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Vaccines and approaches that target trained immunity in COVID-19: Immunological mechanisms of action and delivery

Edited by
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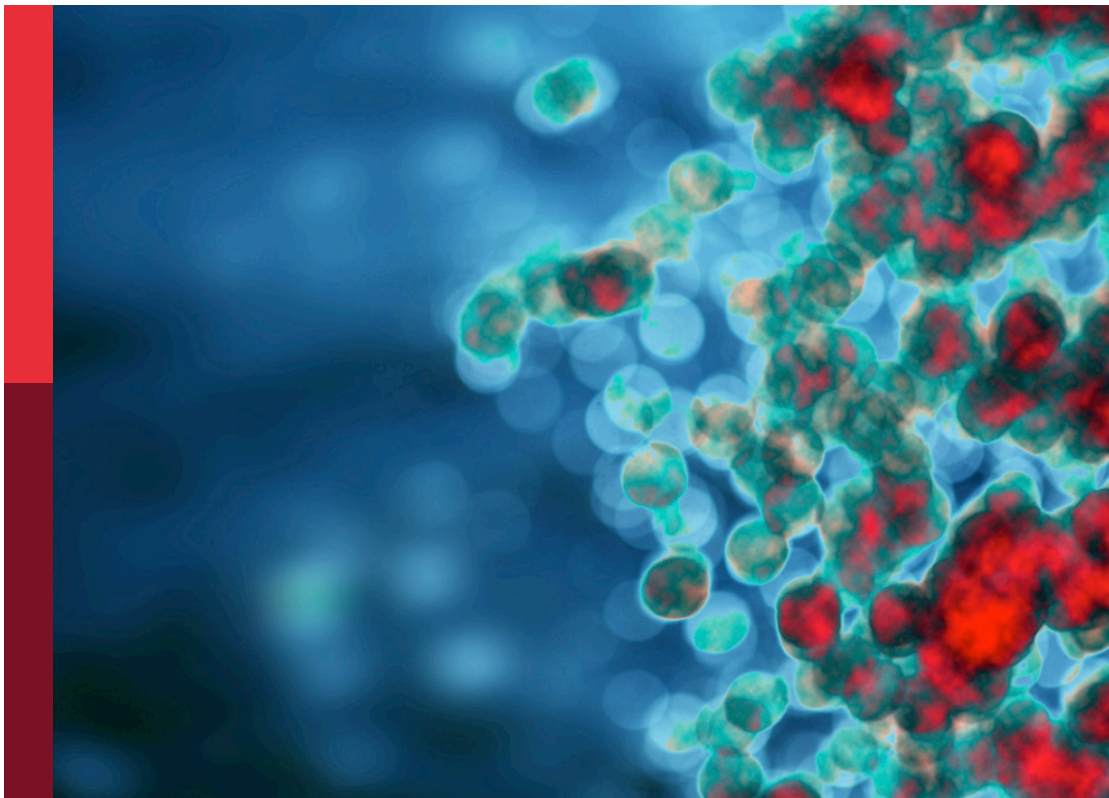

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Vaccines and approaches that target trained immunity in COVID-19: Immunological mechanisms of action and delivery

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COVID-19 Vaccine Hesitancy Among Chinese Population: A Large-Scale National Study

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Globally, vaccine hesitancy is a growing public health problem. It is detrimental to the consolidation of immunization program achievements and elimination of vaccine-targeted diseases. The objective of this study was to estimate the prevalence of COVID-19 vaccine hesitancy in China and explore its contributing factors. A national cross-sectional online survey among Chinese adults (≥18 years old) was conducted between August 6, 2021 and August 9 *via* a market research company. We collected sociodemographic information; lifestyle behavior; quality of life; the knowledge, awareness, and behavior of COVID-19; the knowledge, awareness, and behavior of COVID-19 vaccine; willingness of COVID-19 vaccination; accessibility of COVID-19 vaccination services; skepticism about COVID-19 and COVID-19 vaccine; doctor and vaccine developer scale; and so on. Odds ratios (OR) with 95% confidence intervals (CI) were used to estimate the associations by using logistic regression models. A total of 29,925 residents (48.64% men) were enrolled in our study with mean age of 30.99 years. We found an overall prevalence of COVID-19 vaccine hesitancy at 8.40% (95% CI, 8.09–8.72) in primary vaccination and 8.39% (95% CI, 8.07–8.70) in booster vaccination. In addition, after adjusting for potential confounders, we found that women, higher educational level, married residents, higher score of health condition, never smoked, increased washing hands, increased wearing mask, increased social distance, lower level of vaccine conspiracy beliefs, disease risks outweigh vaccine risk, higher level of convenient vaccination, and higher level of trust in doctor and developer were more willing to vaccinate than all others (all $p < 0.05$). Age, sex, educational level, marital status, chronic disease condition, smoking, healthy behaviors, the curability of COVID-19, the channel of accessing information of COVID-19 vaccine, endorsement of vaccine conspiracy beliefs, weigh risks of vaccination against risks of the disease, making a positive influence on the health of others around you, and lower trust in healthcare system may affect the variation of

willingness to take a COVID-19 vaccine (all $p < 0.05$). The prevalence of COVID-19 vaccine hesitancy was modest in China, even with the slight resulting cascade of changing vaccination rates between the primary and booster vaccination. Urgent action to address vaccine hesitancy is needed in building trust in medical personnel and vaccine producers, promoting the convenience of vaccination services, and spreading reliable information of COVID-19 vaccination *via* the Internet and other media.

Keywords: COVID-19 vaccine hesitancy, China, primary vaccination, booster vaccination, factors (individual factors, contextual factors)

INTRODUCTION

Vaccination is the most cost-efficient method of avoiding infectious diseases and has been one of the most effective public health interventions to date (1–4). The effectiveness of the COVID-19 vaccination depends solely on its uptake. If there are individuals who are hesitant or unwilling to be immunized, the vaccination coverage will be limited. A study indicated that a refusal rate of more than 10% is estimated to be sufficient to weaken the population benefits of vaccination against COVID-19 (5). Since the global outbreak of COVID-19, researchers from all around the world have been working tirelessly and collaboratively to develop the vaccines against the virus. Numerous types of vaccines are currently available including inactivated vaccines, recombinant protein vaccines, adenovirus vector vaccines, attenuated influenza virus vector vaccines, and nucleic acid (mRNA and DNA vaccines) vaccines (6). However, this global effort might be hampered by vaccine hesitancy despite its availability (7).

Vaccine hesitancy has been identified as one of the greatest threats to public health at a global level (8) and a common phenomenon in the developed world for decades (9–11). However, the prevalence of COVID-19 vaccine hesitancy is chaotic globally, posing a significant obstacle to the global efforts to containing the current COVID-19 pandemic. A recent review of vaccine acceptance rates demonstrated that developed countries such as the USA, France, and Italy generally have expected vaccine acceptance rates of less than 60% (ranging from 53.7% to 58.9%). Meanwhile, low rates of COVID-19 vaccine acceptance were reported in the Middle East, Russia, Africa, and several European countries as well (11). However, the current analytics show that countries like China, Malaysia, and Ecuador are expected to have uptake rates of more than 90% (ranging from 91.3% to 97.0%) (12). In addition, another recent survey in the UK showed that 16.9% of respondents were hesitant about the vaccine (13). While a survey conducted in Hong Kong, China, showed a shift in the predicted uptake rate from 44.2% to 34.8% at different waves of local epidemic among the population (14).

The reasons for COVID-19 vaccination hesitancy are varied and, to some extent, unclear. Earlier research examined the complex nature of vaccine hesitancy by examining the epidemiologic triad of environmental, agent, and host factors (i.e., EAH framework) (15). Environmental factors include public health policies, social factors, and media messaging (16, 17) while the agent (vaccine and disease) factors include the perception of vaccine safety and effectiveness, besides the perceived susceptibility to the disease (18). Host factors are dependent on knowledge, previous experience, and educational and income levels (19). Recent research indicates that vaccine hesitation is frequently framed in terms of complacency, confidence, and convenience (3Cs framework). Vaccine hesitation occurs when there is a low perception of the necessity for vaccination (referred to as complacency), worries about the efficacy and safety of the vaccine (referred to as low confidence), and a lack of vaccine accessibility (referred to as convenience) (20). Based on the frameworks, youth, female gender, low income, low education, high informational reliance on social media, low informational reliance on print and broadcast media, membership of other than white ethnic groups, low perceived risk from COVID-19, and low trust in scientists, medics, and biomedical science, as well as (to a much lesser extent) low trust in government were all recognized as relevant factors that may affect COVID-19 vaccine hesitancy. Similar associations have been observed in countries including Canada, USA, Europe, Australia, Japan, Middle East, Russia, and Africa. The incidence of COVID-19 vaccination hesitancy has been researched, as well as how it is influenced by socioeconomic position, particularly individual psychological characteristics. However, such information is lacking in China.

China may be a world leader in COVID-19 vaccination coverage not only because it constitutes one-fifth of the world's population but also because the country is becoming increasingly interconnected with the rest of the world. Although more than 62.4% (880 million) of the Chinese population have been officially confirmed to be vaccinated so far, some residents are still hesitant to get vaccinated (21). We therefore sought to expand on the EAH and 3C frameworks in order to better explain COVID-19 vaccine hesitancy among Chinese. Therefore, we conducted a nation-wide survey in 31 provinces across mainland China during the period of primary and booster vaccination of COVID-19 vaccines. We calculated the level of COVID-19 vaccine hesitancy (delay or refusal) in a large sample by analyzing expressed readiness to get an approved vaccination and identifying subgroups within the population and regions where it may be higher. Our major objective was to gain a better

understanding of vaccine hesitancy on an individual psychological level to guide methods for increasing vaccination acceptance rates.

METHODS

Participants and Procedures

On July 10, 2021, we performed a preliminary online survey in Zhongmou County, Henan Province. We conducted a face-to-face interview with participants from a representative village and community obtained through cluster sampling approach. Basing on the vaccine hesitation rate and the reliability and validity of the questionnaire of the preliminary online survey, we estimated the minimum sample size required for the formal survey to be 6,638 participants, which was based on a prevalence of COVID-19 vaccine hesitancy of 16.57% in a preliminary online survey, an allowable error of 1%, a 95% confidence level, and an anticipated design effect of two. A subsequent national cross-sectional online survey using a snowball sampling method among Chinese adults (≥18 years old) was conducted from August 6, 2021 *via* a market research company. The invited respondents were unaware of the topic prior to their tentative consent to complete the survey. In order to ensure that the sample size for this study was large enough to estimate the prevalence of vaccine hesitancy, a sample saturation was monitored during the investigation. Saturation, in this study, refers to the point at which the sample size reaches a specific size whereby the vaccine hesitancy rate becomes constant and no longer varies considerably with the snowballing sample size growth. We found that when the number of valid questionnaires reached 21,780, the sample began to saturate (**Appendix Figure 1**). We ended the online survey when the valid sample reached 29,925 on August 9, 2021. The flowchart of participant selection and sample saturation monitoring procedures are shown in **Figure 1**.

Assessments

Due to the absence of a uniform COVID-19 vaccine hesitancy scale in China, we designed two items to assess whether there was a delay in immunization acceptance or refusal based on the Oxford COVID-19 Vaccine Hesitancy Scale (13). The items comprised themes "In terms of COVID-19 vaccination in current stage, I would describe myself as" for estimating hesitancy rate in primary vaccination and "I would describe my attitude towards regularly receiving a COVID-19 vaccine in the future as" for predicting hesitancy rate in booster vaccination in future. For each item, item-specific response options coded from 1 to 5 were used, including (1) Vaccination, (2) Willing to get the COVID-19 vaccine, (3) Delay to getting the COVID-19 vaccine, (4) Unwilling to get the COVID-19 vaccine, and (5) Anti-vaccination." Higher scores indicated a higher level of vaccine hesitancy. According to the definition of vaccine hesitancy, options (1) and (2) were merged into "Acceptance" and options (3), (4), and (5) were merged into "Hesitancy" during data analysis. Based on the EAH and 3C frameworks, our questionnaire subsequently collected exploratory and confirmatory factors from four aspects, namely, (1) individual characteristics (i.e., social-demographic information, subjective social status, health status), (2) COVID-19 pandemic progress perception (i.e., awareness of COVID-19 blocking, judgement of the trend, pandemic skepticism), (3) COVID-19 vaccine perception (i.e., general knowledge on vaccine, COVID-19 vaccination perception, vaccine skepticism), and (4) the

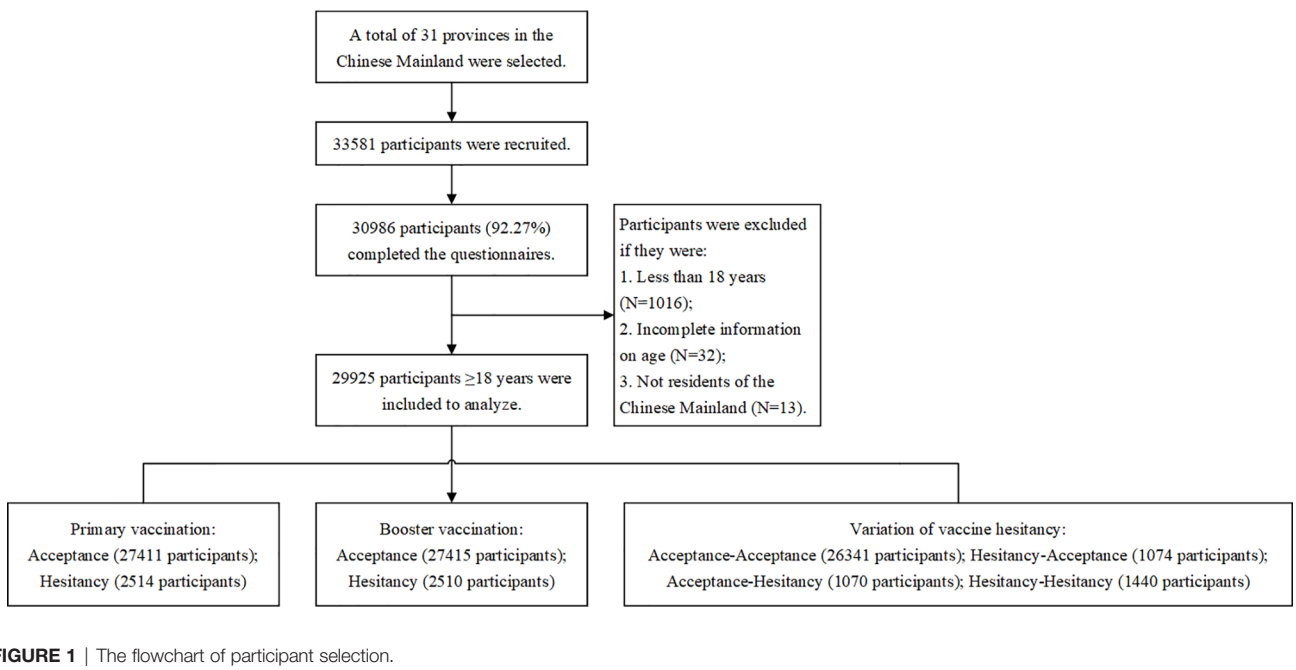

FIGURE 1 | The flowchart of participant selection.

healthcare system dimension (trust in doctors and vaccine developers, convenience of vaccination). All questionnaires are shown in **Appendix 1**.

Statistical Analysis

The Chi-square goodness-of-fit test was used to monitor sample saturation throughout the formal online survey to determine sample underrepresentation error. An independent samples *t*-test or Chi-square test was carried out to test differences in willingness to get vaccinated across groups. Binary logistic regression analyses were conducted to examine factors associated with COVID-19 vaccine hesitancy and COVID-19 pandemic progress perception, COVID-19 vaccine perception, and trust in healthcare system after controlling for demographic and socioeconomic confounders. Multinomial logistic regression model was applied to assess between associated factors and transformations of COVID-19 vaccine hesitancy. The collinearity test was carried out to assess the correlation between independent variables using a variance inflation factor (VIF) <4, and no collinearity was detected. A sensitivity analysis was performed by excluding participants suffered from chronic diseases to test the robustness of model results and assess source of model uncertainty. We did all statistical analyses using SAS 9.4. Differences were regarded as statistically significant if p values were less than 0.05.

Ethical Approval

This study was deemed exempt by the Life Science Ethics Review Committee of Zhengzhou University.

RESULTS

Prevalence of COVID-19 Vaccine Hesitancy

A total of 29,925 residents from 31 provinces of Chinese mainland were included in the current study. A summary of the sociodemographic, awareness of COVID-19 pandemic, COVID-19 vaccine exception, trust in healthcare system, and hesitancy of all study participants is provided in **Table 1**. In all, 2,514 (8.40%, 95% confidence interval (CI) 8.09% to 8.72%) participants endorsed clear vaccine hesitancy response in primary vaccination. Furthermore, 2,510 (8.39%, 95% CI 8.07% to 8.70%) expressed their hesitancy in booster vaccination of COVID-19 vaccine. We found that the prevalence of vaccine hesitancy was higher in men than women in all age groups (**Appendix Figure 2**). Higher prevalence in both phrases were observed among population with elders (age ≥60 years), men, lower educational level, minority, religious beliefs, not in marriage, higher subjective social status, lower self-report health condition, suffered from chronic diseases, current smoker, former drinker, extreme endorsement of COVID-19, vaccine conspiracy beliefs, medium or low possibility of curability of COVID-19, inconvenience of vaccination, and lower trust in healthcare system. The hesitancies in both phases varied substantially by the province in mainland China. More than one in 10 of the study population in Beijing, Hebei, and Tianjin were observed to be hesitant to uptake the vaccine in current stage after standardizing age and sex. In terms of predicted hesitancy in the booster vaccination, the age- and sex-standardized prevalence of hesitancy in Beijing, Tianjin, Hebei, and Hainan were more than 10%, ranging from 10.13% to 15.76% (**Figure 2**; **Appendix Table 1**).

COVID-19 Vaccine Hesitancy and Its Influencing Factors

In the binary logistic regression model among all study participants, age, sex, educational level, marital status, self-report health condition, subjective social status in community level, smoking status, drinking status, healthy behaviors, the risk of COVID-19 infection, the curability of COVID-19, the channel of accessing information of COVID-19 vaccine, endorsement of vaccine conspiracy beliefs, weigh risks of vaccination against risks of the disease, other life/health responsibilities, inconvenience of vaccination, and lower trust in healthcare system were independent factors associated with hesitancy on COVID-19 primary vaccination. A similar pattern of hesitancy in the booster vaccination was also shown among the subjects (**Table 2**).

Variation of COVID-19 Vaccine Hesitancy

Our study highlighted that the prevalence of vaccine hesitancy among Chinese residents remains at a low and stable level, with a slight shift from 8.40% to 8.39% (95% CI, 8.07 to 8.70) that was observed between the primary and booster vaccinations. In detail, 88.02% and 4.81% of the population responded in a consistent acceptance and hesitancy towards taking a COVID-19 vaccine in both phases, respectively. Notably, there were also individuals who showed varying levels of willingness to take vaccines. In summary, 3.58% of respondents declared an acceptance of rejection, whereas 3.59% of those who were previously hesitant became willing to receive vaccination (**Table 3**). The associations of COVID-19 vaccine hesitancy transformations and socio-demographic, awareness of COVID-19 pandemic, COVID-19 vaccine exception, and trust in healthcare system were summarized in **Table 3**. The variation of willingness (i.e., acceptance to hesitancy, hesitancy to acceptance, hesitancy to hesitancy) to take a COVID-19 vaccine was associated with age, sex, educational level, marital status, chronic disease condition, smoking, healthy behaviors, the curability of COVID-19, the channel of accessing information of COVID-19 vaccine, endorsement of vaccine conspiracy beliefs, weigh risks of vaccination against risks of the disease, other life/health responsibilities, and lower trust in healthcare system were independent.

Sensitivity Analysis

In sensitivity analyses, exclusion of cases with chronic disease did not appreciably alter the findings for vaccine hesitancy. The effect estimates remained similar for the main results (**Appendix Table 2**).

TABLE 1 | Sociodemographic, awareness of COVID-19 pandemic, COVID-19 vaccine exception, trust in healthcare system, and COVID-19 vaccine hesitancy of all study participants.

Covariates	Total (%)	*p*-value[a]	Vaccine hesitancy in the primary vaccination (95% CI)	*p*-value[a]	Vaccine hesitancy in booster vaccination (95% CI)	*p*-value[a]
Total participants	29,925 (100)		8.40 (8.09–8.72)[b]		8.39 (8.07–8.70)[b]	
Demographic characteristics						
Age (years)		<0.001		<0.001		<0.001
18–29	13,312 (44.48)		10.84 (10.31–11.37)[b]		10.97 (10.44–11.50)[b]	
30–39	11,911 (39.80)		6.73 (6.28–7.18)[b]		6.63 (6.19–7.08)[b]	
40–49	3,269 (10.92)		4.68 (3.96–5.40)[b]		4.37 (3.67–5.08)[b]	
50–59	1,149 (3.84)		7.05 (5.57–8.53)[b]		7.57 (6.04–9.10)[b]	
60–	284 (0.95)		12.32 (8.50–16.15)[b]		10.56 (6.99–14.14)[b]	
Sex		<0.001		<0.001		<0.001
Men	14,556 (48.64)		11.55 (8.50–16.15)[b]		11.42 (10.91–11.94)[b]	
Women	15,369 (51.36)		5.42 (5.06–5.78)[b]		5.51 (5.15–5.87)[b]	
Educational status		<0.001		<0.001		<0.001
Below high school	3,839 (12.83)		21.36 (20.06–22.66)[b]		20.08 (18.82–21.35)[b]	
High school graduate	7,893 (26.38)		8.40 (7.79–9.01)[b]		7.99(7.40-8.59)[b]	
University graduate	18,193 (60.80)		5.67 (5.33–6.00)[b]		6.09 (5.74–6.44)[b]	
Ethnic groups		<0.001		<0.001		<0.001
Han	28,579 (95.50)		7.76 (7.45–8.07)[b]		7.86 (7.55–8.17)[b]	
Minority	1,346 (4.50)		21.92 (19.71–24.13)[b]		19.54 (17.42–21.66)[b]	
Religion		<0.001		<0.001		<0.001
Atheist	25,424 (84.96)		6.44 (6.14–6.74)[b]		6.69 (6.39–7.00)[b]	
Others	4,501 (15.04)		19.46 (18.31–20.62)[b]		17.95 (16.83–19.07)[b]	
Marital status		<0.001		<0.001		<0.001
Married	18,363 (61.36)		6.42 (6.06–6.77)[b]		6.33 (5.98–6.69)[b]	
Others	11,562 (38.64)		11.56 (10.97–12.14)[b]		11.65 (11.07–12.24)[b]	
Subjective social status						
Society level	6.66 ± 2.09	<0.001	6.98 ± 2.06[c]	<0.001	6.89 ± 2.02[c]	<0.001
Community level	7.00 ± 2.13	<0.001	7.39 ± 2.14[c]	<0.001	7.27 ± 2.12[c]	<0.001
Self-report health condition (EQ-5D)	84.36 ± 14.58	<0.001	75.55 ± 19.68[c]	<0.001	75.70 ± 19.55[c]	<0.001
Chronic condition		<0.001		<0.001		<0.001
0	24,960 (84.95)		5.48 (5.20–5.77)[b]		5.76 (5.47–6.05)[b]	
1	3,245 (11.04)		22.53 (21.09–23.96)[b]		21.23 (19.83–22.64)[b]	
2	891 (3.03)		22.45 (19.71–25.19)[b]		22.11 (19.39–24.83)[b]	
≥3	287 (0.98)		23.34 (18.45–28.24)[b]		23.34 (18.45–28.24)[b]	
Smoking status		<0.001		<0.001		<0.001
Current smoker	9,702 (32.42)		17.99 (17.22–18.75)[b]		17.13 (16.38–17.88)[b]	
Former smoker	1,664 (5.56)		10.88 (9.38–12.37)[b]		9.19 (7.81–10.58)[b]	
Never smoker	18,559 (62.02)		3.17 (2.92–3.42)[b]		3.74 (3.47–4.02)[b]	

(Continued)

TABLE 1 | Continued

Covariates	Total (%)	*p*-value[a]	Vaccine hesitancy in the primary vaccination (95% CI)	*p*-value[a]	Vaccine hesitancy in booster vaccination (95% CI)	*p*-value[a]
Drinking status		<0.001		<0.001		<0.001
Current drinker	18,484 (61.77)		11.04 (10.58–11.49)[b]		10.91 (10.46–11.36)[b]	
Former drinker	1,080 (3.61)		12.96 (10.96–14.97)[b]		11.39 (9.49–13.28)[b]	
Never drinker	10,361 (34.62)		3.22 (2.88–3.56)[b]		3.57 (3.21–3.93)[b]	
Health behaviors						
Washing hands	23,737 (79.32)	<0.001	4.76 (4.49–5.03)[b]	<0.001	5.00 (4.72–5.27)[b]	<0.001
Wearing mask	27,340 (91.36)	<0.001	5.80 (5.52–6.07)[b]	<0.001	6.06 (5.78–6.35)[b]	<0.001
Social distance	12,688 (42.40)	<0.001	2.01 (1.77–2.25)[b]	<0.001	2.47 (2.20–2.75)[b]	<0.001
Awareness of COVID-19 pandemic						
COVID-19 conspiracy beliefs		<0.001		<0.001		<0.001
Level 1	7,224 (24.14)		2.92 (2.53–3.31)[b]		3.06 (2.66–3.46)[b]	
Level 2	6,410 (21.42)		3.67 (3.21–4.13)[b]		3.76 (3.29–4.23)[b]	
Level 3	7,659 (25.59)		8.51 (7.89–9.14)[b]		9.31 (8.66–9.96)[b]	
Level 4	8,632 (28.85)		16.40 (15.62–17.19)[b]		15.47 (14.70–16.23)[b]	
Risk of COVID-19 infection		<0.001		<0.001		<0.001
Very high	2,188 (7.31)		11.38 (10.05–12.71)[b]		11.01 (9.70–12.33)[b]	
High	2,520 (8.42)		21.98 (20.37–23.6)[b]		20.32 (18.75–21.89)[b]	
Medium	4,636 (15.49)		14.75 (13.73–15.77)[b]		14.00 (13.00–15.00)[b]	
Low	15,107 (50.48)		4.41 (4.08–4.74)[b]		4.88 (4.54–5.22)[b]	
No	4,531 (15.14)		5.61 (4.94–6.28)[b]		5.52 (4.85–6.18)[b]	
Not sure	943 (3.15)		11.35 (9.32–13.37)[b]		12.83 (10.70–14.97)[b]	
Curability of COVID-19				<0.001		<0.001
Very high	12,611 (42.14)		3.61 (3.28–3.93)[b]		3.73 (3.40–4.06)[b]	
High	10,916 (36.48)		7.39 (6.90–7.88)[b]		7.20 (6.72–7.69)[b]	
Medium	3,625 (12.11)		21.32 (19.99–22.66)[b]		20.74 (19.42–22.06)[b]	
Low	1,529 (5.11)		20.21 (18.20–22.22)[b]		21.06 (19.02–23.10)[b]	
No	515 (1.72)		15.92 (12.76–19.08)[b]		17.28 (14.02–20.55)[b]	
Not sure	729 (2.44)		12.07 (9.71–14.44)[b]		12.48 (10.08–14.88)[b]	
COVID-19 vaccine exception						
Channel of vaccine information		<0.001		<0.001		<0.001
Community worker	8,416 (28.12)		4.62 (4.17–5.07)[b]		4.72 (4.26–5.17)[b]	
Internet	15,522 (51.87)		7.26 (6.85–7.67)[b]		7.40 (6.99–7.81)[b]	
Others	5,987 (20.01)		16.67 (15.73–17.61)[b]		16.10 (15.17–17.03)[b]	
Vaccine conspiracy beliefs		<0.001		<0.001		<0.001
Level 1	7,033 (23.50)		2.79 (2.40–3.17)[b]		2.70 (2.32–3.08)[b]	
Level 2	6,920 (23.12)		3.18 (2.77–3.59)[b]		3.41 (2.98–3.84)[b]	
Level 3	8,168 (27.29)		7.16 (6.60–7.72)[b]		7.44 (6.87–8.01)[b]	

(Continued)

TABLE 1 | Continued

Covariates	Total (%)	*p*-value[a]	Vaccine hesitancy in the primary vaccination (95% CI)	*p*-value[a]	Vaccine hesitancy in booster vaccination (95% CI)	*p*-value[a]
Level 4	7,804 (26.08)		19.39 (18.51–20.26)[b]		18.91 (18.04–19.78)[b]	
Weigh risks of vaccination against risks of the disease		<0.001		<0.001		<0.001
Disease outweigh vaccine	18,862 (64.27)		5.48 (5.16–5.81)[b]		5.31 (4.99–5.63)[b]	
Vaccine outweigh disease	10,487 (35.73)		13.25 (12.61–13.90)[b]		13.44 (12.78–14.09)[b]	
Other life/health responsibilities		<0.001		<0.001		<0.001
Very high	14,607 (48.81)		2.88 (2.60–3.15)[b]		2.84 (2.57–3.11)[b]	
High	8,821 (29.48)		4.84 (4.39–5.29)[b]		5.76 (5.27–6.25)[b]	
Medium	3,656 (12.22)		22.73 (21.37–24.09)[b]		23.11 (21.75–24.48)[b]	
Low	1,796 (6.00)		32.96 (30.79–35.14)[b]		28.45 (26.37–30.54)[b]	
Very low	1,045 (3.49)		23.35 (20.78–25.91)[b]		22.11 (19.59–24.62)[b]	
Type of vaccination		<0.001		<0.001		<0.001
Unvaccinated	221 (0.74)		76.02 (70.39–81.65)[b]		79.19 (73.83–84.54)[b]	
Viral vector	3,737 (12.49)		6.50 (5.71–7.29)[b]		5.91 (5.16–6.67)[b]	
Inactivated	14,853 (49.63)		6.27 (5.88–6.66)[b]		6.32 (5.93–6.71)[b]	
Protein subunit	4,066 (13.59)		19.43 (18.21–20.65)[b]		19.16 (17.95–20.37)[b]	
Accept all vaccines	7,048 (23.55)		5.42 (4.89–5.95)[b]		5.62 (5.08–6.16)[b]	
Convenience of vaccination		<0.001		<0.001		<0.001
High	28,164 (94.14)		7.28 (6.98–7.59)[b]		7.34 (7.03–7.64)[b]	
Medium	1,401 (4.68)		25.34 (23.06–27.62)[b]		24.20 (21.95–26.44)[b]	
Low	360 (1.20)		30.00 (25.27–34.73)[b]		29.17 (24.47–33.86)[b]	
Trust in health care system						
Trust in doctors		<0.001		<0.001		<0.001
Level 1	8,502 (28.41)		18.22 (17.40–19.04)[b]		18.15 (17.33–18.97)[b]	
Level 2	7,344 (24.54)		7.94 (7.32–8.56)[b]		8.02 (7.40–8.64)[b]	
Level 3	7,092 (23.70)		4.09 (3.63–4.55)[b]		3.95 (3.49–4.40)[b]	
Level 4	6,987 (23.35)		1.32 (1.05–1.58)[b]		1.40 (1.13–1.68)[b]	
Trust in developers		<0.001		<0.001		<0.001
Level 1	8,100 (27.07)		19.85 (18.98–20.72)[b]		19.68 (18.81–20.54)[b]	
Level 2	10,145 (33.90)		7.37 (6.86–7.88)[b]		7.21 (6.70–7.71)[b]	
Level 3	11,680 (39.03)		1.35 (1.14–1.56)[b]		1.58 (1.36–1.81)[b]	

CI, confidence interval.

We categorized the score of COVID-19 conspiracy beliefs by quartiles as level 1 (≤7 points), level 2 (8–13 points), level 3 (14–20 points), and level 4 (≥21 points) and the score of vaccine conspiracy beliefs by quartiles as level 1 (≤7 points), level 2 (8–12 points), level 3 (13–18 points), and level 4 (≥19 points). We categorized the score of trust in doctors by quartiles as level 1 (≤30 points), level 2 (31–34 points), level 3 (35–38 points), and level 4 (≥39 points) and the score of trust in developers by quartiles as level 1 (≤17 points), level 2 (18–21 points), and level 3 (≥22 points).

[a]*Differences between categories within each variable.*

[b]*Row percentages derived from the total number in the corresponding row.*

[c]*The mean ± standard deviation for variables.*

Student's t-tests for continuous variables and Chi-square tests for categorical variables.

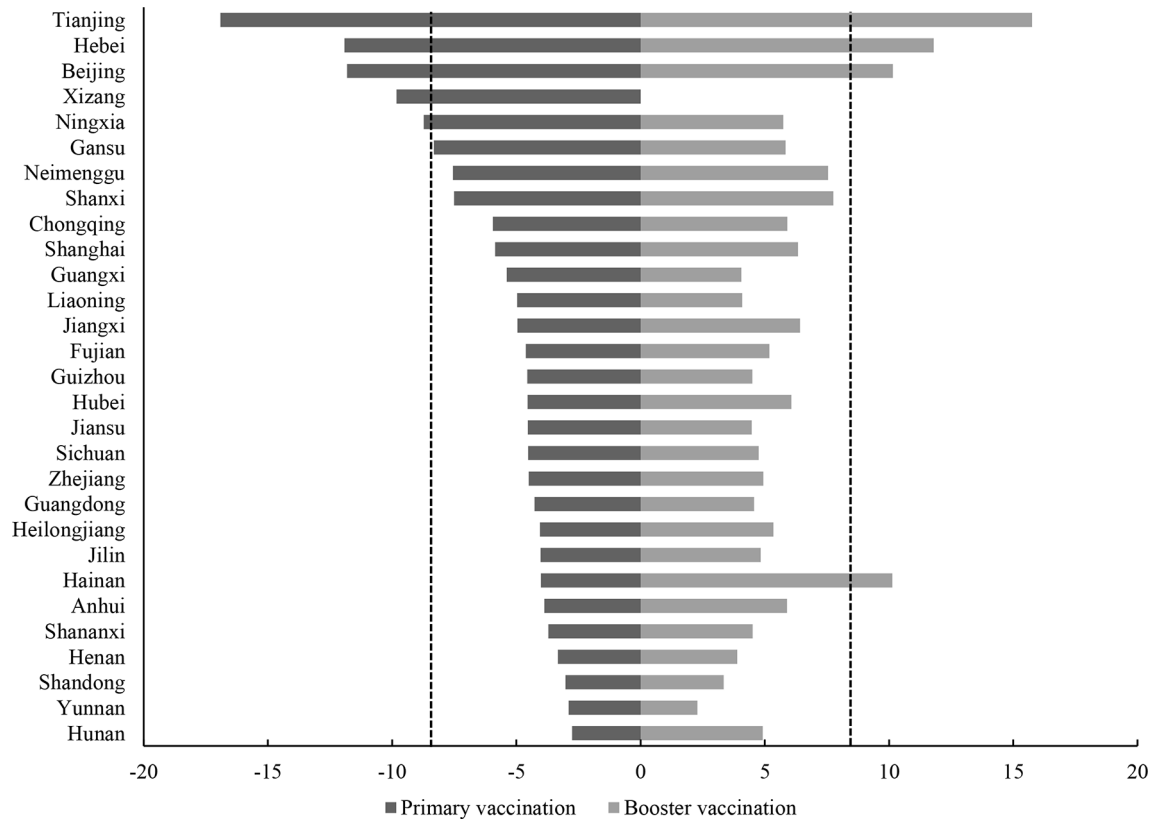

FIGURE 2 | Age- and sex-standardized prevalence of COVID-19 vaccine hesitancy by province in primary vaccination (left) and booster vaccination (right). Qinghai and Xinjiang province were not shown.

DISCUSSION

The current study examined the prevalence of COVID-19 vaccine hesitancy in a large representative sample of 31 provinces of mainland China. Our findings indicated that a sizable majority (88.02%) of mainland Chinese citizens express their readiness to be vaccinated. It is possible that this proportion will remain robust throughout the upcoming COVID-19 vaccine booster, but this is likely to be researched and confirmed. Our predicted vaccine hesitancy rate is comparable with earlier research conducted on the majority of the Chinese residents. According to these surveys, the vaccination rate among Chinese residents was found to be around 80%. The reason for the lower vaccination hesitancy rate, or for the greater vaccine acceptance rate, is mostly attributable to the following factors: Firstly, China has established a vaccine management law and successfully passed the World Health Organization's evaluation of its National Vaccine Regulatory System (NRS) which guarantees its quality and supply of the vaccine (22, 23). Secondly, China has consistently enhanced postmarket surveillance of vaccinations, with an emphasis on the safety and effectiveness of the vaccine while making a consistent follow-up on the incidences of vaccine-preventable disease as well as public acceptance of the vaccines. In addition, tracking the experience of vaccine use together with the development of vaccine big data are still ongoing (24). Thirdly, China has strengthened risk communication to ensure that recipients and the public have a consciousness of the benefits and risks of vaccination and to actively disseminate the scientific concept that the overall benefits of the vaccination greatly outweigh the risks. Finally, China has engaged in expanding vaccine availability, which requires vaccination services to be tailored to the characteristics of the jurisdiction area and population, as well as a reasonable distribution of vaccination clinics.

At the provincial level, the prevalence of COVID-19 vaccine hesitation varies greatly. Our results show that among the 31 provinces, the prevalence of vaccine hesitancy was more than 10% in three provinces. The reason for this level of hesitancy is not yet clear, and a variety of factors may be involved. Since the first vaccine was approved for marketing in mainland China on December 31, 2020, various provinces have made strenuous efforts to increase primary vaccination rates, but there are significant differences in the demographic structure, health literacy, prevalence of chronic diseases, and vaccination service supply capacity among provinces. It is likely that the combination of these factors has led to the uneven distribution of vaccine hesitation rates among provinces. With 4.81% of the population refusing to receive a COVID-19 vaccination, in the current study, the timeline for eradicating the pandemic may be

TABLE 2 | Associations between COVID-19 vaccine hesitancy and sociodemographic, awareness of COVID-19 pandemic, COVID-19 vaccine exception, and trust in healthcare system.

Covariates	Primary vaccination				Booster vaccination			
	Model 1	*p*-value	Model 2	*p*-value	Model 1	*p*-value	Model 2	*p*-value
Sociodemographic								
Age (years)								
18–29	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
30–39	0.59 (0.54–0.65)	<0.001	0.77 (0.68–0.88)	<0.001	0.58 (0.53–0.63)	<0.001	0.79 (0.70–0.90)	0.0002
40–49	0.40 (0.34–0.48)	<0.001	0.75 (0.60–0.93)	0.009	0.37 (0.31–0.44)	<0.001	0.66 (0.54–0.82)	0.0002
50–59	0.62 (0.49–0.79)	<0.001	0.89 (0.66–1.20)	0.451	0.67 (0.53–0.83)	0.0004	0.99 (0.75–1.32)	0.9683
60–	1.16 (0.81–1.65)	0.427	0.90 (0.55–1.46)	0.659	0.96 (0.65–1.41)	0.8292	0.68 (0.41–1.13)	0.1357
Sex								
Men	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Women	0.44 (0.40–0.48)	<0.001	0.80 (0.72–0.89)	<0.001	0.45 (0.42–0.49)	<0.001	0.79 (0.71–0.88)	<0.001
Educational status								
Below high school	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
High school graduate	0.34 (0.30–0.38)	<0.001	0.69 (0.60–0.79)	<0.001	0.35 (0.31–0.39)	<0.001	0.67 (0.58–0.77)	<0.001
University graduate	0.22 (0.20–0.24)	<0.001	0.65 (0.56–0.74)	<0.001	0.26 (0.23–0.29)	<0.001	0.69 (0.60–0.79)	<0.001
Ethnic groups								
Han	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Minority	3.33 (2.91–3.82)	<0.001	1.07 (0.89–1.29)	0.4750	2.85 (2.47–3.28)	<0.001	0.95 (0.79–1.15)	0.5987
Religion								
Atheist	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Others	3.51 (3.21–3.84)	<0.001	1.19 (1.04–1.35)	0.0089	3.05 (2.79–3.34)	<0.001	1.08 (0.95–1.23)	0.2262
Marital status								
Married	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Others	1.91 (1.76–2.07)	<0.001	1.42 (1.27–1.58)	<0.001	1.95 (1.80–2.12)	<0.001	1.39 (1.25–1.56)	<0.001
Score of health condition	0.97 (0.96–0.97)	<0.001	1.00 (0.99–1.00)	0.0014	0.97 (0.96–0.97)	<0.001	1.00 (0.99–1.00)	0.0004
Subjective social status								
In China	1.09 (1.06–1.11)	<0.001	0.98 (0.94–1.02)	0.2301	1.06 (1.04–1.08)	<0.001	0.99 (0.95–1.03)	0.5260
In one's community	1.10 (1.08–1.13)	<0.001	1.05 (1.01–1.09)	0.0173	1.07 (1.05–1.09)	<0.001	1.02 (0.98–1.06)	0.3521
Chronic condition								
0	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
1	5.01 (4.54–5.53)	<0.001	1.57 (1.37–1.80)	<0.001	4.41 (3.99–4.88)	<0.001	1.53 (1.34–1.75)	<0.001
2	4.99 (4.22–5.89)	<0.001	1.08 (0.88–1.34)	0.4637	4.65 (3.93–5.49)	<0.001	1.24 (1.00–1.52)	0.0465
≥3	5.25 (3.97–6.94)	<0.001	0.90 (0.65–1.25)	0.5386	4.99 (3.77–6.59)	<0.001	1.03 (0.74–1.42)	0.8830
Smoking status								
Current smoker	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Former smoker	0.56 (0.47–0.66)	<0.001	1.12 (0.91–1.38)	0.2859	0.49 (0.41–0.58)	<0.001	0.86 (0.70–1.07)	0.1845
Never smoker	0.15 (0.14–0.16)	<0.001	0.61 (0.52–0.70)	<0.001	0.19 (0.17–0.21)	<0.001	0.71 (0.62–0.82)	<0.001
Drinking status								
Current drinker	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Former drinker	1.20 (1.00–1.44)	0.0508	1.55 (1.22–1.96)	0.0003	1.05 (0.87–1.27)	0.6256	1.23 (0.96–1.56)	0.0956
Never drinker	0.27 (0.24–0.30)	<0.001	0.92 (0.78–1.07)	0.2774	0.30 (0.27–0.34)	<0.001	0.88 (0.76–1.02)	0.1011
Health behaviors								
Washing hands								
Increased	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Unchanged/Decreased	5.18 (4.76–5.63)	<0.001	1.75 (1.57–1.97)	<0.001	5.18 (4.76–5.63)	<0.001	1.62 (1.45–1.81)	<0.001
Wearing mask								
Increased	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Unchanged/Decreased	9.12 (8.29–10.03)	<0.001	1.80 (1.58–2.04)	<0.001	7.62 (6.92–8.38)	<0.001	1.71 (1.50–1.94)	<0.001
Social distance								
Increased	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Unchanged/Decreased	7.35 (6.45–8.39)	<0.001	1.73 (1.48–2.03)	<0.001	5.75 (5.10–6.49)	<0.001	1.53 (1.32–1.77)	<0.001
Awareness of COVID-19 pandemic								
COVID-19 conspiracy beliefs								
Level 1	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Level 2	1.27 (1.05–1.53)	0.0148	0.99 (0.79–1.24)	0.9291	1.24 (1.03–1.49)	0.0243	0.93 (0.75–1.15)	0.5166
Level 3	3.09 (2.64–3.63)	<0.001	1.11 (0.89–1.38)	0.3567	3.25 (2.79–3.80)	<0.001	1.14 (0.93–1.41)	0.2122
Level 4	6.52 (5.62–7.57)	<0.001	1.19 (0.94–1.51)	0.1496	5.80 (5.01–6.71)	<0.001	1.05 (0.84–1.31)	0.6929
Risk of COVID-19 infection								
Very high	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
High	2.19 (1.87–2.58)	<0.001	1.57 (1.29–1.91)	<0.001	2.06 (1.75–2.43)	<0.001	1.38 (1.13–1.68)	0.0013
Medium	1.35 (1.15–1.57)	0.0002	1.35 (1.11–1.63)	0.0025	1.32 (1.12–1.54)	0.0007	1.26 (1.04–1.53)	0.0168

(Continued)

TABLE 2 | Continued

Covariates	Primary vaccination				Booster vaccination			
	Model 1	*p*-value	Model 2	*p*-value	Model 1	*p*-value	Model 2	*p*-value
Low	0.36 (0.31–0.42)	<0.001	0.93 (0.77–1.13)	0.4856	0.41 (0.36–0.48)	<0.001	0.98 (0.81–1.19)	0.8654
No	0.46 (0.39–0.56)	<0.001	1.02 (0.82–1.28)	0.8438	0.47 (0.39–0.57)	<0.001	0.98 (0.78–1.23)	0.8688
Not sure	1.00 (0.78–1.27)	0.9784	0.84 (0.59–1.20)	0.3311	1.19 (0.94–1.50)	0.1451	1.05 (0.75–1.46)	0.7910
Curability of COVID-19								
Very high	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
High	2.13 (190–2.40)	<0.001	1.36 (1.19–1.56)	<0.001	2.00 (1.78–2.25)	<0.001	1.27 (1.11–1.46)	0.0004
Medium	7.24 (6.41–8.19)	<0.001	2.43 (2.09–2.82)	<0.001	6.76 (5.98–7.64)	<0.001	2.32 (2.00–2.68)	<0.001
Low	6.77 (5.79–7.91)	<0.001	2.82 (2.32–3.43)	<0.001	6.89 (5.91–8.04)	<0.001	3.01 (2.50–3.63)	<0.001
No	5.06 (3.93–6.52)	<0.001	2.01 (1.44–2.81)	<0.001	5.40 (4.22–6.90)	<0.001	2.57 (1.88–3.51)	<0.001
Not sure	3.67 (2.88–4.67)	<0.001	1.98 (1.42–2.77)	<0.001	3.68 (2.90–4.68)	<0.001	1.83 (1.32–2.54)	0.0003
COVID-19 vaccine exception								
Channel of vaccine information								
Community worker	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Internet	1.62 (1.44–1.82)	<0.001	1.39 (1.21–1.59)	<0.001	1.62 (1.44–1.82)	<0.001	1.36 (1.19–1.56)	<0.001
Others	4.13 (3.65–4.67)	<0.001	2.21 (1.90–2.56)	<0.001	3.88 (3.43–4.38)	<0.001	2.09 (1.81–2.42)	<0.001
Vaccine conspiracy beliefs								
Level 1	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Level 2	1.15 (0.94–1.39)	0.1734	1.07 (0.85–1.36)	0.5446	1.27 (1.05–1.54)	0.0152	1.32 (1.06–1.66)	0.0146
Level 3	2.69 (2.28–3.17)	<0.001	1.24 (1.00–1.55)	0.0507	2.90 (2.45–3.42)	<0.001	1.58 (1.27–1.95)	<0.001
Level 4	8.39 (7.20–9.77)	<0.001	1.37 (1.08–1.74)	0.0090	8.40 (7.20–9.81)	<0.001	1.77 (1.40–2.23)	<0.001
Weigh risks of vaccination against risks of the disease								
Disease outweigh vaccine	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Vaccine outweigh disease	2.64 (2.42–2.87)	<0.001	1.19 (1.07–1.32)	0.0009	2.77 (2.54–3.02)	<0.001	1.33 (1.20–1.47)	<0.001
Other life/health responsibilities								
Very high	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
High	1.72 (1.50–1.97)	<0.001	0.96 (0.82–1.13)	0.6023	2.09 (1.83–2.39)	<0.001	1.16 (0.99–1.35)	0.0598
Medium	9.94 (8.78–11.25)	<0.001	2.14 (1.82–2.51)	<0.001	10.28 (9.08–11.64)	<0.001	2.26 (1.93–2.65)	<0.001
Low	16.61 (14.47–19.07)	<0.001	2.94 (2.48–3.49)	<0.001	13.60 (11.80–15.67)	<0.001	2.41 (2.02–2.86)	<0.001
Very low	10.29 (8.65–12.23)	<0.001	3.16 (2.57–3.90)	<0.001	9.71 (8.14–11.57)	<0.001	3.04 (2.46–3.75)	<0.001
Type of vaccination								
Unvaccinated	1.00 (ref.)				1.00 (ref.)			
Viral vector	0.02 (0.02–0.03)	<0.001	0.03 (0.02–0.04)	<0.001	0.02 (0.01–0.02)	<0.001	0.02 (0.02–0.04)	<0.001
Inactivated	0.02 (0.02–0.03)	<0.001	0.03 (0.02–0.05)	<0.001	0.02 (0.01–0.03)	<0.001	0.03 (0.02–0.05)	<0.001
Protein subunit	0.08 (0.06–0.11)	<0.001	0.08 (0.05–0.12)	<0.001	0.06 (0.05–0.09)	<0.001	0.08 (0.05–0.12)	<0.001
Accept all vaccines	0.02 (0.01–0.03)	<0.001	0.04 (0.03–0.06)	<0.001	0.02 (0.01–0.02)	<0.001	0.04 (0.02–0.06)	<0.001
Convenient vaccination								
High	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Medium	4.32 (3.80–4.91)	<0.001	1.56 (1.32–1.85)	<0.001	4.03 (3.54–4.59)	<0.001	1.42 (1.20–1.67)	<0.001
Low	5.46 (4.34–6.87)	<0.001	2.21 (1.63–3.00)	<0.001	5.20 (4.13–6.56)	<0.001	1.85 (1.37–2.50)	<0.001
Trust in healthcare system								
Trust in doctors								
Level 1	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Level 2	0.39 (0.35–0.43)	<0.001	0.79 (0.69–0.91)	0.0012	0.39 (0.36–0.44)	<0.001	0.80 (0.69–0.91)	0.0009
Level 3	0.19 (0.17–0.22)	<0.001	0.70 (0.57–0.84)	0.0002	0.19 (0.16–0.21)	<0.001	0.65 (0.54–0.78)	<0.001
Level 4	0.06 (0.05–0.07)	<0.001	0.67 (0.50–0.89)	0.0056	0.06 (0.05–0.08)	<0.001	0.68 (0.52–0.89)	0.0045
Trust in developers								
Level 1	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
Level 2	0.32 (0.29–0.35)	<0.001	0.65 (0.57–0.74)	<0.001	0.32 (0.29–0.35)	<0.001	0.62 (0.55–0.71)	<0.001
Level 3	0.06 (0.05–0.07)	<0.001	0.45 (0.35–0.58)	<0.001	0.07 (0.06–0.08)	<0.001	0.49 (0.39–0.62)	<0.001

We categorized the score of COVID-19 conspiracy beliefs by quartiles as level 1 (≤7 points), level 2 (8–13 points), level 3 (14–20 points), and level 4 (≥21 points) and the score of vaccine conspiracy beliefs by quartiles as level 1 (≤7 points), level 2 (8–12 points), level 3 (13–18 points), and level 4 (≥19 points). We categorized the score of trust in doctors by quartiles as level 1 (≤30 points), level 2 (31–34 points), level 3 (35–38 points), and level 4 (≥39 points) and the score of trust in developers by quartiles as level 1 (≤17 points), level 2 (18–21 points), and level 3 (≥22 points).

Model 1: unadjusted.

Model 2: adjusted age, sex, educational status, ethnic groups, religion, marital status, change one's job, family doctor, score of health condition, subjective social status in China, subjective social status in one's community, body mass index, chronic condition, smoking status, drinking status, health behaviors, COVID-19 conspiracy beliefs, risk of COVID-19 infection, curability of COVID-19, channel of vaccine information, vaccine conspiracy beliefs, weigh risks of vaccination against risks of the disease, other life/health responsibilities, trust in doctors, trust in developers, and convenient vaccination.

TABLE 3 | Associations between COVID-19 vaccine hesitancy transformations and sociodemographic, awareness of COVID-19 pandemic, COVID-19 vaccine exception, and trust in healthcare system.

Covariates	Acceptance to hesitancy		Hesitancy to acceptance		Hesitancy to hesitancy	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
Sociodemographic						
Age (years)						
18–29	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
30–39	0.60 (0.52–0.68)*	0.72 (0.61–0.86)*	0.63 (0.56–0.72)*	0.73 (0.61–0.87)*	0.54 (0.48–0.61)*	0.75 (0.64–0.89)*
40–49	0.34 (0.26–0.45)*	0.51 (0.37–0.70)*	0.41 (0.32–0.53)*	0.65 (0.48–0.89)*	0.37 (0.30–0.47)*	0.72 (0.54–0.96)*
50–59	0.49 (0.33–0.72)*	0.74 (0.47–1.14)	0.39 (0.26–0.61)*	0.60 (0.37–0.99)*	0.76 (0.58–1.00)*	1.08 (0.76–1.55)
60–	0.68 (0.35–1.33)	0.64 (0.30–1.39)	1.09 (0.63–1.87)	0.97 (0.50–1.88)	1.17 (0.75–1.84)	0.79 (0.41–1.52)
Sex						
Men	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Women	0.48 (0.42–0.55)*	0.77 (0.66–0.90)*	0.45 (0.40–0.51)*	0.78 (0.67–0.91)*	0.41 (0.37–0.46)*	0.74 (0.64–0.85)*
Educational status						
Below high school	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
High school graduate	0.35 (0.29–0.41)*	0.62 (0.51–0.77)*	0.33 (0.28–0.39)*	0.66 (0.54–0.80)*	0.30 (0.26–0.35)*	0.64 (0.53–0.77)*
University graduate	0.29 (0.25–0.33)*	0.70 (0.58–0.85)*	0.21 (0.18–0.24)*	0.63 (0.52–0.77)*	0.20 (0.18–0.23)*	0.58 (0.48–0.70)*
Ethnic groups						
Han	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Minority	2.62 (2.10–3.26)*	1.00 (0.76–1.31)	3.60 (2.96–4.37)*	1.13 (0.87–1.46)*	3.49 (2.93–4.15)*	0.99 (0.77–1.26)
Religion						
Atheist	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Others	3.02 (2.64–3.46)*	1.17 (0.98–1.39)	3.99 (3.51–4.55)*	1.38 (1.15–1.64)*	3.54 (3.16–3.98)*	1.18 (1.00–1.40)
Marital status						
Married	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Others	1.72 (1.52–1.94)*	1.22 (1.04–1.43)*	1.64 (1.45–1.85)*	1.22 (1.04–1.44)*	2.22 (2.00–2.47)*	1.63 (1.40–1.88)*
Score of health condition	0.97 (0.96–0.97)*	0.99 (0.99–1.00)	0.97 (0.96–0.97)*	0.99 (0.99–1.00)*	0.96 (0.96–0.97)*	1.00 (0.99–1.00)*
Subjective social status						
In China	1.09 (1.06–1.12)*	1.09 (1.05–1.12)*	1.15 (1.11–1.18)*	0.99 (0.94–1.05)	1.05 (1.02–1.08)*	0.97 (0.92–1.02)
In one's community	0.99 (0.99–1.00)*	0.99 (0.99–1.00)*	1.17 (1.13–1.20)*	1.07 (1.01–1.13)*	1.07 (1.04–1.09)*	1.03 (0.98–1.08)
Chronic condition						
0	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
1	3.76 (3.23–4.38)*	1.46 (1.20–1.77)*	4.90 (4.23–5.68)*	1.47 (1.21–1.77)*	5.78 (5.10–6.55)*	1.99 (1.67–2.38)*
2	5.06 (3.99–6.41)*	1.37 (1.03–1.82)*	5.83 (4.61–7.37)*	1.08 (0.81–1.44)	5.35 (4.31–6.64)*	1.22 (0.92–1.62)
≥3	5.26 (3.53–7.83)*	1.04 (0.67–1.63)	5.86 (3.93–8.74)*	0.81 (0.52–1.27)	5.89 (4.13–8.40)*	0.94 (0.61–1.45)
Smoking status						
Current smoker	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Former smoker	0.36 (0.27–0.49)*	0.72 (0.51–1.01)	0.51 (0.40–0.65)*	1.13 (0.85–1.50)	0.54 (0.44–0.67)*	1.16 (0.88–1.52)
Never smoker	0.20 (0.18–0.23)*	0.72 (0.59–0.88)*	0.12 (0.11–0.14)*	0.52 (0.42–0.64)*	0.15 (0.14–0.17)*	0.63 (0.52–0.77)*
Drinking status						
Current drinker	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Former drinker	0.78 (0.56–1.08)	1.04 (0.72–1.50)	1.09 (0.83–1.44)	1.62 (1.16–2.25)*	1.26 (1.00–1.59)	1.62 (1.19–2.22)*
Never drinker	0.31 (0.26–0.36)*	0.77 (0.62–0.95)*	0.23 (0.19–0.28)*	0.85 (0.66–1.08)	0.28 (0.24–0.32)*	0.94 (0.76–1.15)
Health behaviors						
Washing hands						
Increased	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Unchanged/Decreased	4.06 (3.58–4.60)*	1.40 (1.19–1.64)*	5.05 (4.46–5.71)*	1.57 (1.33–1.85)*	7.31 (6.54–8.15)*	2.07 (1.79–2.40)*
Wearing mask						
Increased	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Unchanged/Decreased	6.72 (5.81–7.78)*	1.60 (1.33–1.93)*	9.40 (8.19–10.78)*	1.82 (1.52–2.19)*	11.75 (10.44–13.24)*	2.14 (1.82–2.52)*
Social distance						
Increased	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Unchanged/Decreased	5.08 (4.28–6.02)*	1.58 (1.30–1.93)*	8.69 (7.05–10.71)*	2.12 (1.67–2.70)*	7.13 (6.03–8.43)*	1.49 (1.22–1.82)*
Awareness of COVID-19 pandemic						
COVID-19 conspiracy beliefs						
Level 1	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Level 2	1.44 (1.09–1.90)*	1.01 (0.75–1.36)	1.53 (1.15–2.04)*	1.22 (0.88–1.69)	1.11 (0.86–1.42)	0.89 (0.66–1.21)
Level 3	3.32 (2.62–4.20)*	1.07 (0.80–1.44)	2.96 (2.29–3.81)*	1.09 (0.78–1.52)	3.33 (2.73–4.07)*	1.36 (1.03–1.80)*
Level 4	6.40 (5.12–8.00)*	0.93 (0.68–1.28)	8.21 (6.51–10.36)*	1.23 (0.86–1.75)	6.20 (5.13–7.50)*	1.21 (0.89–1.65)
Risk of COVID-19 infection						
Very high	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
High	1.97 (1.57–2.48)*	1.42 (1.09–1.85)*	2.19 (1.76–2.73)*	1.72 (1.33–2.22)*	2.48 (1.98–3.10)*	1.60 (1.21–2.10)*
Medium	0.96 (0.77–1.21)	1.10 (0.85–1.43)	1.04 (0.83–1.29)	1.19 (0.92–1.54)	1.68 (1.36–2.08)*	1.54 (1.18–2.01)*

(Continued)

TABLE 3 | Continued

Covariates	Acceptance to hesitancy		Hesitancy to acceptance		Hesitancy to hesitancy	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
Low	0.36 (0.29–0.44)*	0.89 (0.69–1.16)	0.26 (0.21–0.33)*	0.79 (0.61–1.03)	0.43 (0.35–0.54)*	1.07 (0.82–1.40)
No	0.35 (0.27–0.46)*	0.84 (0.61–1.14)	0.34 (0.26–0.45)*	0.88 (0.64–1.21)	0.56 (0.43–0.71)*	1.11 (0.81–1.51)
Not sure	0.83 (0.58–1.18)	0.94 (0.58–1.50)	0.53 (0.35–0.79)*	0.55 (0.31–0.97)*	1.48 (1.10–2.00)*	0.83 (0.53–1.31)
Curability of COVID-19						
Very high	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
High	1.90 (1.61–2.24)*	1.30 (1.09–1.56)*	2.14 (1.81–2.53)*	1.47 (1.22–1.78)*	2.20 (1.87–2.58)*	1.35 (1.12–1.63)*
Medium	5.67 (4.75–6.77)*	2.17 (1.77–2.67)*	6.50 (5.44–7.77)*	2.38 (1.92–2.95)*	9.22 (7.83–10.85)*	2.97 (2.44–3.62)*
Low	5.26 (4.17–6.62)*	2.85 (2.18–3.72)*	4.98 (3.91–6.35)*	2.51 (1.88–3.36)*	9.54 (7.84–11.62)*	4.03 (3.15–5.17)*
No	3.63 (2.44–5.40)*	2.02 (1.27–3.21)*	2.94 (1.87–4.60)*	1.11 (0.62–1.99)	7.57 (5.60–10.22)*	2.97 (1.99–4.43)*
Not sure	3.01 (2.10–4.31)*	1.95 (1.23–3.08)*	2.94 (2.02–4.28)*	2.20 (1.36–3.56)*	4.63 (3.41–6.28)*	1.88 (1.22–2.90)*
COVID-19 vaccine exception						
Channel of vaccine information						
Community worker	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Internet	1.52 (1.28–1.79)*	1.34 (1.12–1.61)*	1.51 (1.28–1.80)*	1.40 (1.15–1.69)*	1.75 (1.49–2.05)*	1.54 (1.28–1.86)*
Others	3.15 (2.63–3.76)*	1.99 (1.62–2.44)*	3.59 (3.01–4.30)*	2.15 (1.75–2.66)*	5.02 (4.26–5.91)*	2.69 (2.20–3.28)*
Vaccine conspiracy beliefs						
Level 1	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Level 2	1.44 (1.07–1.93)*	1.52 (1.10–2.09)*	1.13 (0.83–1.53)	1.09 (0.78–1.53)	1.17 (0.91–1.50)	1.16 (0.85–1.57)
Level 3	2.86 (2.20–3.72)*	1.79 (1.31–2.43)*	2.39 (1.84–3.09)*	1.16 (0.84–1.60)	3.00 (2.43–3.71)*	1.51 (1.13–2.00)*
Level 4	10.51 (8.26–13.39)*	2.29 (1.63–3.21)*	10.28 (8.15–12.98)*	1.41 (1.00–2.00)	8.55 (7.01–10.42)*	1.36 (0.99–1.85)
Weigh risks of vaccination against risks of the disease						
Disease outweigh vaccine	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Vaccine outweigh disease	2.82 (2.48–3.19)*	1.25 (1.08–1.44)*	2.53 (2.24–2.87)*	1.04 (0.89–1.20)	2.93 (2.63–3.27)*	1.28 (1.12–1.46)*
Other life/health responsibilities						
Very high	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
High	2.20 (1.83–2.64)*	1.25 (1.02–1.54)*	1.48 (1.21–1.81)*	0.87 (0.69–1.09)	2.02 (1.67–2.43)*	1.05 (0.85–1.31)
Medium	8.80 (7.35–10.52)*	2.04 (1.63–2.55)*	8.21 (6.86–9.83)*	1.77 (1.41–2.22)*	13.93 (11.80–16.45)*	2.70 (2.18–3.34)*
Low	12.01 (9.74–14.81)*	2.19 (1.71–2.80)*	16.97 (14.00–20.56)*	2.89 (2.29–3.65)*	21.57 (17.94–25.95)*	3.24 (2.57–4.08)*
Very low	9.24 (7.17–11.91)*	2.85 (2.12–3.84)*	10.24 (8.03–13.06)*	3.09 (2.32–4.12)*	12.81 (10.18–16.11)*	3.88 (2.92–5.15)*
Type of vaccination						
Unvaccinated	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Viral vector	0.05 (0.03–0.09)*	0.11 (0.05–0.23)*	0.09 (0.05–0.16)*	0.24 (0.10–0.57)*	0.01 (0.00–0.01)*	0.01 (0.00–0.01)*
Inactivated	0.05 (0.03–0.09)*	0.15 (0.07–0.30)*	0.08 (0.04–0.14)*	0.27 (0.12–0.63)*	0.01 (0.00–0.01)*	0.01 (0.01–0.02)*
Protein subunit	0.14 (0.08–0.24)*	0.29 (0.15–0.60)*	0.21 (0.11–0.39)*	0.54 (0.23–1.25)	0.03 (0.02–0.05)*	0.04 (0.02–0.06)*
Accept all vaccines	0.03 (0.02–0.05)*	0.13 (0.06–0.26)*	0.04 (0.02–0.08)*	0.22 (0.09–0.52)*	0.01 (0.01–0.01)*	0.02 (0.01–0.03)*
Convenient vaccination						
High	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Medium	3.03 (2.46–3.75)*	1.21 (0.94–1.55)	3.55 (2.91–4.33)*	1.41 (1.10–1.81)*	5.52 (4.73–6.45)*	1.71 (1.39–2.11)*
Low	3.36 (2.26–5.01)*	1.47 (0.90–2.38)	3.78 (2.58–5.54)*	2.07 (1.31–3.27)*	7.66 (5.87–9.99)*	2.81 (1.95–4.06)*
Healthcare system						
Trust in doctors						
Level 1	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Level 2	0.40 (0.34–0.46)*	0.78 (0.64–0.94)*	0.38 (0.33–0.44)*	0.78 (0.64–0.95)*	0.35 (0.31–0.40)*	0.78 (0.65–0.94)*
Level 3	0.17 (0.14–0.21)*	0.52 (0.40–0.69)*	0.18 (0.15–0.22)*	0.58 (0.44–0.77)*	0.17 (0.15–0.20)*	0.70 (0.54–0.91)*
Level 4	0.07 (0.06–0.10)*	0.56 (0.39–0.82)*	0.06 (0.05–0.09)*	0.55 (0.36–0.83)*	0.05 (0.04–0.07)*	0.72 (0.49–1.06)
Trust in developers						
Level 1	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Level 2	0.30 (0.26–0.34)*	0.66 (0.55–0.79)*	0.31 (0.27–0.36)*	0.71 (0.59–0.87)*	0.29 (0.26–0.33)*	0.58 (0.49–0.69)*
Level 3	0.08 (0.07–0.10)*	0.68 (0.49–0.93)*	0.06 (0.05–0.08)*	0.61 (0.43–0.87)*	0.04 (0.03–0.05)*	0.36 (0.26–0.51)*

*$p < 0.05$.

We categorized the score of COVID-19 conspiracy beliefs by quartiles as level 1 (≤7 points), level 2 (8–13 points), level 3 (14–20 points), and level 4 (≥21 points) and the score of vaccine conspiracy beliefs by quartiles as level 1 (≤7 points), level 2 (8–12 points), level 3 (13–18 points), and level 4 (≥19 points). We categorized the score of trust in doctors by quartiles as level 1 (≤30 points), level 2 (31–34 points), level 3 (35–38 points), and level 4 (≥39 points) and the score of trust in developers by quartiles as level 1 (≤17 points), level 2 (18–21 points), and level 3 (≥22 points).

Model 1: unadjusted.

Model 2: adjusted age, sex, educational status, ethnic groups, religion, marital status, change one's job, family doctor, score of health condition, subjective social status in China, subjective social status in one's community, body mass index, chronic condition, smoking status, drinking status, health behaviors, COVID-19 conspiracy beliefs, risk of COVID-19 infection, curability of COVID-19, channel of vaccine information, vaccine conspiracy beliefs, weigh risks of vaccination against risks of the disease, other life/health responsibilities, trust in doctors, trust in developers, and convenient vaccination.

delayed, resulting in widespread of vaccine hesitancy, wreaking havoc on individuals and the healthcare systems. Thus, in the future, policy development in China should prioritize minimizing existing inequalities among provinces when it comes to vaccination.

COVID-19 vaccine hesitancy increases with inconvenience of vaccination. Although China established tens of thousands of temporary vaccination sites in a relatively short time, vaccination service is provided through appointments and the waiting time at the vaccination site is frequently longer due to limited health personnel resources and a shortage of vaccines. This situation brings a lot of inconvenience to vaccinators and may have played a significant role towards vaccine acceptance rate. In order to effectively prevent the spread of the virus, vaccinators have to be registered through a reliable and user-friendly appointment system. Additionally, the majority of residents in China are not yet accustomed to vaccine appointments, which creates a considerable "sense of inconvenience" for vaccinators, which in turn causes some people to have doubts about whether to receive a vaccination. Therefore, for improving COVID-19 vaccination uptake, it is particularly important to improve the experience of vaccination services. Key measures that should also be considered include increasing the number of vaccination personnel, vaccine supply, encouraging qualified medical institutions to provide vaccination services, and actively using digital technologies to reduce waiting time (25–27).

There is a considerable link between doctor and vaccine developer distrust and COVID-19 vaccine hesitation. In essence, willingness to take a vaccination is a matter of trust: that the vaccine is necessary, that it will function, and that it is safe. Due to recent vaccination-related adverse events and instances of counterfeit vaccine, the public's trust in medical professionals and vaccine developers has decreased significantly (28). To build faith in the vaccine, the vaccination service organization should, on the one hand, expand the number of vaccination medical personnel, train and develop doctor-patient communication skills, and improve the quality of vaccination service evaluation. China should also accelerate the development of a vaccine industry credibility system, encourage vaccine production, and encourage companies to take the lead in vaccine production and circulation while ensuring the quality and safety of vaccine products from development stage to circulation. In addition, recommending the one vaccine in which at the given moment is with the highest level of the public willingness will likely result in a less prevalence of COVID-19 vaccination hesitancy.

It is worth mentioning that when the role of gender in COVID-19 vaccine hesitancy was assessed, males were shown to be more likely to reject the vaccine. The finding was consistent with a previous research in which a higher vaccine acceptance rate was associated with men's increased perception of COVID-19 vaccine and decreased belief in disease-related conspiracy theories (11). However, our research was carried out at the stage when the vaccination rate had already exceeded 60%. As information about the COVID-19 vaccination circulated, women may have become fully aware of the implications of the disease and so lost belief in conspiratorial claims, implying that their vaccine hesitancy rate was found to be lower than that of males. In the future COVID-19 vaccination, attention should be paid to increasing the vaccination rate of men. Additionally, age, men, educational level, marital status, self-report health condition, subjective social status, smoking status, healthy behaviors, the curability of COVID-19, the channel of accessing information of COVID-19 vaccine, endorsement of vaccine conspiracy beliefs, weigh risks of vaccination against risks of the disease, and other life/health responsibilities were all found to be independently associated with COVID-19 vaccine hesitancy. Numerous reports show that the mechanism underlying vaccine hesitancy is exceedingly complicated, and that effective countermeasures should be implemented concurrently (17, 20, 29, 30). Firstly, we think that assisting persons with poor information and insufficient health literacy in obtaining a correct understanding of vaccines through education may play a critical role. Government authorities should also communicate clearly and consistently in order to instill public confidence in vaccination programs. This involves describing how vaccines function and are created, from recruiting to regulatory approval based on safety and efficacy. Effective campaigns should also carefully describe the level of effectiveness of a vaccine, the duration of protection (with multiple doses if necessary), and the critical nature of population-wide coverage in order to attain the herd immunity. Secondly, vaccine information transmitted by the Internet and the media should be effectively identified and any misleading information must be eliminated. The Internet and other forms of media should serve as a link between vaccination services and the general population through disseminating vaccine knowledge received through official channels and eradicating social misconceptions about the vaccines.

Strengths and Weaknesses of the Study

This is the first large-scale study to assess the prevalence and associated factors of COVID-19 vaccination hesitancy in a large, saturated sample of the Chinese population. Due to the saturation of the sample, we can be certain that our estimate of vaccine hesitancy is accurate. To provide more extensive explanatory variables, we adopted the most widely accepted international definition of vaccine hesitancy and collected data using the EAH and 3C frameworks. One of the major limitations of the current study is that it relies on self-reports of willingness to take a COVID-19 vaccination to assess vaccine hesitancy, and we were unable to develop a standard for validation due to the lack of a universal scale to assess COVID-19 vaccine hesitancy in China. Due to the fact that an accurate assessment of COVID-19 vaccine hesitancy can serve as an important basis for vaccine development and production, as well as the estimation of market demand, the development of a global scale for COVID-19 vaccine hesitancy assessment will become one of the important directions of future research. However, the COVID-19 vaccine hesitancy was assessed from a reliable questionnaire and the results of it was similar with previous studies according to the Oxford COVID-19 Vaccine Hesitancy Scale (13, 31). Another

shortcoming of the study includes its cross-sectional design, which precluded the establishment of a cause-and-effect link. Finally, despite the fact that we used data from a large saturation sample of the population from 31 provinces, due to the epidemic, we were forced to collect data *via* online questionnaires utilizing the snowball sampling approach. Therefore, these research findings may differ from those estimated using probability sampling. In addition, the influence of socioeconomic level on COVID-19 vaccination hesitancy observed in this study may not be applicable to persons without Internet access.

CONCLUSIONS

Despite the aforementioned constraints, COVID-19 vaccination hesitancy prevalence in China is modest in comparison with other countries. This will lay a solid foundation for future booster vaccinations. However, interprovincial disparities in COVID-19 vaccine hesitation may delay the onset of herd immunity, and local vaccination efforts should be stepped up in Tianjin, Hebei, Beijing, and Hainan provinces due to their significantly greater frequency of COVID-19 vaccine hesitancy. Emphasis should be placed on building trust in medical personnel and vaccine producers, promoting the convenience of vaccination services, and spreading reliable COVID-19 vaccine information *via* the Internet and other media.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

Conceptualization: YM, JW, QL, MW, and JG. Data curation: YM, QL, WW, and MM. Formal analysis: JW, QL, and YM. Funding acquisition: MW and JG. Investigation: JW, YM, QL, MM, LZ, and ZM. Methodology: YM, JW, QL, and CT. Project administration: JW. Resources: JW and YM. Software: QL, LZ, and ZM. Writing—original draft: JW, YM, QL, and CT. Writing—review and editing: YM, CT, MW, and JG. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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The Humoral Immune Response to BNT162b2 Vaccine Is Associated With Circulating CD19+ B Lymphocytes and the Naïve CD45RA to Memory CD45RO CD4+ T Helper Cells Ratio in Hemodialysis Patients and Kidney Transplant Recipients

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Background: The humoral and cellular immune responses to SARS-COV-2 vaccination remain to be elucidated in hemodialysis (HD) patients and kidney transplant recipients (KTRs), considering their baseline immunosuppressed status. The aim of our study was to assess the associations of vaccine-induced antibody responses with circulating lymphocytes sub-populations and their respective patterns of alterations in maintenance HD patients and KTRs.

Materials and Methods: We included 34 HD patients and 54 KTRs who received two doses of the mRNA-vaccine BNT162b2. Lymphocyte subpopulations were analyzed by flow cytometry before vaccination (T0), before the second vaccine dose (T1) and 2 weeks after the second dose (T2). The anti-SARS-CoV2 antibody response was assessed at T1 and at T2.

Results: 31 HD patients (91.8%) and 16 KTRs (29.6%) became seropositive at T2. HD patients who became seropositive following the first dose displayed higher CD19+ B lymphocytes compared to their seronegative HD counterparts. A positive correlation was established between CD19+ B cells counts and antibody titers at all time-points in both groups ($p < 0.001$). KTRs showed higher naïve CD4+CD45RA+ T helper cells compared to HD patients at baseline and T2 whereas HD patients displayed higher memory CD45RO+ T cells compared to KTRs at T2. The naïve CD4+CD45RA to memory CD4+

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CD45RO+ T helper cells fraction was negatively associated with antibody production in both groups.

Conclusions: Our study provides a potential conceptual framework for monitoring vaccination efficacy in HD patients and KTRs considering the correlation established between CD19+ B cells, generation of memory CD4+ T helper cells and anti SARS-CoV2 antibody response to vaccination.

Keywords: SARS-COV-2 vaccination, hemodialysis, kidney transplant recipients, anti-SARS-CoV2 antibodies, CD19+ B lymphocytes, naïve CD4+CD45RA+ T helper cells, memory CD4+CD45RO+ T helper cells

INTRODUCTION

The COVID-19 pandemic poses unique challenges to patients undergoing maintenance renal replacement therapy and kidney transplant recipients (KTRs) with available evidence until now indicating a higher morbidity and mortality trend following infection compared with the general population (1, 2). Despite increased rates of vaccination among these vulnerable populations, the adequacy of the respective generated immune responses remains a subject of concern and ongoing evaluation. The complex derangement of the immune system as occurs both in end-stage kidney disease (ESKD) and kidney transplantation has been directly associated with an increased susceptibility to infections and impaired response to vaccination in these patients (3, 4). The uremic milieu of ESKD and the immunosuppressive and immunomodulatory medications administered in the setting of kidney transplantation affect directly both the humoral and cell-mediated immunity (5–8). Overall, decreased numbers of circulating T, B and NK lymphocytes as well as altered CD4+ and CD8+ T cell responses and low antibody production by B lymphocytes following stimulation have been found in hemodialysis patients (5, 6). Likewise, altered T-cell activation, proliferation, cytokine production and cytotoxicity and B-cell lymphopenia represent the hallmark of the immunosuppressed state of kidney transplantation (7, 8).

Available reports regarding the humoral response to COVID-19 vaccination in patients receiving maintenance hemodialysis, show better results compared to the poor antibody response of KTRs (9). Yet lower overall antibody titers in maintenance hemodialysis patients as compared to individuals without kidney disease have been reported (10–12).

Specific T-cell memory responses elicited by the SARS-CoV-2 infection might play a significant protective role even in the absence of specific antibodies (13, 14). Despite an abundance of data regarding generation of anti-S protein IgG and virus-specific neutralizing antibody responses, T cell responses following vaccination, including patterns of naive T cell activation and differentiation into effector cells have not been fully evaluated. Reduced numbers of NK cells in peripheral blood together with NK cell hyperactivation and dysfunction have been found in patients with severe Covid-19 disease, whereas there is scarce and controversial evidence regarding NKT cells responses in this setting (15, 16). Furthermore models of vaccine-dependent generation of antigen-specific memory NK cells and the utilization of NKT cell agonists as novel immune adjuvants in the setting of vaccination have received increasing attention recently (17, 18). With regard to the baseline immunosuppressed state associated with ESKD and transplantation, there is a paucity of data regarding the analysis of peripheral blood lymphocyte subpopulations, their patterns of change following vaccination against SARS COV-2 as well as their respective immunologic and clinical significance in such context.

Considering that the immune response is orchestrated by the specialized subpopulations of lymphocytes, the aim of our study was to evaluate and compare the antibody response status together with vaccine-induced alterations in circulating lymphocytes subsets, including B cells, CD4+ and CD8+ T cells, naïve and memory T lymphocytes subpopulations, as well as well as NK and NKT cells, following the administration of two doses of the BNT162b2 vaccine in a cohort of maintenance hemodialysis (HD) patients and KTRs.

