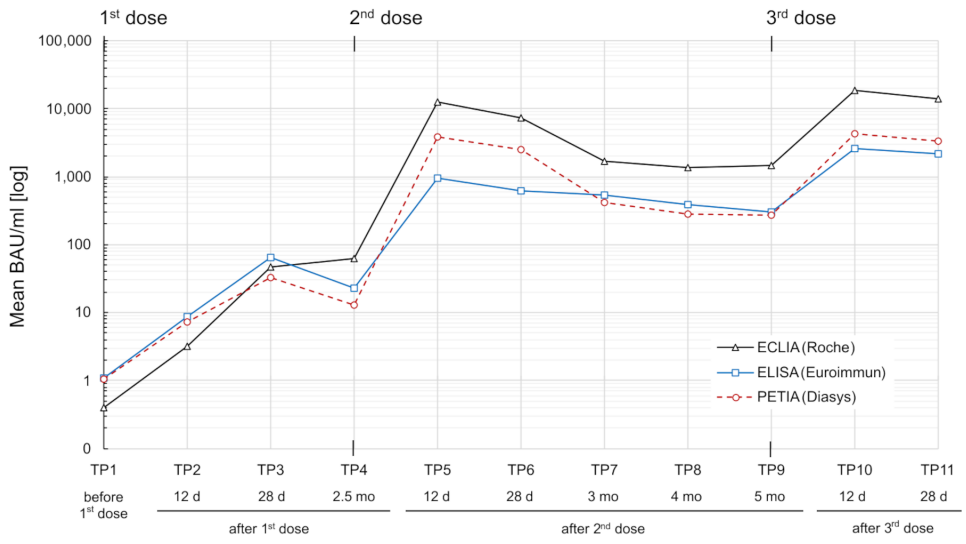

FIGURE 1
SARS-CoV-2 S1-specific antibody response in a heterogeneous vaccination cohort (n = 76) over 8.5 months. Serum samples were measured by three routine immunoassays: ECLIA (Roche; black line), ELISA (Euroimmun; blue line), and PETIA (Diasys; red dotted line). Mean binding antibody units per milliliter (BAU/ml) for each time point (TP1–TP11) are plotted in logarithmic scale.

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TABLE 1 SARS-CoV-2 S1-specific antibody titers (Mean BAU/ml).

Time point	TP1	TP2	TP3	TP4	TP5	TP6	TP7	TP8	TP9	TP10	TP11
ECLIA	0.4	3.2	46.5	62.7	12,704.4	7,410.1	1,705.0	1,379.8	1,456.9	1,8564.4	13,793.6
ELISA	0.4	8.5	61.0	21.8	959.7	582.5	522.6	373.7	284.0	2,601.9	1,766.5
PETIA	1.0	7.1	32.6	13.1	3,885.9	2,491.9	416.6	280.2	267.7	4,267.6	3,347.8
Sample size	68	68	62	59	62	63	59	53	57	54	41

Passing–Bablok regression analysis. Slope and intercept were calculated with their respective 95% confidence intervals (CI), representing the systematic and proportional differences between the assays. Two methods can be considered to have no significant proportional differences if the 95% CI of the slope includes the value 1, e. g., slope = 1.01 (95% CI: 0.99–1.02). All three binding assays showed good overall correlation, with Spearman's rank correlation coefficients (ρ) ranging from 0.77 (ELISA/ECLIA) to 0.92 (PETIA/ELISA) (Figure 2, Table 2). However, Passing–Bablok regression revealed significant proportional differences (deviation of slope from 1.00) between all three anti-S1 assays to varying degrees: 0.06 (ECLIA/ELISA; 95% CI: 0.05–0.07), 0.19 (PETIA/ELISA; 95% CI: 0.16–0.22), and 3.12 (PETIA/ECLIA; 95% CI: 2.80–3.47) (Figure 2, Table 2). The largest proportional difference was observed for ECLIA (anti-RBD) and ELISA (anti-S1), despite improvement by WHO harmonization (from 0.02 to 0.06; see Supplementary Table S6). Of note, the ELISA assay is specific for IgG antibodies, whereas ECLIA and PETIA do not discriminate by isotype, according to the manufacturers. Given the difference in antibody response after SARS-CoV-2 infection versus vaccination, this strongly suggests that calibration to the WHO IS does not improve the comparability of anti-S1 binding assays, especially if the assays are sensitive to differences in the IgG/IgM/IgA ratio.

3.3 Correlation of antibody titers (BAU/ml) and neutralizing potential (sVNT IH%)

Next, we inquired whether we could still define a universal threshold in BAU/ml for all anti-S1 binding assays that correlate with humoral immune protection. Therefore, we analyzed the serum level of neutralizing anti-SARS-CoV-2 antibodies in each sample using a surrogate virus neutralization test (sVNT; SARS-CoV-2 NeutraLISA; Euroimmun).

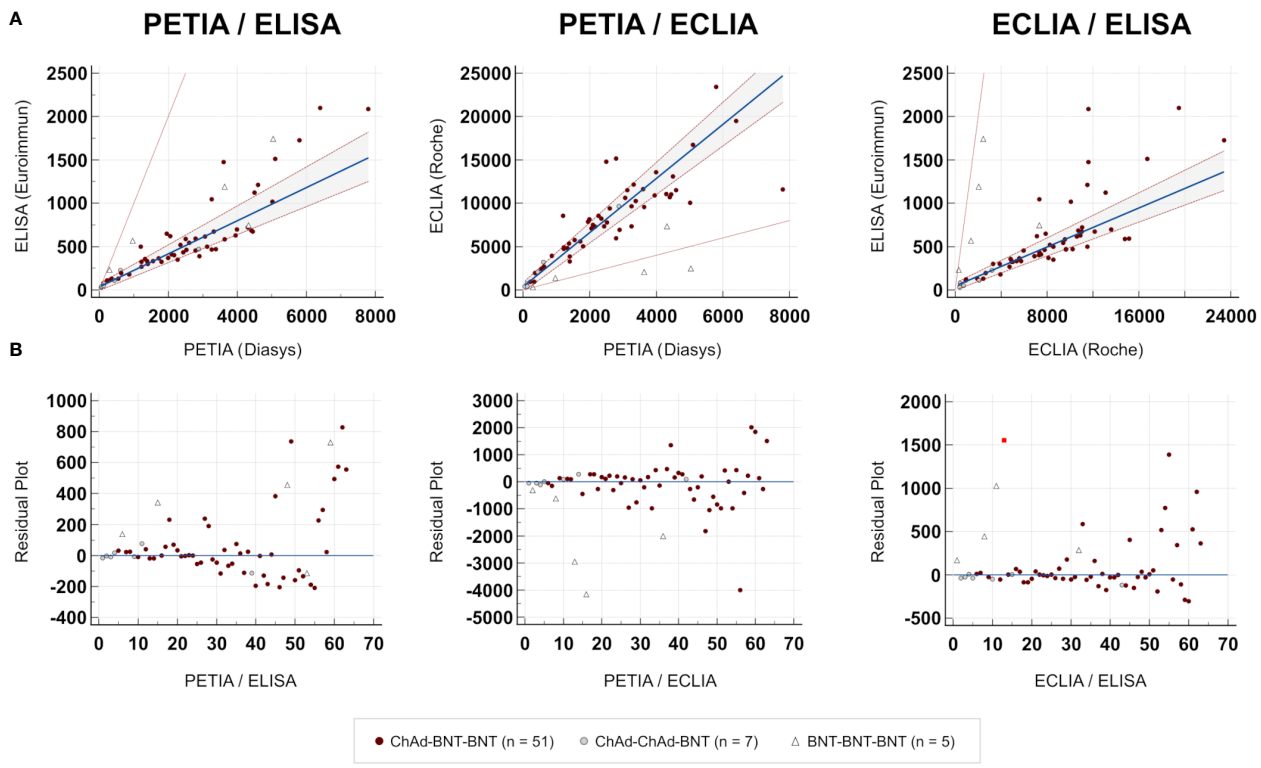

FIGURE 2
Comparison of immunoassays. Passing–Bablok regression analysis (TP6; n = 63). **(A)** Scatter diagram. Regression line (blue line), 95% CI of the regression line (dotted red lines), and identity line (thin red line). **(B)** Residual plot. Distribution of differences from the regression line (blue line). The red square indicates an outlier.

TABLE 2 Passing–Bablok regression analysis (TP6).

	Spearman rank correlation	Passing–Bablok regression		Cusum test
	ρ (95% CI)	Intercept (95% CI)	Slope (95% CI)	P
PETIA/ELISA	0.92 (0.88–0.95)	34.50 (-7.67–71.60)	0.19 (0.16–0.22)	0.24
PETIA/ECLIA	0.82 (0.71–0.89)	367.74 (-200.60–830.25)	3.12 (2.80–3.47)	0.39
ECLIA/ELISA	0.77 (0.65–0.86)	45.73 (-0.70–79.77)	0.06 (0.05–0.07)	0.80

In Figure 3, the percentage inhibition of RBD-ACE2 interaction (IH%) is plotted against the respective antibody titer at five selected time points (TP1, TP3, TP5, TP9, and TP11). In general, the kinetic of neutralizing potential paralleled the observed kinetic of antibody response; and both IH% and BAU/ml mean values peaked within 4 weeks after the second (TP5/TP6) and third (TP10/TP11) vaccinations, respectively (Supplementary Figure S6). The proportion of subjects above the positive sVNT cut-off (≥35 IH%) increased from 0% at TP1 to 98.4% at TP5 (12 days after the second dose; Figure 4A). At TP11 (28 days after the third dose), all subjects, regardless of vaccination schedule, had a neutralizing potential well above the positive cut-off (90% are ≥90 IH%, 100% are >60 IH%, Figure 4A; Supplementary Figure S7).

Interestingly, the decline in neutralizing potential did not parallel the waning in antibody titers after the second vaccination (from TP5 to TP9). While the mean IH% declined by 30% (from 93.8 to 64.4 IH%), the mean BAU/ml decreased more drastically during the same period: by 70% for ELISA, 89% for ECLIA, and 93% for PETIA (Figure 4A; Supplementary Figure S4). Furthermore, the proportion of subjects with neutralizing potential decreased substantially (from 90% of subjects >90 IH% at TP5 to 19% at TP5), but only 14% of the subjects fell below the positive neutralization cut-off at TP9 (from 98% of subjects ≥35 IH % to 84%) (Figure 4B). Despite the large relative change, the mean BAU/ml values did not fall below 100 BAU/ml at TP9 for all three assays, suggesting a potential threshold for immune protection (Figure 1; Supplementary Figure S6).

Given the heterogeneity of our cohort, we compared the kinetics of antibody titers and neutralizing potential by vaccination scheme (Supplementary Figure S7). Subjects receiving homologous prime-boost vaccination (ChAd-ChAd) had lower BAU/ml and IH% values after the second vaccination (TP5) than subjects receiving heterologous vaccination (ChAd-BNT). However, only one of the 76 subjects was clearly below the positive sVNT cut-off at TP5. The same subject was below 100 BAU/ml when measured by ECLIA (Supplementary Figure S7). Thus, for Elecsys® Anti-SARS-CoV-2-S (Roche), antibody titers above 100 BAU/ml may indicate immune protection in our cohort. However, for ELISA and PETIA, 5% and

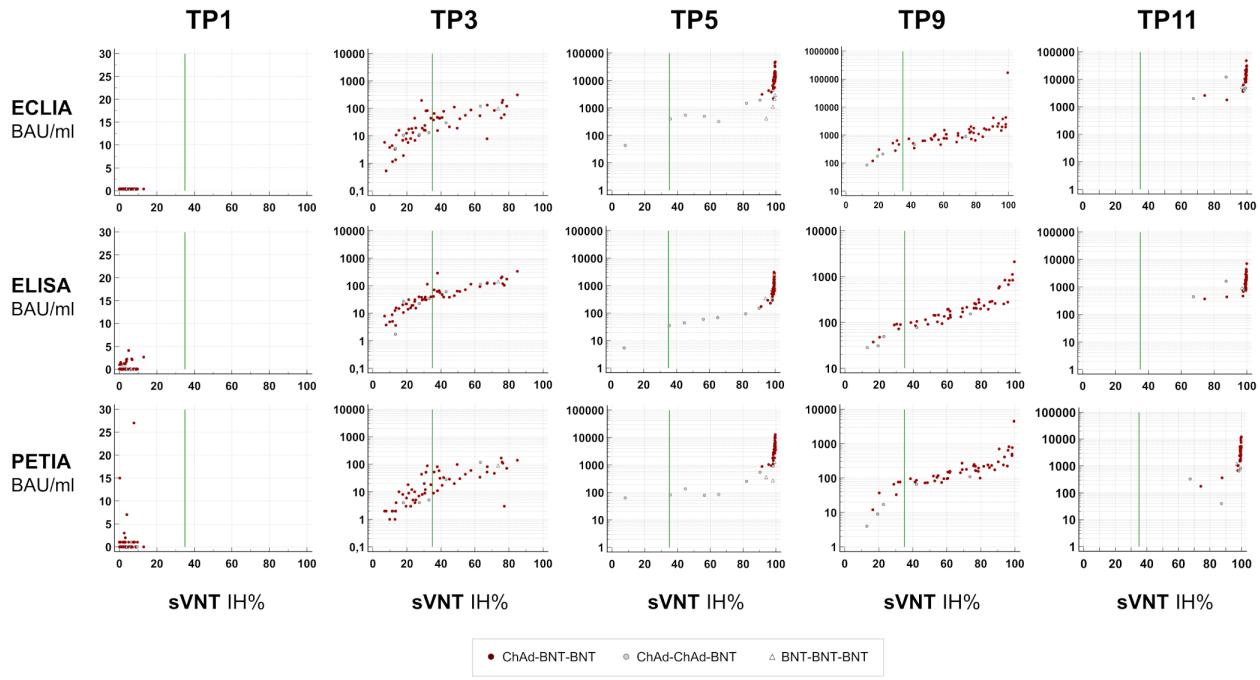

FIGURE 3
Neutralizing potential over time. S1-specific antibody titers (BAU/ml) for five selected time points are plotted against percentage inhibition (IH%) measured by a surrogate virus neutralization test (sVNT; SARS-CoV-2-NeutraLISA; Euroimmun). Negative cut-off, <20 IH%; positive cut-off, ≥35 IH% (green line).

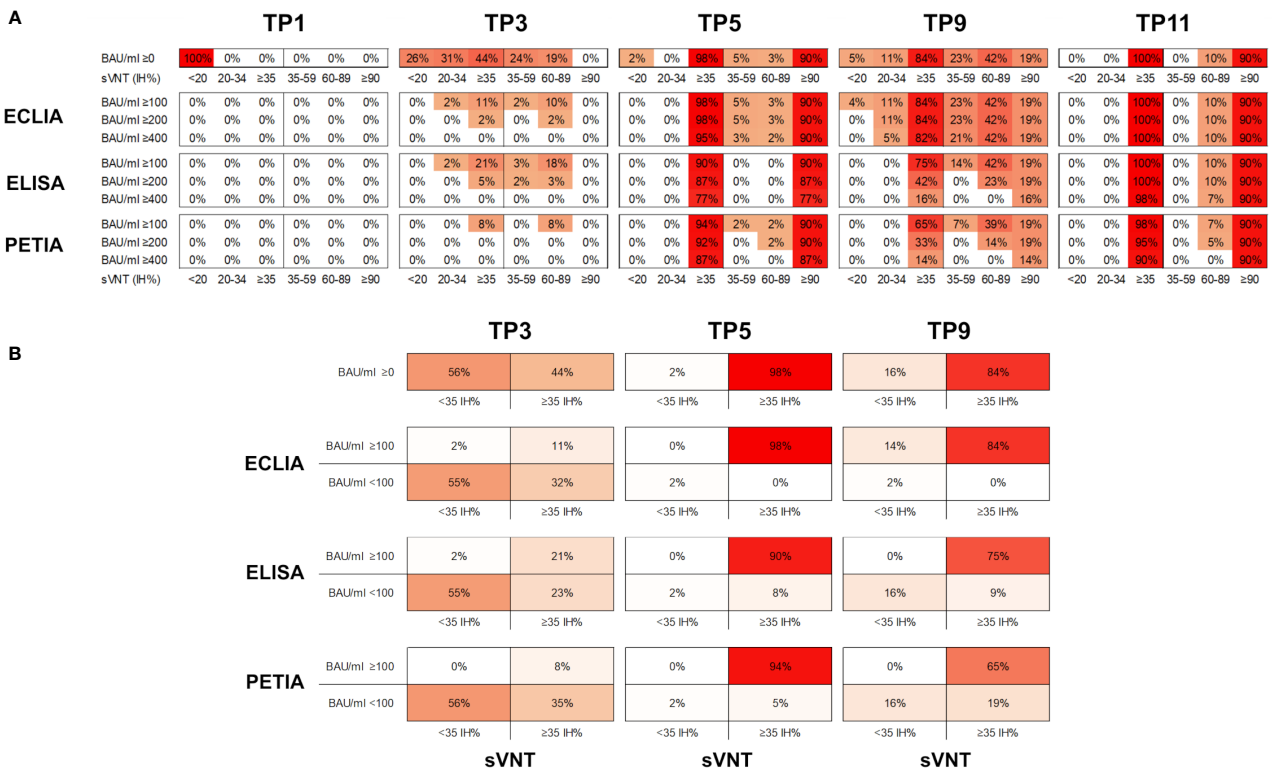

FIGURE 4
(A) Proportion of samples (%) in six different sVNT categories (<20; 20–45; ≥35, 35–59; 60–89; and ≥90 IH%) and above three potential BAU/ml thresholds (≥100; ≥200; and ≥400 BAU/ml) for five selected time points. **(B)** Proportion of samples (%) in four different categories (<35 IH%/<100 BAU/ml; <35 IH %/≥100 BAU/ml; ≥35 IH%/<100 BAU/ml; ≥35 IH%/≥100 BAU/ml) for three selected time points (TP3, TP5, and TP9).

8% of all subjects were still below 100 BAU/ml at TP5, respectively, despite neutralizing potentials ≥35 IH% (Figure 4A; Supplementary Figure S7).

Five months after the second dose (TP9), 84% of all subjects were ≥35 IH% and all had antibody titers >100 BAU/ml as measured by ECLIA (Figure 4B). For ELISA and PETIA, only 75% and 65%, respectively, exceeded both thresholds. By contrast, the BAU/ml threshold failed to identify subjects without immune protection at TP9 for ECLIA: 16% of all subjects were <35 IH% but only 2% were <100 BAU/ml (Figure 4B). For ELISA and PETIA, the 100 BAU/ml threshold predicted subjects without immune protection (all subjects <35 IH% are <100 BAU/ml) but failed to identify subjects ≥35 IH% (i.e., not all subjects ≥35 IH% are >100 BAU/ml).

As seen at TP5, homologous prime-boost vaccination with ChAd-ChAd resulted in lower antibody titers and neutralizing potential than ChAd-BNT (Figure S7). Five months after the first booster (TP9), only 20% of the subjects vaccinated with ChAd-ChAd (4/5) showed antibody titers >100 BAU/ml, as measured by ELISA and PETIA. For ECLIA, 80% of subjects receiving ChAd-ChAd were >100 BAU/ml (100% for ChAd-BNT) (Figure S7). It is worth noting that we do not report significant differences between vaccination regimens due to the inherent limitations of our cohort. Therefore, we cannot draw any conclusions about the superiority of heterologous prime-boost vaccination with ChAd-BNT over BNT-BNT.

4 Discussion

In summary, our results indicate that all S1-specific binding assays facilitate monitoring of the antibody response in vaccinated individuals. All assays resulted in similar antibody kinetics, and increasing antibody titers were associated with increasing inhibitory potential. However, we were unable to define a clear cut-off value in BAU/ml across all methods that would help distinguish subjects above and below 35 IH%, mainly due to the large proportional differences between the binding assays.

The heterogeneity of vaccination schemes and individual immune responses adds another layer of complexity that further complicates the definition of a common threshold in BAU/ml. As previously reported, homologous prime-boost vaccination (ChAd-ChAd) appears to result in lower antibody titers and neutralizing potential than heterologous vaccination (ChAd-BNT). For example, 5 months after the second dose (TP9), all subjects are >100 BAU/ml for ECLIA, whereas all ChAd-ChAd vaccinated subjects are <100 BAU/ml when measured by PETIA, although both are anti-RBD total Ig assays that should correlate comparably with sVNT (Supplementary Figure S7). Interestingly, the substantial decline of S1-specific antibodies observed within 5 months after the first booster vaccination did not reflect a similar decline in inhibitory potential (sVNT IH%). It should be noted that neutralizing potential has been reported for anti-S1 antibodies raised against epitopes outside the RBD, whereas surrogate neutralization assays

such as sVNT are limited to neutralizing anti-RBD antibodies. This may partially explain the observed differences in antibody waning between ECLIA/PETIA (anti-RBD) and ELISA (anti-S1) after the first booster.