MATERIALS AND METHODS

This prospective study was conducted in the Hemodialysis Unit of the Nephrology Department and the Kidney Transplant Unit of the University Hospital of Ioannina. The study protocol was approved by the Ethical Committee of our hospital (8/14-4-2021) and has been registered on ClinicalTrials.gov (NCT04932876). We included in our study 34 chronic HD patients and 54 KTRs with no previous history of SARS-CoV2 infection, who received two doses of the mRNA-vaccine BNT162b2. The two doses of the BNT162b2 vaccine were administered intramuscularly and 21 days apart. All patients provided signed informed consent for participation in the study. Exclusion criteria included presence of active autoimmune disease, active malignancy and chronic infections (HBV, HCV, HIV). In addition, patients with acute infections, recent surgical procedures within the last 2 weeks from vaccination or organ transplantation within the last six months from vaccination were excluded from the study.

Clinical data, including the maintenance immunosuppressant regimen were recorded from the patients' medical files. Furthermore, baseline routine laboratory tests and blood levels of immunosuppressive medications (tacrolimus and cyclosporine) were obtained at all time points. In KTRs estimated glomerular filtration rate (eGFR) was calculated

using the CKD-EPI formula and 24-hours protein urine excretion was assessed.

Anti-SARS-CoV2 Antibody Response

Serologic response was assessed by using the ARCHITECT IgG II Quant test (Abbott). Titers >50 arbitrary units (AU)/ml were considered positive for seroconversion (detection range, 6.8–80,000 AU/ml); positive agreement, 99.4%; negative agreement, 99.6%. The anti-SARS-CoV2 antibody response against the spike protein was assessed at two time points, immediately before the second vaccine dose (T1) and 2 weeks after administration of the second dose (T2).

Flow Cytometry Analysis

Conjugated monoclonal antibodies were used for four-color flow cytometric analysis performed in a FACScalibur cytometer (Becton Dickinson). The particular anti-human antibodies used were: CD3-FITC (Clone UCHT1), CD4-PE (Clone MEM-241), CD4-APC (Clone MEM-241), CD8-APC (Clone MEM-31), CD16-PE (Clone MEM-154), CD19-APC (Clone LT19), CD45-PerCP (Clone MEM-28), CD45RA-FITC (Clone MEM-56), CD45RO-PE (Clone UCHL1) and CD56-PE (Clone LT56), purchased from EXBIO, Praha SA. 100 μl of whole-blood was added to flow cytometry (FC) tubes and incubated with respective antibodies according to manufacturer's instructions. 500 μl of Versalyse (Beckman Coulter) was added and incubated for 10 minutes at room temperature (18-25°C) protected from light, to lyse red blood cells. Samples were processed immediately for four-color FC analysis. The data were analyzed using the CellQuest V3.1 software (Becton Dickinson). Lymphocyte subpopulations, including NKT cells and NK cells, CD19+ B lymphocytes, CD45RA+ (naïve) CD45RA+CD45RO+ (transient) and CD45RO+ (memory) T cell isoforms, CD4+ T helper cells, CD8+ T cells, CD4+CD45RA (naive) T helper cells, CD4+CD45RO (memory) T helper cells and their ratio were analyzed by FC within 24 hours from sampling at three time points, at baseline, i.e. before vaccination (T0), immediately before the second vaccine dose (T1), and 2 weeks following administration of the second vaccine dose (T2) (**Figure 1**). In specific and as previously described, whole blood from each individual was analyzed by flow cytometry, for the presence of specific lymphocyte subpopulations at T1 and T2, at the same time points that antibody response was evaluated.

Statistical Analysis

Means and standard deviations were used to express all outcome measures while the median and IQR were reported in cases where normality was not met, under the Shapiro Wilk criterion. A mixed models approach was adopted to examine differences within the levels of each outcome across the three time points but also between KTRs and HD patients. Differences between responders and non responders were also examined where the sample size was not too small. The Tukey's HSD criterion was applied to adjust for the significance level after multiple comparisons. Correlations between different parameters were established using Spearman's Rho criterion. Depiction of correlations was performed using Regression Variable Plots. The SPSS v23.0 software was applied to analyze all data and the significance level was set at 0.05 in all cases.

RESULTS

The main demographics, clinical and laboratory parameters of the HD patients and KTRs are depicted in **Table 1**. Hemodialysis patients were significantly older than KTRs (69.4 ± 12.3 vs 58.2 ± 9.7, $p<0.001$) and had significantly lower levels of hemoglobin in comparison to KTRs (10.8 ± 1.1 vs 13.5 ± 1.9 g/dl, $p<0.001$).

Overview of the Humoral Immune Response Following Administration of BNT162b2 Vaccine

With regard to antibody response status in patients undergoing maintenance dialysis, 17 (50%) of them became seropositive following administration of the first vaccine dose (T1), which increased to overall 31 patients (91.8%) becoming seropositive two weeks after administration of the second vaccine dose (T2). On the other hand, only 3 (5.6%) patients achieved seropositivity in the KTRs group after administration of the first vaccine dose (T1) and subsequently 16 KTRs (29.6%) following the second vaccine dose (T2) (**Figure 2**). In the same line, the mean level of antibody titers was significantly higher in HD patients in comparison to KTRs in both measurements, following the first and second vaccine doses respectively (156.9 ± 279.8 vs 16.9 ± 74.6 g/dl, $p <0.001$ and 5759.9 ± 6771.6 vs 113.9 ± 300.0 g/dl, $p < 0.001$).

Table 2 presents the main demographics, clinical and laboratory parameters of the HD patients and KTRs with regard to their antibody response status and comparisons within each group between patients who converted and those who did not convert following the second vaccine dose. In specific, among KTRs, non-responders displayed lower eGFR levels (48.9 ± 17.2 vs 61.6 ± 15.4 ml/min per 1.73m^2, $p = 0.014$) and their regimen included more immunosuppressive medications (Tacrolimus+MMF/MPA+ Steroids 28, 73.7% vs 7, 43.8%, $p = 0.035$) as compared to responders.

Distribution of Specific Immune Cell Subsets Before and After Vaccination Against SARS-CoV-2

CD19+ B lymphocytes produce antibodies and are in control of the humoral immune response. CD19+ B lymphocyte counts (normal reference values 6-22%) before vaccination were 5.35% and 5.45% of total lymphocytes in the HD patients and KTRs, respectively. No significant differences were found between the two patients groups regarding CD19+ B cell counts at any time points with p-values exceeding 0.96 in all cases. Yet, HD patients who developed an antibody response with IgG antibodies against the spike receptor-binding domain (RBD) of SARS-CoV-2 above 50 AU/ml following administration of the first vaccine dose, had at all times higher levels of CD19+ B cell counts in comparison to HD patients who failed to generate an antibody response at this time point. Accordingly, the mean differences for CD19+ B cell

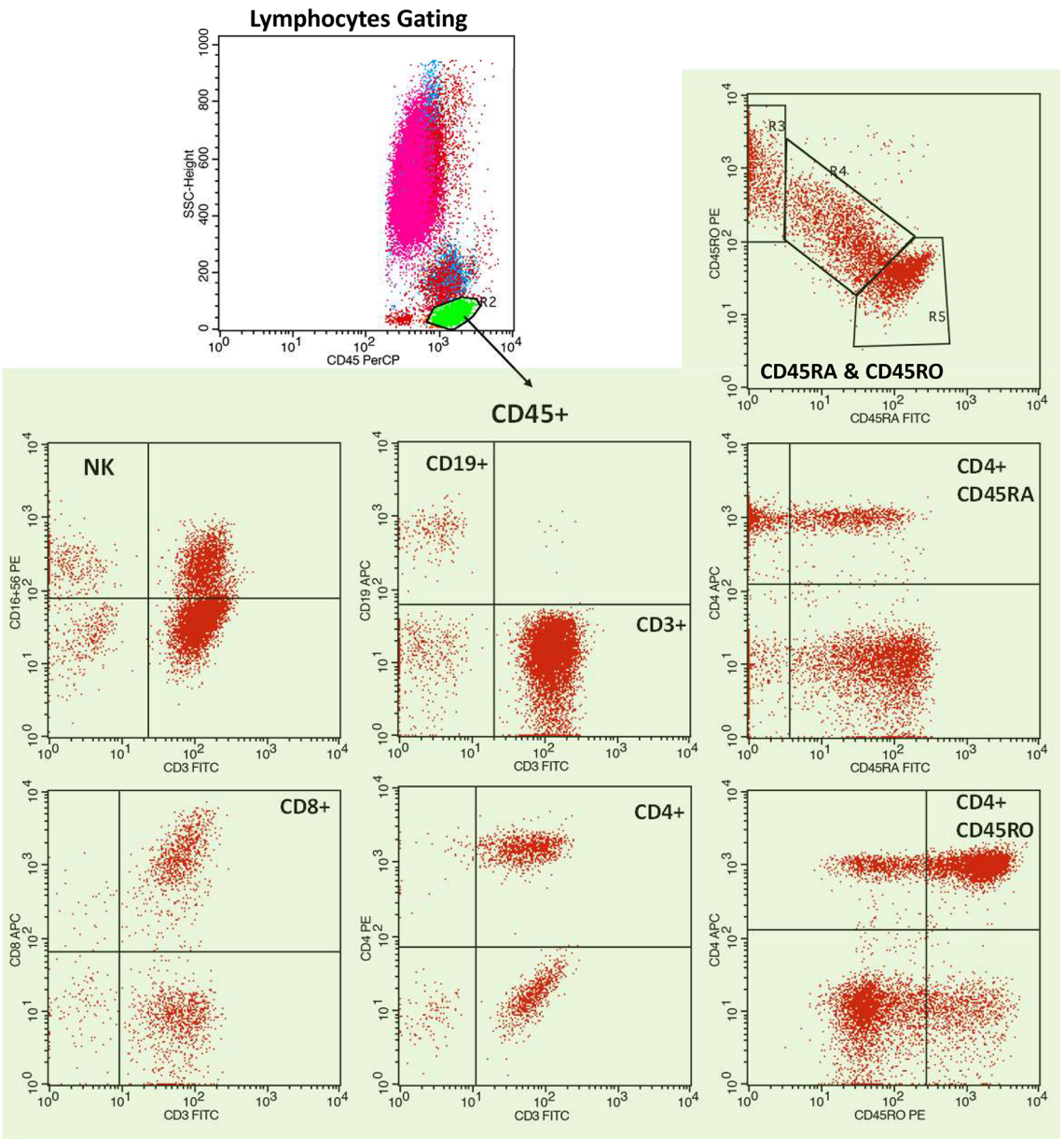

FIGURE 1 | Representative flow cytometric analysis of a hemodialysis patient. Representative dot plots depicting lymphocyte gating with B-lymphocytes (CD19+), and T lymphocytes (CD3+), CD4+ T cells, CD8+ T cells, naïve (CD45RA+) and memory (CD45RO+) T cell isoforms, naïve (CD4+CD45RA) T helper cells and memory (CD4+CD45RO) T helper cells, NK cells (CD3-CD16+CD56+) and NKT cells (CD3+CD16+CD56+).

counts at T0, T1 and T2 respectively were (3,118 ± 1.759% vs 7.588±,3.355% p=0.015), (3.117 ± 1.484% vs 7.118 ± 2.601%, p=0.049), (3.323 ± 1.446% vs 8.147 ± 3.081%, p=0.006) (**Figure 3**). However, this difference was not maintained with regard to antibody response following the second vaccine dose, neither did we detect any differences between CD19+ B lymphocytes and antibody response status in KTRs. Yet, one should take into consideration the fact that nearly half of our HD cohort already had a positive serology following the first vaccine dose as compared to only 4 (7%) of KTRs.

We further distinguished the T cell compartment in naïve T cells (CD45RA+) which are unprimed lymphocytes and memory T cells (CD45RO+) which have encountered antigen and respond faster and with increased intensity on antigenic stimulation compared with (CD45RA+) naïve T cells (19). Analysis of the CD45RA+ (naïve) CD45RA+CD45RO+ (transient) and CD45RO+ (memory) isoforms as expressed by T cell subsets, revealed no differences with regard to either CD45RA+ or CD45RA+CD45RO+ levels between KTRs and HD patients at any time point, neither within each subgroup of responders and non-responders. On the other hand,

TABLE 1 | Demographics, clinical and laboratory parameters in hemodialysis patients and kidney transplant recipients.

	Hemodialysis patients (n = 34)	Kidney Transplant Recipients (n = 54)	p
Age, yr	69.4 ± 12.3	58.2 ± 9.7	<0.001
Male gender	23, 67.6%	38, 70.4%	0.35
BMI, kg/m^2	25.9 ± 4.0	25.1 ± 4.1	0.35
Diabetes mellitus	9, 26.5%	10, 18.5%	0.38
History of Cancer	1, 5.6%	5, 9.3%	0.62
Time on dialysis, years	12.39 ± 8.36	5.71 ± 4.68	<0.001
Kt/V	1.6 ± 0.3		
Time from Kidney Transplant, years		11.9 ± 8.3	
Donor type (deceased, live, both)		35, 64.8%/18, 33.3%/1, 1.9%	
ABO group			
O	11, 32.4%	22, 42.3%	0.44
A	12, 35.3%	21, 40.4%	
B	9, 26.5%	7, 13.5%	
AB	2, 5.9%	2, 3.8%	
Hb, g/dl	10.8 ± 1.1	13.5 ± 1.9	<0.001
WBC, 10^3/ml	8184 ± 2667	8165 ± 9648	0.99
Induction, Anti-CD25		54, 100%	
CNI		54, 100%	
Tacrolimus		41, 75.9%	
Cyclosporine		13, 24.1%	
MMF/MPA		50, 92.6%	
Steroids		46, 85.2%	
Tacrolimus+MMF/MPA		39, 72.2%	
Tacrolimus+MMF/MPA+Steroids		35, 64.7%	
Tacrolimus levels, ng/ml		6.6 ± 1.4	
Cyclosporine T0 levels, ng/ml		103.7 ± 22.8	
eGFR, ml/min per 1.73m^2		52.7 ± 17.5	
Urine Protein, mg/24h		329.6 ± 411.3	

BMI, body mass index; CNI, calcineurin inhibitor; eGFR, estimated glomerular filtration rate; MMF, mycophenolate mofetil; MPA, mycophenolic acid.

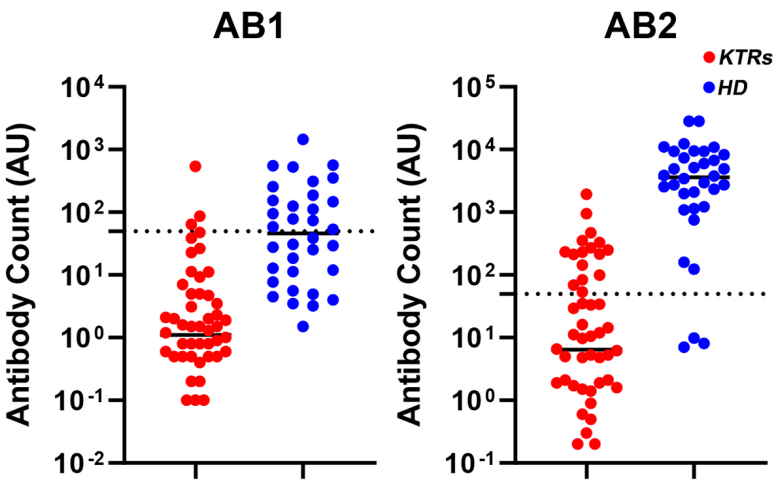

FIGURE 2 | Scatter plots representing individual values for each patient of the anti-SARS-CoV2 antibody titers against the spike protein immediately before the second vaccine dose (T1) and 2 weeks after administration of the second dose (T2) in hemodialysis patients and kidney transplant recipients. AB1, antibody response at T1; AB2, antibody response at T2; HD, hemodialysis patients; KTRs, kidney transplant recipients.

hemodialysis patients displayed higher mean levels of the CD45RO+ T cells compared to KTRs at T2 (39.706 ± 8.792% vs 33.185 ± 9.481%, p=0.020) independently of the antibody response status.

CD4+ T helper cells are regarded as the orchestrators of cellular immunity, with several roles in antiviral responses, including the assistance to B cell activation, generation of antibody-producing plasmocytes and memory B cells, the expression of cytokines as well as the generation of cytotoxic and memory CD8+ T cell subpopulations (20). CD4+ T helper cells significantly increased in KTRs at T2 as compared to

TABLE 2 | Demographics, clinical and laboratory parameters in hemodialysis patients and kidney transplant recipients presented as responders versus non responders in each group after the second dose of vaccine.

	Hemodialysis patients (n = 34)		p	Kidney Transplant Recipients (n = 54)		p
	Responders (n= 31)	Non-Responders (n=3)		Responders (n=16)	Non-Responders (n=38)	
Age, yr	69.2 ± 12	71.3 ± 17.6	0.78	55.01 ± 2.23	59.5 ± 7.48	0.18
Male sex	22, 71.0%	1, 33.3%	0.24	13, 81.3%	25, 65.8%	0.26
BMI, kg/m^2	25.23 ± 4.19	23.77 ± 2.86	0.56	25.85 ± 3.99	25.98 ± 4.05	0.92
Diabetes mellitus	7, 22.6%	2, 66.7%	0.16	3, 18.7%	7, 18.4%	0.98
History of Cancer	1, 6.7%	–		1, 6.2%	4, 10.5%	0.62
Time on dialysis, years	5.81 ± 4.88	4.66 ± 1.53	0.69			
Time from Transplant, years				12.94 ± 9.62	11.45 ± 7.70	0.55
Donor type						
Deceased				9, 56.2%	26, 68.4%	0.55
Live				7, 43.8%	11, 28.9%	
Both				–	1, 2.6%	
ABO group			0.57			0.27
A	10, 32.2%	2, 66.7%		4, 25.0%	17, 47.2%	
AB	2, 6.5%	–		–	2, 5.6%	
B	8, 25.8%	1, 33.3%		2, 12.5%	5, 13.9%	
O	11, 35.5%	–		10, 62.5%	12, 33.3%	
Hb, g/dl	10.97 ± 1.00	9.37 ± 1.01	0.013	14.11 ± 1.85	13.30 ± 1.88	0.15
WBC, 10^3/ml	8326 ± 10050	6496 ± 4010	0.76	7961 ± 2751	8277 ± 2663	0.69
Tacrolimus				10, 62.5%	31, 81.6%	0.13
Cyclosporine				6, 37.5%	7, 18.4%	0.13
MMF/MPA				12, 75.0%	38, 100.0%	0.006
Steroids				12, 75.0%	34, 89.5%	0.17
Tacrolimus+MMF/MPA				8, 50.0%	31, 81.6%	0.018
Tacrolimus+MMF/MPA+Steroids				7, 43.8%	28, 73.7%	0.035
Tacrolimus levels, ng/ml				6.2 ± 1.7	6.8 ± 1.2	0.24
Cyclosporine T0 levels, ng/ml				102.6 ± 19.2	104.5 ± 26.0	0.88
eGFR, ml/min per 1.73m^2				61.6 ± 15.4	48.9 ± 17.2	0.014
Urine Protein, mg/24h				307 ± 550	339 ± 345	0.80

BMI, body mass index; CNI, calcineurin inhibitor; eGFR, estimated glomerular filtration rate; MMF, mycophenolate mofetil; MPA, mycophenolic acid.

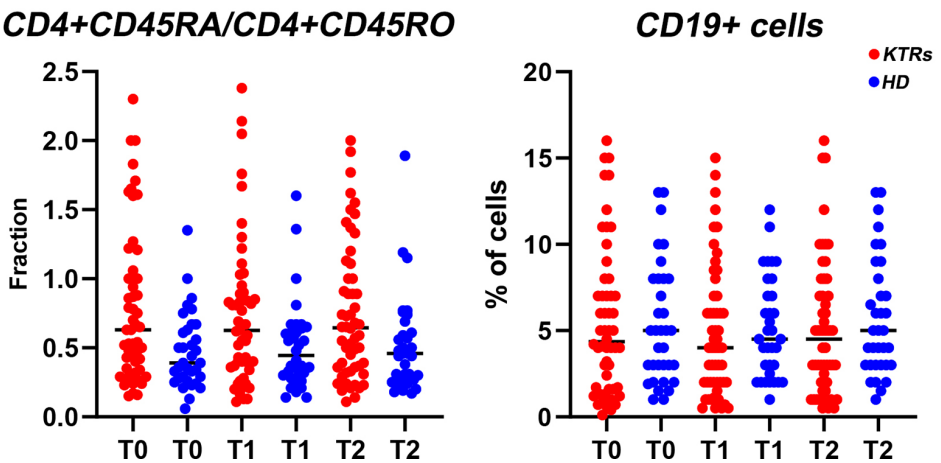

FIGURE 3 | Scatter plots representing individual values for each patient of the fraction of CD4+CD45RA/CD4+CD45RO T-helper cells and the percentage of CD19+ B cells respectively at baseline (T0), immediately before the second vaccine dose (T1) and 2 weeks after administration of the second dose (T2) in hemodialysis patients and kidney transplant recipients. HD, hemodialysis patients; KTRs, kidney transplant recipients.

baseline (54.185 ± 11.63% vs 49.389 ± 10.967%, p=0.004). With regard to HD patients, a declining trend of CD4+ T cells from T0 to T1 (44.000 ± 9.032% vs 42.853 ± 7.207%) was observed which was subsequently followed by a significant increase of CD4+ T cell counts at T2 as compared to T1 (48.294 ± 11.559% vs 42.853 ± 7.207%, p=0.043). Overall, KTRs showed higher CD4+ T cell counts in comparison to the HD patient group at the T2 time point.

The central role of CD4+ cells in immunity led us to further study the transition from CD4+CD45RA (naive) to CD4+CD45RO (memory) T helper cells during the vaccination period and in conjunction to the antibody response. Accordingly, KTRs showed higher CD4+ CD45RA+ T helper cell counts in comparison to HD patients at T0 (18.852 ± 9.252% vs 12.618 ± 6.642%, p=0.015) as well as at T2 (19.167 ± 10.136% vs 13.824 ± 7.129%, p=0.057) though marginally significant at this time point, independently of the antibody response status. No differences were found between KTRs and HD patients at any time point, nor between the responders and non-responders within each group with regard to CD4+CD45RO T helper cells.

CD8+ T cells showed a decreasing trend in both the HD and KTRs patient groups at T2 as compared to baseline. Thus, CD8+ T cells counts at T2 as compared to T0 respectively were 24.296 ± 11.409% vs 30.167 ± 11.119% (p <0.001) in KTRs and 23.206 ± 9.984% vs 28.118 ± 9.546% (p=0.008) in HD patients. Furthermore, the CD4+/CD8+ ratio significantly increased at T2 compared to baseline in both HD (2.722 ± 2.051 vs 1.885 ± 1.085, p=0.029) and KTRs (2.941 ± 2.017 vs 1.929 ± 0.986, p<0.001).

Natural killer cells and NKT cells, are considered to bridge the innate and acquired arms of the immune system, thus organizing adaptive immune responses and immunoregulation (21). Regarding NKT-cells (CD3+CD16+CD56+) counts, an increase was observed in KTRs at T1 (5.967 ± 6.705) and T2 (7.520 ± 6.273%) as compared to baseline (3.978 ± 4.377%). Hemodialysis patients displayed a different pattern of change in NKT cell counts. NKT cells levels increased substantially from T0 to T1 (2.465 ± 1.583% vs 6.180 ± 7.186% p=0,014), whereas a decrease was subsequently observed at T2 (2.941 ± 2.373%), with their levels albeit remaining higher than baseline. In addition, KTRs displayed higher levels of NKT cells at T2 compared to their hemodialysis counterparts (7.520 ± 6.2735 vs 2.941 ± 2.373%, p=0.001). NK cells (CD3-/CD16+/CD56+) remained stable at all time points within each sub-group, with HD patients in general displaying higher NK cell counts compared to KTRs.

The Antibody Response Status Is Associated With the CD19+ Lymphocyte Subpopulation and the Fraction of CD45RA Naïve to CD45RO Memory CD4+ T Helper Cells

To gain further insight into the cellular immune responses that lead to COVID-19 antibody production, we examined whether antibody levels were associated with CD19+ B lymphocyte counts. Accordingly, a positive correlation was established between CD19+ B cells counts in the circulation and the IgG antibody levels at all time-points (p <0.001) (**Supplementary Table 1**). Collectively, our data support that even though CD19+ B cell counts are below the normal reference values in HD patients and KTRs, their positive correlation to antibody production supports the induction of the humoral immune response following BNT162b2 vaccination.

Furthermore, in order to study the transition between naïve and memory CD4+ T helper cells we analyzed the fraction of CD4+CD45RA/CD4+CD45RO cells, with a lower fraction signifying a larger percentage of memory cells compared to naïve cells (**Figure 3**). We found that the CD4+CD45RA/CD4+CD45RO fraction was negatively associated to antibody production, in both time-points (**Supplementary Table 2**), thus allowing us to conclude that the production of memory T helper cells is directly associated with the antibody response status.

A summary of our findings regarding the correlations between the percentages of CD19+ B cells in the circulation, the fraction of CD4+CD45RO/CD4+CD45RO T helper cells and the antibody levels for HD patients and KTRs at T0, T1 and T2 is depicted in **Figure 4**. Based on a collective view of our results, even though a high variability is observed between different patients, the strongest humoral immune responses are clustered in the upper left quadrant of each diagram, which correspond to individuals with a higher percentage of CD19+ B cells as well as a larger fraction of memory CD4+ CD45RO T helper cells. HD patients tend to exhibit stronger humoral responses than KTRs. When comparing the results in the histograms corresponding to T0, T1 and T2 respectively, we conclude that the percentage of CD19+ cells did not significantly change between T0-T2. Interestingly, there were some patients who despite lower CD19+ B cell counts (depicted in the lower left quadrant), succeed in generating anti- SARS-CoV2 antibodies. In conclusion, a strong correlation appears to exist between antibody-producing CD19+ B cells, memory CD4+ T cells and anti SARS-CoV2 antibody counts.

DISCUSSION

The results of our study regarding the immunogenicity of the BNT162b2 vaccine in terms of eliciting an antibody response in maintenance HD patients and KTRs are in accordance with the until now available evidence regarding immunization rates in these vulnerable populations (10–12, 22–28). Notably, the findings of our study suggest that evaluation of both CD19+ lymphocytes and CD4+CD45RO helper T cell subsets in the peripheral circulation might serve as a means for estimation of the subsequent immune responses to vaccination in these patients. In particular, our findings regarding the induction of CD4+CD45RO memory T helper cells in HD patients and KTRs following vaccination is an indicator, albeit indirect, which allows us to speculate that administration of the BNT162b2 vaccine elicits a cellular immune memory response in addition antibody production.

With regard to antibody response in our patients undergoing maintenance dialysis, our findings are consistent with published data from other centers reporting that although less than one-third of HD patients develop antibodies after the first dose of BNT162b2 COVID-19 mRNA vaccine, still the overall seropositivity rate exceeds 80% after 2 doses of the BNT162b2 mRNA vaccine (22, 23). Likewise, the low seropositivity rates in our KTRs, further confirm the results from other centers reporting a weak antibody response to SARS-COV-2 vaccination with immunization rates varying from 20-50% (24–26). Furthermore, the mean levels of

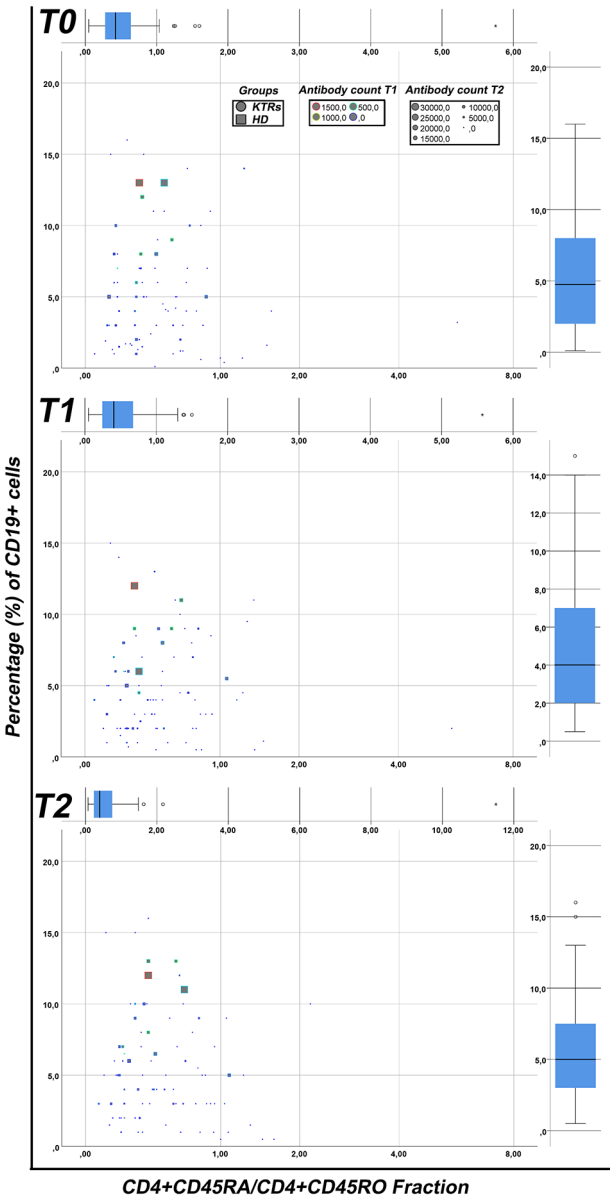

FIGURE 4 | Correlation between the humoral immune response with different lymphocyte subsets. The fraction of CD4+CD45RA/CD4+CD45RO (X-axis) has been plotted against the percentage of CD19+ cells (Y-axis) in kidney transplant recipients (circular points) and hemodialysis patients (square points) patients. The antibody counts following the first (0-100 grey, 101-500 green, 501-1500 yellow, >1500 red) and the second (different sizes from small to large from 0-30000) dose of vaccine were also depicted in each point. The results are presented for T0, T1 and T2 respectively. HD, hemodialysis patients; KTRs, kidney transplant recipients; HD, hemodialysis patients; KTRs, kidney transplant recipients.

antibody titers detected in our HD patients who achieved a humoral immune response to vaccination, are in line with other reports, confirming substantially lower antibody titers in HD patients compared to the general population (27). Yet, in order to correctly interpret these data, one should take into account that evidence from the available studies is heterogenous in terms that antibody assays from different manufacturers were utilized, most but not all studies examined the immune response to the BNT162b2 COVID-19 mRNA vaccine whereas a few of these studies included dialysis patients with previous COVID 19 infection as well.

The classical immunosuppressive drug regimen of kidney transplantation consists of the combination of three classes of drugs, i.e. corticosteroids, a calcineurin inhibitor and an antimetabolite, which leads to improved graft survival rates yet in the setting of simultaneous profound immunosuppression. In specific, available data from research studies regarding successful implementation of vaccination strategies in organ transplantation, suggest that glucocorticoids alone dot not seem to affect vaccine efficacy (4). On the other hand, CNIs directly suppress the early antigen-dependent T-cell activation whereas the antimetabolite mycophenolate mofetil (MMF) inhibits antibody production in a T-cell independent manner, with relative sparing of T-cell responses, thus potentially affecting vaccine efficacy (4, 29–31). In specific, evidence from previous studies of influenza vaccine immunogenicity in transplant recipients indicates that MMF reduced the interleukin-4+ CD4+ T-cell frequencies as well as inhibited HLA-DR expression on B-cells together with B-cell activation, as a result decreasing production of immunoglobulin G (31, 32). In line with the above, the utilization of an intensive immunosuppressive regimen including MMF or mycophenolic acid (MPA) in our KTRs was associated with an attenuated antibody response status following vaccination against SARS COV-2.

A noteworthy observation of our study is kidney graft function was significantly related to the antibody response status in KTRs. Until now, there are scarce and controversial data in the literature regarding a possible relationship between the GFR and seroconversion rates following vaccination in patients with chronic kidney disease (33–35).

Although the evaluation of the humoral immune responses following vaccination is rather accessible and convenient, the assessment of the cellular immune responses is crucial, especially in immunosuppressed individuals such as HD patients and KTRs. Earlier studies have shown a significant reduction of total B-cell counts in the peripheral blood both in ESKD patients and KTRs (8, 36). In addition, CD19+ B-cell lymphopenia has recently been suggested as an independent predictor of all-cause and cardiovascular mortality in HD patients (8, 36). Thus, in line with previous evidence, both HD patients and KTRs in our study displayed lower levels than normal of CD19+ B lymphocytes in the circulation. A notable finding of our study is that the percentage of CD19+ B cells in the peripheral circulation is directly associated to higher antibody production following vaccination against SARS-COV2. Considering that both groups exhibit lower than normal baseline CD19+ B cell levels, our results suggest that even in such conditions of immunosuppression, there is a possibility of achieving efficient humoral responses that might ultimately offer protection against severe COVID-19 disease. Likewise, a pilot-study regarding SARS COV-2 vaccination in individuals

receiving B-cell depleting treatment with rituximab, which showed that generation of an adequate antibody response coincided with the detection of peripheral CD19+ B cells (37).

Impaired renal function has been directly associated with depletion of naïve T cells, including naïve CD4+ and naïve CD8+ T lymphocytes subsets due to increased apoptosis of these cells (5, 38). Moreover, it has been shown that HD patients mount a defective effector memory CD4+ T helper cell response following vaccination with hepatitis B surface antigen (39). With regard to organ transplantation, the status of memory T cells is currently a subject of ongoing research, including their role in protective immunity as well as potential implications in allograft rejection (40). Available data regarding T cell responses following administration of the influenza vaccine in transplant recipients indicates that vaccination elicited CD4+ and $CD8^+$ T-cell responses at a similar level compared to influenza infection whereas humoral immunity had a significant correlation with a CD4+ response. In addition, it appears that memory T cells may be less susceptible to the effects of conventional immunosuppression and booster immunizations are generally beneficial in these transplant recipients (41–43).

We found that the fraction of CD4+CD45RA+/CD4+CD45RO + to be negatively associated with antibody production, thus confirming the concordance between cellular and humoral immunityand hinting that concomitant production of specialized memory immune cells against the SARS-COV2 antigens occurs together with the generation of the humoral immune response following vaccination. A similar immune response pattern has been previously observed in patients who recovered from mild-to-moderate COVID-19 disease (44). Such assumption requires further validation with measurement and characterization of specific memory immune cells with a SARS-COV2 antigen specific assay.,

Interestingly, the ratio of CD4+ to CD8+ T-helper cells progressively increased following vaccination in both hemodialysis patients and KTRs, which should be ascribed to an increasing trend of CD4+ T helper cells counts together with a simultaneous reduction of CD8+ T cells following the second vaccine dose. On the other hand, the SARS-CoV-2 infection itself has been associated with the depletion of both CD4+ and $CD8+^+$ T cells counts (45, 46). Available evidence suggests that an inverted CD4+ to CD8+ T –cell ratio may indicate an impaired ability to respond to repeated antigenic stimulation in the setting of influenza vaccination (47). An inverted CD4+ to CD8+ T-cell ratio is a common finding in HD patients and if present before transplantation, it may be a risk factor for post-transplant infections (48, 49).

With regard to the other cell sub-populations, we found lower NKT cell counts in HD patients compared to KTRs following the second vaccine dose, whereas NKT cell counts increased in both patient groups following administration of the first and the second vaccine dose. Available evidence indicates that patients with ESKD display depleted invariant NKT cells, which however return to normal levels within one year following kidney transplantation (50). It should be noted that low levels of NKT cells in peripheral blood of COVID-19 subjects have been associated with the severity of the disease in these patients (51).

A remarkable finding of our study is the variability in the immune responses between immunosuppressed individuals following vaccination, a known issue concerning the general population as well (52). This variability may be attributed, among others, to the genetic background, age and the specific underlying patterns of immune-compromise, as occur in ESKD and organ transplantation. Such variable and "patient-specific" responses require sensitive techniques for the follow-up of the immune response in the setting of vaccination.

The Covid-19 pandemic disclosed the current lack of knowledge of the efficacy of vaccination strategies in the immunosuppressed individuals. There is evidence that administering a third vaccine dose in immunosuppressed populations might further enhance their immunogenicity (53). Follow-up data regarding immune responses in the setting of supplemental SARS-COV-2 vaccine doses in our patient cohorts following approval by national committees shall be made available in the future.

In conclusion, taking into account the clinical outcome of severe COVID-19, the monitoring of vaccination success in high-risk individuals such as HD patients and KTRs is of paramount importance (54). Herein, we provided a general scheme of the background immune profile of the vulnerable populations of HD patients and KTRs as well as highlighted specific traits of the humoral and cellular immune alterations following SARS COV -2 vaccination with an mRNA vaccine, thus aiming to improve the understanding of both the ability and defects of the suppressed immune system to respond to vaccination. In addition, we brought to the forefront the variation in individual immune responses which characterizes these two vulnerable patient cohorts. Thus, our methodology provides a conceptual framework for the analysis of humoral responses following BNT162b2 vaccination, based on antibody counts and flow cytometric analysis of major lymphocyte sub-populations, for monitoring vaccination success in high-risk individuals. Despite the challenges lying ahead, future research studies will further elucidate the intricate mechanisms linking background immune phenotypes to vaccine responsiveness in order to identify appropriate markers of immunogenicity and efficacy in immunosuppressed patients.

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 human participants were reviewed and approved by Ethical Committee of the University Hospital of Ioannina. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Individual contribution of each co-author is as follows: AD, design of the study, analysis and interpretation of data, drafting the article. GM, conducted flow cytometry analyses, analysis and interpretation of data, drafting and revising the article. IM, recruitment of patients, patients' data recorded and entry, drafting the article. HP, conception and design of the study, patients' recruitment. EP, recruitment of patients, patients' data recorded and entry. VK, recruitment of patients, patients' data recorded and entry. ET, recruitment of patients, patients' data recorded and entry. GB, conducted flow cytometry analyses, analysis and interpretation of data. SP, handling and preparing patients' blood samples, data entry. KG, conducted anti-SARS-CoV2 antibody measurements. MM, conception of the study. ED, conception and design of the study, interpretation of data, drafting and revising the article. All authors contributed to the article and approved the submitted version.

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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.2021.760249/full#supplementary-material

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Characterization of SARS-CoV-2-Specific Humoral and Cellular Immune Responses Induced by Inactivated COVID-19 Vaccines in a Real-World Setting

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While the immunogenicity of inactivated vaccines against coronavirus disease 2019 (COVID-19) has been characterized in several well-conducted clinical trials, real-world evidence concerning immune responses against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) raised by such vaccines is currently missing. Here, we comprehensively characterized various parameters of SARS-CoV-2-specific cellular and humoral immune responses induced by inactivated COVID-19 vaccines in 126 individuals under real-world conditions. After two doses of vaccination, S-receptor binding domain IgG (S-RBD IgG) and neutralizing antibody (NAb) were detected in 87.06% (74/85) and 78.82% (67/85) of individuals, respectively. Female participants developed higher concentrations of S-RBD IgG and NAb compared to male vaccinees. Interestingly, a longer dosing interval between the first and second vaccination resulted in a better long-term SARS-CoV-2 S-RBD IgG response. The frequencies of CD4+ T cells that produce effector cytokines (IFN-γ, IL-2, and TNF-α) in response to stimulation with peptide pools corresponding to the SARS-CoV-2 spike (S), nucleocapsid (N) or membrane (M) protein were significantly higher in individuals received two doses of vaccine than those received one dose of vaccine and unvaccinated individuals. S, N, or M-specific CD4+ and CD8+ T cell responses were detectable in 95.83% (69/72) and 54.16% (39/72) of double-vaccinated individuals, respectively. The longitudinal analysis demonstrated that CD4+ T cell responses recognizing S, N, and M waned quickly after a single vaccine dose, but were boosted and became more sustained following a second dose. Overall, we provide a comprehensive characterization of immune responses induced by inactivated COVID-19 vaccines in real-world settings, suggesting that both humoral and cellular SARS-CoV-2-

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specific immunity are elicited in the majority of individuals after two doses of inactivated COVID-19 vaccines.

Keywords: COVID-19, SARS-CoV-2, inactivated vaccine, humoral immune responses, cellular immune responses

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an unprecedented burden to global healthcare systems and causes severe economic havoc e.g., through prolonged lockdowns. Effective COVID-19 vaccines already start to mitigate these problems – especially in regions with high vaccination coverage, suggesting that effective global vaccination has the potency to eventually terminate the COVID-19 pandemic. The astonishingly rapid implementation of COVID-19 vaccination programs is unprecedented in the history of vaccine development and application (1). As of August 2021, more than 5 billion doses of COVID-19 vaccines have been administered globally (2). This number comprises at least 18 different COVID-19 vaccines, which utilize a broad range of vaccine principles such as inactivated virus particles, mRNAs and viral vectors expressing the viral spike protein, or adjuvanted spike protein subunits (3). Inactivated vaccines belong to the most frequently used types of COVID-19 vaccines, and as of August 2021, over 2 billion doses of inactivated COVID-19 vaccines have been administered just in China (2, 4). The development of inactivated vaccines is a mature technology, which is widely used for the prevention and control of emerging infectious diseases (5). Inactivated vaccines are produced by growing SARS-CoV-2 in cell culture, usually on Vero cells, followed by chemical inactivation of the virus (6). Because the whole virus is presented to the immune system, immune responses are likely to target not only the spike protein of SARS-CoV-2 but also the matrix, envelope and nucleoprotein (7).

The effectiveness and immunogenicity of the 3 inactivated COVID-19 vaccines currently in use in China, namely "BBIBP-CorV", "CoronaVac", and "WIBP-CorV", have been demonstrated in several clinical trials (5, 8–12). In each of these studies, standardized vaccination protocols were precisely followed, using inactivated vaccines from identical companies at well-defined intervals between first and second vaccination. Obviously, authentic real-world practice is more flexible, pragmatic, and diverse various combinations of inactivated vaccines from different companies are applied for the first and second vaccination, and the intervals between the two vaccinations vary to a certain degree. To our knowledge, data of SARS-CoV-2-specific humoral and cellular immune responses induced by inactivated COVID-19 vaccines in the real-world practice are not available so far. To generate such information, we recruited volunteers who received inactivated COVID-19 vaccines in real-world practice, and characterized their antibodies and T cell responses recognizing SARS-CoV-2. Our data suggest that both the humoral and the cellular SARS-CoV-2-specific immune responses are elicited in the majority of individuals after two doses of inactivated vaccines.

MATERIALS AND METHODS

Study Design and Participants

Healthy adults, aged 19 to 79 years, without history of SARS-CoV-2 infection (*via* serological and nucleic acid test) were eligible for enrollment in the study. Exclusion criteria were as follows: confirmed natural SARS-CoV-2 infection; working in an environment posing a high risk for an exposure to SARS-CoV-2; symptoms indicating acute infections such as fever, cough, runny nose, sore throat, diarrhoea, dyspnoea, or tachypnoea during a 14 day period prior to sampling; abnormalities in laboratory tests; pregnancy or lactation; a history of autoimmune diseases; and prior or ongoing use of immunotherapy. All vaccinated participants and vaccinees immunized with one or tow doses of "BBIBP-CorV" (6.5 U per 0.5 ml of aluminium hydroxide per dose), or "CoronaVac"(600 SU per 0.5 ml of aluminium hydroxide per dose), or "WIBP-CorV" (200 WU per 0.5 ml of aluminium hydroxide per dose) were recruited at the Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology from December 2020 to July 2021. Informed written consent was obtained from each participant before sampling, and the study protocol was approved by the local medical ethics committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (2021-0570) and Chinese Clinical Trial Registry (ChiCTR2100048837). The study was conducted in accordance with the guidelines of the Declaration of Helsinki.

Preparation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation (DAKEWE Biotech, China) and rapidly assessed by flow cytometry analysis without intermittent cryo-preservation.

Detection of SARS-CoV-2 Neutralizing Antibody and S-RBD IgG

Antibodies disturbing the binding of S to the ACE2 [for simplicity referred to as neutralizing antibody (NAb)], and IgG antibody recognizing the SARS-CoV-2 receptor binding domain (RBD) of the S protein (for simplicity refer to as S-RBD IgG) were tested by competitive or indirect chemiluminescence immunoassay (CLIA), respectively, by MAGLUMI^{TM} 4000 Plus (Snibe, Shenzhen, China). The SARS-CoV-2 NAb assay described above is a qualitative detection of NAb in human serum and plasma, which is mainly used for the evaluation of NAb in patients recovering from COVID-19 or the auxiliary evaluation of the effect of the COVID-19 vaccines.

For the detection of SARS-CoV-2 NAb, in brief, human plasma, buffer, and magnetic microbeads coated with ACE2 antigen and ABEI labeled with recombinant SARS-CoV-2 S-RBD antigen are

mixed thoroughly and incubated. SARS-CoV-2 NAb present in the plasma compete with ACE2 antigen immobilized on magnetic microbeads for binding recombinant SARS-CoV-2 S-RBD antigen labeled with ABEI. After precipitation in a magnetic field, chemiluminescent reaction were initiated and the light signal is measured by a photomultiplier as relative light units (RLUs), which is inversely proportional to the concentration of SARS-CoV-2 NAb presented in the plasma.

For the detection of S-RBD IgG, in brief, human plasma, buffer, and magnetic microbeads coated with S-RBD recombinant antigen are mixed thoroughly and incubated, forming immune-complexes. After precipitation in a magnetic field, ABEI labeled with anti-human IgG antibody were added to incubate to form complexes. Then, chemiluminescent reaction were initiated and the light signal is measured by a photomultiplier as RLUs, which is proportional to the concentration of S-RBD IgG presented in the plasma.

A study performed with the SARS-CoV-2 NAb and S-RBD IgG was obtained by testing 381 individuals neither SARS-CoV-2 infection nor vaccination, 99% SARS-CoV-2 NAb values were ≤ 0.05 μg/ml and 99% S-RBD IgG values were < 1.0 AU/ml. Cut-off value for SARS-CoV-2 NAb was 0.05 μg/ml and 1.0 AU/ml for S-RBD IgG.

Analysis of Effector T Cell Responses

Three pools of lyophilized peptides, consisting mainly of 15-mer sequences with 11 amino acids (aa) overlap, that cover the entire sequences of the surface glycoprotein (S, Cat No.RP30027, Genscript Biotech Corporation, Nanjing, China), the nucleocapsid phosphoprotein (N, Cat No. RP30013, Genscript Biotech Corporation, Nanjing, China) or the membrane glycoprotein (M, Cat No. 30022, Genscript Biotech Corporation, Nanjing, China) of SARS-CoV-2, were used for cell stimulation. On day 1, PBMCs were cultivated in complete medium [RPMI 1640 containing 10% (v/v) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer] with recombinant interleukin (IL)-2 (20 U/ml; Hoffmann-La Roche, Italy). Cells without anti-CD3, anti-CD28 and peptide stimulation served as negative control (unstimulated control). Cells with anti-CD3 (1 μg/ml; Invitrogen, USA) and anti-CD28 (1 μg/ml; Invitrogen, USA) stimulation served as positive control. Cells stimulated with S, N, or M peptide pools (1 μg/ml) in the presence of anti-CD28 served as peptide stimulation groups. Fresh medium containing IL-2 was added on day 4 and 7. On day 10, cells were restimulated for 5 hours with the same peptide pool in the presence of brefeldin A (BD Biosciences, San Diego, CA). Cells previously stimulated by anti-CD3 and anti-CD28 were restimiulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin (Iono) for 5 hours served as positive controls. Cells were then tested for IFN-γ, IL-2, and TNF-α expression by intracellular cytokine staining. Specific cytokine responses were calculated by subtracting the background activation i.e. the percentage of cytokine positive cells in the unstimulated control. T cell responses were defined as being detectable in the case that the frequency in specifically stimulated cultures exceeded the unstimulated control at least two-fold (stimulation index > 2). Samples with responseless positive controls were excluded from further analysis.

Flow Cytometry

Surface and intracellular staining for flow cytometry analysis were performed as described previously (13, 14). For surface staining, cells were incubated with relevant fluorochrome-labeled antibodies (eFluor 780-anti-CD3, PE-Cy7-anti-CD8, and PerCP-Cy5.5-anti-CD4) for 30 min at 4°C in the dark. For intracellular cytokine staining, cells were fixed and permeabilized using the Intracellular Fixation & Permeabilization Buffer Set (Invitrogen, USA) and subsequently stained with FITC-anti-IFN-γ, PE-anti-IL-2 and APC-anti-TNF-α (Invitrogen, USA). Approximately 100,000 PBMCs were acquired for each sample using a BD FACS Canto II flow cytometer. Data analysis was performed using the FlowJo software V10.0.7 (Tree Star, Ashland, OR, USA). Cell debris and dead cells were excluded from the analysis based on scatter signals and Fixable Viability Dye eFluor 506.

Statistical Analysis

Statistical analyses were performed using the SPSS statistical software package (version 22.0, SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk method was used to test for normality. Mann-Whitney U-test, one-way ANOVA and Pearson product-moment correlation coefficient were used where appropriate. All reported *P* values were two-sided, and a P value below 0.05 was considered as hallmark of statistical significance (*,$P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$).

Data Availability

We support data sharing of the individual participant data. The individual participant data that underlie the results reported in this Article, after deidentification will be shared. Researchers who provide a scientifically sound proposal will be allowed to access the de-identified individual participants data. Proposal should be sent to the corresponding authors, at xinz@hust.edu.cn or jialiu77@hust.edu.cn. The proposals will be reviewed and approved by the funders, investigator and collaborators on the basis of scientific merit. To gain access, data requestors will need to sign a data access agreement.

RESULTS

Characterization of the Study Cohort

The vaccine-induced SARS-CoV-2-specific humoral and cellular immune responses were characterized in 168 blood samples collected from 126 healthy individuals, among which 32 samples were collected prior to vaccination and 136 samples were collected after inoculation with inactivated COVID-19 vaccines (BBIBP-CorV, CoronaVac, and/or WIBP-CorV). The demographic profiles of all participants are shown in **Table 1**. The age of participants received two doses of vaccines was older than the individuals received single dose, and no difference was observed in sex of the three groups. The dosing interval between the first and second vaccination was 21-63 days (median: 43 days). In 104 participants, blood samples were collected at a

TABLE 1 | Baseline characteristics of the cohort.

Characteristic	Baseline	After 1st vaccination	After 2nd vaccination		*P* value
No. of participants	32	41	85		NA
Sex (Male/Female)	10/22	15/26	29/59		NS*
Age, median, year-old	28 (21-79)	27 (22-55)	34 (19-77)		0.02#
Manufacturer information of each vaccination					NA
			1st vaccination	2nd vaccination	
BBIBP-CorV		5.00% (2/40)	17.33% (13/75)	25.68% (19/74)	
CoronaVac		22.50% (9/40)	10.67% (8/75)	54.05% (40/74)	
WIBP-CorV		72.50% (29/40)	72.00% (54/75)	20.27% (15/74)	
Manufacturer information of the two vaccinations					NA
Single manufacturer			22.97% (17/74)		
BBIBP-CorV			5.41% (4/74)		
CoronaVac			4.05% (3/74)		
WIBP-CorV			13.51% (10/74)		
Mixed manufacturers			77.03% (57/74)		
BBIBP-CorV+ CoronaVac			10.81% (8/74)		
BBIBP-CorV+ WIBP-CorV			20.27% (15/74)		
CoronaVac+ WIBP-CorV			45.95% (34/74)		
Overall adverse reactions			34.26% (34/101)		NA
Injection site adverse reactions					
Pain			30.69% (31/101)		
Redness and swelling			0		
Itch			0		
Induration			0.99% (1/101)		
Systemic adverse reactions					
Fever			0.99% (1/101)		
Fatigue			0.99% (1/101)		
Somnolence			0		
Headache			0.99% (1/101)		
Muscle pain			1.98% (2/101)		
Rash			0		
Vomiting			0		
Diarrhea			0.99% (1/101)		
Underlying diseases					NA
Hypertension	3.13% (1/32)	0	2.70% (2/74)		
Diabetes	0	2.44% (1/41)	2.70% (2/74)		
Cardiovascular diseases	0	0	1.35% (1/74)		
COPD	0	0	0		
Tumor	0	0	0		
Others	0	4.88% (2/41)	2.70% (2/74)		
Sampling times					NA
1		82.54% (104/126)			
2		7.94% (10/126)			
3		3.17% (4/126)			
4		6.35% (8/126)			

**Chi-square test was used to test the statistical significance.*

#One-way ANOVA followed by Turkey's multiple comparisons test was used to test the statistical significance.

COPD, chronic obstructive pulmonary disease; NA, not available; NS, not significant.

single time point before or after vaccination. In 22 participants, blood samples were collected at 2 to 4 different time points before and after the first and second vaccination. The interval time between the blood donation and the first and second vaccination was 10-45 days (median: 29 days) and 10-57 days (median: 30 days), respectively. In the first and second vaccination, 22.97% (17/74) of the participants were inoculated with vaccines from the same manufacturer (5.41%, 4/74, BBIBP-CorV; 4.05%, 3/74, CoronaVac; 13.52%, 10/74, WIBP-CorV), and 77.03% (57/74) of the vaccinees were immunized with vaccines of different manufactures (10.81%, 8/74, BBIBP-CorV+ CoronaVac; 20.27%, 15/74, BBIBP-CorV+ WIBP-CorV; 45.95%, 34/74, CoronaVac+ WIBP-CorV). Overall adverse reactions were reported in 34.26% (34/101) of the participants within 7 days of each injection, among which the most common were injection site pain (30.69%, 31/101), followed by induration (0.99%, 1/101), fever (0.99%, 1/101), muscle pain (1.98%, 2/101), fatigue (0.99%, 1/101), and headache (0.99%, 1/101). No serious adverse reactions occurred.

Characterization of Vaccine-Induced Antibody and T Cell Responses Specific to SARS-CoV-2

Firstly, vaccine-induced humoral responses were characterized by measuring serum SARS-CoV-2 S-RBD IgG and neutralizing

antibody (NAb) concentrations. At baseline, none of the participants had detectable neutralizing antibodies. Concentrations of S-RBD-specific IgG were significantly higher after the second vaccination (median: 13.3 AU/ml) compared to baseline levels (0.5 AU/ml) (**Figure 1A**). The concentrations of NAb were significantly higher after the second vaccination (median: 0.25 μg/ml) compared to the baseline (median: 0.03 μg/ml) and compared to the response after the first vaccination (median: 0.03 μg/ml) (**Figure 1A**). The seropositivity rates of S-RBD-specific IgG at baseline, after the first and the second vaccination, were 15.79% (3/19), 22.58% (7/31) and 87.06% (74/85), respectively (**Figure 1B**). The seroconversion rates regarding NAb after the first and the second vaccination were 9.68% (3/31) and 78.82% (67/85), respectively (**Figure 1B**).

Next, we examined the vaccine-induced SARS-CoV-2-specific cellular immunity by stimulating fresh isolated PBMCs

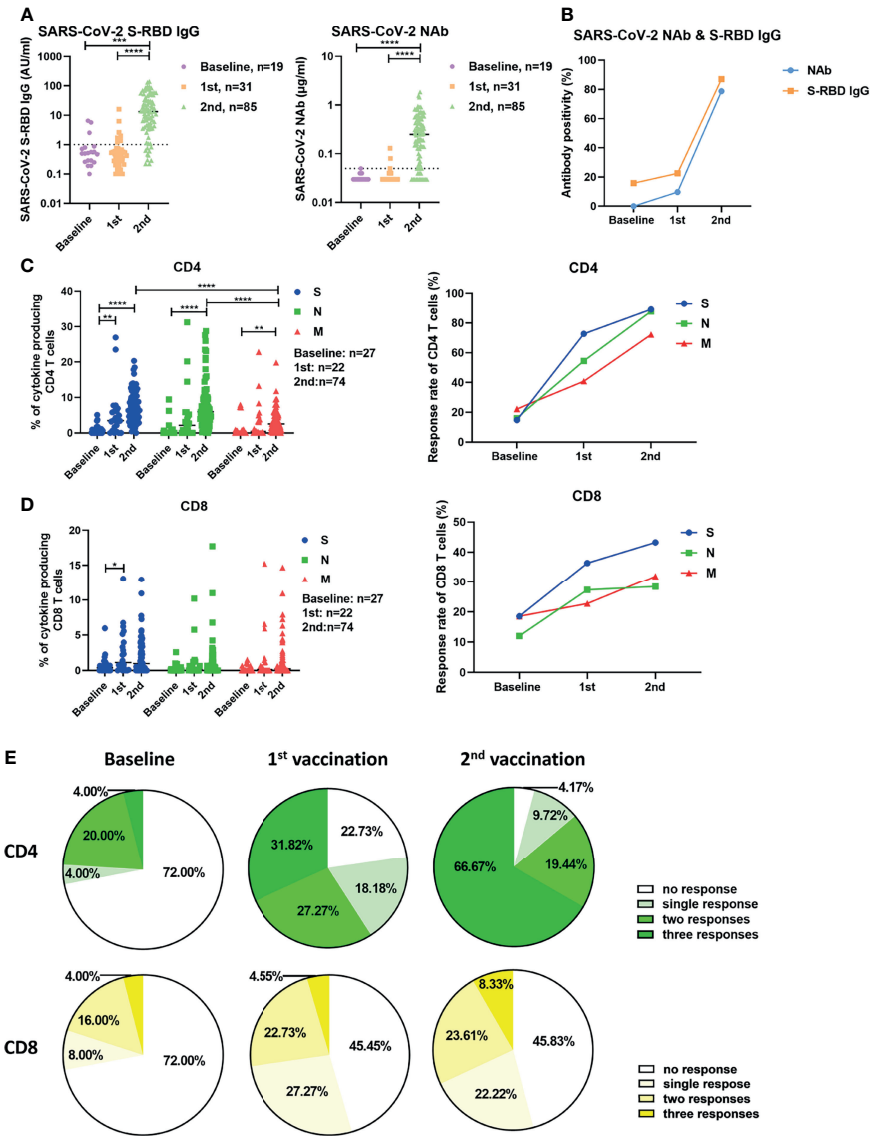

FIGURE 1 | Characterization of SARS-CoV-2-specific antibodies and T cell responses in individuals before and after vaccination. **(A)** SARS-CoV-2 S-RBD IgG and NAb levels in serum before and after vaccination. The dotted line indicates the cut-off value of the antibody. **(B)** The antibody positivity for SARS-CoV-2 S-RBD IgG and NAb before and after vaccination. **(C)** The magnitude and response rate of overall cytokine responses of CD4+ T cells against S, N, and M of SARS-CoV-2 in the participants before and after the first and second vaccination. **(D)** The magnitude and response rate of overall cytokine responses of CD8+ T cells against S, N, and M of SARS-CoV-2 in the participants pre- and post-vaccination. **(E)**The breadth of CD4+ and CD8+ T cell responses before and after the first and second vaccination. Baseline: before vaccination; 1st: after the first vaccination; 2nd: after the second vaccination. Each symbol represents an individual donor with a line indicating the median of each group. One-way ANOVA followed by Turkey's multiple comparisons test was used to test the statistical significance of data shown in **(A)**, **(C, D)**. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

with 3 panels of overlapping peptides spanning the SARS-CoV-2 proteins S, N, and M. We used an intracellular cytokine staining flow cytometry assay (**Figure S1**), and the magnitude of overall cytokine responses (IFN-γ, IL-2, and TNF-α) for CD4+ and CD8+ T cells for all participants are shown in **Figure 1C, D**. The frequencies of effector cytokine-producing CD4+ T cells in response to S, N, or M peptide pool stimulation were significantly higher after the second vaccination compared with the baseline responses and after the first vaccination (**Figure 1C**). The intensities of CD4+ T cell responses against S and N were significantly higher compared to those against M after two doses of vaccination (**Figure 1C**, left). In contrast to the observation with CD4+ T cells, the first dose of vaccination only induced a significant increase in the intensity of S-specific CD8+ T cell responses in participants (**Figure 1D**, left). The S-, N-, and M-specific CD8+ T cell responses were detected in 18.52% (5/27), 12.00% (3/25), and 18.52% (5/27) of participants at baseline, respectively (**Figure 1D**, right). These rates reached 36.36% (8/22), 27.27% (6/22), and 22.73% (5/22) after the first vaccination, but did not further increase after the second vaccination (**Figure 1D**, right). No significant differences were observed between the intensities of CD8+ T cell responses against S, N, and M (**Figure 1D**, left). Similar results were observed when the intensities of T cell responses were measured by single effector cytokine (IFN-γ, IL-2, or TNF-α) expression (**Figure S2**). Additionally, we analyzed the breadth (to how many peptide pools the T cells responded) of SARS-CoV-2-specific T cell responses induced by vaccination. At baseline, CD4+ T cell responses against a single, two, or three peptide pools of the different proteins were detected in 4.00% (1/25), 20.00% (5/25), and 4.00% (1/25) of participants, respectively (**Figure 1E, left**). These ratios were 18.18% (4/22), 27.27% (6/22), and 31.82% (7/22) in participants who received the first vaccination (**Figure 1E**, middle), and reached 9.72% (7/72), 19.44% (14/72), and 66.67% (48/72) in participants who received the second vaccination, respectively (**Figure 1E**, right). There were only 4.17% (3/72) of participants, who did not mount detectable CD4+ T cell responses after the second vaccination (**Figure 1E**, right). At the baseline, CD8+ T cell responses against a single, two, or three peptide pools of the different viral proteins were detected in 8.00% (2/25), 16.00% (4/25), and 4.00% (1/25) of participants, respectively (**Figure 1E**, left). These ratios were 27.27% (6/22), 22.73% (5/22), and 4.55% (1/22) in participants following the first vaccination (**Figure 1E**, middle), and were 22.22% (16/72), 23.61% (17/72), and 8.33% (6/72) in participants who received the second vaccination (**Figure 1E**, right). However, there were 45.83% (33/72) of participants, who showed no detectable CD8+ T cell responses after the second vaccination (**Figure 1E**, right).

Next, we explored whether the intensities of vaccine-induced humoral and cellular immune responses were correlated. In general, there were no significant correlations between the intensities of S-, N-, or M-specific CD4+ or CD8+ T cell responses and the serum concentrations of S-RBD IgG or NAb after the first or second vaccination (**Figures S3A–D**). We only observed that the intensity of S-specific CD8+ T cell response was very weakly, but borderline statistical significantly, correlated with the concentrations of NAb after two doses of vaccination (**Figure S3D**).

Taken together, these data suggest that two doses of inactivated vaccine elicit SARS-CoV-2-specific antibodies and CD4+ T cell responses in most individuals.

Influences of Sex and Age on Vaccine-Induced SARS-CoV-2-Specific Antibody and T Cell Responses

We next explored whether sex and age influence the intensities of vaccine-induced SARS-CoV-2-specific antibody and T cell responses. As shown in **Figure 2A**, after two doses of inactivated vaccine, female participants showed significantly higher concentrations of SARS-CoV-2-specific S-RBD IgG and NAb than male participants did. No significant differences in the intensities of both CD4+ and CD8+ T cell responses were observed between females and males irrespective of the number of applied vaccine doses (**Figure 2B**). After two doses of vaccine, SARS-CoV-2-specific CD4+ T cell responses were detectable in 98.00% of female participants versus 90.91% of male participants (**Figure 2C**). Moreover, we also examined potential correlations between age and the intensities of vaccine-induced SARS-CoV-2-specific antibody and T cell responses. As shown in **Figure S4**, we only observed that the breadth of CD8+ T cell responses was negatively correlated with age after the first vaccination (**Figure S4B**), while no obvious correlations between age and the concentrations of SARS-CoV-2 S-RBD IgG and NAb, or the intensities of S-, N-, and M-specific CD4+ and CD8+ T cell responses were observed after the second vaccination.

Correlation Between Vaccine-Induced Immune Responses and the Time Post Vaccination

Next, we analyzed how the intensities of vaccine-induced SARS-CoV-2-specific antibody and T cell responses change over time post vaccination. The concentrations of serum S-RBD IgG and NAb showed no significant correlation with the time after the second vaccination up to 57 days (**Figure S5A**). The frequencies of effector cytokine-producing CD4+ T cells in response to S (r^2 = 0.137, *P*=0.026) and N (r^2 = 0.196, *P*=0.007) peptide pool stimulation were significantly inversely correlated with the days post the first vaccination (**Figure 3A**). After the second vaccination, we observed that only the frequency of effector cytokine-producing CD4+ T cells in response to N (r^2 = 0.090, *P*=0.010) stimulation was negatively correlated with days post the second vaccination (**Figure 3A**). Like for CD4+ T cell responses, the frequency of effector cytokine-producing CD8+ T cells in response to N (r^2 = 0.142, *P*=0.024) peptide pool stimulation was significantly inversely correlated with the days post the first vaccination (**Figure S5B**, left). In contrast, no significant negative correlation between the intensities of CD8+ T cell responses and the time post the second vaccination was observed (**Figure S5B**, right). No significant correlation between the breadth of SARS-CoV-2 T cell responses and the days post the first or second dose of vaccine was observed (**Figure S5C**). Similar results were observed when the magnitude of T cell

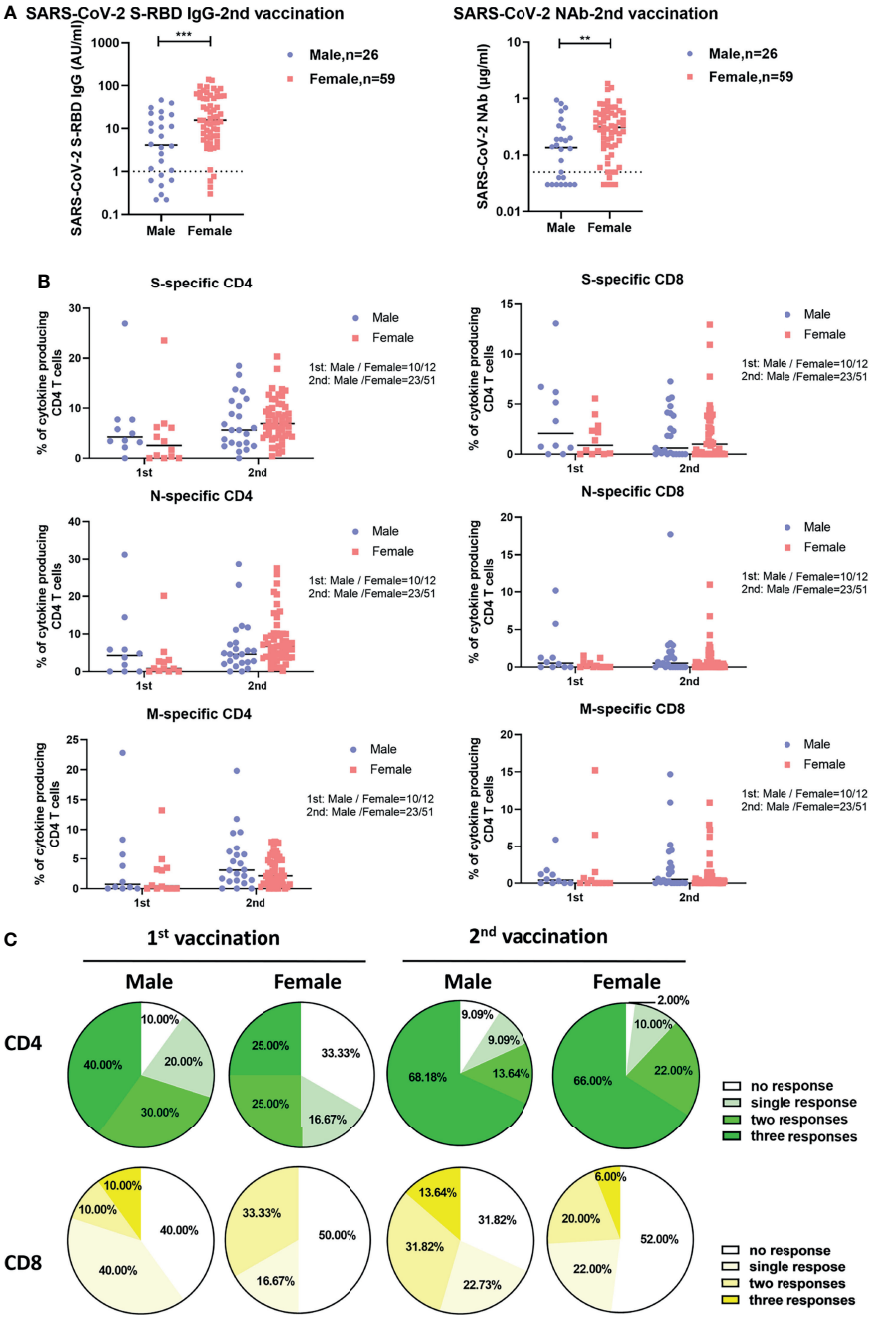

FIGURE 2 | Differences in SARS-CoV-2-specific humoral and cellular immune responses between male and female vaccinees. **(A)** Comparison of the SARS-CoV-2 S-RBD IgG and NAb levels in serum after the second vaccination between male and female vaccinees. The dotted line indicates the cut-off value of the antibody. **(B)** Comparison of the magnitude of CD4+ and CD8+ T cell responses against S, N, and M of SARS-CoV-2 between male and female vaccinees. **(C)** Comparison of the breadth of CD4+ and CD8+ T cell responses between male and female vaccinees. Each symbol represents an individual donor with a line indicating the median of each group. Mann Whitney U test was used to test the statistical significance of data shown in **(A, B)**. $^{**}P < 0.01$; $^{***}P < 0.001$.

responses was analyzed according to single effector cytokine (IFN-γ, IL-2, or TNF-α) expression levels (**Figure S6**). We next stratified the participants into two groups regarding to the time post their last vaccination: more or less than 30 days. We observed that the intensities of CD4+ T cell responses against S, N, and M were significantly lower in participants who received the first dose of vaccine more than 30 days before as compared to those individuals who got it less than 30 days ago (**Figure 3B**). After the second vaccination, only the intensities of CD4+ responses against N were significantly lower in participants

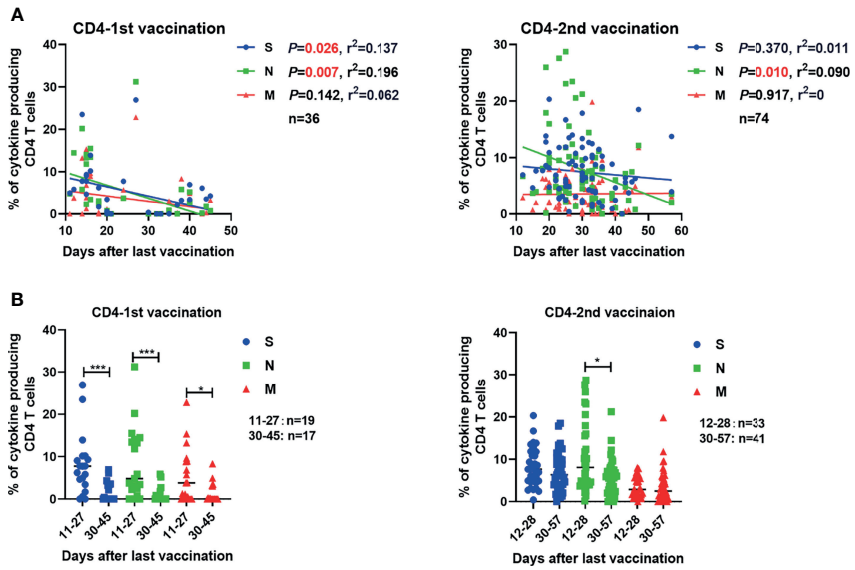

FIGURE 3 | Correlation between the SARS-CoV-2-specific T cell responses and the time that had elapsed from the last vaccination. **(A)** The correlations between SARS-CoV-2-specific CD4+ T cell responses and days after the first and second vaccination are shown. **(B)** Comparison of the magnitudes of SARS-CoV-2-specific CD4+ and CD8+ T cell responses before and after 30 days after the first or second vaccination. Pearson product-moment correlation coefficient test was used to test the significance of data shown in **(A)** and P value and r^2 value (correlation coefficient) are indicated in each panel. Each symbol represents an individual donor with a line indicating the median of each group. Mann Whitney U test was used to test the statistical significance of data shown in **(B)**. *$P < 0.05$; ***,$P < 0.001$.

who received the second dose of vaccine over 30 days compared to those with less than 30 days (**Figure 3B**). Taken together, these results indicated that the SARS-CoV-2-specific CD4+ T cell responses induced by a single dose of inactivated COVID-19 vaccine were short-lived, but can be strengthened and perpetuated by the booster vaccination.

Kinetics of Vaccine-Induced SARS-CoV-2-Specific Antibody and T Cell Immune Responses

We further assessed the longitudinal changes of SARS-CoV-2-specific S-RBD IgG and NAb concentrations, as well as the intensities of SARS-CoV-2-specific T cell responses in 22 participants who had been sampled 2-4 times during the observation period. 2 of the 22 participants received vaccines of the same manufacturer (WIBP-CorV, VA-2 and VA-22) and the other 20 participants were inoculated with vaccines from different manufacturers (WIBP-CorV+ CoronaVac, VA-1, VA-3~VA-7, VA-9, VA-10, VA-12~VA-16, VA-18~VA-21; WIBP-CorV+ BBIBP-CorV, VA-8, VA-11, VA-17). In agreement with the aforementioned results of our cross-sectional analysis, the longitudinal analysis demonstrated that the second vaccination was required to increase the serum concentrations of SARS-CoV-2-specific S-RBD IgG and NAb (**Figure 4A**). Increased intensities of S-, N-, and M-specific CD4+ T cell responses were observed in the majority of participants two weeks after the first vaccination, however, these responses quickly decreased to undetectable levels 2-3 weeks later (**Figures 4B–D**). The first vaccination induced increased intensities of S-, N-, and M-specific CD8+ T cell responses in some participants two weeks after the vaccination. Similar to the CD4+ T cells, the CD8+ T cell responses also started to decline shortly afterwards (**Figures 4B–D**). In contrast to the observation that T cell responses decreased significantly at the later time point after a single dose of vaccination (**Figure 4E**), concentrations of SARS-CoV-2-specific S-RBD IgG and NAb started to increase slightly at the later time point in some of the participants (**Figure 4F**). Nevertheless, increased S-RBD IgG and NAb responses, as well as SARS-CoV-2-specific CD4+ T cell responses were observed in the majority of participants after the second vaccination compared to the baseline (**Figures 4A–D**). Taken together, the results of the longitudinal analysis further underlined the importance of boost vaccinations for generating effective and sustained humoral and cellular immunity against SARS-CoV-2.

Influence of the Dosing Interval on Vaccine-Induced SARS-CoV-2-Specific Antibody and T Cell Immune Responses

In our real-world study, the dosing interval between the first and second vaccination varied greatly (21-63 days). Therefore, we examined whether the dosing interval had an impact on vaccine-induced SARS-CoV-2-specific antibody and T cell immune responses. No significant correlation between the intensities of antibodies or T cell responses after the second vaccination and the days of dosing interval was observed in overall participants (**Figure S7A**). Since the time that elapsed from the last vaccination may also influence the intensity of SARS-CoV-2-specific immunity, we next stratified the participants into two groups according to the time post the second vaccination (2-4

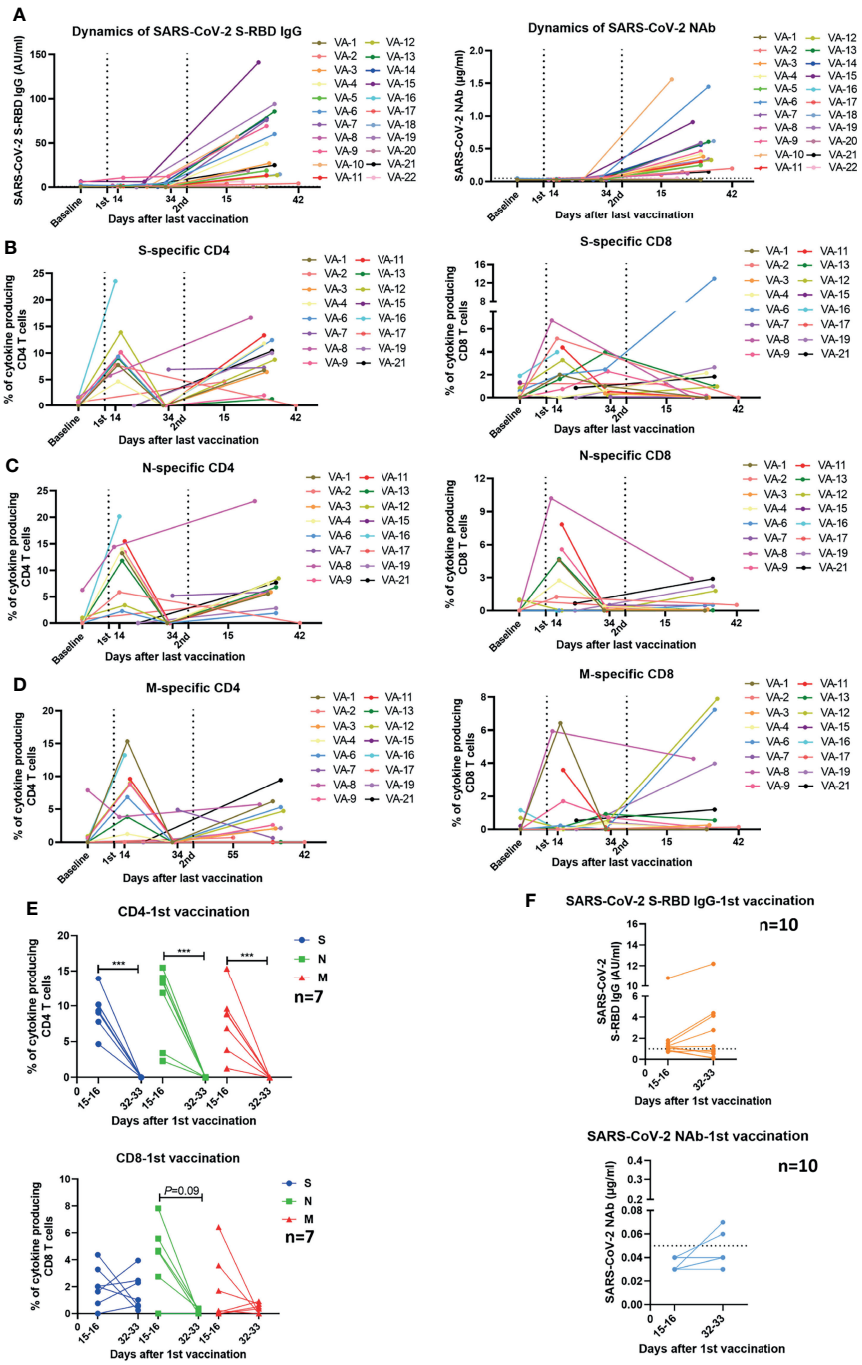

FIGURE 4 | Kinetics of humoral and cellular immune responses in individuals who received vaccines. **(A)** Dynamic changes of SARS-CoV-2 S-RBD IgG and NAb levels in 22 vaccinees. The dotted line indicates the cut-off value of the antibody. **(B–D)** Dynamic changes of the magnitude of T cell responses against S, N, and M in 16 vaccinees. The dynamics of different individuals are exhibited by lines of different colors. The numbers noted under the X-axis demonstrate the starting and ending days post the last vaccination. **(E)** Comparison of the magnitudes of T cell responses in 7 vaccinees detected at different time points after the first vaccination. **(F)** Comparison of the SARS-CoV-2 S-RBD IgG and NAb levels in 10 vaccinees at different time points after the first vaccination. The dotted line indicates the cut-off value of the antibody. Baseline: before vaccination; 1st: after the first vaccination; 2nd: after the second vaccination. Each symbol represents an individual donor with a line indicating the median of each group. Mann Whitney U test was used to test the statistical significance of data shown in **(E, F)**. ***$P < 0.001$.

weeks and 4-6 weeks). No significant correlation between the intensities of antibody or T cell responses after the second vaccination and the length of the dosing interval was observed in the participants of 2- to 4-week group (**Figure S7B**). However, a significantly positive correlation between the concentration of SARS-CoV-2 S-RBD IgG and the dosing interval was observed in the participants of the 4-6-week group (**Figure 5A**). No significant correlation between the concentration of NAb or the intensities of T cell responses and the days of dosing interval was observed in this group (**Figures 5A, B**). Taken together, these results suggested that a longer dosing interval might favor better long-term SARS-CoV-2 S-RBD IgG responses after the second vaccination.