The adaptive immune response to SARS-CoV-2 infection results in the activation and clonal expansion of virus-specific B cells. These differentiate into plasma cells and secrete soluble immunoglobins (Ig) into circulation that have different affinities for different viral proteins, mainly the viral spike (S) and nucleocapsid (N) protein in the case of SARS-CoV-2. Although pentameric IgM (low affinity/high avidity) provides the first line of defense, the subsequent seroconversion and production of high-affinity IgG are critical for long-term immune protection (49, 50). Neutralizing antibodies can inhibit the essential interaction between the RBD, located in the S1 subunit of the viral S protein, and the host cell receptor ACE2. Of note, IgA antibodies, which are responsible for mucosal immune defense, have been reported to exhibit ever higher neutralizing potential against SARS-CoV-2 than IgG antibodies (51). Immunization with vector or mRNA vaccines, on the other hand, results in S protein-specific antibodies raised against various epitopes in the S1 and S2 subunits.

The ability of different heterologous and homologous ChAd/BNT vaccination schemes to reduce SARS-CoV-2 infections or severe COVID-19 cases (VE) has been studied in large clinical trial populations (21–26, 52). A correlate of protection (CoP), on the other hand, is a measurable parameter that allows the prediction of immune protection in vaccinated individuals. Although spike-specific antibody titers have been proposed as a promising CoP for COVID-19, it is challenging to define which antibody titers are sufficient for immune protection (34, 37, 53). Several groups compared the antibody response in different ChAd/BNT vaccination cohorts using different routine binding assays (49, 54–56). These assays vary widely in antigen and isotype specificity as well as assay design and detection method. Spaeth et al. and Brehm et al. compared the performance of different N- and S-specific assays in SARS-CoV-2- positive subjects and patients with mild COVID-19 disease, respectively (57, 58). Here, we compared three anti-S1 binding assays in a heterogeneous vaccination cohort that use different parts of the same spike protein S1 subunit as antigen (RBD vs. full S1). It was not the aim of this study to compare the efficacy of different prime-boost vaccination regimens. However, it is worth noting that our results are consistent with previous reports, as homologous prime-boost vaccination with ChAd-ChAd seems to result in lower antibody titers than vaccination with ChAd-BNT or BNT-BNT (TP5). These differences are almost equalized after the third vaccination with BNT (TP11; Supplementary Figure S7) (59–63).

According to the WHO, an arbitrary unit of 1,000 BAU/ml can be used to assist the comparison of binding assays that detect "the same class of immunoglobulins with the same specificity" (45). Therefore, we asked ourselves the following question: How similar must anti-S1 binding assays be— in terms of isotype discrimination and assay principle— to meet this definition?

The ELISA assay (Euroimmun) detects IgG antibodies raised against the entire S1 subunit, whereas the ECLIA and PETIA assays both detect anti-RBD IgG, IgM, and IgA antibodies. Accordingly, PETIA and ECLIA show the smallest proportional difference, whereas ELISA and ECLIA show the lowest correlation and the largest proportional difference. Interestingly, PETIA and ECLIA interact similarly with the convalescent plasma-derived WHO IS (Roche U/ml = BAU/ml and Diasys AU/ml = BAU/ml) but yielded considerably different BAU/ml values in the heterogeneous vaccination cohort (Supplementary Figure S1, Supplementary Table S2) (39, 40). This might be explained by the difference in assay principles: in the Elecsys® Anti-SARS-CoV-2 S assay (Roche), RBD-specific IgG, IgM, and IgA bind to a mix of biotinylated and ruthenylated RBD antigen. The resulting double-antigen sandwich (DAGS) complexes are immobilized on the solid phase via streptavidin-coated microparticles and quantified by electrochemiluminescence measurement. In the turbidimetric SARS-CoV-2 UTAB FS PETIA assay (Diasys), the RBD-antigen is coupled to polystyrene beads and binds to IgM, IgG, and IgA antibodies in the sample. The homogeneous PETIA assay can be more prone to non-specific reactions than heterogeneous technologies such as ELISA or (E)CLIA (64). Pentameric IgM tends to form larger antigen-antibody complexes than monomeric IgG or IgA and may result in a higher signal (65). In conclusion, the differential interaction of all three assays with the reference material, which is derived from SARS-CoV-2 infected individuals, versus vaccine serum strongly suggests that an alternative approach is required to harmonize different anti-S1 assays in a vaccination cohort.

The clinical benefits and intrinsic limitations of serological SARS-CoV-2-specific immunoassays are still vividly discussed (30, 66, 67). In particular, the repeated emergence of highly mutated VOCs, such as the *Omicron* variants, raised concerns that commercially available binding assays may become obsolete too quickly (66, 68, 69). More than thirty alterations have been identified within the *Omicron* spike protein, resulting in significantly reduced anti-RBD antibody binding and immune evasion (70–72). Wey et al. recently reported that the RBD-specific PETIA assay can quantify the antibody response to *Alpha* (B.1.1.7) and *Kappa* (B.1.617.1), while cross-reactivity to *Omicron* (B.1.1.529) is reduced by approximately 50% compared with wild-type virus and all other VOCs (64). In this study, we analyzed serum from subjects vaccinated in 2020/21, before the emergence of *Omicron* variants. We did not systematically compare the performance of all four binding assays in serum from patients infected with SARS-CoV-2 VOCs.

Other groups pointed out the inherent limitations of harmonization to the WHO IS, especially for SARS-CoV-2 binding assays that differ significantly in target antigen (N vs. S protein) and isotype specificity (IgG vs. IgM) (40, 59, 73–75). However, the early and widespread adoption of the WHO standard and the wide availability of conversion factors for commercial SARS-CoV-2 assays led to the following erroneous conclusion: conversion to BAU/ml allows the harmonization of two given SARS-CoV-2 binding assays. Of note, the WHO Expert Committee on Biological Standardization expressed concern that assigning the same unitage for binding assays based on different antigens would allow for an inappropriate use of the WHO IS (76). Our results confirm the distinct behavior of different anti-S1 binding assays: 1) assays that discriminate by isotype (IgG specific) but less by epitope (whole S1 subunit), and 2) assays that are more epitope specific (RBD only), but

less isotype specific (total antibodies). Therefore, SARS-CoV-2 binding assays with the same antigen specificity and similar interaction with the WHO IS interact differently with vaccine serum. Furthermore, the correlation and proportional differences between ECLIA (heterogenous double-antigen sandwich assay) and PETIA (homogeneous turbidimetric assay) seem to change during the 8.5-month vaccination course, indicating a high susceptibility to the serum immunoglobulin composition (changing IgG/IgM/IgA ratio) (Supplementary Figure S7). This discrepancy, which is most likely due to different assay principles, adds another item to the list of hurdles we must overcome if we are to achieve proper harmonization of binding assay results, especially in populations that have received different vaccine regimens of varying efficacy.

Although all COVID-19 vaccines are based on the full-length S protein, the presentation of antigen-derived peptides is strikingly different —not only between protein-based and nucleic acid-based vaccines but also between mRNA (BNT) and vector (ChAd) vaccines. This in turn leads to different CD8+ and CD4+ T cell activation, which shapes the subsequent antibody response (77–80). It is conceivable that the heterogeneity of current vaccine platforms negatively affects the comparability of binding assays that detect total antibodies directed against the same antigen.

A viable way to overcome this limitation in the future would be to harmonize against the material of defined antigen specificity and/or isotype composition. Of note, Freeman et al. characterized five antigen-specific fractions of a serum-based reference material, containing antibodies against the SARS-CoV-2 S protein (anti-S1/S2, -S1, -S2, and anti-RBD) and N protein, for the standardization of IgG and total Ig serological assays (81). Consistent with our observations, the anti-RBD IgG assay (sCOVG) values were approximately the same for anti-RBD and anti-S1 sera, whereas the anti-RBD total antibody assay (COV2T) values were doubled for anti-S1 serum. Interestingly, the authors conclude that it is unlikely that IgM and IgA antibodies contribute to this discrepancy, as the serotiters of both have been reported to decline substantially by 6 weeks after symptom onset when sample collection for the reference material began (80). Nevertheless, the substantial discrepancy between the two anti-RBD total antibody assays, which varies over the course of vaccination in our cohort (Supplementary Figure S7), may still be due to differences in the two detection methods (PETIA vs. ECLIA).

The serum samples used for assay comparison were derived from a small non-representative cohort (n = 76) with a high female/male ratio and variable sample size per time point (n = 41–68; 32 subjects with ≥10 samples). Therefore, we do not report any significant difference between vaccination regimes, nor do we draw any conclusions about the superiority of heterologous prime-boost vaccination (ChAd-BNT vs. ChAd-ChAd). However, this assay comparison study has several limitations. Owing to the limited sample volume provided by the WHO, the 7-point WHO standard dilutions (3.9–250 BAU/ml) were assayed in singlets, which limits the accuracy of measurement. In addition, neutralizing antibodies were assessed by sVNT (inhibition of the RBD-ACE2 interaction), which does not reflect antiviral activity *in vivo*. However, surrogate assays are the only feasible way to estimate the neutralizing capacity of serum samples in clinical routine. The gold standard plaque reduction neutralization test (PRNT) is labor-intensive, time-consuming, and requires Biosafety Level 3 (BSL-3) facilities. Furthermore, sVNT is limited to neutralizing anti-RBD antibodies as detected by ECLIA and PETIA, whereas the ELISA assay detects antibodies against the entire S1 subunit (6–8).

Vaccine efficacy must be assessed using gold standard methods and studies must demonstrate a significant reduction in COVID-19 cases and/or severe disease progression in large study populations. However, in routine clinical practice, we must rely on cost-effective surrogate markers and surrogate assays to assess and evaluate the individual immune response in vaccinated individuals. Nevertheless, it is still under debate which marker(s) should be used for monitoring and what cut-off indicates adequate immune protection.

As discussed above, an *in vitro* CoP will never accurately predict vaccine efficacy and vaccine-induced immune protection in individuals, especially for highly evolving viruses, such as *Coronaviridae*. The recent COVID-19 pandemic has highlighted the need for rapid and flexible vaccine development and manufacturing, to ensure immune protection against emerging VOCs.

In summary, our results underscore the urgent need for rapidly evolving technology, not only for vaccines but also for serological binding assays, and for the continued development of both —bioanalytical methods and dedicated higher-order reference materials— to keep pace with rapidly mutating viruses. For future viral pandemics, if we are to use total antibody assays to monitor the vaccine-induced immune responses and predict immune protection in vaccinated individuals, we should strive to be more aware of method-specific differences and focus on the development of higher-order reference standards. Each reference material should be appropriate for the diagnostic task at hand, e.g., monitoring the antibody response post-infection versus post-vaccination.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ärztekammer Nordrhein, Tersteegenstr. 9, 40474 Düsseldorf, Germany. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SS: Formal Analysis, Writing – original draft, Writing – review & editing, Investigation, Methodology, Visualization. CL: Writing – review & editing, Conceptualization, Data curation, Supervision. YS: Conceptualization, Data curation, Supervision, Writing – review & editing, Resources. BK: Writing – review & editing, Investigation, Methodology. JA: Investigation, Methodology, Writing – review & editing. HS: Investigation, Methodology, Writing – review & editing,

Validation. TM: Investigation, Validation, Writing – review & editing, Data curation, Formal Analysis. CP: Validation, Writing – review & editing, Resources. MG: Resources, Writing – review & editing, Conceptualization, Data curation, Formal Analysis, Supervision, Writing – original draft.

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Conflict of interest

Authors SS, CL, YS, BK, JA, and HS are employed at Medizinisches Versorgungszentrum für Labormedizin und Mikrobiologie Ruhr GmbH Essen, Germany. MG is employed at DiaServe Laboratories GmbH Iffeldorf, Germany. MG and TM are employees of DiaSys Diagnostic GmbH Holzheim, Germany and are named as inventors on a patent application Deutsche Patentanmeldung 10 2020 122 593.8 claiming the manufacturing and use of the described PETIA for serological quantification of anti-SARS-CoV-2 antibodies.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1257265/full#supplementary-material

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Immunological insights into COVID-19 in Southern Nigeria

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Introduction: One of the unexpected outcomes of the COVID-19 pandemic was the relatively low levels of morbidity and mortality in Africa compared to the rest of the world. Nigeria, Africa's most populous nation, accounted for less than 0.01% of the global COVID-19 fatalities. The factors responsible for Nigeria's relatively low loss of life due to COVID-19 are unknown. Also, the correlates of protective immunity to SARS-CoV-2 and the impact of pre-existing immunity on the outcome of the COVID-19 pandemic in Africa are yet to be elucidated. Here, we evaluated the natural and vaccine-induced immune responses from vaccinated, non-vaccinated and convalescent individuals in Southern Nigeria throughout the three waves of the COVID-19 pandemic in Nigeria. We also examined the pre-existing immune responses to SARS-CoV-2 from samples collected prior to the COVID-19 pandemic.

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Methods: We used spike RBD and N- IgG antibody ELISA to measure binding antibody responses, SARS-CoV-2 pseudotype assay protocol expressing the spike protein of different variants (D614G, Delta, Beta, Omicron BA1) to measure neutralizing antibody responses and nucleoprotein (N) and spike (S1, S2) direct ex vivo interferon gamma (IFNγ) T cell ELISpot to measure T cell responses.

Result: Our study demonstrated a similar magnitude of both binding (N-IgG (74% and 62%), S-RBD IgG (70% and 53%) and neutralizing (D614G (49% and 29%), Delta (56% and 47%), Beta (48% and 24%), Omicron BA1 (41% and 21%)) antibody responses from symptomatic and asymptomatic survivors in Nigeria. A similar magnitude was also seen among vaccinated participants. Interestingly, we revealed the presence of preexisting binding antibodies (N-IgG (60%) and S-RBD IgG (44%)) but no neutralizing antibodies from samples collected prior to the pandemic.

Discussion: These findings revealed that both vaccinated, non-vaccinated and convalescent individuals in Southern Nigeria make similar magnitude of both binding and cross-reactive neutralizing antibody responses. It supported the presence of preexisting binding antibody responses among some Nigerians prior to the COVID-19 pandemic. Lastly, hybrid immunity and heterologous vaccine boosting induced the strongest binding and broadly neutralizing antibody responses compared to vaccine or infection-acquired immunity alone.

KEYWORDS

COVID-19, SARS-CoV-2, immunity, vaccine, Nigeria, pre-pandemic, preexisting

Introduction

Given the pace of SARS-CoV-2 transmission, its relatively high morbidity and mortality rate, and its global impact, COVID-19 has recently become one of the most severe pandemics. Over six hundred and ninety million people were infected, and almost seven million died globally (1). This virus's indiscriminate and rapid spread across international borders resulted in mild, moderate, and severe outcomes, necessitating a variety of public health responses in different countries and among different demographics. Nigeria, the most populated country in Africa with many highly populated cities and fragile health care, is poised for an explosive spread of SARS-CoV-2. Interestingly, as of October 2023, Nigeria's reported confirmed infections (266675 cases) and mortality in only (3155 individuals), which were significantly lower than other highly populated countries (1). The reasons for this are not entirely clear, but certain factors, such as the mixing of the population (increased exposure) and the immunological status of its population, may be responsible for the differences in the outbreak in Nigeria (2). With the availability of vaccines, the global morbidity and mortality from COVID-19 has been greatly reduced. Efforts are directed towards understanding the features of natural, vaccine-induced and combined (hybrid) protective immunity that will provide greater insight and understanding to increase preparedness, prevent or contain future pandemics (3, 4). Studies have shown that the humoral (neutralizing antibody) responses are important in blocking the entry of SARS-CoV-2 and reducing fatal COVID-19 diseases, but lower titres do not prevent SARS-CoV-2 infection (5). With numerous variants of concern capable of evading the antibody (neutralizing) response, the T-cell response has been shown to be an important second barrier to disease and more durable (5–7). Moreso, combined or hybrid immunity, the acquisition of both vaccine and naturally acquired immunity through exposure and infection, has been documented to induce the most robust immune responses and provided the greatest cross-protection against the different variants of SARS-CoV-2 (8). Preexisting immunity from seasonal coronavirus may potentially result in cross-protection against SARS-CoV-2 in different regions of the world (9). It has also been proposed that immunity from burden of concurrent exposure to other diseases has been

responsible for the less catastrophic outbreak in Africa compared to other parts of the world. However, the impact of both pre-existing and community acquired immunity during the pandemic is yet to be elucidated in Nigeria. Notably, the vast majority of SARS-CoV-2 immune correlates data from the COVID-19 pandemic are based on reports from developed nations, with a paucity of immunological data emanating from Africa where the pandemic was less catastrophic than in other parts of the world (10).

This study assessed both the natural and vaccine-induced immune responses from vaccinated, non-vaccinated and convalescent individuals in Southern Nigeria throughout the three waves of the pandemic in Nigeria. Also, we assessed pre-existing immune response from patient's samples collected prior to the COVID-19 pandemic. Our data provides immune correlate data from Africa's most populous nation and measures the impact of pre-existing immunity on the outcome of the COVID-19 pandemic.

Methods

Study participants

The study participants were enrolled through ACEGID Clinical site networks in Abakaliki (Alex Ekwueme Federal University Teaching Hospital Abakaliki (AE-FUTHA), Ebonyi State), Owo (Federal Medical Centre (FMC) Owo, Ondo State), Osun State University Teaching Hospital (UNIOSUNTH) Osogbo, Osun State, Nigeria between February 2021 and December 2022. The study comprised four categories of participants: (i) hospitalized and COVID-19 convalescent patients (survivors N=89) with a negative SARS-CoV-2 polymerase chain reaction [PCR]/RDT tests at the time of sample collection, (ii) exposed non hospitalized asymptomatic contacts (exposed asymptomatic participants N=34), (iii) SARS-CoV-2 vaccinees (N=517) and (iv) prepandemic sera (N=64) (Tables 1, 2). The inclusion criteria is that the participant is able and willing to provide written consent or assent (if underage) to participate in the study and willing to share contact and location for follow up study and for the vaccinated cohorts, must have received a single or complete dose(s) of either the AstraZeneca (AZD-1223), or Janssen (Ad26.CoV2.S) vaccine, Pfizer or Moderna COVID-19 vaccines administered preferably within three months but not more than six months prior to study enrollment. The exclusion criteria on the other hand, is that the participation do not have a positive or confirmed SARS-CoV-2 polymerase chain reaction [PCR]/RDT tests at the time of sample collection. Also, that the participant do not have any significant condition (medical, psychological, psychiatric, or social), which, in the judgment of the study investigator, might interfere with the conduct of the study. There were more females (60%, 62%) than males (40%, 38%) in both COVID-19 survivors and vaccinees. The mean age for both survivors and the vaccinees were 36.03(± 14.89 years) and 38.09(± 10.76 years) at 95% confident interval respectively.

PBMC isolation and serum separation

Plasma and peripheral blood mononuclear cells (PBMCs) were separated immediately following manufacturer instructions (Sigma-Aldrich, Z642843). 10mL of whole blood was transferred from the EDTA tubes into LeucoSep-tube containing ficoll-hypaque at a ratio of 2:1. The tube was centrifuged at 800 x g for 30 minutes at room temperature in a swinging-bucket rotor with no break. The top layer of plasma was removed, and the buffy coat interface was collected, washed twice with PBS-EDTA (10 mM), and centrifuged for 10 minutes at 250 x g with the brake on. The pelleted cells were suspended in red blood cell lysis buffer (1 mM KHCO3, 0.15 M NH4Cl, 0.1 mM EDTA, HCl pH 7.2 to 7.4) at room temperature for 5 minutes. The cells were washed again with PBS-EDTA, centrifuged at 250 x g for 10 minutes at 4°C and resuspended in appropriate medium (Leibovitz medium, Sigma-Aldrich, L1518) for further assay (ELISpot). The plasma was centrifuged at 250 x g for 5 minutes at 4°C and transferred to a new 15 mL tube to remove cells and debris. Both the PBMCs and plasma were transferred to 2mL cryotubes for further assay (ELISpot and ELISA) and storage at -80°C.