DISCUSSION

The establishment of antigen-specific T- and B-cell responses is essential for sustained protection against viral diseases. NAbs generated by B cells are able to bind to SARS-CoV-2 and directly interfere with viral entry into target cells, whereas T cell responses are thought to limit viral replication by diminishing the number of infected cells, and reduce COVID-19 severity (15). Here we provide, to our knowledge, the first analysis of SARS-CoV-2-specific antibody and T cell responses in a cohort of vaccinees who received inactivated COVID-19 vaccines in real-world settings. We show that seroconversion rates concerning S-RBD IgG and NAb were 87.06% and 78.82%, respectively, at 10-57 days post the second dose of vaccination. In previous phase 1/2 clinical trials that assessed inactivated COVID-19 vaccines, seroconversion rates of S-RBD IgG and NAb ranged from 83% to 100% and from 25 to 100%, respectively (8–11). The dosing interval seems to be an important factor that influences the seroconversion rates of NAb and S-RBD IgG since in the phase 1 clinical trials of CoronaVac, the 28-day-interval group showed higher seroconversion rates of NAb and S-RBD IgG than the 14-day-interval group (9). It was also reported that extension of the interval between vaccine doses for the BNT162b2 mRNA vaccine from the conventional 3-4 week regimen to 6-14 weeks resulted in higher NAb levels (16). This is consistent with our findings in the real-world settings that a longer interval between the first and second vaccination results in higher concentrations of S-RBD IgG 4 weeks after the second vaccination. Moreover, we also observed that females showed superior antibody responses than males after receiving two doses of inactivated vaccines. Interestingly, a trend for more robust SARS-CoV-2-specific humoral responses in females was also observed in individuals who received anti-COVID-19 mRNA vaccines (17, 18). It is known that females generally exhibit greater humoral and cell-mediated immune responses to antigenic stimulation, vaccination, and infection than males do (19–21). In line with our current observation, a very recent study reported that the efficacy of inactivated vaccines against COVID-19 was higher in females than in males during the during the outbreak of the Delta variant in May 2021 in Guangzhou city, China (22).

So far, the virus-specific T cell immunity induced by inactivated COVID-19 vaccines is far from being well defined. Yao Deng et al. recently reported that BBIBP-CorV recipients raise specific T cell responses that recognize multiple structural proteins (S, N, and E proteins) of SARS-CoV-2 (23). However, the study only enrolled 10 healthy individuals and T cell responses were only characterized by ELISpot, which does not allow distinguishing CD4+ and CD8+ T cell responses. Here, we

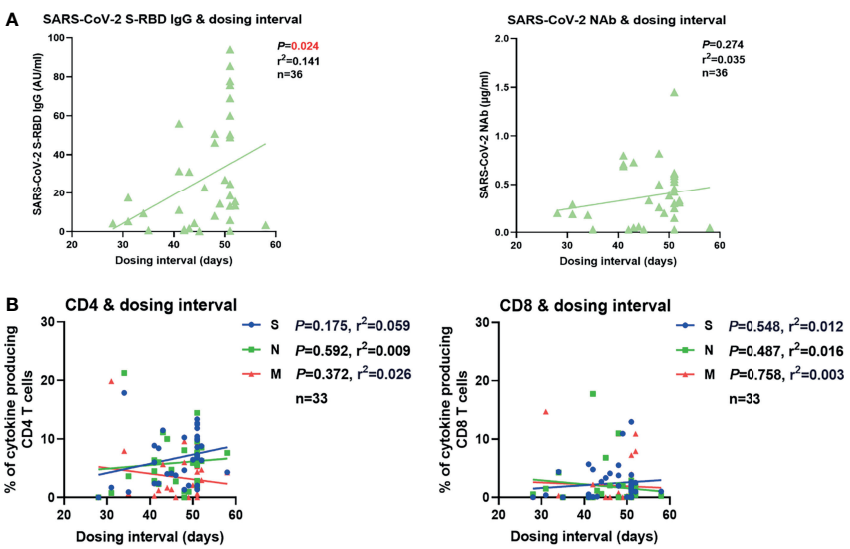

FIGURE 5 | Correlation between SARS-CoV-2-specific humoral and cellular immune responses and dosing interval. **(A)** Correlation between the SARS-CoV-2 S-RBD IgG (left) and NAb (right) levels 4-6 weeks after the second vaccination and the days of dosing interval. **(B)** Correlation between the magnitudes of SARS-CoV-2-specific CD4+ (left) and CD8+ (right) T cell responses 4-6 weeks after the second vaccination and the days of dosing interval. Pearson product-moment correlation coefficient test was used to test the significance and P value and r^2 value (correlation coefficient) are indicated in each panel.

analyzed the SARS-CoV-2-specific T cell responses induced by inactivated COVID-19 vaccines in more detail and more comprehensively. We show that the magnitude of SARS-CoV-2-specific CD4+ T cell responses was already significantly increased after a single dose vaccination and further increased after the second administration. The SARS-CoV-2-specific CD4+ T cell responses were detectable in over 95% of participants after two doses and S- and N-specific CD4+ T cell responses were significantly stronger than M-specific CD4+ T cell responses. Similarly, it has been reported that in COVID-19 patients the CD4 T cell responses to S are the most abundantly detected responses, followed by the responses to N and M (24–26).Corresponding mechanisms should be addressed in future studies. However, SARS-CoV-2-specific CD8+ T cell responses induced by inactivated vaccines were rather weak and less frequently observed in the participants compared to the aforementioned CD4+ T cell responses. This finding is in accordance with our expectations since inactivated vaccines tend not to induce strong CD8+ T cell responses (9).

Another key issue that needs to be characterized is the duration of humoral and cellular immunity generated by inactivated vaccines. Our data suggest that virus-specific CD4+ T cell responses generated by single-dose vaccinations are extremely short-lived and last less than 5 weeks. A boost vaccination generates more robust S-, N-, and M-specific CD4+ T cell responses, which in most individuals lasted at least up to 2 months post the second administration. However, the intensities of SARS-CoV-2-specific CD4+ T cell responses induced by two doses of vaccination were still only one-third of the responses that we detected in convalescent individuals after COVID-19. Moreover, we observe that the intensity of N-specific CD4+ T cell response is negatively correlated with the time post the second vaccination, suggesting vaccine-induced cellular immune responses may already start to wane during our short observation period. This is in line with our recent finding that SARS-CoV-2-specific memory T cell responses in convalescent individuals are not long-lasting and wane profoundly 10 months after infection (data in submission). In this regard, our results argue in favor of booster immunizations of inactivated vaccines in order to maintain effective and lasting immunity including CD4+ T cells against SARS-CoV-2 infections.

There are several limitations in the current study. First, although the local spread of SARS-CoV-2 in Wuhan and the surrounding areas was prevented by strict non-pharmacologic intervention methods during the period in which the study was conducted, it is still difficult to absolutely exclude the pre-existing SARS-CoV-2-specific immunity in vaccinees induced by virus contact (e.g. by household contacts of asymptomatically infected persons or before initiation of the intervention methods). Second, other regions outside of S-RBD could also be neutralizing, however, only SARS-CoV-2 NAb and S-RBD IgG were detected in the current study. SARS-CoV-2-specific antibodies such as anti-S IgG and anti-N IgG should also be characterized in future study. Third, SARS-CoV-2-specific T cell responses targeting other structural and accessory proteins such as envelope (E) protein and open reading frame (ORF) 3,6,7 and 8 (27) were not characterized in the current study.

Collectively, we provide a comprehensive characterization of the immune responses induced by inactivated COVID-19 vaccines in real-world settings. While a single vaccination was insufficient to induce robust immune responses, both humoral and cellular SARS-CoV-2-specific immunity could be elicited in the majority of individuals who received two inactivated COVID-19 vaccine doses.

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 local medical ethics committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZL, TX, BL, XZ, and JL planned the experiment. ZL, TX, and BL performed the detection of T cell responses, clinical data collection and data analysis. HW conducted the detection of SARS-CoV-2 NAb and S-RBD IgG and XQ helped with the data collection of the antibodies. XF involved in the collection of blood samples. ZL and JL wrote the manuscript. HD, XW, SML, SHL, XY, BW, GZ, MT, KS, ML, UD, DY, and XZ revised the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.802858/full#supplementary-material

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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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Kinetics of the Antibody Response to Boostering With Three Different Vaccines Against SARS-CoV-2

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Background: Heterologous vaccinations against SARS-CoV-2 with ChAdOx1 nCoV-19 and a second dose of an mRNA-based vaccine have been shown to be more immunogenic than homologous ChAdOx1 nCoV-19. In the current study, we examined the kinetics of the antibody response to the second dose of three different vaccination regimens (homologous ChAdOx1 nCoV-19 vs. ChAdOx1 nCoV-19 + BNT162b2 or mRNA-1273) against SARS-CoV-2 in a longitudinal manner; whether there are differences in latency or amplitude of the early response and which markers are most suitable to detect these responses.

Methods: We performed assays for anti-S1 IgG and IgA, anti-NCP IgG and a surrogate neutralization assay on serum samples collected from 57 participants on the day of the second vaccination as well as the following seven days.

Results: All examined vaccination regimens induced detectable antibody responses within the examined time frame. Both heterologous regimens induced responses earlier and with a higher amplitude than homologous ChAdOx1 nCoV-19. Between the heterologous regimens, amplitudes were somewhat higher for ChAdOx1 nCoV-19 + mRNA-1273. There was no difference in latency between the IgG and IgA responses. Increases in the surrogate neutralization assay were the first changes to be detectable for all regimens and the only significant change seen for homologous ChAdOx1 nCoV-19.

Discussion: Both examined heterologous vaccination regimens are superior in immunogenicity, including the latency of the response, to homologous ChAdOx1 nCoV-19. While the IgA response has a shorter latency than the IgG response after the first dose, no such difference was found after the second dose, implying that both responses are driven by separate plasma cell populations. Early and steep increases

in surrogate neutralization levels suggest that this might be a more sensitive marker for antibody responses after vaccination against SARS-CoV-2 than absolute levels of anti-S1 IgG.

Keywords: SARS-CoV-2, vaccination, B-cell response, immune response, kinetics

INTRODUCTION

Vaccinations against the Severe acute respiratory syndrome coronavirus 2 (SARS−CoV−2) have been approved and administered since the late year 2020 as a promising measure to contain the further spread of the virus as well as to prevent severe cases of coronavirus disease 2019 (COVID-19). Among the first vaccines to be approved were the two mRNA-based vaccines BNT162b2 (Comirnaty; BioNTech/Pfizer, Germany/USA) (1) and mRNA-1273 (Spikevax; Moderna, USA) (2) and the adenoviral vector vaccine ChAdOx1 nCoV-19 (Vaxzevria; Oxford-AstraZeneca, UK/Sweden) (3). Emerging data on efficacy and reactogenicity of these vaccines (4–7) led to changes in official recommendations concerning the administration of these vaccines. Especially reports of cases of vaccine-induced immune thrombotic thrombocytopenia (VITT), mainly in female recipients of ChAdOx1 nCoV-19 below the age of 60 (7), caused the German permanent commission on vaccination (ständige Impfkommission) to recommend that all individuals who had received a first dose of ChAdOx1 nCoV-19 were to receive a second dose of either BNT162b2 or mRNA-1273, while the use of ChAdOx1 nCoV-19 is generally recommended for recipients ≥ 60 years only.

Studies examining the effect of the different vaccination regimens that resulted from these recommendations (homologous ChAdOx1 nCoV-19 or ChAdOx1 nCoV-19 plus either BNT162b2 or mRNA-1273) have unanimously found heterologous vaccination with ChAdOx1 nCoV-19 and an mRNA-based vaccine induces greater immune responses than homologous ChAdOx1 nCoV-19 (8–11). For the comparison between heterologous regimens, there is still a scarcity of data. However, we were able to find in a previous study (manuscript currently under review) that ChAdOx1 nCoV-19 plus mRNA-1273 induces slightly higher levels of antibodies against SARS-CoV-2 than the respective combination with BNT162b2. As many of these studies have been conducted cross-sectionally, examining only one or two time points since the second vaccination, questions remain whether these differences are caused by different latencies of the responses to the different vaccines or whether they are independent of the time point of sample collection.

In the current study, we examined the intraindividual kinetics of the antibody response to different doses of the second vaccination against SARS-CoV-2. We collected and examined serum samples over the period of 8 days, starting on the day of the second dose, from recipients of anti-SARS-CoV-2 vaccines. As a study collective, individuals who had received ChAdOx1 nCoV-19 as a first dose (and could therefore be expected to exhibit comparable baseline values at the time of the second dose) and were due to receive either ChAdOx1 nCoV-19, BNT162b2, or mRNA-1273 as a second dose were chosen.

With the collected data, we addressed the following questions:

-Do all vaccination regimens induce detectable antibody responses in the examined period of time?

-Are there differences in the kinetics of the antibody response between recipients of different vaccines as second dose? And if yes, are they differences in latency or amplitude of the response?

-What markers are suitable to detect an early response to the second dose and at what point in time can a response be expected?

-Does the recipients' sex or age influence the kinetics of the antibody response?

METHODS

Study Population

Participants were recruited from health care professionals working at the University Hospital Schleswig-Holstein (Lübeck, Germany) who received a second dose of a vaccine against SARS-CoV-2 after having received a first dose of ChAdOx1 nCoV-19. The interval between the two doses were twelve weeks for these vaccinees (see **Figure 1**). Despite the official recommendation that these individuals should receive either BNT162b2 or mRNA-1273 as a second dose, recipients were also free to make an informed decision to receive a second dose of ChAdOx1 nCoV-19 (as only this regimen was approved by the European Medical Agency). Participants who accepted the offer to receive a second dose of an mRNA-based vaccine were not able to choose between BNT162b2 or mRNA-1273.

Prior to participation, all participants gave written informed consent to all procedures they underwent. The study was approved by the University of Kiel institutional review board (AZ: D499/20) and performed in accordance with the declaration of Helsinki (12).

Sample Characteristics

Serum samples were collected at eight different time points: on the day of the second vaccination (but immediately before it) and on each of the following seven days. Subsequent to collection, the samples were pseudonymized, centrifuged and stored at 4°C until assays were performed. All reported assays (anti-S1 IgA and IgG, anti-NCP IgG and the surrogate neutralization assay) were performed from serum.

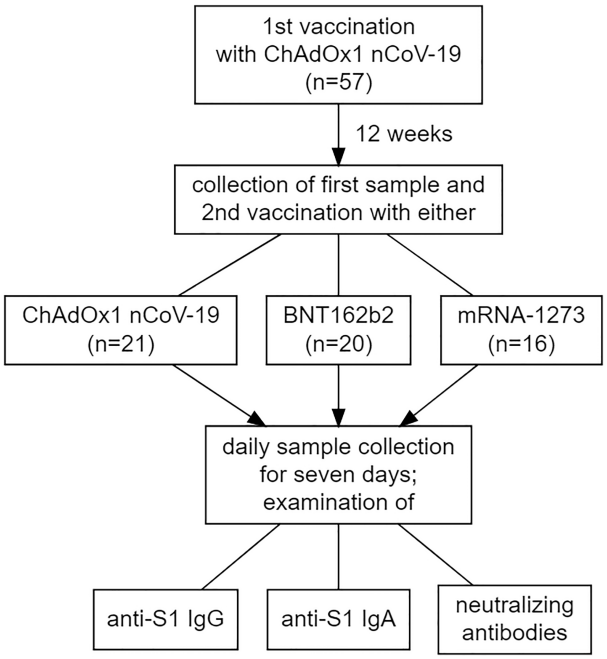

FIGURE 1 | Visualization of the study design, including the makeup of the study cohort concerning the vaccination regimens administered.

Anti-SARS-CoV-2 Antibodies

Antibodies of the classes IgA and IgG against the S1 subunit of the Spike protein of SARS-CoV-2 (anti-S1) as well as IgG against the nucleocapsid (anti-NCP) antigen were measured from the serum samples using the Anti-SARS-CoV-2-ELISA IgA and Anti-SARS-CoV-2-QuantiVac-ELISA (IgG) test kits by EUROIMMUN (Lübeck, Germany) according to the manufacturers' instructions. Anti-S1 IgA was performed as a possible early marker of the B-cell response to exposure to SARS-CoV-2 specific antigens. Anti-NCP IgG was performed to exclude any past exposure to the virus itself. More information on the dimension of the reported results as well as their interpretation can be found in the supplement.

Surrogate Neutralization Assay

The capacity of the anti-S1 antibodies to potentially neutralize SARS-CoV-2 was tested *via* a surrogate neutralization assay (NeutraLISA, EUROIMMUN, Lübeck, Germany). A more detailed description of this assay is contained in the supplement. This test yields a quantitative result reported as a calculated percentage of antibody-induced neutralization. According to the manufacturer, there is a concordance of 98.6% between this method and the examination of neutralizing antibodies *via* plaque reduction neutralization test (PRNT50) (13). The choice to perform a surrogate neutralization assay rather than a neutralization assay was motivated both by reasons of practicability and by the fact that, due to its very limited availability, assays like the PRNT are not likely to be included in routine examinations of vaccine response, which might be different for surrogate neutralization assays which are much easier to implement on a larger scale.

Statistical Analysis

To analyze the influence of one or more factors on continuous variables, analyses of variance (ANOVAs) were calculated. If the influence of more than one factor was examined, the resulting p-values were adjusted for multiple comparisons using the method described by Benjamini and Yekutieli (14). If exploratory analyses revealed significant main or interaction effects, *post-hoc* testing *via* Tukey's honest significant differences, a single-step statistical test adjusting for multiple comparisons, was applied. To analyze differences in the distribution of categorically scaled variables between groups, Pearson's Chi-squared test was used. To analyze the association between two continuous variables, correlations using Spearman's rho were calculated. For the interpretation of Spearman's rho, the rule of thumb suggested by Rea and Parker (15) was used ($0.0 < 0.1$: negligible; $0.1 < 0.2$: weak; $0.2 < 0.4$: moderate; $0.4 < 0.6$: relatively strong; $0.6 < 0.8$: strong; $0.8 < 1.0$: very strong). Statistical significance was assumed for p-values <0.05. Average values with corresponding measures of dispersion are reported as medians with the median absolute deviation (MAD), unless otherwise stated. All statistical analyses were performed using the open-source software for statistical computing and graphics R (version 4.1.0) with the integrated development environment RStudio (Version 1.4.1717) (16).

RESULTS

Study Population

For the current study, 57 participants were included, of which 34 (59.6%) were female. Their median age was 40 years old (± 17.8; range: 21-63 years old). Of the 57, 21 (36.8%) received ChAdOx1 nCoV-19 as second vaccine, 20 (35.1%) received BNT162b2 and 16 (28.1%) received mRNA-1273 (See **Table 1** for the numerical makeup of the study cohort, including median ages). A two-way ANOVA with the factors sex and type of second vaccine revealed no significant main or interaction effects of either of these factors on participants' age (i.e. there was no significant difference in age between recipients of the different vaccination regimens as well as between the two sexes). Pearson's chi-squared test revealed a slight imbalance in the distribution of sexes between the different vaccination regimens (chi-squared = 6.5101, df = 2, p = 0.03858). This is due to only 38.1% of recipients of ChAdOx1 nCoV-19 as second dose being female, compared to 70% for BNT162b2 and 75% for mRNA-1273. None of the participants showed an anti-NCP IgG response at any time point, suggesting that no participant was exposed to SARS-CoV-2, neither prior to or during the study. Information on missing data can be found in the supplement.

Kinetics of the Antibody Response Depending on the Vaccination Regimen

Three-way ANOVAs with the factors second vaccine, days since second dose and sex revealed statistically highly significant main effects for the factors second vaccine and days since second dose, as well as a significant interaction effect between these two factors for all examined markers (p (adj.) for all comparisons < 0.0001). *Post-hoc* testing (Tukey's honest significant differences) showed that this was due to the levels of all markers rising significantly in

TABLE 1 | Overview of the number and respective median ages (including the median absolute deviation as a measure of dispersion), both of the whole cohort and each individual subgroup (recipients of the different vaccination regimens and members of the two sexes).

	Second vaccine:			Whole cohort
	ChAdOx1 nCoV-19	BNT162b2	mRNA-1273	
n (total)	21	20	16	57
n (female)	8	14	12	34
n (male)	13	6	4	23
Median age (total)	47 ± 20	38.5 ± 8.9	39 ± 10.4	40 ± 17.8
Median age (female)	47 ± 17.8	40 ± 10.4	41 ± 13.3	42 ± 13.3
Median age (male)	53 ± 13.3	33 ± 10.4	32 ± 14.8	40 ± 23.7

the course of the seven days post second dose. Further exploring the data, it can be seen, however, that this significant rise happens only after second vaccination with BNT162b2 or mRNA-1273, for which a significant increase in observed levels can be detected after six days (anti-S1 IgG and IgA) or five days (neutralizing antibodies) after the second dose with highly significant correlations of strong to very strong effect size between days since second dose and the respective marker (See **Figures 2B, C, E, F, H, I**). For second vaccination with ChAdOx1 nCoV-19, a significant increase in levels from day 0 to day 7 post second dose could only be shown for neutralizing antibodies and correlations between days since second dose and the examined markers, while statistically significant, were only weak to moderate (See **Figures 2A, D, G**).

The *post-hoc* testing further showed that there were no statistically significant differences between recipients of the different second vaccines in any of the observed markers until (and including) day four post second dose. From day five onward, recipients of mRNA-1273 as second dose develop significantly higher levels of all examined markers compared to recipients of ChAdOx1 nCoV-19. For BNT162b2, the same is true at day six (anti-S1 IgG and IgA) or day five (neutralizing antibodies), respectively (See **Figure 3**). The comparison between both mRNA-based vaccines mRNA-1273 and BNT162b2 shows that the former intermittently induces higher levels of all examined markers at day five (neutralizing antibodies), day six (anti-S1 IgA), or both day five and six (anti-S1 IgG), while at day seven after the second dose, there is no statistically significant difference anymore (although a visual trend in favor of mRNA-1273 is still discernible, see **Figure 3**). Of note, a visualization of the same comparisons for days 0-3 can be found in **Figure S1** of the supplement.

Influence of Sex on the Immune Response

The aforementioned three-way ANOVAs with the factors second vaccine, days since second dose and sex reveals a significant main effect of sex ($F = 9.332$, $df = 1$, $p\ (adj.) = 0.006$) only on neutralizing antibodies. *Post-hoc* testing shows that this significant main effect of sex is due to men exhibiting slightly higher levels neutralizing antibodies than women (25.2 ± 22.5% vs. 20.9 ± 20.7%), if values are viewed across all time points and vaccination regimens. However, this difference was not found either for one of the three vaccination regimens or one of the eight time points separately.

For anti-S1 IgG and IgA, there was no significant main or interaction effect of sex.

Influence of Age on the Immune Response

Due to the strong influences of the day since second dose and the vaccine administered as second dose, a possible influence of age on the kinetics of the antibody response to the second vaccination against SARS-CoV-2 was difficult to analyze. Correlations of all examined markers across all vaccination regimens calculated for each day since the second dose revealed significant negative correlations of weak to moderate effect size only between age and anti-S1 IgG on day six ($R = -0.29$, $p = 0.0329$) as well as between age and anti-S1 IgA on days six ($R = -0.33$, $p = 0.0165$) and seven ($R = -0.32$, $p = 0.019$).

DISCUSSION

Our results show that all of the examined vaccination regimens elicit a detectable antibody response within seven days after the administration of the second dose. Further inspection, however, reveals significant differences in between the three examined vaccination regimens: For the mRNA-based vaccines mRNA-1273 and BNT162b2, significant increases in all examined markers can already be seen at day six after the second dose (or even day five for neutralizing antibodies), only a very weak increase can be detected for anti-S1 IgG and IgA seven days after the second dose of ChAdOx1 nCoV-19. There is, however, a significant increase in levels of neutralizing antibodies at day seven for ChAdOx1 nCoV-19.

Therefore, the differences between the second doses of mRNA-1273 and BNT162b2 that can be seen at day five and six (and that continue to be detectable at 14 days after the second dose (manuscript currently under revision) are a matter of the amplitude of the antibody response (with a stronger response for mRNA-1273), and not its respective latency. The difference between ChAdOx1 nCoV-19 and either of the mRNA-based vaccines (but especially mRNA-1273) is both in latency and in amplitude of the measured responses. One explanation for this difference might be found in the possibility of immune responses against the adenoviral vector impairing the induction of the desired immune response to the vaccine. While the use of a chimpanzee adenoviral vector all but precludes the possibility of preexisting immunity against the vector of ChAdOx (17), the reutilization of the same vector for the second dose might give rise to antivector

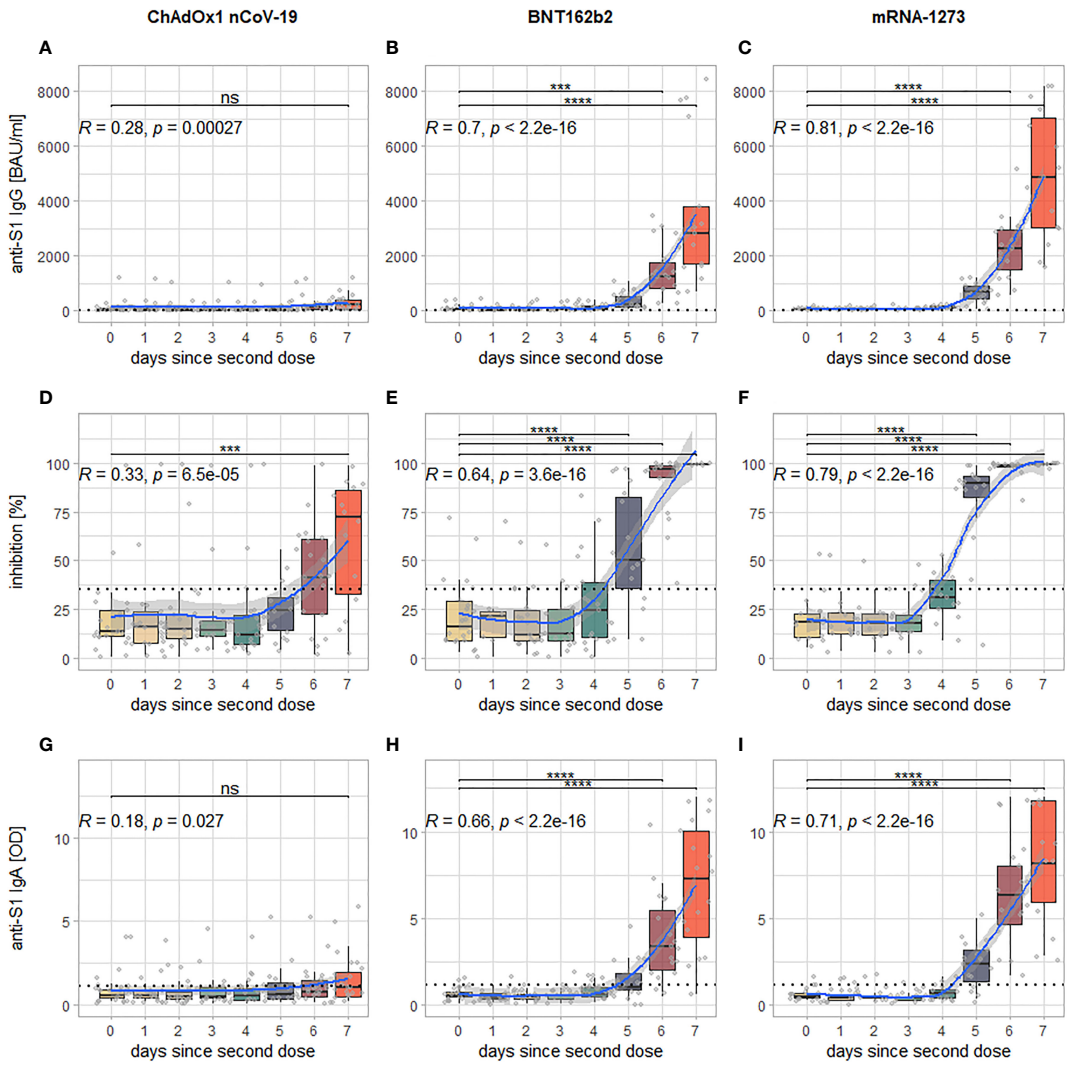

FIGURE 2 | Visualization of the kinetics of all examined markers for the different vaccination regimens: each column of panels represents data from participants who have received either ChAdOx1 nCoV-19 **(A, D, G)**, BNT162b **(B, E, H)** or mRNA-1273 **(C, F, I)** as second dose; each row represents one examined marker: anti-S1 IgG **(A–C)**, inhibition *via* surrogate neutralization assay **(D–F)**, and anti-S1 IgA **(G–I)**. Within each panel, every boxplot represents one time point of sample collection, the individual results are additionally plotted as grey dots. The blue line indicates the smoothed means with a 95% confidence band in light grey. The dotted horizontal line indicates the cutoff for positivity for each assay. In the upper left-hand corner is Spearman's rho of the correlation between measured levels of the examined marker and days since second dose (along with the associated p-value), the brackets above the boxplots indicate which comparisons between individual time points reveal significant differences (corrected for multiple comparisons). Levels of significance: ****p < 0.0001; ***p < 0.001; ns, not statistically significant.

immunity that interferes with vaccine delivery (18). Other manufacturers have employed different adenoviral vectors for prime and boost doses to circumvent this phenomenon (19, 20). A possible implication is that a second vaccination with an mRNA-based vaccine provides clinical protection earlier than ChAdOx1 nCoV-19, as it has been shown that clinical protection correlates well with the measured levels of anti-S1 IgG (21). Whether or not the observed differences between both mRNA-based vaccines are clinically relevant remains debatable.

There are some surprising findings in the data: While we found in an earlier study that anti-S1 IgA responses after a first dose of an mRNA based vaccine precedes the anti-S1 IgG response (22), we could not find any difference in latency between the IgG and the IgA response in the current study. A possible explanation is the recent finding that a first dose of BNT162b2 induces an IgA-dominant plasmablast response (mainly against the S2 epitope), which might represent a recall response of mucosal memory B-cells formed in response to previous pulmonary coronavirus infections, whereas the (neutralizing) anti-S1 response (IgA and IgG) most likely stems from naïve B-cells which are recruited after the first dose and boostered after the second (23). The role of IgA in respiratory infections is not completely understood, but it is assumed that it acts as a first line of defense on muscosal tissues (24).

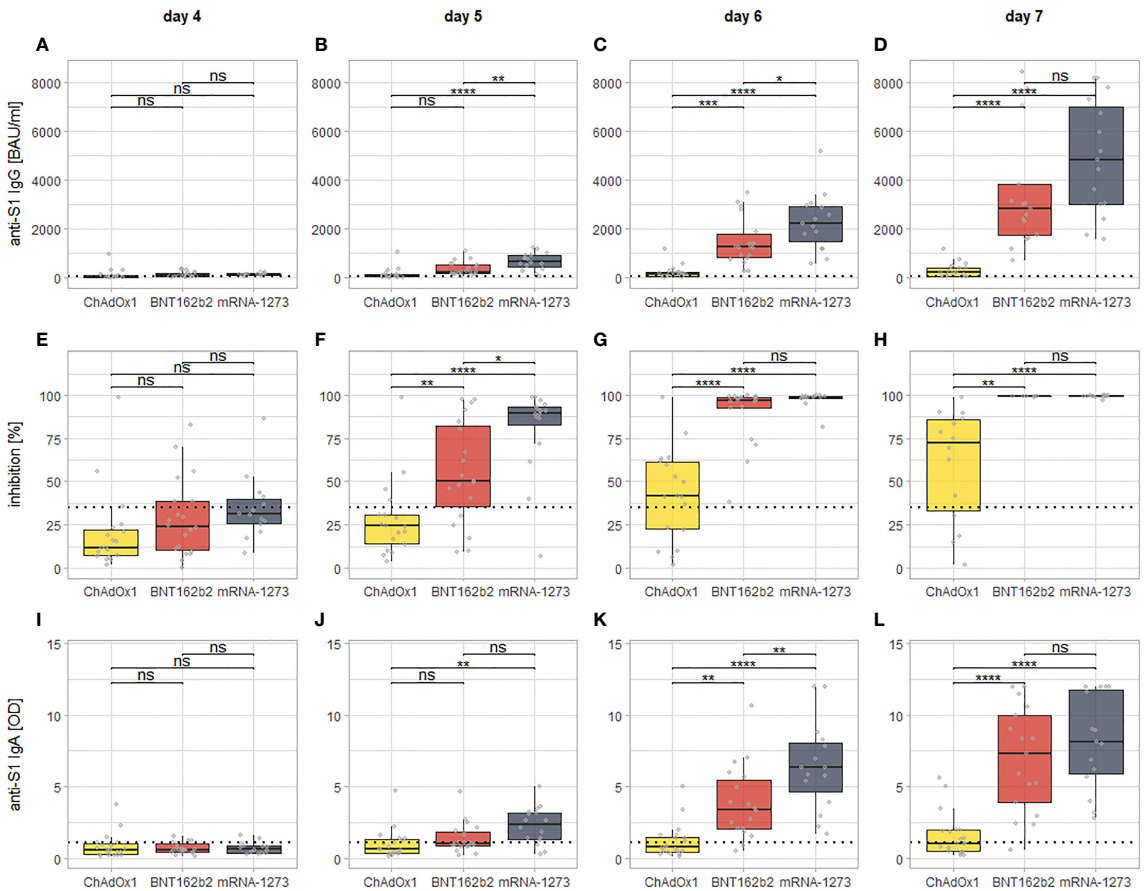

FIGURE 3 | Visualization of the day by day comparison of all examined markers for all of the three vaccination regimens from day four since the second dose onward. Each column of panels represents results for a single time point since the second dose (day 4: **A, E, I**; day 5: **B, F, J**; day 6: **C, G, K**; day 7: **D, H, L**), while each row represent results for a specific assay (anti-S1 IgG: **A–D**; inhibition *via* surrogate neutralization assay: **E–H**; anti-S1 IgA: **I–L**). The dotted lines indicate the cutoffs for positivity for each assay. The brackets indicate the results of *post-hoc* testing for statistically significant differences (via Tukey's Honest significant differences). Levels of significance: ****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05; ns, not statistically significant.

More research is certainly warranted on the effects of vaccine-induced anti-S1 IgA more, as the focus of research to date was mainly on IgG.

Another intriguing finding is that the ability of the induced antibodies to inhibit binding between S1 and ACE2 *in vitro* during the surrogate neutralization assay increases earlier and more strongly than the overall antibody response (anti-S1 IgG and IgA). For ChAdOx1 nCoV-19 this is even the only response for which significant increases can be shown, with only weak increases of anti-S1 IgG or IgA. This suggests that the second exposure to the S1-antigen *via* the second vaccination preferably induces the production of antibodies with a high affinity to the S1-antigen of which smaller quantities are needed to inhibit the binding between S1 and ACE2. It is possible, therefore, that apart from the quantitative IgG response, as measured *via* international binding antibody units per milliliter, the qualitative ability to inhibit the binding of the virus might be a more sensitive marker of the immune response after vaccination, especially as quantitative levels of IgG wane over time. This assumption is supported by the finding that results of neutralization assay permit a good prediction of protective immunity against SARS-CoV-2 (25, 26). It is important to note however, that to date there are no reliable thresholds for either anti-S1 IgG or neutralizing antibodies (via surrogate neutralization assay) above which a certain degree of clinical protection can be assumed.

Our study has several limitations: Due to considerations of practicability, we did not examine any part of the T-cell response after the second vaccination. Data we gathered for the first dose of the vaccine suggest that the T-cell response might be detectable even earlier after the second dose than the examined antibody response (22). We did not perform a neutralization assay in the proper sense, but rather a surrogate neutralization assay. However, as mentioned, according to the manufacturer, there is a very good concordance between the assay we used and PRNT, one of the methods of choice for neutralization assay. Further, our sample size was quite small, therefore it is possible that we overlooked smaller differences between certain groups.

Whether such differences are of clinical significance might be debated however. Lastly, both due to the sample size and because of the fact that the oldest participant was 63 years of age, possible effects of old age might have been overlooked in this study.

In conclusion, we were able to show that all three examined vaccination regimens are able to induce a significant antibody response within a short period of time after the second dose. In between the different vaccination regimens, there are significant differences in latency and amplitude of the response (for the comparison between ChAdOx1 nCoV-19 and both mRNA-based vaccines) or mainly of the amplitude of the response (for the comparison between mRNA-1273 and BNT162b2). Whether these differences are of clinical significance for the protection against SARS-CoV-2 is unclear, however. Lastly, our data suggest that surrogate neutralization assays like the one we used might be used as an early and sensitive marker of the antibody response to the second dose of the vaccination against 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 studies involving human participants were reviewed and approved by University of Kiel institutional review board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RM, MZ, DJ, DP, SS, K-PW, BS, FL, and RJ designed the study. RM, MZ, DJ, CB, JD, SE, JR, and SG contributed to recruitment of participants. The described vaccinations were performed under the direction of SG, MZ, and DJ. The assays were performed by RM, MZ, DJ, DP, KS, VH, DZ, and CK. Data analysis was performed by RM, MZ, DJ, KS, VH, and DZ. The manuscript was written by RM, with support from all authors. All authors have read and approved the final version of the manuscript.

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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.2022.811020/full#supplementary-material

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REVIEW
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mRNA Vaccine: How to Meet the Challenge of SARS-CoV-2

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Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) with high infectivity, pathogenicity, and variability, is a global pandemic that severely affected public health and the world economy. The development of safe and effective vaccines is crucial to the prevention and control of an epidemic. As an emerging technology, mRNA vaccine is widely used for infectious disease prevention and control and has significant safety, efficacy, and high production. It has received support and funding from many pharmaceutical enterprises and becomes one of the main technologies for preventing COVID-19. This review introduces the current status of SARS-CoV-2 vaccines, specifically mRNA vaccines, focusing on the challenges of developing mRNA vaccines against SARS-CoV-2, and discusses the relevant strategies.

Keywords: SARS-CoV-2, COVID-19, mRNA vaccine, challenges, strategies

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1 INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel positive-sense single-stranded RNA coronavirus, which belongs to the *Betacoronavirus* genus (1, 2). Coronavirus disease 2019 (COVID-19) caused by this virus has spread rapidly throughout the world (3, 4), threatening public health and the world economy. Globally, as of December 25 in 2021, more than 278 million confirmed cases of COVID-19, including more than 5 million deaths, have been reported to the World Health Organization (WHO) (5). According to the severity of the disease, COVID-19 is divided into different clinical classifications: asymptomatic, mild, moderate, severe, and critical symptoms (3, 6, 7). Of note, SARS-CoV-2 has high transmission efficiency for asymptomatic or mild cases (8). The clinical symptoms of COVID-19 include fever, sore throat, dry cough, pneumonia symptoms (with or without hypoxemia), and so on. In addition, the complications of patients with COVID-19, such as acute respiratory distress syndrome, sepsis, acute liver and kidney injury, multisystem inflammatory syndrome, autoimmune hemolytic anemia, and neurological complications, are also worthy of attention (9).

The epidemiological studies of COVID-19 have demonstrated that airborne transmission is the major mode in the spread of SARS-CoV-2. Significantly, fecal–oral transmission and transplacental transmission are possible (10–12). On the other hand, SARS-CoV-2 can spread between humans and animals (13). Recent studies have suggested that severe COVID-19 is not limited to the elderly; children and young adults are also at risk (14, 15). Furthermore, the clinical characteristics vary with age. Altogether, SARS-CoV-2 has high infectivity, pathogenicity, and mutability, making the prevention and control of COVID-19 difficult.

Vaccination is one of the most effective and economical ways to prevent and control infectious diseases. It is essential to develop safe and effective vaccines for implementing mass vaccination to prevent and control the COVID-19 pandemic. In recent years, mRNA vaccines have been studied extensively for the prophylaxis and control of infectious diseases (16–18). Compared with other vaccines like whole bacteria, subunit, and DNA vaccine, the mRNA vaccine can induce T-cell and B-cell immune response and is non-integrating and naturally degradable (19–22). In addition, the fast and simple production procedures, free of eggs and cells, make the mRNA vaccine a promising and attractive vaccine candidate that potentially fills the gap between the emerging epidemic and the urgent need for effective vaccines (23). So far, it has been demonstrated that BNT162b2 (BioNTech/Pfizer) and mRNA-1273 (Moderna), granted authorization for emergency use, are generally safe and efficacious to prevent COVID-19 (24–26).

Worldwide, despite the measures taken to control COVID-19 disease spread, infections still occur, suggesting that more effective vaccines will be an immediate need to end the pandemic. Here, we focus on the current challenges of the mRNA vaccine against SARS-CoV-2 and discuss possible countermeasures to contain the continuing spread of the disease.

2 CHALLENGES

With the continued global epidemic, the COVID-19 mRNA vaccines still face several important challenges: SARS-CoV-2 variants, protective immunity, immune evasion, vaccinated population, and adverse reactions after vaccination. Undoubtedly, more in-depth knowledge on these challenges will contribute to the design of safer and more effective mRNA vaccines in the future.

2.1 Variants

Generally, the RNA virus has a high mutation rate. In addition, all viruses, including SARS-CoV-2, may change over time (27, 28). Most changes have little impact on the properties of the virus. However, some changes may affect the properties of the virus reported to the WHO, such as transmission capacity, related disease severity, or the performance of vaccines, therapeutic medicines, diagnostic tools, and so on (29). For SARS-CoV-2, multiple selective pressure may develop novel variants. It is considered that the most likely selective pressure is to increase the inherent adaptability of the virus by directly replicating within the host or spreading between the hosts (30, 31). In addition, variants may include mutations that change interactions with key host components (32). Of note, selective pressure can induce mutations that permit variants to escape from adaptive immune responses (30).

2.1.1 Antibody Neutralization and Immune Evasion

In the changing global SARS-CoV-2 pandemic context, several variants including B.1.1.7 (Alpha), B.1.351 (Beta), B.1.617.2 (Delta), etc. showed different sequence variations and amino acid sequence changes of the spike protein, which has been reported and has aroused wide concern due to their widespread transmission and possible immune evasion (33).

For the Lambda variant, the receptor-binding domain (RBD) mutation can weaken the ability to recognize broadly neutralizing antibodies. Mutations of T76I and L452Q induce higher infectiousness (34). Besides, the 7-amino-acid deletion within the N-termini domain outside the RBD domain leads to immune evasion. Notably, antibodies from Pfizer-BioNTech vaccinated individuals drop about 3.5-fold for the Delta variant after 6 months (35). The Delta variants have the characteristics of several spike protein mutations, which may affect immune responses and vaccine potency, including T19R, Δ157-158, L452R, D950N, T478K, P681R, and D614G (36, 37). Significantly, SARS-CoV-2 strains with mutations at P681R may enhance viral replication, resulting in higher viral loads and increased spread (38). Also, laboratory data suggested that D614G substitution, which is prevalent among SARS-CoV-2 strains, improves the competitive fitness, infectivity, and transmission of the strains in primary human cells and animal models (31, 39). Besides, the reorganized receptor-binding interface of the Delta variant attenuates interactions with some neutralizing antibodies, resulting in immune evasion (36).

As an important strategy to control the COVID-19 pandemic, mRNA vaccination remains effective in preventing symptomatic and severe COVID-19 associated with infection from several variants. However, whether the effectiveness of mRNA vaccines will be reduced due to variants is still a concern. Using a TNCC (a test negative case control) analysis, the estimated vaccine effectiveness against symptomatic disease with Delta infection for two doses of BNT162b2 is approximately 88% (95% CI, 85.3 to 90.1) (40). Due to concerns about thrombotic events after vaccination with ChAdOx1 nCov-19, heterologous mRNA boost strategies have been recommended and implemented (41, 42). Laboratory data have indicated that heterologous BNT162b2 boost after ChAdOx1 nCov-19 consistently leads to higher neutralizing titers against the Alpha, Gamma, and Beta variants compared with homologous BNT162b2 vaccination. Interestingly, homologous BNT162b2 prime boost seems to be more efficient in generating neutralizing antibodies against the Delta variant (43, 44). Similarly, it has been reported that an mRNA-1273 boost can induce the production of antibodies that neutralize the B.1.351 variant. In addition, mRNA-1273 vaccine can stimulate the SARS-CoV-2-specific memory B cell produced by the first dose of ChAdOx1 nCov-19 vaccine. Compared with a ChAdOx1 nCov-19 boost, an mRNA-1273 boost may provide better immune protection against the B.1.351 variant (45).

2.1.2 Vaccine Breakthrough Infections

The major factor of the vaccine breakthrough infections of variants may be obtaining new antigenic properties to circumvent the recognition of broadly neutralizing antibodies generated from vaccination, which may be enhanced by the weakening of immune protection of the vaccination over time (35). With the emergency and wide spread of variants and the occurrence of vaccine breakthrough infections, some studies have compared the protective efficiency of mRNA vaccines against SARS-CoV-2 variants of concern, assessing variant-

specific viral loads or neutralizing antibodies in cases of vaccine breakthrough infections, to prevent and control the global SARS-CoV-2 pandemic better (46, 47).

A case-to-case study, comparing the effectiveness of mRNA vaccines against Delta versus Alpha variants, has found higher infectivity with the Delta variant infections and significantly higher odds of vaccine breakthrough infections in Delta cases when compared with Alpha cases (47). Additionally, a multicenter retrospective cohort study of patients with B.1.617.2 infection showed that the odds of severe COVID-19 needing supplemental oxygen were significantly lower following vaccination compared with unvaccinated individuals in the vaccine breakthrough group (33). Interestingly, vaccinated and unvaccinated groups at diagnosis have similar PCR cycle threshold values, but viral loads declined faster in vaccinated patients.

Variants may cause adverse effects on protective efficacy of currently available mRNA vaccines. The emergence and transmission of variants present a grand challenge for the prevention and control of the SARS-CoV-2 pandemic *via* mRNA vaccination (48–50). Differences in the number of patients with variant infections, age, individuals receiving mRNA vaccines, follow-up of vaccinated individuals, sensitivity or specificity of PCR testing, and other aspects may have an impact on evaluating the effectiveness of mRNA vaccines (40). Therefore, study protocols and data collection assessing the effectiveness of mRNA vaccines should be carefully designed and implemented. Detecting the SARS-CoV-2 nucleic acid of patients and reviewing the existing variants on neutralizing antibodies are required to understand the transmissibility, infectivity, virulence, and immune escape of variants. Evaluating the effectiveness of current mRNA vaccines against SARS-CoV-2 will contribute to develop elaborate strategies for more effective mRNA vaccines against the COVID-19 pandemic.

2.2 Protective Immunity

Although much remains to be determined about immune-related factors that protect against SARS-CoV-2 infection, emerging data have demonstrated the importance of humoral and cellular immunity in terms of protection (51, 52). Therefore, it is important to ensure the development of effective mRNA COVID-19 vaccines.

Generally speaking, mRNA vaccines have the unique ability to induce the innate immune system to regulate antigen-specific immune responses. mRNA vaccines can be identified by MHC I and MHC II, which led to antigen-specific humoral and cellular immune responses (53). Meanwhile, it has adjuvant features which can timulate immune cells to secrete tumor necrosis factor-α (TNF-α), interferon-α (IFN-α), and other cytokines (CK) that activate the consuming adaptive immune responses (54). Besides, the mRNA vaccine produces high levels of virus-blocking antibodies, known as neutralizing antibodies (nAb), so many scientists believe that it is superior to other vaccines in preventing infection (55). However, the results of several mRNA candidate vaccine studies show that mRNA may stimulate excessive immune response, which will stimulate cells to secrete large amounts of interferon, thereby inhibiting the effect of mRNA translation and ultimately leading to immune response termination (56, 57). In addition, there is currently a lack of information on the protective life span caused by mRNA vaccines. Effective measurements of vaccines and definitive experimental descriptions of duration do not yet exist. Experience with other human coronaviruses has shown that reinfection is possible due to reduced antibody response. Special attention must be paid to mRNA potential problems, which will help us use mRNA more effectively (58, 59).

In the absence of data for humans, animal studies can help to identify potential correlates of protection. However, there is still a lack of animal models that can fully simulate human immune responses, and animal studies cannot fully predict efficacy in humans (60). The results of H10N8 and H7N9 mRNA vaccines showed that mRNA vaccines could stimulate the human immune response to produce higher neutralizing antibodies, but the animal immune response could only produce lower neutralizing antibodies (21), which proved this point of view. Moreover, since SARS-CoV-2 is a novel pathogen, any surrogate endpoints identified in animal studies would ideally need validation in clinical trials. Researchers need to continue to seek clinical evidence of the effectiveness of mRNA vaccines in human studies (24).

2.3 Vaccinated Population

As we all know, the immune system is related to multiple factors which obviously have roles both in innate and adaptive immunity. The monitoring of protective immunity in different immunized populations with vaccination is one of the most important factors in the effectiveness of vaccine. The physical conditions of the recipient as well as sex and age also affect the effectiveness of vaccine.

SARS-CoV-2 is extremely infectious. Everyone can be considered a susceptible group, and the infected individuals present a variety of symptoms (61). Some initial observations have indicated that adults are more likely to contact SARS-CoV-2 than children (62); however, limitation in testing availability in many countries during the pandemic has made it difficult to accurate quantify the true risk of infection in individuals. It is certain that patients with other clinical diseases may be more susceptible to infection (63). Recently, the WHO has shown that young people are becoming the main spreader of SARS-CoV-2, and people under 40 are more susceptible to infection, which will increase the risk of infection for the most vulnerable groups, especially the elderly and patients (64). Additional clinical trials are needed to better establish the infection rate of SARS-CoV-2.

Vaccines have made a huge contribution in preventing infection. It is crucial to understand the difference between the antibody response of different immune populations to vaccination and infection as soon as possible. Compared with traditional vaccines, there are unique advantages of the use of mRNA-based antiviral vaccines (65). The mRNA-based vaccines mRNA-1273 and BNT162b1 are two mRNA vaccines that are currently progressing rapidly. The phase 3 trial of mRNA-1273 (the fastest-growing mRNA vaccine currently in development, which stimulates the expression of a target antigen after vaccination) was launched in late July 2020. The trial was mainly for people who have no infection history at the age of 18 or older and included some persons with different racial and

ethnic backgrounds. The results have shown that the safety of the mRNA-1273 vaccine is 94.1% effective in preventing SARS-CoV-2 when an individual is completely vaccinated (25). Infection and the safety of mRNA-1273 are not affected by age (66). However, the trial did not precisely assess the immunogenicity of different populations, and pregnant women and children were not included in the trial. Additional evaluation of vaccines needs to be planned (25, 67). Consistent with mRNA-1273, the BNT162b2 vaccine showed 94% efficacy in preventing SARS-CoV-2 infection, while there is still controversy about the immunization effect in different populations (68, 69). Taken together, although the mRNA vaccine under development provides great hope for the prevention of COVID-19, there is still a lack of strong proof of the immune effect in different populations. More in-depth research should be conducted to understand the vaccination status in different people. Understanding the mechanisms involved in individual disparity in the effectiveness of mRNA vaccines will contribute to improve the development of mRNA vaccines.

2.4 Adverse Reactions

As of December 21, 2021, there are over 300 COVID-19 vaccine candidates being developed. Of these, at least 137 candidates derived from different platforms, including the whole-microbe approach (inactivated vaccine, live-attenuated vaccine, and viral vector vaccine), the subunit approach, and the genetic approach (nucleic acid vaccine), are currently in clinical development (70, 71). Like with any vaccine, mild to moderate side effects may emerge after being vaccinated against COVID-19 (72). It is a normal sign of the immune response of the body to the vaccine (73). However, severe side effects or adverse reactions could be experienced, causing fear and anxiety about vaccination in the population (72, 74). Therefore, evaluating safety is more conducive to the development and emergency use of COVID-19 vaccines.

Theoretically, mRNA vaccine has greater security compared with other types of vaccines, for instance, without infectious virus in the production process, a lower risk of virulence reversion, and insertional mutagenesis (75). Nevertheless, high-quality real-world safety data on mRNA-based COVID-19 vaccines are still relatively scarce in the literature. Common adverse reactions to mRNA-based COVID-19 vaccines include allergic reaction and some other side effects such as arm pain, fatigue, and a mild fever (**Table 1**). Also, serious adverse reactions following BNT162b2 mRNA COVID-19 vaccination including pericarditis, arrhythmias, deep vein thrombosis, pulmonary embolism, myocardial infarction, intracranial hemorrhage, and thrombocytopenia need more emphasis (82) (**Table 1**). A study demonstrated a possible association between Bell's palsy and vaccination with BNT162b2 and mRNA-1273 COVID-19 mRNA vaccine (82). Regarding other mRNA vaccines like CVnCov, Arct-021, and LNP-nCovsaRNA, the main adverse reactions are mild (**Table 1**). Clinical trials may be inherently limited in assessing vaccine safety because of the small number of participants and the inadequate representation of the sample. Hence, long-term and comprehensive monitoring of vaccine safety is required.

We found an interesting fact that adverse reactions appear within different time periods after injection of some vaccines by data analysis. What could be the reason? Allergic reactions caused by vaccination are usually lgE-mediated and occurred within the first 30 min after vaccination (83). Not all immediate reactions associated with vaccines are true allergic reactions (84). The allergic reactions are usually related to vaccine components, which include excipients, inactive ingredients, and liposomal delivery vehicles (84, 85). The possibility that differential composition may make adverse events happen at different temporal stages after vaccination still need to be confirmed. Besides, some studies suggest that the incidence of adverse reactions is associated with dosage (82, 86). Whether dosage has an impact on the occurrence of adverse reactions still need to be explored. Meanwhile, interindividual differences in innate immune system should not be disregarded (87).

It is noteworthy that some differences in the safety of vaccines from various platforms have been recognized. An observational study assessed cases of cerebral vein thrombosis and attributed the rates to four COVID-19 vaccines: tozinameran (Pfizer-BioNTech, mRNA vaccine), CX-024414 (Moderna, mRNA vaccine), ChAdOx1 nCov-19 (AstraZeneca, chimpanzee adenoviral vector vaccine), and AD26.COV2.S (Janssen, adenoviral vector vaccine). It has been found that cases of cerebral vein thrombosis events (with or without thrombocytopenia) were observed for all these four vaccines. Furthermore, compared with adenoviral vaccines, a lower reporting rate of thrombocytopenia and unusual site thrombosis adverse drug reaction was observed for the mRNA vaccine (88). Moreover, a systematic review shows that adverse events associated with serious metabolic, immune system, musculoskeletal, and renal disorders were seen more often with inactivated vaccine recipients; the occurrence of serious gastrointestinal complications and infections was more frequent among viral vector and inactivated vaccine recipients than mRNA vaccines; and serious vessel disorders were observed more in mRNA vaccine recipients (89). Research has found that the common adverse events of ZF2001 (Longcom, a recombinant protein subunit vaccine) were mild or moderate, including injection site pain, swelling, redness, fever, and fatigue. The incidence of fever and fatigue was lower among ZF2001 vaccine recipients than mRNA-based vaccines or adenovirus-vectored vaccines (90). Similarly, the first-in-human study of ZyCoV-D (Cadila Healthcare Limited, DNA vaccine) shows that adverse reactions following vaccination including systemic symptoms (headache, fever, fatigue, nausea, vomiting, diarrhea, arthralgia, and muscle pain) and local reactions (injection site pain and pruritus) were mild to moderate in severity (91).

However, these evaluations of the safety profile of COVID-19 vaccines based on different platforms may have several limitations which are related to sample size, comparator group, follow-up time, age, gender, and ethnic group of vaccinated persons. Further exploring the exact associations between vaccination and associated adverse events will lead to a greater and more comprehensive safety assessment of COVID-19 vaccine candidates. Understanding how serious or life-threatening adverse events could be is helpful to avoid the potential risk of vaccination for patients with related diseases.

TABLE 1 | The common adverse reactions of mRNA vaccine candidates in clinical trials.

Vaccine name	BNT162b2 (26, 69)	mRNA-1273 (25, 76)	CVnCoV (77, 78)	ARCT-021 (79)	LNP-nCoVsaRNA (80)	ChulaCov19 (81)
Phase	III/IV	IV	III	II	I	I
Developers	BioNTech/Fosun Pharma/ Pfizer	Moderna/NIAID	CureVac AG	Arcturus/Duke-NUS	Imperial College London	Chulalongkorn University
Age at vaccination	≥16 years	≥18 years	18–60 years	20–80 years	18–75 years	18–75 years
Route of administration	IM	IM	IM	IM	IM	IM
Number of doses	2	2	2	2	2	2
Duration	The second injection was given after 28 days	The second injection was given after 28 days	The second injection was given after 28 days	–	The second injection was given after 28 days	The second injection was given after 21 days
Time of adverse reaction	Mostly start about 15 min	Mostly start about 15 h after vaccination	Mostly start about 24 h after immunization	–	–	–
Adverse reaction	Right axillary lymphadenopathy; paroxysmal ventricular arrhythmia; right leg paresthesia	Pneumonia; immediate systemic allergic reactions	Moderate headache; injection site pain; moderate headache; transient lymphopenia	ARCT-021 was generally well tolerated; most adverse reactions are mild	Purpura on the skin; soreness of the arms; blockage of a vein; small nerve injury	Pain; tenderness; induration/swelling; ulceration; scabs; hypersensitivity
	The injection site: mild-to-moderate injection site pain Respiratory system: cough; shortness of breath Digestive system: decrease appetite; nausea, vomiting; diarrhea Nervous system: fatigue; lethargy; paralysis; headache; dizziness Systemic reaction: asthenia, malaise Skin: night sweats, hyperhidrosis					

Phase: a stage in the development of mRNA vaccine clinical trials.
IM, intramuscular injection.

3 POSSIBLE STRATEGIES

Since the outbreak of the COVID-19 pandemic, scientists around the world have been together seeking for a longer-term vaccine solution in order to reduce the risk of SARS-CoV-2 transmission as well as lower COVID-19 morbidity and mortality. Despite significant progress and promising results being gained, the current COVID-19 mRNA vaccine still faces the above intractable challenges. Significantly, the selection of antigen and routes of administration, influencing vaccine efficacy and security, should be taken into account when finding some effective vaccine solution strategies.

3.1 Antigen Selection

The core principle of mRNA vaccine is to deliver the mRNA sequence encoding target antigen into the host cell cytoplasm and translate the corresponding antigen by using the host cell machinery, thereby inducing immune responses for the prevention or treatment of disease (85, 92). Some features of antigen, such as immunogenicity and specificity, have an impact on the effectiveness of the mRNA vaccine (18). Therefore, the selection of antigens plays an essential role in designing and developing an mRNA vaccine.

As one of the major structural proteins of SARS-CoV-2, the S (spike) protein plays a crucial role in virus entry and infection and contains several B-cell and T-cell epitopes, which can trigger neutralizing antibodies and immune protection (93, 94). Thus, the S protein is considered a dominant antigen candidate of mRNA vaccine against COVID-19 (95). Both the mRNA-1273 vaccine and BNT162b2 encode the prefusion-stabilized full-length spike protein of SARS-CoV-2 and contribute to prevent and control SARS-CoV-2 and its variant infectiona (25, 96). A study (97) established three mRNA vaccine candidates encoding different antigens for COVID-19, showing that only RQ3013-VLP (encodes the S, M, and E proteins to form SARS-CoV-2 virus-like particles) induced humoral and T-cell immune responses in mice, for RQ3012-Spike (encodes the full-length wild-type S) and RQ3013-VLP contain the same amount of S mRNA. Notably, RQ3011-RBD (2 μg RNA/dose, RNA encoding the RBD of the S glycoprotein (residues 331–524) of SARS-CoV-2 with both an N-terminal signal peptide and a C-terminal membrane-anchoring helix) failed to elicit sufficient immunity in mice. However, some improvements may strengthen the immunogenicity of RBD-based mRNA vaccine. Sun et al. (98) found that TF-RBD, an mRNA vaccine based on the trimeric RBD fused to ferritin-formed nanoparticles, induced a stronger

humoral immunity response and produced RBD-specific antibodies and neutralizing antibodies as well as Th1-biased cellular response in mice, when compared with T-RBD (an mRNA vaccine based on the trimeric RBD). Furthermore, the mRNA vaccine has a high flexibility, because mRNA(s) encoding single or multiple antigens can be co-delivered to enhance and broaden immune responses (99, 100). The TF-RBD multivalent vaccine targeting SARS-CoV-2 variants (B.1.1.7 and B.1.351) can elicit a broad spectrum of neutralizing antibodies (98). The TF-RBD mRNA vaccine strategy contributes to establish multivalent vaccines against SARS-CoV-2 mutations. Besides the S protein and RBD, other antigens, containing the S1 subunit and N-terminal domain of the S protein, are used in the studies of immunogenicity as antigen candidates for vaccine against COVID-19 (93, 101, 102). However, whether these antigen candidates are suitable for use in mRNA vaccine for preventing and controlling COVID-19 still needs to be evaluated.

3.2 Mucosal Vaccination

So far, most of the vaccine candidates, including mRNA vaccine in clinical phase against COVID-19, can select intramuscular administration (IM) to induce both humoral and cellular immune responses for preventing and controlling COVID-19 (103). However, IM seems to only prevent lower respiratory tract infections but fails to elicit sterilizing immunity in the upper airway, because it induces a strong serum IgG reflex but does not elicit epithelial cell IgA responses (58, 104). Generally, the ideal vaccine against SARS-CoV-2 infection through mucosal transmission should be able to elicit not only systemic but also mucosal immune responses (105, 106). Recent research suggested that intranasal administration (IN) can elicit high neutralizing antibody generation, mucosal IgA, and T-cell responses to avoid SARS-CoV-2 infection (107). Besides, Du et al. (108) have found that IN shows an excellent profile in inducing mucosal and humoral immune responses in mice, through comparing the immunological potency induced by IN, IM, and ID (intradermal) administration with an RBD-based SARS-CoV-2 vaccine. Furthermore, IN vaccination has some other unique strengths, including non-invasiveness, easy administration, and self-administration, which is a more cost-effective and efficient way of administration during the COVID-19 pandemic. Interestingly, the self-assembling nanocomplex formulated with cationic cyclodextrin-polyethylenimine 2k conjugate (CP 2k) is a safe and effective delivery vector for intranasal mRNA vaccine, which can overcome the nasal epithelial barrier and enhance the intranasal and paracellular delivery of mRNA encoding antigen and induce strong mucosal and systemic immune responses (109). Thus, IN administration may be an advantageous immunization route of COVID-19 mRNA vaccine.

4 CONCLUSION

Over the past several years, it has become clear that mRNA-based vaccines have promising prophylactic applications. The ongoing mRNA vaccines against SARS-CoV-2 play an important role in maintaining public health, being the most recent example of critically important advancements in the field of mRNA vaccines. Some potential risks, such as the emergence of novel SARS-CoV-2 variants, increased immune evasion, and serious adverse effects, may have an impact on the effectiveness or promotion of current available COVID-19 mRNA vaccines. It is necessary to further improve mRNA-based vaccines. In addition to choosing the dominant antigen, modifying nucleosides and optimizing sequences are also possible approaches to strengthen protein translation expression and immunogenicity. Vaccines targeting multivalent antigens and combined vaccines may be used to strengthen protective immunity. The mRNA-based vaccine combined with other vaccines will most likely be able to deal with COVID-19 mutations. Nevertheless, this would increase the complexity of the vaccine and any changes to these parameters may have implications on vaccine production and the interaction of vaccines may maximize adverse events after administration. Additional research will need to define the variation trend of SARS-CoV-2 and prepare for long-term coexistence of SARS-CoV-2. Furthermore, the optimal immunization route should be selected to enhance the protective efficiency of mRNA vaccine. Besides intramuscular, intranasal immunization might also be advantageous for inducing immune response. Taken together, prior work involving mRNA-based vaccines, together with current studies of COVID-19 mRNA vaccines, provides evidence for the viability of this novel vaccine modality. There is still a lack of effective data to show the duration of mRNA vaccines under the changing epidemic situation, and a more complete understanding of COVID-19 mRNA vaccine protection still needs to be pursued. This review is focused on the challenges and possible development strategies of mRNA vaccine against SARS-CoV-2 and its variants, to provide a theoretical basis for preventing and controlling the COVID-19 pandemic. In summary, efficacy, security, production capacity, and costs need to be carefully evaluated so as to determine whether these strategies for improving mRNA vaccine are feasible and effective.

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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Seroreactivity of the Severe Acute Respiratory Syndrome Coronavirus 2 Recombinant S Protein, Receptor-Binding Domain, and Its Receptor-Binding Motif in COVID-19 Patients and Their Cross-Reactivity With Pre-COVID-19 Samples From Malaria-Endemic Areas

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Despite the global interest and the unprecedented number of scientific studies triggered by the COVID-19 pandemic, few data are available from developing and low-income countries. In these regions, communities live under the threat of various transmissible diseases aside from COVID-19, including malaria. This study aims to determine the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) seroreactivity of antibodies from COVID-19 and pre-COVID-19 samples of individuals in Mali (West Africa). Blood samples from COVID-19 patients (n = 266) at Bamako Dermatology Hospital (HDB) and pre-COVID-19 donors (n = 283) from a previous malaria survey conducted in Dangassa village were tested by ELISA to assess IgG antibodies specific to the full-length spike (S) protein, the receptor-binding domain (RBD), and the receptor-binding motif ($RBM_{436–507}$). Study participants were categorized by age, gender, treatment duration for COVID-19, and

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comorbidities. In addition, the cross-seroreactivity of samples from pre-COVID-19, malaria-positive patients against the three antigens was assessed. Recognition of the SARS-CoV-2 proteins by sera from COVID-19 patients was 80.5% for S, 71.1% for RBD, and 31.9% for RBM ($p < 0.001$). While antibody responses to S and RBD tended to be age-dependent, responses to RBM were not. Responses were not gender-dependent for any of the antigens. Higher antibody levels to S, RBD, and RBM at hospital entry were associated with shorter treatment durations, particularly for RBD ($p < 0.01$). In contrast, higher body weights negatively influenced the anti-S antibody response, and asthma and diabetes weakened the anti-RBM antibody responses. Although lower, a significant cross-reactive antibody response to S (21.9%), RBD (6.7%), and RBM (8.8%) was detected in the pre-COVID-19 and malaria samples. Cross-reactive antibody responses to RBM were mostly associated ($p < 0.01$) with the absence of current *Plasmodium falciparum* infection, warranting further study.

Keywords: SARS-CoV-2 S protein, seroreactivity, COVID-19 samples, cross-reactivity, Pre-COVID-19 samples, malaria endemic-area

INTRODUCTION

Coronaviruses are a group of enveloped viruses containing a single-stranded RNA genome with positive polarity (1). They include severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome (MERS-CoV) (1–3), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or COVID-19 (4, 5). COVID-19 affects people of all ages, but morbidity and mortality are more significant in the elderly and those with chronic diseases (6–9). Emerging in China in 2019 (10), COVID-19 rapidly spread worldwide and was declared a pandemic by the WHO in March 2020[1,2]. More than 200 million cases and over 4 million deaths have been reported worldwide, affecting 220 countries and territories[3], generating massive economic and social consequences. The first COVID-19 case diagnosed in Mali was reported on March 25, 2020, and Malian health authorities quickly established a strategy to control the disease[4]. In addition, the authorities have promoted the harmonization of research activities by leveraging research laboratory capacities and strengthening relationships among local and international stakeholders (11–13).

Despite considerable global efforts to study the immune responses elicited by SARS-CoV-2 and their role in clinical protection and pathogenesis (14–17), the host factors leading to low or moderate clinical manifestations, as well as completely asymptomatic infections, are not well understood. Initial analysis indicates that certain populations have been exposed to other microorganisms, either pathogenic or non-pathogenic, which appear to induce immune responses against COVID-19 (i.e., antibodies or potentially other immune effectors that contribute to reducing or preventing COVID-19 clinical manifestations (18–25)).

Specific antibody responses to COVID-19 have been reported in moderately and severely symptomatic SARS-CoV-2-positive individuals (26–32). However, there are few data available linking symptomatic disease and duration of hospitalization or treatment with specific antibodies to SARS-CoV-2 antigens. Such antibodies may be detected as early as the end of the first week of illness; however, they may also take weeks to appear, giving rise to different clinical outcomes (29, 33). In addition, the presence or absence of protective immunity due to infection or vaccination may affect future transmission and disease severity (29).

Of notable importance, it has been observed that there are significantly lower COVID-19 clinical cases and fatalities in malaria-endemic regions than in non-endemic areas (19, 22, 34). Several host factors, including sociodemographic conditions, genetic background, and immune status, could be influencing the COVID-19 clinical evolution. Moreover, other SARS cases, induced by viruses potentially sharing common immunodominant antigens, might affect the outcome of the disease (18, 20, 35, 36).

Considering the burden of malaria in Mali (37) and the potential for clinical overlap with COVID-19, efforts to both study diseases and understand the potential immunological interplay are ongoing (19, 22). This potential relationship has tremendous epidemiological relevance not only for understanding clinical outcomes in malaria-endemic and non-endemic regions but also for COVID-19 vaccination efforts. In the absence of a specific anti-SARS-CoV-2 treatment, research into this area is of considerable importance.

[1] World Health Organization: October 22, 2020|COVID-19: Surveillance, case investigation and epidemiological protocols. https://www.who.int/publications/i/item/considerations-in-the-investigation-of-cases-and-clusters-of-covid-19: [Accessed on October 12, 2021].

[2] World Health Organization: March 19, 2020| COVID-19: Infection prevention and control / WASH. https://www.who.int/publications/i/item/10665-331495. [Accessed on October 12, 2021]

[3] World Health Organization: Weekly epidemiological update—February 9, 2021. https://www.who.int/publications/m/item/weekly-epidemiological-update—9-february-2021: [Accessed on December 12, 2021].

[4] Institut National de Prévoyance Sociale: Rapport de situation COVID-19 au Mali, 10 Janvier 2021 / 04 au 10 Janvier 2021 / N°136. https://covid19-ml.org/: [Accessed January 11, 2021].

The spike (S) protein is encoded by a systematic interplay between the SARS-CoV-2 genome, the nucleocapsid (N), the membrane (M), the envelope (E), and various additional structural proteins. It plays a crucial role in viral infection and pathogenesis of COVID-19 (38, 39), as it is essential for the viral invasion of the host cell, mainly through its RBD domain (5, 9, 37). Both RBD and its ligand, the human angiotensin-converting enzyme-2 (ACE2), are crucial research targets for developing COVID-19 therapeutic antibodies, vaccines, and serological tests (2, 40–45). Currently, most COVID-19 vaccines in use or development are based on the S protein; however, the different vaccine platforms have demonstrated a variety of strengths and weaknesses[5]. In addition to the commonly used S protein and its RBD, we designed (manuscript submitted) and studied the S protein's receptor binding motif ($RBM_{436–507}$) that interacts with ACE2.

Vaccine success is likely associated with the specificity and strength of the immune response it triggers against the S protein, specifically against its RBD. However, this immune response may also correlate with factors like age, gender, ethnicity, disease experience (i.e., disease evolution), treatment duration, and comorbidities, among others (6–8).

In light of all these issues, this study aimed to assess the natural antibody response specific to the full-length S protein, its functional domains RBD (protein), and RBM (peptide) using plasma collected from COVID-19-positive patients and pre-COVID-19 participants from a malaria-endemic region. The epidemiological paradox observed in COVID-19 and malaria patients in the initial phase, and in the dynamics of infection in malaria-endemic countries (19, 22), promotes the need for further studies in this area to produce a better understanding of the genetic and immunological factors involved.

METHODS

Study Type, Periods, and Sites

A cross-sectional study was conducted to assess the seroreactivity of COVID-19 patients and pre-COVID-19 donors against the SARS-CoV-2 full-length recombinant S protein and its binding domains RBD and RBM. Samples were collected from the Dermatology Hospital of Bamako (HDB) in Mali (West Africa); sociodemographic and epidemiological surveys were also carried out. While all COVID-19 blood samples were collected from patients confirmed to harbor SARS-CoV-2 by RT-PCR test, pre-COVID-19 plasma samples were gathered in 2019—before the onset of the COVID-19 pandemic—and therefore were not tested by COVID-19 RT-PCR. The latter were collected from donors living in the Village of Dangassa in Mali, a malaria-endemic zone, and were stored frozen at −20°C. All laboratory tests were performed at the Laboratory of Immunogenetic and Parasitology, at the International Centre of Excellence in Research (ICER-Mali) of the University of Sciences, Techniques and Technologies of Bamako (Mali). The data management and sample processing were carried out from May 2021 to September 2021.

Study Population

The study population included COVID-19-infected patients (n = 266; sex *ratio = 1.2 in favor* of men) with SARS-CoV-2 confirmed by RT-PCR and admitted to the HDB for inpatient care. The pre-COVID-19 population consisted of volunteers (n = 283; sex *ratio = 1.1 in favor* of women) who had participated in a previous malaria survey study in 2019, before the onset of COVID-19 in Mali. The study population (COVID-19 and pre-COVID-19 participants) were stratified by age groups 1–4, 5–9, 10–14, 15–19, 20–29, 30–39, 40–49, 50–59, 60–69, and 70+ years. This adjusted for the age structure of the population as recommended by the WHO guidelines on population-based sero-surveys of SARS-CoV-2 infection[6]. COVID-19 participants provided sociodemographic and epidemiological data, including comorbidities and length of treatment duration. Pre-COVID-19 participants had records of sociodemographic and epidemiological data, and current *Plasmodium falciparum* infection (parasitemia) was confirmed by microscopic examination after Giemsa staining of blood smear (BS) slides. None of the participants had a history of COVID-19 vaccination.

Ethical Considerations

This study was approved by the Institutional Review Board (Ethics Committee, EC) of the Faculties of Medicine and Odontostomatology and of the Pharmacy of Bamako (with reference N°2021/25/CE/USTTB). Written informed consent (IC) was obtained from each COVID-19 patient for the collection of blood samples, sociodemographic information, and clinical data for future investigative purposes. The authorization of the use of pre-COVID-19 samples and data was also obtained from the same EC and under the reference cited above. The current study was based on available data from participants whose plasma samples and related data were available and accessible. The confidentiality of the participants' data was preserved throughout this study.

Variables, Data, and Sample Collections

Data analysis was carried out using medical records from the HDB data register. Data were collected at the time of hospital admission (on week 1) and during hospitalization at HDB in 2020. Data were collected using a paper questionnaire developed for this purpose, including 1) sociodemographic information; 2) symptoms and severity of disease; 3) comorbidities or factors such as diabetes, hypertension, asthma, and body weight; 4) clinical evolution of the disease's form; and 5) duration of hospital stay or treatment. The pre-COVID-19 participant samples were collected from the village of Dangassa in 2019 before the onset of COVID-19 in Mali. The variables in the pre-COVID-19 group included sociodemographic (age and gender) and epidemiological data such as the presence

[5] World Health Organization: COVID-19 vaccines. https://www.who.int/emergencies/diseases/novel-coronavirus-2019/covid-19-vaccines: [Accessed on September 11, 2021].

[6] Population-based age-stratified seroepidemiological investigation protocol for coronavirus 2019 (COVID-19) infection, May 26, 2020, version 2.0. https://apps.who.int/iris/handle/10665/332188: [Accessed on September 29, 2021].

and density of current *P. falciparum* infection. A BS slide was performed and examined by microscopy for the presence and density of *P. falciparum* [positive (BS+) or negative (BS–) for each pre-COVID-19 sample].

Whole blood (5–10 ml) was collected from each COVID-19 patient by venipuncture upon admission to HDB, and the sample transportation to the laboratory was carried out following the WHO guidelines for Infectious Substances 2019–2020 (46). Trained biologists were responsible for ensuring compliance with these guidelines.

Protein Sequence Analysis, Design, and Antigen Production

Sequences of the S protein were downloaded from the National Center for Biotechnology Information (NCBI) SARS-CoV-2 Resources[7]. Recombinant proteins from the full-length S and RBD were provided by ExcellGene SA (Monthey, Switzerland) and Protein Production and Structure Core Facility, EPFL (Lausanne, Switzerland)[8]. Proteins were produced according to the manufacturer's recommendations[9]. A peptide covering the receptor-binding interface (receptor binding motif, $RBM_{436–507}$) of the S protein was synthesized at the Chemistry Department, Florence University, Florence, Italy. RBM is known to undergo some post-translational modifications (PTMs) such as glycosylation, but this does not directly contribute to the binding affinity between SARS-CoV-2 S and ACE-2 (47). In addition, as it is a synthetic product used in ELISA, RBM is not expected to undergo any further modification. The 3D images were generated using PyMol software, an open-source molecular graphics tool (48) using the atomic coordinates from PDB entry 6ZOY (49). The illustrative diagram of domains, amino acid sequences, and the 3D structure of the S protein displaying both the RBD and RBM sequences are all shown in **Supplementary Figure 1**.