ELISpot

PBMCs were re-suspended in 10 mL of media (500 mL Leibovitz media supplemented: 5 mL Pen/Strep, 5 mL L-glutamine, 12.5 mL HEPES, 0.5 mL 2-mercaptoethanol) and were plated onto customized ELISpot plates (Catalogue no: 10602KMM) coated with IFNγ ($2x10^5$ cells/well) according to manufacturer's instructions. 100µl (1µg/mL) of PepMixTM SARS-CoV-2 spike peptides (JPT, PM-WCPV-S-1 (pooled into S1 and S2 covering

TABLE 1 The seroprevalence of SARS-CoV-2 S-RBD and N binding (IgG) and neutralizing antibody response among survivors and their contacts in Southern Nigeria.

	ELISA			Virus Pseudotype Neutralization		
Subpopulation	N-IgG (%)	S-RBD IgG (%)	D614G (%)	Beta (%)	Delta (%)	Omicron BA1(%)
Survivor	74	70	49	48	56	41
Contact	62	53	29	24	47	21
Pre-pandemic	60	44	0	0	0	0

TABLE 2 Vaccine and convalescent sera distribution.

Vaccines	Doses	Negative to SRBD and N	Positive to N	Positive to SRBD	Positive to SRBD and N	Total
AstraZeneca	Single	10	4	21	24	59
	Double	2	3	17	45	67
	Third			1		1
Pfizer	Single	7	7	14	41	69
	Double	1	5	15	43	64
	Third		2	15	40	57
Moderna	Single	6	7	10	29	52
	Double	4	7	24	39	74
	Third				1	1
Janssen	Single	6	1	14	24	45
	Double	3	2	8	15	28
	Third					
		39	38	139	301	517
%Total		7.54	7.35	26.89	58.22	

the entire SARS-CoV-2 spike) and the nucleoprotein peptides (JPT, PM-WCPV-NCAP1) were added to each well according to the plate map (see Supplementary Table 1). All peptides were 15mer with 11 amino acid overlaps. anti CD3 and vehicle control (media) was then added separately to individual wells of the customized ELISpot plates containing the PBMC (anti CD3 and vehicle control (media) were used as both positive and negative controls respectively). The plate was incubated in the hood for 20-24 hours at 37°C and 5% CO_2 with no disturbance. After incubation, 80µL of detection solution was added to each well and incubated for 2 hours at room temperature following washing twice with 0.05% Tween-PBS. Thereafter, the detection solution was decanted and wells were washed three times with 0.05% Tween-PBS and incubated with 80uL of tertiary solution for another 30 minutes at room temperature. The plate was later washed two times with 0.05% Tween-PBS and two times with distilled water, 200µL/well each time. 80uL/well of blue developer solution was added and incubated for 15 minutes at room temperature. The reaction was stopped by gently rinsing the membrane with tap water, and decanting; this step was repeated three times. The protective underdrain was removed, and the back of the plate was also rinsed with tap water. The plate was air-dried for 24 hours face down on paper towels on the bench top. Scanning and plate count was done using CTL immunospot counter. For all wells, the numbers of spot forming units (SFU) were determined using SmartCountTM and Autogate. Tests and controls were carried out in duplicates for each sample. Counts per sample were obtained by subtracting the mean of background SFU (Media) from the mean of peptide specific SFU then expressed as SFU/million PBMCs. The threshold for detection of a positive response was assigned 40 SFU/million PBMCs; this is the mean SFU multiplied by 3x standard deviation from three known negative samples.

ELISA

ELISA was performed on human plasma using ReSARSCoV-2 (S-RBD) and ReSARSCoV-2 N IgG ELISA Test Kit (10180 and 10166, Zalgen Labs, LLC) with either S-RBD or N as the capture antigens according to the manufacturer's instructions. Lyophilized human monoclonal calibrator and negative control plasma were reconstituted with 0.10 mL and 0.25 mL laboratory-grade water respectively. Calibrator was diluted 1:101 (0.01 mL/1.0 mL followed by four threefold serial dilutions to create a calibration curve for antibody concentration estimation. Calibrator (or Reference) dilutions, diluted negative control and patient samples (1:100) were transferred (0.1 mL/well) in duplicate wells. Microwell plates were incubated at ambient temperature (18–30°C) for 30 minutes. Microwell plates were washed four times with 0.05% Tween-PBS wash buffer. Anti-human IgG or IgM-horse- radish peroxidase conjugated reagent was added to each well (0.1 mL/well) followed by a 30 minute incubation at ambient temperature. After repeating the PBS-Tween wash, 3,3′,5,5′-Tetramethylbenzidine (TMB) Substrate was added to each well (0.1mL/well). The TMB substrate was incubated for 10 minutes followed by the addition (0.1 mL/well) of Stopping Solution (2% Methane sulfonic Acid). Developed ELISA plates were read at 450 nm (with 650 nm reference). IgG concentration was estimated using the Optical Density (OD) reading from the ELISA plate reader. The negative cut-off (0.3) was determined as the mean multiplied by three standard deviations of

three known negative samples (mean(3SD) three samples from participants with no prior exposure to SARS-CoV-2).

Virus Pseudotype Neutralization Assay

We used SARS-CoV-2 pseudotype assay protocol described by Di Genova et al. (2020) (11). To produce the pseudotyped Viruses (PVs) expressing the spike protein of different variants (D614G, Delta, Beta, Omicron BA1), HEK293T/17 cells were transfected with HIV Gag-pol, pCSFLW firefly luciferase and the SARS-CoV-2 spike plasmids using FuGENE-HD, incubated for 48 hours at 37°C and 5% CO2, and the supernatant harvested. To determine the titre of the PVs, on day 1 the HEK293T/17 cells were transfected with ACE-2 and TMPRSS2 plasmids to be used as target cells. On day 2, on a 96-well plate, the PV supernatants were serially diluted 1:2 in DMEM. The target cells were added to the 96-well plate at 10,000 cells/well. The PV production titre (in relative light units per ml; RLU/ml) was calculated from the luciferase expression measured on day 4 using Bright-Glo (Promega) reagent with the luminometer GloMax Explorer (Promega). The neutralising IC_{50} of the human sera was determined by serially diluting the samples 1:5 in DMEM in a 96-well plate and incubating for one hour with $5x10^5$ – $5x10^6$ RLU per well of PV. Transfected target cells (as above) were added at a density of 10,000 cells per 96-well and incubated for 48 hours. The RLU was measured as above, and the IC_{50} calculated using GraphPad Prism according to Ferrara and Temperton (2018) (12).

Data analysis and statistical methods

Data was analyzed using Microsoft Excel (version 16.39, Microsoft, Redmond, WA) and GraphPad Prism (version 8.4.2, 2020, GraphPad Software, Inc., San Diego, CA). Discrete and categorical variables were presented as frequencies and percentages and were compared using test of proportion by calculating chi-square. Continuous variables were presented as geometric mean (GM) and 95% confidence interval of the GM and compared using a non-parametric test (Mann-Whitney U test). All tests of significance were two-tailed and values of $P < 0.05$ were indicative of statistical significance.

Ethical approval

All methods were carried out in accordance with relevant guidelines and regulations. All subjects enrolled in this study and/or their legal guardians provided written informed consent. Human subjects testing and sample collection, was approved by the Redeemer's University Institutional Review Board, the Nigerian National Health Research Ethics Committee (SIP-NG-NHREC/01/01/2007-12/01/2021, ARISE-NHREC/01/01/2007-11/02/2022), Federal Medical centre (FMC), Owo, Alex Ekwueme Federal Teaching Hospital (AE-FUTHA) Ethics and Scientific Research Committee and the University of Cambridge Institutional Review Board. Once informed consent is obtained from the participants, blood samples were collected from study participants and processed in the Virology Laboratory at the Alex Ekwueme Federal University Teaching Hospital (AE-FUTHA) Abakaliki Ebonyi State and Federal Medical Centre (FMC) Owo, Ondo State. Only qualified Nigerian medical personnel and laboratory staff were involved in the administration of questionnaire and sample collection from the participants.

Results

Both hospitalized convalescent and non-hospitalized exposed contacts make similar binding and neutralizing antibody responses to SARS-CoV-2 antigens.

Using standardised protocols and validated kits (ReSARSCoV-2 (S-RBD and N) Kit (10180 and 10166, Zalgen Labs, LLC)), we estimated the binding antibody responses (IgG) of both hospitalized COVID-19 survivors and their non-hospitalized exposed contacts (exposed to SARS-CoV-2 positive individuals but with no history of COVID-19 symptoms or a positive SARS-CoV-2 polymerase chain reaction [PCR] test at the time of sample collection). This group included individuals who managed or provided care to COVID-19 acute or convalescent patients such as family members, clinicians, and nurses. Of the 123 sera tested (89 survivors and 34 contacts), both survivors and contacts had similar percentages of IgG responders to SARS-CoV-2 S-RBD and N protein (Table 1). Interestingly, the mean OD was not significantly different (Mann–Whitney test, p=0.55,0.09) between the survivors and the contacts both for S-RBD IgG and N-IgG (Figure 1A). From these, we selected those with binding antibodies and measured their neutralizing potential against four different SARS-CoV-2 pseudotype viruses (PV) expressing the full-length spike of the original Wuhan-Hu-1 isolate, and the successive variants- beta, delta and omicron BA1 respectively. Neutralizing antibodies against these selected SARS-CoV-2 PVs were similar to the binding antibody response data. Notably, both survivors and their contacts' sera had neutralizing antibodies against one or more of the SARS-CoV-2 PVs, and no significant difference (Mann–Whitney test, p=0.94,0.23,0.81,0.25) was detected in the mean IC50 of both the survivor and contacts. (Figure 1B).

Hybrid immunity and with different vaccines induced stronger binding and neutralizing IgG antibody responses to the SARS-CoV-2 RBD antigens.

Similar to the standardised protocols and validated kits described above, we estimated the binding and neutralizing antibody responses from COVID-19 vaccinated participants' sera. We compared sera from different participants (n=517) that received four different vaccine types at different doses (AstraZeneca (ADV1222), Janssen (Ad26.COV2.S), Pfizer (BNT162b2) and Moderna (mRNA-1273). Not unexpectedly, we found that many vaccinated participants' sera had positive binding antibody responses to both SARS-CoV-2 S-RBD and N antigens. The positive N IgG responses evidenced that vaccinated participants had prior exposure to either SARS-CoV-2 or cross-reactive coronaviruses as vaccines used were devoid of N antigens, thus providing evidence of previous infection and hybrid immunity. Interestingly, when we measured the binding antibody responses to S-RBD IgG in the SARS-CoV-2 vaccinated sera, participants with hybrid immunity and those

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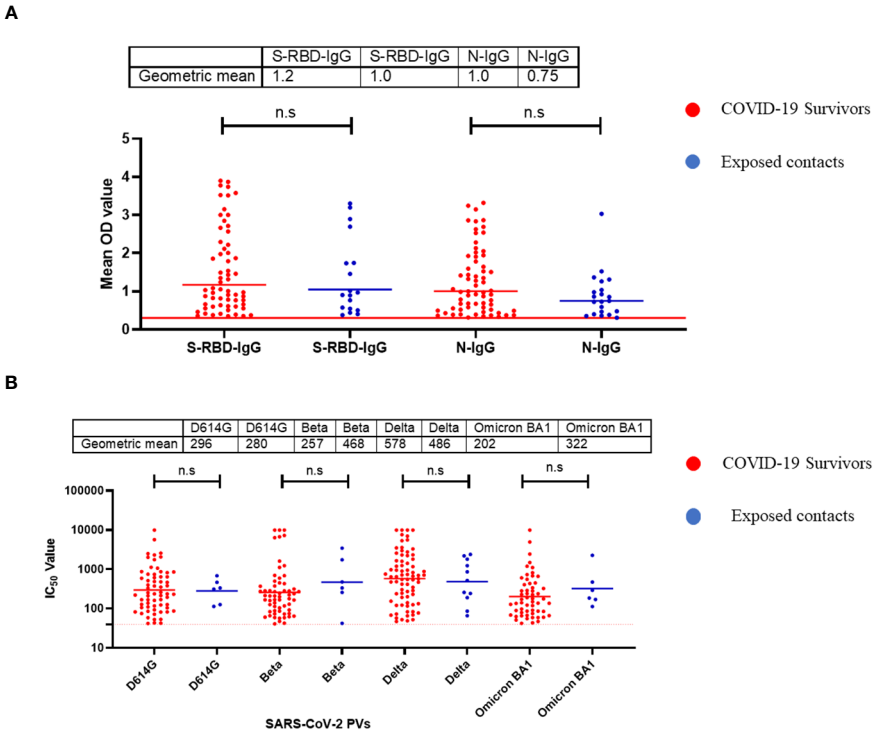

FIGURE 1
Binding antibody responses (IgG) to SARS-CoV-2 S-RBD and N proteins **(A)** and neutralizing antibody response to SARS-CoV-2 PVs **(B)** from COVID-19 survivors and their contacts in Southern Nigeria. Specific SARS-CoV-2 S-RBD and N antigen ELISA were used to measure the binding antibody response (IgG) from hospitalized COVID-19 convalescent survivors (89) and non-hospitalized asymptomatic contacts (34). We used a 1:100 dilution of the serum sample. Among those with binding antibody response, we selected some sera and measured the neutralizing titre (IC50) against SARS-CoV-2 PVs expressing the S-RBD of different variants (D614G, Beta, Delta and Omicron). The OD of the negative cutoff was selected as the mean multiplied by three standard deviations of three known negative samples (0.3) for binding antibody and (40) for neutralizing antibody response (limit of detection). The table shows the geometric mean at 95% CI. Statistical significance was calculated by Mann–Whitney test and p values are indicated. (Capped line with * indicating significance.

that received different vaccine booster combinations (i.e. ADV1222 and BNT162b2) over three dosages had the strongest binding antibody IgG response to S-RBD (Mann–Whitney test, p=0.16) compared to those that received booster vaccinations with just one type of vaccines alone (Figure 2A). We further measured the neutralizing antibodies against the SARS-CoV-2 PV panels. Not unexpectedly, sera from vaccinated participants and those with hybrid immunity also had significantly stronger (Mann–Whitney test, p=0.01) neutralizing antibody responses to all the SARS-CoV-2 S-RBD PVs compared to the sera from convalescent participants (Figure 2B).

Binding and neutralizing antibody responses from convalescent participants correlated with waves of COVID-19 in Southern Nigeria.

Nigeria experienced four major waves of COVID-19 to date. Wave one was from February 2020 to August 2020 and was dominated by the ancestral Wuhan strain (D614G), wave two from September 2020 to March 2021, was dominated by the eta and alpha variants, while the delta variant was the dominant wave three and lasted from April 2021 to November 2021 (13). Wave four, the omicron wave, began in December 2021 until today (Figure 3A). We evaluated the binding and neutralizing antibody responses from convalescent individuals' sera collected during three of the four major waves of COVID-19 in Nigeria. By the fourth wave, samples were not prospectively collected due to extensive vaccine coverage, and relatively few people presented to the hospitals during the omicron wave. Samples collected were subdivided into different wave groups based on the date of COVID-19 diagnosis or hospital admission. Our results revealed that sera collected during the third wave, dominated by the delta variants, had the strongest binding antibody response. This was corroborated by our pseudotype-neutralizing antibody assay, which also showed that sera collected during the third wave had the strongest neutralizing antibody responses and the delta variant was the most frequently neutralized of the SARS-CoV-2 variants tested while the omicron was the least neutralized (Figure 3B, C).

Pre-pandemic sera's cross-reactive binding antibody responses to SARS-CoV-2 did not neutralize any of the SARS-CoV-2 or variants.

Randomly selected sera (n=79) from positive and negative Lassa fever patients collected before the pandemic (2018-2019) were

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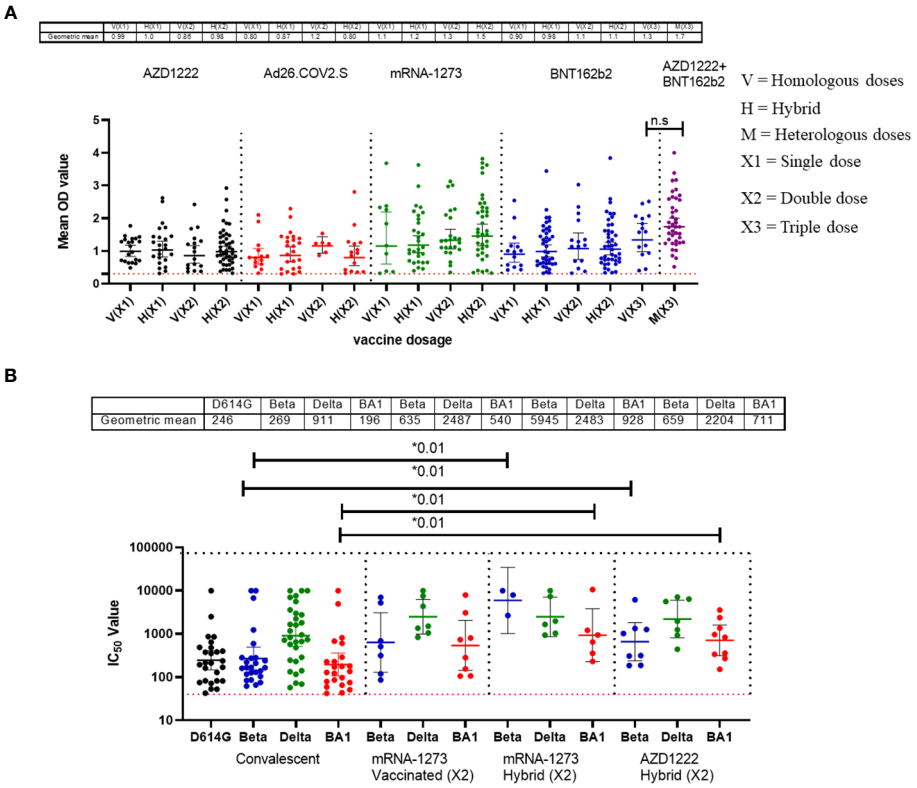

FIGURE 2
Binding antibody responses (IgG) to SARS-CoV-2 S-RBD proteins **(A)** and neutralizing antibody response to SARS-CoV-2 PVs **(B)** from COVID-19 vaccinees in Southern Nigeria. Specific SARS-CoV-2 S-RBD antigen ELISA was used to measure the binding antibody response (IgG) from COVID-19 vaccinees (521). We used a 1:100 dilution of the serum sample. Among those with binding antibody response, we selected some sera (50) and measured the neutralizing titre (IC50) against SARS-CoV-2 PVs expressing the S-RBD of different variants (D614G, Beta, Delta and Omicron). The OD of the negative cutoff was selected as the mean multiplied by three standard deviations of three known negative samples (0.3) for binding antibody and (40) for neutralizing antibody response (limit of detection). The table shows the geometric mean at 95% CI. Statistical significance was calculated by Mann–Whitney test and p values are indicated. (Capped line with * indicating significance).

tested for binding antibody response to SARS-CoV-2 S-RBD and N antigens and neutralizing antibody responses to the same panel of SARS-CoV-2 pseudotype viruses (PVs) expressing the spike of the ancestral strain (D614G), beta, delta and omicron BA1. Notably, cross-reactive serological antibody responses to both S-RBD and N protein of SARS-CoV-2 were identified in 35(44%) and 47(60%) pre-pandemic sera available (Table 1). Interestingly, these responses were in the same range as binding antibody responses from documented COVID-19 survivors (Figure 4). However, none of the pre-pandemic sera had detectable neutralizing antibody responses to any SARS-CoV-2 S-RBD PVs in contrast to sera from COVID-19 survivors (Data not shown).

Hospitalized COVID-19 survivors had T cell responses to both S and N proteins of SARS-CoV-2

T cell responses among COVID-19 survivors were evaluated using direct ex vivo interferon gamma (IFNγ) T cell ELISpot. SARS-CoV-2 spike (S1, S2) and N 15mer peptides from D614G strain were generated and used for this assay. Peptides were incubated with PBMCs from survivors (n=89) in culture overnight, and IFNγ spots were counted as a read-out for active T cell response (spot forming unit per 1 million cells). The average limit of detection (red line=40) was calculated as the mean multiplied by three standard deviations of the three known negative/naïve samples (individuals with no known previous exposure to SARS-CoV-2). Of the 89 samples analyzed for their T cell responses, 69% of the survivors were found to have T cell responses to either spike (S1, S2 peptides and N, or both peptides' pools above detectable threshold for confirmed naive/negative individuals. The highest frequency of T cell response were observed in the S2 pool amino acid regions compared to the S1 and N peptides (Figure 5).