Enzyme-Linked Immunosorbent Assay

Sample seroreactivity was studied using an ELISA with 96-well plates (type of plate, Ref 442404). Plates were coated with 1 µg/ml of S, RBD, or RBM (antigen coating) or not coated with an antigen (non-antigen coating) and then incubated overnight (O/N) at 4°C. The plates were then blocked for 1 h at room temperature (RT) with phosphate-buffered saline (PBS) 1× (3% milk) before being incubated for 2 h at RT with COVID-19 and pre-COVID-19 plasma samples at a dilution of 1:100. Goat anti-human IgGs, conjugated to horseradish peroxidase (HRP), were used as secondary antibodies, diluted to 1:5,000 (Life Technologies, Carlsbad, CA, USA; Ref H10307), and incubated for 1 h at RT. Signals were revealed using TMB substrate reagent (BD OptEIA, cat 555214; BD Biosciences, San Jose, CA, USA) for 20 min in the dark at RT, and the reaction was stopped using 1 M of sulfuric acid (Merck, Darmstadt, Germany; 1.00731.1000). Optical density (OD) was measured at 450/630 nm in a microplate ELISA-Reader (SoftMax®Pro Software). Samples were considered positive when their mean OD was ≥mean OD + 3SD of the negative control samples (indicated as the cutoff). The cross-reactivity of pre-COVID-19 samples was considered significant for the samples with a mean OD ≥ mean OD + 3SD of the negative controls with a dilution of 1:100 (indicated as the cutoff). Non-specific binding samples (i.e., samples with antibody responses in non-antigen-coated plates), were determined to be samples with an OD against non-coated plates greater or equal to the same sample's response against antigen-coated plates (i.e., responder sample).

Data Management and Statistical Analysis

Data from the coded questionnaires were directly entered into the electronic data entry system during data and sample collection. Each participant was assigned a number that was known only to the investigators. The information was entered in Excel 2013, and ELISA data were imported directly into Excel and associated with the participants' sociodemographic and epidemiological data. The analysis and generation of figures were done with Stata and Prism 5 software. The unpaired t-test, chi-squared test, and Fisher's exact test were used to compare groups with a significance threshold of 5%.

RESULTS

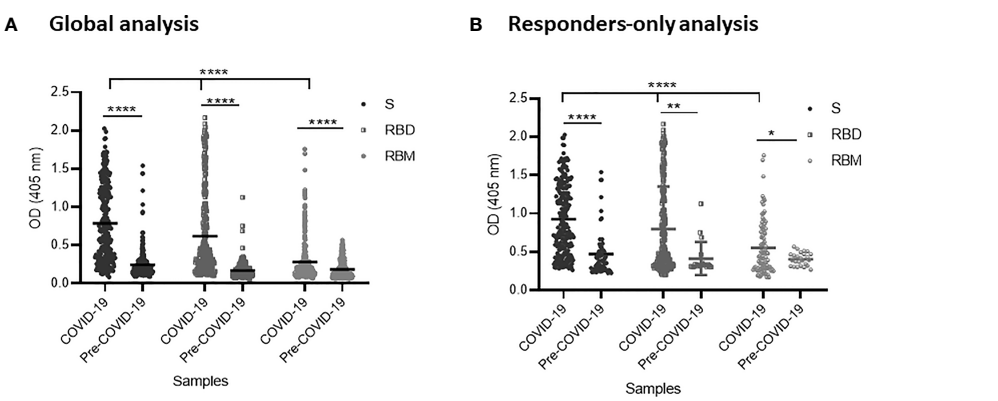
Sequences and 3D Structures of S Protein, and the Receptor-Binding Domain and Receptor-Binding Motif Domains

Three antigens, namely, the full-length S protein (1250 aa), its RBD (211 aa), and a synthetic peptide covering the binding interface (RBM; 72 aa) of RBD, were used in this study (**Supplementary Figure 1**). The S protein plays a crucial role in viral infection and pathogenesis, as it mediates the SARS-CoV-2 binding to human ACE2. It comprises two functional subunits: S1, which harbors the N-terminal domain (NTD) and the receptor-binding domain (RBD), responsible for binding to the host cell receptor; and the S2, which harbors the heptad repeat 1 (HR1) and 2 (HR2), responsible for the fusion of viral and cell membranes (39) (**Supplementary Figure 1A**). The full-length sequence of the S protein of SARS-CoV-2 was obtained using the BLASTP search program (50, 51). The SARS-CoV-2 RBD shows significant sequence homology (~73%) with seasonal phylogenetically related coronaviruses (25, 52–54) (**Supplementary Figure 1B**). The RBM is a segment representing approximately 6% of the S protein's length, located within the RBD domain. It is recognized by the ACE2 protein and not only represents the most variable region of the protein but is also highly specific to SARS-CoV-2 (**Supplementary Figure 1C**). The 3D image of the SARS-CoV-2 S protein structure was made while displaying the RBD and RBM locations (48, 49) (**Supplementary Figure 1D**).

Seroprevalence of Antibodies Against S, Receptor-Binding Domain, and Receptor-Binding Motif in COVID-19 Patients

Overall, all three antigens were well recognized by the COVID-19 samples but with significant variation among the S, RBD, and RBM antigens ($p < 0.0001$; **Figure 1A**). In terms of antibody

[7] https://www.ncbi.nlm.nih.gov/sars-cov-2/: [Accessed on September 30, 2021].
[8] https://www.epfl.ch/research/facilities/ptpsp/: [Accessed on September 29, 2021].
[9] https://www.excellgene.com: [Accessed on September 29, 2021].



Samples	S median OD (Q1; Q3)	RBD median OD (Q1; Q3)	RBM median OD (Q1; Q3)
COVID-19 (N=266)	0.685 (0.335; 1.217)	0.378 (0.225; 0.880)	0.177 (0.126; 0.277)
Pre-COVID-19 (N=283)	0.191 (0.150; 0.260)	0.1415 (0.110; 0.201)	0.139 (0.088; 0.266)

Samples	S responders median OD (Q1; Q3)	RBD responders median OD (Q1; Q3)	RBM responders median OD (Q1; Q3)
COVID-19	0.834 (0.509; 1.324)	0.5268 (0.340; 1.194)	0.436 (0.283; 0.773)
Pre-COVID-19	0.365 (0.276; 0.521)	0.324 (0.308; 0.3510)	0.391 (0.315; 0.467)

FIGURE 1 | Distribution of antibody responses against S, receptor-binding domain (RBD), and receptor-binding motif (RBM) antigens in COVID-19 and pre-COVID-19 samples. **(A)** Global analysis of samples (positive and negative in ELISA) shows that antibody (Ab) levels (mean OD shown as a horizontal black line in the dot plots) for S, RBD, and RBM were significantly higher in COVID-19 patient samples as compared to pre-COVID-19 donor samples ($p < 0.0001$). Also, the Ab levels varied significantly ($p < 0.0001$) among S, RBD, and RBM in COVID-19 samples. The table shows the median OD, Q1, and Q3 values of antibodies for S, RBD, and RBM in COVID-19 and pre-COVID-19 samples. **(B)** Levels of Ab responses in responder-only COVID-19 samples were significantly higher than in responder-only pre-COVID-19 samples (cross-reactive responders) for S ($p < 0.0001$), RBD ($p < 0.01$), and RBM ($p < 0.05$). The table shows the median OD, Q1, and Q3 of antibodies for S, RBD, and RBM of responder-only samples in COVID-19 and pre-COVID-19 participants. The unpaired t-test and ANOVA were performed to compare the mean ODs of antibodies between the two groups and within the groups themselves, respectively. *$p < 0.05$; ** $p < 0.01$; ****$p < 0.0001$; OD, optical density; Q1, quartile 1; Q3, quartile 3.

prevalence, of the 266 samples studied, 214 samples (80.5%) recognized S, 189 (71.1%) recognized RBD, and 85 (31.9%) recognized RBM (**Table 1**). In terms of antibody level, the S protein showed a two-fold higher antibody OD than RBD, which in turn showed a two-fold higher antibody OD than RBM; the median OD and interquartile 1 and 3 (Q1; Q3) were 0.685 (0.335; 1,217), 0.378 (0.225; 0.880), and 0.177 (0.126; 0.277), respectively (**Figure 1A**).

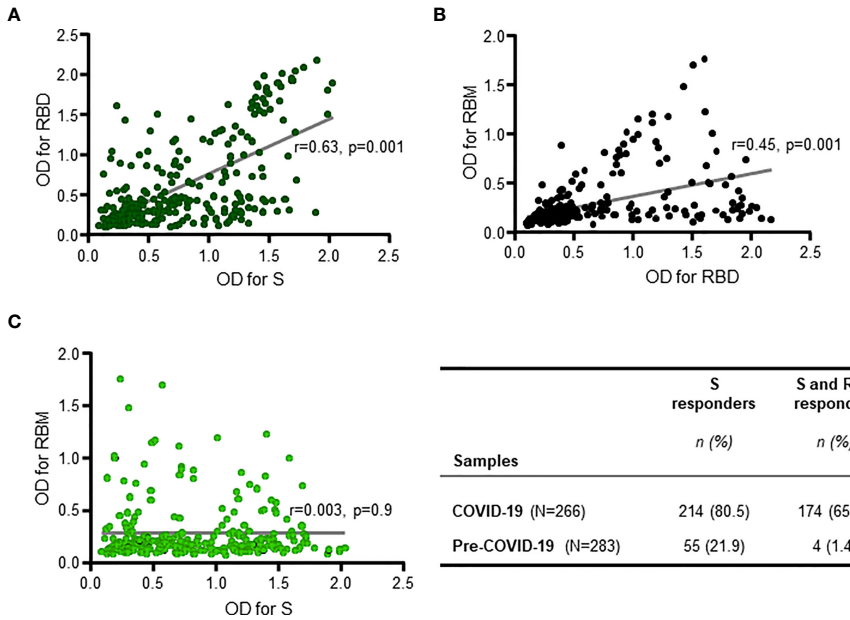
When only the reactive samples (responders) were assayed, the S protein showed a higher median OD for Q1 and Q3 [0.834 (0.509; 1.324)] than did RBD [0.5268 (0.340; 1.194)] or RBM [0.436 (0.283; 0.773)] (**Figure 1B**). While reactivity with S and RBD was observed in 65.5% (174/266), only 27.1% (72/266) of COVID-19 donors recognized all three antigens (**Figures 2A**–**C**). This reactivity would be relevant in selecting antibody donors and antigens for further analysis. The recognition of S correlated with recognition of RBD (r = 0.63, $p = 0.001$; **Figure 2A**), and recognition of RBD correlated with recognition of RBM (r = 0.45, $p = 0.001$; **Figure 2B**). In contrast, there was little correlation between the recognition of S and the recognition of RBM (r = 0.003, $p = 0.9$; **Figure 2C**). Although samples from pre-COVID-19 volunteers (n = 283) presented lower reactivity frequencies and ODs than the COVID-19 samples ($p < 0.05$; **Figures 1**, **2**; **Table 1**), they still displayed a significant level of cross-reactivity against the three antigens (see **Figure 3** and **Supplementary Figure 4**).

The analysis of IgG antibody levels by gender (male (M) and female (F)) in the COVID-19 patient group indicated comparable results between the two genders for each antigen

TABLE 1 | Frequency of responders against S, RBD, and RBM in COVID-19 and pre-COVID-19 donors.

Samples	S responder *n (%)*	RBD responder *n (%)*	RBM responder *n (%)*
COVID-19 (N = 266)	214 (80.5)	189 (71.1)	85 (31.9)
Pre-COVID-19 (N = 283)	62 (21.9)	19 (6.7)	25 (8.8)
p	**	**	**

The proportion of responder samples against S, RBD, and RBM was calculated using the samples showing an antibody mean OD ≥ mean OD + 3SD of the negative controls at the dilution 1:100 (indicated as the ELISA cutoff). Fisher's exact test was used to compare the proportion of responders between the COVID-19 and pre-COVID-19 groups. N, total number of samples; n, number of responder samples; %, percent of responder samples; RBD, receptor-binding domain; RBM, receptor-binding motif; OD, optical density.
***$p \leq 0.01$.*



Samples	S responders *n (%)*	S and RBD responders *n (%)*	S, RBD and RBM responders *n (%)*
COVID-19 (N=266)	214 (80.5)	174 (65.4))	72 (27.1)
Pre-COVID-19 (N=283)	55 (21.9)	4 (1.4)	1 (0.4)

FIGURE 2 | Positive responder samples from COVID-19 patients simultaneously recognizing two or all three antigens. There was a significant positive correlation between antibody responses (antibody optical density (OD)) against S and receptor-binding domain (RBD) (R = 0.63, p = 0.001 **(A)**), and between antibody responses against RBD and receptor-binding motif (RBM) ((R = 0.45, p = 0.001 **(B)**), but not for antibody responses against S and RBM (R = 0.003, p = 0.9 **(C)**). The two-sided Spearman's rank correlation test was used to determine p- and R-values. The gray lines are the lines of best fit for each scatter diagram. The table shows the number (n) and prevalence (%) of responder samples recognizing only S, or only S and RBD, or recognizing all three antigens simultaneously.

(**Supplementary Figures 2A–C**). In the COVID-19 patient group, the median OD (Q1; Q3) for M vs. F was 0.609 (0.333; 1.260) vs. 0.712 (0.339; 1.193), 0.371 (0.229; 0.873) vs. 0.390 (0.213; 0.887), and 0.174 (0.130; 0.251) vs. 0.186 (0.123; 0.300) for S, RBD, and RBM, respectively (**Supplementary Figures 2A–C**). The frequency of responders and antibody OD were both similar between M and F ($p > 0.05$) in both COVID-19 and pre-COVID-19 groups, except for the cross-reactive response to RBM (**Supplementary Figure 2C**) in the pre-COVID-19 group (**Table 2**). Furthermore, the non-specific binding of antibody samples in COVID-19 patients accounted for 8.9% (17 out of 189), and 14.1% (12 out of 85) of the seroreactive samples for S, RBD, and RBM, respectively (**Table 3**).

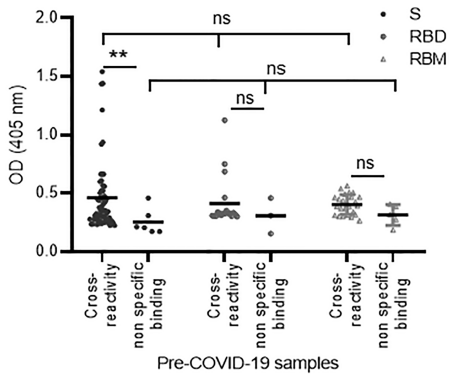
Overall, antibody levels increased as a function of age—particularly for S and RBD—but not for the RBM fragment (**Figure 4**). Furthermore, antibody levels to S and RBD were comparable at the earlier ages under 19 and above 59 years and were significantly greater than those against RBM.

Levels of Anti-S, Receptor-Binding Domain, and Receptor-Binding Motif Antibodies at Hospital Admission and Duration of Remission From the Symptomatic COVID-19

Here, we analyze the association between antibody levels toward S, RBD, and RBM at the time of hospital admission and duration of treatment (i.e., the remission of symptomatic forms). Duration of remission was thus defined as the estimated time in days (≤30 or >30 days) from hospital admission to recovery from symptomatic SARS-CoV-2 infections, as confirmed by at least two negative RT-PCRs. Overall, the duration of treatment was shorter for participants who had higher antibody levels at admission for all three antigens, especially for RBD ($p < 0.01$) (**Figure 5**). In addition, for the patient group with treatment periods ≤30 days, Ab levels for S, RBD, and RBM varied more significantly from each other ($p < 0.0001$) than among those hospitalized for longer periods ($p = 0.037$) (**Figure 5**). However, the proportion of responder samples for S, RBD, or RBM was comparable between the ≤30- and >30-day treatment groups (**Figure 5**).

Preexisting Comorbid Conditions and Elicitation of Anti-S, Receptor-Binding Domain, and Receptor-Binding Motif Antibodies Among COVID-19 Patients

Comorbidities such as diabetes, hypertension and asthma, and high body weight were evaluated as factors that may impact the effective development of antibodies against S, RBD, and RBM in COVID-19 patients. The antibody levels (mean OD) for S, RBD, and RBM were similar between the patient groups with and without arterial hypertension (AHT) and were slightly higher in the patient groups not suffering from diabetes or asthma



Antigens	Cross-reactivity in pre-COVID-19 samples (N=283)		Non-specific binding in pre-COVID-19 & cross-reactive samples	
	Frequency	*Median OD*	*Frequency*	*Median OD*
	n (%)	*(Q1; Q3)*	*n (%)*	*(Q1; Q3)*
S	62 (21.9)	0.347 (0.269; 0.521)	6 (9.6)	0.209 (0.171; 0.346)
RBD	19 (6.7)	0.324 (0.308; 0.351)	3 (15.8)	0.308 (0.154; 0.458)
RBM	25 (8.8)	0.391 (0.315; 0.467)	5 (20.0)	0.318 (0.231; 0.396)

FIGURE 3 | Cross-reactivity and non-specific binding against S, receptor-binding domain (RBD), and receptor-binding motif (RBM) in pre-COVID-19 and endemic malaria samples. The cross-reactive antibody levels (mean optical density (OD) shown as a horizontal black line in the dot plots) for S, RBD, and RBM were demonstrably higher than in non-specific binding antibody levels; this was significant for S ($p < 0.01$). The table shows the number and proportion (frequency) of samples showing cross-reactions or non-specific binding for S, RBD, and RBM. N, total number of pre-COVID-19 samples; n, number of cross-reactive or non-specific binding samples; %, percent of cross-reactive or non-specific binding samples; Q1, quartile 1; Q3, quartile 3. The unpaired t-test and ANOVA were used to compare mean antibody ODs between different groups and within the groups themselves, respectively. $^{**}p \leq 0.01$; ns, not significant.

TABLE 2 | Prevalence of antibody responders against S, RBD, and RBM according to gender in COVID-19 and pre-COVID-19 sample groups.

Samples	S responders			RBD responders			RBM responders		
	Male n (%)	*Female n (%)*	*p*	*Male n (%)*	*Female n (%)*	*p*	*Male n (%)*	*Female n (%)*	*p*
COVID-19 (N = 266)	116 (79.5)	98 (81.7)	*ns*	105 (71.9)	84 (70.0)	ns	44 (30.1)	41 (34.2)	ns
Pre-COVID-19 (N = 283)	32 (23.7)	30 (20.3)	ns	7 (5.2)	12 (8.1)	ns	7 (5.2)	18 (12.1)	*

The proportions of S, RBD, and RBM responders in COVID-19 samples as compared to pre-COVID-19 samples were determined. Fisher's exact test was used to compare the proportion of responders between COVID-19 and pre-COVID-19 samples.

N, total number of samples; n, number of responders; %, percentage of responders; RBD, receptor-binding domain; RBM, receptor-binding motif; ns, not significant.

$^{*}p \leq 0.05$.

(**Figures 6A**–**C**). Similarly, the prevalence of antibody responders for S and RBD remained similar between patient groups with or without comorbidity ($p > 0.05$; **Table 4**), whereas COVID-19 patient groups suffering from asthma and diabetes showed no positive antibody responses against RBM (**Table 4**). In addition, increasing body weight was associated with a significant decrease in antibody responses to S and a slight decline in antibody response to RBD (**Figure 6D**). The occurrence of two or more simultaneous comorbidities in a COVID-19 patient did not significantly impact the level of anti-S- and RBD-specific antibodies; however, there was no correlation between two comorbidities in COVID-19 patients and the response against RBM (**Supplementary Figures 3A**–**C**).

Level of Anti-S, Receptor-Binding Domain, and Receptor-Binding Motif Cross-Reacting Antibodies and Active Malaria Infection in the Pre-COVID-19 Malaria Infection Samples

The cross-reactivity of S, RBD, and RBM among the pre-COVID-19 samples from donors living in malaria-endemic areas (Dangassa village) was studied. The antibody OD

TABLE 3 | Proportion of non-specific binding antibodies against S, RBD, and RBM responders in COVID-19 patients.

COVID-19 samples (N = 266)				
Antigens	***Responders***		***Non-specific Ab binding from responders***	
	n (%)	***Median OD (Q1; Q3)***	***n (%)***	***Median OD (Q1; Q3)***
S	214 (80.5)	0.834 (0.509; 1.324)	19 (8.9)	0.664 (0.504; 0.781)
RBD	189 (71.1)	0.527 (0.340; 1.194)	17 (8.9)	0.728 (0.626; 0.884)
RBM	85 (31.9)	0.436 (0.283;0.773)	12 (14.1)	0.737 (0.642; 0.866)

The proportion of samples showing non-specific binding antibodies for S, RBD, and RBM was determined in COVID-19 patient samples. The non-specific binding antibody samples are those showing in no antigen-coating, i.e., in plates coated with no antigen, a mean OD of antibody ≥ mean OD in antigen coating. The median OD and interquartile (Q1 and Q3) are illustrated.

N, number of COVID-19 samples; n, number of responders or non-specific binding samples, %, the proportion of responders or non-specific binding samples; RBD, receptor-binding domain; RBM, receptor-binding motif.

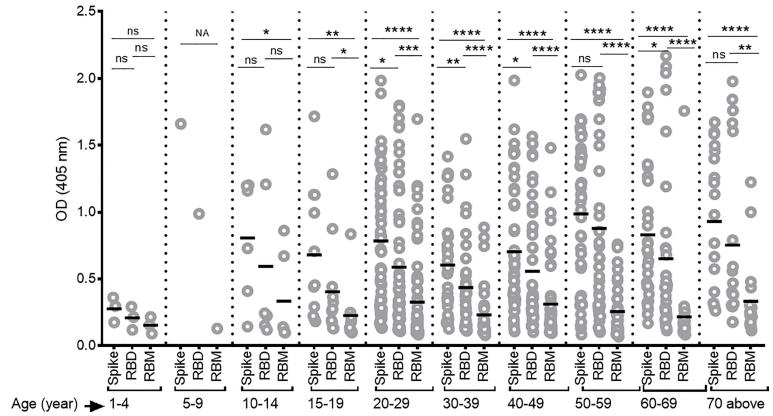
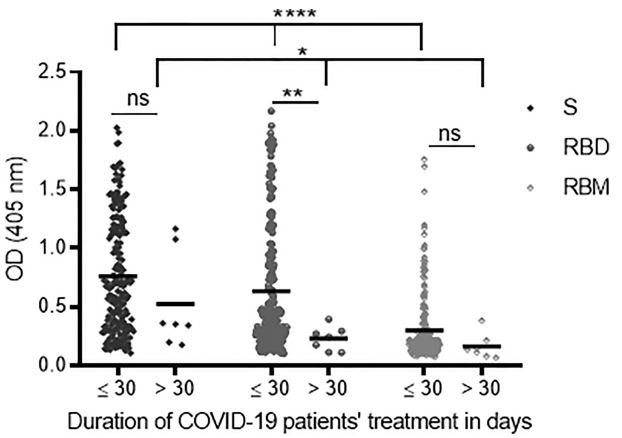


FIGURE 4 | Differing antibody responses against S, receptor-binding domain (RBD), and receptor-binding motif (RBM) according to different age groups of COVID-19 patients. Antibody responses against S, RBD, and RBM were studied for each age group of COVID-19 patients. A correlation was observed between increasing antibody levels and increasing age. The average Ab response (mean optical density (OD)) against each antigen was calculated for each age group. Comparisons were made using an unpaired t-test to study the difference in responses against each antigen within each age group. NA, not applicable; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. ns, not significant; Age (year), age ranges in years.

distribution was similar among the S, RBD, and RBM ($p > 0.05$; **Figure 3**) with respective median antibody ODs (Q1; Q3) of 0.347 (0.269; 0.521), 0.324 (0.308; 0.351), and 0.391 (0.315; 0.467). There was a higher frequency of cross-reactive samples for S (21.9%) than for RBD (6.7%) or RBM (8.8%) (**Figure 3**). In addition, cross-reactive antibodies against all three antigens were present in all age groups; however, they were higher for S and RBM in most age ranges than they were for RBD (**Supplementary Figure 4**). No significant correlation was found between the density of malarial parasitemia and the level of antibodies cross-reacting with S (r = 0.10 $p = 0.09$; **Figure 7A**), RBD (r = 0.06, $p = 0.35$; **Figure 7B**), or RBM (r = −0.07 $p = 0.27$; **Figure 7C**). In contrast, cross-reacting antibodies appeared to be more common in samples without parasitemia (i.e., without active *P. falciparum* infection, or BS− samples), representing 77.4% (42 out of 62), 100% (19 out of 19), and 88% (22 out of 25) of the cross-reactive samples against S, RBD, and RBM, respectively (**Figure 7D**). This correlation is made evident by the fact that BS− samples demonstrated significantly higher mean antibody ODs against RBM than BS+ samples (**Figure 7D**).

DISCUSSION

Despite the extraordinary breadth of scientific studies on COVID-19, limited data are available from regions where populations are being exposed to additional severe and lethal diseases, such as malaria. This study has demonstrated a high level of seroreactivity for both COVID-19 samples and pre-COVID-19 samples from a malaria-endemic area (Mali) against the SARS-CoV-2 S protein. For the COVID-19 patients (n = 266), most samples reacted with the full-length protein and its internal domain RBD, although responses to the RBM were notably lower. Higher antibody levels at the time of hospital admission were associated with shorter treatment durations for COVID-19. Furthermore, certain comorbidities and the presence of high body weights appeared to be associated with a weaker



Patients' treatment duration	Responder samples for: S n (%)	RBD n (%)	RBM n (%)
≤ 30 days	161 (80.1)	147 (73.1)	69 (34.3)
> 30 days	5 (71.4)	3 (42.9)	1 (14.3)
P	ns	ns	ns

FIGURE 5 | Association of anti-S, receptor-binding domain (RBD), and receptor-binding motif (RBM) antibodies at the time of hospital admission with the duration of treatment for symptomatic COVID-19. The lowest antibody levels for S, RBD, and RBM at the time of hospital admission were associated with increased patient treatment time for symptomatic forms of COVID-19 (i.e., >30 days) as shown in the graph. The correlation was strongest with RBD recognition. The table shows the proportions of S, RBD, and RBM responders as a function of their treatment duration, but no significant difference was observed between the three antigens and the treatment duration time. The unpaired t-test and ANOVA were used to compare the mean Ab optical density (OD) between the two treatment duration groups and between antigens, respectively, and Fisher's exact test was used to determine the proportion of responders with a treatment duration of ≤30 or >30 days. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$. ns, not significant.

antibody response to S, RBD, and RBM. The positive response of COVID-19 plasma against different sequence domains (RBD and RBM) of S protein highlights peptide synthesis as an effective vaccine approach, which could ultimately contribute to the mass production of crucial COVID-19 good manufacturing practice (GMP) products (55–57).

Overall, our data demonstrate the importance of RBD—which showed comparable antibody responses (71.9%) to the full-length S protein (80.5%)—as an alternative target for vaccinations and antiviral therapies (58, 59). However it should be noted that we observed a relatively low prevalence of S antibodies (the most prevalent antigen); various other studies observed an antibody response of 95% from their COVID-19 patients (31, 60–64), indicating that our value of 80.5% is lower than expected. This may have been caused by a lack of seroconversion in some patients, as plasma was collected within the first week after hospital admission. According to the literature, at least 11–14 days after the onset of the disease is reported to be necessary to observe an average seroconversion rate of approximately 90%–100% for antibodies (IgM or IgG) against the SARS-CoV-2 S and N proteins (31, 60–69). Future investigations of the antibody dynamics, including in the early (acute) and late (convalescent) phases of COVID-19 infection, may provide more insight into this issue.

Antibody responses to SARS-CoV-2 antigens increased with age but were not associated with gender. Indeed, the S antigen showed a higher antibody level than RBD or RBM across all age groups. The same was observed for RBD as compared to RBM. Some studies have indicated that immunity and COVID-19 infection correlate positively with age (27, 70, 71), while others have suggested that aged patients are more prone to developing an uncontrolled and ineffective immune response, thus increasing disease severity (27, 70, 71). Our data strengthen the argument for inadequate antibody immunity as the cause of higher incidence of hospitalization in elderly patients despite high antibody levels in such groups. Regarding gender, it has been suggested that an immune response to COVID-19 may differ between men and women, thus influencing their ability to recover from a severe infection (72–77). Indeed, in women, higher IgG levels in the early phase and during COVID-19 (72–77) appear to play an essential role in reducing severe disease and mortality (78). However, this study analyzed samples only once, enabling the comparison of antibody levels in mild, severe, and convalescent cases. Still, studies on the dynamics of antibody responses to S, RBD, and RBM—controlling for variables like age and gender—are now necessary. Moreover, it was not possible to determine whether the SARS-CoV-2 antibody levels at hospital admission were correlated with recent exposure to COVID-19, which might explain the benign outcome of the disease in this group of patients.

Concerning treatment duration, patients with stronger responses to S, RBD, or RBM experienced remission in a shorter time period (≤30 days), supporting the idea that S- and RBD-specific antibodies play a crucial role in controlling the severity of SARS-CoV-2 infections. These findings are consistent with other studies that showed that the failure to develop antibodies against SARS-CoV-2 was an essential factor in worsening the disease (79) and was problematic for serodiagnosis tests (30).

This study shows that an accurate assessment of the interactions between preexisting comorbidities and antibody elicitation in the onset of SARS-CoV-2 is essential for existing vaccination strategies and especially to protect those at higher risk from severe forms of COVID-19. Preexisting comorbidities such as diabetes, hypertension, and asthma did not appear to influence antibody response against S and RBD. However, it is interesting that asthma and diabetes seemed to impede the elicitation of antibodies against RBM (the more specific domain for SARS-CoV-2) and that higher body weights appeared to weaken the antibody responses against S in COVID-19 patients. Altogether, these data suggest that preexisting comorbidities—which are associated with disease severity—may be directly impacting the immune responses to SARS-CoV-2 (80–83).

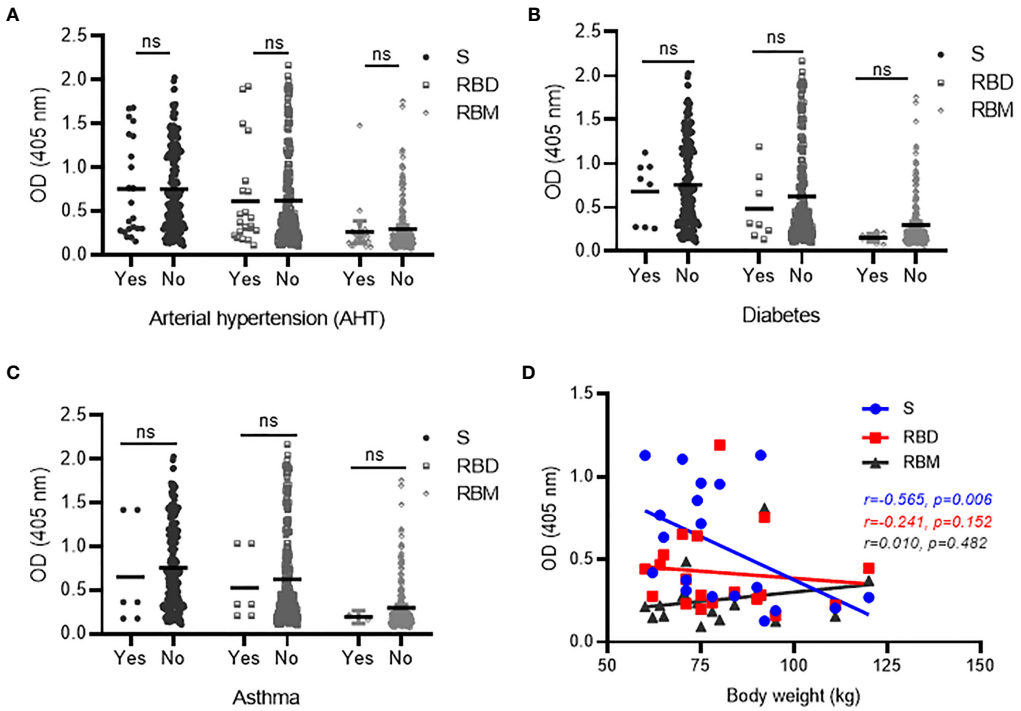
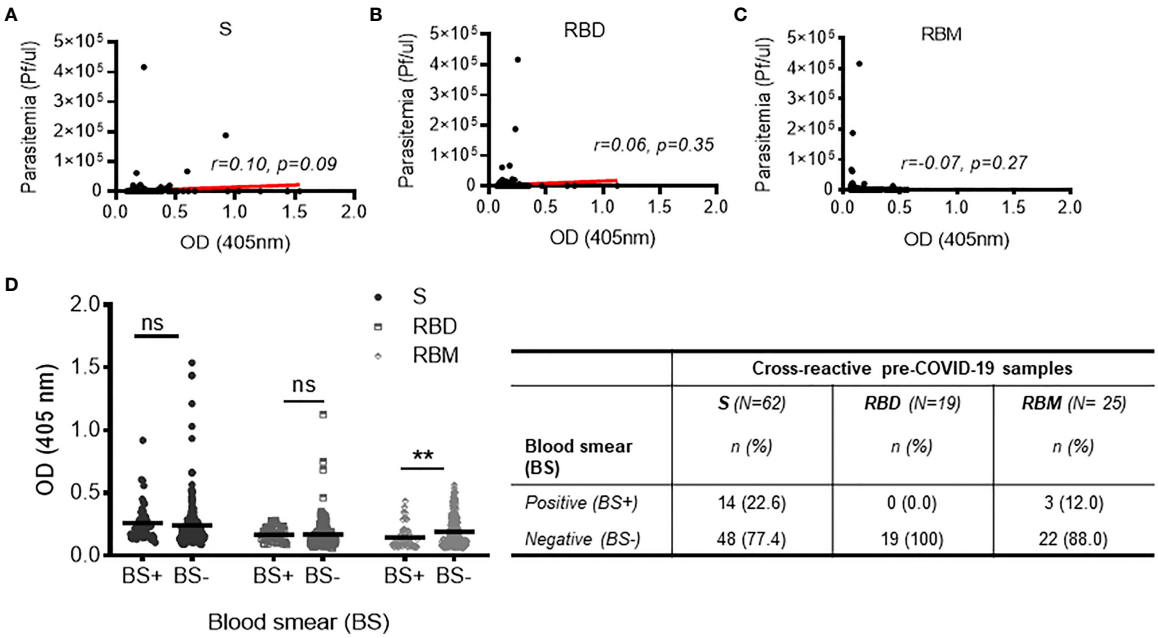


FIGURE 6 | Correlation between antibodies against S, receptor-binding domain (RBD), and receptor-binding motif (RBM) and comorbid conditions in COVID-19 patients. No significant variation was observed in antibody responses against S, RBD, or RBM between COVID-19 patients with the presence (Yes) vs. absence (No) of comorbid conditions, such as AHT (hypertension) **(A)**, diabetes **(B)**, and asthma **(C)**. However, a trend toward increased antibody levels for all three antigens was observed in the COVID-19 patient groups with no diabetes **(B)** or asthma **(C)**. **(D)** Spearman's rank analysis shows a significant negative correlation between antibody levels for S and body weight but showed no significant impact on antibodies against RBD and RBM in COVID-19 patients. ns, not significant.

Additionally, our findings imply that even with a lack of specific binding, there is still a high degree of cross-recognition for the SARS-CoV-2 antigens among populations not infected with SARS-CoV-2 living in malaria-endemic areas. Cross-reactive as high as 21.9% against S (highest) is consistent with previous studies, where it reached 17% or even upwards of 20% in malaria-endemic areas (23, 24). This cross-reactivity between malaria and SARS-CoV-2 raises the question of whether other SARS or malaria infections can produce similarly cross-reactive antibodies, playing a role in SARS-CoV-2 infection. In this regard, there is evidence for a cross-neutralization reaction between SARS-CoV and SARS-CoV-2, albeit controversial (25, 84). Malarial infections may also elicit a wide range of immune responses that could also be cross-reactive for COVID-19 antigens (18–22). In addition, antigen cross-reactivity (85, 86) may be due to a non-specific, antigen-independent antibody binding. In pre-COVID-19 volunteers, we observed false positivity against the three antigens in 9.6% to 20.0% of the cross-reactive samples, potentially indicating a non-specific antibody binding. These findings further confirm that anti-SARS-CoV-2 antibody tests may exhibit some false positives, as revealed by ELISA after removing the antigen coating (87, 88). Also, several proteins, present in human plasma at high concentrations—such as albumin (89)—can interfere with the detection of low abundance analytes (90) by increasing background signals and non-specific antibody binding (91).

Moreover, no correlation was found between the cross-recognition of SARS-CoV-2 antigens and current malaria infection. In contrast, the most cross-reactive antibodies were mainly associated with the absence of acute malarial infections, indirectly indicating a protective antibody response to malaria that cross-reacts with SARS-CoV-2. The cross-reactivity is more than likely to occur, since non-specific or poly-specific activation of B cells may occur during or before the process of induction of etiologic antibodies (92–95). Therefore, the coinfection of malaria and COVID-19, their impact on each other (in terms of clinical issues), and the cross-reactivity of COVID-19 antigens with malaria-endemic samples may help to explain the paradox in the incidence of COVID-19 in malaria-endemic areas (20–22, 96–98). Further study is necessary to assess how the coinfection of malaria and SARS-CoV-2 can impact the clinical outcomes of each disease.

In conclusion, the characterization of the individual antibody target domains/epitopes (like RBD and RBM) present in the SARS-CoV-2 S—in both naturally COVID-19 exposed patients and malaria exposed donors without COVID-19 infection—not only would contribute to our understanding of the fine specificity



	Cross-reactive pre-COVID-19 samples		
	S *(N=62)*	***RBD*** *(N=19)*	***RBM*** *(N= 25)*
Blood smear (BS)	*n (%)*	*n (%)*	*n (%)*
Positive (BS+)	14 (22.6)	0 (0.0)	3 (12.0)
Negative (BS-)	48 (77.4)	19 (100)	22 (88.0)

FIGURE 7 | Relationship between cross-reactivity against S, receptor-binding domain (RBD), and receptor-binding motif (RBM) and active malaria infection among pre-COVID-19 donors. **(A–C)** Non-significant correlations of cross-reactive antibodies to S, RBD, or RBM with present malarial infection (i.e., *Plasmodium falciparum* parasitemia in the pre-COVID-19 donor groups). Red lines indicate the best-fit relationship between data points. *p*- and R-values were calculated using the two-tailed Spearman's rank correlation tests. **(D)** The graph shows no significant variation in cross-reacting antibodies against S and RBD in pre-COVID-19 samples with (blood smear positive (BS+)) or without (blood smear negative (BS–)) present malarial infections; on the other hand, the high level of cross-reactive antibody against RBM was strongly associated ($p < 0.01$) with the absence of malarial infection (BS–). The table shows the proportions of BS+ or BS– cross-reactive samples against S, RBD, and RBM. N, total number of cross-reactive samples; n, number of BS+ or BS– cross-reactive samples. Comparisons of the mean optical density (OD) for BS+ and BS– sample groups were made using the unpaired t-test. $^{**}p < 0.01$; ns, not significant.

TABLE 4 | Proportion of antibody responders for S, RBD, and RBM in conjunction with the presence or absence of comorbid conditions among COVID-19 patients.

	S responders		RBD responders		RBM responders	
	n (%)	**p**	**n (%)**	**p**	**n (%)**	**p**
AHT						
Yes	18 (81.8)	*ns*	15 (68.2)	*ns*	5 (22.7)	*ns*
No	148 (79.6)		135 (72.6)		65 (34.9)	
Diabetes						
Yes	6 (75)	*ns*	5 (62.5)	*ns*	0 (0.0)	*NA*
No	160 (80)		145 (72.5)		70 (35)	
Asthma						
Yes	2 (66.7)	*ns*	2 (66.7)	*ns*	0 (0.0)	*NA*
No	164 (80)		148 (72.2)		70 (34.2)	

The proportions of responders against S, RBD, and RBM in COVID-19 samples were determined according to the presence (Yes) or the absence (No) of comorbidities (arterial hypertension (AHT), diabetes, and asthma). Fisher's exact test was used to compare the proportion of S, RBD, or RBM responders in groups with or without comorbidities.
N, total number of samples; n, number of responders; %, percentage of responders; ns, not significant; NA, not applicable; RBD, receptor-binding domain; RBM, receptor-binding motif.

of SARS-CoV-2 antigens and their cross-reactivity observed in these populations but also may offer strategies for designing a second-generation of vaccines. The cross-reactivity of the SARS-CoV-2 antigens was evident in pre-COVID-19 infected samples, as was the impact of protective malarial infection on said cross-reactivity. It can be noted that the early development of high antibody levels against RBD was essential in shortening treatment durations for SARS-CoV-2 infections. Furthermore, factors such as asthma, diabetes, and weight may adversely affect antibody responses to SARS-CoV-2.

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 in the article/ **supplementary material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee, EC of the Faculties of Medicine and Odontostomatology, and the Pharmacy of Bamako at the University of Science Technical and Technologies of Bamako, Mali. Written informed consent to participate in this study was provided by the participant's legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GC and SB designed the experiment. AT, MG, DK, BT, SD, SK, AD, AC, and SB performed most experiments, tests, and analyses. AK, MH, SH, GC, and SB wrote the manuscript. NI, FP, PM, AP, LP, PR, and FE contributed to antigen processing and manuscript revisions. MK, YC, OF, and MD contributed to sample processing and manuscript revisions. All authors read and approved the submitted version.

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SUPPLEMENTARY MATERIALS

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.856033/full#supplementary-material

Supplementary Figure 1 | Structure and amino acid sequences of the S and RBD proteins, and RBM peptide of SARS-CoV-2. **(A)** Structural features diagram of the SARS-CoV-2 spike (S) protein showing the subunit ectodomains S1 and S2; NTD, the N-terminal domain; RBD, the receptor-binding domain; FP, the fusion peptide; HR1 and HR2, the heptad regions 1 and 2; TM, the transmembrane domain; IC, the intracellular tail, (Yang et al., 2021) [39]. The sequence of ~1250 amino acid (aa) covering the full-length Spike protein is below. The sequence of residues in RBD is shown in green. The full-length sequence of the Spike (S) protein of SARS-CoV-2 is obtained using the BLASTP search program (50, 51). **(B)** The sequence of the SARS-CoV-2 RBD (aa319-529; ~211aa, in green) and several other RBD sequences from different SARS and viruses are provided in parallel for comparison. The portion in magenta, which is more variable than other parts of the RBD domain, is illustrated. * Indicates identical residues; similar residues are green while different ones are red. **(C)** Shows a synthetic peptide sequence (aa436-507; ~72aa) covering the binding segment (RBM, receptor biding motif) of the SARS-CoV-2 RBD. **(D)** Illustrates the 3D structure of SARS-CoV-2 spike (S) protein trimer with an S monomer outlined by blue color, and RBD and RBM in green and magenta, respectively. The other two monomers of S are in grey.

Supplementary Figure 2 | Antibody responses against S, RBD and RBM according to gender in COVID-19 and pre-COVID-19 donors. **(A–C)** Show respectively not significant antibody responses (OD) against S **(A)**, RBD **(B)** and RBM **(C)** between male and frmale in COVID-19 samples. Whereas, in pre-COVID-19 samples, the antibody level (cross-reactive antibody) for RBM was significantly higher in female group ($p<0.01$). The table shows median OD;s and interquartiles (Q1 and Q3) for antibody responses against S, RBD and RBM in COVID-19 and pre-COVID-19 groups. **$p\leq 0.01$; ns, not significant.

Supplementary Figure 3 | Analysis of antibody responses to S, RBD, and RBM according to the presence of multiple comorbid conditions in COVID-19 patients. **(A–C)** Show not significant variation of antibody responses against S, RBD and RBM according to the presence or absense of various comorbidities in COVID-19 patients, respectively. Unlike S and RBD, no association was found between two comorbidities and response to MBR **(C)**. CMB, comorbidity; ns, not significant.

Supplementary Figure 4 | Cross-reactivity of S, RBD and RBM according to age group in pre-COVID-19 samples. Cross-reactive antibody levels (in pre-COVID-19 samples) for spike (S) and MBR were comparable, but significantly higher than for MBR in most of the different age groups. Comparison of antibody levels between different antigens in the same age group was determined in unpaired t-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NA, not applicable; n, not significant; Age (year), age ranges.

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Design of a Recombinant Multivalent Epitope Vaccine Based on SARS-CoV-2 and Its Variants in Immunoinformatics Approaches

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The development of an effective multivalent vaccine against SARS-CoV-2 variants is an important means to improve the global public health situation caused by COVID-19. In this study, we identified the antigen epitopes of the main global epidemic SARS-CoV-2 and mutated virus strains using immunoinformatics approach, and screened out 8 cytotoxic T lymphocyte epitopes (CTLEs), 17 helper T lymphocyte epitopes (HTLEs), 9 linear B-cell epitopes (LBEs) and 4 conformational B-cell epitopes (CBEs). The global population coverage of CTLEs and HTLEs was 93.16% and 99.9% respectively. These epitopes were spliced together by corresponding linkers and recombined into multivalent vaccine. In silico tests, the vaccine protein was a non-allergen and the docking with TLR-3 molecule showed a strong interaction. The results of immune simulation showed that the vaccine may be helpful to initiate both cellular and humoral immunity against all VOC. The optimistic immunogenicity of the vaccine was confirmed *in vivo* and *in vitro* finally. Therefore, our vaccine may have potential protection against SARS-CoV-2 and its variants.

Keywords: SARS-CoV-2, COVID-19, variant, vaccine, epitope, immunoinformatics

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1 INTRODUCTION

In December 2019, the world witnessed the first coronavirus pandemic in human history. The coronavirus is a novel virus which was named as the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses (ICTV), and caused corona virus disease 2019 (COVID-19) which was named by the World Health Organization

(WHO) (1). The SARS-CoV-2 can invade lung and other multiple organs, causing severe pathological damage (2). Most of the young adults have no serious clinical symptoms, but the elderly and patients with underlying diseases are more serious after infected by SARS-CoV-2 (3, 4). The human individual is generally vulnerable to the SARS-CoV-2. The Worldometer database showed that more than 240 million novel coronavirus pneumonia cases had been confirmed and the cumulative death toll was more than four million all over the world as of October 2021. The COVID-19 had triggered serious public health problem and additional financial burdens around the world (5, 6).

The SARS-CoV-2 is a single and positive stranded RNA virus with an enveloped structure (7). The length of the virus genome is 26-32 kb and diameter of virus particles is between 70-120 nm. The SARS-CoV-2 encodes replicase, spike protein (S protein), envelope protein (E protein), membrane protein (M protein) and nucleocapsid protein (N protein) successively in sequence from genome 5' to 3' end (8). The S protein, especially, plays a key role in SARS-CoV-2 infection for it can bind human angiotensin converting enzyme 2 (ACE2) to infect target cells. As RNA virus, SARS-CoV-2 mutation probability is much higher than DNA virus (9). Up to now, some mutants of SARS-CoV-2 have attracted great attention. They are uniformly called variants of human concern (VOC), because the transmission, virulence and sensitivity of VOC have changed significantly compared with the original strain (10).

The variant named B.1.1.7 first appeared in the United Kingdom and had 10 characteristic mutations in S protein, including D614G and N501Y mutations in ACE2 receptor binding domain (RBD) and two characteristic mutations in Protein N (11, 12). It was reported that the transmission rate of B.1.1.7 variant increased by 35-45% and the mortality of patients infected with B.1.1.7 variant can be increased by 30% (13, 14). The variant B.1.351 had three mutations combination of N501Y, E484K and K417N on RBD in South Africa and then spread around the world (15). It was later confirmed that the binding strength of B.1.351 variant to ACE2 was three times stronger than that of the wild type (16). The variant P.1 first reported in Brazil and Japan that derived from Lineage B.1.1.28, and it had 12 S protein missense mutations, including three RBD mutations of E484K, K417T and N501Y (17). Paiva et al. (18) recently found that P.1 variant existed in cases of reinfection with SARS-CoV-2, which suggested that P.1 can escape from the antibody produced by the infection of the original strain. The variant B.1.429 in California contained four missense mutations, of which the L452R mutation is located in RBD (11). Recently, researchers at the University of California found that the variant B.1.429 can affect the neutralization efficiency of protective antibodies, so the B.1.429 variant is more contagious in people. Los Alamos National Laboratory in New Mexico also found that B.1.1.7 and B.1.429 variant can recombine and fuse with each other, which warns us that people may have entered a new phase of the SARS-CoV-2 pandemic. The variant B.1.617.2 with 13 amino acid mutations was first discovered in India in October 2020, including E484Q, L452R and P681R in its spike protein (19). Therefore, the mutations weakened the binding between SARS-CoV-2 S protein and existing antibodies, and potentially reduced the protection of the vaccine (13, 20).

At present, there are many kinds of vaccines on the market globally, such as BioNTech vaccine (Pfizer Corp), mRNA vaccine (Moderna Corp), AZD1222 adenovirus-based vaccine (Astrazeneca Corp), JNJ-78436735 adenovirus-based vaccine (Johnson & Johnson Corp), NVX-CoV2373 nanoparticle-based vaccine (Novavax Corp), etc. However, with the emergence of SARS-CoV-2 variants, the epitopes of original virus have also changed, threatening the effectiveness of existing vaccines (21). In view of the variation characteristics of SARS-CoV-2, it is a particularly urge task to further develop efficient COVID-19 vaccine for preventing this serious infectious disease (22). Currently, it has been proved that the SARS-CoV-2 variants can escape the humoral immunity induced by the vaccine, so the development of specific vaccines against virus mutants is particularly important for the prevention and control of COVID-19 epidemic. The research and development of global vaccine involve a variety of technical schemes, among which the recombinant protein vaccine made *in vitro* is the most widely used (23). Recombinant protein vaccine can contain different specific pathogen proteins and stimulate human body to produce corresponding antibodies. Because the spike protein coupled with ACE2 receptor mediates the viral entry, it plays a crucial function in the SARS-CoV-2 infection and has been used the top candidate antigen for the development of vaccine. With the immunoinformatics, the activation of immunogenic epitopes on the S protein may be a more useful approach. We have researched scientifically rigorous strategy of multi-epitope peptides based on different proteins against parasitic and bacteria diseases, such as hydatid and brucellosis (24–28). In this study, we have used immunoinformatics to predict and design a multivalent and multi-epitope vaccine that derived from the S proteins of prevalent SARS-CoV-2 and its variants for conferring optimistic protection.

2 MATERIALS AND METHODS

2.1 The Acquisition of Vaccine Candidate Antigens

2.1.1 Screening SARS-CoV-2 Variants

China National Center for Bio-information (CNCB) (https://bigd.big.ac.cn/ncov/variation/annotation) had classified the SARS-CoV-2 variants. This study was graded according to the assessment of CNCB website, and the Class I variants (the population incidence was greater than 0.05) were selected to screen antigens for vaccine design. Finally, we selected 5 SARS-CoV-2 variants of different lineages with the best quality evaluation.

2.1.2 Obtaining S Protein Sequences

Because of the important role of S protein in the SARS-CoV-2 infecting host process, the Protein S of SARS-CoV-2 and its variants were used to identify antigen epitopes and to design vaccine. The S protein sequences of Wuhan-Hu-1 and B.1.1.7,

B.1.351, P.1, B.1.429, B.1.617.2 etc. variants were obtained from National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). In addition, antigenicity of all S proteins was predicted using online software VaxiJen 2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). The VaxiJen was the first server that did not rely on protein sequence alignment and only classified proteins according to their physicochemical properties, so as to predict protective antigens (29).

2.1.3 The Sequence Alignment of Protein S

The sequence alignment of all S proteins of Wuhan-Hu-1 and B.1.1.7, B.1.351, P.1, B.1.429 B.1.617.2 variants were performed using software SnapGene, which could screen out the mutated amino acid sites.

2.2 The Prediction of Signal Peptide

To determine whether there are signal peptide regions in the candidate antigen proteins, the signal peptides of S proteins in different SARS-CoV-2 lineages were predicted using SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/) (30).

2.3 The Identification of Antigen Epitopes

2.3.1 The Identification of CTL Epitopes

The antigenic epitopes were restrictive in binding to human leukocyte antigen (HLA) molecules. In order to make the vaccine effect cover the global population more widely, we expanded the selection of HLA allele. According to the HLA allele reference set from the IEDB website (http://tools.iedb.org/mhci/) database (31), there are 27 high frequency HLA-I alleles (HLA-A*01:01, HLA-A*02:01, HLA-A*02:03, HLA-A*02:06, HLA-A*03:01, HLA-A*11:01, HLA-A*23:01, HLA-A*24:02, HLA-A*26:01, HLA-A*30:01, HLA-A*30:02, HLA-A*31:01, HLA-A*32:01, HLA-A*33:01, HLA-A*68:01, HLA-A*68:02, HLA-B*07:02, HLA-B*08:01, HLA-B*15:01, HLA-B*35:01, HLA-B*40:01, HLA-B*44:02, HLA-B*44:03, HLA-B*51:01, HLA-B*53:01, HLA-B*57:01, HLA-B*58:01) were used to predict the SARS-CoV-2 cytotoxic T lymphocyte (CTL) antigenic epitopes. In this study, the online software NetCTLpan was used to predict the CTL epitopes (CTLEs). The online Server NetCTLpan 1.1 (http://www.cbs.dtu.dk/services/NetCTLpan/) used artificial neural networks (ANNs), and it can simulate the binding between antigen peptide and major histocompatibility complex (MHC) for the prediction of MHC class I antigenic epitopes (32). The signal peptide sequences were removed when predicting epitopes in S proteins. In each virus strain, the CTLEs that appeared in four HLA-I alleles at the same time were selected as the dominant epitopes. And the dominant epitopes of six virus strains were compared and integrated for vaccine design. The antigenicity of epitopes screened out was further predicted using VaxiJen 2.0, and the antigen epitopes that its antigenicity value greater than threshold were used for the construction of the final vaccine.

2.3.2 The Identification of HTL Epitopes

According to the HLA allele reference set from IEDB (http://tools.iedb.org/mhcii/) (32), there were also 27 high frequency HLA-II alleles (HLA-DRB1*01:01, HLA-DRB1*03:01, HLA-DRB1*04:01, HLA-DRB1*04:05, HLA-DRB1*07:01, HLA-DRB1*08:02, HLA-DRB1*09:01, HLA-DRB1*11:01, HLA-DRB1*12:01, HLA-DRB1*13:02, HLA-DRB1*15:01, HLA-DRB3*01:01, HLA-DRB3*02:02, HLA-DRB4*01:01, HLA-DRB5*01:01, HLA-DQA1*05:01/DQB1*02:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DQA1*03:01/DQB1*03:02, HLA-DQA1*04:01/DQB1*04:02, HLA-DQA1*01:01/DQB1*05:01, HLA-DQA1*01:02/DQB1*06:02, HLA-DPA1*02:01/DPB1*01:01, HLA-DPA1*01:03/DPB1*02:01, HLA-DPA1*01:03/DPB1*04:01,HLA-DPA1*03:01/DPB1*04:02, HLA-DPA1*02:01/DPB1*05:01, HLA-DPA1*02:01/DPB1*14:01) were used to predict the SARS-CoV-2 helper T lymphocyte (HTL) antigen epitopes. The online Server NetMHCIIpan 4.0 (https://services.healthtech.dtu.dk/service.php?NetMHCIIpan-4.0) (33) was used to predict the HTL epitopes (HTLEs). In each virus strain,the HTLEs that appeared in six HLA-II alleles at the same time were selected as the dominant epitopes and the prediction method of the final epitope was the same as that of CTLEs.

2.3.3 The Identification of Linear B-Cell Epitopes

The online Server BepiPred-2.0 (https://services.healthtech.dtu.dk/service.php?BepiPred-2.0) (34) was used to predict the B-cell epitopes (BEs). The BEs were divided into linear epitopes and conformational epitopes. And the BepiPred-2.0 predicted that was linear B-cell epitopes (LBEs). The BEs of six virus strains were compared and synthesized, and the finally screened epitopes were used for vaccine construction. The signal peptide sequences were removed when predicting epitopes in S proteins.

2.3.4 The Identification of Conformational B-Cell Epitopes

The online software IEDB (http://tools.iedb.org/ellipro/) was used to predict the conformational B-cell epitopes (CBEs). The predicted conformational epitopes of six virus strains were compared and synthesized. The predicted conformational epitopes of six virus strains were compared and synthesized, and finally used in the construction of vaccine. And the signal peptide sequences were removed when predicting epitopes in S proteins.

2.4 Design and Construction of Vaccine

All the filtered CTLEs, HLTEs, LBEs and CBEs were placed and used in the multivalent vaccine construct. In light of the research of Dong (35), our study selected reasonable Linker-sequence to connect the SARS-CoV-2 antigen epitopes respectively. First, the AAY linkers were inserted between CTLEs, the GPGPG linkers were inserted between HTLEs and the KK linkers were inserted between BEs. Then the TAT-sequence (TGALLAAGAAA) was attached to the C-terminal of merged epitopes, which can make the vaccine deliver intracellularly (36). The recombinant SARS-CoV-2 multivalent epitope vaccine was named as *rSMEV* in the study.

2.5 Secondary and Tertiary Structure Prediction of *rSMEV*

When the antigen epitopes were connected sequentially by Linkers, the secondary structure of *rSMEV* was predicted by online software SOMPA (npsa-prabi.ibcp.fr/cgi-bin/

npsa_automat.pl?page=npsa_sopma.html). The SOMPA adopted the self-optimized prediction method (SOPM) to improve the accuracy of prediction by multiple alignment with protein sequences of the same family (37). And then the tertiary structure was predicted by software Rosettafold. The Rosettafold had ultra-high tertiary structure prediction accuracy by successively converting and integrating the three-track network information of 1D sequence, 2D distance and 3D coordinates (38).

2.6 Structure Optimization and Quality Validation

After primary 3D modeling, the initial vaccine 3D model was further optimized by GalaxyRefine Server (http://galaxy.seoklab.org/). The GalaxyRefine adopted ab initio modeling, and refined the loop or terminus regions in the primary protein 3D model (39). At the same time, the rationality of the optimized protein tertiary structure needs to be further verified. The ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php) and the Structure Assessment service of SWISS-MODEL (https://swissmodel.expasy.org/assess) were used to verify the tertiary structure quality of vaccine construct. The ProSA-web could evaluate the overall model quality by Z-Score as well as the local model quality. In addition, the SWISS-MODEL had drawn Ramachandran plot that displayed favorable areas of backbone dihedral angles against amino acid residues in tertiary structure (40).

2.7 Analysis and Evaluation of *rSMEV*

2.7.1 Analysis of *rSMEV* Physicochemical Properties

The online tool ProtParam (https://web.expasy.org/protparam/) (41) was used to predict the physical and chemical properties of *rSMEV*. The important physicochemical properties that ProtParam had performed included molecular weight, theoretical isoelectric point (PI), charged polar, atomic composition, half-life, stability, hydrophobicity and so on. According to the physicochemical properties of vaccine protein, it can guide the preparation of vaccine solution and the vaccination strategy *in vivo* experiment.

2.7.2 Analysis of *rSMEV* Allergenicity and Antigenicity

In order to ensure the safety of the vaccine, we predicted its allergenicity. The online software AllerTOP 2.0 (http://www.ddg-pharmfac.net/AllerTOP/) was used to predict the allergenicity of *rSMEV*. This prediction tool is based on auto cross covariance (ACC) transformation of protein sequences into uniform equal-length vectors, and the target protein was compared with allergens and non-allergens data sets for predicting allergen properties (29). Meanwhile, the VaxiJen 2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) (34) was used to predict the antigenicity of *rSMEV*.

2.7.3 Evaluation of *rSMEV* Population Coverage

The alleles and genotype frequencies were different among different populations in the world, and the polymorphism of HLA alleles affects the binding ability of antigen peptides to HLA-I or HLA-II molecules (42). The *rSMEV* had contained both HLA-I and HLA-II antigen epitopes. In addition, a wide range of HLA-I and HLA-II alleles were selected when predicting antigen epitopes. In order to verify the coverage of *rSMEV* to the world population, the IEDB (http://tools.iedb.org/population/) (32) was used to analyze the *rSMEV* population coverage.

2.8 Molecular Docking of *rSMEV* With TLR-3

The toll like receptor (TLR) was a class of important protein molecules involved in innate immunity, and it was also a bridge between nonspecific immunity and specific immunity (43). The TLR-3 molecule was found in a wide range of antigen presenting cells (APCs), such as tissue dendritic cells and monocytes, and TLR-3 could activate the specific recognition response of body cells to RNA virus infection (44). In the study, the binding affinity that *rSMEV* with TLR-3 was confirmed by the molecular docking approach. The tertiary structure of TLR-3 molecular was obtained from the PDB database (http://www.wwpdb.org/). The molecular docking works were performed by ClusPro Server (https://cluspro.bu.edu/home.php) (45). And the interaction interface residues analysis was performed by software PyMol and Ligplot.

2.9 Molecular Dynamics Simulation

After molecular docking, the best-docked system was solvated in a rectangular box of TIP3P (46) waters extending up to minimum cutoff of 15 Å from the protein boundary. Cl^- or Na^+ ions were added into the protein surface to neutralize the total charges of the systems. The Amber ff14SB force field (47) was employed for the protein in all of the molecular dynamics (MD) simulations. The initial structures were fully minimized using combined steepest descent and conjugate gradient method. Then, the systems were gently annealed from 10 to 300 K under canonical ensemble for 0.2 ns with a weak restraint of 15 kcal/mol/Å. The 500 ps of density equilibration was performed under isothermal-isobaric ensemble at target temperature of 300K and the target pressure of 1.0 atm using Langevin-thermostat (48) and Berendsen barostat (49) with collision frequency of 0.002 ns and pressure-relaxation time of 0.001 ns. After proper minimizations and equilibrations, a productive MD run of 100 ns was performed for all the complex systems. The MD simulations were performed with GROMACS 2021.3 (50), and calculated MMGBSA through MMPBSA.py by Amber tools (51).

2.10 Immune Simulation

To detect the immune response of *rSMEV* to the host, the C-ImmSim Server (https://kraken.iac.rm.cnr.it/C-IMMSIM/) was used to perform the immune simulation (52). The C-ImmSim could simulate the cellular and humoral immunity in the immune response of vaccine to mammals. It provided a fast Position Specific Scoring Matrix (PSSM) to predict the epitopes that bound to HLA molecules (53). In this simulation work, the HLA heterozygous combination of host was HLA-A (HLA-A*01:01, HLA-A*03:01), HLA-B (HLA-B*15:01, HLA-B*35:01) and HLA-DR (HLA-DRB1*0401, HLA-DRB1*1302). The selection of host HLA was determined according to the highest

frequency HLA molecules of all epitopes in *rSMEV*. The injection procedure was once every four weeks, a total of three injections. The time steps were 1, 84 and 168 (one time step corresponds to 8 h) (54). In addition, the Random Seed (12345), the Simulation Volume (10), the Adjuvant dose (100) and Number of antigens injected (1000) were system default. The simulation step was set as 1050 (53).

2.11 Codon Optimization

In order to better express the vaccine protein for subsequent animal *in vivo* experiment, we analyzed the adaptability and preference of the codon of *rSMEV*. The online tool JCat (JAVA Codon Adaptation Tool) (http://www.jcat.de/Start.jsp) was used to optimize the codon (55). The Escherichia coli (Strain K12) was chosen to express the *rSMEV* protein. Meanwhile, avoided rho-independent transcription terminators option, avoid prokaryotic ribosome binding sites option and avoided cleavage sites of restriction enzymes et al. options were selected for generating optimized DNA sequence corresponding to *rSMEV*.

2.12 In Silico Cloning of *rSMEV*

The pET-28a (+) was selected as the vector that expressed the *rSMEV* protein, and it was introduced into Escherichia coli. Before inserting the DNA sequence of *rSMEV* into the vector, it had been confirmed that there were no specific restriction enzyme recognition sites in the target gene sequence. Finally, the corresponding restriction endonuclease sequences were inserted at the N- and C-terminal of the DNA sequence of *rSMEV*. The in silico cloning work was performed by SnapGene. Finally, the *rSMEV* vaccine protein was synthesized and purified by SynPeptide Co Ltd (Shanghai, China).

2.13 *In Vivo* and *In Vitro* Experiments

2.13.1 Animal

The eight-week-old SPF BALB/c mice provided by the Animal Experiment Center of Xinjiang Medical University were randomly divided into rSMEV and Healthy control (HC) two groups. The mice were dripped intranasally with vaccine protein in rSMEV group (n=8), and the mice was dripped intranasally with normal saline in HC group (n=8). The concentration of vaccine protein solution was 500 μg/mL, each mouse was given 60 μL each time (30 μL each nostril), once every 2 weeks, 3 times in total and the vaccine protein was detected as endotoxin-negative by limulus amebocyte lysate (LAL) before using. Two weeks after the third immunization, the mouse splenocytes were collected aseptically in rSMEV and HC groups for experimental detection. The study was approved by the animal ethics committee of Xinjiang Medical University, and the whole animal experiment process was carried out in strict accordance with the experimental requirements and operation guidelines of the animal ethics committee of Xinjiang Medical University.

2.13.2 ELISPOT

The Enzyme-Linked Immunospot (ELISPOT) experiment was performed for evaluating the specific B-cell response in mice. First, the *rSMEV* vaccine protein solution (10 μg/mL) was coated on the ELISPOT plate and incubated overnight at 4 °C. Then the plate was washed for 5 times by Microplate washer (Thermo Fisher, FI-01620 Vantaa, Finland) and blocked with 200 μl RPMI-1640 medium (Sigma Aldrich, St. Louis, UA) containing 10% fetal calf serum (FCS) (Sigma) for 30 minutes at 37 °C. After the plate was washed by PBS containing 0.05% Tween 20, the vaccine protein and mice splenocytes suspension prepared (2×10^5) were added into the ELISPOT plate3. Three repeat wells were set for each sample and the plate was incubated for 20 hours in a 37 °C humidified incubator with 5% CO2. After the plate was washed for 5 times, the biotinylated detecting antibody (anti-mouse IgG) was added and the plate was incubated for 2 hours at room temperature. After the plate was washed, the Streptavidin-ALP was added and incubated at room temperature for 1 hour. The BCIP/NBT solution (MlBio) was added and incubated for chromogenic reaction. The plate was rinsed and dried, the antibody secreting cell (ASC) spot were counted using EliSpot Reader (AID, D 72479, Germany). In this experiment work, the Mouse IgG ELISpot BASIC kit (ALP) (3825-2A, Mabtech, Stockholm, Sweden) was adopted for the ELISPOT experiment.

2.13.3 ELISA

The enzyme-linked immunosorbent assay (ELISA) experiment was performed for evaluating the $CD4^+$ T-cell response in mice. First, the splenocytes in HC and rSMEV groups were co-cultured with the rSMEV vaccine protein (5 μg/mL). The splenocytes were incubated with RPMI-1640 medium containing 10% FCS for 48 hours in a 37 °C under 5% CO_2 humidified incubator. Then the culture supernatant was collected, and the samples and standards were added into the IFN-γ and IL-4 ELISA plates after plates were washed. The ELISA plates were incubated at 37 °C for 1.5 hours and washed for 5 times. The biotinylated detecting antibody was added to the plates and incubated at 37 °C for 1 hour. After the plates were washed for 5 times, the Streptavidin-ALP was added and incubated at 37 °C for 30 minutes. The BCIP/NBT solution were added for chromogenic reaction at 37 °C away from light for 15 minutes. Finally, the termination solution was added, and the OD450nm value was measured immediately by Spectrophotometer (Thermo Fisher, FI-01620 Vantaa, Finland) for detecting the corresponding level of IFN-γ and IL-4. The Mouse IFN-γ ELISA Kit (SEKM-0031, Solarbio Science & Technology Co., Ltd, Beijing, China) and Mouse IL-4 ELISA Kit (SEKM-0005, Solarbio Science & Technology Co., Ltd, Beijing, China) were adopted for ELISA experiment.

3 RESULTS

The vaccine construction strategy of this study was shown in **Figure 1**.

3.1 The Acquisition of Vaccine Candidate Antigens

3.1.1 Screening SARS-CoV-2 Variants

The GenBank search number of the six SARS-CoV-2 virus strains was NC_045512 (Wuhan-Hu-1), MW642026 (Lineage B.1.1.7),

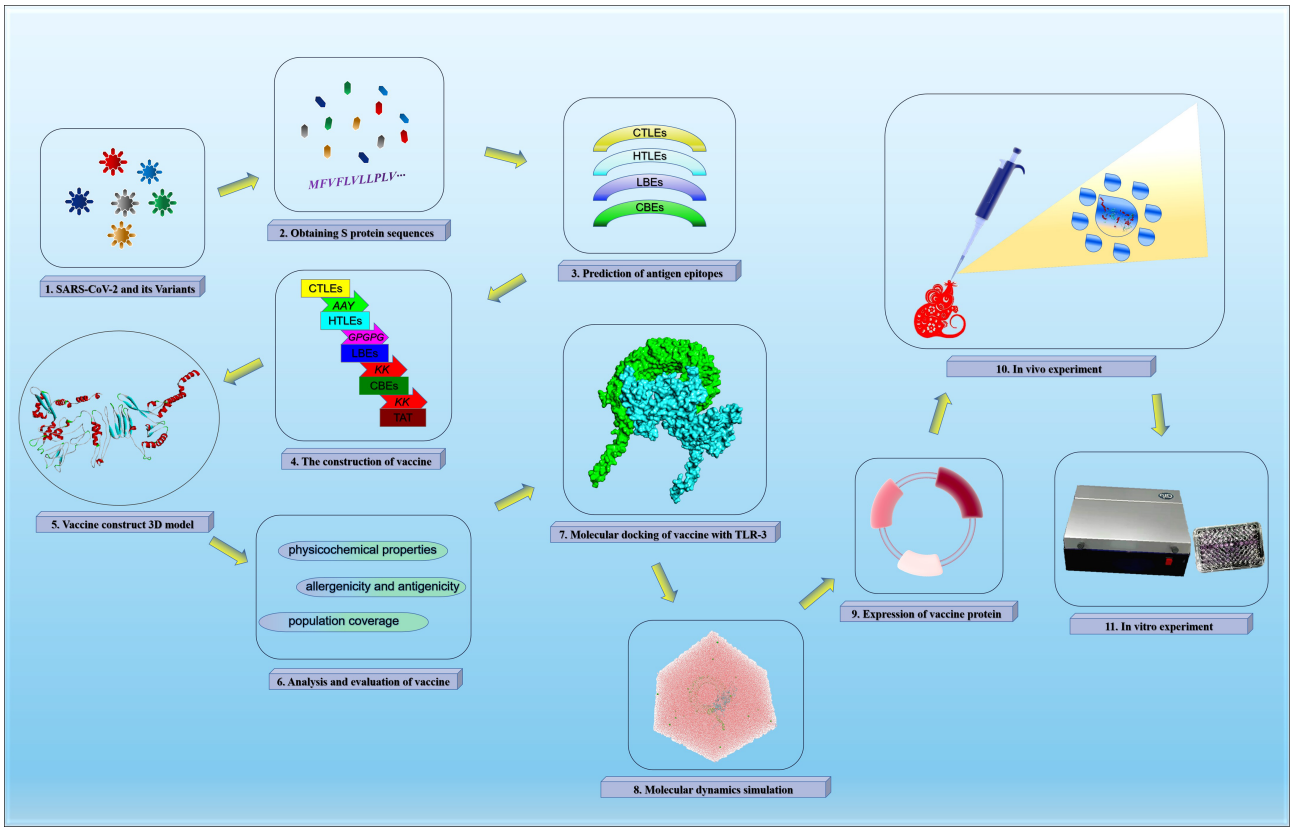

FIGURE 1 | The vaccine construction strategy.

MW621453 (Lineage B.1.351), MW642250 (Lineage P.1), MW631888 (Lineage B.1.429) and MZ491545 (Lineage B.1.617.2) separately.

3.1.2 Obtaining S Protein Sequences

The GenBank accession number of S protein derived from six SARS-CoV-2 virus strains was YP_009724390 (Wuhan-Hu-1), QRX39358 (Lineage B.1.1.7), QRV12312 (Lineage B.1.351), QRX39425 (Lineage P.1), QRW35522 (Lineage B.1.429) and QXF88313 (Lineage B.1.617.2). The result was shown in **Table 1** and the amino acid sequence of these proteins was shown in **Supplementary Data**.

3.1.3 The Sequence Alignment of Protein S

Compared with the original SARS-CoV-2 virus strain, the five virus variants had a total of 37 mutation sites. There were 25 mutation sites in the SARS-CoV-2 S1 region, among them, 20 mutation sites were in N-terminal domain (NTD) and five mutation sites in RBD. There were 17 mutation sites in the SARS-CoV-2 S2 region. Among them, two mutation sites were in heptad repeat region 1 (HR1) and one mutation site in HR2 (**Figure 2**).

3.2 The Prediction of Signal Peptide

After the analysis, the S proteins of six different SARS-CoV-2 lineages all had signal peptide regions. The signal peptide region of Lineage Wuhan-Hu-1 was 1-15 (MFVFLVLLPLVSSQC), the signal peptide region of Lineage B.1.1.7 was 1-15 (mfvflvllplvssqc), the signal peptide region of Lineage B.1.351 (mfvflvllplvssqc), the signal peptide region of Lineage P.1 was (mfvflvllplvssqc), the signal peptide region of Lineage B.1.429 was 1-15 (mfvflvllplvsiqc), the signal peptide region of Lineage B.1.617.2 was (mfvflvllplvssqc) (**Figure 3**).

3.3 The Identification of Vaccine Antigen Epitopes

3.3.1 The Identification of CTL Epitopes

In this part, the CTLEs of six SARS-CoV-2 virus strains were analyzed by online Server NetCTLpan 1.1 with each virus strain having 27 kinds of CTLEs of HLA-I alleles. The Rank of CTLEs was less than 1% in the prediction using IEDB. All CTLEs were ranked from small to large according to the sequence start position (**Figure 4A**). For each virus strain, the CTLEs that appeared in four HLA-I alleles at the same time were selected as the dominant CTLEs (**Figure 4B**). And the dominant CTLEs of

TABLE 1 | The antigenic value of SARS-CoV-2 S proteins.

Lineage	Protein	Accession number [a]	Residue length	Predictive value [b]
Wuhan-Hu-1	S	YP_009724390	1273aa	0.4646
B.1.1.7	S	QRX39358	1270aa	0.4742
B.1.351	S	QRV12312	1270aa	0.4656
P.1	S	QRX39425	1273aa	0.4723
B.1.429	S	QRW35522	1273aa	0.4783
B.1.617.2	S	QXF88313	1271aa	0.4695

[a]*The accession number from National Center for Biotechnology Information (NCBI).*

[b]*The Virus was selected as the protective antigen prediction model in VaxiJen 2.0, and the default threshold of protective antigen prediction is 0.4.*

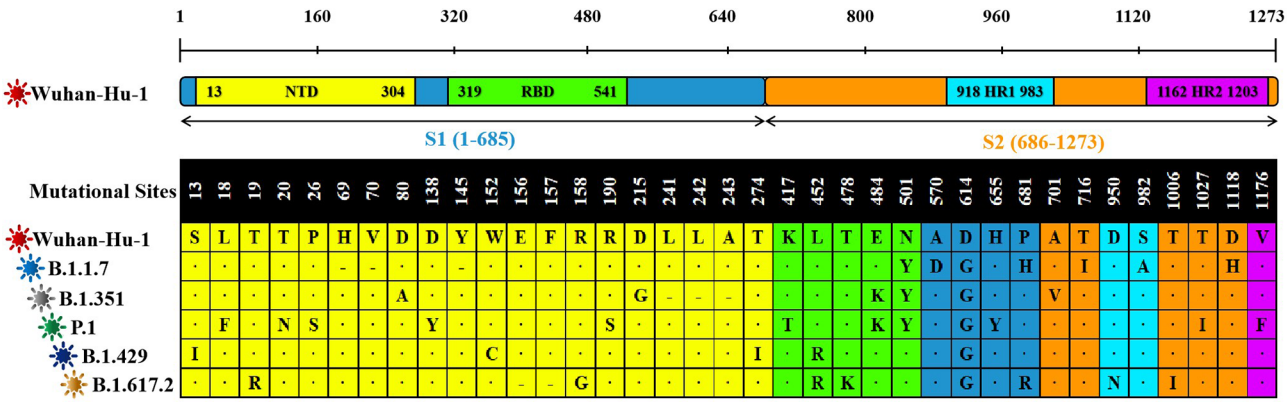

FIGURE 2 | The sequence alignment of S proteins in different virus strains. The study had screened S proteins of six kinds of different SARS-CoV-2 virus strains. The Wuhan-Hu-1 strain was selected as the reference sequence in the sequence alignment. In this figure, we had used different colors to represent the different domains of the S protein. From the N-terminal to the C-terminal of the S protein, a total of 37 amino acid positions had been changed. Among these variations, "·" was omission, which represented the same amino acid as the reference sequence. "-" was loss, which represented the deletion of amino acid sequence. And the remaining mutation sites were amino acid missense mutation.

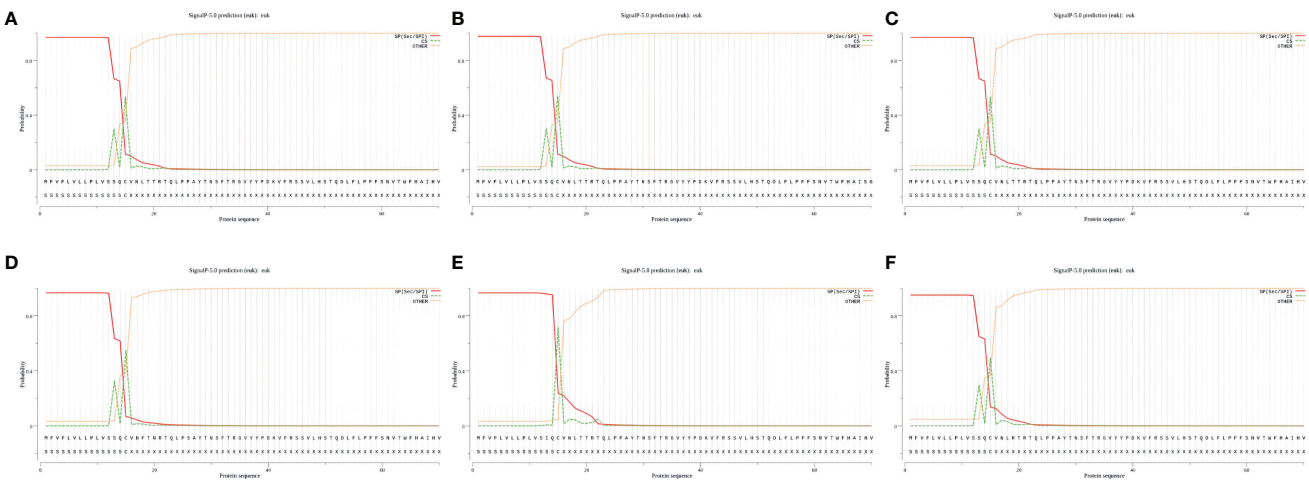

FIGURE 3 | The signal peptides of S proteins. **(A)** The probability of signal peptide is 96.886%. **(B)** The probability of signal peptide is 97.653%. **(C)** The probability of signal peptide is 96.886%. **(D)** The probability of signal peptide is 96.685%. **(E)** The probability of signal peptide is 96.625%. **(F)** The probability of signal peptide is 95.053%. Because the virus strains Wuhan-Hu-1 and B.1.351 have high homology, and the server can only give the signal peptide probability of the first 60 amino acid residues, so **(A)** and **(C)** are very similar.