Discussion

Details of the COVID-19 pandemic in Africa remain to be elucidated. The impact of SARS-Cov-2 was not as severe as in many other parts of the world (14, 15). For example, Nigeria, Africa's most populous nation and 6th most populous country in the world had less than one percent (0.01%) of the global COVID-19 morbidity and mortality (1). The factors responsible for the relatively

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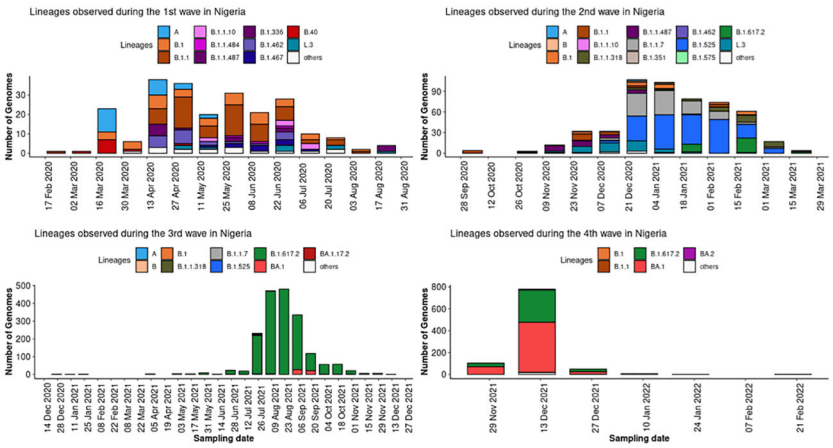
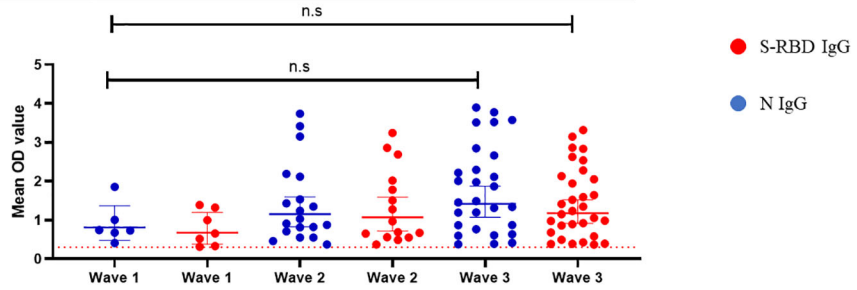
A



B

	Wave 1	Wave 1	Wave 2	Wave 2	Wave 3	Wave 3
Geometric mean	0.81	0.68	1.2	1.1	1.4	1.2



C

	D614G	D614G	D614G	Beta	Beta	Beta	Delta	Delta	Delta	BA2	BA2	BA2
Geometric mean	294	196	247	169	224	184	116	449	1355	93	172	243

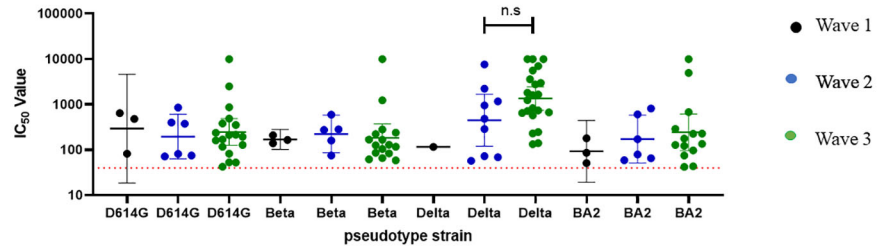


FIGURE 3
Binding antibody responses (IgG) to SARS-CoV-2 S-RBD and N proteins **(B)** and neutralizing antibody response to SARS-CoV-2 PVs **(C)** from COVID-19 survivors based on the waves of pandemic **(A)** in Southern Nigeria. Specific SARS-CoV-2 S-RBD and N antigen ELISA were used to measure the binding antibody response (IgG) from hospitalized COVID-19 convalescent survivors (89). The serum was grouped based on the time of infection/diagnosis into the three waves of the COVID-19 pandemic. We used a 1:100 dilution of the serum sample. We also measured the neutralizing titre (IC50) against SARS-CoV-2 PVs expressing the S-RBD of different variants (D614G, Beta, Delta and Omicron). The OD of the negative cutoff was selected as the mean multiplied by three standard deviations of three known negative samples (0.3) for binding antibody and (40) for neutralizing antibody response (limit of detection). The table shows the geometric mean at 95% CI. Statistical significance was calculated by Mann–Whitney test and p values are indicated. (Capped line with * indicating significance).

low morbidity and mortality in Nigeria are not clear. The presence of uncharacterized endemic Coronaviruses or concurrent infections in Africa, have been postulated (16). Other factors such as differences in age distribution dynamics, testing have also been hypothesized as the drivers of the relatively low morbidity and mortality in Africa (2). As we enter the post-pandemic phase, opportunities are directed towards understanding such questions concerning natural and acquired immunity to SARS-CoV-2 in Africa and how it impacted different populations globally. Unfortunately, there is a paucity of information on the immunity to SARS-CoV-2 from the African continent as the majority of the COVID-19 research has come from resource rich and research intensive nations (17, 18). Here, we present our first insight into both natural and vaccine-acquired immune response in a small cohort of individuals in Southern Nigerians. Our cohorts include

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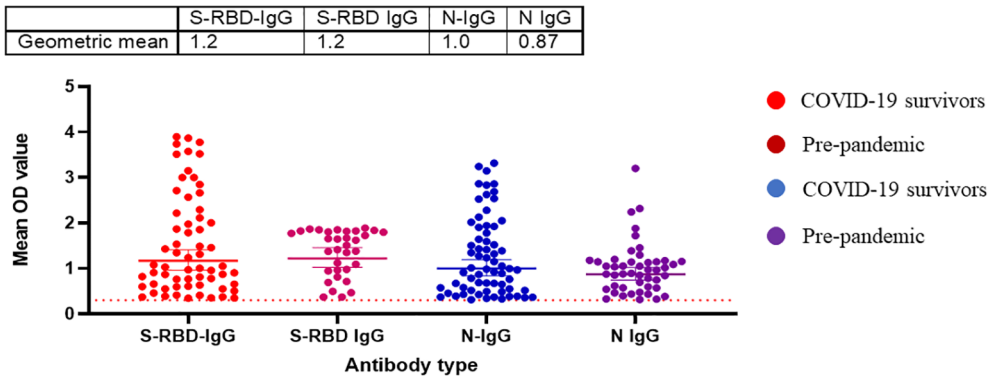

FIGURE 4
Pre-pandemic sera has detectable binding antibody responses (IgG) to SARS-CoV-2 S-RBD and N proteins similar to sera from COVID-19 survivors in Southern Nigeria. Specific SARS-CoV-2 S-RBD and N antigen ELISA were used to measure the binding antibody response (IgG) from sera (N=64) collected prior to the COVID-19 pandemic. We used a 1:100 dilution of the serum sample. The OD of the negative cutoff was selected as the mean multiplied by three standard deviations of three known negative samples (0.3).The table shows the geometric mean at 95% CI. Statistical significance was calculated by Mann–Whitney test and p values are indicated. (Capped line with * indicating significance).

known survivors of COVID-19, vaccinated individuals, non-hospitalized asymptomatic but exposed individuals (contacts) and pre-pandemic samples.

Our data revealed a similar magnitude of binding and neutralizing antibody responses to both S-RBD and N antigens of SARS-CoV-2 from hospitalized survivors of COVID-19 and non-hospitalized asymptomatic survivors (Figures 1A, B). This corroborated previous seroprevalence data from both Nigeria and Ghana that identified high-binding antibody responses in asymptomatic individuals with no positive diagnosis of SARS-CoV-2 infection (19, 20). Here we extended these observations to demonstrate that sera from asymptomatic participants neutralized SARS-CoV-2 Spike PVs to the same level as sera from symptomatic COVID-19 survivors. This contrasted our observation in Lassa fever disease immunity where both hospitalized and non hospitalized patients generate binding antibody response to the Lassa virus GP and NP protein but neutralizing antibody response was mainly from the hospitalized patients (21). The binding antibody response to N antigens observed in both sera from symptomatic and asymptomatic individuals needs to be differentiated by peptide-based assays to determine if the antibody response is from SARS-CoV- related or other Coronaviruses. Interestingly, sera collected prior to the COVID-19 pandemic have binding antibody responses to both N and S-RBD antigens and none had neutralizing antibody response to any of SARS-CoV-2 Spike PVs, unlike sera from COVID-19 symptomatic and asymptomatic participants with both binding and neutralizing antibody responses to SARS-CoV-2 PVs. The presence of cross-reactive binding antibody responses but with no neutralizing function have been observed in pre-pandemic sera in other African countries such as SierraLeone and Uganda (16, 22). Future efforts to explore the non-neutralizing function of the pre-pandemic sera such as antibody dependent cytotoxicity (ADCC) and peptide arrays will

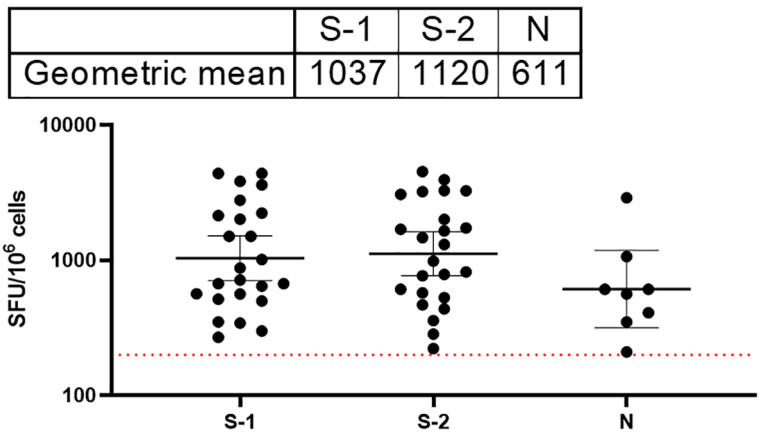

FIGURE 5
Stronger T cell responses to the SARS-CoV-2 spike proteins compared to the Nucleoprotein in COVID-19 survivors. The result (table showing geometric mean at 95% CI) showed stronger T cell response to both S1 and 2 compared to the N peptides. The average limit of detection (red dotted line=200) was calculated as the mean multiplied by one standard deviation of the three known negative samples.

help to determine the specificity and if pre-existing, cross-reactive bystander immunity may have played a role in reducing morbidity and mortality in Africans during the COVID-19 pandemic.