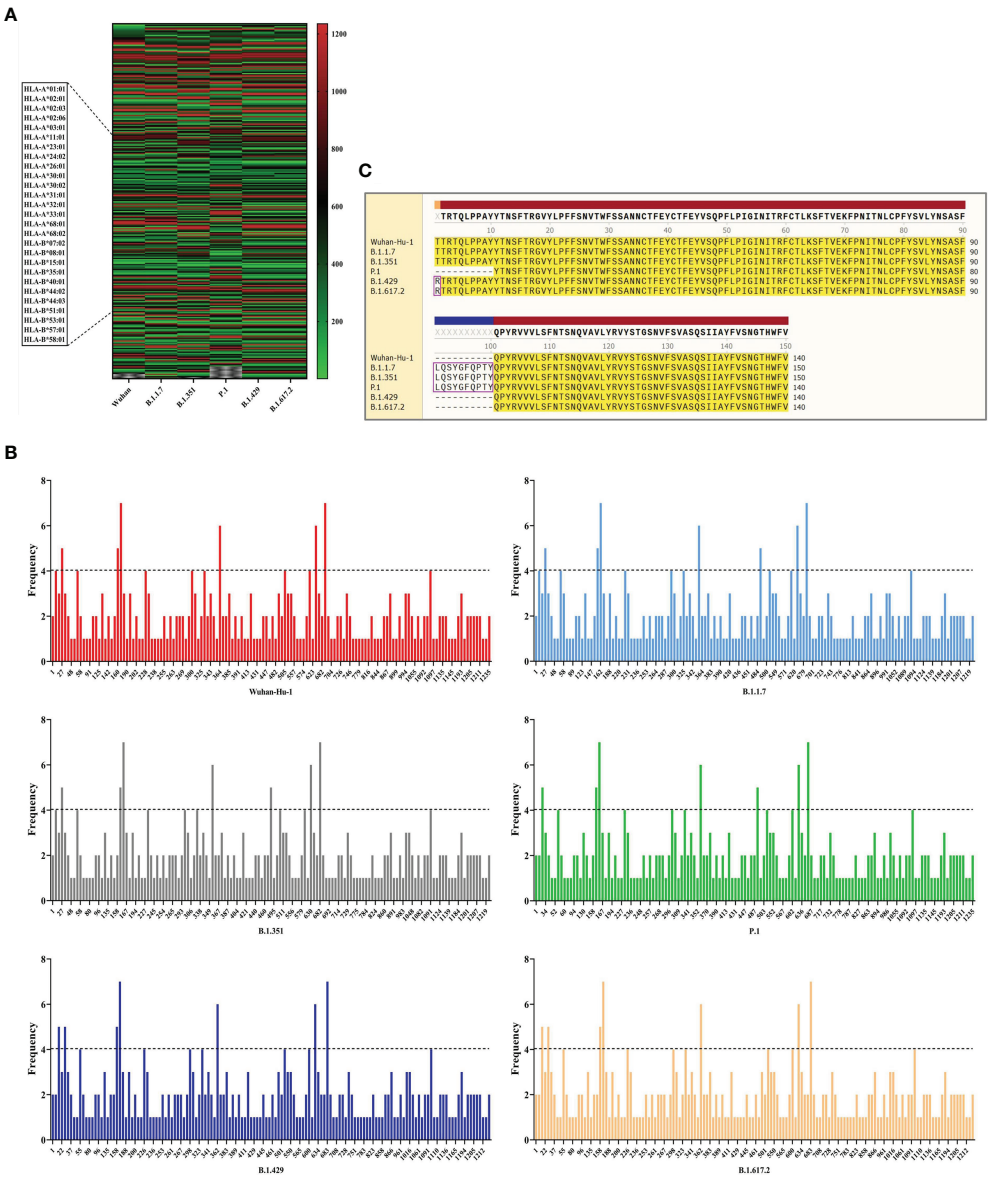

FIGURE 4 | The identification of CTL epitopes. **(A)** After identifying the 27 kinds of HLA-I alleles, all the CTLEs of the S protein in the 6 SARS-CoV-2 strains were shown here. The abscissa represents the six virus strains and the ordinate represents the position of the beginning of the epitope peptide. **(B)** The abscissa represented the position of the beginning of the epitope, and the ordinate represented the frequency of the epitope in the 27 alleles. **(C)** All epitopes of each virus strain were connected in sequence according to the size of the starting position, and the epitopes that had undergone mutation were in the "pink" box. Through sequence alignment, all the common and variant epitopes of all strains were used for vaccine construction.

six virus strains were compared and integrated for vaccine design (**Figure 4C**). The antigenicity of epitopes screened out was further predicted, and the antigen epitopes that antigenicity value greater than the threshold were used for the construction of the final vaccine (**Supplementary Table 1**).

3.3.2 The Identification of HTL Epitopes

The HTLEs of six SARS-CoV-2 virus strains were analyzed by online Server NetMHCIIpan 4.0 and each virus strain also had 27 kinds of HTLEs of HLA-II alleles. The Rank of HTLEs was less than 2% in the prediction using IEDB. All HTLEs were ranked from small to large according to the sequence start position (**Figure 5A**). For each virus strain, the HTLEs that appeared in six HLA-II alleles at the same time were selected as the dominant HTLEs (**Figure 5B**). And the dominant HTLEs of six virus strains were compared and integrated for vaccine design (**Figure 5C**). The antigenicity of epitopes screened out was further predicted, and the antigen epitopes that antigenicity

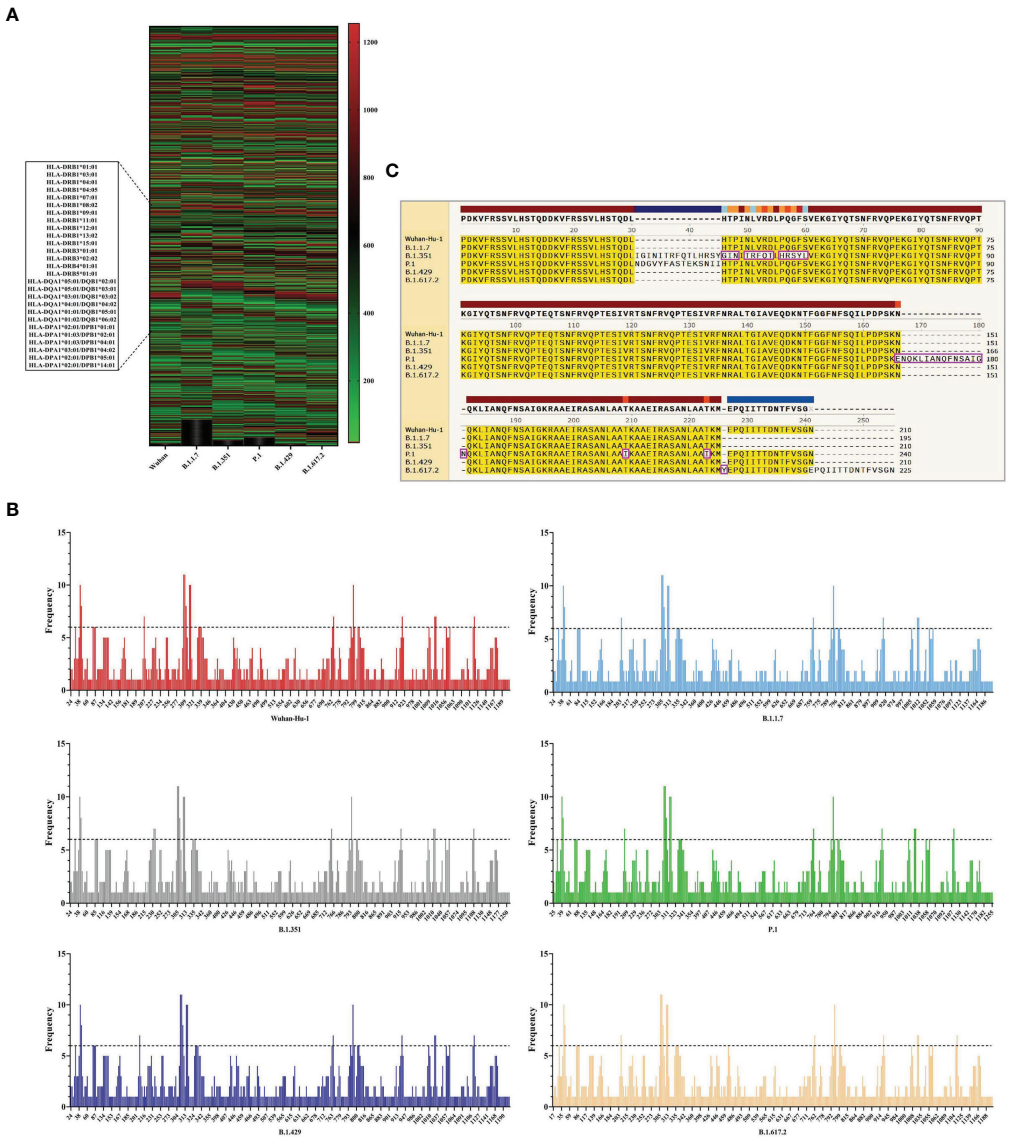

FIGURE 5 | The identification of HTL epitopes. **(A)** After identifying the 27 kinds of HLA-II alleles, all the HTLEs of the S protein in the 6 SARS-CoV-2 strains were shown here. The abscissa represents the six virus strains and the ordinate represents the position of the beginning of the epitope peptide. **(B)** The abscissa represented the position of the beginning of the epitope, and the ordinate represented the frequency of the epitope in the 27 alleles. **(C)** All epitopes of each virus strain were connected in sequence according to the size of the starting position, and the epitopes that had undergone mutation were in the "pink" box. Through sequence alignment, all the common and variant epitopes of all strains were used for vaccine construction.

value greater than the threshold were used for the construction of the final vaccine (**Supplementary Table 2**).

3.3.3 The Identification of Linear B-Cell Epitopes

The BepiPred-2.0 predicted the LBEs. In the result outputted from BepiPred-2.0, when the threshold of the peptide was greater than 0.8, the peptide sequence was recognized as an epitope (**Figure 6A**). The BEs of six virus strains were compared and synthesized, and the finally screened epitopes were identified as the dominant LBEs (**Figure 6B**). After all dominant LBEs were predicted for their antigenicity, the antigen epitopes that antigenicity value greater than the threshold were used for the construction of the final vaccine (**Supplementary Table 3**).

3.3.4 The Identification of Conformational B-Cell Epitopes

The software IEDB had predicted the CBEs of S proteins from different SARS-CoV-2 lineages. We selected the best ranked CBEs among the six virus strains (**Figure 7**). After comparison, the sequences of CBEs were used to the construction of final vaccine (**Supplementary Table 4**).

3.4 Design and Construction of Vaccine

The *rSMEV* construct contained the best candidate antigen epitopes. There were 8 CTLEs, 17 HTLEs, 9 LBEs and 4 CBEs in the *rSMEV*. The Linker-AAY had connected all CTLEs, which can enhance the presentation of epitopes and help to produce suitable sites for antigen epitopes to bind to TAP transporters (35). The Linker-GPGPG had connected all HTLEs, the Linker-KK had connected all LBEs and CBEs (**Figure 8**). All the antigen epitopes for construction of *rSMEV* were shown in the **Table 2**. The number of vaccine residues of final designed multivalent vaccine was 614 aa.

3.5 Secondary and Tertiary Structure Prediction of *rSMEV*

In the result of secondary prediction, there were 20.03% Alpha helix (Hh), 23.94% Extended strand (Ee), 6.51% Beta turn (Tt) and 49.51% Random coil (Cc) (**Figure 9A**). The tertiary structure was shown in secondary structure style (**Figure 9B**), and the proportion of Hh, Ee, Tt and Cc was consistent with the secondary structure. This indirectly showed that the prediction of secondary structure and tertiary structure was reasonable.

3.6 Tertiary Structure Optimization and Quality Validation

After the initial tertiary structure was refined by GalaxyRefine Server, the server had outputted five optimized models. All models were further verified by ProSA-web, the website had calculated the Z-Score of models. The Z-Score of initial *Model* was −6.89, the optimized *Model 1* was −6.88, the optimized *Model 2* was −6.96, the optimized *Model 3* was −6.93, the optimized *Model 4* was −7.02 and the optimized *Model 5* was also −6.87. Through observation, it was found that the optimized *Model* 4 was more in line with the Z-score range of similar protein size. So, the optimized *Model* 4 was selected as the final tertiary structure. The Ramachandran plot drawn by SWISS-MODEL showed that there were 91.03% Ramachandran favored region, 2.45% Ramachandran Outliers region and 0.00% Rotamer Outliers region in initial structure, and there were 92.65% Ramachandran favored region, 1.47% Ramachandran Outliers region and 0.21% Rotamer Outliers region in final structure (**Figure 10**).

3.7 Analysis and Evaluation of *rSMEV*

3.7.1 Analysis of *rSMEV* Physicochemical Properties

The vaccine protein had 614 amino acids, the total number of atoms was 9122 and the formula was $C_{2904}H_{4540}N_{822}O_{850}S_6$. The molecular weight was 65 KD, and it was an acceptable vaccine since that the molecular weight of protein less than 110 KD could be easily purified (56). The theoretical pI was 10.04, and it included 29 acidic (negatively charged residues) amino acids (Asp + Glu) and 69 alkaline (positively charged residues) amino acids (Arg + Lys). The instability index (II) was computed to be 27.49 (when it<40, the protein is stable) (57), so the vaccine was classified as stable protein. The aliphatic index was 64.28 and Grand average of hydropathicity (GRAVY) was −0.481 (the range of GRAVY is −2∼2, negative value means that protein is hydrophilic) (58), so the vaccine belonged to hydrophilic protein.

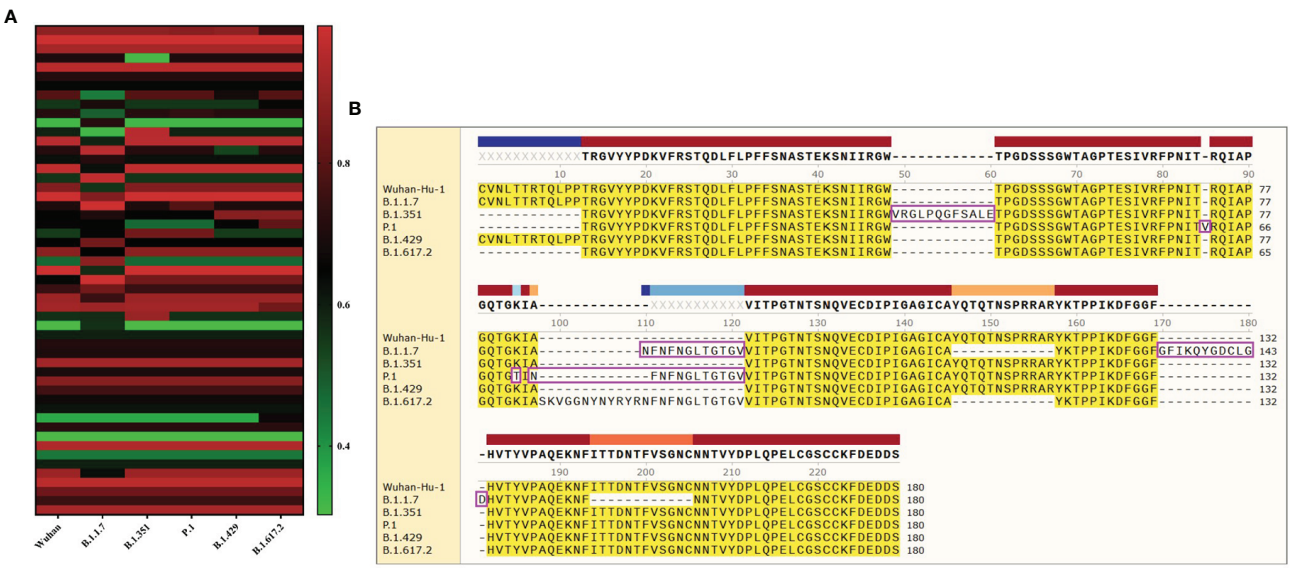

FIGURE 6 | The identification of B-cell epitopes. **(A)** All the LBEs of the S protein in the 6 SARS-CoV-2 strains were shown here. The abscissa represents the six virus strains and the ordinate represents the Rank of the epitope peptide. **(B)** All epitopes of each virus strain were connected in sequence according to the size of the starting position, and the epitopes that had undergone mutation were in the "pink" box. Through sequence alignment, all the common and variant epitopes of all strains were used for vaccine construction.

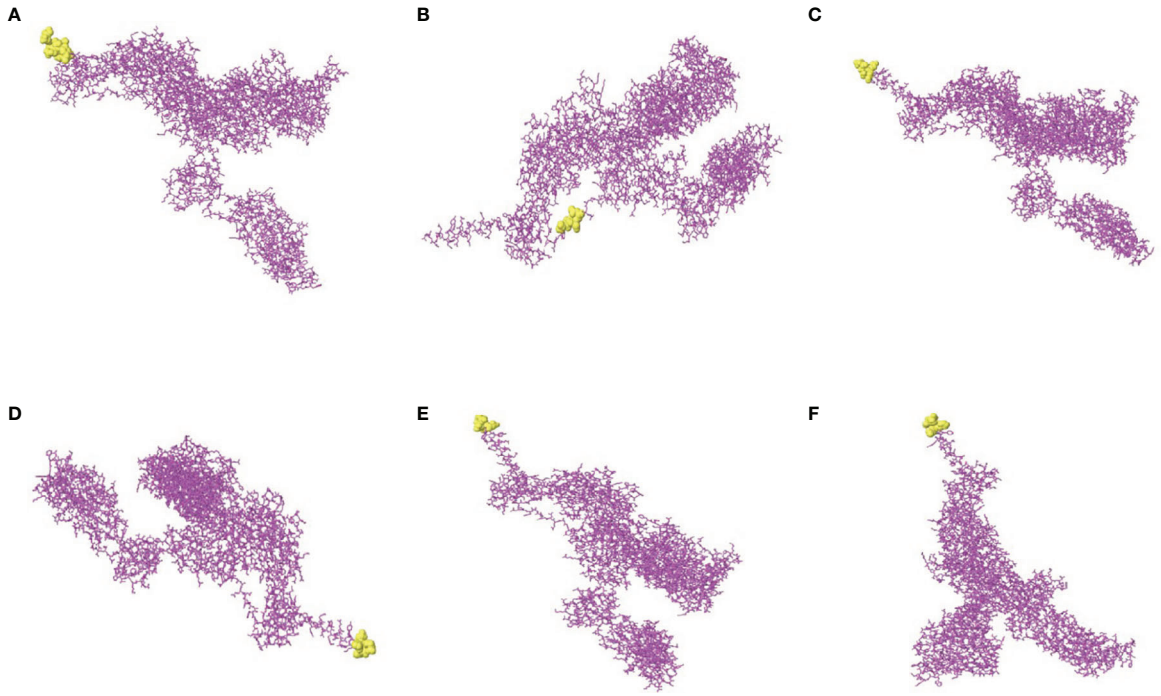

FIGURE 7 | The conformational B-cell epitopes of six SARS-CoV-2 lineages. **(A)** The "yellow" region is the predicted best conformational B-cell epitope of S protein of virus strain Wuhan-Hu-1. **(B)** The "yellow" region is the predicted best conformational B-cell epitope of S protein of virus strain B.1.1.7. **(C)** The "yellow" region is the predicted best conformational B-cell epitope of S protein of virus strain B.1.351. **(D)** The "yellow" region is the predicted best conformational B-cell epitope of S protein of virus strain P.1. **(E)** The "yellow" region is the predicted best conformational B-cell epitope of S protein of virus strain B.1.429. **(F)** The "yellow" region is the predicted best conformational B-cell epitope of S protein of virus strain B.1.617.2.

3.7.2 Analysis of *rSMEV* Allergenicity and Antigenicity

The online software AllerTOP 2.0 defined the *rSMEV* as non-allergen, the UniProtKB accession number of the protein nearest to *rSMEV* was P46379 and P46379 was also probable non-allergen. The VaxiJen 2.0 showed that the overall prediction value for the protective antigen was 0.6858, the Virus was selected as the prediction model and the threshold for this

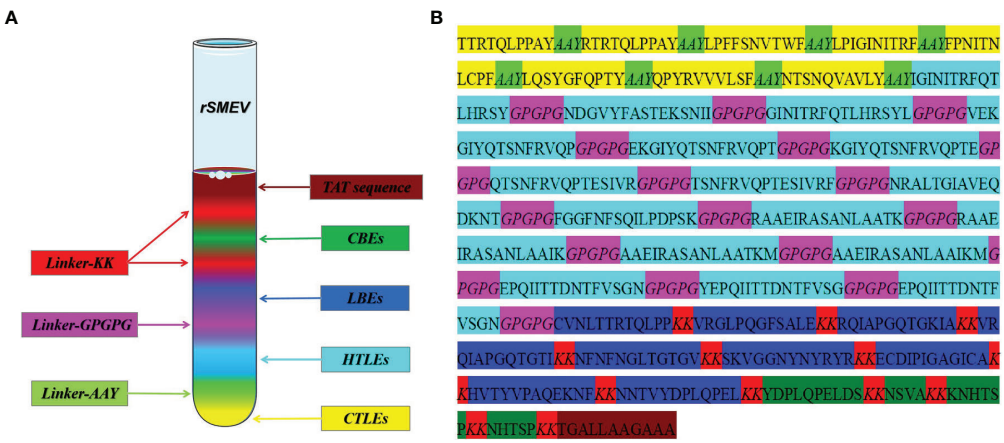

FIGURE 8 | The design and construction of *rSMEV*. **(A)** Schematic diagram of all the components needed in vaccine construction, and the different colors represented different epitopes and linkers. **(B)** The specific amino acid sequence of all the epitopes and linkers required for vaccine construction, and the color of each part corresponded to the color in the **(A)**.

TABLE 2 | The antigen epitopes for vaccine construction.

NO.	Antigen epitope	Variant epitope	Type	Variation rate [a]
1	TTRTQLPPAY	RTRTQLPPAY	CTLEs	25.0%
2	RTRTQLPPAY	LQSYGFQPTY		
3	LPFFSNVTWF			
4	LPIGINITRF			
5	FPNITNLCPF			
6	LQSYGFQPTY			
7	QPYRVVVLSF			
8	NTSNQVAVLY			
9	IGINITRFQTLHRSY	IGINITRFQTLHRSY	HTLEs	37.5%
10	NDGVYFASTEKSNII	NDGVYFASTEKSNII		
11	GINITRFQTLHRSYL	GINITRFQTLHRSYL		
12	VEKGIYQTSNFRVQP	RAAEIRASANLAAIK		
13	EKGIYQTSNFRVQPT	AAEIRASANLAAIKM		
14	KGIYQTSNFRVQPTE	YEPQIITTDNTFVSG		
15	QTSNFRVQPTESIVR			
16	TSNFRVQPTESIVRF			
17	NRALTGIAVEQDKNT			
18	FGGFNFSQILPDPSK			
19	RAAEIRASANLAATK			
20	RAAEIRASANLAAIK			
21	AAEIRASANLAATKM			
22	AAEIRASANLAAIKM			
23	EPQIITTDNTFVSGN			
24	YEPQIITTDNTFVSG			
25	CVNLTTRTQLPP	VRGLPQGFSALE	LBEs	33.3%7
26	VRGLPQGFSALE	VRQIAPGQTGTI		
27	RQIAPGQTGKIA	NFNFNGLTGTGV		
28	VRQIAPGQTGTI			
29	NFNFNGLTGTGV			
30	SKVGGNYNYRYR			
31	ECDIPIGAGICA			
32	HVTYVPAQEKNF			
33	NNTVYDPLQPEL			
34	YDPLQPELDS	NSVA	CBEs	75.0%
35	NSVA	KNHTSP		
36	KNHTSP	NHTSP		
37	NHTSP			

[a]*Among the corresponding types of epitopes, the proportion of epitopes appearing in the virus variant.*

model was 0.4. Therefore, in this allergenicity and antigenicity analysis, the *rSMEV* was identified as safe protective antigen.

3.7.3 Evaluation of *rSMEV* Population Coverage

In the analysis of population coverage, the Class I and Class II T-cell epitopes were calculated separately. The IEDB showed that the HLA-I T-cell epitope covered 93.16% and HLA-II T-cell epitope covered 99.9% world population in the *rSMEV*. The detailed results were showed in **Supplementary Tables 5, 6** and **Supplementary Figure 1**.

3.8 Molecular Docking of *rSMEV* With TLR-3

The PDB ID of TLR-3 was 7c76 (59) in the PDB database and the TLR-3 molecular obtained was a complex that blended with UNC93B1. The TLR-3 was isolated from the complex using software Discovery Studio for molecular docking. In this molecular docking work, the TLR-3 molecules acted as the receptor, while the *rSMEV* acted as ligand. The ClusPro Server can output some clusters after docking work, and each cluster has a different number of members. In the *rSMEV*-TLR-3 complex formed by the combination of vaccine molecule *rSMEV* and antigen recognition molecule TLR-3, the lowest complex energy weighted score was −1175.3 cal/mol and all the docking results were shown in **Supplementary Table 7**. The interaction interface residues analysis from PyMol was 3D and Ligplot was 2D format. The results show that there were two ionic bonds and four hydrogen bonds at the docking interface, which participated in the interaction between the subunits of the complex (**Figure 11**).

3.9 Molecular Dynamics Simulation

In order to evaluate the structural stability of *rSMEV*-TLR-3 complex, the best docking pose (**Figure 12A**) of *rSMEV* and TLR-3 was simulated by MD. The docking complex was solvated in a rectangular box of TIP3P waters (**Figure 12B**). After running the MD simulation of 100 ns, the results showed that the root mean square displacement (RMSD) value rose sharply to 1nm within 30

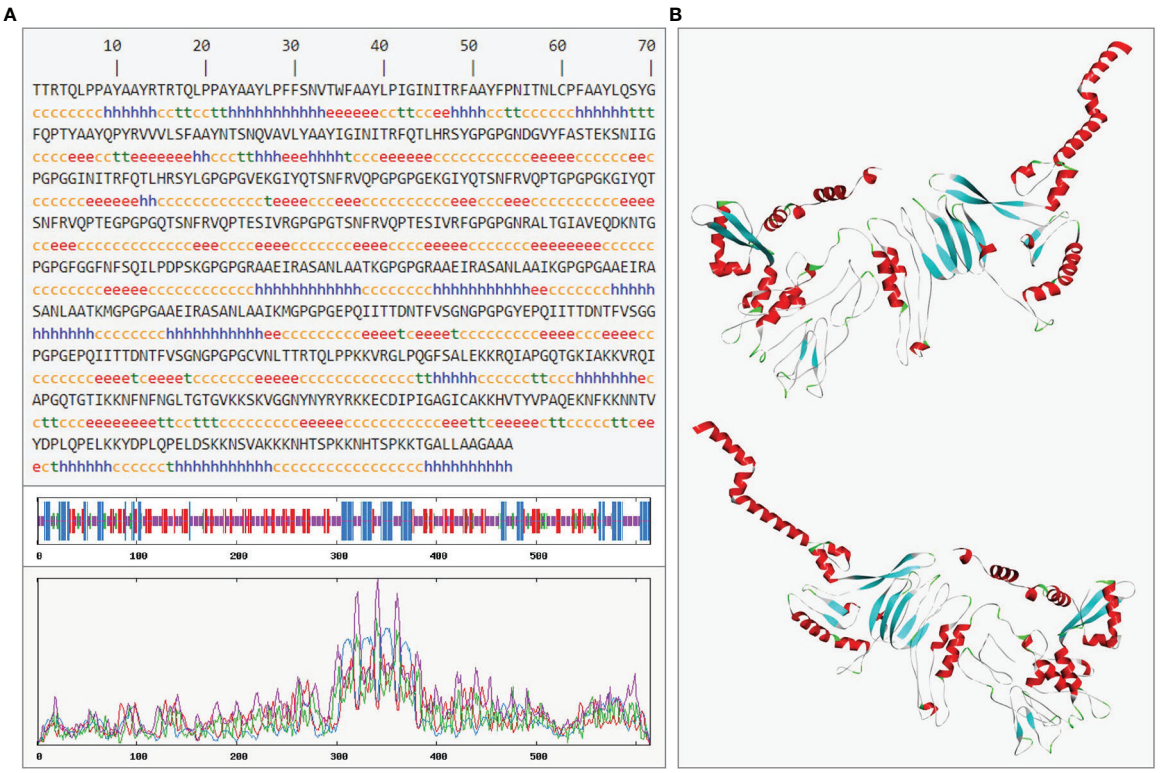

FIGURE 9 | The prediction of *rSMEV* secondary and tertiary structure. **(A)** In the prediction of *rSMEV* secondary structure, the "h" represented the Alpha helix, the "e" represented the Extended strand, the "t" represented the Beta turn and the "c" represented the Random coil. **(B)** In this 3D model, the tertiary structure was displayed in front view and back view. The "red" part was Alpha helix, the "cyan" part was Extended strand, the "green" pare was Beta turn and the "gray" part was Random coil in 3D model.

ns. After 30 ns, the RMSD value tended to be stable, and the floating range of the overall operation was about 1 nm (**Figure 12C**). The root mean square mobility (RMSF) value showed that the two chains of the complex had a lower value (<1 nm) between residues 0-650, while the residues after site 650 had a larger RMSF value (>1 nm) (**Figure 12D**). Next, the average binding free energy was −88.54 ± 6.91 kcal/mol of complex detected by MMPBSA tool of the GROMACS. (**Supplementary Table 8**).

3.10 Immune Simulation

The C-ImmSim Server conducted the immune stimulation of the *rSMEV* vaccine. The Immune simulation results showed that the antigen count decreased with the increase of antibody level in the immune response, which was mainly due to the production of total B-lymphocytes (**Figure 13A**) and T-lymphocytes. The IgM + IgG and IgM antibodies were found in the primary immunization. And the levels of IgM + IgG, IgM, IgG1 and IgG1 + IgG2 antibodies increased in the secondary and tertiary immune responses compared to primary immune response (**Figure 13B**). Besides B-cell, total and memory TH-cell (helper T cell) (**Figure 13C**) populations along with active TH-cell (**Figure 13D**) populations increased during the secondary and tertiary immune responses. The active TC-cell (cytotoxic T lymphocyte) count was sustained growth after each immunization (**Figure 13E**). And in the three stages after immunization, the level of IFN-γ had increased (**Figure 13F**).

3.11 Codon Optimization

The *rSMEV* protein had 614 amino acids, and the DNA sequence of *rSMEV* had 1842 nucleotides after adaptability and preference analysis of codon. The codon adaptation index (CAI) of the improved sequence was 0.93. When the CAI-Value>0.8, the codon improved was considerate to have a high adaptability to the DNA sequence (**Supplementary Figure 2**). The GC-Content of the improved codon was 54.51% and the GC-Content of *Escherichia coli* strain K12 used in the study was 50.73%.

3.12 In Silico Cloning of *rSMEV*

The DNA sequence of *rSMEV* was inserted to the sites between XhoI (site 158) and BamHI (site 2006) in the pET-28a (+) vector. The DNA sequence of *rSMEV* was written in **Supplementary Data**. The XhoI and BamHI were common restriction endonucleases in molecular cloning, and the DNA sequence did not contain these two digestion sites (**Supplementary Figure 3**).

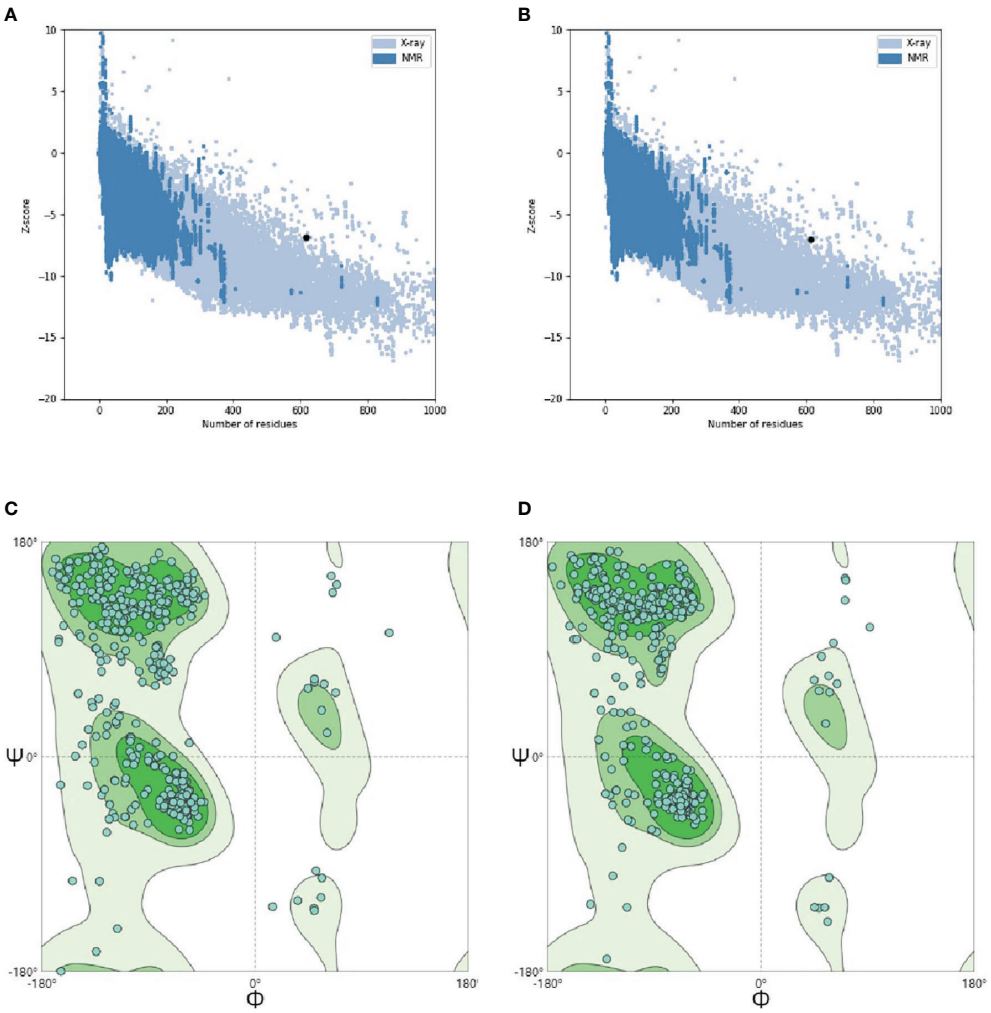

FIGURE 10 | Tertiary structure optimization and quality validation. **(A)** and **(B)** are the Z-score evaluation results. The abscissa represents the number of amino acids of the protein and the ordinate represents the score. The region formed by blue and gray blots in the figure are the reasonable scoring areas of the protein, and the black dot represents the target protein. The good target protein with the corresponding number of amino acids should fall in reasonable area. **(C)** and **(D)** are Ramachandran plot. In the figure, dark green is favored regions, green is additional allowed regions, light green is generously allowed regions and white is disallowed regions. The rationality of amino acid residues in these regions decreased in turn. When evaluating the quality of 3D model, it is hoped that the proportion of amino acid residues in favored regions is larger.

3.13 The Results of ELISPOT and ELISA Experiments

In the study, the ELISPOT experiment was performed to evaluate the *rSMEV* induced B-cell response. The splenocytes of rSMEV group mice could secrete specific antibodies induced by vaccine protein, while the splenocytes of HC group mice did not secrete specific antibodies (**Figure 14A**). After statistical test, the specific antibody secreting cells (ASCs) in rSMEV group was significantly higher than that in HC group, and the difference was statistically significant ($P<0.001$) (**Figure 14B**). This suggested that *rSMEV* protein could activate B-cell reaction. The ELISA experiment was performed to evaluate the *rSMEV* induced $CD4^+$ T-cell response. The $CD4^+$ T-cell was activated by vaccine protein, so the content of IFN-γ (**Figure 14C**) and IL-4 (**Figure 14D**) in rSMEV group was significantly higher than that in HC group, and the difference was statistically significant (*P*-value of IFN-γ was less than 0.0001 and *P*-value of IL-4 was less than 0.001). This suggested that *rSMEV* protein could activate $CD4^+$ T-cell reaction.

4 DISCUSSION

As you known, the decrease of vaccine protection ability elicited mainly by S protein mutation of SARS-CoV-2. There was no a multivalent epitope vaccine for S protein to resist the infection of SARS-CoV-2 and its variants at present. Therefore, we had contrived a multivalent epitope vaccine of S protein to cope

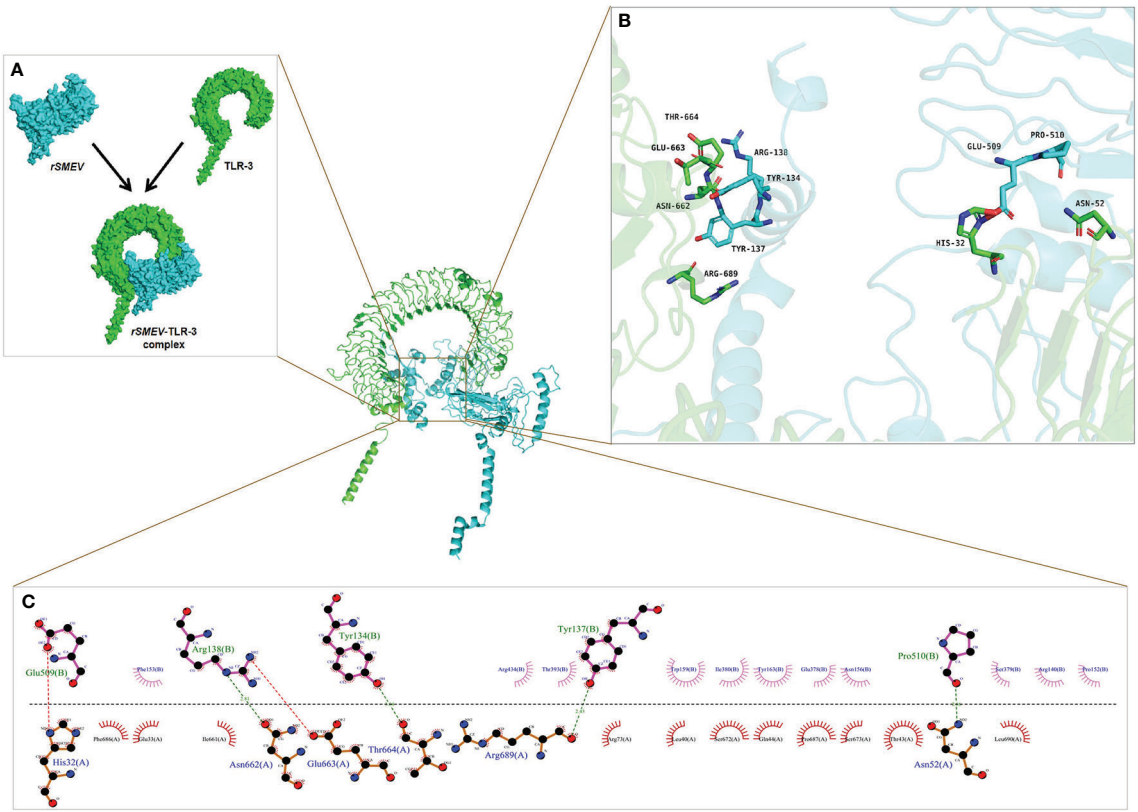

FIGURE 11 | The molecular docking of *rSMEV* with TLR-3. **(A)** Schematic diagram of docking between vaccine molecule *rSMEV* and antigen recognition molecule TLR-3. The *rSMEV* is light blue and TLR-3 is green. After docking, the *rSMEV*-TLR-3 complex was formed. **(B)** The interaction interface predicted by the PyMol is three-dimensional. In this three-dimensional interaction interface, it is mainly ionic bond and hydrogen bond that participate in the interaction. **(C)** The interaction interface predicted by the Ligplot is two-dimensional. And in this two-dimensional interaction interface, the red dotted line represents ionic bond and the green dotted line represents hydrogen bond, so there are 2 ionic bonds and 4 hydrogen bonds involved in the interaction.

with the problem of virus mutation more effectively. In this study, we took the VOC including Wuhan-Hu-1 and SARS-CoV-2 variants B.1.1.7, B.1.351, P.1, B.1.429, B.1.617.2, etc as the objects of study, and took S proteins of virus strains as the research target. Meanwhile, the immunogenicity of the vaccine was confirmed *in vivo* and *in vitro* study. Theoretically, the vaccine designed by this study has a broader protective effect for SARS-CoV-2 infection.

When recognizing the antigenic peptide presented by APCs or target cells, the T cell receptor (TCR) should recognize both the antigenic peptide and the polymorphic parts of its MHC molecules. Therefore, MHC molecular restriction was fully considered when screening the $CD8^+$ and $CD4^+$ T-cell epitopes for activating the cellular immunity. In order to expand population coverage of the vaccine, we predicted 27 kinds of HLA-I and 27 kinds of HLA-II T-cell epitopes respectively (60). By comparing the predicted epitope sequences of each virus strain, the common epitopes of different virus strains with the highest frequency were screened and selected. Ultimately, the global population coverage of CTLEs and HTLEs was 93.16% and 99.9% separately in our *rSMEV*. That means the epitopes-based vaccine might be employed by more population in different regions.

To ensure the SARS-CoV-2 vaccine more reliable and effective, we spliced together the chosen epitopes that included 8 CTLEs, 17 HTLEs, 9 LBEs and 4 CBEs by linker sequences. The appropriate linkers could avoid the formation of new epitopes (Linker-epitope) and enhance the presentation of antigen epitopes by ensuring the full exposure of the screened target epitopes (61, 62). Then, the TAT sequence (11aa) was attached to the N-terminal of vaccine construct simultaneously. The TAT sequence can carry macromolecular substances, making it easier to penetrate the cell membrane, so as to promote the phagocytosis of vaccine protein by APCs (36). After the design and construction of the vaccine, the secondary structure of *rSMEV* was analyzed. The secondary structure analysis showed that the vaccine protein had 6.51% Beta turn and 49.51% Random coil. Because the spatial structure of Beta turn and random coil is loose, it is easy to form epitopes. Therefore, the *rSMEV* that containing a large amount of this structure has a good vaccine structural basis.

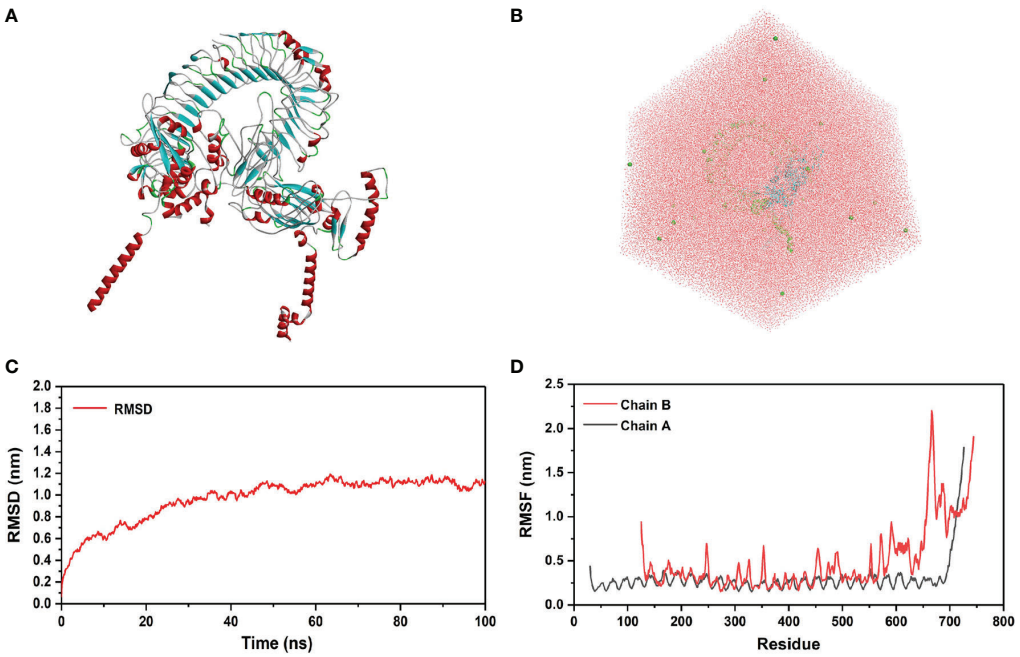

FIGURE 12 | Molecular docking of *rSMEV* with TLR-3. **(A)** This is a three-dimensional model of *rSMEV* and TLR-3 complex, and its color is displayed in the form of secondary structure style. **(B)** This is the rectangular box of TIP3P waters, and it extends up to minimum cutoff of 15 Å from the protein boundary. After molecular docking, the best-docked system was solvated in it. **(C)** This is the RMSD running diagram of MD simulation. The abscissa represents the running time of MD simulation, and the ordinate is the value of RMSD. **(D)** This is the RMSF running diagram of MD simulation. The chain A is TLR-3 and the chain B is *rSMEV*. The abscissa represents each amino acid residue in the complex, and the ordinate represents the RMSF value.

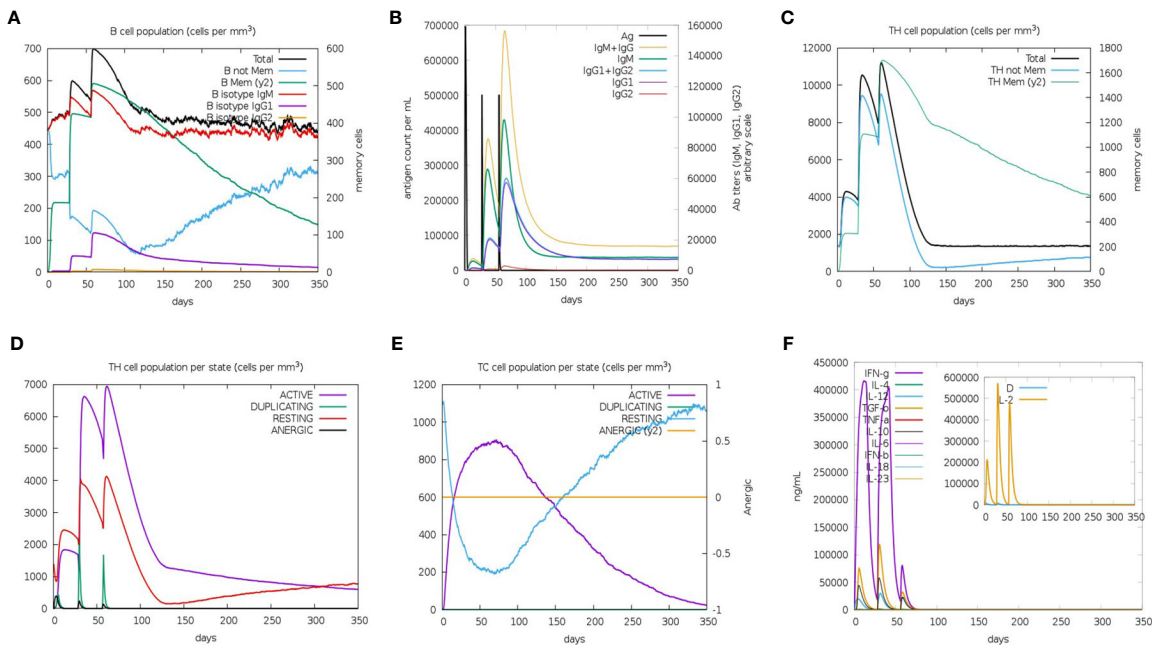

FIGURE 13 | The immune simulated response spectrum. **(A)** The B-cell populations that could produce antibodies of various subtypes after vaccination. **(B)** The production of antibodies that represented the proliferation of the immune response after vaccination, and the various subtypes of immunoglobulins were shown in different colors. **(C)** The generation of memory and non-memory HTL populations after vaccination. **(D)** The HTL populations in various states. **(E)** The CTL populations in various states. **(F)** The cytokine profile showed the production of various cytokines after vaccination.

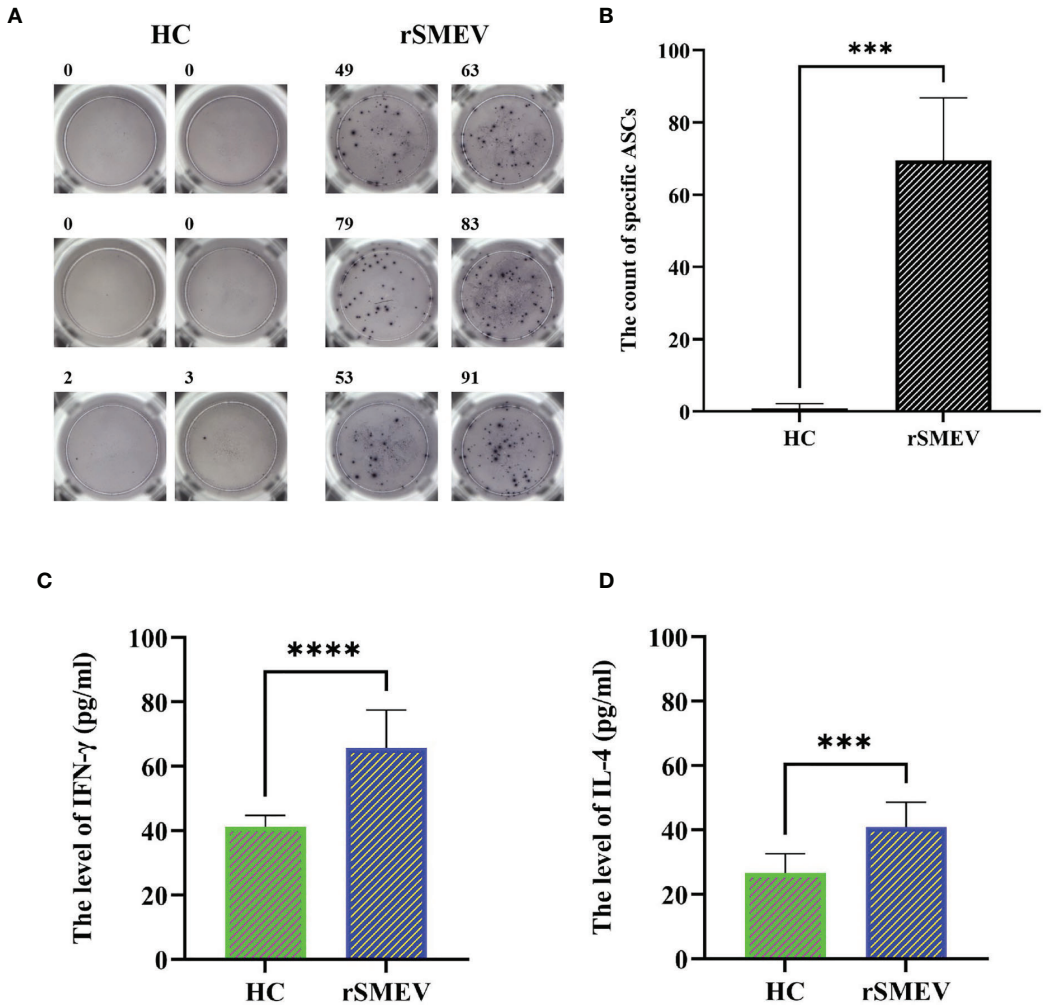

FIGURE 14 | The results of ELISPOT and ELISA experiments. **(A)** The representative ELISPOT spot diagram in HC and rSMEV groups. **(B)** The count of ASCs in the HC and rSMEV groups were significantly different (***P<0.001) after using the two independent sample t-test, and the statistical test was two-tailed. The t-value was 9.671 and the degree of freedom was 5.059 (***P < 0.001). **(C)** The two independent sample t-test of the level of IFN-γ between HC group and rSMEV group, and the difference was statistically significant (****P<0.0001), the t-value was 6.247 and the degree of freedom was 18. **(D)** The two independent sample t-test of the level of IL-4 between HC group and rSMEV group, and the difference was statistically significant (***P<0.001), the t-value was 4.652 and the degree of freedom was 18.

In view of Rosettafold's excellent performance in protein tertiary structure prediction (38), we used it to predict the high-quality tertiary structure of *rSMEV*. Meanwhile, the unreliable loops or termini of the predicted 3D structure were reconstructed and refined by the GalaxyRefine Server. The good tertiary structure of *rSMEV* would be used for later molecular docking and MD simulation. We further analyzed the physicochemical properties and allergenicity of *rSMEV*. The physicochemical properties results showed that molecular weight of *rSMEV* was 65 KD. Previous studies have reported that it is reasonable when the molecular weight of vaccine protein is less than 110 KD (56), which hinted that the *rSMEV* was suitable for vaccine. In addition, the analysis of theoretical pI, amino acid acidity, alkalinity, stability, hydrophilicity and allergenicity also displayed that the *rSMEV* was reasonable and safe.

When microorganisms break through the mucosal barrier, TLR can recognize them and initiating the adaptive immune response (63). Because TLR-3 molecule plays an important role in innate immunity against virus, the binding of antigen and TLR-3 can help APCs to present antigen and release local cytokines. For the sake of proving that the *rSMEV* had strong affinity with TLR-3 molecule, we conducted the molecular docking using ClusPro Server. The results showed that lowest energy value in the all outputted *rSMEV*-TLR-3 complexes was −1175.3 kcal/mol. The capacity of the system is lower, the structure of object is more stable (45). Therefore, the structure of *rSMEV*-TLR-3 complex was quite stable. Moreover, the

interaction interfaces proved that the binding affinity between *rSMEV* and TLR-3 was ionic and hydrogen bonds mainly. Due to the interaction of ionic bond and hydrogen bond is strong, this also indirectly indicates that the binding between *rSMEV* and TLR-3 is very close (64).

We also used MD simulation to analyze the structural stability and agility of *rSMEV*-TLR-3 complex. The RMSD value represented the structural fluctuation of the overall structure of the vaccine and TLR-3 complex, which can evaluate the stability of the system during interaction. In this part, the floating range of 100 ns RMSD value was about 1 nm, suggesting that the structure of the complex is relatively stable. The RMSF value represented the flexibility of amino acid residues in the docking complex, which can evaluate the agility of each residues in the complex. The results suggested that the agility of receptor and ligand sites after 650 was higher than that of residues 0-650. The average binding free energy of the system calculated by the MMGBSA tool was −88.54 ± 6.91 kcal/mol. Therefore, MD simulation proved that *rSMEV* and TLR-3 docking complex in this study had reasonable structural stability and agility. The above results showed that *rSMEV* can closely bind to TLR-3, and the complex has high stability and agility.

That is more important, we had verified the immunogenicity of the *rSMEV in vivo* and *in vitro*. Firstly, the *rSMEV* can stimulate an excellent immune response confirmed by immune simulation. Then the mice were immunized with *rSMEV* through experiments *in vivo* and *in vitro*. The mice can produce specific ASCs and high levels of IFN-γ and IL-4, the result confirmed that the *rSMEV* can induce cellular and humoral immunity and has satisfied immunogenicity.

In many current studies that based on S proteins to design SARS-CoV-2 vaccine (46–51, 65), there are few studies on designing for multiple variants at the same time. Starting from considering the mutation of SARS-CoV-2, to the restriction of HLA alleles, and then to the binding between vaccine protein and TLR-3 molecule. Our research had made innovative and reasonable design in the above aspects for improving the immunogenicity and applicability of SARS-CoV-2 vaccine. At the same time, we also evaluated the immunogenicity of the vaccine by experiments *in vivo* and *in vitro*. Compared with other studies, our *rSMEV* is more significant in the current perspective. Obviously, the *rSMEV* designed by the study estimated that it may have a high potency against SARS-CoV-2 and its variants theoretically. However, this study did not include other antigens with important immunogenicity of SARS-CoV-2. At the same time, this study still needs to further carry out human clinical trials to ensure the effectiveness of the vaccine. All in all, we provided an effective immunological strategy and mean for the active prevention and control of COVID-19 worldwide.

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 authors.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Experiment Medical Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University.

AUTHOR CONTRIBUTIONS

MY, YZ, YL, ZC, ZWL, JW, and ZNL performed the experiments. MY and YZ wrote and edited the paper. YL, ZWL, JW, and ZNL collated and verified the experimental data. FZ and JD provided experimental design and ideas, and reviewed and modified the paper. All the authors agreed to publish the final version of the paper.

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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.2022.884433/full#supplementary-material

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Prior Vaccination Exceeds Prior Infection in Eliciting Innate and Humoral Immune Responses in Omicron Infected Outpatients

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Antibody response following Omicron infection is reported to be less robust than that to other variants. Here we investigated how prior vaccination and/or prior infection modulates that response. Disease severity, antibody responses and immune transcriptomes were characterized in four groups of Omicron-infected outpatients (n=83): unvaccinated/no prior infection, vaccinated/no prior infection, unvaccinated/prior infection and vaccinated/prior infection. The percentage of patients with asymptomatic or mild disease was highest in the vaccinated/no prior infection group (87%) and lowest in the unvaccinated/no prior infection group (47%). Significant anti-Omicron spike antibody levels and neutralizing activity were detected in the vaccinated group immediately after infection but were not present in the unvaccinated/no prior infection group. Within two weeks, antibody levels against Omicron, increased. Omicron neutralizing activity in the vaccinated group exceeded that of the prior infection group. No increase in neutralizing activity in the unvaccinated/no prior infection group was seen. The unvaccinated/prior infection group showed an intermediate response. We then investigated the early transcriptomic response following Omicron infection in these outpatient populations and compared it to that found in unvaccinated hospitalized patients with Alpha infection. Omicron infected patients showed a gradient of transcriptional response dependent upon whether or not they were previously vaccinated or infected. Vaccinated patients showed a significantly blunted interferon response as compared to both unvaccinated Omicron infected outpatients and unvaccinated Alpha infected hospitalized patients typified by the response of specific gene classes such as OAS and IFIT that control anti-viral responses and IFI27, a predictor of disease outcome.

Keywords: SARS-CoV-2 omicron infection, prior COVID-19 infection, antibody responses, immune transcriptome, COVID-19 alpha infection, interferon response, anti-viral response

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INTRODUCTION

The highly transmissible Omicron (B.1.1.529) variant is less susceptible to neutralizing antibodies elicited by previous vaccination or other variant infection (1–4), thus resulting in a continuation of the COVID-19 pandemic. While recent studies have investigated the antibody response to breakthrough infections (5, 6), there is a knowledge gap about the humoral and genomic immune response to Omicron infection in outpatients that had been vaccinated, previously infected with another SARS-CoV-2 variant or both. Specifically, the impact of previous infection as compared to vaccination in the generation of anti-Omicron spike antibodies upon Omicron infection has yet to be determined.

While Omicron infections generally result in a more moderate symptomology compared to other variants, the genomic immune response in this patient population has not been investigated. Transcriptome studies on hospitalized patients infected with the Alpha (7) or Beta (8) variants have revealed the activation of interferon pathway genes with an emphasis of the JAK/STAT pathway. While the interferon response in severely ill patients has been reported, it is not clear if Omicron patients with a generally lighter symptomology have a different immune transcriptome which can be further modulated by vaccination and prior infection.

To address these questions, we investigate the transcriptional and humoral immune response in 83 outpatients with documented Omicron infection. Thirty-four had no prior infection and were not vaccinated, 23 persons had been vaccinated and boosted with the BNT162b2 mRNA vaccine (Pfizer–BioNTech), 19 had a history of prior infection by other SARS-CoV-2 variants and 7 had been vaccinated and had a prior infection. This study permitted an understanding of how vaccination and prior infection impact the immune response to Omicron infection and we identified a gradient response that corresponds to the humoral profile.

RESULTS

Study Design

The first case of Omicron infection in Austria was reported at the end of November 2021 (9) and recruitment of outpatients took place between December 2021 and March 2022. Here, we investigate the humoral and transcriptional immune response in 83 persons with documented Omicron infection. Four groups were studied: no vaccination/no prior infection (n=34), vaccination/no prior infection (n=23), no vaccination/prior infection (n=19) and vaccination/prior infection (n=7) (**Figure 1A**; **Table 1**). Demographic and clinical characteristics of the study population are provided in **Table 1**. The transcriptional response of the first three groups was investigated at days 1-3 following initial SARS-CoV-2 RT-PCR test and the humoral response at two timepoints (range of group means days 1-3 and 12-15 following initial SARS-CoV-2 RT-PCR test). The humoral response for the fourth group

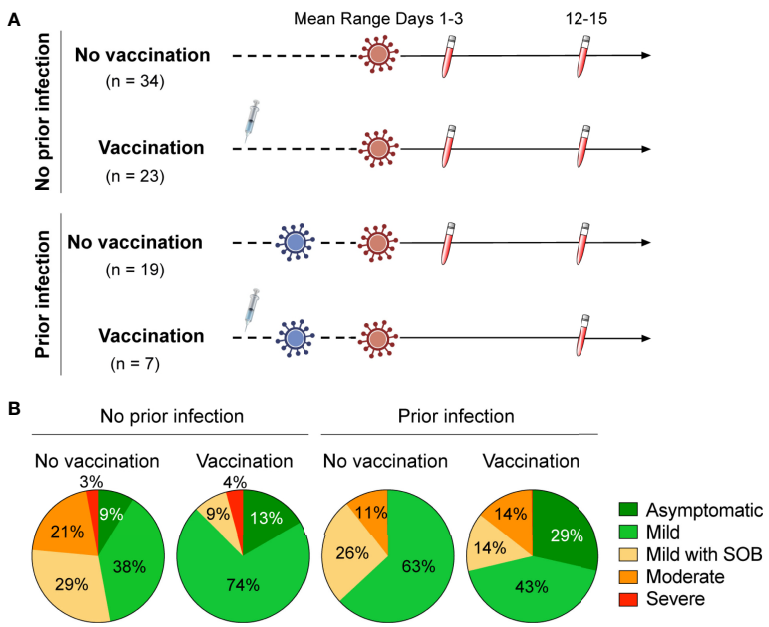

FIGURE 1 | Study design and outpatient symptomology. **(A)** Schematic presentation of the experimental workflow. All 83 study subjects were infected by the SARS-CoV-2 Omicron variant with or without prior infection with another SARS-CoV-2 variant. Four groups were studied: no vaccination/no prior infection (n=34), vaccination/no prior infection (n=23), no vaccination/prior infection (n=19) and vaccination/prior infection (n=7) (**Table 1**). Blood was collected from the outpatients from three groups at two timepoints and from the fourth group at one time point after testing PCR positive. The range of the means of the different groups are shown. **(B)** Symptomology of the study cohorts. SOB, shortness of breath.

TABLE 1 | Characteristics of Omicron study population.

	No prior infection		Prior infection		
	No vaccination	**Vaccination**	**No vaccination**	**Vaccination**	**Chi-Square**
Age (years), mean (range)	45 (9-83)	38 (17-82)	33 (10-63)	36 (24-52)	9.07
Gender Female					
	26 (76%)	13 (57%)	10 (53%)	5 (71%)	0.87
Male	8 (24%)	10 (43%)	9 (47%)	2 (29%)	2.78
Medical condition	16 (47%)	8 (35%)	5 (26%)	1 (14%)	1.98
Auto-immune Disease	1	2	3	0	
Chronic Heart Disease	1	2	0	0	
Chronic Pulmonary Disease	2	1	0	0	
Dementia	1	0	0	0	
Diabetes	1	0	0	0	
Gout	1	0	0	0	
Hypertension	3	1	0	0	
Hypothyroid	3	0	2	0	
Kidney Disease	1	0	0	0	
Multiple Sclerosis	1	2	0	0	
S/P Cancer	1	0	0	1	
COVID-19 vaccine history	0	23	0	7	
3 doses	0	10	0	2	
2 doses	0	13	0	5	
Days from last vaccine dose to positive PCR test, mean (range)	N/A	111 (1-294)	N/A	126 (34-303)	
Days from prior infection dose to positive PCR test, mean (range)	N/A	N/A	98 (21-289)	124 (60-172)	
Days from positive PCR test to sampling, mean (range)					
1st sampling	2 (0-5)	1 (0-3)	3 (0-5)	0	
2nd sampling	13 (6-17)	13 (6-14)	12 (6-18)	15 (6-19)	

N/A, not applicable.

(vaccination/prior infection) was measured at range of group means days 12-15 following initial SARS-CoV-2 RT-PCR test for comparison. Due to recruitment challenges and to the low numbers of these people in the community studied, only samples for the later timepoint were available for that group. Transcriptional studies were limited to days 1-3, the point of highest immune transcriptional response. The percentage of patients with asymptomatic or mild disease without respiratory symptoms was highest in the vaccination/no prior infection group (87%), followed by the no vaccination/prior infection group (63% with mild disease, no asymptomatic), the no vaccination/no prior infection (47% with either mild disease or asymptomatic) group and the vaccination/prior infection (72% with either mild disease or asymptomatic) group (**Figure 1B**). Moderate and severe cases accounted for 24% in the no vaccination/no prior infection group but only 4% of the vaccinated group, 11% in the no vaccination/prior infection group and 14% in the vaccinated/prior infection group (**Figure 1B**).

Vaccination Is Superior to Prior Infection in Preparing the Immune Response to Omicron

First, we measured circulating anti-spike antibody levels in serum samples obtained from the Omicron outpatients within the first three days (days 1-3) following initial SARS-CoV-2 RT-PCR test (**Figure 2**). The anti-Omicron spike IgG levels were highest in the vaccinated patients without prior infection and approximately 10-fold lower in the unvaccinated patients with and without prior infection (**Figure 2A**). An equivalent pattern was obtained for the ancestral strain (**Figure 2A**) and other variants (**Figure S1A**). A significant increase of anti-Omicron spike, but not anti-ancestral spike, antibodies was observed in the vaccinated and the previously infected group within 12-15 days following Omicron infection (**Figure 2A**). Antibody levels did not increase in the naïve group. These findings are mirrored by anti-spike antibody levels from other variants (**Figure S1A**).

At this point in the pandemic, a critical question is whether prior BNT162b2 vaccination can prompt development of neutralizing antibodies in Omicron infected individuals. Here, we assessed neutralization capacity using the angiotensin-converting enzyme 2 (ACE2) binding inhibition assay, against the Omicron spike protein and those from other variants. We measured neutralization within three days following initial SARS-CoV-2 RT-PCR test (Days 1-3) and after 12-15 days (**Figure 2B**). Significant (approximately 11-fold higher in the vaccinated group compared to the no vaccinated group) Omicron neutralizing activity was seen in the vaccinated group, which further increased after 12-15 days following initial SARS-CoV-2 RT-PCR test. Significant neutralizing activity was detected in the previously infected group, with diverse increase, but less than the vaccinated group, at day 12-15 following initial SARS-CoV-2 RT-PCR test. These findings parallel those seen for other variants (**Figure S1B**).

Vaccination but Not Prior Infection Blunts Interferon Responses Elicited by Omicron Infection

To understand the impact of prior vaccination or prior infection on the genomic immune response to Omicron infection, we investigated the immune transcriptome (**Figure 3**; **Table S1**).

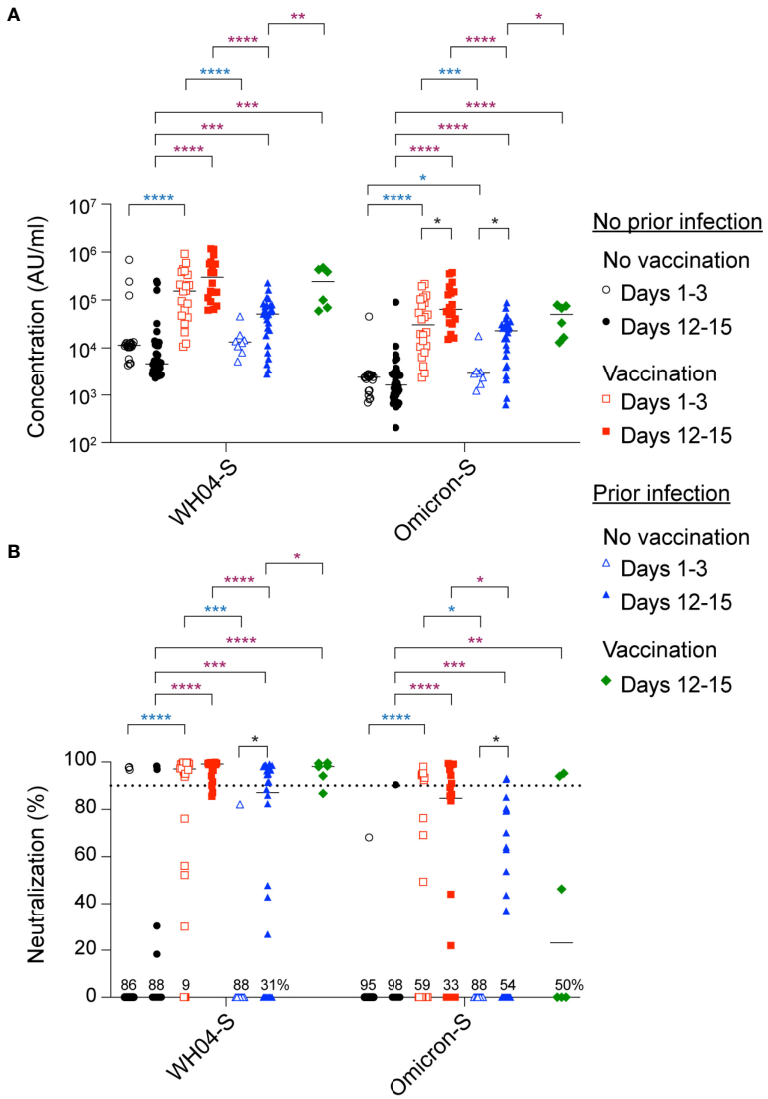

FIGURE 2 | Antibody analysis. **(A)** Plasma IgG antibody binding the SARS-CoV-2 RBD (spike) from the ancestral and Omicron strains in the unvaccinated and vaccinated Omicron patients without or with prior infection experience. **(B)** Neutralization response to virus spike protein of the ancestral and Omicron variants. *p*-value between two groups is from one-tailed Mann-Whitney t-test. Black asterisks indicate significance between days within the same group, blue asterisks indicate significance between groups (range of group means days1-3), purple asterisks indicate significance between groups (range of group means days 12-15). Percentage of samples with zero neutralization activity for each group are indicated over the X axis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$P < 0.0001$. Line at median, dotted line at 90%.

These data sets were also compared to a naïve reference population (no previous SARS-CoV-2 infection and no vaccination) (10) and to hospitalized patients that had been infected with the Alpha variant (7). Bulk RNA-seq on buffy coats isolated within the first two days after validated Omicron infection was performed with an average sequencing depth of 200 million reads per sample. Because no day 1-3 samples were available for the prior infection/no vaccination group (**Figure 1**), the analysis was limited to the no prior infection/no vaccination, no prior infection/vaccination and prior infection/no vaccination groups. First, we directly compared the transcriptomes of the vaccinated and unvaccinated Omicron cohorts without prior infection with a reference cohort of 30 healthy individuals from the same geographic area (Tyrol Control Transcriptomes, TCT). Expression of 489 and 732 genes was induced significantly in the no vaccination and vaccination group, respectively. Expression of 146 and 246 genes was reduced. Next, we compared the transcriptomes between TCT and no vaccination/prior infection group and found 356 significantly induced and 153 reduced genes. GSEA analyses linked the induced genes in three groups to innate immune responses including interferon response and cytokine signaling through the JAK/STAT pathway (**Figures 3A, B**). Among the JAK/STAT responsive genes activated in the Omicron patients is the interferon induced

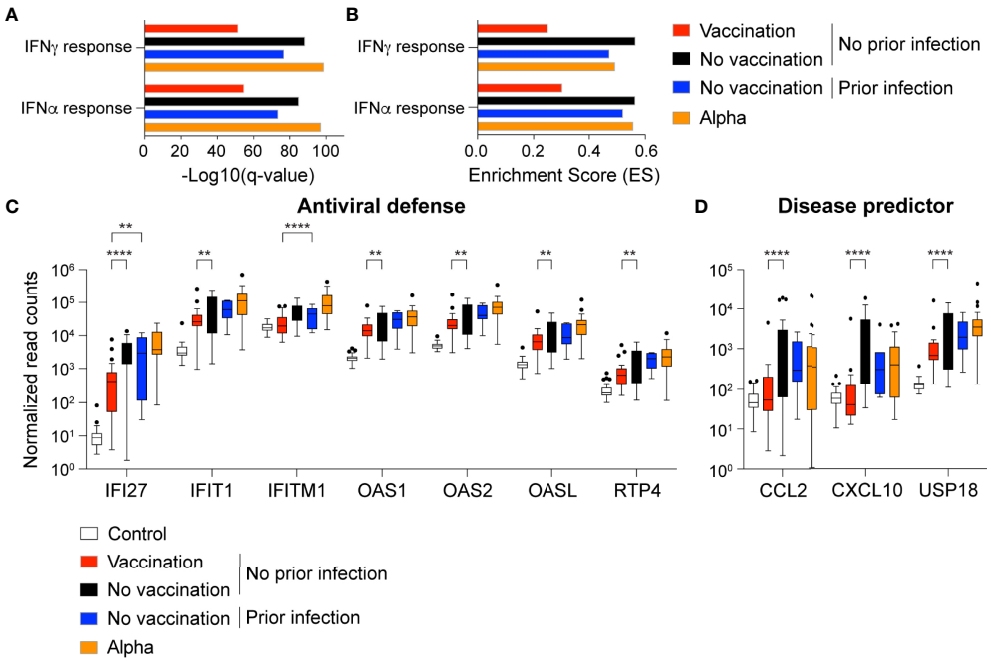

FIGURE 3 | Immune transcriptomes following Omicron infection. **(A, B)** Gene categories expressed at significantly higher levels in unvaccinated and vaccinated Omicron patients without prior SARS-CoV-2 infection, unvaccinated Omicron patients with prior SARS-CoV-2 infection, and a reference group of patients infected by the SARS-CoV-2 Alpha variant were significantly enriched in interferon-activated and inflammatory pathways. X-axis denotes statistical significance as measured by minus logarithm of FDR q-values **(A)** and enrichment score (ES) **(B)**, respectively. Y-axis ranked the terms by q values **(A)** or enrichment score **(B)**. **(C, D)** Bar plots with the normalized read counts to mRNA levels of fifteen innate immune response genes of the 23 genes that are significantly induced in all cohorts and higher in the 'no vaccination group' compared to 'vaccination group' **(C)** and seven that are significantly induced in the 'no vaccination' group, but not 'vaccination group', and higher in No vaccination group compared to Vaccination group **(D)**. Asterix shows significance between Omicron groups, and significance between healthy control and COVID-19 groups left out. *p*-value are from 2-way ANOVA with Tukey's multiple comparisons test. $^{**}p < 0.01$, $^{****}p < 0.0001$. Median, middle bar inside the box; IQR, 50% of the data; whiskers, 1.5 times the IQR.

family (IFI) and the antiviral OAS genes (**Figure 3C**). Overall, the differences observed between the cohorts were of a quantitative rather than a qualitative nature.

We directly explored those genes that were differentially expressed between vaccinated and unvaccinated outpatients depending on their prior infection status. (**Figures 3C, D**, **Table S1**). In general, the activation of genes linked to antiviral defense programs and COVID-19 disease predictors was lowest in the vaccinated patients without prior infection and highest in the patients infected with the Alpha variant (**Figures 3C, D**). The antiviral defense programs encompass interferon-induced gene families, including the OAS family that counteract viral attacks by degrading viral RNAs. We also identified genes that had not previously linked to COVID-19, such as USP18, an IFN-induced gene encoding a negative regulator of type I IFN signaling (11), which highly activated in unvaccinated Omicron patients and Alpha patients.

Of particular interest is the OAS family of antiviral enzymes where hypomorphic mutations have been associated with susceptibility to viral infection and activating mutations with autoimmune disease. The OAS1 member of this family is of particular interest as it harbors a mutation traced back to the Neanderthal genome that results in a splice variant associated with protection from severe COVID-19 (12). RNA-seq data revealed that the rs10774671 haplotype was found in 83% of our control population (TCT cohort), 86% and 85% in the unvaccinated and vaccinated Omicron population, respectively and 96% of the Alpha population. Induction of OAS1 expression is highest in unvaccinated Omicron patients and the Alpha patients (**Figure 3C**).

DISCUSSION

Omicron infection of unvaccinated and vaccinated people has been reported to result in milder disease relative to previous variants (13). However, unlike the response to Delta breakthrough infection (5, 6, 14), Omicron breakthrough infections might result in lower levels of neutralizing antibodies (15). The muted antibody response may be due to the high share of asymptomatic and mild infections as is also indicated by a less active immune transcriptome shown in our study. Notably, individuals with prior vaccination showed significant Omicron neutralization activity, even in the presence of a blunted transcriptional response. Naïve individuals demonstrated significantly higher transcriptional

response but a less robust humoral response. The response in vaccinated or prior infection groups was quantitatively less, but similar in unvaccinated/no prior infection group, when compared to the transcriptional response of hospitalized individuals with Alpha infection. Vaccination was vastly superior to prior infection in promoting neutralizing Omicron antibodies and subsequent Omicron infection yielded an elevated antibody response in both groups. In contrast, Omicron infection in the antigen naïve population did not result in any significant antibody production within a time window of two weeks, suggesting that the initial exposure to Omicron spike proteins does not elicit a substantial immune response.

The study documented that both antibody and immune transcriptional responses to Omicron infection are modified by prior vaccination more significantly than by prior infection. Antibody response is higher in concert with a blunted immune transcriptional response. In this group of largely previously healthy individuals there were no significant differences in symptomatology that correlated with either antibody or transcriptional response, but it needs to be noted that few patients in the cohorts developed severe disease and all were outpatients. It is established that prior vaccination is protective against hospitalization for Omicron infection (16) and known that antibody generation is impacted by antigen exposure history and presentation through vaccination as compared to infection (17). While other studies have similarly examined antibody titers and neutralizing responses in Omicron breakthrough infections, here we integrated the innate immune transcriptome response. This added information layer enabled us to reveal that vaccination, but not previous infection, blunted the Omicron-induced immune transcriptome response at the same time it augmented the antibody response. There are relatively few studies comparing transcriptome response following SARS-CoV-2 infection in vaccinated and unvaccinated individuals. In a previous study, we examined the comparative response with vaccination prior to infection and found an enhanced JAK-STAT-mediated response in vaccinated as compared to unvaccinated patients (8). There are three significant differences between this previous study and the present one. First, here we examined the response a mean of 111 days following vaccination whereas in the previous study it was only 11 days. Second, here all patients were outpatients while in the previous study all patients were hospitalized with infection. Third, in the previous study patients were infected with the SARS-CoV-2 Beta variant while in the present study we examined the transcriptional response to Omicron. We found an important difference, while JAK-STAT signaling was enhanced in the vaccinated patients in the previous study, here we found a blunted response. It is possible this is due to a difference in disease severity or the timing of the vaccination prior to infection or a variant-specific because breakthrough Omicron infection has been reported to be less immunogenic than infection with other variants (15, 18, 19). Other groups as well as we have examined the immune transcriptional response post vaccination (10, 20–22) and document that, like acute SARS-CoV-2 infection, innate immunity pathways are activated.

The role of prior vaccination in augmenting antibody response to Omicron infection has been reported across a range of available vaccines but not all studies examined the response following Omicron infection. Here we specifically focused on the response following documented Omicron infection. A study available in preprint form similarly reports comparing 14 vaccinated and 7 unvaccinated Omicron infected patients show higher convalescent titers and neutralization activity than unvaccinated patients (23). Omicron-infected patients with prior vaccination with both Pfizer-BNT162b2 or J&J-Ad26 were shown to develop higher Delta virus neutralization titers (24). Broader T cell immunity following Omicron infection in Pfizer/BioNTech BNT162 vaccinated individuals is reported (25).

Higher neutralization activity against Omicron was found in Ad26.COV2.S vaccinated individuals who then experienced breakthrough Delta infection (26, 27). Prior Comirnaty or Coronvac vaccination results in higher neutralization titers against Omicron subvariants as well as other SARS-CoV-2 variants (28). The ability of Omicron to evade the immune system is both an individual clinical concern as well as an issue for population-based infection control. Breakthrough Omicron infections are documented in individuals with a range of vaccine-induced protective antibody titers (29). Booster vaccination can increase antibody levels and activity but cannot absolutely prevent breakthrough infection (30). Overall, it has been observed that Omicron is the most neutralization resistant SARS-CoV-2 variant of concern (VOC) (31).

A recent study determined that neutralizing antibody titers tracked with clinical severity of Omicron breakthrough infections in previously vaccinated patients (15). We did not see any significant differences in antibody levels between asymptomatic to mild breakthrough infections compared to moderate or severe infections. These differences might lie in the demographics of the two populations. Our cohort was significantly younger with few underlying health conditions and milder disease. We also did not observe a correlation between antibody levels and disease severity in Omicron breakthrough infections of patients that had previous documented infections by other SARS-CoV-2 variants. Interferon type I IFN (IFN-I) signaling has been previously reported to correlate with higher disease severity in a study of five unvaccinated patients admitted to hospital with the ancestral COVID-19 strain (32, 33) and correlated with progression to pulmonary fibrosis in a study of 12 unvaccinated hospitalized severely ill patients infected with the ancestral strain (34). In our larger study of outpatients infected with Omicron the blunted interferon gamma response found in vaccinated individuals was not correlated with more severe disease. The SARS-CoV-2 pandemic has been typified by progression through evolving variants. Our study recruited patients during the initial BA.1 phase of Omicron infection. It has been reported that neutralization evasion is similar for the different Omicron variants (35), suggesting that results reported here may be predictive of response to the newer variants as well.

Identification of biomarkers predictive of disease progression has the possibility of contributing positively to clinical

management of patients. Type I Interferon response, RIG-I signaling, and multiple proteins known to be induced by interferon signaling including CXCL10 (also known as Interferon Gamma-induced protein, IP-10), MCP-1, MCP-2 and CXCL11 have been reported in a recent preprint to be associated with COVID-19 disease progression in a study of eight patients (22). As discussed above, in the cohort study here we had insufficient numbers of patients with progressive severe disease to test for biomarkers of progression. We did, however, note that CXCL10, identified as a marker of disease progression in this cited study was significantly elevated in the unvaccinated as compared to vaccinated patients in our study. We also found significant elevations in CCL2 and USP18. CCL2 has been linked to influenza induced disease severity in an animal model (36) and documented as one of the significantly increased chemokines in severe COVID-19 hyperinflammation syndrome (37). USP18 is capable of attenuating Type 1 Interferon signaling, which has been associated with persistence of both Hepatitis B and C infection in liver (38). It is possible that elevations of USP18 might contribute to SARS-CoV-2 persistence as well, an aspect of SARS-CoV-2 infection that was not examined in this study but could be approached in a systematic way in a prospective study designed around the question of SARS-CoV-2 viral persistence.

In summary, vaccination prior to Omicron infection modifies humoral and transcriptional responses with higher antibody and neutralization titers and lower interferon gamma activation than that found in unvaccinated individuals either with or without prior infection. Transcriptome studies drew attention to specific genes of interest for future studies of the impact of vaccination on SARS-CoV-2 disease presentation and progression.

METHODS

Ethics

This study was approved (EK Nr: 1064/2021) by the Institutional Review Board (IRB) of the Office of Research Oversight/ Regulatory Affairs, Medical University of Innsbruck, Austria, which is responsible for all human research studies conducted in the State of Tyrol (Austria). The investigators do not need to have an affiliation with the University of Innsbruck. Participant information was coded and anonymized.

Study Population, Study Design and Recruitment

A total of 83 patients infected with Omicron were recruited for the study under informed consent. Thirty-four with no history of prior vaccination or prior infection with other SARS-CoV-2 variants, 23 patients who had received 2 or 3 doses of the BNT162b2 vaccine, 19 unvaccinated patients with prior infections and 7 vaccinated patients with prior infection (**Table 1**). Recruitment and blood sample collection took place between December 2021 and March 2022. Patients were enrolled after either attending an outpatient clinic at Krankenhaus St. Vinzenz Zams for SARS-CoV-2 PCR testing, or, as a family contact with a positive SARS-CoV-2 PCR test. Variant PCR screening for single nucleotide polymorphisms characteristic for Omicron was used to detect Omicron infection. Symptomatic patients presented themselves to the clinic within a few days of initial symptoms. Single or serial blood samples were collected from consenting patients. Day numbers for samples refers to the number of days following initial positive SARS-CoV-2 RT-PCR test. Patient recruitment was performed by a medic who assessed clinical status including performance of an oxygen saturation (SpO2) test. The clinical spectrum of the patients' SARS-CoV-2 symptoms were classified based on the National Institutes of Health (NIH) treatment guidelines (https://files.covid19treatmentguidelines.nih.gov/guidelines/section/section_43.pdf). A subset of patients across groups reported subjective shortness of breath (SOB) but did not show clinical evidence of lower respiratory disease during clinical assessment or by imaging. These patients were classified as mild with shortness of breath for this study. Information on prior infection was obtained from medical records. No patient was admitted to hospital with their prior infection. Disease severity with the prior infection in these patients ranged from mild to moderate to severe based on the NIH guidelines. Prior infection variant and number of days (mean and range) infected prior to Omicron infection for the no vaccination/prior infection group are as follows: Delta (n=9, mean 79 days, range 42-189 days, D614G (n=4, mean 181 days, range 55-289 days, unknown variant n=6, mean 70 days, range 21-158 days. Prior infection variant and number of days (mean and range) infected prior to Omicron infection for the vaccination/prior infection group are as follows: Delta (n=4, mean 55 days, range 34-64 days, D614G (n=2, mean 181 days, range 67-295 days, Alpha n=1, 303 days. The reference "Alpha" cohort consists of individuals (n=36, mean age 71 years) infected with the Alpha variant in the spring of 2021 that were hospitalized after developing COVID-19 (7). Blood samples were collected within 10 days of verified SARS-CoV-2 infection and the RNA was prepared by the same person, who isolated the RNA for the current Omicron study. RNA-seq was conducted using the same supplies and equipment as in the current Omicron study and data analysis was performed by the same person (HKL). A waiver of informed consent was obtained from the Institutional Review Board (IRB) of the Office of Research Oversight/Regulatory Affairs, Medical University of Innsbruck (https://www.i-med.ac.at/ethikkommission/). Written informed consent was obtained from all subjects. This study was determined to impose minimal risk on participants. All methods were carried out in accordance with relevant guidelines and regulations. All research has been have been performed in accordance with the Declaration of Helsinki (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/). In addition, we followed the 'Sex and Gender Equity in Research – SAGER – guidelines' and included sex and gender considerations where relevant.

Antibody Assay

Whole blood was collected by medical personnel after subjects had tested positive for SARS-CoV-2. Antibody containing sera were obtained by centrifuging EDTA blood samples for 10 min at 4,000g. End-point binding IgG antibody titers to various SARS-CoV-2–derived antigens were measured using the Meso Scale Discovery (MSD) platform. SARS-CoV-2 spike, nucleocapsid, Alpha, Beta, Gamma, Delta, and Omicron spike subdomains were assayed using the V-plex multispot COVID-19 serology kits (Panel 23 (IgG) Kit, K15567U). Plates were coated with the specific antigen on spots in the 96 well plate and the bound antibodies in the samples (1:50000 dilution) were then detected by anti-human IgG antibodies conjugated with the MSD SULPHO-TAG which is then read on the MSD instrument which measures the light emitted from the tag.

ACE2 Binding Inhibition (Neutralization) ELISA

The V-PLEX COVID-19 ACE2 Neutralization kit (Meso Scale Discovery, Panel 23 (ACE2) Kit, K15570U) was used to quantitatively measure antibodies that block the binding of ACE2 to its cognate ligands (SARS-CoV-2 and variant spike subdomains). Plates were coated with the specific antigen on spots in the 96 well plate and the bound antibodies in the samples (1:10 dilution) were then detected by Human ACE2 protein conjugated with the MSD SULPHO-TAG which is then read on the MSD instrument which measures the light emitted from the tag.

Extraction of the Buffy Coat and Purification of RNA

Whole blood was collected, and total RNA was extracted from the buffy coat and purified using the Maxwell RSC simply RNA Blood Kit (Promega) according to the manufacturer's instructions. The concentration and quality of RNA were assessed by an Agilent Bioanalyzer 2100 (Agilent Technologies, CA).

mRNA Sequencing (mRNA-Seq) and Data Analysis

The Poly-A containing mRNA was purified by poly-T oligo hybridization from 1 mg of total RNA and cDNA was synthesized using SuperScript III (Invitrogen, MA). Libraries for sequencing were prepared according to the manufacturer's instructions with TruSeq Stranded mRNA Library Prep Kit (Illumina, CA, RS-20020595) and paired-end sequencing was done with a NovaSeq 6000 instrument (Illumina) yielding 200-350 million reads per sample.

The raw data were subjected to QC analyses using the FastQC tool (version 0.11.9) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). mRNA-seq read quality control was done using Trimmomatic (39) (version 0.36) and STAR RNA-seq (40) (version STAR 2.5.4a) using 150 bp paired-end mode was used to align the reads (hg19). HTSeq (41) (version 0.9.1) was to retrieve the raw counts and subsequently, Bioconductor package DESeq2 (42) in R (https://www.R-project.org/) was used to normalize the counts across samples (43) and perform differential expression gene analysis. Additionally, the RUVSeq (44) package was applied to remove confounding factors. The data were pre-filtered keeping only genes with at least ten reads in total. The visualization was done using dplyr (https://CRAN.R-project.org/package=dplyr) and ggplot2 (45). The genes with log2 fold change >1 or <-1 and adjusted p-value (pAdj) <0.05 corrected for multiple testing using the Benjamini-Hochberg method were considered significant and then conducted gene enrichment analysis (GSEA, https://www.gsea-msigdb.org/gsea/msigdb).

Quantification and Statistical Analysis

Differential expression gene (DEG) identification used Bioconductor package DESeq2 in R. P-values were calculated using a paired, two-side Wilcoxon test and adjusted p-value (pAdj) corrected using the Benjamini–Hochberg method. The cut-off value for the false discovery rate was pAdj > 0.05. Genes with $\log_2$ fold change >1 or <-1, pAdj <0.05 and without 0 value from all sample were considered significant. For significance of each GSEA category, significantly regulated gene sets were evaluated with the Kolmogorov-Smirnov statistic. P-values of antibody between two groups were calculated using one-tailed Mann-Whitney t-test on GraphPad Prism software (version 9.0.0). A value of $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ was considered statistically significant.

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 in the article/**Supplementary Material**. RNA-seq data generated from this study were deposited under the accession GSE201530 in the Gene Expression Omnibus (GEO). RNA-seq data from buffy coat of healthy control and COVID-19 Alpha patients were obtained GSE189039, GSE190747 and GSE190680.

ETHICS STATEMENT

This study was approved (EK Nr: 1064/2021) by the Institutional Review Board (IRB) of the Office of Research Oversight/Regulatory Affairs, Medical University of Innsbruck, Austria, which is responsible for all human research studies conducted in the State of Tyrol (Austria). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HL, LK, PF, and LH designed the study. LK Sr, MF, YC, CJ, PK, MO, CB, NK, AZ, and GW recruited patients and collected material. HL analyzed RNA-seq data. MW and YD conducted IgG antibody and neutralization assay. HL, PF, and LH analyzed data. HL administrated project. HL, PF, and LH wrote the paper. All authors read and approved the manuscript.

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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.2022.916686/full#supplementary-material

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High baseline expression of IL-6 and IL-10 decreased CCR7 B cells in individuals with previous SARS-CoV-2 infection during BNT162b2 vaccination

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The current pandemic generated by SARS-CoV-2 has led to mass vaccination with different biologics that have shown wide variations among human populations according to the origin and formulation of the vaccine. Studies evaluating the response in individuals with a natural infection before vaccination have been limited to antibody titer analysis and evaluating a few humoral and cellular response markers, showing a more rapid and intense humoral response than individuals without prior infection. However, the basis of these differences has not been explored in depth. In the present work, we analyzed a group of pro and anti-inflammatory cytokines, antibody titers, and

cell populations in peripheral blood of individuals with previous SARS-CoV-2 infection using BNT162b2 biologic. Our results suggest that higher antibody concentration in individuals with an earlier disease could be generated by higher production of plasma cells to the detriment of the presence of memory B cells in the bloodstream, which could be related to the high baseline expression of cytokines (IL-6 and IL-10) before vaccination.

KEYWORDS

SARS-CoV-2, BNT162b2, Interleukin 6, Interleukin 10, CCR7 B cells

Introduction

The SARS-CoV-2 infection has revealed gaps in the immune response concerning coronaviruses affecting human populations (1–3). Many publications have evaluated the antibody production in infected and vaccinated people (4–6). Antibody levels vary from very low in patients with mild or asymptomatic infections to high levels in hospitalized infected patients (7–10). The massive vaccination with different biologics has also shown wide variations among human populations depending on the origin and vaccine formulation (5, 6, 11). However, there are scarce studies where antibody titers have been measured by comparing healthy people with vaccinated people who have suffered a SARS-CoV-2 infection (12–14). These studies have shown that a previous infection correlates with higher antibody titers. However, the bases for these differences have not been explored in depth.

This work determined the antibody responses in a sample of people vaccinated with the biological BNT162b2, separating the population into people who previously suffered or did not have an infection with SARS-CoV-2. In addition to the antibody titers, various cell populations and pro- and anti-inflammatory cytokines were analyzed. Given the number of parameters studied in this work, we decided to use a principal component analysis (PCA), looking for those parameters that could best correlate with the differences in antibodies that PCA allowed by concentrating on a few parameters, simplifying the analysis.

The population of B lymphocytes expressing the chemokine receptor CCR7 decreased in those who previously had an infection with SARS-CoV-2. Likewise, people showed increased IL-10, IL-12p70, and IL-6 levels once infected. Interleukins 6 and 10 participate in the differentiation of activated B lymphocytes towards plasma cells, which could correlate with higher antibody titers. In contrast, IL-12p70 could participate, *via* gamma interferon stimulation, in the change of isotype towards IgG.

Our results suggest that people who suffered a previous SARS-CoV-2 infection once vaccinated with the biological BNT162b2 generate a more significant production of plasma cells to the detriment of the generation of memory B lymphocytes circulating through the secondary lymph nodes.

Materials and methods

Study population and sampling

This work was a longitudinal observational study in a single health center, including adults with previous SARS-CoV-2 infection and naïve. The local Ethical Committee approved the study (CE/FESI/022021/1380). A total of 65 individuals from the staff of the Health Institutes of Veracruz, Mexico, vaccinated with the Pfizer-BioNTech biological (BNT162b2) were selected 20 with a previous SARS-CoV-2 infection (pre-infected) and 45 naïve (Table 1). The peripheral blood samples for the analysis were taken on day 0, before the first dose (T1), the day of the second dose (T2), and the third sample was obtained 14 days after the boost (T3).

Cytokine quantification by flow cytometry

The panel of cytokines IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, CXCL8 (IL-8), CXCL10 (IP-10), CCL2 (MCP-1), IFN-γ, TNF-α, and TGF-β1 were measured in the peripheral blood plasma using LEGENDplex™ HU Essential Immune Response Panel (13-plex) kits (Biolegend), according to the manufacturer's instructions to quantify the absolute values of cytokines. The flow cytometry data acquisition was performed with (CytoFLEX S, Beckman Coulter) equipment, and the results were analyzed using the LEGENDplex™ software.

Cell population quantification by flow cytometry

Multicolor staining with monoclonal antibodies and flow cytometry was used to identify subpopulations of B lymphocytes (CD19+, CD20+, CD19+ CD27+, CD20+ CD27+, CD19+ CCR7+, CD20+ CCR7+), T lymphocytes (CD3+ CD4+, CD3+ CD8+, CD4+ CD25+ CD127Low, CD3+ CD4+ CD45Ra+, CD3+ CD4+

TABLE 1 General data of the study population.

	Naïve	Pre-infected
Number of individuals	45	20
Women(%)	67	65
Men (%)	33	35
average age (years)	42.2 (12)	39.9 (13)
minimum age (years)	24	29
maximum age(years)	57	58
Median age (years)	44	39
1st Quartile age (years)	37	36
3rd Quartile age (years)	49	49
Mean time from the onset of infection to the first dose (months)	NA	6.6 (6)
Minimum time from onset of infection to the first dose (months)	NA	1
Maximum time from onset of infection to the first dose (months)	NA	10
Median time from onset of infection to the first dose (months)	NA	7
1st Quartile time from onset of infection to the first dose (months)	NA	3
3rd Quartile time from onset of infection to the first dose (months)	NA	9

The study population is described, including the mean and, in parentheses, the ICR of the quantitative variables.
NA, not applicable.

CD45Ra+ CCR7+, CD3+ CD8+ CD45Ra+, CD3+ CD8+ CD45Ra+ CCR7+), NK lymphocytes (CD56+, CD57+, CD3- CD16+ CD56+, CD3- CD16+ CD57+) and monocytes (CD14+, CD14+ CCR7+, CD14+ TLR4+, CD14+ TLR4+ CCR7+ CD11c+, HLA-DR+) (Supplementary Figure 1). A volume of whole blood with 1×10^6 white blood cells (previously counted with hemocytometer) were stained with the four antibody panels and incubated for 30 minutes at room temperature in darkness, washed with phosphate-buffered saline containing 0.1% bovine serum albumin, and lysed with the OptiLyse C reagent (Beckman Coulter) following the manufacturer's recommendations. Samples were analyzed with the Cytoflex S system (Beckman Coulter), 100,000 events acquired in each of the four panels used, and data was analyzed with the Kaluza C software (Beckman Coulter).

Anti-SARS-CoV-2 quantification by flow cytometry

The anti-SARS-CoV-2 anti-spike and anti-RBD antibodies were quantified in plasma using the LEGENDplex™ SARS-CoV-2 Serological IgG Panel Detection Abs (Biolegend), following the manufacturer's instructions for determining the absolute antibody values. The acquisition was carried out by flow cytometry (Cytoflex S), and the results were analyzed with the LEGENDplex software.

Principal component analysis (PCA)

Through the FactoMineR package in R software, we performed a PCA, which summarizes and visualizes the information in all our data sets to describe multiple inter-correlated quantitative variables; we also added a concentration ellipse around pre-infected and naïve clusters from a mean point using the default confidence level (0.95) underlying Gaussian distribution. We used PCA to extract the essential variables to express the principal components (variables) involved in differentiating the response to the BNT162b2 vaccine between pre-infected and naïve individuals.

Statistical analysis

After the PCA analysis, we selected the normalized group data, compared the most representative variables between the different clusters, and did a Student's t-test. Values with a confidence interval of 95 and P-values ≤ 0.05 were considered statistically significant.

Results

Antibodies production

Anti-RBD and anti-Spike antibodies were determined at the three-time points, T1, T2, and T3. In pre-infected SARS-CoV-2 individuals, anti-RBD (4.32 ng/mL on average) and anti-Spike (19.84 ng/mL antibodies were identified on average from the T1 moment of the first vaccination (Figures 1A, B). In the case of pre-infected and naive individuals, the concentration of anti-Spike and anti-RBD antibodies increased steadily during follow-up, reaching an average concentration of 2930 ng/mL and 559.03 ng/mL, respectively at the last sampling. In the case of the anti-Spike antibodies of the pre-infected individuals, 2930 ng/mL and 2325.1 ng/µl for the naïve individuals (Figure 1B); while the average values reached for the anti-RBD antibodies were 334.921 ng/µL for the naive individuals and 559.03 ng/mL in the case of pre-infected individuals (Figure 1A).

Principal component analysis (PCA)

To reduce the dimensionality of the multivariate and to address the complexity of the immune response generated by the vaccine in the population studied with minimal loss of information, we constructed a PCA at each of the three moments (T1, T2, and T3), using values of all the evaluated subpopulations cells and the determination of cytokines

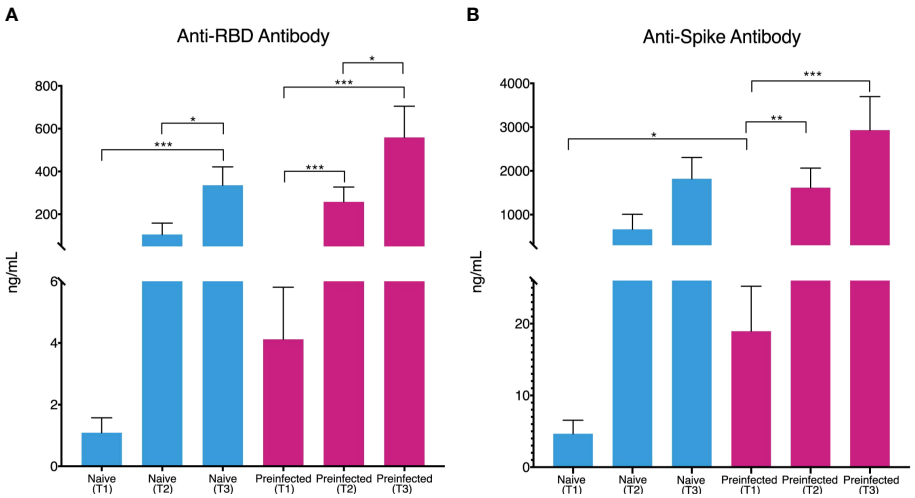

FIGURE 1
Production of Anti-RBD and Anti-Spike antibodies during vaccination. Individuals without previous infection (blue bars, n= 45) and with previous SARS-CoV-2 infection (red bars, n = 20) showed an increase in the concentration (ng/mL) of Anti-RBD antibodies **(A)** throughout the three times evaluated [T1 (1.084 and 4.12 mean), T2 (105.32 and 258.11 mean) and T3 (334.91 and 559.03 mean)]. Anti-Spike antibody concentration **(B)** showed the same behavior [T1 (4.65 and 18.94 mean), T2 (664.71 and 1615.25 mean), and T3 (1819.72 and 2930.08 mean)]. (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, two-sided t-test). vertical lines show the standard error.

(Figure 2). Considering that the size of the constructed ellipse depends directly on the variance between the data, we can assume that the differences in cell subpopulations and cytokine concentration between pre-infected and naive clusters at times T1 and T2 (Figures 2A, B) are less than the variation that occurs between these two groups in T3 (Figure 2C).

Modified cell populations

The PCA allowed us to summarize and identify the most critical parameters that differentiate the response generated by the vaccine in pre-infection compared with naive individuals. Of these principal components, the cell populations that showed statistically significant differences are B lymphocytes,

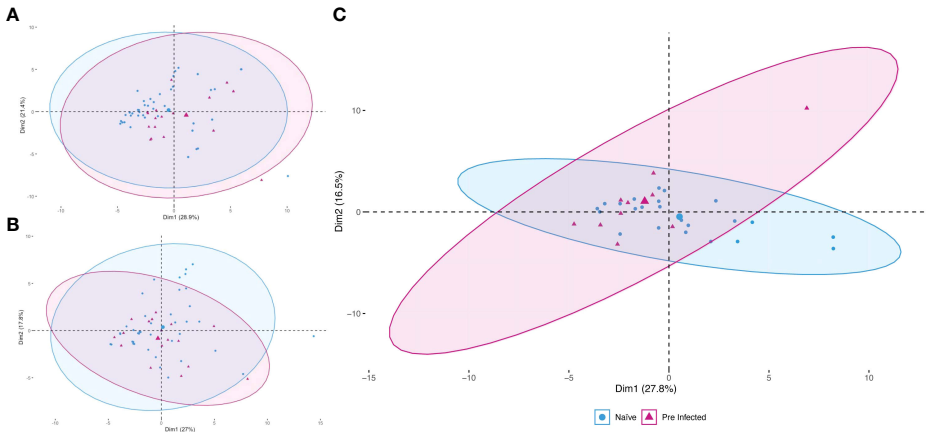

FIGURE 2
Principal component analysis during vaccination. The study was applied during the three times evaluated [T1 **(A)**, T2 **(B)**, and T3 **(C)**]. Red triangles represent pre-infected individuals (n = 20). Blue circles represent individuals without prior SARS-CoV-2 infection (n = 45). The more prominent symbols represent each population's centroid (mean), and the concentration ellipses represent the estimates according to a Gaussian distribution at a 95% confidence level for each group.

specifically the populations of CD19+ CCR7+, CD20+ and CD20+ CCR7+ cells (Figure 3A). In addition, all these populations were reduced in individuals with a previous infection compared to naive individuals. Both results were observed at the second vaccine dose (T2) and on day 14 after the boost (T3) (Figures 3A, B), meanwhile on day zero, before the application of the first dose (T1), no significant difference was observed in these cell markers (data not shown), this could be due to the variation in the time of infection and vaccination in the population studied (Table 1).

Modified cytokines

Of the principal components obtained from PCA that correspond to cytokines, IL-10 (32.5 pg/mL from naïve and 29.3 pg/mL from pre-infected average concentration), IL-12p70 (28.9 pg/mL from naïve and 29.4 pg/mL from pre-infected average concentration), and IL-6 (62.5 pg/mL from naïve and 64.0 pg/mL from pre-infected average concentration) showed significant differences ($p \leq 0.005$) (Figure 4), the concentration is higher in individuals with an infection before vaccination. These

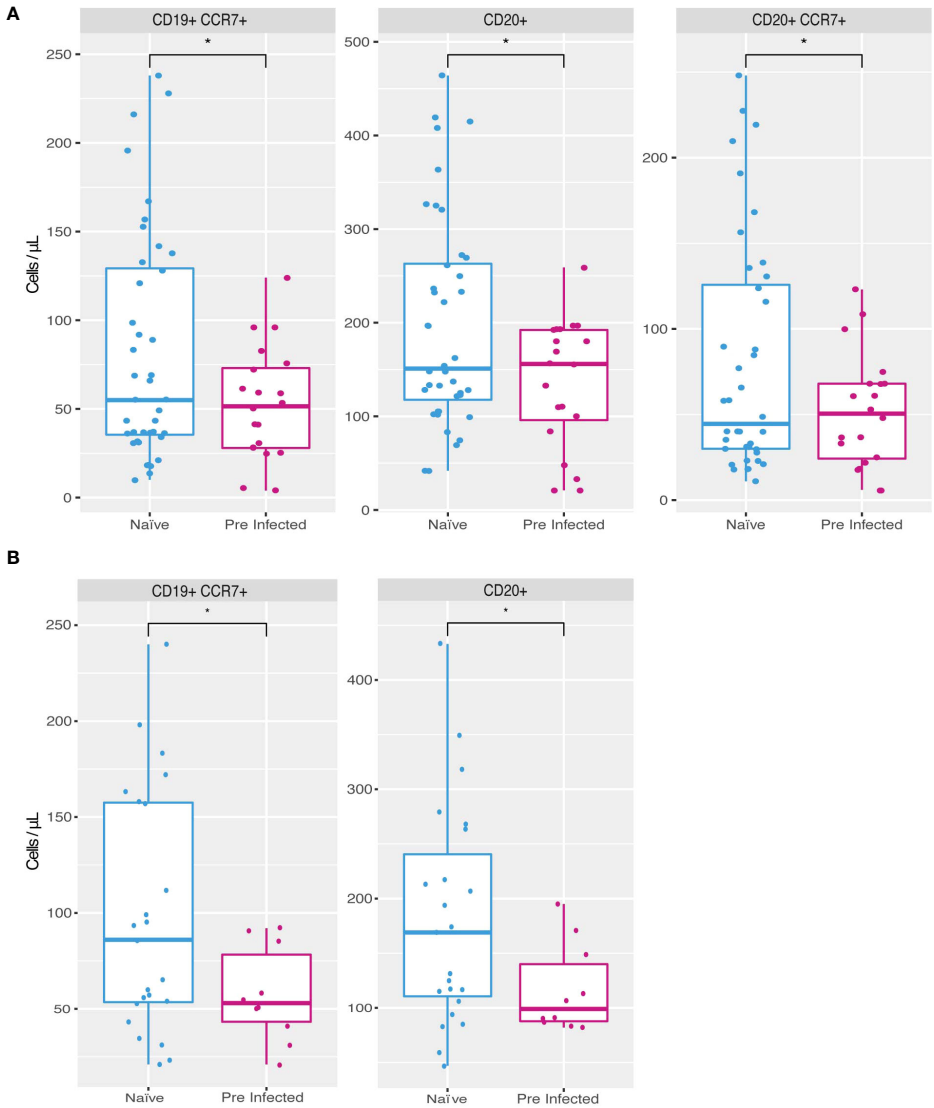

FIGURE 3
Increased cell populations in individuals without previous SARS-CoV-2 infection. Naive individuals (n = 45) showed higher counts of CD19+ CCR7+ (81.9 cells/µl mean), CD20+ (196.87 cells/µl mean) and CD20+ CCR7+ (79.95 cells/µl mean) cells during time 2 **(A)** compared to previously infected individuals (n = 20) (52.85, 136.6 and 51.8 cells/µl mean respectively). During time 3 **(B)** naive individuals presented a higher count of CD19+ CCR7+ (98 cells/µl on average) and CD20+ (181.04 cells/µl mean) populations compared to previously infected individuals (57.5 and 116.8 cells/µl mean, respectively) (*$p \leq 0.05$, two-sided t-test).

differences are observable only before administering the vaccine's first dose.

Discussion

Consistently, with prior studies, we have found that individuals with a previous infection show anti-Spike and anti-RBD antibody titers before administering the first dose of the vaccine, unlike individuals without the previous disease who showed lower values (14, 15). Individuals in the first group (previous infection) tested positive for SARS-CoV-2 before the vaccine and generated a humoral immune response. After the first dose, antibody production is higher in individuals with previous immunity (Figure 1), consistent with findings suggesting that a seropositive state causes a more rapid antibody response to vaccination and reinforces the justification for considering a one-dose vaccine regimen in this population (14, 15).

Our monitoring of antibody titers throughout the vaccination process (including the second dose and 14 days later) showed that the response in individuals with immunity before vaccination is more remarkable, as reported by previous work (13, 16). In addition, individuals with prior infection showed anti-Spike and anti-RBD antibody titers two-fold higher than individuals without disease before vaccination (Figure 1), suggesting that immunity before immunization led to a more intense response, not only after the first dose but throughout the entire vaccine-induced response, providing more robust and longer-lasting protection against infection (17).

Although this more intense response in the production of antibodies has been previously reported by (14, 15, 18, 19), it is clear that there may be more differences in the immune response mounted by individuals with natural infection and without previous natural disease, and some of them could even help to explain the disparity in the humoral response. We evaluated a panel of cytokines and cell populations in peripheral blood and the follow-up to identify these differences. These data were analyzed using a PCA which allowed us to explore and reduce this large set of data, increasing the interpretability and minimizing the loss of information; showing that the study groups begin to show variance from T2, increasing in T3 (Figure 2), a result that coincides with reports where the immune response due to the immunization process becomes evident from day 14 (20).

The resulting principal components and their pattern throughout the follow-up differed only by a couple of elements between the two working groups; however, a higher concentration of pre-vaccination of IL-6 IL-10 and IL12p70 in naturally infected individuals (Figure 4). Although these cytokines have not been previously analyzed in response to vaccination, recent work has shown that these cytokines

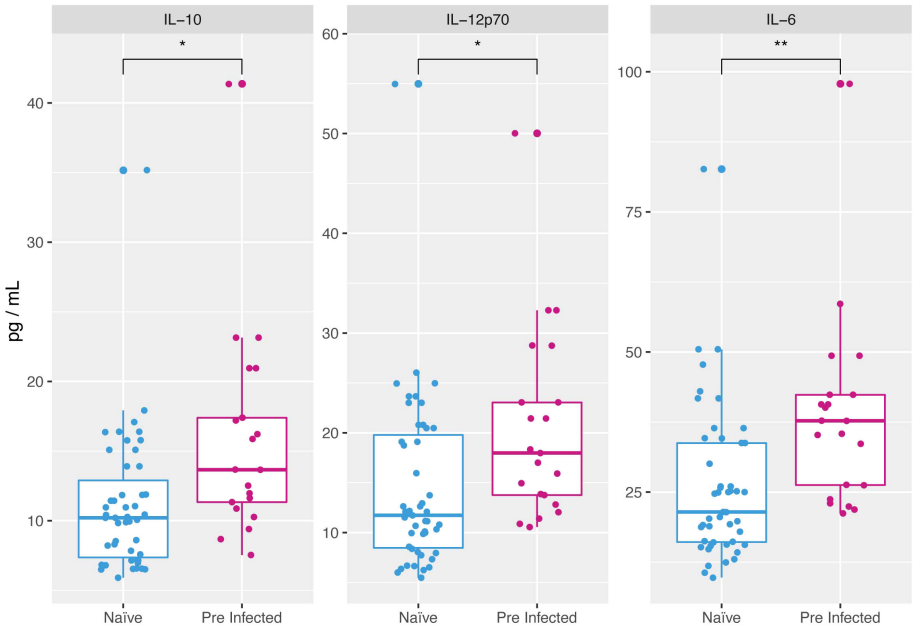

FIGURE 4
Cytokines overexpressed at the beginning of vaccination in individuals with previous SARS-CoV-2 infection. At time 1 naive individuals presented a lower concentration of cytokines IL-10 (11.08 pg/ml mean), IL-12p70 (14.4 pg/ml mean) and IL-6 (25.94 pg/ml mean) compared to individuals with pre-vaccination SARS-CoV-2 infection (15.96, 20.50, and 38.36 cells/µl mean respectively) (*p ≤ 0.05, **p ≤ 0.01, two-sided t-test).

increased their concentration very early during COVID-19 and that their function could be related to the severity of the SARS-CoV-2 infection (21–24).

In the case of IL-6, it is thought that this pro-inflammatory cytokine could be part of an innate inflammatory response that precedes an adaptive response in natural infection, including SARS-CoV-2 infection (24, 25). Thus, the basal concentration is higher in individuals with the previous disease (Figure 4), i.e., it could be related to an earlier induction of an adaptive response, promoting a rapid humoral reaction mediated by the differentiation and proliferation of B cells (24), and therefore with the higher concentration of antibodies in these same individuals. To analyze this possible correlation, we performed a linear regression analysis between IL-6 production in pre-infected individuals and antibody production, finding a Correlation Coefficient with an R value lower than 0.5 (Anti-RBD/IL-6 R = 0.006 and Anti-Spike/IL-6 R = 0.003). Therefore, there is no direct correlation between I-L6 concentration, and the antibody titer found, which is evidence that the increase of IL-6 in the pre-infected individuals may be participating in the increase of the antibody production seen. However, they do not seem to be the only signals responsible for this process. Some other cytokines, receptors, and signaling pathways must be involved.

IL-10 has recently been reported as a crucial biomarker of severity and mortality in patients with COVID-19 disease (21, 26). The early expression of IL-10 could have an anti-inflammatory or immunosuppressive effect, preventing the hyper inflammation that characterizes SARS-CoV-2 infection (24). However, it has been reported that when secreted by regulatory T lymphocytes in patients with severe COVID-19 disease, it would decrease the immune response mediated by T lymphocytes and even their depletion in peripheral blood (27–30). Thus, in the current case of vaccinated individuals with previous infection and high basal levels of IL-10 (Figure 4), this cytokine could decrease the immune response mediated by T lymphocytes to the vaccine but, on the other hand, polarized it to a strong response mediated by B lymphocytes.

Although other studies have shown a differential effect at the serological level of vaccination in individuals with a previous infection by SARS-CoV-2 (13–16), few studies have addressed the differences *in vivo* in the quantification of leukocyte populations in peripheral blood.

In vitro evidence of a response mediated by T lymphocytes in individuals with previous infection and the application of a single dose of BNT162b2 is absent or minimal in individuals without previous disease (31, 32). Our analysis considered the identification of different subpopulations of T lymphocytes. However, no significant differences were observed between the two study groups throughout the follow-up. This result is possibly due to the need to look for other subpopulations of T cells, which may be analyzed by future research.

In the case of other cell populations, our work did identify modifications in B lymphocyte populations in individuals with a previous infection to vaccination, specifically a decrease in the count of B lymphocytes in peripheral blood that express the chemokine receptor CCR7 (Figure 3).

The expression of CCR7 has been previously reported in mature B cells on the way to differentiate into antibody-secreting plasmablasts (33, 34). Therefore, the lower count of these cells in peripheral blood could be related to their migration to the secondary lymphoid organs. They differentiate into plasmablasts and thus increase the concentration of antibodies, which coincides with our finding of a higher average concentration of individuals with the previous infection. However, monitoring these memory plasma cells in peripheral lymphoid organs is almost impossible due to the difficulty of obtaining lymphoid samples from voluntary individuals. For this reason, it could be confirmed using model organisms in future works.

Our work is not the first to report a differential response of B cells from individuals with previous infection and the use of the vaccine (4, 12). However, it does coincide with reports where infection induced a modification in the production of antibodies. This result is possibly related to the activation of memory B lymphocytes (12), which, as our findings suggest, could be generated at the expense of a decrease in circulating mature B lymphocytes (Figure 3).

More importantly, this increased immune response in previously infected individuals could be related to the higher baseline of specific cytokines before vaccination. An analysis with a broader panel of cytokines, including those reported here that participate in the natural response to SARS-CoV-2 infection (24), might reveal a mechanism to explain the higher response and possibly more protective in those suffering SARS-CoV-2 infection before the use of the vaccine.

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 work was approved by the local ethics committee under registration number (CE/FESI/022021/1380). The participants provided their written informed consent to participate in this study.

Author contributions

AP-G: First authorship. MV: Equal contribution and first authorship. MC-A, RJ, EH, RR, CB, GP-G, RÁ, AG, MS, WT-S, CD-M, SO-Q, SS-F, IG-O, RV-M, AM-C, RJ-F, LS-A, JL, HR-R, and VR-G: Equal contribution. BA-B: Senior authorship. 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.2022.946770/full#supplementary-material

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Single-cell analysis of the adaptive immune response to SARS-CoV-2 infection and vaccination

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Amid the ongoing Coronavirus Disease 2019 (COVID-19) pandemic, vaccination and early therapeutic interventions are the most effective means to combat and control the severity of the disease. Host immune responses to SARS-CoV-2 and its variants, particularly adaptive immune responses, should be fully understood to develop improved strategies to implement these measures. Single-cell multi-omic technologies, including flow cytometry, single-cell transcriptomics, and single-cell T-cell receptor (TCR) and B-cell receptor (BCR) profiling, offer a better solution to examine the protective or pathological immune responses and molecular mechanisms associated with SARS-CoV-2 infection, thus providing crucial support for the development of vaccines and therapeutics for COVID-19. Recent reviews have revealed the overall immune landscape of natural SARS-CoV-2 infection, and this review will focus on adaptive immune responses (including T cells and B cells) to SARS-CoV-2 revealed by single-cell multi-omics technologies. In addition, we explore how the single-cell analyses disclose the critical components of immune protection and pathogenesis during SARS-CoV-2 infection through the comparison between the adaptive immune responses induced by natural infection and by vaccination.

KEYWORDS

SARS-CoV-2, infection, vaccine, adaptive immune response, antibody production

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Introduction

Single-cell (sc) technologies, including flow cytometry (FACS), mass cytometry (CyTOF), cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), single-cell RNA sequencing (scRNA-seq), single-cell assay for transposase-accessible chromatin using sequencing (scATAC-seq), single-cell T cell receptor sequencing (scTCR-seq), and single-cell B cell receptor sequencing (scBCR-seq), are well reviewed to characterize the heterogeneity of innate or adaptive immune responses to vaccination, infection, and cancer (1, 2). Briefly, they can 1) address immune heterogeneity and identify novel cell subsets; 2) offer more accurate descriptions of cell phenotypes and their responses to vaccination and infection; 3) deduce the transition, differentiation or developmental relationships between cell subsets; 4) explore the function of antigen-specific cells; 5) characterize the immune repertoire such as functional TCRs and BCRs. These single-cell technologies have been extensively adopted to study the protective and pathogenic mechanisms and to develop new strategies to prevent and treat COVID-19 (3–8).

Especially, the antigen-specific adaptive immune responses, which generally play a vital role in controlling most viral infections (9), are revealed by single-cell technologies in SARS-CoV-2 infection and vaccination. This role is reflected in its critical impact on the various clinical outcomes of SARS-CoV-2 infection and on the efficacy of vaccination. B cells, $CD4^+$ T cells, and $CD8^+$ T cells are three critical arms of the adaptive immune system, contributing to the control of SARS-CoV-2 and the development of immune memory (10–12). $CD4^+$ T cells perform effector functions and provide assistance for other immune cells (13), while $CD8^+$ T cells are important tissue-resident lymphocytes which are ready to eradicate virus-infected cells (14). B cells mainly produce antibodies to neutralize viruses or target virus-infected cells in an Fc-mediated manner (15). Both SARS-CoV-2 infection and vaccination effectively elicited T cell and B cell responses against SARS-CoV-2 (3, 16), providing a high level of protection against symptomatic SARS-CoV-2 infection (17), despite the challenges posed to the waned immunity by the immunity-escaping emerging viral variants. Of note, single-cell analysis together with the classic immunological assay, have greatly contributed to deeper understanding of the dynamics of SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cells, B cells and neutralizing antibodies (18–23) during natural infection and various vaccination (Table 1) (24–36). A comprehensive comparison of the adaptive immune responses with natural infection and vaccination is required to dissect the protective immune properties against SARS-CoV-2 infection. Here, we will review the current knowledge of SARS-CoV-2-specific immune responses during natural infection or vaccination and highlight future directions.

T cell response against SARS-CoV-2

Studies have shown that both SARS-CoV-2 infection and vaccination elicited robust anti-viral T cell responses, showing an increased expression of T-cell relevant cytotoxic signatures (3, 16). A variety of $CD4^+$ and $CD8^+$ T cell subsets were characterized by scRNA-seq analyses. The reported $CD4^+$ T cell subsets by scRNA-seq mainly include naïve (*CCR7*$^+$, *TCF7*^{high}, *LEF1*^{high}), T-helper 1 like cells (T_H1, *TBX21*$^+$ *GNLY*$^+$), T-helper 2 like cells (T_H2, *GZMK*$^+$ *GATA2*$^+$), proliferative cells (*MKI67*$^+$), T follicular helper cells (T_{FH}, *ICOS*$^+$), T-helper 17 like cells (T_H17, *RORC*$^+$ *CCR6*$^+$), T-regulatory cells (Treg, *CTLA4*$^+$ *FOXP3*$^+$), and interferon-stimulated genes (ISGs^{high}) cells. The reported $CD8^+$ T cell subsets by scRNA-seq mainly included naïve (*CCR7*$^+$), effector memory (T_{EM}, *GZMK*$^+$ *LTB*$^+$), cytotoxic terminal effector (T_{TE}, *GNLY*$^+$ *GZMB*$^+$ *PRF1*$^+$), exhausted-like (T_{EX}, *PDCD1*$^+$ *LAG3*$^+$), tissue-resident memory (T_{RM}, *ITGAE*$^+$ *CXCR6*$^+$ *ZNF683*$^+$), and proliferative (*MKI67*$^+$) cells (4, 5, 37–53) (Table 2). The antigen specificity, cross-reactivity, composition and transcription features of these T cell subsets have been extensively explored.

$CD4^+$ T cell responses

SARS-CoV-2 specific $CD4^+$ T cells

Using activation-induced markers (AIM) or MHC-II tetramers, circulating SARS-CoV-2 specific $CD4^+$ T cell responses have been extensively monitored by FACS (54). SARS-CoV-2 infection and vaccination induce robust SARS-CoV-2 specific $CD4^+$ T cell responses that occur prior to the

TABLE 1 The approved COVID-19 vaccines explored by single-cell technologies.

Vaccines	Vaccine type	Immunogens	Reference
mRNA-1273	mRNA	Spike protein	(24–26)
BTN162b2	mRNA	Spike protein	(24, 27–33)
AZD1222	Non-replicating viral vector	Spike protein	(32)
Ad5-nCoV	Non-replicating viral vector	Spike protein	(34)
BBIBP-CorV	Inactivated SARS-CoV-2 virus	Multiple viral antigens	(35)
CoronaVac	Inactivated SARS-CoV-2 virus	Multiple viral antigens	(36)

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TABLE 2 The T cell and B cell subsets identified by single cell technologies and their canonical markers.

Cell subsets	Trait	scRNA-seq marker	Flow cytometry/Mass spectrometry marker	Reference
$CD4^+$ T cell subsets	T_H1-like	*GZMB, GNLY, PRF1, TBX21*	$CD45RA^-$, $CXCR5^-$, $CCR6^-$, $CXCR3^+$, $CCR4^-$	(4, 42–45)
	T_H2-like	*GATA3, GZMK*	$CD45RA^-$, $CXCR5^-$, $CCR6^-$, $CXCR3^-$, $CCR4^+$	(4, 45, 46)
	T_H17-like	*CCR6, RORC*	$CD45RA^-$, $CXCR5^-$, $CCR6^+$	(4, 5)
	T_{FH}	*ICOS, CXCR5*	$CD45RA^-$, $CXCR5^+$	(4, 38)
	Treg	*FOXP3, CTLA4*	$CD25^+$, $CD127^-$	(4, 38, 44)
	Naive	*CCR7, SELL, TCF7*	$CD45RA^+$, $CD62L^+$	(4, 43–45)
	Proliferative	*MKI67, TOP2A*	$Ki67^+$	(4, 6)
	ISGs+ subsets	*ISG15, ISG20, IFI6*	-	(4, 16)
$CD8^+$ T cell subsets	Naive	*CCR7, SELL, TCF7*	$CD45RA^+$, $CD62L^+$, $CD95^-$	(43–45)
	T_{TE}	*GZMB, GNLY, PRF1*	$CD244^+$, $KLRG1^+$	(43–45)
	T_{EM}	*GZMK, LTB*	$CD45RA^-$, $CD127^+$, $CD28^+$, $CD95^+$	(40, 43–45)
	T_{RM}	*CXCR6, ITGAE, ZNF683*	$CXCR6^+$, $CD103^+$	(5, 40, 41)
	T_{EX}	*PDCD1, LAG3*	$PD\text{-}1^+$	(40, 43, 47)
	Proliferative	*MKI67, TOP2A*	$Ki67^+$	(4, 6, 43)
	ISGs+ subsets	*ISG15, ISG20, IFI6*	-	(6, 16)
B cell subsets	Naive	*TCL1A, BACH2*	IgD^+, $CD27^-$	(4, 45, 48, 49)
	Class-switched MBC	*IGHD*$^-$, *CD27*$^+$	IgD^-, $CD27^+$	(42, 45, 49, 50)
	Not-class-switched MBC	*IGHD*$^+$, *CD27*$^+$	IgD^+, $CD27^+$	(42, 49)
	Plasma cells	*MZB1, CD38, SDC1*	$CD27^+$, $CD38^+$	(42, 48, 49)
	Plasmablasts	*MZB1, CD38, MKI67*	$Ki67^+$	(42, 45, 48, 49)
	Immature B cell	*IGHM, MME*	$CD20^+$, $CD24^+$, $CD38^+$	(49, 51)
	Exhausted B cell	*CD27*$^-$, *CR2*$^-$, *CD22*^{high}	-	(49, 52)
	Atypical MBC	*TBX21, FCRL5, ITGAX*	$T\text{-}bet^+$, $CD11c^+$	(28, 50)
	Germinal center B	*MS4A1, NEIL1, BCL6, AICDA*	$CD20^+$, $Bcl6^+$	(45, 53)

generation of high antibody titers and persist for at least 8-12 months (55). The reactivity of spike-specific $CD4^+$ T cells to various SARS-CoV-2 variants including Omicron is well preserved (56, 57). Both FACS and scRNA-seq studies revealed that infection-induced specific (mainly spike-specific) $CD4^+$ T cell responses showed memory characteristics, including subsets with T_H1, T_H17 and T_{FH} phenotypes (25, 58, 59). Notably, the vaccination-induced spike-specific $CD4^+$ T cells were predominately T_H1-like rather than T_{FH}-like phenotype despite the robust and persistent human T_{FH} cell responses in the draining lymph nodes, whereas these SARS-CoV-2-reactive $CD4^+$ T cells following infection are enriched for both T_{FH} and T_H1-like phenotype (60, 61). Moreover, following booster immunization, examined by AIM assay, convalescent individuals mount more robust circulating T_{FH} cell response and generate a distinct spike-specific $CD4^+$ T cell population expressing both IFN-γ and IL-10, compared with those uninfected individuals, which was consistent with better recall responses induced by the hybrid immunity (54, 62) (Figure 1).

T_H1 and T_H17 cells

Revealed by CyTOF and scRNA-seq analysis, T_H1-like and tissue-resident T_H17-like cells were increased and more clonal expanded in patients with mild COVID-19 (5, 43, 63), suggesting virus reactivity of these cells. T_H1-like cells from patients with mild COVID-19 exhibited a T_H1 polarization state, characterized by upregulation of effector marker genes (*PDCD1*, *CCL5*, *CXCR2*, *CCR2*, *GZMA/B*, *NKG7*, *PRF1*, *IFNG*, and *CCL4*) (5, 63) and genes involved in effector function such as *CXCR4*, *CXCL2*, *ANXA1*, *SOCS2* and *LTB* (5). Whereas in critical COVID-19 cases, T_H1-like cells were predominant with the expression of activation markers (*HLA-DR*, *CXCL13*), auto-reactive markers (*LGALS1*, *CCL3*), stress-response markers (*PDIA6*, *HSBP1*, *VDAC3*, *PARP1*), mitochondrial stress genes (*LDHA*, *PKM*, *COX17*, *VDAC1*, *COX8A*), IL-2 withdrawal-associated stressed genes (*MT1E*, *MT1X*), proteotoxic stress genes (*PSMB3/B6/D4/A7/C3*, *HSPB11*, *PARK7*, *EIF4EBP1*) and glycolysis involved genes (*PGAM1*), suggesting functional dysregulation of theses T_H1-like cells. Tissue-resident T_H17-like cells were thought to play protective roles (63, 64), and T_H17 response was found suppressed in severe COVID-19 cases, along with the reduced expression of typical T_H17-associated genes including *RORC*, *IL17A*, *IL17F*, and *CCR6* (46). In vaccination recipients characterized by scRNA-seq and FACS, BNT162b2, mRNA-1273, or CoronaVac primarily induces T_H1-like cell responses. These cells express T_H1-related cytokines, including IL-2, IFNγ or TNFα, activation markers *CD38* and *HLA-DRA*, cytotoxic molecules *GZMK* and *PRF1*, and transcription factor gene *TCF7*

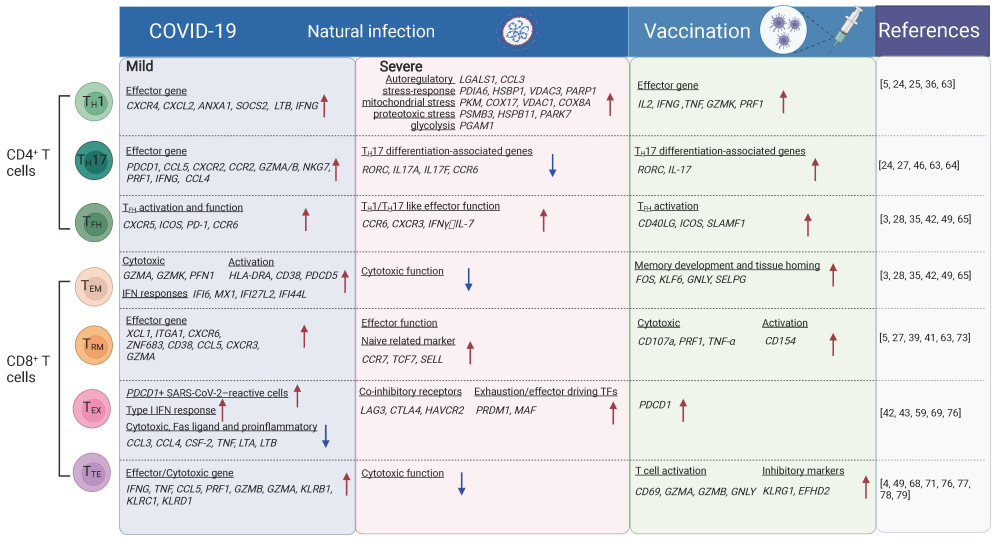

FIGURE 1
The T cell subsets and their transcriptional changes after SARS-CoV-2 vaccination and infection. The red arrows indicate the genes or pathways that were up-regulated in individuals with COVID-19 or vaccinated compared to the healthy donors, while blue arrows indicate the genes or pathways that were down-regulated. T_H1, T-helper 1 like cells; T_{FH}, T follicular helper cells; T_H17, T-helper 17 like cells; T_{EM}, effector memory; T_{TE}, cytotoxic terminal effector; T_{EX}, exhausted-like; T_{RM}, tissue-resident memory cells. The figure was created using Biorender.

(24, 25, 36) (Figure 1). Moreover, T_H1 cell responses were induced rapidly following infection or vaccination followed by the production of high antibody titers. Robust polyfunctional T_H17 responses were also observed within $CD4^+$ T cell compartments following vaccination (24, 27).

T_{FH} cells

Studies of FACS, scRNA-seq, and CITE-seq reported that T_{FH} cells were significantly expanded in asymptomatic and symptomatic cases compared with uninfected people (42, 49), which is indicative of recent antigen encounters and emigration from the germinal center (GC). Spike-specific circulating T_{FH} cells positively correlate with plasma neutralizing activity in COVID-19 patients (65). These cells are enriched for genes involved in type I and type II IFN responses which were notably absent in circulating T_{FH} (cT_{FH}) cells from uninfected people (28). Early T_{FH} cell responses correlate with the neutralizing antibody levels after the first dose of vaccination and with the $CD8^+$ T cell responses after the second dose of vaccination (3). Significant increase in the expression of signature genes for T_{FH} responses (*CD40LG*, *ICOS*, *SLAMF1*, etc.) and the B cell activation (*TLR7*, *CD80/CD86*, *BCL6*) were observed after vaccination (35) (Figure 1). These genes were known to be related to the production of neutralizing antibodies. Moreover, cT_{FH} from vaccinated individuals showed an increased transcriptional signature of TNF-NFκB pathway activation (28), which is linked to improved cT_{FH} survival and robust humoral immune responses in a previous study of influenza vaccination (66).

Pathogenic $CD4^+$ T cells

Some $CD4^+$ T cell subsets are reported to be involved in COVID-19 pathogenesis. Both FACS and scRNA-seq analysis revealed that T_H2 cells were increased and more clonally expanded in patients with severe COVID-19 (4, 67, 68) and they expressed higher levels of *IL4R* and *MAF* associated with T_H2 responses. In contrast, lower levels of T_H2 (*IL4*) responses were observed after vaccination (29). The expression of *GATA3* (T_H2 transcription factor) was also decreased following SARS-CoV-2 infection and vaccination (24). In addition, ex vivo studies showed an absence of T_H2 responses to spike peptides (24). IL-22-expressing $CD4^+$ T cells (T_H22-like) were relatively enriched in asymptomatic or mild COVID-19 cases. These cells could be associated with tissue-protective responses which could limit immunopathology (49). Proliferative $CD4^+$ T cells expressing the proliferation marker Ki67 were enriched in cases with greater severity. The negative feedback mechanisms induced by FoxP3 in Tregs are altered in the lung, which may also contribute to immunopathology (46).

In summary, T_H1, tissue-resident T_H17, and T_{FH} play a protective role in early SARS-CoV-2 infection. These cells could also be elicited by SARS-CoV-2 vaccination. Vaccine-induced spike-specific $CD4^+$ T cell responses peaked more rapidly than

antibody responses after the two-doses vaccination. By contrast, the unregulated T_H2 and Treg cell responses may contribute to the immunopathology in severe COVID-19 cases.

$CD8^+$ T cell responses

SARS-CoV-2 specific $CD8^+$ T cells

SARS-CoV-2 specific $CD8^+$ T cells have been extensively characterized in recent single-cell analyses by FACS and scRNA-seq. SARS-CoV-2-specific $CD8^+$ T cells show a predominantly effector memory phenotype, in a less terminally differentiated state (69, 70), although terminal effector memory cells and transitional memory cells were also observed for SARS-CoV-2-specific $CD8^+$ T cells. These SARS-CoV-2-specific memory $CD8^+$ T cells are still present at least one year after the infection (71). ScRNA-seq studies revealed that SARS-CoV-2-specific $CD8^+$ T cells during the acute phase of infection expressed genes associated with cytotoxicity (*GZMA*, *GZMK*, and *PFN1*), activation (*HLA-DRA*, *CD38*, and *PDCD5*), proliferation (*MKI67*, *MCM7*, and *NUDC1*), and IFN responses (*IFI6*, *MX1*, *IFI27L2*, and *IFI44L*), compared with that in the recovery phase (71). More SARS-CoV-2 specific $CD8^+$ T_{EM} cells are accumulated in asymptomatic/mild cases compared with severe/critical cases. In airways, $CD8^+$ T_{EM} cells showed elevated levels of HLA-DR, PD-1 and reduced levels of CD127, suggesting the activated status *in situ* (Figure 1). SARS-CoV-2 specific $CD8^+$ T_{EM} cells in vaccinees were significantly expanded after the second dose of vaccination examined by scRNA-seq (24). Recently, SARS-CoV-2 specific $CD8^+$ T cells from individuals with vaccination, infection and breakthrough infection were extensively profiled using MHC-I multimers and scRNA-seq. It was reported that breakthrough infection mounted vigorous non-spike-specific responses, while vaccination among previously infected individuals led to the expansion of spike-specific T cells and continued differentiation to $CCR7^-CD45RA^+$ phenotype. More importantly, TCR analysis of SARS-CoV-2 reactive $CD8^+$ T cells showed no evidence of repertoire narrowing following repeated exposure (72).

Tissue-resident $CD8^+$ T cells

In addition to SARS-CoV-2-specific $CD8^+$ T cells identified in blood, these cells were also readily detected in tonsils and displayed tissue-resident memory phenotypes with high expression of CD103 and CD69 (73). Analyzed by scRNA-seq, scTCR-seq and CITE-seq, $CD8^+$ T_{RM} cells showed increased proportion and clonal expansion in the airways of mild COVID-19 cases compared with those in severe/critical cases (5, 39, 41, 63). Along the pseudotime differentiation trajectory, $CD8^+$ T_{RM} cells were enriched at the end of the lineage in mild COVID-19 cases, indicating a terminally differentiated phenotype. Moreover, these cells express higher levels of the effector molecules *XCL1*, *ITGAE*, *CXCR6*, and *ZNF683* in mild COVID-19 patients than that in severe cases (5, 41) (Figure 1). SARS-CoV-2-specific $CD8^+$ T_{RM} cells persisted at least 2 months after viral clearance in the nasal mucosa (74). Accumulation of both $CD8^+$ T_{EM} cells and $CD8^+$ T_{RM} cells is generally associated with lower disease severity, suggesting that they contribute to better outcomes for COVID-19 cases (49, 75). Interestingly, $CD8^+$ T_{RM} cells in the nasal mucosa were expanded up to 12 days post the first and second doses of SARS-CoV-2 mRNA vaccination (27). Thus, $CD8^+$ T_{RM} cells are important protective cells against the infection of SARS-CoV-2 and other pathogens.

Exhaustion of $CD8^+$ T cells

Studies of FACS, scRNA-seq and CITE-seq also revealed that $CD8^+$ T_{EX}-like cells were phenotypically heterogeneous among patients with different outcomes (42, 43, 76). In addition to the increased expression of the inhibitory checkpoint and cytotoxic markers, $CD8^+$ T_{EX}-like cells were characterized by proliferation with G2M and S gene scores, especially in critical COVID-19 cases (43). Among the co-inhibitory receptors, *LAG3*, *CTLA4*, and *HAVCR2* were enriched in $CD8^+$ T cells from COVID-19 patients that eventually succumbed to the disease, but *PDCD1* and *TIGIT* were enriched in $CD8^+$ T cells from COVID-19 patients that eventually recovered and discharged (76). The exhaustion/effector driving TFs (*PRDM1*, *MAF*) were also upregulated in COVID-19 patients that eventually succumbed to the disease. However, there are evidences that the PD-1-expressing SARS-CoV-2-specific $CD8^+$ T_{EX}-like cells are functional rather than exhausted (69). In addition, SARS-CoV-2 vaccination can also induce PD-1 expression in $CD8^+$ T cells (59) (Figure 1). Hence, whether $CD8^+$ T cell is truly exhausted requires further discussion. Transcriptional signatures alone are not sufficient to indicate whether the cells were more exhausted or merely more activated.

Pathogenic $CD8^+$ T cells

CyTOF and scRNA-seq studies reported the early upregulation of effector molecules by $CD8^+$ T_{TE}-cells, typically observed within 7 days from symptoms onset and peaking at 14 days following SARS-CoV-2 infection. $CD8^+$ T_{TE}-cells are associated with effective SARS-CoV-2 clearance and improved clinical outcomes (68, 77). Since $CD8^+$ T_{TE} cells are less enriched in SARS-CoV-2 specific $CD8^+$ T cells (71), they may be bystander-activated and contribute to disease control in other settings (77, 78). In the late stage of infection, $CD8^+$ T_{TE} cells were enriched in cases with greater severity (49, 79), and they are the most proliferative compartments in COVID-19 patients, especially in severe cases (4). The kinetics of T_{TE}-cell responses were prolonged and continued to increase up to day 40 after symptom onset (80). Effector genes expressed by $CD8^+$ T_{TE} cells such as *GZMB* drive a clear separation between stable and progressive COVID-19 patients (76). In the patients with moderate COVID-19, $CD8^+$ T_{TE} cells showed higher expression

of *IFNG*, *TNF*, *CCL5*, *PRF1*, *GZMB*, and *GZMA*, together with genes encoding cytotoxic receptors (*KLRB1*, *KLRC1*, and *KLRD1*) in comparison with severe cases. Besides decreasing cytotoxic function, pro-inflammatory cytokines were also poorly expressed in these cells in patients with severe COVID-19. Hence, peripheral leukocytes are not a major contributor to the putative cytokine storm in COVID-19 cases (81).

Overall, various single-cell technologies revealed that infection elicits more diverse immune phenotypes than vaccination, in which $CD4^+$ and $CD8^+$ T cells activation increased from day 0 to day 14, peaked at day 28, and decreased from day 28 to day 35 (3, 24, 36). Most vaccines elicited T_H1-skewed and $CD8^+$ T_{EM} responses (24). Early T_{FH} and T_H1 cell responses correlate with effective neutralizing antibody responses after the first dose, whereas $CD8^+$ T_{EM} cell responses were elicited after the second dose (3). Breakthrough infection and booster immunization can induce recall response and continued T cell differentiation.

B cell responses to SARS-CoV-2

B cell subsets

Various B cell subsets were identified using FACS, CyTOF, scRNA-seq, and CITE-seq in COVID-19 patients, including naïve B cells (*TCL1A*$^+$ *SELL*$^+$), atypical memory B cells (atMBC, *ITGAX*$^+$ *FCRL5*$^+$), activated B cells (ABC, $CD21^{low}$ $CD27^+$ $CD10^-$), memory B cells (MBC, $CD21^+$ $CD27^+$ $CD10^-$), *etc.* According to their class switching states, memory B cells also comprised class-switched (IgD^- $CD27^+$) and non-class-switched (IgD^+ $CD27^+$) subsets, where the class-switched memory B cells are thought to have undergone affinity maturation through GC reactions. The identification of antibody-secreting cells (ASCs), like plasmablast (PB, *CD79*$^+$ *MS4A1*$^-$ *MKI67*$^+$), 'active' plasma cells (*XBP1*^{high} *MS4A1*$^-$ *PRDM1*$^+$), and 'terminal' plasma cells (*XBP1*^{high} *MS4A1*$^-$ *PRDM1*$^-$ *CCL5*^{high}) were also reported following SARS-CoV-2 infection and vaccination (5, 79, 82) (Table 2).

Previous studies have shown that the proportion of $CD19^+$ B cells was increased in severe COVID-19 cases, while transitional B cell subsets were increased in mild/moderate cases. High-dimensional FACS analysis revealed that the proportion of memory B cells was decreased but that of ASCs was increased in severe cases (83). Severe/critical COVID-19 cases also displayed hallmarks of extrafollicular B cell activation which was similar to previously reported in lupus (84) (Figure 2). Expansion of atMBC is also a feature of COVID-19 (85). The increased atMBC proportion was positively correlated with COVID-19 severity and decreased to normal levels after recovery (43, 85). AtMBCs in COVID-19 showed impaired proinflammatory effector functions (86). However, spike-specific atMBCs were abundantly produced during secondary immune responses and SARS-CoV-2 infection induces more atMBC than vaccination (26), suggesting that atMBC are functional in these individuals. Therefore, it is unclear whether atMBC is correlated with impaired humoral immune responses during COVID-19. In contrast to atMBC, vaccine-induced ABCs had a similar capacity to bind to specific viral antigens in both SARS-CoV-2-convalescent individuals and naïve subjects (87).

Plasma cells

Plasma cells produce antibodies against antigens. Both plasma cells and plasmablasts were more expanded in moderate and severe COVID-19 cases compared to mild cases and uninfected controls (50, 81). Antibodies produced shortly after infection are mostly derived from short-lived plasma cells that are developed through an extrafollicular response. Whereas in the later stage, a smaller population of long-lived plasma cells

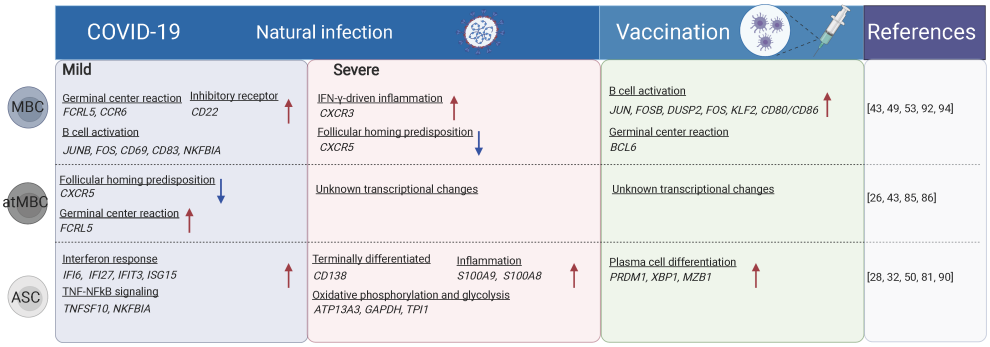

FIGURE 2
The B cell subsets and their transcriptional changes after SARS-CoV-2 vaccination and infection. The red arrows indicate the genes or pathways that were up-regulated in individuals with COVID-19 or vaccinated compared to the healthy donors, while blue arrows indicate the genes or pathways that were down-regulated. MBC, memory B cells; atMBC, atypical memory B cells; ASC, antibody secreting cells. The figure was created using BioRender.

is generated residing in the bone marrow (88). The PRDM1$^-$ plasma cells are characterized by sub-optimal differentiation or activation, which may be defective or even inhibit productive antibody responses in COVID-19. In contrast, PRDM1$^+$ plasma cells were supposed to be long-lived populations (89). Plasmablasts are more expanded in hospitalized patients compared to mild/asymptomatic COVID-19 patients (90). The proportions of circulating plasmablasts/plasma cells were positively correlated with the serum levels of TNF, IL-10, and IL-21, which are factors critically involved in B cell differentiation to plasmablasts (42, 90, 91). ScRNA-seq and CITE-seq analyses revealed that these ASCs displayed a high metabolic activity (including oxidative phosphorylation, type I and type II IFN responses, fatty acid metabolism, and mTORC1 signaling), which was reduced following recovery. Meanwhile, memory and naïve B cells showed no significant difference in overall metabolic activity in patients with different disease severity (28). Following vaccination, CD27$^+$CD38^{bright} plasmablasts were substantially increased one week later as examined by FACS (32, 50). Besides, plasmablasts from both COVID-19 patients or individuals following SARS-CoV-2 vaccination were enriched for genes involved in IL-6 receptor signaling (JAK/STAT) and inflammatory response, which is consistent with their roles in promoting plasmablast differentiation (28) (Figure 2).

Germinal center B cells

GC reaction is important and capable of determining the quality of B cell response upon SARS-CoV-2 infection (21). The GC-derived memory B cells and plasma cells are more stable and long-lived (50), and capable of producing high-affinity antibodies (Figure 3). The memory B cells can rapidly differentiate into ASCs upon antigen re-encounter. On the contrary, extrafollicular B cell response leads to the production of low-affinity antibodies and wanes rapidly. ScRNA-seq and multi-color immunofluorescence showed that severe COVID-19 cases showed impaired GC reactions than mild cases (43, 92). Thus, the elevated antibody levels and memory B cell responses in severe COVID-19 could be explained by stronger extrafollicular B cell responses. Recently, using fine-needle sampling of lymph nodes and single-cell analyses, some interesting aspects of GC reactions in SARS-CoV-2 infection and vaccination are revealed (21, 93). ScRNA-seq and FACS analysis showed that SARS-CoV-2 vaccination induced a robust specific GC B cell response in the draining lymph nodes, and these GC B cells were maintained for at least 6 months in some individuals, indicating that they are likely to be undergoing further affinity maturation (21, 53, 94). Consistently, it is shown that SARS-CoV-2 memory B cells undergo continued evolution following vaccination or infection, mainly reflected by memory B cells with the increased somatic hypermutation (SHM) and the encoding of the high-affinity and broadly-reactive antibodies (12, 95–97).

SARS-CoV-2 specific B cells

The spike-specific class-switched memory B cells and neutralizing antibodies appeared to be stable up to 6 months after infection in COVID-19 patients (22, 98, 99). Vaccination also elicits robust specific B cell responses (88). Single-cell BCR tracing found that the switched memory B cells increased on day 7, and sustained until day 28 (36). In healthy individuals, SARS-CoV-2-specific antibody responses were enhanced upon the second dose of vaccination. Besides, SHM levels of unswitched memory B cells on day 0 are similar to the clonally expanded switched memory cells on days 7-28, suggesting that vaccine-activated unswitched memory B cells can differentiate into

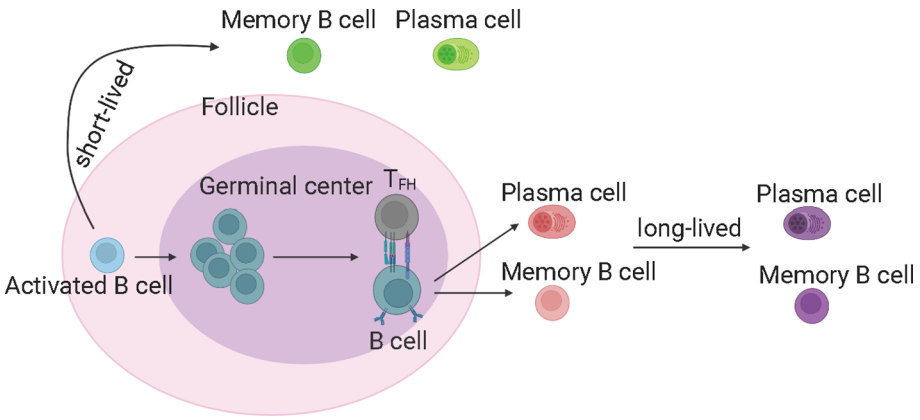

FIGURE 3
The germinal center reaction and the production of long-lived memory B cells and antibodies. The germinal center derived memory B cells and plasma cells are more stable and long-lived, and able to produce high-affinity antibodies. T_{FH}, T follicular helper cells. The figure was created using BioRender.

switched memory B cells independent of the GC (36, 50, 100, 101). Recently, SARS-CoV-2 specific B cell responses have been comprehensively probed using oligonucleotide-conjugated SARS-CoV-2 proteins at the single-cell resolution. During the early phase of SARS-CoV-2 infection, the pre-existing seasonal coronavirus cross-reactive memory B cells were activated, whereas the SARS-CoV-2-specific B cell clones were gradually accumulated with time and contributed to the majority of memory B cell pool later. The SARS-CoV-2 specific memory B cells maintained the evolution and affinity-mature over several months, by progressively acquiring SHM in GC (21). Over the course of infection and recovery, the number of activated specific B cells decreases while the resting MBCs increase, irrespective of disease severity.

FACS accompanied with biotinylation labeling analysis revealed that spike-specific MBCs were also robustly induced by SARS-CoV-2 vaccination. The induced specific MBCs persist and increase over time after immunization (102). Moreover, SARS-CoV-2 vaccination in recovered individuals induces a significant increase in the antibody response and B cell response, whereas the second dose of vaccine does not induce any further increase in antibody responses. Spike-specific MBCs were not reduced in individuals who had breakthrough infections. In contrast, 5 to 8 months after breakthrough infections, salivary anti-spike IgA levels declined compared with that before SARS-CoV-2 infection. But these salivary anti-spike IgA levels remained significantly higher than in fully vaccinated individuals who never experienced SARS-CoV-2 infection, possibly due to response to a novel antigen (102).

In summary, both SARS-CoV-2 infection and vaccination induce robust humoral immune responses. Severe COVID-19 is usually accompanied by an elevated level of humoral immune responses, likely due to a more robust extrafollicular B cell response. Although the SARS-CoV-2 antibody levels wane rapidly, the SARS-CoV-2 specific memory B cell responses increase over the first several months following the infection and vaccination. Memory B cells progressively acquire somatic mutations in their immunoglobulin heavy variable genes and continue to undergo clonal evolution due to an ongoing GC response. Finally, The booster immunization was effective in increasing neutralizing antibody titers and breadth encoded by vaccine-induced memory B cells against the SARS-CoV-2 variants.

T cell and B cell immune repertoire

Single-cell based analyses (scTCR-seq and scBCR-seq) have provided key information on the dynamics of TCR and BCR repertoire following SARS-CoV-2 infection or vaccination.

TCR repertoire

TCRs are capable of recognizing fragments of antigen as peptides bound to MHC molecules to target virus-infected cells (103). Identification of SARS-CoV-2 T-cell epitopes, generation of MHC multimers, together with single-cell immune repertoire sequencing will facilitate analyses of SARS-CoV-2-specific TCR repertoires (2, 72, 104, 105). The V(D)J genes usage and immune characteristics of TCRs on SARS-CoV-2-specific T cells have been described by several studies, but their function during SARS-CoV-2 infection needs to be further validated.

SARS-CoV-2 immunodominant epitopes may drive the molecular patterns of T cell responses in COVID-19 patients (105). $CD8^+$ T-cell repertoire to SARS-CoV-2 was heavily skewed by 70% of all TCR mappings to 5.2% (14/269) of candidate antigen pools (106), suggesting that the T cell response is dominated by recognizing a few specific epitopes linked to distinct HLA types. Indeed, TCRs recognizing HLA-A*01:01-restricted TTDPSFLGRY epitope displayed enrichment for the TRBV27 segment (107). YLQPRTFLL-specific TCRs showed a biased usage of TRAV12-1 (71%) and TRBV7-9 (16%), and RLQSLQTYV-specific TCRs use TRAV13-2 (15%) and TRBV6-5 (25%) more frequently as compared with only 3%-4% gene usage in the control TCRs (108). Although TCRs recognizing HLA-A*02:01-restricted YLQPRTFLL and RLQSLQTYV epitopes were observed in convalescent patients, those TCRs were undetectable or at a very low frequency in the total TCR repertoire of the peripheral blood. It is possible that clones specific to the two antigens are both localized in the tissues, and only a limited number of cells are present in the circulation, which was in accordance with our previous findings that $CD8^+$ T_{RM} cells were highly clonally expanded (39, 41). TCRs recognizing HLA-A*11:01-restricted KTFPPTEPK epitope are cross-reactive to various SARS-CoV-2 variants and could confer cytotoxic responses upon encounter with target cells, providing support for developing T-cell epitope incorporating vaccines to prevent continuously emerging SARS-CoV-2 variants (109). By contrast, the antigen-specific TCR repertoire of $CD4^+$ T cells was less studied and understood. It was reported that SARS-CoV-2-specific $CD4^+$ T cells were increased in patients with severe COVID-19, but these cells displayed low functional avidity and clonality in severe cases than those in mild cases (110), which should be investigated to acquire a better understanding of the underlying mechanisms in future studies.

Various RBD- and spike-specific T cell clones were found from different memory subsets of convalescent COVID-19 patients and individuals receiving SARS-CoV-2 mRNA vaccination. However, there is a limited overlap of TCRs between SARS-CoV-2 infection and vaccination. TRAV29/DV5, TRBV5-1, TRAV29/DV5, and TRBV6-5 are biased in convalescent COVID-19 patients. In contrast, TRAV29/DV5, TRBV11-2, TRAV29/DV5, TRBV7-9, TRAV12-2, and TRBV6-2 are frequently used following vaccination (24). These distinct TCR patterns highlight the differences in the breadth of the epitopes recognized in SARS-CoV-2 infections versus

vaccinations (111). These immunodominant epitopes and the TCRs confirmed with functional superiority can better inform next-generation vaccine designs.