As expected, sera from individuals vaccinated with combinations of the different vaccines deployed in Nigeria demonstrated binding to S-RBD antigens and neutralizing antibody responses. Interestingly, sera from those with previous infection (either SARS-CoV-2 or seasonal coronaviruses) as well as vaccination (hybrid immunity) elicited stronger binding and broader neutralizing antibody responses compared to those with infection or vaccination alone (Figures 2A, B). Our data corroborated other studies that demonstrated the quality of hybrid immunity in contrast to either infection or vaccine induced immunity alone (23, 24). It will be interesting to determine whether the boosting immunity in documented hybrid immune cases is acquired by previous infection by SARS-CoV-2 variants or certain seasonal coronavirus. Interestingly Amanat et al. (2022) demonstrated that mice that were previously exposed to seasonal coronavirus had no boosting or inhibitory effects on subsequent vaccine immunity so-called immune imprinting (25). Given that much of the population have now been infected as well as vaccinated, future studies will be needed to elucidate the nature of prior coronavirus infection that caused this pre-pandemic 'immune imprinting' like phenomenon on subsequent SARS-CoV-2 immunity in Africans in an effort to understand why COVID-19 was less severe in a carefully documented populations in Africa.

Another outcome of this study was the observation of incremental binding antibody responses with frequency of vaccine boosters. Three doses of BioNTech/Pfizer vaccines elicited stronger binding antibody responses compared to the first and second vaccine doses (Figure 2A). However, for those that received three different vaccines, the heterologous vaccine prime and boost combinations resulted in stronger binding antibodies compared to homologous vaccine booster immunizations (Mann–Whitney test, p=0.16). Interestingly, the majority of vaccine failure (vaccinees with no binding antibody responses at the time of sampling) were most frequent in individuals that only received a single dose of the vaccine compared to those with multiple booster immunizations. More than two thirds' sera with negative binding antibody responses from people documented to have been vaccinated, were in individuals with a single immunization, while one-third were in individuals that had received two immunizations, and none was found among those that have been immunized 3 or more times (Figure 6). No relationships were found in documented cases of vaccine failures or negative binding antibody responses between vector-based vaccines and mRNA-based vaccines both in the single and the double immunizations.

Nigeria experienced four major waves of COVID-19. Not surprisingly, our binding antibody and neutralizing antibody responses followed the waves of infection in Nigeria over time (Figures 3A, B, C). Not unexpectedly, (as has been previously reported in non-Africa nations) sera collected during the most recent wave was associated with the strongest binding and neutralizing antibody responses compared to those collected in the first and second wave. The third wave was dominated by the delta variant which was reflected in the titres of neutralizing antibodies to this variants corroborating similar studies in India showing stronger antibody response in the second wave dominated by the delta variants (26). The reason for the increase in antibody response post-delta wave may be due to the fact that delta variants infected more people than other variants in Nigeria. It could also be a function of boosting from prior infection or immunizations with repeated exposure and the resultant maturation of antibody affinity over time. Individuals in the third wave were likely to have had more exposure, or re-exposure to SARS-CoV-2 than second and first waves.

With regard to T -cell mediated immunity, our convalescent participants also had T cell responses to both S and N antigens. The

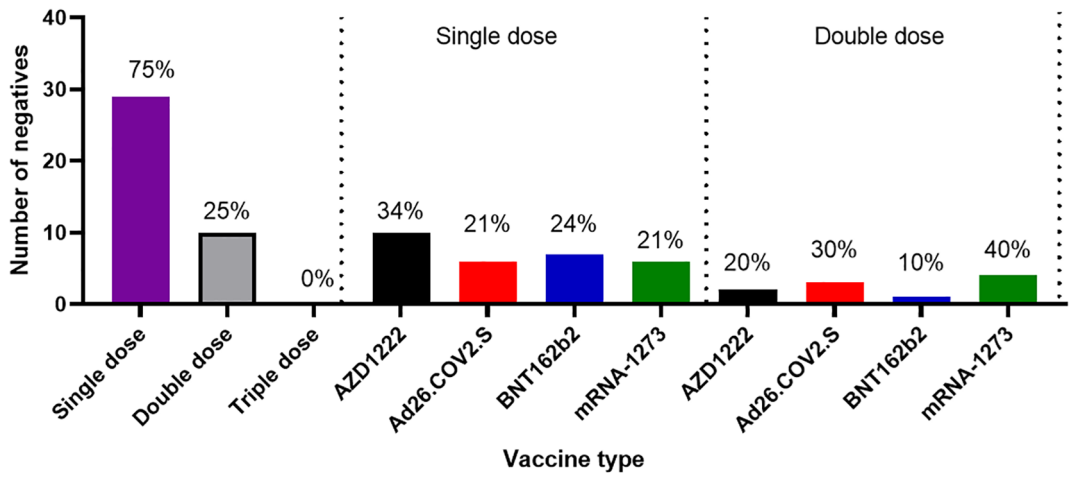

FIGURE 6
Cases of vaccine failures among COVID-19 vaccinees in Southern Nigeria. Vaccine failures were most frequent in individuals who only received a single dose of the vaccine compared to those with multiple booster immunizations, and no difference in cases of vaccine failure among those who received different types of vaccines.

strongest T cell response was observed against the highly conserved S-2 region of the Spike protein (Figure 5). Due to cold chain logistic and technical reasons, limitation of PBMC sampling made it difficult to have sufficient samples to draw further conclusions or to follow the kinetics of T cell response in vaccinated participants.

In summary, our data revealed that asymptomatic cases of COVID-19 generate similar magnitudes of both binding and neutralizing antibody responses to individuals with symptoms of COVID-19 in Nigeria. Notably, we were able to clearly separate the asymptomatic COVID-19 antibody responses from pre-existing antibody response by absence of neutralizing antibodies in cases in which we found to have a pre-pandemic pre-existing or cross-reactive immunity to SARS-CoV-2. We also showed that both vaccine and convalescent antibody responses were able to cross neutralize different circulating VOCs in Nigeria. In addition, convalescent antibody responses were found to correlate with the waves of SARS-CoV-2 in Nigeria. Lastly, hybrid immunity and heterologous vaccine boosting induced the strongest binding and broadly neutralizing antibody responses compared to vaccine or infection acquired immunity alone. This data is the first detailed study of SARS-CoV-2 immune responses acquired throughout the COVID-19 pandemic in Nigeria. Understanding the nature of the pre-existing cross-reactive SARS-CoV-2, non-neutralizing antibodies in African populations, and their possible impact on the relative COVID-19 disease resistance is a question that remains to be elucidated.

Limitation of the current study

This study sampling was mainly from Southern Nigeria and sample sizes were variable for each study groups. Due to logistic and social security circumstances, we were unable to recruit participants from Northern Nigeria. Our data focused heavily on antibody immune responses. Due to lack of cold-chain resources we were unable to measure T cell response from immunized participants. Due to increased vaccination and population complacency, convalescent sera were largely unavailable during the 4th wave of the pandemic.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Nigerian National Health Research Ethics Committee and Redeemer's University Institutional Review Board (SIP-NG-NHREC/01/01/2007-12/01/2021, ARISE-NHREC/01/01/2007-11/02/2022). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

CU: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. OA: Data curation, Investigation, Methodology, Project administration, Writing – review & editing. OJ: Data curation, Investigation, Methodology, Project administration, Writing – review & editing. BA: Data curation, Investigation, Methodology, Project administration, Writing – review & editing. IA: Data curation, Investigation, Methodology, Project administration, Writing – review & editing. OO: Data curation, Investigation, Project administration, Writing – review & editing. IB: Data curation, Investigation, Writing – review & editing. AC: Investigation, Methodology, Writing – review & editing. JC: Investigation, Methodology, Writing – review & editing. JA: Investigation, Methodology, Writing – review & editing. IB: Data curation, Visualization, Writing – review & editing. KA: Data curation, Formal analysis, Methodology, Project administration, Writing – review & editing. JO: Formal analysis, Project administration, Supervision, Writing – review & editing. PE: Methodology, Supervision, Writing – review & editing. PO: Investigation, Supervision, Validation, Writing – review & editing. AO: Investigation, Writing – review & editing. IA: Investigation, Writing – review & editing. VA: Investigation, Writing – review & editing. EB: Investigation, Writing – review & editing. OA: Investigation, Writing – review & editing. NA: Investigation, Project administration, Supervision, Validation, Writing – review & editing. EO: Investigation, Project administration, Supervision, Validation, Writing – review & editing. KU: Investigation, Project administration, Supervision, Writing – review & editing. SO: Investigation, Project administration, Writing – review & editing. OO: Investigation, Writing – review & editing. OK: Supervision, Validation, Writing – review & editing. ME: Investigation, Methodology, Writing – review & editing. VO: Investigation, Methodology, Writing – review & editing. OA: Investigation, Project administration, Supervision, Writing – review & editing. FE: Investigation, Writing – review & editing. MO: Investigation, Writing – review & editing. VI: Investigation, Writing – review & editing. OA: Investigation, Project administration, Supervision, Validation, Writing – review & editing. AC: Investigation, Project administration, Supervision, Writing – review & editing. SO: Investigation, Project administration, Supervision, Writing – review & editing. NO: Investigation, Methodology, Project administration, Writing – review & editing. SA: Investigation, Methodology, Project administration, Writing – review & editing. WI: Investigation, Methodology, Project administration, Writing – review & editing. MO: Investigation, Project administration, Supervision, Writing – review & editing. AA: Investigation, Project administration, Supervision, Writing – review & editing. AN: Data curation, Formal analysis, Methodology, Visualization, Writing – review & editing. EA: Data curation, Formal analysis, Methodology, Visualization, Writing – review & editing. GC: Data curation, Formal analysis, Methodology, Supervision, Visualization, Writing – review & editing. NK: Data curation, Formal analysis, Methodology, Writing – review & editing. AC: Formal analysis, Methodology, Supervision, Validation, Writing – review & editing. CG: Methodology, Supervision, Validation, Writing – review &

editing. RK: Project administration, Supervision, Validation, Writing – review & editing. PT: Methodology, Project administration, Supervision, Validation, Writing – review & editing. NT: Methodology, Supervision, Validation, Visualization, Writing – review & editing. JH: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. CH: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1305586/full#supplementary-material

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