BCR repertoire

Antibodies, also called immunoglobulins, comprise 5 different classes: IgM, IgD, IgG, IgA, and IgE (112). Different classes of antibodies may form synergy to achieve maximum immune activity against SARS-CoV-2 infection (113). BCR repertoire is the genetic source of neutralizing antibodies. Single-cell analyses have revealed BCR repertoires for total B cells, SARS-CoV-2 specific B cells or plasma cells, facilitating the identification of neutralizing antibodies.

BCR clonality was sharply increased at the early stage of infection and then gradually decreased in the convalescent phase to normal levels (79). BCRs in COVID-19 patients exhibited biased VDJ usage compared with that of healthy controls. The reported SARS-CoV-2 neutralizing antibodies are frequently derived from IGHV3 and IGHV1 family, and more than 40 neutralizing antibodies used IGHV3-53 have been identified. Using single-cell BCR sequencing, it was found that the genes of IGHV3 family including IGHV3-7, IGHV3-15, IGHV3-21, IGHV3-23, and IGHV3-30 were over-represented in COVID-19 patients compared with that in the controls. Besides, the preferred IGKVs were IGKV1-17, IGKV2-28, and IGKV3-15, and the preferred IGLVs were IGLV1-44, IGLV2-8, and IGLV3-27 (51). In contrast, after vaccination, IGHV3-33, IGHV3-43, and IGHV3-49 in the IGHV3 family and IGHV1-69D, IGKV1D-39, and IGLV5-45 were preferentially expanded (24). The cause for different V-gene usage between infection- versus vaccine-induced BCRs is unclear, possibly related to different epitope breadth. Infection elicited B cell clones targeting more epitopes on various SARS-CoV-2 proteins, whereas vaccination mainly induces narrowed antibody responses against S1 and RBD domains.

SHM reduction in COVID-19 patients was consistent with an extrafollicular B cell response mentioned above. RBD-specific clones have been shown to display a low level of SHM (below 5%) during the early stages of infection irrespective of disease severity (114). IgG1, IgG3, and IgA1, and to a lesser extent IgA2 and IgE were dominant isotypes that were rarely mutated or unmutated, indicating that they were derived from an early extrafollicular class switching event. This SHM level increases over time, suggesting an ongoing and persistent GC response.

Perspectives on adaptive immune responses to COVID-19 at single-cell solution

We have reviewed the broad applications of single-cell analyses in dissecting adaptive immunity in SARS-CoV-2 infection and vaccination. However, the studies of SARS-CoV-2-specific adaptive immunity are still ongoing and many questions remain unresolved.

Breadth, diversity, magnitude and lifespan of adaptive immunity

These aspects are the most important characteristics of immune defenses against SARS-CoV-2 infection. The SARS-CoV-2 genome encodes six functional open reading frames (ORFs): replicase (ORF1a/ORF1b), spike (S), envelope (E), membrane (M) and nucleocapsid (N), and seven putative ORFs encoding accessory proteins that are interspersed between the structural genes. More than 2, 000 different SARS-CoV-2-derived epitopes have been curated and reported (IEDB; www.iedb.org), exhibiting great epitope diversity. Structural proteins (S, M, E and N) are predominant targets of T cell and B cell responses (115).

Among these reported epitopes, 95% of reported class II and 98% of class I epitopes were fully conserved in different SARS-CoV-2 variants (Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Mu (B.1.621), Delta (B.1.617.2), and Omicron (B.1.1.529)) (30). For S protein, $CD8^+$ T cell epitopes are homogeneously distributed, whereas only a few immunodominant regions were observed for $CD4^+$ T cells (116). While the immunodominant epitopes for $CD4^+$ and $CD8^+$ T cells were noted to be similar in M and N proteins (within 7-101 and 131-213 residues for M protein, and within 31-173 and 201-371 residues for N protein) (105). T cell epitopes in nonstructural proteins show a similar pattern to that of the S protein. $CD8^+$ T cell recognition showed more homogeneous patterns, but $CD4^+$ T cell epitopes were more evident in nsp3 and nsp12 protein (105).

By contrast, B cell epitopes were prone to immune evasion, especially for the key mutations on spike protein which significantly reduced the neutralization activity of antibodies against SARS-CoV-2 variants (56). B cell epitopes have been extensively mapped for the structural proteins including S, N, M, and E proteins, especially the S. Full-length S or S1 domain which contains RBD were considered a good vaccine antigen as they are the main target to induce neutralizing antibody. Considering the broadly reactive T-cell response versus the waning humoral immunity, we need to incorporate these T-cell and B-cell epitopes outside of spike proteins to develop potentially more effective vaccines.

The magnitude of T cell and B cell responses varied when it comes to SARS-CoV-2 infection and vaccination, and is correlated with the disease severity (Figures 4A, B). Within T cells, the memory response is skewed toward more $CD4^+$ T cell responses than that of $CD8^+$ T cells, despite their similar levels immediately after infection (117). Moreover, the S-specific $CD4^+$ T cells showed a limited increase after vaccination compared with $CD8^+$ T cells (59), while the S-specific $CD8^+$ T cell responses after vaccination are weaker. For humoral

immunity, vaccination generally induces higher amounts of circulating antibodies compared with infection (Figure 4C).

In terms of the duration of memory responses, T cell immunity persists better than antibody responses. However, both SARS-CoV-2 specific memory T cells and SARS-CoV-2 neutralizing antibodies can be detected one year after infection or vaccination (118). Patients with severe COVID-19 showed the delayed engagement of anti-viral $CD8^+$ T cell responses compared with mild cases. $CD4^+$ T cell responses are more robust than that of $CD8^+$ T cells and may even increase in frequency over time, potentially reflecting antigen persistence. However, there was no difference in the magnitude of T-cell responses or neutralizing antibodies in patients with different disease severity one year after infection (118). Vaccines can substantially induce S-specific $CD8^+$ T cell responses which peak in most donors at 9-12 days after immunization (59). These S-specific $CD8^+$ T cells showed effector memory phenotype with expansion capacity, cytokine production, and degranulation capacity, and remained stable irrespective of vaccine booster (Figure 4B). The percentage of B cells increased from day 0-14 but decreased from day 14-28 after immunization. RBD-specific and S1-specific memory B cells may be observed on day 21 and increased gradually over the period of vaccination.

In summary, due to the aforementioned difference in breadth, activity and duration between the SARS-CoV-2 specific T-cell and B-cell responses, and between infection- and vaccination-induced immunity. We propose to improve current vaccination strategies by adding flavors of T-cell components and using heterologous immunization to mimic hybrid immunity to induce more effective, durable and broadly reactive immune responses.

High-throughput and accurately dissecting antigen-specific immune responses

Single-cell analyses have revealed various aspects of adaptive immune response to SARS-CoV-2 infections and vaccinations, as described above. However, a deep understanding of how these adaptive immune cells are molecularly regulated remains largely unclear. Single-cell multi-omic technologies such as CITE-seq, scATAC-seq, and scBCR/TCR-seq combined with peptide-MHC multimers will help reveal the underlying regulatory mechanisms driving COVID-19 pathology or prompting long-term protective immunity after vaccination (2, 76). After antigen exposure, immune cells are quickly differentiated into different subtypes to eliminate pathogens through various mechanisms (15, 119). Cell state transition or differentiation between different cell subsets is a multistage and multifactorial process, and single-cell multi-omics can facilitate the exploration of the regulatory mechanism underlying these processes, which is now a challenge due to technological limitations (120, 121). For example, combined scRNA-seq and scATAC-seq can simultaneously profile gene expression and open chromatin from the same cell, enabling deeper characterization of cell types/states and uncovering new gene regulatory mechanisms underlying antigen-specific T/B cell activation, differentiation and memory formation (122). While multi-omic single-cell

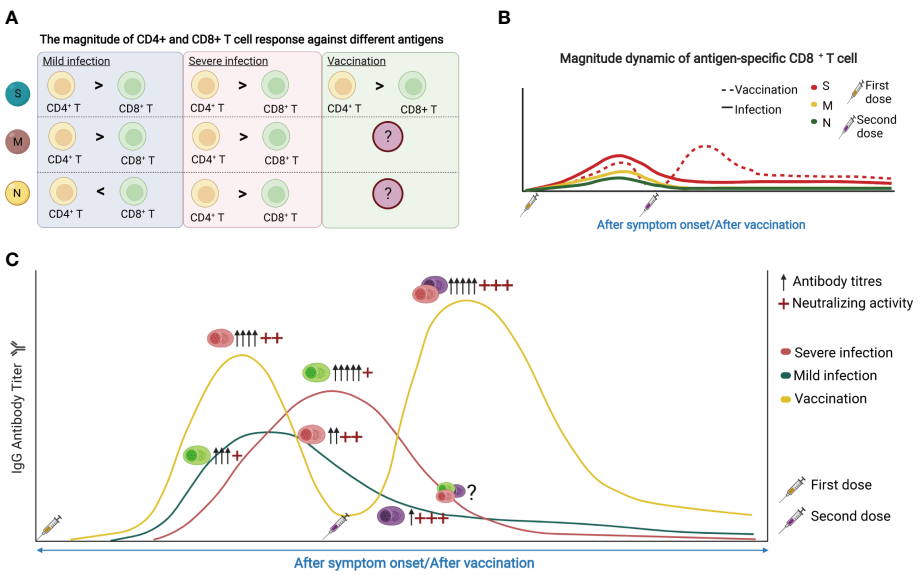

FIGURE 4
(A) The magnitude of $CD4^+$ and $CD8^+$ T cell response against different antigens. S, spike protein; M, membrane; N, nucleocapsid. **(B)** The dynamic of SARS-CoV-2 reactive $CD8^+$ T cell response following SARS-CoV-2 infection and vaccination. **(C)** The dynamic of SARS-CoV-2 reactive antibodies and memory B cells in response to SARS-CoV-2 infection and vaccine. The figure was created using BioRender.

immune profiling can provide full-length V(D)J sequences for TCRs/BCRs, cell surface protein expression, antigen specificity, and gene expression all from a single cell, allowing clonal tracing of antigen-specific T/B response to infection or vaccination (123). Finally, T and B cells respond to antigen stimulation by metabolic remodeling to execute their functions. Recent advances in single-cell metabolomic analyzing techniques will greatly increase our ability to interrogate these metabolic pathways at single-cell level in antigen-specific lymphocytes (124). Together, the integration of these new technologies can accurately dissect the cellular state transition processes and provide detailed temporal resolution of dynamic changes during infection and vaccination.

Furthermore, identifying and/or isolating antigen-specific T cells or B cells is important not only for the understanding of SARS-CoV-2 induced immune response, but also for providing direct solutions for adoptive immunotherapy (125, 126). However, current protocols for characterizing the immune phenotypes of antigen-specific reactions, including activation-induced marker, degranulation, proliferation, ELISA, ELISpot, intracellular cytokine staining, cytotoxicity, and multimer-based assays, are low-throughput, time-consuming and labor-intensive. New high-throughput single-cell based screening technologies are emerging. LIBRA-seq, an emerging multi-omics application, can simultaneously link antigen specificity with BCR sequencing at single-cell solution (82, 127). Other technologies linking antigen specificity with TCR sequencing will be possibly developed in the future (72). These antigen-specific single-cell sequencings provide effective means to comprehensively characterize the B cell or T cell responses and identify neutralizing antibodies or epitope-specific TCRs.

Spatial-temporal immune response

The tissue-resident immunity fights against pathogens at the front line of the host (128–130). Upon SARS-CoV-2 infection, circulating T cells need to be quickly activated and deployed to the site of infection, playing either protective and/or pathogenic roles (131). As the infection resolved, some of these protective effector T cells transformed to long-lived resident T cells, safeguarding the tissues from the potential pathogen re-encounter. In another line of defense, some B cells were initially engaged at the extrafollicular zones where they differentiated into short-lived plasmablasts to provide early antibody responses. Whereas other B cells may undergo GC reactions to produce both long-lived plasma cells and memory B cells (132). Understanding those spatial-temporal processes of SARS-CoV-2 specific T and B cell responses is important but challenging. Although memory T cells and memory B cells were disseminated throughout the body, the source and the distribution of pathogen-specific memory T cell or B cell population have not been systemically elucidated. Single-cell TCR and single-cell BCR sequencing, together with antigen-specific immune cell isolation strategies, can be used to trace the immune cell distribution from the circulation to peripheral tissues, to trace the pathogen-specific memory T (or B) cell turnover, and their differentiation from effector memory phenotype to central memory phenotype over time (4, 28, 72, 123).

In conclusion, the merging multi-omics single-cell technologies will continuously aid the efforts to study adaptive immunity against SARS-CoV-2. The power of these deep immune profiling techniques has already enabled simultaneous analyses of epigenomic, transcriptomic, proteomic, immune repertoire and spatial characteristics of rare populations of SARS-CoV-2 reactive T and B cells. We believe that these advances will greatly prompt both the fundamental and applied studies of SARS-CoV-2 infection and vaccination in the future.

Author contributions

FQ, SZ, and ZZ wrote and edited the manuscript. FQ and YC created the figures and tables. 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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Developing dendritic cell for SARS-CoV-2 vaccine: Breakthrough in the pandemic

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Finding a vaccine that can last a long time and effective against viruses with high mutation rates such as SARS-CoV-2 is still a challenge today. The various vaccines that have been available have decreased in effectiveness and require booster administration. As the professional antigen presenting cell, Dendritic Cells can also activate the immune system, especially T cells. This ability makes dendritic cells have been developed as vaccines for some types of diseases. In SARS-CoV-2 infection, T cells play a vital role in eliminating the virus, and their presence can be detected in the long term. Hence, this condition shows that the formation of T cell immunity is essential to prevent and control the course of the disease. The construction of vaccines oriented to induce strong T cells response can be formed by utilizing dendritic cells. In this article, we discuss and illustrate the role of dendritic cells and T cells in the pathogenesis of SARS-CoV-2 infection and summarizing the crucial role of dendritic cells in the formation of T cell immunity. We arrange the basis concept of developing dendritic cells for SARS-CoV-2 vaccines. A dendritic cell-based vaccine for SARS-CoV-2 has the potential to be an effective vaccine that solves existing problems.

KEYWORDS

dendritic cells, immunotherapy, T cells, SARS-CoV-2, vaccine candidate, vaccine approach

Introduction

COVID-19, which WHO declared a pandemic in March 2020, remains the focus of world problems (1). The infection is caused by the SARS-CoV-2 virus, a positive-strain RNA virus that belongs to the beta coronavirus family (2). SARS-CoV-2 conveys a genome resemblance to the MERS-CoV and SARS-CoV viruses (3). SARS-Cov-2 continues to mutate, giving rise to various variants of this virus. Some emerging variants classified as Variance of Concern (VoC) include the alpha, beta, delta, and omicron variants (4).

The SARS-CoV-2 infection manifests into various organ system abnormalities such as the respiration, cardiovascular, nervous, and digestive systems with a broad spectrum of symptoms ranging from mild to severe (5). In SARS-CoV-2 infection, various pathology findings were documented, such as a decrease in the number of lymphocytes to an increase in inflammatory cytokines production that led to cytokine storm in severe symptomatic patients (6). These findings indicate the failure of human immune response in SARS-CoV-2 infection. The immune system failure is attributed to the ability of SARS-CoV-2 to evade the human immune response. Specifically, T cell dysfunction was found in SARS-CoV-2 infection, which is essential in eliminating SARS-CoV-2 in the body (7).

To date, various types of vaccines have been developed and approved to prevent SARS-CoV-2 infection. All of these vaccines are oriented to produce antibodies that can neutralize SARS-CoV-2. However, studies show that there is a decline in antibodies several months after vaccination and also a decrease in the effectiveness of existing vaccines against the evolving variants of SARS-CoV-2 (8). This has implications for the need of the novel effective vaccine development to protect against the emergence of SARS-CoV-2 variants. Meanwhile, it has been known that memory T cells are capable of lasting longer than the antibodies formed and have the capability to recognize the SARS-CoV-2 variants (9). Therefore, the development of a T cell-oriented vaccine is a promising approach for the generation of effective and long-lasting immunity against SARS-CoV-2.

Dendritic cells (DC) have a pivotal role in the immune system, which connects the activation of the innate and adaptive immune systems. In addition, DC is well-known for its ability to activate and differentiate naïve T cells (10). DC has been developed as an immunotherapy or vaccine for cancer and infections (11). DC's ability to activate the immune system, the successful development of DC-based immunotherapy in other diseases, and also considering the role of DC in the COVID-19 can be the cornerstone for the development of DC-based vaccine for SARS-CoV-2. Therefore, this article discuss the potential development of DC as a SARS-CoV-2 vaccine by focusing on the role of T cells and DC in SARS-CoV-2 infection, the formation of immunity in SARS-CoV-2 infection, and the role of DC in shaping immunity which is the foundation for the development of DC as a SARS-CoV-2 vaccine.

Immune system dysfunction in SARS-CoV-2 infection

Viruses that invade the body first will activate an innate immune response that aims to eliminate the virus and then trigger an adaptive immune response. RNA Viruses such as SARS-CoV-2 have Pathogens Associated Molecular Patterns (PAMPs) that can be recognized and bonded to Patterns Recognition Receptors (PRR) in the cytosol and endosomal phagocytic cell (12). This process leads to polynuclear lymphocyte cells, monocytes, Natural Killer (NK) cells along with DC recruitment (13). Recruitment of these cells is a crucial process that intends to eliminate the virus and stop the disease progression. Antigen Presenting Cell (APC) captures incoming viral particles to be introduced to naïve T cells (14). Naïve T cells then differentiate into specific CD4+ and CD8+ T cells (15). There are two kinds of CD8+ T cells: effector T cells or cytotoxic T cells (Tc) and memory cells. These formed Tc cells are responsible for eliminating the virus. CD4+ T cells or T helper (Th) assist the role of Tc and contribute to the formation of the humoral immune system by differentiating B cells into B cell-producing specific antibodies (16).

There are several immunopathologies found in COVID-19. Studies revealed the presence of lymphopenia and increased activation of T cells, which are the characteristics of lymphocyte dysfunction, abnormalities in monocytes and granulocytes, increased cytokines production, and the generation of specific antibodies, especially in patients with severe symptoms (17, 18). All these hallmarks correlate to severity degree and survival rate (19). These conditions also indicate the presence of both innate and adaptive immune dysfunctions by which the SARS-CoV-2 capability to evade the immune responses (20).

The invading SARS-CoV-2 will be identified by Retinoid-acid Inducible Gene-1 (RIG-1), Melanoma-Differentiation Associated protein 5 (MDA-5), Toll-like Receptor 7 (TLR-7), and TLR-4 which specifically recognize S SARS-CoV-2 glycoprotein (21). The process activates the transcription of Nuclear Factor kappa-B (NF-kB), Interferon Regulatory Factor 3 (IRF-3), and IRF-7 (22). Under normal circumstances, the invading virus initiates the provision of type I interferon (IFN-I), IFN-III, pro-inflammatory cytokines, in conjunction with chemokines (6). At the early phase of the disease, IFN-I plays a critical role in eliminating and inhibiting viral replication and assisting in activating adaptive immune responses (23). However, delays in the provision and activity of IFN-I will trigger the progressivity of SARS-CoV-2 infection (24). In SARS-CoV-2 infection, there was a suppression and delay in the IFN-I provision (25). It is caused by inhibition of signaling pathways by Open Reading Frame 3b (ORF3b), ORF4a, ORF4b, ORF5, ORF6, Non-specific protein 1 (Nsp1), Nsp2, Nsp14, M, and N SARS-CoV-2 (21). The suppression of IFN-I is a mechanism by which SARS-CoV-2 avoids the immune system that leads to unrestrainable viral replication and disease progressivity (26).

Failure to eliminate SARS-CoV-2 leads to an increase in activation of Nod-like Receptor Family Pyrin Domain Containing 3 (NLRP3) inflammasome (27). This condition contributes to severe inflammatory reactions and severe progressivity of the disease. In COVID-19, NLRP3 activation involves the appearance of programmatic cell death through the

production of interleukin 1β (IL-1β) and IL-18, which induces leucopenia (28). NLRP3 activation also increases macrophage activation, thus, increasing the production of IL-1RA, IL-6, IL-8, IL-10, Tumor Necrosis Factor-Alpha (TNF-α), and chemokine C-X-C ligand 10 (CXCL-10) (29). This process is one of the factions of the occurrence of cytokine storms in COVID-19 patients (see Figure 1) (30).

Cellular adaptive immune responses play an important role in the pathogenesis of COVID-19, which involves SARS-CoV-2-specific CD4+ and CD8+ T cell activity (31). T cells will respond to SARS-CoV-2 through the recognition of the SARS-CoV-2 epitope presented by MHC (32). The main targets of T cells are the M, N, S, and other various epitope proteins expressed by ORF3, ORF8, Nsp2, and Nsp4 SARS-CoV-2 (33). Approximately, there are 1.400 SARS-CoV-2 epitopes recognizable by T cells (34). Studies have shown that most epitopes are retained in various variants of SARS-CoV-2 (35).

Earlier induction of CD8+ T cell was found in the patients with mild symptoms (36). This demonstrates the critical role of CD8+ T cells in eliminating the SARS-CoV-2. In the severe patients, there was an escalation in T cells activation, especially CD8+ T, which was characterized by an increase in the expression of several activation markers (CD38, Human Leukocyte Antigen-DR isotype/HLA-DR, Ki-67) and cytotoxic proteins (perforin and granzyme B) (37). T cells activation leads to the T cells fatigue. This condition is characterized by increased inhibitor receptors expression such as Lymphocyte Activation Gene 3 (LAG-3), T-cell Immunoglobulin and Mucin Domain-Containing Protein 3 (TIM-3), and also Programmed Cell Death Protein-1 (PD-1) (37, 38). The fatigue T cells will have a reduction in their cytotoxic ability thus, they are ineffective in eliminating the virus.

There were CD4+ and CD8+ T cell numbers declining peculiarly in severe patients, indicating the presence of T cell dysfunction in COVID-19 infection (39). Several mechanisms have been thought to cause the decrease in the T cell counts. First, it is caused by viral infection directly through the ACE receptors owned by T cells (35). Second, it is caused by the suppression of the infected lymphoid organs so that there is a decrease in lymphocyte production (40). Third, it is caused by the process of T cell apoptosis mediated by the bond of Fas and Fas Ligand (FasL). In COVID-19, Fas expression on the surface of T cells and plasma FasL production was found to increase (41). Fourth, the presence of T cell pyroptotic induced by the upregulation of NLRP-3 (29). Fifth, direct cytopathic effects on T cells by IL-6 and TNF-α (42). Sixth, T cell apoptosis mediated by infected DC, characterized by an increase in Tumor Necrosis Factor-related Apoptotic Inducing Ligand (TRAIL) in the DC (43).

SARS-CoV-2 has been shown to have the ability to infect DC, causing a decrease in the DC's number and DC's function impairment. SARS-CoV-2 infection can reduce the number of mononuclear DC (moDC) by 10-20% (44). Studies in COVID-19 patients in acute and convalescent-phase showed a decrease in the conventional DC (cDC) and plasmacytoid (pDC) number accompanied by an increase in the cDC/pDC ratio, especially in patients with severe symptoms (45). There was also a pDC decrease in pediatric patients who experienced Multisystem

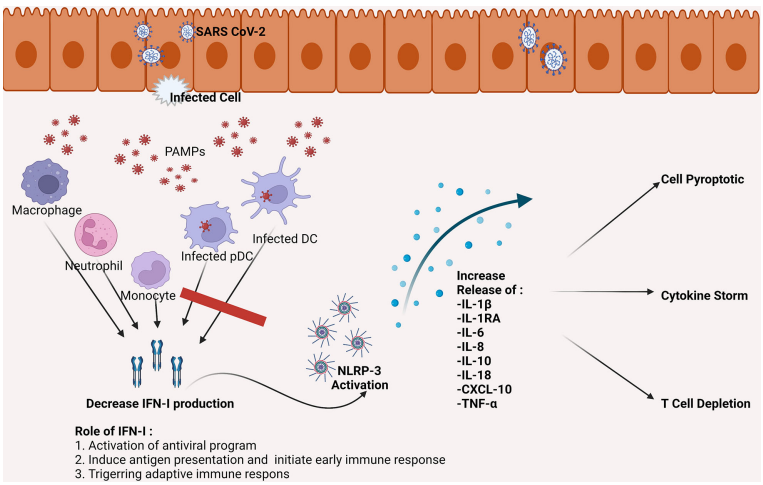

FIGURE 1
SARS-CoV-2 can infect DC, including pDC, which is the primary producer of IFN-I. The SARS-CoV-2 infection causes a decrease in the number of DC as well as a decrease in IFN-I production. Inadequate IFN-I leads to failed elimination of SARS-CoV-2. The failure eventually increased the activity of NLRP-3, which leads to pro-inflammatory cytokines increase which then triggers cell apoptotic, cytokine storms, and depletion of T cells. CXCL, the chemokine C-X-C motif ligand; DC, dendritic cell; IFN, interferon; IL, interleukin; NLRP-3, NLR family pyrin domain containing 3 inflammasome; pDC, plasmacytoid dendritic cell; TNF, tumor necrosis factor.

Inflammatory Syndrome in Children (MIS-C) due to SARS-CoV-2 infection (46). Depletion in cDC and pDC number remained found until seven months post-infection (47).

SARS-CoV-2 infection also causes DC maturity impairment. Examination of patient alveolus tissue showed an increase in DC recruitment that did not have maturity molecules (48). Studies showed a decrease in Human Leucocyte Antigen – DR isotype (HLA-DR) and CD80 expressions, which are the markers of DC maturity, and a reduction in STAT2 activity, which correlates with correlates to the ability of DC to activate CD8+ T cells (43, 49, 50). The immature DC is unable to present antigens to T cells, so the differentiation and production of specific T cells are inadequate (51).

The decrease and dysfunction of DC caused by SARS-CoV-2 infection results in an IFN-I reduction. SARS-CoV-2 inhibits the phosphorylation of STAT1 in moDC and pDC, which leads to suppression and delaying the production of IFN-I (44). The infected DC also produced pro-inflammatory cytokines (IL-6, TNF-α) as well as chemokines (Interferon gamma-induced Protein 10/IP-10, Macrophage Inflammatory Protein 1 alpha/MIP-1α, Monocyte Chemoattractant Protein1/MCP-1 (see Figure 1) (51). Thus, SARS-CoV-2 infection in DC has responsible for immune system dysfunction.

Specific immunity against SARS-CoV-2

Antibodies will be formed when SARS-CoV-2 infection occurs. Immunoglobulin M (IgM) and IgG that are specific to the N and S protein begin to be measured on day 2 of symptoms. IgM peaks on day 11-13 then decrease after 3 weeks besides IgG will be observed entirely on day 17-19 (52). The increase in IgG is followed by the formation of memory B cells for up to 3 months in length (53). Nevertheless, some patients with mild or asymptomatic symptoms were not found to be any seroconversion of these antibodies (54). Studies have also shown a decrease in these antibodies in the 3-6 months (55). Tiandan et al. found that the IgG ability to neutralize SARS-CoV-2 in 1-year post-onset was only 43% subjects, and its antibody ability would decrease against new variants of SARS-CoV-2 (56).

SARS-CoV-2 infection also forms a T-cell response (57). The CD4+ T-cell response was detected in all patients, while CD8+ T cells were found in most patients, not in all patients (33). CD8+ T-cells can be observed on day seven and peak until day 14 (58). T cell responses also remained to be found in mild or asymptomatic patients, despite absent antibodies seroconversion (54). The detected T cell response was characterized by the formation of effector and memory T cells. The formed memory T cells are capable of recognizing various epitopes of SARS-CoV-2 (59). formation of specific memory T cells forms immunity and prevention against reinfection. This finding indicates the superiority of T cell immunity compared to antibodies in preventing the infection.

The memory CD8+ T cells were found to be diverse, ranging from central memory (Trm), effector memory (Tem), resident memory (Trm), even into polyfunctional memory cells or memory T cells that can act as *stem cells* (Tscm) (60). The ability of memory CD8+ T cell formation is attributed to the recognition and elimination ability of SARS-CoV-2 (61). Transient T cell formation CD4+ memory is correlated with the presence of B cells and the production of IgG (53). The specific T cells remain observed for up to 6 months post-infection (62). While polyfunctional T cells remain detected for up to 10–12 months (60). This suggests that SARS-CoV-2 specific T cells can persist for an extended period. This condition shows similarities to SARS-CoV infection in which specific memory T cells remain detected for 17 years (63).

Currently, various vaccines have been developed and used to strengthen immunity against SARS-CoV-2. There are several types of vaccines in circulation, such as protein-based vaccines, messenger ribonucleid acid (mRNA), viral vectors, and inactivated viruses (8). All types of vaccines have the formation of specific antibodies that can neutralize SARS-CoV-2 with varying efficacy. mRNA-based vaccines show effectiveness above 90% (64, 65), virus vector-based vaccines 66-91% (66, 67), inactivated virus-based vaccines can reach 80% (68), while protein-based vaccines are currently still being developed (69). However, research shows a decrease in the effectiveness of all these vaccines against VoC by 0.5–11 times (8).

Role of dendritic cell in shaping T cell immunity

DC is well-known as the most potent APC and plays a pivotal role in innate and adaptive human immune systems (10). In the innate immune system, DC introduces and determines the body's response to DAMP or PAMP. In the adaptive immune system, DC is responsible for presenting antigens to naïve T cells (70). DC exposed to the antigen will maturate and drain to the lymphoid organs, then present the antigen to the naïve T cells leading to T cell differentiation (71). Therefore, DC has a role in connecting the innate and the adaptive immune system.

DC is derived from Lymphoid Primed Multi-Potent Progenitor (LMPP) which differentiates into Granulocyte-Macrophage DC progenitor (GMDP) and then becomes macrophage DC progenitor (MDP). MDP will be a Common DC Progenitor (CDP) that will differentiate into pDC, cDC1, and cDC2 (72). In addition, there is DC derived from monocytes (moDC) and DC subset known as Langerhans cells (10). In general, there are five types of DC. pDC, cDC1, and cDC2 are DC found under any conditions, while Langerhans cells are specified in the skin, while moDC is only produced when there is

inflammation. DC can be found in the lymphoid organs, circulation, and specific tissues or organs such as the lungs, liver, and digestive tract (73).

The critical role of DC in the immune system is to perform priming cell T (Figure 2). This process differentiates naive T cells into antigens or pathogen-specific T cells (10). Memory T cells will cause pathogen elimination to occur faster and prepare the body for repeated pathogens exposure (74). DC presents antigens to CD4+ through MHC-II molecules and CD8+ *via* MHC-I (75). Activation of CD4+ T cells by DC will induce the formation of plasma cells so that specific antibodies are formed (Figure 2B). In addition to the ability to recognize external antigens, DC can also recognize self-antigens in the body to prevent the occurrence of autoimmune through priming T cell becomes cell T regulator (Treg) (71). T cells priming process is affected by the presence of antigen presentations, co-stimulating molecules, and the presence of cytokine production (70).

Each type of DC has its function (Table 1). pDC can be found in the circulation and lymphoid organs and plays a crucial role in the body's immune mechanism against viruses because it has TLR that can recognize RNA and DNA (81). Besides as primary producer of IFN-I (such as IFN-α), pDC also produces IFN-III, TNF-α, IL-6, and granzyme B *(72)*. CD4+ T cells can be primed by pDC by CD-303 and CD-367 molecules, while CD8+ T cells are primed by pDC through antigen transfer to cDC and the resulting IFN-I activity (76).

Conventional dendritic cells 1 (cDC1) are more prevalent in tissues than blood (73). cDC1 activates effector CD8+ T cells and NK cells through the C-X-C chemokine Ligand motif 9 (CXCL9), CXCL10, and XC 1 chemokine receptors (CXR1) expression so that it can regulate cytotoxic cells (77). In addition, cDC1 can also activate Trm through CD-24 expression and the production of IL-12 and IL-15 (70). These cytokine productions can also activate Th1 cells (72). Studies

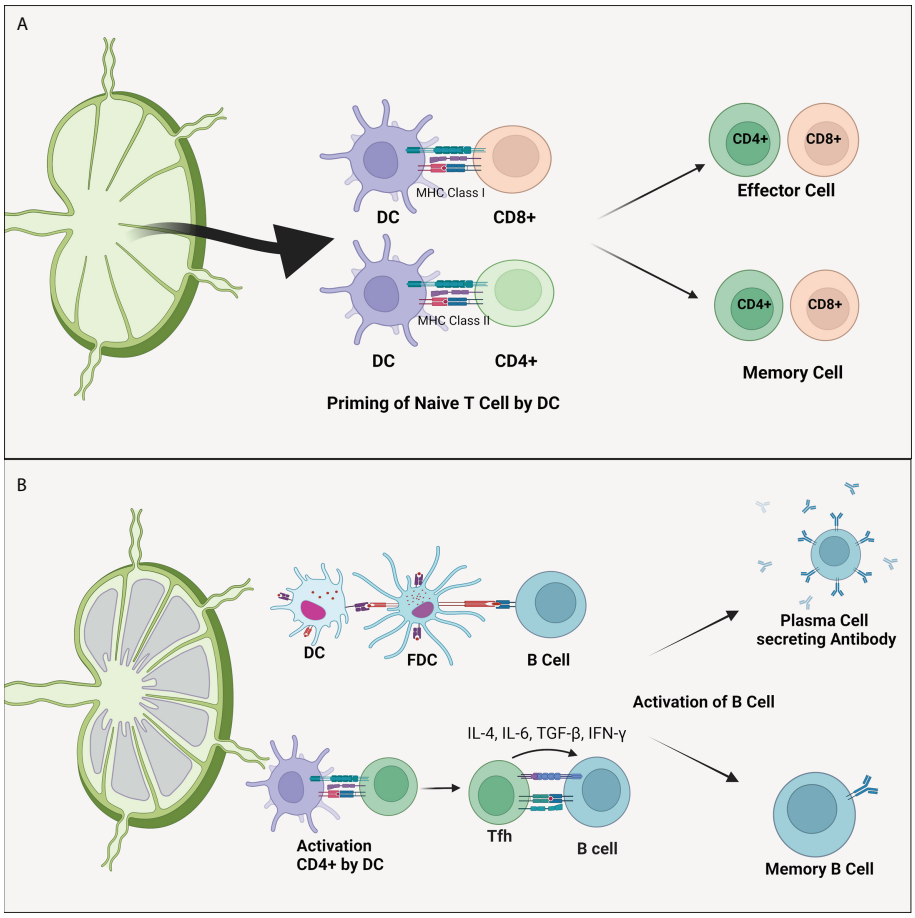

FIGURE 2
(A) Dendritic cell's ability to differentiate naïve T cells. As APC, DC presents antigens to CD4+ and CD8+ T cells through MHC-II and MHC-I, respectively. This process forms antigen-specified effector and memory T cells. **(B)** Dendritic cell also plays a vital role in the B cells activation. Activation of B cells can be directly carried out by DC cells or by the intermediately by CD4+ T cells. Activated CD4+ T cells will migrate to the follicular area to activate specific B cells of both plasma B cells that produce antibody and memory B cells. DC, dendritic cell; FDC, follicular dendritic cell; IL, interleukin; MHC, major histocompatibility complex; TGF-β, transforming growth factor β.

TABLE 1 Types of dendritic cells and their functions.

Dendritic Cell Types	Function	References
Plasmacytoid Dendritic Cell (pDC)	Priming of CD8+ through IFN-I production and antigen transfer to cDC and priming CD4+ through regulation of CD-303 and CD-367 molecules	(72, 76)
Conventional Dendritic Cell 1 (cDC1)	Regulate and prime CD8+ by IFN-III, CXCL 9/10, and IL-12 production	(77)
	Priming Trm by the production of CD-24, IL-12, IL-15	(70)
	Differentiation Th1 and Tfh that induced of B cell	(16, 72)
Conventional Dendritic Cell 2 (cDC2)	Potent activator of Th1, Th2, Th17 through IL-1β, IL-6, IL-12, dan IL-23 production	(73)
	Differentiates CD8+ and regulating Tcf1	(78)
	The efficient inductor of Tfh	(16)
	Differentiates Treg through the production of IL-10 and TGF-β	(72)
Monocyte derived Dendritic Cell (moDC)	CD4+ and CD8+ T cells priming through regulation of Tbet, Tcf1 and by producing cytokines in inflammation states.	(72, 79)
	Differentiates long term memory T cells by producing IL-15	(80)
Langerhans Cell	Specific immune responses in the skin	(10)

show that cDC1 also plays a role in the activation of Tfh. In addition, Th1 and Tfh produce cytokines IL-4, IL-21, and IFN-γ which activate B cells that are capable of producing antibodies (16). Thus, cDC1 contributes to the formation of the humoral immune system.

Conventional dendritic cell 2 (cDC2) is a DC that has a broader cross-presentation capability to CD4+ and CD8+ T cells compared to other DCs (82). This DC is the leading producer of IL-1β, IL-6, IL-12, and IL-23 that makes DC as the most potent activator of Th1, Th2, and Th17 (73). The produced IL-12 is capable of regulating Transcription factor 1 (Tcf1) which is a regulator for the differentiation of CD8+ into effector cells as well as memory cells (78). Based on research, cDC2 is also an efficient Tfh inducer, thus making these cells have an essential role in antibody generation (16). In addition, cDC2 also plays a role in Tregs differentiation through the IL-10 and Transforming Growth Factor-β (TGF-β) production (72).

Monocyte derived dendritic cell (moDC) originate from monocytes during infection and inflammation (73). *In vitro*, moDC can be formed by administering Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and IL-4 stimulation through the IRF-4 signaling pathway (75). Like other types of DCs, moDC has the ability to prime T cells through T-bet and Tcf1 regulation in line with the production of cytokines IL-1, IL-23, and TNF-α (72, 79). moDC also produces IL-15 causes memory CD8+ T cells last a long time (80). In addition, moDC also secretes IL-12 which can activate T cells that become Th1 cells (71).

Rationale of dendritic cell based vaccine for SARS-CoV-2 infection

Dendritic cells have been widely developed and researched as immunotherapy in managing various diseases. DC-based immunotherapy has been tested on breast, prostate, melanoma, kidney, glioblastoma, ovarian, and lung cancers (83). Clinical trial studies of DC-based vaccines arrayed promising results, with a marked rise in the count of anti-tumor-specific CD8+ T cells (84). As an example, clinical trials in patients with advanced ovarian cancer given autologous DC vaccines pulsed with HOCl-oxidized tumor lysate (OC-DC) showed an increase in T cell response and a lengthening of the survival rate for two years to 100% accompanied by low side effects (85).

DC-based immunotherapy was also developed for infectious diseases. In HIV trials, DC-based vaccines increased specific T cells response, although the effectiveness of reducing viral load was still not conclusive (86). Clinical trials for hepatitis C also showed an upsurge of specific cellular immunity to HCV in the absence of severe side effects (87). Further, DC-based vaccines were also developed for hepatitis B, malaria, as well as influenza (11, 88, 89).

The success of DC-based cancer immunotherapy and infection vaccines suggests the potential for DC development as a SARS-CoV-2 vaccine. This approach utilizes the ability to present antigens and induce the immune system possessed by DC (90). Immature DCs can be introduced with SARS-CoV-2 antigens, for example, S protein which has proven to elicit an immune response (91). This process can be developed both *in-vivo* and *ex-vivo*, but the *ex-vivo* approach can be an option in developing this vaccine because of its feasibility and shortening of the processes that should occur in the body (92). The DCs that have been exposed to the antigen will undergo maturation and drain to the lymphoid organs, then present the antigen to the naïve T cells so that specific immunity to SARS-CoV-2 is formed (71). This approach is currently being developed in Indonesia and commonly known as Nusantara Vaccine.

There are four main reasons that can support the utilization of DC as a SARS-CoV-2 vaccine, including (Figure 3):

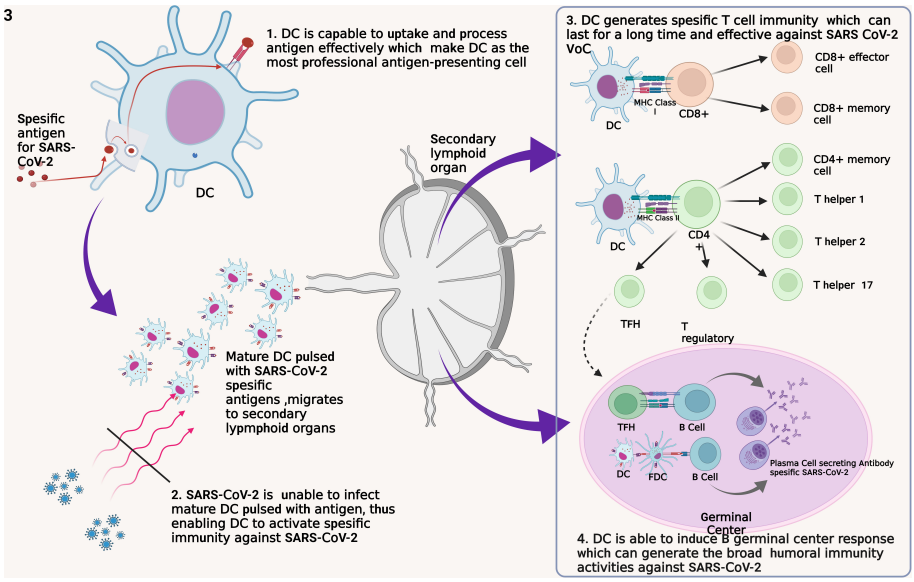

FIGURE 3
Four fundamental reasons for the development of DC as a SARS-CoV-2 vaccine. DC, dendritic cell; FDC, follicular dendritic cell; MHC, major histocompatibility complex; TFH, T follicular helper.

First, DC is a professional APC that captures, processes, and exposes antigens efficiently and effectively to other immune cells such as T cells (32). DC recognizes and internalizes antigens by endocytosis or by direct contact with gap junction and by cross-dressing (93). This method allows the DC to be able to identify and capture antigens in other infected cells and those that have experienced apoptotic. DC has a lower lysosome protease enzyme and the ability to neutralize pH well to maintain the antigens captured until the exposure process to other immune cells (32). In addition, DC has Gamma Interferon-Induce Lysosomal Thiolreductase (GILT), whose function is to maintain intracellular processes in the DC so that pyroptotic does not occur due to inflammasome activation (94). Thus, the use of DC as a vaccine will ensure the process of introduction and presentation of SARS-CoV-2 antigens so that specific immunity formation occurs.

Second, DC is a cell that SARS-CoV-2 weakens to evade the body's immune response thus, DC is a plausible vaccination target (95). In the acute phase, the significant decrease of pDC leads to IFN-I depletion thus, causes a failure of the innate immune response (96). In addition, SARS-CoV-2 infection also inhibits adaptive immune responses through impairing DC maturation characterized by a decrease in Human Leucocyte Antigen – DR isotype (HLA-DR) and CD80 expressions (49, 50). In COVID-19 patients, it was found that the reduction of DC was correlated with the depletion of T cell numbers (97). Altogether, this condition leads to the failure to transition an innate immune response into an adaptive immune response. Therefore, vaccination with a focus on improving and protecting DC function has the potential to provide better results.

Third, DC has a good ability for T cell activation. As previously explained, DC will activate various types of T cells. Naïve CD8+ T cells will be activated into effector and memory T cells (76). Formed Th2 and Tfh cells play a role in the differentiation of B cells into antibody-producing cells, while Treg cells control the function of other lymphocytes (75). Evidence that formed SARS-CoV-2-specific memory T cells persist for an extended period implies this vaccine can prevent infection and replication of SARS-CoV-2 in the long term (53, 60). In addition, studies have shown that memory T cells remain effective against VoC thus, this DC-based vaccine has the potential to persist effective against various mutated virus variants (35). All of these things are also supported by studies that show that T cells play an essential role in SARS-CoV-2 infection. Therefore, the ability of DC to activate T cells is the basis of the use of DC for SARS-CoV-2 vaccines that potential to have good effectiveness.

Fourth, the DC-based vaccine has the potential to trigger the formation of germinal center (GC) cell responses so that B cells are formed and can recognize virus variants. DC induces the response of B GC cells through the activation of naïve T cells into Tfh cells, which will then activate B cells (98, 99). The activation process triggers the formation of plasma and memory B cells that undergo affinity maturation and clonal evolution so that a broad B cell response is formed to fight viruses with an immense mutation rate such as SARS-CoV-2 (100). Through this mechanism, antibodies that can neutralize SARS-CoV-2 widely will be generated so that they are effective against various virus variants.

For these four reasons, DC can be used as a SARS CoV-2 vaccine. The immunity generated through this approach is oriented towards forming T cells so that the vaccine can last a long time and remain effective against the developing variants of SARS-CoV-2. DC-based vaccines also have the potential to create antibodies that have a broad response. The integration in producing specific T cells and antibodies is the main key to developing DC as a potential SARS-CoV-2 vaccine. For this reason, further studies need to be executed to prove the safety and effectiveness of DC-based vaccines.

Translation of DC-based vaccine for SARS-CoV-2: Challenges and future perspective

DC-based vaccine translation depends on various factors, DC type selection and processing, antigen loading selection, and administration methods of DC-based vaccines (101). As already mentioned above, there are various subtypes of DC present in the human body. pDC is often associated and fights an important immunity protection to viral infections (96). cDC that is able to activate T cells widely so that it is postulated is able to activate CD4+ which plays a role in the formation of antibodies (77). However, the utilization of both subsets requires a more invasive procedure, and its proportion in the body <1% in the blood becomes an obstacle in its utilization (102). moDC is a DC subtype that is widely chosen because it is easily accessible from peripheral blood which is then incubated with GM-CSF and IL-4 (103). Vaccines using moDC in cancer have been shown to be able to form T cell immunity. However, some studies have shown the potential for moDC inferiority in priming T cells compared to cDC and pDC (104). However, some studies have also shown that the ability of antigen transfer or cross-presentation that moDC then introduce antigens to endogenous cDC in the body so that it is able to produce cytokines (IL-12) that are able to priming CD4 cells (105). As well as a study also showed the cancer DC vaccine that the activation ability of CD8+ is also spaced by endogenous DC interacting with the DC vaccine (106). Thus moDC is potential candidate in the development of a vaccine for SARS-CoV-2.

Selection of loaded-proteins or antigens is also critical issue since the protein is determinator of a specific immune response. In this case, the selection of specific proteins capable of triggering a strong immune response to SARS-CoV-2 must be determined properly because it is related to its effectiveness even against virus variants that continue to develop. Currently the S-protein is widely used as a target in vaccine development. Utilization of this protein includes the use of full-length SARS-CoV-2 S-proteins, specific sub-units of S-protein (S1, S2), and specific RBD S-protein SARS-CoV-2. S-protein plays important role in the entry of viruses, and several loci of its RBD are targets of the SARS-CoV-2 immunoglobulin antibody (107). This is supported by the results of research that S-protein is able to trigger specific immunity to SARS-CoV-2 (108). However, evidence also shows the occurrence of mutations in some loci in the S-protein which results in a decrease in the effectiveness of various other vaccines where there is a decrease in the affinity of the antibodies produced (109, 110).

The S-protein can still be an option in the development of DC-based vaccines. Given that DC-based vaccines are oriented towards the formation of T Cell immunity. As outlined, that the SARS-CoV-2 variant retains most of its epitope, specific research into epitope in the delta and omicron variants also shows that both variants still retain T cell epitopes by 75-90% (111). Therefore, the utilization of the S protein as a loaded-antigen in DC-based vaccines has the potential to maintain the effectiveness of the vaccine against the evolving SARS-CoV-2 variant according to the orientation of DC-based vaccines is the formation of T cells immunity. Determination of loaded-antigens in DC-based vaccines remains an opportunity for the foreseeable future. Determination of loaded-antigens in addition to affecting effectiveness, can also affect the load and cost of vaccine production.

Conclusion

The development and discovery of effective and enduring vaccines remain a challenge in conquering the COVID-19 pandemic. Although various types of vaccines have been distributed, these vaccines still have limitations. The known professional ability of DC in activating T cells and their involvement in SARS-CoV-2 infection encourage the development of DC-based vaccines that have the potential to have good effectiveness. However, more research is still needed to get a safe and effective DC-based vaccine so that in the end it can be a breakthrough to overcome the ongoing pandemic.

Author contributions

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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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Non-replicative antibiotic resistance-free DNA vaccine encoding S and N proteins induces full protection in mice against SARS-CoV-2

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SARS-CoV-2 vaccines currently in use have contributed to controlling the COVID-19 pandemic. Notwithstanding, the high mutation rate, fundamentally in the spike glycoprotein (S), is causing the emergence of new variants. Solely utilizing this antigen is a drawback that may reduce the efficacy of these vaccines. Herein we present a DNA vaccine candidate that contains the genes encoding the S and the nucleocapsid (N) proteins implemented into the non-replicative mammalian expression plasmid vector, pPAL. This plasmid lacks antibiotic resistance genes and contains an alternative selectable marker for production. The S gene sequence was modified to avoid furin cleavage (Sfs). Potent humoral and cellular immune responses were observed in C57BL/6J mice vaccinated with pPAL-Sfs + pPAL-N following a prime/boost regimen by the intramuscular route applying *in vivo* electroporation. The immunogen fully

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protected K18-hACE2 mice against a lethal dose (10^5 PFU) of SARS-CoV-2. Viral replication was completely controlled in the lungs, brain, and heart of vaccinated mice. Therefore, pPAL-Sfs + pPAL-N is a promising DNA vaccine candidate for protection from COVID-19.

KEYWORDS

SARS-CoV-2, DNA vaccine, S protein, N protein, mouse model, pPAL, furin

Introduction

Vaccines are considered the most effective method to control the COVID-19 pandemic and have helped restore the global economy (1, 2). However, efficacy of the protective immune response induced by these vaccines against variants of concern (VOCs) is problematic (3). New VOCs present a high mutation rate in the receptor binding domain (RBD) and the N-terminal domain sequences of the SARS-CoV-2 spike glycoprotein (S). Antibody-based immunity against these VOCs is less effective compared to the original strain, Wuhan-Hu-1 (4, 5). Nonetheless, the T-cell response remains robust to these VOCs (6). T cells recognize more epitopes on the S protein than antibodies do (6). T-cell responses do not fade as quickly as antibody responses, and are, therefore, more effective at protecting from emerging variants (3, 7–9). Vaccines expressing the S protein from the Omicron variants don't improve efficacy when compared to the original S protein. This indicates that altering the vaccine antigen to the most recent S form may not necessarily correlate with improved efficacy (3). Developing highly efficacious vaccines to prevent COVID-19 remains a global need. Vaccines that elicit an adequate cellular immune response will be necessary in the future to limit the impact of new VOCs on health systems (10).

DNA vaccines have been used in veterinary medicine and induce specific protective immune responses against pathogens. They are easily modifiable allowing for quick testing of multiple vaccine candidates against new virus strains. One of the benefits of DNA vaccine production is that it can easily be scaled up. DNA vaccines are thermotolerant, and, consequently, cold chain maintenance is not required for long-term storage or worldwide distribution. Developing countries would benefit from this feature (11). Several DNA vaccine candidates are being tested in clinical trials against COVID-19 (12), although most contain an antibiotic resistance gene as a selectable marker for the manufacturing process (12). We have developed a new DNA vaccine candidate for the prevention of SARS-CoV-2 infection that consists of the complete S and the nucleocapsid (N) gene sequences cloned into the non-replicative antibiotic resistance gene-free pPAL plasmid (13). The S gene was modified to stabilize the protein product against furin cleavage (Sfs) and includes the RBD, which mediates virus entry into the host cell (14). The vaccine candidate, pPAL-Sfs + pPAL-N, is composed of a 1:1 mass ratio mixture of each plasmid dissolved in sterile water. We decided to include the genes in separate plasmids to avoid a single larger plasmid, which would result in reduced production yield. The pPAL plasmid vehicle includes a long CpG island (13) and does not require additional adjuvants. The N protein was chosen as it remains significantly conserved among betacoronaviruses (15). A protective role for the N protein against SARS-CoV-2 infections has been recently proposed (16). The N protein is the most abundant viral protein, highly immunogenic in coronavirus infections, and may contribute to broadening the T-cell response, improving cross-reactivity.

Therefore, we assessed the efficacy of the pPAL-Sfs + pPAL-N vaccine candidate against SARS-CoV-2 using a prime/boost administration regime by the intramuscular route followed by *in vivo* electroporation (11). Humoral and cellular immunity were evaluated in wild-type C57BL/6J mice and the level of protection against a lethal challenge of SARS-CoV-2 was assessed in the mouse line B6Cg-Tg(K18-hACE2)2Prlmn/J (17). The present study provides evidence that pPAL-Sfs + pPAL-N is a promising vaccine candidate against SARS-CoV-2 infection.

Materials and methods

pPAL constructs

DNA vaccines require a vector to replicate genes and express encoding antigens. Antibiotic resistance genes are often used as selection markers, which must not be released into the environment upon final product commercialization. Considering this, the use of antibiotic resistance-free vectors is imperative. The pPAL mammalian expression plasmid vector is based on the cytomegalovirus enhancer and promoter sequences. This plasmid does not replicate in mammalian cells and does not contain selectable markers based on antibiotic resistance. The selectable marker is the *E. coli fab*I gene, which encodes for the enoyl-ACP reductase. This enzyme is inhibited by the bacteriostatic compound triclosan, which is the selection agent at an optimal concentration of 3 μM (13). The pPAL-Sfs

construct (Figure 1A) contains a modified version of the SARS-CoV-2 S-encoding gene with NCBI acc. no. NC045512, gene ID 43740568. First, the sequence was optimized by the Monte Carlo approach according to relative codon usage frequencies. Second, the cleavage site was modified to avoid furin cleavage (PRRA → PGGS; 681-684). For this purpose, the nucleotide sequence CCTCGGCGGGCA was replaced by CCAGGCGGCAGC (2041–2052). The pPAL-N construct contains the SARS-CoV-2 N protein with NCBI acc. no. NC045512, gene ID 43740575. The KpnI-site-flanked pPAL-Sfs and pPAL-N constructs were obtained by gene synthesis in the pGH vector (ATG Biosynthetics, Merzhausen, Germany) and transferred to the *E. coli* SURE2 (Agilent, Santa Clara, CA) strain by electroporation at 1,800 V, 200 Ω, and 25 µF. Selection in LB-agar medium was performed with 3 µM triclosan (Sigma-Aldrich, St. Louis, MO, United States). pGH was excised by KpnI (NEB, Ipswich, MA) digestion, and the vaccine constructs were circularized with T4 DNA ligase (NEB). Endotoxin-free pPAL, pPAL-Sfs, and pPAL-N plasmid preparations were obtained with PureLink™ Expi Endotoxin-Free Giga Plasmid Purification Kit (Invitrogen, Waltham, MA) following the manufacturer's instructions.

Antigen gene expression in transfected HEK293 cells

HEK293 cells (CRL-1573™, ATCC®, Manassas, VA) were grown at 37 °C in a 5% CO_2 atmosphere in complete medium (CM) containing DMEM supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) (Sigma-Aldrich, Burlington, MA), 100 IU penicillin – 100 µg/mL streptomycin. Semi-confluent HEK293 cells were detached by mild pipetting and washed once with 1 mL sterile PBS and once with 0.3 mL GTporator®-M (Protean, Ceske Budejovice, Czech Republic) per 3 x 10^6 cells. The cells were transfected by electroporation with pPAL, pPAL-S, pPAL-Sfs, or pPAL-N, and a mock transfection control that didn't contain DNA was included. Transfection of 1.2 x 10^6 cells with 5 µg of DNA was performed at 220 V, 25 Ω, and 950 µF in 80 µL of GTporator®-M solution in a sterile 2 mm-gap cuvette (BTX, Cambridge, United Kingdom) using an ECM 630 Electro Cell Manipulator Precision Plus® (BTX, Cambridge, United Kingdom). 1.2 mL of pre-warmed medium was immediately added to the cells. 0.2 mL of the cell suspension were placed in an 8-well culture slide (Nunc® Lab-Tek® Chamber Slide™, Sigma-Aldrich, Burlington, MA) and 1 mL in a 24-well plate. The cell suspensions were incubated at 37 °C for 24 h in a 5% CO_2 atmosphere.

Transfected HEK293 cells in 24-well plates were washed twice with 1 mL CM and lysed with 50 µL of a buffer containing 25 mM Tris-HCl pH 7.8, 2 mM EDTA, 2 mM DTT, 1% glycerol, and 1% Triton-X100. The protein extracts were quantified by the Bradford method. Each 20 µg protein extract was treated with 8.3 U/µL of TurboNuclease from *Serratia marcescens* (Sigma-Aldrich, Cambridge, United Kingdom) at room temperature for 10 min and prepared for SDS-PAGE in Laemmli buffer, heated at 95 °C for 5 min, and run at 30 mA for 90 min in 8-20% TGX precast SDS-PAGE gels (BioRad, Hercules, CA). Semi-dry transfer was performed on 0.45 µm nitrocellulose membranes (BioRad, Hercules, CA) at 1.3 A and 25 V for 10 min (high molecular weight transfer) in a TransBlot Turbo device (BioRad, Hercules, CA) following the manufacturer's instructions. The membranes were blocked with 5% skimmed milk in PBS-0.1% Tween 20 (PBS-Tween) at room temperature under mild shaking for 1 h and washed thrice in PBS-Tween, 15, 5, and 5 min, respectively. The membrane was incubated at room temperature under mild shaking for 90 min with the primary antibodies prepared at the appropriate dilutions in blocking buffer. The rabbit anti-SARS-CoV-2 S polyclonal antibody #ab272504, specifically recognizing the S2 subunit (Abcam, Cambridge, United Kingdom), was used at a 1:750 dilution in blocking solution. The anti-SARS-CoV-2 N polyclonal antibody, kindly provided by Mercedes Domínguez and Inmaculada Moreno (Centro Nacional de Microbiología, Virología e Inmunología Sanitarias, Instituto de Salud Carlos III), was used at a 1:500 dilution in blocking solution. After washing three times, a 1 h incubation was carried out with the secondary HRP-conjugated goat anti-rabbit whole IgG polyclonal antibody (DAKO Agilent Technologies, Santa Clara, CA) at a 1:2,000 dilution in blocking solution. After repeating the wash steps, chemoluminescence was developed with ECL Western Blotting Reagent (Thermo Fisher Scientific, Waltham, MA) for 1 min. The images were acquired with a ChemiDoc MP Image System (BioRad, Hercules, CA). The colorimetric and the chemoluminescence images were merged using ImageLab 6.1. software (BioRad, Hercules, CA).

Transfected cells in 8-well slides were washed once with 200 µL of a hypotonic solution (11:9 water:DMEM) and twice with 1:1 acetone:methanol. Fixation and permeabilization was performed with 1:1 acetone:methanol at -20 °C for 10 min. The preparations were air-dried and the wells were carefully removed from the slides. Three 5 min washes with 0.22 µm-filtered PBS were carried out in a Coplin jar. The preparations were then air-dried and blocked with 20 µL of a 5% skimmed milk solution in 0.22 µm-filtered PBS-Tween at 37 °C in a humid chamber for 1h. After removing the excess blocking solution, 20 µL of a 1:50 dilution in blocking buffer of the anti-S2 and anti-N primary antibodies mentioned above were added to the corresponding preparation and incubated for 1 h. A single 5 min wash step was applied. Then, the cells were incubated with 20 µL of 1:200-diluted Alexa Fluor® 488-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) at room temperature in the dark for 1 h. 10 min before the secondary antibody incubation was completed, 20 µL of 10 µg/mL DAPI in 0.22 µm-filtered PBS were added. The

slides were washed four times with 0.22 µm-filtered PBS, 5 min per wash, and were then mounted with 50 µL Mowiol 4-88 and left to dry at 4 °C for 16 h in the dark. The fluorescence images were acquired with an SP8 STED 3X confocal microscope (Leica Wetzlar, Germany).

Immunization with pPAL-Sfs + pPAL-N

87 eight-week-old wild-type C57BL/6J female mice (Charles River, Wilmington, MA) were used for immune response analysis, and 140 eight-week-old B6Cg-Tg(K18-hACE2) 2Prlmn/J female mice (Jackson ImmunoResearch, West Grove, PA) were employed in protection experiments. The animals were generally lodged in groups of five, except for four groups of eight C57BL/6J mice in the dose-response experiment, always following the space requirements specified in legislation (EU Directive 2010/63 and Spain regulation RD53/2013, modified by RD1386/2018). The mandatory permits to perform the experiments were approved by the Consejo Superior de Investigaciones Científicas (CSIC) Ethics Committee and the Comunidad Autónoma de Madrid. Experimentation with infected mice was carried out in BSL3+ laboratories (CISA-INIA-CSIC). All animals received food and water *ad libitum*. Animal welfare measures were applied, considering replacement, reduction, and refinement. Environmental enrichment was implemented. Anesthesia with isofluorane (3% for induction, 1.5% for maintenance) was administered while the vaccine inoculation followed by *in vivo* electroporation were being applied. 0.2 mg/Kg ibuprofen was then added to the drinking water. The same anesthesia was administered upon sacrifice by intracardiac puncture. All other measures specified in the regulations were applied. The endpoint criterion was adopted when appropriate. In the specific case of the K18-hACE2 mice after the viral challenge, euthanasia was immediately applied when animal weight decreased 20% or more and when any incipient sign of suffering was detected. The procedures applied in challenge experiments were then subject to retrospective evaluation.

C57BL/6J and K18-hACE2 mice were immunized by the IM route with the pPAL-Sfs + pPAL-N vaccine, which is an endotoxin-free DNA mixture composed of 20 µg pPAL-Sfs and 20 µg pPAL-N in sterile CHROMASOLV^{TM} water (Honeywell Riedel-de Haën, Charlotte, NC). Control mice received 40 µg of pPAL. Electroporation was applied using two 30G (0.3 x 13 mm) electrode needles connected to an ECM 830 Square Wave Electroporation System (BTX, Harvard Bioscience Inc., Cambridge, MA, USA), which were placed equidistant to the inoculation point in the direction of the muscle fibers, leaving a ~5 mm separation between them. The negative pole was placed in the posterior position. Six 50 ms 100 V pulses were applied in 1 s intervals. The animals were immobilized on an electrically isolated surface and were kept under isofluorane anesthesia (3% induction, 1.5% maintenance) during the procedure. 0.2 mg/Kg ibuprofen was administered in drinking water after the procedure. Wild-type C57BL/6J mice were euthanized a week after the booster dose for cellular immune response evaluation in the spleen. K18-hACE2 mice were subjected to challenge 2 weeks after the booster dose following humoral immune response evaluation.

ELISA

100 µL of peripheral blood was collected 1 week before vaccination and 15 days post-vaccination. Blood was left to clot at 4 °C for 5 h. Serum samples were obtained by centrifugation at 8,000 x*g* for 10 min and stored at -20 °C until use. Serial dilutions (1/3) of sera were prepared with 1% BSA in PBS-0.05% Tween 20. 96-well flat-bottom microplates were coated with 50 µL of 50 µg/mL recombinant S1+S2 ECD-His protein (Sino Biological, Beijing, China) and RBD (RayBiotech Life, Peachtree Corners, GA) in 3.36 mM carbonate–10 mM bicarbonate buffer at 4° C for 16 h. After three washes with 1% BSA in PBS-0.05% Tween 20, blocking was performed in PBS-0.05% Tween 20 containing 3% BSA for 1 h at room temperature. Next, the washes were repeated, and 100 µL of diluted serum samples were added, incubating for 1 h. After repeating the wash step, incubations with 1:8,000-diluted HRP-conjugated protein A (Invitrogen, Waltham, MA), 1:20,000 goat anti-mouse IgG1, or 1:20,000 goat anti-mouse IgG2c (Bethyl Laboratories, Montgomery, TX) were performed for 1 h, followed by three final washes. Color development was executed with TMB Substrate Kit (Thermo Scientific, Waltham, MA) for 10 min in 100 µL. The reactions were stopped by adding 100 µL of 2 N H_2SO_4. A_{450} was registered with Microplate Reader 680 (BioRad, Hercules, CA) and Microplate Manager 5.2.1 software (BioRad, Hercules, CA).

SARS-CoV-2 neutralization assays

The SARS-CoV-2 MAD6 viral strain and B.1.617.2 (Delta) variant were kindly provided by Dr. Luis Enjuanes and Dr. Juan Francisco García-Arriaza (CNB-CSIC, Madrid, Spain), respectively. The MAD6 SARS-CoV-2 genome sequence is identical to the Wuhan-Hu-1 isolate (GenBank MN908947). Serial dilutions of heat-inactivated mouse sera (starting at 1:10) were incubated with 100 PFU of SARS-CoV-2 MAD6 or B.1.617.2 (Delta) for 1 h at 37 °C in 96-well flat-bottom plates. 2×10^4 Vero E6 cells per well were then seeded on the sera/virus mixture and incubated for 3 days at 37 °C, 5% CO_2. Culture media was then removed, and cells were fixed with 2% paraformaldehyde prior to staining with 2% crystal violet. Neutralizing antibody (NAb) titers were calculated as the serum dilution at which less than 50% cytopathic effects were observed in replicate wells.

ELISpot and intracellular cytokine staining

Splenocyte suspensions were obtained in 15 mL of PBS containing 0.3 mM EDTA and 2% HIFBS (wash solution) using 0.45 µm BD Falcon Cell Strainers (BD Biosciences, San Jose, CA). The cells were harvested by centrifugation at 500 x*g* for 7 min and erythrocytes were lysed at room temperature for 2 min with 5 mL of 1X RBC Lysis Buffer (pluriSelect Life Science, Leipzig, Germany). 25 mL of wash solution were immediately added, and the cells were centrifuged at 500 x*g* for 7 min. After an additional wash, the cells were resuspended in 5 mL of proliferation medium containing RPMI supplemented with 10% HIFBS, 100 UI penicillin – 100 µg/mL streptomycin, and 4.5 µM β-mercaptoethanol. The cell suspensions were filtered through 0.45 µm BD Falcon Cell Strainers (BD Biosciences, San Jose, CA) and an aliquot was 1:1 diluted with 0.4% Trypan Blue solution (Sigma-Aldrich, St. Louis, MO) for live cell counting in TC10 Cell Counting Slides using a TC10 Automated Cell Counter (BioRad, Hercules, CA).

ELISpot assays were performed with the Murine IFN-γ ELISpot Kit (Abcam, Cambridge, United Kingdom) following the manufacturer's instructions. In the stimulation step, 2 x 10^5 splenocytes were seeded in 200 µL of proliferation medium containing the appropriate stimulus. Stimulation was performed in triplicate at 37 °C for 20 h in a 5% CO_2 atmosphere. The stimuli were: i) 1 µg/mL concanavalin A (Sigma-Aldrich, St. Louis, MO); ii) an equimolar peptide mixture at a final concentration of 25 µg/mL representing the whole S protein sequence prepared from the PepTivator® SARS-CoV-2 Prot_S1 and PepTivator® SARS-CoV-2 Prot_S (Miltenyi Biotec, Bergisch Gladbach, Germany) mixtures; and iii) an equimolar peptide mixture representing the whole N protein sequence at a final concentration of 25 µg/mL prepared from the PepTivator® SARS-CoV-2 Prot_N (Miltenyi Biotec, Bergisch Gladbach, Germany) mixture.

Splenocytes were plated in 96-well flat-bottom tissue culture plates (1x10^6 per well) and stimulated with S peptide pool (25 µg/mL) (Peptivator® SARS-CoV-2 Prot_S Miltenyi Biotec, Bergisch Gladbach, Germany), or with phorbol 12-myristate 13-acetate (50 ng/mL) (Sigma-Aldrich, St. Louis, MO) and ionomycin (1 µg/mL) (Sigma-Aldrich, St. Louis, MO) as the positive control, or left unstimulated as the negative control. Brefeldin-A (5 µg/mL), monensin (2 µM), and anti-CD107a antibody (1 µg/mL) (PE anti-mouse CD107a; clone 1D4B) (Biolegend, San Diego, CA) were also added to all wells at this stage. Splenocytes were incubated for a further 4 h in a humidified incubator at 37 °C, 5% CO_2. Cells were then washed with PBS and stained with viability marker LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Thermofisher, Waltham, MA) as described in the manufacturer's protocol for 20 min on ice. Cells were then washed in staining buffer (PBS + 2% HIFBS + 0.02% sodium azide) and stained for surface cell markers (Alexa Fluor® 488 anti-mouse CD8a, clone 53-6.7; Brilliant Violet 510™ anti-mouse CD4, clone GK1.5; and PerCP/Cyanine5.5 anti-mouse CD3, clone 17A2; all from Biolegend, San Diego, CA) for 20 min on ice. Cells were washed twice in staining buffer, and fixed and permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions for 20 min on ice, followed by staining for intracellular cytokines for 30 min on ice (Brilliant Violet 421™ anti-mouse TNF-α, clone MP6-XT22; Alexa Fluor® 647 anti-mouse IFN-γ, clone XMG1.2; and Brilliant Violet 785™ anti-mouse IL-2; all from Biolegend, San Diego, CA). After washing as indicated in the Cytofix/Cytoperm Fixation/Permeabilization kit, cells were resuspended in staining buffer and acquired with a FACSCelestaSORP flow cytometer (BD Biosciences, San Jose, CA). Data analysis was performed with FlowJo Software (BD Biosciences, San Jose, CA).

SARS-CoV-2 maintenance and infectious challenge

The Calu3 cell line was grown in DMEM medium supplemented with 20% HIFBS and 10 mM HEPES. This cell line was kindly provided by Luis Enjuanes (CNB-CSIC, Madrid, Spain) and was used for viral propagation at a multiplicity of infection (MOI) of 0.001 PFU per cell. The supernatant was harvested 72 h after infection (hpi), subjected to three freeze/thaw cycles, and clarified by centrifugation at 1,970 x*g* for 10 min. The virus was titrated and stored at -80 °C until use. Titration was performed using the Vero E6 cell line (CRL-1586, ATCC®, Manassas, VA), which was maintained in DMEM medium supplemented with 5% HIFBS. Viral adsorption was allowed for 1 h in 70-80% confluent Vero E6 cell culture monolayers. 2 mL of 0.5% semisolid agar in DMEM medium supplemented with 2% HIFBS were then added. The PFU count was performed after 6 days.

Mice were challenged with 10^5 PFU of MAD6 or Delta SARS-CoV-2 by the intranasal route 15 days after the booster dose. Thereafter, body weight and clinical profiles were followed daily. Body weight and clinical score follow-up included 10 K18-hACE2 mice per group (pPAL control and pPAL-Sfs + pPAL-N) for each experiment. The clinical score was calculated as explained in Supplementary Table 1. 20 mice per group in each experiment were employed for viral burden evaluation in subgroups of five on days 2, 4, 7, and 14 post-challenge.

Clinical score evaluation

Mice were observed and weighed daily post-challenge, and clinical signs were scored according to Table S1. The sum score

in clinical signs (based on body weight, appearance, motility, and respiration) was used to evaluate disease severity. A humane endpoint was implemented when this score reached >50 to reduce animal suffering.

Viral load evaluation in target organs

Samples of the target organs (lung, heart, and brain) obtained on days 2, 4, 7, and 14 post-challenge were lysed applying three freeze-thaw cycles and 20 sonication cycles/min at 5 W for 2 min. The lysates were centrifuged at 220 x*g* for 5 min to obtain clarified viral stocks. Total RNA extraction was performed with Trizol Reagent (Invitrogen, Waltham, MA) following the manufacturer's instructions. Two-step qRT-PCR was performed using SuperScript III reverse transcriptase (Invitrogen, Waltham, MA) and EHF DNA polymerase (Roche, Basel, Switzerland) as described by Toussaint et al. (18) using the N1-F (GACCCCAAAATCAGCGAAAT) and N1-R (TCTGGTTACTGCCAGTTGAATCTG) primers, and the N1-P (FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1) probe. The primers and probe for the β-actin reference gene were ACT_F_1005-1029 (CAGCACAATGAAGATCAAGATCATC), ACT_R_1135-1114 (CGGACTCATCGTACTCCTGCTT), and ACT_P_1081-1105 (JOE-TCGCTGTCCACCTTCCAG CAGATGT-BHQ1). The same clarified viral stocks from target organs were used to titrate viral replication by plaque assays in VERO cells measured as PFU/g tissue.

Statistical analysis

Two-way ANOVA with Fisher's LD *post-hoc* test was applied to intracellular cytokine staining experiments only. In all other experiments, statistical inference was performed with the Student's t-test applying p-value adjustment by the Holm-Sidak method.

Results

HEK293 cells transfected with the pPAL-Sfs + pPAL-N vaccine candidate expresses the SARS-CoV-2 Sfs and N genes

S and N protein gene sequences were retrieved from the SARS-CoV-2 Wuhan-1 isolate genome sequence (GenBank Acc. No. MN908947). Sfs is a modified version of the S gene generated to avoid furin cleavage (2041 CCTCGGCGGGCA → CCAGGCGGCAGC; 681 PRRA → PGGS). Endogenous furin from HEK293 cells only cleaves the S glycoprotein and not Sfs as the S2 fragment is only observed in pPAL-S transfectants (Figure 1A). The Sfs and the N clones were separately cloned into the pPAL mammalian expression plasmid vector under the control of the CMV enhancer/promoter (13), obtaining the pPAL-Sfs + pPAL-N vaccine candidate (Figure 1). HEK293 cells transfected with each DNA construct express the respective antigen genes according to Western blot (Figures 1A, B). Indirect immunofluorescence experiments using anti-S2 and anti-N polyclonal antibodies confirmed Sfs and N expression (Figures 1C, D).

pPAL-Sfs + pPAL-N vaccination induces SARS-CoV-2-specific humoral and cellular immune responses in mice

C57BL/6J (B6) mice were immunized with pPAL-Sfs + pPAL-N (20 μg each) by the intramuscular route by applying an *in vivo* electroporation procedure following a 15-day-interval prime-boost homologous regimen. The average titers of circulating anti-S IgG, anti-RBD IgG, and anti-RBD IgG2c were >2,000. The IgG2c/IgG1 ratio was ~750 in pPAL-Sfs + pPAL-N vaccinated mice (Figure 2A). All vaccinated animals were positive for neutralizing antibodies (NAb). The average NAb titers were ~100 after the booster dose (Figure 2B). Hence, the pPAL-Sfs + pPAL-N vaccine candidate elicits a SARS-CoV-2-specific neutralizing humoral immune response in mice.

According to an ELISpot assay, all vaccinated mice produced >500 IFN-γ-secreting spot-forming colonies (SFC) per million splenocytes on day 7 post-boost, specifically against an 11-mer peptide pool representing the whole SARS-CoV-2 S protein sequence with an overlap of 7 amino acids. The average was ~1,600, and the maximum was ~3,100 (Figure 3A). The mean of IFN-γ-secreting SFC per million splenocytes specific to a peptide pool representing the whole SARS-CoV-2 N protein sequence with the same characteristics is much lower (~200) (Figure 3A). A second independent ELISpot assay confirmed high levels of IFN-γ-secreting splenocyte clones and showed that pPAL-Sfs + pPAL-N response is dose-dependent (>2,000, ~1,500, ~1,300, and 0 in mice immunized with 20, 10, 5, and 1 μg of each plasmid, respectively) (Figure 3B). The T-cell response was assessed by intracellular cytokine staining (ICS). The CD107a, IFN-γ, IL-2, and TNF-α markers were monitored in $CD4^+$ and $CD8^+$ T cell populations in splenocyte preparations stimulated with the S and N peptide pools. All vaccinated mice produced $CD4^+$ and $CD8^+$ T cells stimulated specifically against the S and N peptide pools (Figure 4A). $CD4^+$ and $CD8^+$ T cell populations activated against the S peptide pool for the CD107a, IFN-γ, IL-2, and TNF-α markers were registered, except for IL-2 in $CD8^+$ T cells (Figure 4B). Activation against the N peptide pool was lower compared to the S peptide pool for all markers, except for CD107a in $CD4^+$ T cells. Particularly, no activation against the N peptides was observed for IFN-γ and IL-2 (Figure 4B). $CD8^+$ T cells showed higher polyfunctionality than $CD4^+$ T cells.

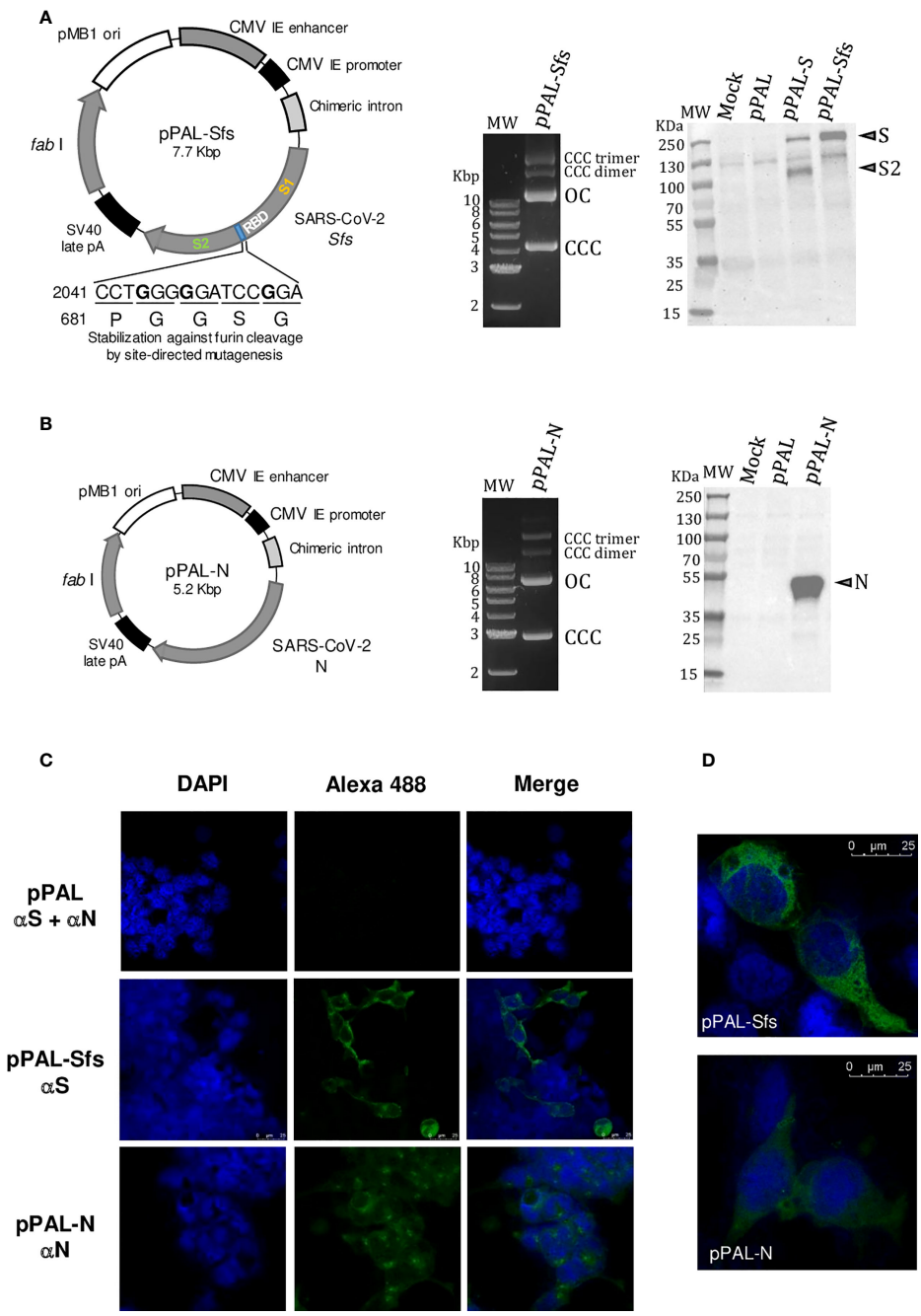

FIGURE 1
The pPAL-Sfs + pPAL-N vaccine candidate. **(A)** pPAL-Sfs map. Sfs is a codon-optimized modified version of the SARS-CoV-2 Wuhan reference sequence that contains a modification to avoid furin cleavage of the protein product. Large-scale laboratory preparation of the pPAL-Sfs plasmid containing supercoiled (CCC), open-circular (OC), CCC-dimer, and CCC-trimer conformers. Western blot of whole protein extracts from HEK293 cells transfected with pPAL, pPAL-S, and pPAL-Sfs. The primary antibody was 1:800-diluted goat anti-S polyclonal antibody (Abcam #ab272504, Cambridge, United Kingdom), which only recognizes the S2 subunit. The secondary antibody was 1:2,000-diluted HRP-conjugated goat polyclonal anti-rabbit Ig. Furin cleaves the S protein obtained from pPAL-S but not Sfs from pPAL-Sfs. **(B)** Large-scale laboratory preparation of the pPAL-N plasmid containing CCC, OC, CCC-dimer, and CCC-trimer conformers. Western blot of whole protein extracts from HEK293 cells transfected with pPAL and pPAL-N. The primary antibody was 1:500-diluted rabbit anti-N polyclonal antibody. The secondary antibody was 1:2,000-diluted HRP-conjugated goat polyclonal anti-rabbit Ig. **(C, D)** immunofluorescence of cultured HEK293 cells transfected with pPAL, pPAL-Sfs, and pPAL-N plasmids. The anti-S and anti-N antibody dilutions were 1:50. The pPAL control was incubated with both antibodies. The secondary antibody was Alexa Fluor® 488-conjugated goat anti-rabbit IgG diluted to 1:200. Sfs and N expression is observed.

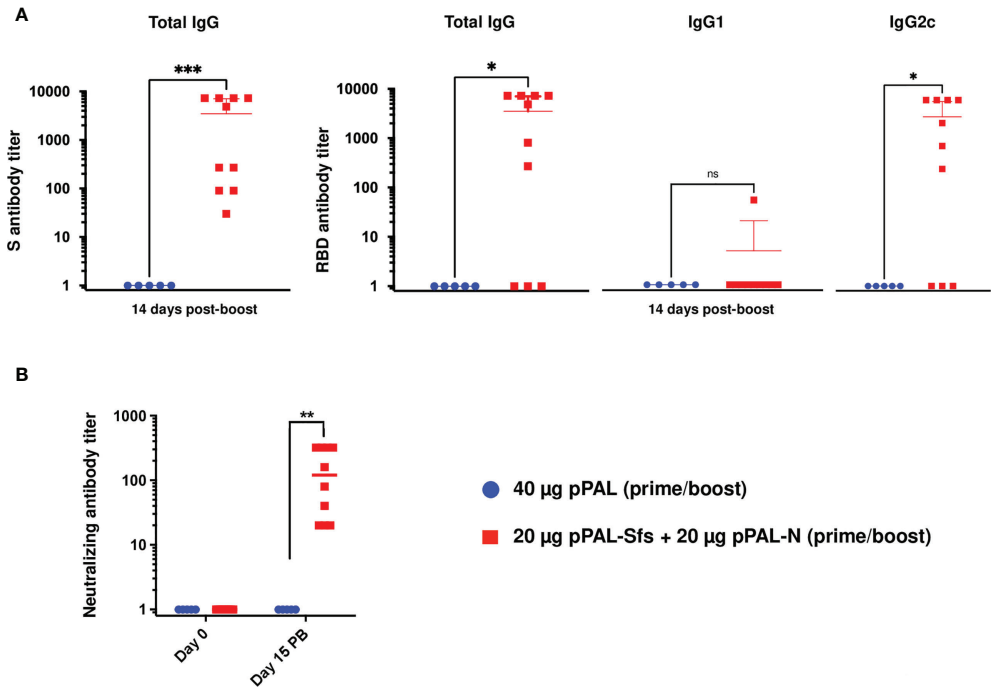

FIGURE 2
The pPAL-Sfs + pPAL-N vaccine candidates activate SARS-CoV-2 S- and RBD-specific humoral immunity in C57BL/6J mice. The serum samples were collected 15 days after the booster dose. **(A)** ELISA determinations of individual and average titers of total circulating anti-S and anti-RBD IgG determined by ELISA using HRP-conjugated protein **(A)** ELISA determinations of individual and average titers of circulating anti-RBD IgG, IgG1, and IgG2c using HRP-conjugated goat anti-mouse IgG1, anti-IgG2, and anti-IgG2c. The average anti-S and anti-RBD IgG titers are ≥2,000. The IgG2c/IgG1 ratio is ~750 in vaccinated animals with respect to the pPAL control group. **(B)** The neutralizing antibody titer average is >100. Statistical inference was performed using the Student's t-test applying p-value adjustment by the Holm-Sidak method ($\alpha = 0.05$; *p<0.05; **p<0.01; ***p<0.001; ns, non-significant).

Patients positive for $CD8^+$ T cells are less sensitive to reinfection (19), highlighting the need for vaccines eliciting a potent cellular immune response. Approximately 30% of $CD8^+$ T cells were positive for three markers (Figure 5). S-specific $CD8^+$ T cells expressing CD107a, IFN-γ, and TNF-α were the most abundant. In summary, vaccination with pPAL-Sfs + pPAL-N triggers robust T-cell activation including Th1, cytotoxic $CD4^+$, and polyfunctional cytotoxic $CD8^+$ T cell populations in vaccinated animals. The presence of cytotoxic $CD4^+$ T cells in vaccinated mice is consistent with previous studies (20).

pPAL-Sfs + pPAL-N vaccination induces complete protection in K18-hACE2 mice

The pPAL-Sfs + pPAL-N vaccine candidate induces a specific immune response against SARS-CoV-2. Therefore, a protection efficacy experiment was performed in B6Cg-Tg (K18-hACE2)2Prlmn/J mice following the same vaccination protocol as for WT B6 mice (see below). 15 days after the booster dose, all vaccinated animals were positive for total anti-RBD IgG, IgG1, and IgG2c, with high average titers (~10,000, ~600, and ~7,000, respectively). The IgG2c/IgG1 titer ratio was ~12 (Figure 6A). SARS-CoV-2 antibody neutralization titers were also high except in one mouse (Figure 6A). Therefore, the animals were challenged with a lethal dose (10^5 PFU) of the SARS-CoV-2 MAD6 isolate intranasally. Clinical signs and body weight were monitored after the challenge to evaluate protection. Vaccinated mice did not show any weight loss compared with control mice, which started to lose weight by day 3 post-challenge (Figure 6B). Furthermore, clinical signs were significantly reduced in vaccinated mice, whereas the clinical score of non-vaccinated controls increased on day 3 post-challenge (Figure 6C). All control mice were sacrificed by day 7 post-challenge (Figure 6B). Hence, complete protection was achieved in mice vaccinated with two doses of the pPAL-Sfs + pPAL-N plasmid mixture (20 µg each) administered by the intramuscular route applying *in vivo* electroporation.

Subgroups of five mice were sacrificed on day 4, day 7, and day 14 post-challenge to quantify viral replication levels. Lungs, heart, and brain were collected from each mouse to evaluate viral

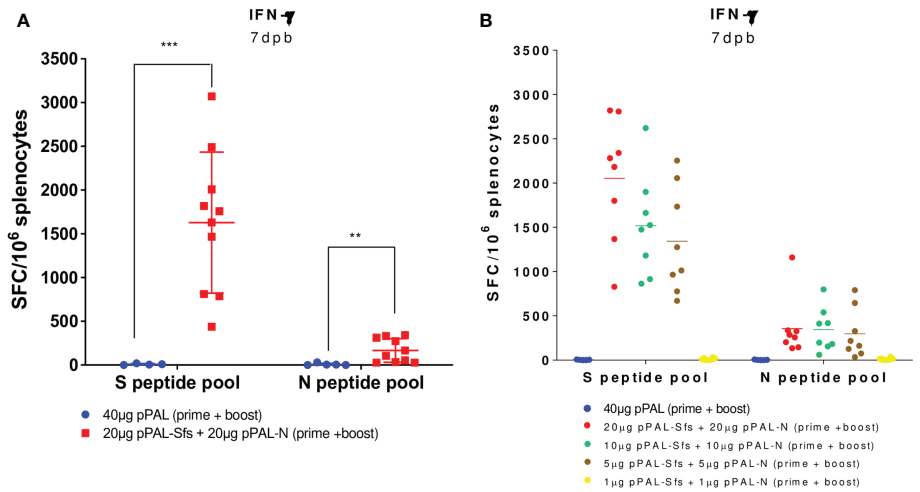

FIGURE 3
S- and N-specific activation of IFN-γ-producing splenocytes induced by the pPAL-Sfs + pPAL-N vaccine candidate in C57BL/6J mice is dose-dependent. Splenocytes were obtained 7 days post-boost after prime/boost inoculation of pPAL and pPAL-Sfs + pPAL-N. Splenocytes (2x10^5 cells/well) were stimulated at 37 °C in IFN-γ ELISpot plates for 16 h with 11-mer 8 amino acid-overlap peptide pools (25 µg/well) representing the whole S and N amino acid sequences in equimolar amounts. Negative stimulation controls were subtracted from the SFC per million cell values. Statistical inference was performed using the Student's t-test applying p-value adjustment by the Holm-Sidak method (α = 0.05; **p<0.01; ***p<0.001). **(A)** Robust S- and N-specific activation of IFN-γ-producing splenocytes is observed with a prime/boost immunization regime with pPAL-Sfs + pPAL-N. **(B)** S-specific activation of IFN-γ-producing splenocytes with pPAL-Sfs + pPAL-N is dose dependent.

load by qRT-PCR and to titrate viral replication by plaque assay. Vaccinated mice showed a significant reduction in SARS-CoV-2 RNA levels in all evaluated organs compared with control mice (Figure 6D). The Ct values in target organs notably decreased in most vaccinated mice by day 4 post-challenge, and no viral RNA was detected on day 14 post-challenge. Quantification of replicative infectious virus in the different tissues indicated a significant reduction in vaccinated mice, with undetectable levels in most cases (Figure 6E). Infectious virus was only detected in the lungs of 2 out of 5 mice on day 7 post-challenge and in the hearts of 3 out of 5 mice on day 4 post-challenge. However, viral load was significantly lower compared to control animals (2.2 x 10^3 in vaccinated mice versus 9 x 10^3 PFU/g). The levels of infectious virus in the brain were considerably higher in control mice. The data indicates that there was initial viral replication in the lungs of control animals, followed by dissemination to the heart and brain, whereas vaccination completely controlled viral replication.

An important issue regarding new SARS-CoV-2 vaccines is long-term memory (21). To evaluate long-term protection, K18-hACE2 mice were vaccinated with two doses of 20 µg of pPAL-Sfs + 20 µg of pPAL-N with a 15-day interval between prime and boost, and lethally challenged with SARS-CoV-2 3 months after the booster dose. Antibody titers were still significantly higher in vaccinated mice compared with control mice (Figure 7A) after 3 months, with a value of IgG2c higher than IgG1, indicating a Th1 immune response. Protection was determined by daily registration of weight and clinical signs. Control mice lost weight continuously during the 7-day period post-challenge, and all had to be sacrificed by day 7. On the contrary, pPAL-Sfs + pPAL-N vaccinated mice did not lose weight post-challenge (Figure 7B). Furthermore, the clinical score was significantly higher in the control group than in the vaccinated group (Figure 7C). These data indicate that the pPAL-Sfs + pPAL-N vaccine induces long-term immunity in mice and, accordingly, confers protection.

pPAL-Sfs + pPAL-N vaccination is effective against the dominant B.1.617.2 (Delta) variant

Efficacy of the same prime-boost pPAL-Sfs + pPAL-N vaccination protocol was evaluated in K18-hACE2 mice against 10^5 PFU of the B.1.617.2 (Delta) variant. The circulating anti-RBD IgG, IgG1, and IgG2c titers were high in vaccinated animals (>10,000, ~1,000, and 8,000, respectively). The IgG2c/IgG1 ratio was ~8, suggesting a Th1 immune response (Figure 8A). Weight and clinical signs were monitored daily after challenge with the SARS-CoV-2 Delta variant. All vaccinated mice maintained their initial weight, whereas the control group continuously lost weight post-challenge (Figure 8B). Vaccinated mice did not show significant clinical signs compared with the control group (Figure 8C). Therefore, the pPAL-Sfs + pPAL-N vaccine protected mice against a lethal challenge with the Delta variant.

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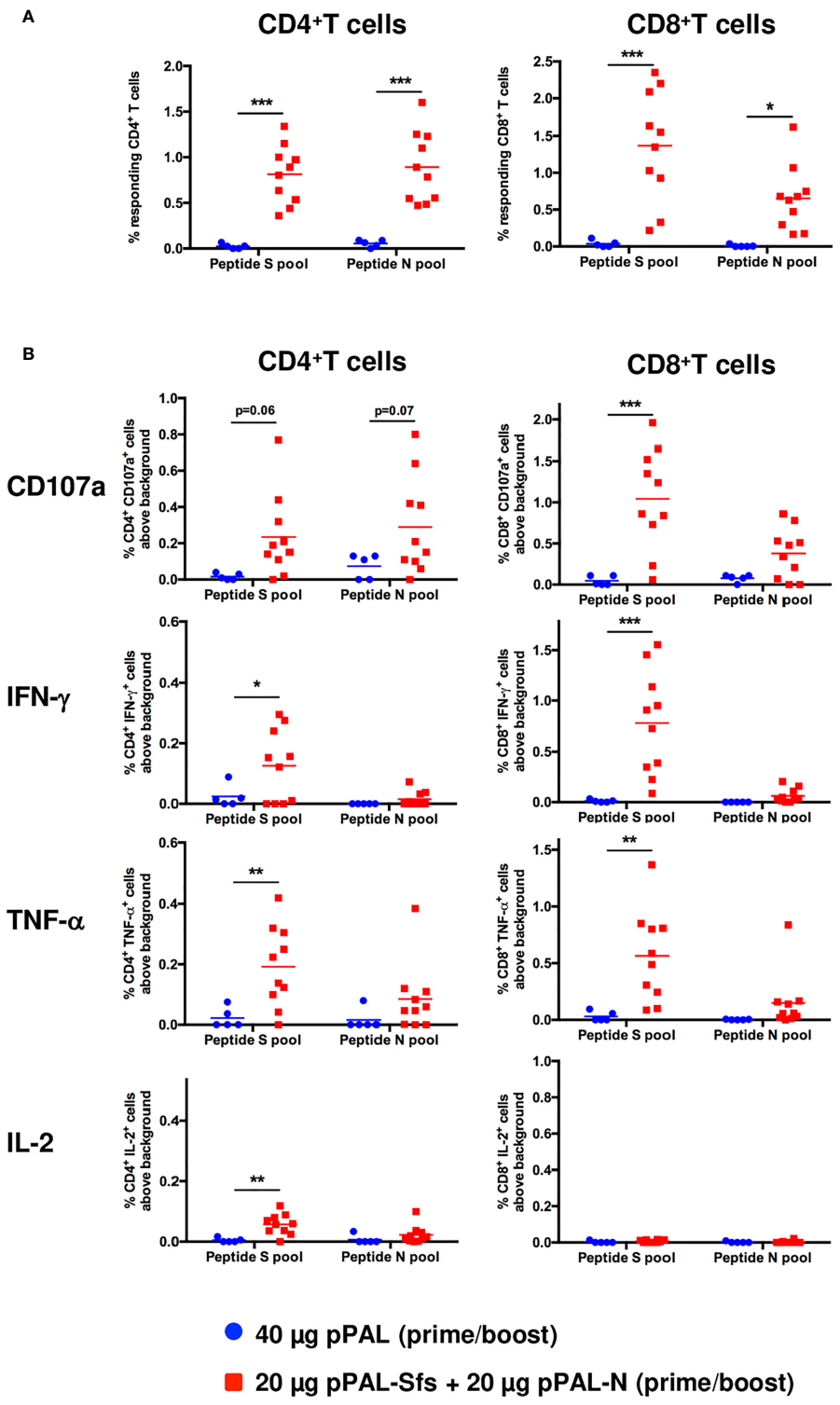

FIGURE 4
Specific $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cell response induced by pPAL-Sfs + pPAL-N in C57BL/6J mice. Splenocytes were obtained from mice after prime/boost inoculation with pPAL and pPAL-Sfs + pPAL-N. Splenocytes were stimulated for 4 h with 11-mer 8 amino acid-overlap peptide pools representing the whole S and N amino acid sequences in equimolar amounts. **(A)** Percentage of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells producing CD107a, IFN-γ, TNF-α, and IL-2 in response to stimulation with peptide S or N pools. **(B)** Percentage of $CD4^+$ and $CD8^+$ T cells responding to S and N peptide pools above background, calculated as the sum of T cells positive for CD107a and/or IFN-γ and/or TNF-α and/or IL-2. Statistical inference using Two-way ANOVA with Fisher's LD *post-hoc* test (*p<0.05; **p<0.01; ***p<0.001).

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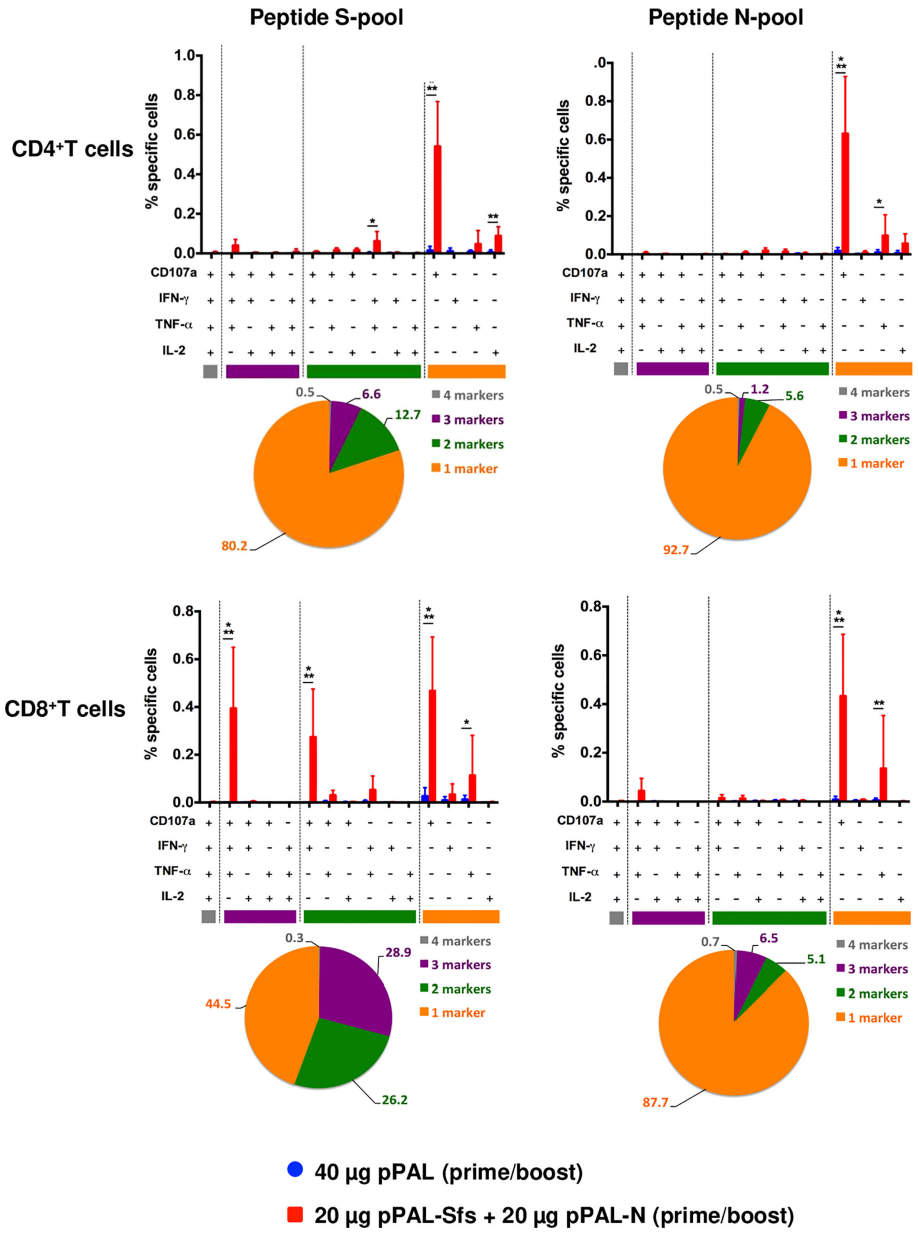

FIGURE 5
Polyfunctionality analysis of specific $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell populations stimulated by pPAL-Sfs + pPAL-N immunization in C57BL/6J mice. Analyses of concomitant expression of CD107a, IFN-γ, TNF-α, and IL-2 in $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells in response to N peptide pool or S peptide pool stimulation. Pie charts split the percentage of T cells expressing either 1, 2, 3, or 4 markers upon stimulation. Statistical inference using Two-way ANOVA with Fisher's LD *post-hoc* test (*p<0.05; **p<0.01; ***p<0.001).

Discussion

The COVID-19 pandemic continues to be a primary concern worldwide even though a high proportion of individuals in developed countries have been vaccinated with vaccines that protect from severe symptoms. COVID-19 vaccines currently in use are based on the S antigen delivered in adenoviral vectors, or as mRNA or recombinant protein formulations. Most are effective at containing the severity of the disease (22). mRNA vaccines are usually included in lipid nanoparticles and induce high protection levels (23–27). These vaccines require the strict maintenance of the cold chain (-80 or -20 °C), which is a drawback for delivery in developing countries. Adverse effects such as anaphylaxis and myocarditis of these mRNA vaccine

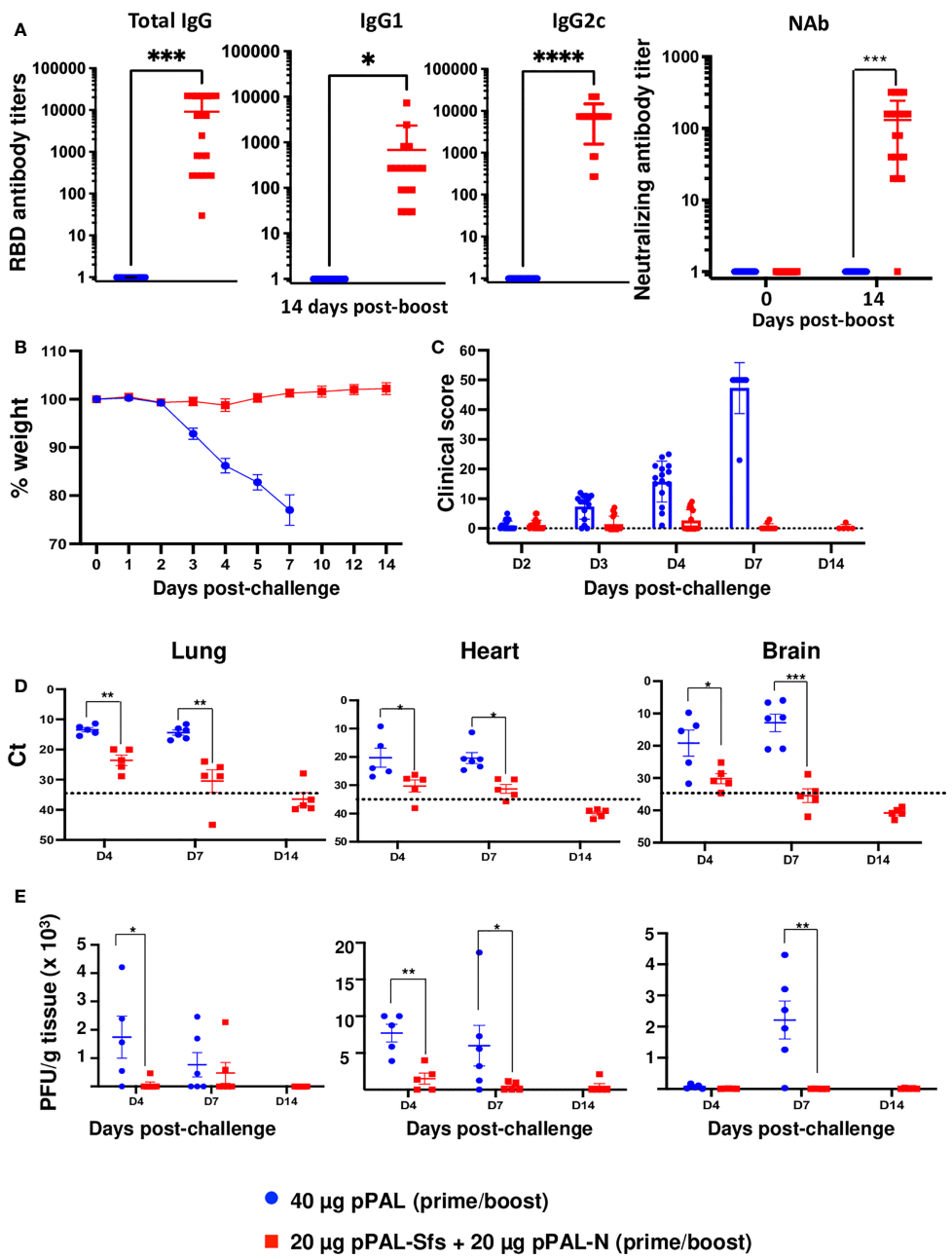

FIGURE 6
The pPAL-Sfs + pPAL-N vaccine confers full protection against challenge with 10^5 PFU of the MAD6 SARS-CoV-2 isolate in the K18-hACE2 murine model. **(A)** Titration of circulating anti-RBD IgG, IgG1, IgG2c, and neutralizing antibodies 15 days after prime/boost immunization with pPAL-Sfs + pPAL-N. **(B)** Body weight evolution after 10^5 PFU SARS-CoV-2 challenge. Vaccinated animals maintained their weight throughout the experiment. Weight decreased in the control group until the endpoint criteria had to be applied (20% of weight loss). **(C)** Clinical sign follow-up after challenge. **(D)** Reduction of the viral load in target organs. ΔCt accounting for SARS-CoV-2 mRNA levels. β-actin was the reference gene. **(E)** Reduction of viral replication (PFU/g tissue) in VERO cells. Statistical inference was performed using the Student's t-test applying p-value adjustment by the Holm-Sidak method ($\alpha = 0.05$; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

formulations are rare (reviewed in (22)). Non-replicating viral vector vaccines also require the cold chain (2-8 °C) and cause rare adverse events, such as thrombosis with thrombocytopenia and the Guillain-Barré syndrome, and proinflamatory response (22, 28). Second generation vaccines based on recombinant proteins with adjuvants lead to high specific antibody responses but confer low T-cell activation levels (29, 30). Although existing vaccines protect against severity of clinical signs, currently dominant

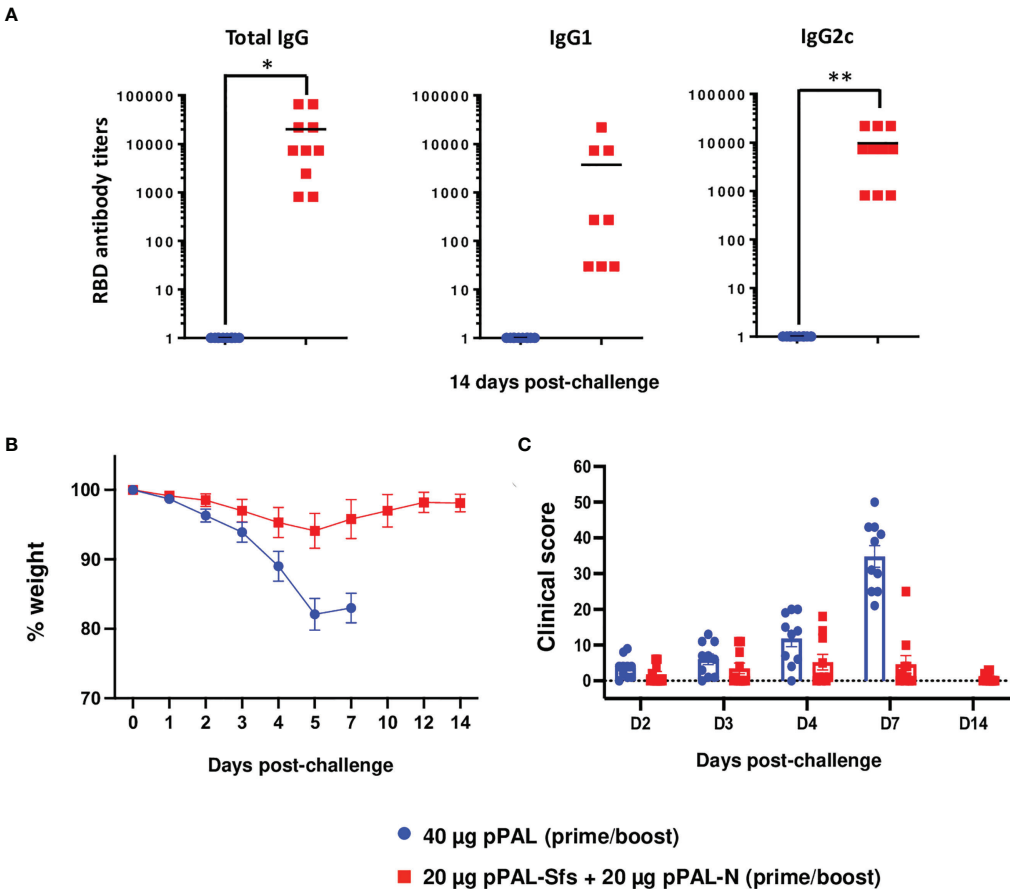

FIGURE 7
Full protection of K18-hACE2 mice after three months of vaccination with pPAL-Sfs+ pPAL-N against challenge with 10^5 PFU of the MAD6 SARS-CoV-2 isolate in the K18-hACE2 murine model. **(A)** Titration of circulating anti-RBD IgG, IgG1, and IgG2c three months after the pPAL-Sfs + pPAL-N booster dose administration. **(B)** Body weight evolution after 10^5 PFU SARS-CoV-2 challenge. Vaccinated animals maintained their weight throughout the experiment, whereas weight decreased in the control group. **(C)** Clinical sign follow-up after challenge with 10^5 PFU SARS-CoV-2. Statistical inference was performed using the Student's t-test applying p-value adjustment by the Holm-Sidak method (α = 0.05; *p<0.05; **p<0.01).

variants, such as Omicron (B.1.1.529), are not very sensitive to these vaccines (5). Additional vaccine doses will unlikely tackle the problem of vaccine escape variants (31). New vaccines providing protection against a broader spectrum of VOCs are required. DNA vaccines are a promising method for COVID-19 control given their ability to be efficiently modified to achieve protection against new variants, their low cost, the relatively simple manufacturing process, and their thermotolerance, which would facilitate worldwide distribution (32). Low-income countries would particularly benefit from this vaccine because the cold chain isn't required for storage and distribution (12). In this study, we describe a DNA vaccine against SARS-CoV-2 delivered by *in vivo* electroporation after injection *via* the IM route. This vaccine elicits a robust SARS-CoV-2-specific humoral and cellular immune response that protects mice from a lethal challenge with the Wuhan strain and the Delta variant.

The N protein in coronaviruses is a critical structural protein (33, 34) whose genetic stability and conservation among coronaviruses, including new putative variants, make it a suitable vaccine candidate (34). The S gene was modified to Sfs to avoid furin cleavage. HEK293 cells transfected with pPAL-Sfs + pPAL-N properly express the codon-optimized Sfs and N genes from the Wuhan strain. C57BL/6J mice vaccinated with two doses of pPAL-Sfs + pPAL-N by electroporation induced a robust humoral response, producing specific IgGs against the S protein and its RBD domain. Anti-RBD IgG subclass analysis showed the predominance of IgG2c, indicating a Th1/Th2 balance skewed towards Th1. Most of the asymptomatic SARS-CoV-2 infected mice exhibited an effective and robust Th1 response, which favors infection clearance (35). Several studies have shown that the initial establishment of a robust Th1 immune

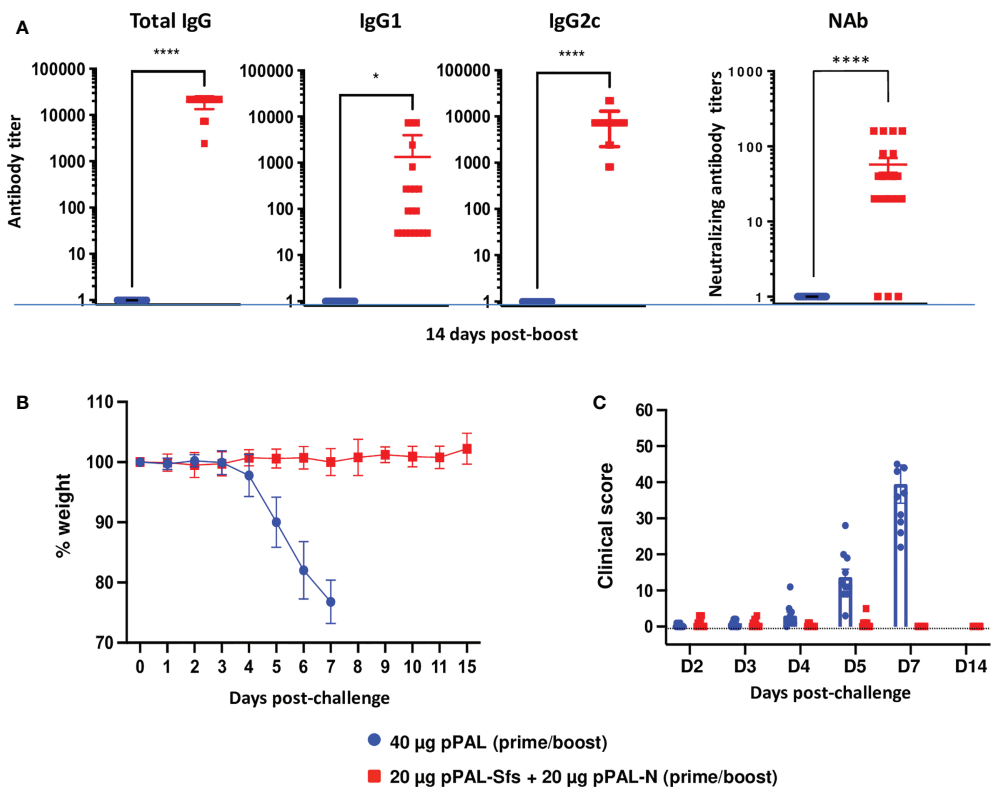

FIGURE 8
The pPAL-Sfs + pPAL-N vaccine confers full protection against challenge with 10^5 PFU of the SARS-CoV-2 Delta variant in the K18-hACE2 murine model. **(A)** Titration of circulating anti-RBD IgG, IgG2c, and neutralizing antibodies 15 days after prime/boost immunization with pPAL-Sfs + pPAL-N. **(B)** Body weight evolution after 10^5 PFU SARS-CoV-2 challenge. Vaccinated animals maintained their weight throughout the experiment, whereas weight decreased in the control group. **(C)** Clinical sign follow-up after challenge with 10^5 PFU SARS-CoV-2. Statistical inference was performed using the Student's t-test applying p-value adjustment by the Holm-Sidak method ($\alpha = 0.05$; *p<0.05; ****p<0.0001).

response leads to control of viral replication, whereas a potent Th2 response correlates with severe forms of the disease (36, 37). In addition to the generation of anti-S IgG antibodies, we have demonstrated that immunization with pPAL-Sfs + pPAL-N also induced production of neutralizing antibodies. Virus neutralization is a measure of antibody efficacy (38) and a correlate of vaccine-induced protection (39). Therefore, pPAL-Sfs + pPAL-N induces a humoral response compatible with protection against SARS-CoV-2.

T-cell responses likely play a significant role in SARS-CoV-2 infection. Strong T-cell responses correlated with recovery of patients that suffered mild disease (40, 41). A robust $CD8^+$ T-cell response with broad specificity is considered a signature of successful protective immunity against SARS-CoV-2 (42). Patients who tested positive for $CD8^+$ T cells are less sensitive to reinfection (19), highlighting the need for vaccines that elicit a potent cellular immune response. One of the main advantages of DNA vaccines is their ability to stimulate a strong T-cell immune response. Vaccination with pPAL-Sfs + pPAL-N activates specific T-cell responses to the SARS-CoV-2 S and N proteins. We assessed four functional parameters: CD107a, IFN-γ, IL-2, and TNF-α, to better understand the function and phenotype of the anti-S and anti-N T cells elicited by vaccination. The finding that cytotoxic $CD4^+$ T cells are present in vaccinated mice is consistent with past studies (20). Cytokine profile analysis of antigen-specific $CD4^+$ and $CD8^+$ T cells indicated that pPAL-Sfs + pPAL-N vaccination induced polyfunctional T cells to different extents depending on the T cell compartment and the antigen. Very few activated cells expressing the four parameters were detected. Nonetheless, we found that more than 50% of $CD8^+$ T cells specific to the S peptide pool were positive for two or more functional parameters, which indicates that these cells are polyfunctional effectors. Curiously, anti-N $CD8^+$ T cells were less polyfunctional and most exclusively displayed a cytotoxic phenotype. Similarly, both anti-S and anti-N $CD4^+$ T cells showed limited polyfunctionality. Still, both cell populations expressed CD107a in response to peptide stimulation, which demonstrates that these $CD4^+$ T cells possess a cytotoxic phenotype. The presence of cytotoxic $CD4^+$ T cells specific for

SARS-CoV-2 in murine models has been reported (20), and it correlates with protection in some viral infections, such as West Nile fever (43). In future work, it would be interesting to determine to which extent these cytotoxic anti-SARS-CoV-2 $CD4^+$ T cells contribute to protection. Overall, the data indicate that vaccination induces cellular immune responses against the SARS-CoV-2 antigens included in the vaccine formulation and that anti-S $CD8^+$ T cell effectors are polyfunctional. Since T cell polyfunctionality can be a correlate of protection (44), pPAL-Sfs + pPAL-N vaccination could activate potent T cell effectors capable of recognizing the viral infection.

As previously stated, the pPAL-Sfs + pPAL-N vaccine triggers specific T-cell activation against N. In some viral infections, non-neutralizing antibodies directed to the nucleoprotein can help clear the infection of enveloped viruses (45–47). One of the mechanisms behind the protective role of these anti-N antibodies has been recently described (15). The E3 ubiquitin ligase TRIM21 may target the N protein for proteasomal degradation through anti-N antibodies, thus triggering the activation of effective cytotoxic T-cell responses against the N antigen (15). This suggests that including the SARS-CoV-2 N gene in the vaccine formulation may improve protection as it could provide T cell reactivity against this highly conserved antigen through multiple mechanisms.

The pPAL-Sfs + pPAL-N vaccine was capable of protecting mice against the Wuhan strain and the Delta VOC. Therefore, the humoral and cellular immune response triggered against the S and N antigens of SARS-CoV-2 by pPAL-Sfs + pPAL-N was likely capable of protecting mice from the disease. Our viral load analysis by qRT-PCR and PFU titration in lung, heart, and brain tissue homogenates indicate that pPAL-Sfs + pPAL-N vaccination prevented the virus from spreading to organs, such as the heart (19) or the brain, linked to serious long-term side effects of COVID-19. Noteworthy, protection was achieved in all vaccinated animals, including those with low specific antibody titers, indicating that the pPAL-Sfs + pPAL-N vaccine produces humoral and cellular immune responses, thus enhancing protection. Additionally, protection was achieved in all vaccinated animals when the SARS-CoV-2 challenge was performed 3 months after the last vaccination. Hence, pPAL-Sfs + pPAL-N vaccination induces long-term immunity against the disease and fully protects mice from the SARS-CoV-2 Wuhan strain and the Delta VOC. Therefore, the pPAL-Sfs + pPAL-N vaccine is ready to advance to human clinical trials.

In summary, homologous prime/boost administration of sterile-water-dissolved pPAL-Sfs + pPAL-N (20 μg per plasmid per dose) administered by the IM route and applying subsequent *in vivo* electroporation fully protects mice against challenge with 10^5 PFU of the Wuhan-Hu-1 MAD6 isolate and the Delta variant. pPAL-Sfs + pPAL-N triggers a Th1 cell response, a polyfunctional $CD8^+$ cytotoxic T-cell response, and the production of neutralizing antibodies. This DNA vaccine is safe, easy to produce at the industrial scale, and suitable for distribution and storage at room temperature.

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 authors.

Ethics statement

The animal study was reviewed and approved by Consejo Superior de Investigaciones Científicas (CSIC) Ethics Committee and the Comunidad Autónoma de Madrid (Proex 254.6/21 and 295.6/21).

Author contributions

PA, NS, and VL contributed to conception and design of the study. PA, JL, DR-M, AA, FL, JR, SR-G, AL-L, AC, PS-C, VM and NS performed the experiments. NS, VL, PA, AA and JR wrote the first draft. FL edited 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.2022.1023255/full#supplementary-material

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Persistence of spike-specific immune responses in BNT162b2-vaccinated donors and generation of rapid *ex-vivo* T cells expansion protocol for adoptive immunotherapy: A pilot study

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Introduction: The BNT162b2 mRNA-based vaccine has shown high efficacy in preventing COVID-19 infection but there are limited data on the types and persistence of the humoral and T cell responses to such a vaccine.

Methods: Here, we dissect the vaccine-induced humoral and cellular responses in a cohort of six healthy recipients of two doses of this vaccine.

Results and discussion: Overall, there was heterogeneity in the spike-specific humoral and cellular responses among vaccinated individuals. Interestingly, we demonstrated that anti-spike antibody levels detected by a novel simple

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automated assay (Jess) were strongly correlated (r=0.863, P<0.0001) with neutralizing activity; thus, providing a potential surrogate for neutralizing cell-based assays. The spike-specific T cell response was measured with a newly modified T-spot assay in which the high-homology peptide-sequences cross-reactive with other coronaviruses were removed. This response was induced in 4/6 participants after the first dose, and all six participants after the second dose, and remained detectable in 4/6 participants five months post-vaccination. We have also shown for the first time, that BNT162b2 vaccine enhanced T cell responses also against known human common viruses. In addition, we demonstrated the efficacy of a rapid ex-vivo T cell expansion protocol for spike-specific T cell expansion to be potentially used for adoptive-cell therapy in severe COVID-19, immunocompromised individuals, and other high-risk groups. There was a 9 to 13.7-fold increase in the number of expanded T cells with a significant increase of anti-spike specific response showing higher frequencies of both activation and cytotoxic markers. Interestingly, effector memory T cells were dominant in all four participants' CD8+ expanded memory T cells; CD4+ T cells were dominated by effector memory in 2/4 participants and by central memory in the remaining two participants. Moreover, we found that high frequencies of CD4+ terminally differentiated memory T cells were associated with a greater reduction of spike-specific activated CD4+ T cells. Finally, we showed that participants who had a CD4+ central memory T cell dominance expressed a high CD69 activation marker in the CD4+ activated T cells.

KEYWORDS

SARS-CoV-2, COVID-19 vaccine, spike-specific immune responses, surrogate neutralization, spike-specific T cells expansion

Introduction

In order to limit the rapid spread of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), and its consequences across the globe, many efforts have been focused on developing safe and effective prophylactic vaccines (1). The BNT162b2 vaccine (Pfizer-BioNTech) was the first vaccine to be authorized for emergency use (2). BNT162b2 is a lipid nanoparticle formulated nucleoside-modified messenger RNA (mRNA) encoding the SARS-CoV-2 full-length spike (S) glycoprotein in a prefusion stabilized conformation (2). The vaccine was found safe and demonstrated 95% efficacy for protection against COVID-19 in phase II/III clinical trials (3). Observational data showed that BNT162b2 is highly effective in preventing SARS-CoV-2 infection, related hospitalization, and death (4). However, limited data exist about the persistence of the humoral and T cell responses and the duration of the vaccine-induced protection after the two-dose mRNA vaccination.

Neutralizing antibodies are the best indicators of protective immunity, therefore the quantification of SARS-CoV-2 neutralizing antibody levels induced by vaccination or infection constitutes a critical parameter to determine the protection level against the virus and to assess the potential vaccine effectiveness (5–7). The conventional virus/pseudovirus neutralization assays are considered the reference methods to determine the functional neutralizing ability of antibodies (8, 9). However, these methods require the use of specialized facilities, trained personnel, are time-consuming (2-4 days), and relatively expensive (8, 9). Several surrogates of neutralization cell-based assays have been developed and evaluated to overcome these limitations (10–13). Most of these assays use ELISA or similar platforms requiring multiple time-consuming binding and washing steps (10–13), thus preventing high-throughput screening. Therefore, simple, rapid, and accurate serological tests measuring neutralizing activity are urgently needed to assess the duration of humoral protective immunity in vaccine recipients and in recovered COVID-19 patients. Various new techniques have been employed globally for antibody response monitoring following SARS-CoV-2 infection or immunization (14). Jess Simple Western system is a novel fully automated assay, from Protein Simple, that detects human serum/plasma binding antibodies reactive to five different SARS-CoV-2 viral antigens in a large number of samples in only three hours (15). At the beginning of the pandemic, this assay showed high utility in COVID-19 diagnosis with a sensitivity and specificity of 94% and 93%, respectively (16). Moreover, Jess revealed a substantial agreement of 90% between the results obtained using Jess and ELISA for SARS-CoV-2 Immunoglobulin G (IgG) detection, which substantiates its implementation as a first-line serological test for clinical diagnostics and vaccination monitoring (16). Subsequently, Jess was then used in several studies to characterize SARS-CoV-2 specific humoral response in animal and human systems (17–19).

Alongside the antibody response, recent studies have shown that T cell response plays a dominant role in SARS-CoV-2 viral clearance

and protection (20–24). Indeed, several reports indicated that COVID-19 patients with undetectable or impaired humoral responses could recover from the disease, highlighting the importance of the T cell response in virus clearance (20–22). In addition, Hurme et al. demonstrated that T cell memory response in COVID-19 vaccinated, and convalescent individuals could be more persistent than antibody response leading to a more durable source of protection (23, 24). Furthermore, recent studies indicated that T cell response and functionality against SARS-CoV-2 were not affected by the mutations or antigenic variation of the emerging variants of concern as the humoral response (25–27). These findings provide direct evidence that a lack or impairment of the T cell response could be associated with an elevated risk of SARS-CoV-2 infection and severe COVID-19 disease outcome. In the same line, numerous studies indicated that severe outcome in COVID-19 patients was associated with lymphopenia, reduction or disability of the T cell cytotoxic potential, and elevated exhaustion markers (28–30). On the other hand, recent studies demonstrated that immunocompromised patients and the elderly have poor immune responses to the BNT162b2 vaccine, indicating that these patients may not be sufficiently protected against SARS-CoV-2 infection (31–33). Therefore, the development of new therapies that support the cellular response to SARS-CoV-2 by preventing the defect of T cell function may have a significant impact on the outcome of the elderly, immunocompromised, and severe COVID-19 patients after infection or vaccination.

We herein report the dynamics and persistence of antibody and T cell responses in a small cohort of healthy adult recipients of two doses of BNT162b2-mRNA vaccine in the state of Qatar. In addition, we explored the interpersonal variation of the humoral and cellular immune response elicited by BNT162b2 immunization among vaccinated healthy individuals. We further evaluated the Jess technology as a surrogate assay for SARS-CoV-2 antibodies neutralizing activity estimation. Finally, we explored the feasibility and efficacy of a rapid *ex-vivo* T cell expansion protocol for spike-specific T cell expansion to be potentially used for adoptive-cell therapy in severe COVID-19, immunocompromised patients, and elderly persons.

Material and methods

Study population and sample collection

This study was conducted at the Translational Cancer Facility, National Center for Cancer Care and Research, Hamad Medical Corporation (HMC), Qatar. A total of six healthy participants with no history of SARS-CoV-2 infection, eligible to receive two doses of the BNT162b2 mRNA vaccine (Pfizer-BioNTech) three weeks apart, were enrolled. For each participant, peripheral blood samples were obtained on day 0 (prior vaccination), day 20 (pre-boost), day 34 (14 days post-boost), and day 150 (five months after the first vaccination dose). Peripheral Blood Mononuclear Cells (PBMCs) and serum were isolated and used for serological and T cell responses analysis as reported in supplement 1 (See supplementary material). Demographic characteristics of enrolled participants have been shown in supplement 2 (See supplementary material). This study was approved by the Institutional Review Board (IRB) committee of HMC (Project number MRC-01-21-113), and informed consent was obtained from all study participants.

PBMCs and sera isolation

Peripheral blood samples were collected in EDTA and serum separator tubes at the different time points reported above. Serum was separated by centrifugation at 3200 rpm and stored at -80°C. PBMCs were isolated by density gradient centrifugation using Ficoll Paque Premium (GE Healthcare) and SepMate tubes (STEMCELL Technology) according to the manufacturer's instructions. Isolated PBMCs were then cryopreserved in a cell recovery medium (Fetal Bovine Serum (FBS, Gibco) supplemented with 10% DMSO (Millipore Sigma) and stored in vapor phase liquid nitrogen until used.

ELISA binding assay

Initially, sera samples were screened for the presence of IgG antibodies against the SARS-CoV-2 recombinant S1 subunit (S1) of the spike protein, using a commercial semi-quantitative ELISA kit (Lionex COVID-19 ELISA-human IgG) as per the manufacturer's instructions. Briefly, sera samples were diluted at 1:50 in a sample diluent and then added to the microtiter plate (coated with SARS-CoV-2 S1 protein) for 60 mins incubation at room temperature. After a washing step, the conjugate (peroxidase-coupled anti-human antibody) and its substrate (TMB) were added to the wells. The optical density (OD) was measured with an ELISA reader (Epoch Biotek) at 450 nm wavelength. Each sample OD was normalized according to the kit calibrator value, and this normalized value determines the test result. Values below 0.8 were considered negative, values between 0.8 and 1.1 were considered borderline, and values above 1.1 indicate a positive anti-SARS-CoV-2 S1 subunit IgG. All samples were run in duplicates and borderline samples were repeated for confirmation.

Detection of SARS-CoV-2 specific antibodies using jess simple western system

The detection and quantification of anti-SARS-CoV-2 IgG antibodies among vaccinated donors' sera were assessed using the Jess Simple Western system (Protein Simple). This system enables the detection of human IgG antibodies reactive against five viral antigens simultaneously: S1 Receptor Binding Domain protein (S1-RBD), S1 subunit full length (S1), S2 subunit full length (S2), Spike protein (S), and Nucleocapsid Protein (N) recombinant antigens as reported in supplement 3 (see supplementary material). Samples were run following the manufacturer's protocol for the 12-230-kDa Jess separation module (Protein Simple). Briefly, the SARS-CoV-2 antigens (Protein Simple) were mixed with 0.1X Sample buffer (Protein Simple) and Fluorescent 5X Master mix (Protein Simple) in the presence of fluorescent molecular weight markers (Protein Simple) and denatured at 95°C for 5 mins. Sera were diluted at 1:10 in

the sample buffer. Ladder (12-230-kDa PS-ST02EZ, Protein Simple) and SARS-CoV-2 proteins were run in capillaries. The SARS-CoV-2 specific human antibodies present in the serum samples serve as primary antibodies that were then detected with anti-goat HRP-conjugated anti-human IgG antibody (R&D Systems). The chemiluminescent revelation was established with peroxide/luminol-S (Protein Simple). The digital image of the capillary chemiluminescence was captured with Compass Simple Western software (version 4.1.0, Protein Simple) that automatically calculated the area of the signal (chemiluminescence intensity). Results are represented as the chemiluminescence intensity of each antigen separately.

Generation of SARS-CoV-2 pseudotyped vesicular stomatitis virus and neutralization assay

For the determination of neutralizing antibodies to SARS-CoV-2, we utilized a recombinant ΔG-Vesicular stomatitis virus (VSV) system to generate SARS-CoV-2 pseudovirus as previously described by Whitt (34). Briefly, HEK293T cells were grown in DMEM medium (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco) and 1% Pen/Strep (Gibco) to reach 80-90% of confluence on the day of the experiment. The following day, cell culture media was replaced with Opti-MEM (Gibco) and incubated for 20 mins before transfecting cells with SARS-CoV-2 Spike-TM plasmid (provided by the Viral Pathogenesis Laboratory, Vaccine Research Center, National Institute of Health). After 4 hours, transfection media was replaced with DMEM (Gibco) supplemented with 5% FBS (Gibco), and cells were incubated at 37° C with 5% CO_2. After 24 hours, cells were examined for the presence of syncytia due to the expression of the envelope protein. Subsequently, transfected cells were infected with pseudotyped ΔG-luciferase (G*ΔG) (Kerafast, Ref. no. EH1025-PM) at a multiplicity of ~3–5. When most of the cells showed a cytopathic effect (24-30 hours), SARS-CoV-2 VSV pseudovirus was harvested by collecting the supernatant. Supernatants were clarified by centrifugation at 300×g for 10 mins before aliquoting and storing at −80°C. For the titration of pseudotyped viruses, HEK293T cells expressing angiotensin-converting enzyme 2 (ACE2) (BEI) were used. Cells were prepared at 1×10^6 cell/ml in complete DMEM (Gibco) and added to serially diluted pseudovirus (50 µl of diluted virus added to 50 µl of cells in suspension) in a 96-well cell culture plate and incubated for 2 hours. 100 µl of complete DMEM (Gibco) was then added to the cells and incubated for 48 hours. After incubation, cells were lysed using 30 µl of 1X cell lysis buffer (Promega), and 50 µl of luciferase reagent (Promega) was added. The titer of the pseudovirus was determined by measuring luminescence using a plate reader (Tecan Infinite). To assess the neutralization of SARS-CoV-2 pseudotyped VSV in sera samples, heat-inactivated serum samples (50 to 200-fold) were serially diluted in 60 µl of DMEM media and then incubated with 100 µl pseudovirus (titer $1\text{-}2\times10^6$ RLU/100 µl) for 30 mins at room temperature. The final volume (160 µl) was then distributed into 3 wells (triplicates) of a 96-cell culture plate. HEK293T-ACE2 cells were then added at 1×10^6 cells/ml and incubated for 48 hours before reading out luminescence using a plate reader (Infinite 200 PRO). A positive response was defined as a neutralizing activity of 20% or more.

Interferon-γ Enzyme-Linked ImmunoSpot assay

The spike-specific T cell responses to the BNT162b2 vaccine were assessed using the T-spot Discovery SARS-CoV-2 kit (Oxford Immunotec), a modified enzyme-linked immunospot technology. This kit is designed to measure interferon-γ responses to overlapping peptide pools covering peptide sequences of five different SARS-CoV-2 antigens, without HLA restriction. The test specificity to SARS-CoV-2 has been enhanced by removing high homology peptide sequences that are potentially cross-reactive with other coronaviruses. The T-spot discovery SARS-CoV-2 kit was used according to the manufacturer's protocols. Briefly, 250 000 PBMCs suspended in AIM-V medium (Gibco) were plated into each well of the T-spot plate in duplicates, stimulated with 3 different antigens: S1 spike subunit peptides, peptides coding for sequences with high homology to other coronaviruses, positive control (phytohemagglutinin), and negative control (AIM-V medium) then incubated for 18 hours (37°C, 5% CO_2). The interferon-γ secreting T cells were detected using an automated ELISpot reader (Autoimmun Diagnositka GMBH). Results are presented as the mean of the number of spots forming cells (SFC) per 250 000 cells for each panel, subtracting the background (negative control) count. A positive response was defined as an SFC of 10 or more.

Ex-vivo spike-specific T cells expansion

The spike-specific T cells were expanded from vaccinated donors' PBMCs (collected five months post-vaccination) using a modified protocol for expansion of multivirus-specific T cells targeting cytomegalovirus (CMV), Epstein-Barr virus (EBV), BK virus (BKV), human herpes virus (HHV)-6, respiratory syncytial virus (RSV), adenovirus (Adv) and influenza previously described by Gerdemann et al. (35). Briefly, fresh PBMCs were pulsed with the spike peptide pools at 1 µg of antigen/15 x 10^6 PBMCs for 30 mins at 37°C. The spike peptide pools (JPT Peptide Technologies) contain a pool of 315 overlapping peptides encompassing the full spike protein. After incubation, cells were resuspended in a virus-specific T cells (VST) medium consisting of 45% Advanced RPMI 1640 (Gibco) supplemented with 45% Click's medium (Irvine Scientific), 2 mM GlutaMAX (Gibco), 10% FBS (Gibco), 10 ng/ml interleukin 7 (IL-7, Peprotech), and 400 U/ml IL-4 (Peprotech) and transferred to a G-Rex 10 device (Wilson Wolf Manufacturing Corporation). Cells were counted on day six and fresh culture media with cytokines was added. Cells were harvested and evaluated for antigen specificity and functionality on day 11.

Flow cytometry

Expanded spike-specific T cells and PBMCs collected five months post-vaccination were stimulated with the S1 peptide pools (1µg/ml, Oxford Immunotec) for 18 hours. Stimulated PBMCs and T cells were

stained with fluorophore-conjugated monoclonal antibodies against CD3 (BD Biosciences), CD4 (BD Biosciences), CD8 (BD Biosciences), CD45RA (BD, Biosciences), CD69 (BD Biosciences), CD107 (BD Biosciences), CD134 (Thermo Fisher), CD137 (Thermo Fisher), and CD197 (BD Biosciences) for phenotypical characterization. All samples were acquired using a Fortessa flow cytometer (BD Biosciences) and the data was analyzed using FlowJo V10 software (BD Biosciences).

Statistical analyses

All statistical analyses and graphs were performed using GraphPad Prism Software (version 9.2.0). The characterization of the humoral and T cell responses dynamics over time was assessed using One-way ANOVA multiple comparison test. The T cell response to S1 antigen before and after expansion was evaluated using the student t test. Correlations between Jess, neutralization, and ELISA immunoassays were analyzed by Pearson correlation and linear regression models. The scatter point represents serum samples (n=24) collected from BNT162b2 vaccinated healthy donors at the baseline, 20-, 34-, and 150-days post-vaccination. The coefficient of correlation (r) represents the strength of the linear relationship between the different immunoassays. The coefficient of determination R squared (R2) represents the percentage of variance in the given data set. The P-value tests whether the regression equation is significant. P-value was considered statistically significant when $P \leq 0.5$.

Results

Heterogeneity of the spike-specific antibody response among BNT162b2 vaccinated individuals

The anti-spike (anti-S) binding and neutralizing antibody responses induced by the BNT162b2 vaccine over time were characterized. In this, serum samples were collected from vaccinated participants at four different time points as reported in supplement 1 (See supplementary material). The anti-S and anti-S1 IgG levels were assessed using Jess and ELISA, respectively (Figures 1A, B, 2A, B and supplement 4). The anti-S neutralizing activity was measured using the neutralization assay (Figures 1C and 2C). Overall, our data showed an interpersonal heterogeneity in the vaccine-elicited humoral response among vaccinated individuals. This interpersonal variation was observed at three stages: the induction detected on day 20, the peak response reached on day 34, and the response decline detected 150 days post-vaccination (Figures 1A–C, 2A–C). Jess results showed that the anti-S IgG antibody response (presented by chemiluminescence intensity (CI)) was induced on day 20 in all six participants with different levels, ranging from 502947 to 6719958 CI (Figures 1A and 2A). This response was boosted on day 34 (after the second dose) in all six participants with varying levels, ranging from 5495488 to 12954728 CI (Figures 1A and 2A). However, 150 days post-vaccination a decline in the anti-S IgG levels was observed in 5/6 participants with different magnitudes ranging from 3344080 to 7009985 CI (Figures 1A and 2A). In contrary, VAC-HD1 showed an increase in this response 150 days post-vaccination (Figures 1A and 2A).

In addition, ELISA results showed that the first vaccination induced the anti-S1 IgG antibody response on day 20 in all six participants with varying degrees of optical density (OD) ranging from 1.58 to 6.75 (Figures 1B and 2B). The second dose increased this response in all six participants with an OD ranging from 7.73 to 9.13 (Figures 1B and 2B). However, five months post-vaccination a decline in the anti-S1 IgG levels was observed in all six participants with an OD ranging from 5.07 to 8.06 (Figures 1B and 2B). We have found also that females (VAC-HD2, 4, 5, and 6) had a stronger anti-S1 IgG antibody response on days 20 and 34 compared to their counterparts in males (VAC-HD1 and 3) (Figures 1B and 2B).

Furthermore, Neutralization results showed that anti-S neutralizing antibodies response was induced on day 20 in all six participants with different levels of neutralizing activity ranging from 6.53 to 75.23% (Figures 1C and 2C). This response was boosted after the second dose in 5/6 participants with a neutralizing activity ranging from 44.27 to 84.76% (Figures 1C and 2C). However, five months post-vaccination a decline in the anti-S neutralizing activity was observed in all six participants with varying magnitudes ranging from 0 to 56.84% (Figures 1C and 2C). Interestingly, we demonstrated that individuals who had low Anti-S neutralizing activity (<25%) on day 20 (VAC-HD3 and 6) tended to have also low response after the second dose and lost this response five months post-vaccination (Figures 1C and 2C). However, individuals who had high anti-S neutralizing activity (>36%) on day 20 (VAC-HD1, 2, 4, and 5) tended to have also high response after the second dose and were able to maintain this response five months post-vaccination (Figures 1C and 2C).

Dynamics of S-specific binding and neutralizing antibody responses following BNT162b2 vaccination

Our data showed that anti-S binding and neutralizing antibodies responses dynamics followed the same trend (Figures 2D, E). Indeed, the anti-S IgG binding antibodies (BAbs) response was induced 20 days after the first dose (26526 CI on day 0 versus 2678053 CI on day 20), significantly boosted with the second dose (2678053 CI on day 20 versus 8519960 CI on day 34, ***P=0.0003), then significantly declined 150 days post-vaccination (8519960 CI on day 34 versus 5205907 CI on day 150, *P=0.0426) (Figure 2D). Identically, we found that the anti-S neutralizing antibodies (NAbs) response was significantly induced 20 days after the priming dose (0% on day 0 versus 38.8% on day 20, *P=0.0136) and was further increased with the booster dose (38.8% on day 20 versus 70.51% on day 34) (Figure 2E). However, the anti-S neutralizing activity had significantly decreased five months post-vaccination as compared to their peak levels at two weeks after the second dose (70.51% on day 34 versus 34.77% on day 150, *P=0.0243) (Figure 2E). Furthermore, we demonstrated that all six participants maintained a detectable anti-S BAbs response five months post-vaccination (Figures 2A and 2D), whereas only four of them maintained the NAbs response (except VAC-HD3 and VAC-HD6) (Figures 2C, E).

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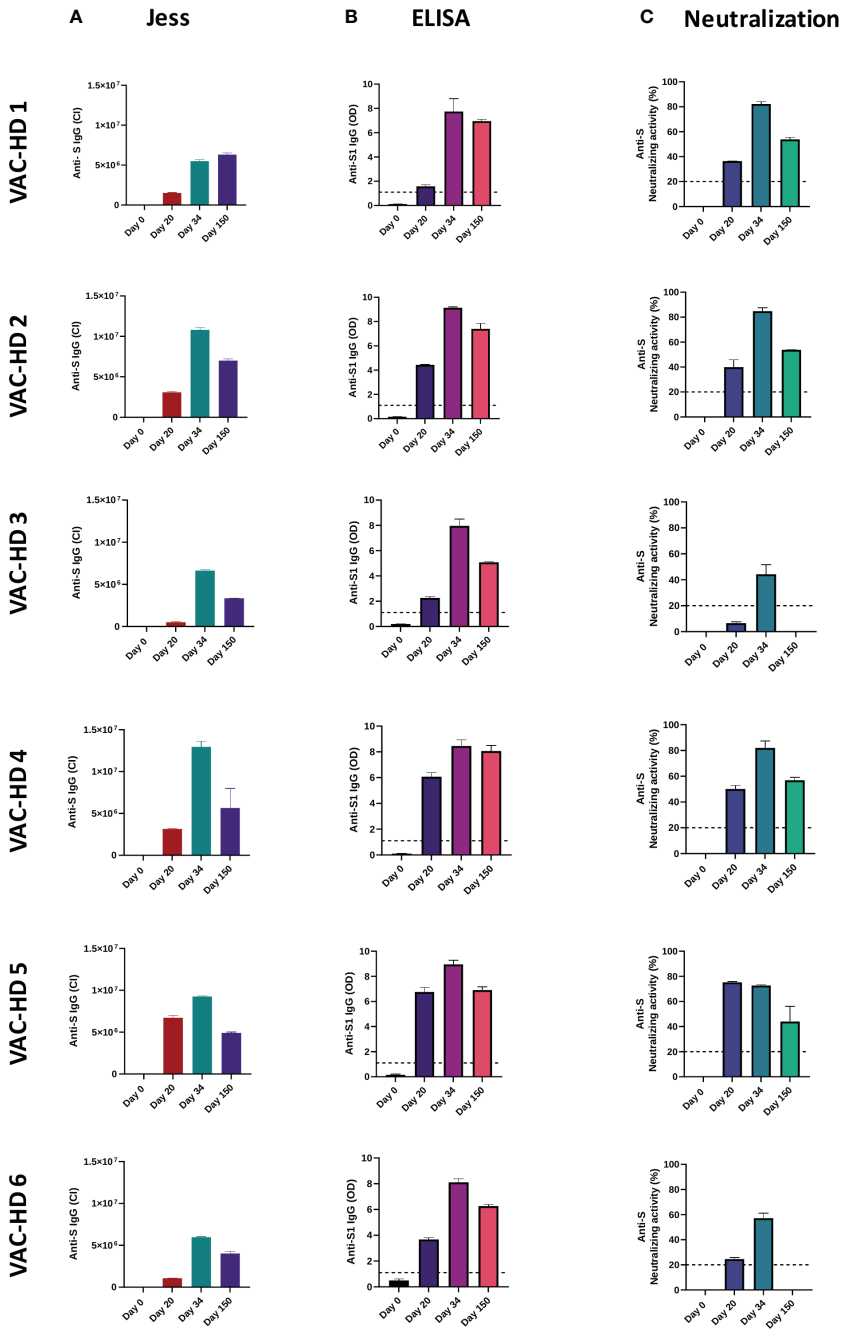

FIGURE 1
Interpersonal variation of anti-spike binding and neutralizing antibodies responses over time among BNT162b2 vaccinated participants. Serum samples were collected from six BNT162b2 vaccinated participants at the baseline, 20-, 34-, and 150-days post-vaccination. The anti-S and anti-S1 IgG levels were assessed using Jess and ELISA, respectively. The anti-S neutralizing activity was measured using the neutralization assay. **(A)** Anti-S IgG response in six BNT162b2 vaccinated participant over time (Jess). **(B)** Anti-S1 IgG response in six BNT162b2 vaccinated participant over time (ELISA). **(C)** Anti-S neutralizing activity in each BNT162b2 vaccinated participant over time (Neutralization assay). The lines indicate the cut-off value of a positive antibody response.

Evaluation of Simple automated immunoassay Jess as an alternative to neutralization cell-based assay for SARS-CoV-2 neutralizing activity estimation

We investigated whether the anti-S IgG BAbs levels measured by Jess can substitute the neutralization cell-based assay for the estimation of neutralizing activity in vaccinated individuals. For this purpose, the anti-S BAbs levels and neutralizing activity of anti-S NAbs were measured in serum samples (n=24) collected at the baseline, 20-, 34-, and 150-days post-vaccination using Jess, ELISA, and neutralization assays in order to evaluate the degree of correlation between these 3 immunoassays. We first performed correlation and linear regression analysis on the four different BAbs (anti S, anti-S1 RBD, anti-S2, and anti-S1 IgG) levels detected by Jess and neutralizing activity measured by neutralization assay (Figures 2F–I). Among the four BAbs, anti-S IgG showed a strong positive, statistically significant correlation (r=0.8630, R^2 = 0.7448, P

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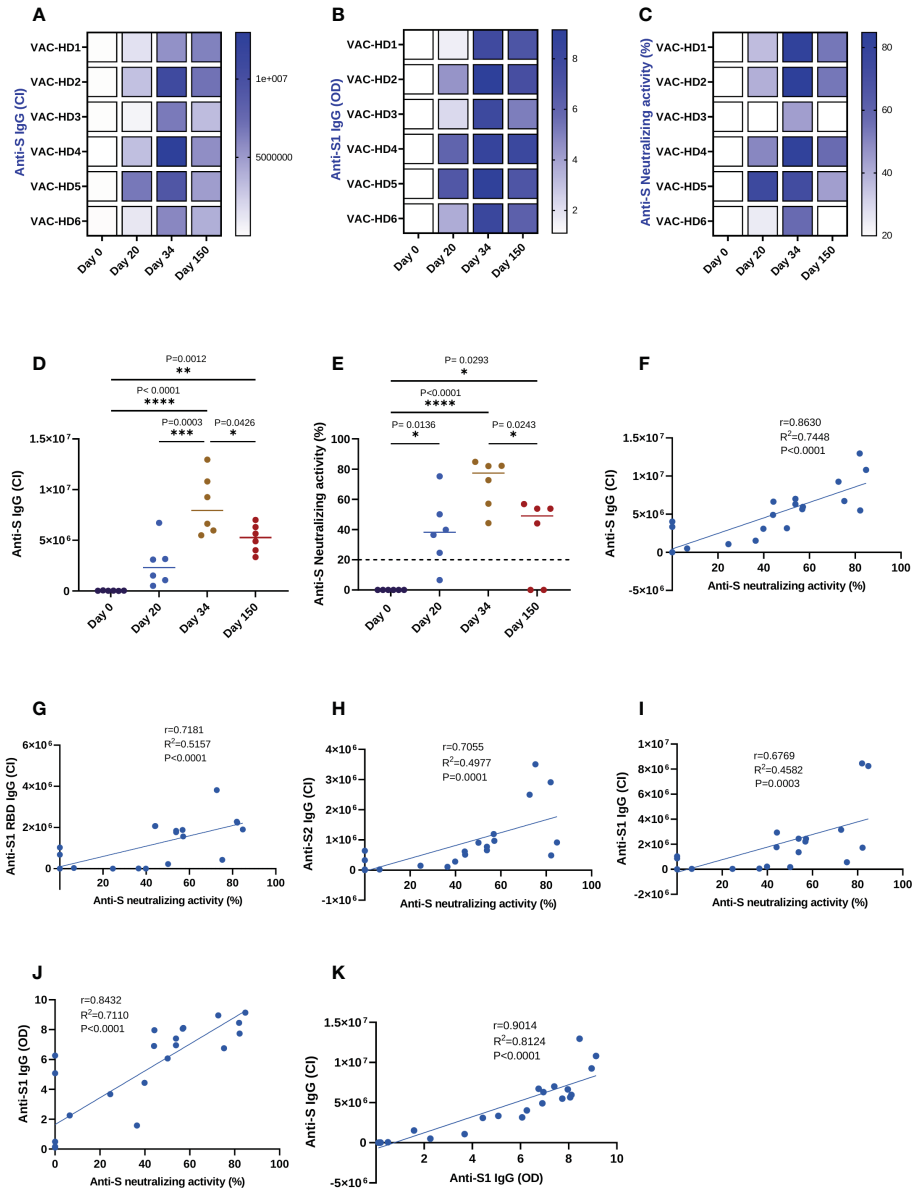

FIGURE 2
Correlation between the anti-spike binding and neutralizing antibodies responses induced by BNT162b2 vaccination and detected by three different immunoassays. Serum samples were collected from six BNT162b2 vaccinated participants at the baseline, 20-, 34-, and 150-days post-vaccination. The anti-S and anti-S1 IgG levels were assessed using Jess and ELISA, respectively. The anti-S neutralizing activity was measured using the neutralization assay. **(A–C)** Heat-map of anti-S IgG, anti-S1 IgG, and anti-S neutralizing activity responses in six BNT162b2 vaccinated participants over time. **(D)** Dynamics of the anti-S IgG levels in six BNT162b2 vaccinated participants over time (Jess). **(E)** Dynamics of the anti-S neutralizing activity in six BNT162b2 vaccinated participants (Neutralization assay). Each symbol represents an individual participant with a line indicating the median of each time point. One-way ANOVA test was used, P value was considered statistically significant when *P ≤ 0.05. All samples were run in duplicates. **(F–I)** Correlation between anti-S, S1 RBD, S2, and S1 IgG levels detected by JESS and neutralizing activity, respectively. **(J)** Correlation between anti-S1 IgG levels detected by semi-quantitative ELISA and neutralizing activity. **(K)** Correlation between anti-S1 IgG levels detected by ELISA and anti-S IgG levels detected by JESS. All correlations were analyzed by Pearson statistical test and linear regression models. The scatter point represents serum samples (n=24) collected from six BNT162b2 vaccinated participants at the baseline, 20-, 34-, and 150-days post-vaccination, and the blue error band represents the 95% confidence interval. The coefficient of correlation (r) represents the strength of the linear relationship between the different immunoassays. The coefficient of determination (R2) represents the percentage of variance in the given data set. The P-value tests whether the regression equation is significant. P value was considered statistically significant when *P ≤ 0.05. The stars present the level of significance. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

<0.0001) with the neutralizing activity (Figure 2F), whereas a moderate correlation (r ranging between 0.6769 and 0.7181) was observed for the remaining IgGs (anti-S1 RBD, anti-S1, and anti-S2) (Figures 2G–I). We then evaluated whether Jess is a better surrogate test for neutralizing activity prediction as compared to another common commercial semi-quantitative ELISA test. Linear regression analysis showed that the linear fit between anti-S IgG detected by Jess and neutralizing activity (R^2 = 0.7448, P<0.0001) was substantially higher as compared to the one obtained between anti-S1 IgG levels detected by ELISA and neutralizing activity (R^2 = 0.7110,

P<0.0001) (Figure 2J). Similarly, a distinguished positive correlation (r=0.9014, R^2 = 0.8124, P<0.0001) between anti-S1 IgG and anti-S IgG levels detected by ELISA and Jess respectively was observed (Figure 2K). Overall, these results indicate that the detection of anti-S IgG levels by Jess could be a better surrogate for neutralizing activity estimation compared to ELISA. Moreover, Jess could potentially be a promising alternative that is quicker, cheaper, and easier than the conventional cell-based assays for neutralizing activity estimation.

BNT162b2-induced T cells response to SARS-CoV-2 and cross-reactivity with other viral antigens

The cellular immune responses induced by BNT162b2 vaccination were characterized by the measurement of interferon-γ responses to S1 peptide pools (the immunodominant subunit of the S protein) using a relatively novel T-spot assay. This assay is highly specific since the SARS-CoV-2 epitopes having a high degree of homology with other endemic human coronaviruses (huCoVs) were removed from the SARS-CoV-2 antigens panels enabling a specific SARS-CoV-2 response determination. Similar to the antibody response, we observed an immense variation in the T cell responses among the vaccinated participants (Figures 3A, B). T-spot results showed that S1-specific T cell response was significantly induced on day 20 in 5/6 participants with variable levels ranging from 2 to 85 SFC (Figures 3A, B). An increase in this response was observed in all six participants on day 34 with an S1-specific T cell response ranging from 14 to 150 SFC (Figures 3A, B). However, 150 days post-vaccination a decline in the S1-specific response was observed in all six participants with different magnitudes ranging from 6 to 47 SFC (Figures 3A, B). We have found that females (VAC-HD 2, 4, 5, and 6) had a stronger S1-specific T cell response on days 20 and 34 compared to their counterparts in males (VAC-HD1 and 3) (Figures 3A, B).

On the other hand, we have demonstrated that the priming dose was able to induce a detectable T cell response (≥ 10 SFCs) against the S1 antigen in only 4/6 participants, whereas after the booster dose, all six participants presented a detectable T cell response ranging from 14 to 150 SFC (Figure 3C). Interestingly, we have shown that T cell response against the S1 antigen was induced 20 days after the priming dose (1 SFC on day 0 versus 25 SFCs on day 20) and significantly increased two weeks after the second dose (1 SFC on day 0 versus 71 SFC on day 34, **P=0.0034) in all the participants (Figure 3C). However, we have observed that T cell response to the S1 antigen was significantly decreased 150 days post-vaccination (71 SFC on day 34 versus 15.83 SFC on day 150, * P=0.0226) (Figure 3C). This decline was observed in all six participants, whereas this T cell response remained detectable 150 days after vaccination in 4/6 participants (Figures 3B, C).

Given the fact that SARS-CoV-2 displays a high level of homology to other human coronaviruses (huCoVs), we evaluated whether the BNT162b2 vaccination could induce a cellular immune response against other huCoVs strains than SARS-CoV-2. Therefore, we compared the T cell response, using high homology peptide pools, on day 0 and day 34 post-vaccination. Interestingly, all six participants showed an increase in T cell response against cross-reactive sequences between SARS-CoV-2 and other huCoVs after the second dose (Figure 3D) with 4/6 participants demonstrated a significant increase in such response (Supplement 5). This result suggests that the booster dose activated and enhanced the T cell responses against other huCoVs strains (priming at day 20 did not enhance this response, data not shown). We further evaluated T cell responses against five different human common viruses peptide pools: CMV, EBV, BKV, Adv 3, and 5 on day 0 and day 34 post-vaccination. As expected, all six participants showed T cell responses to all of these viruses at baseline (day 0 before vaccination), ranging between 87 and 236 SFC due to previous exposure to these viruses (Figure 3E). Importantly, we have demonstrated that this T cell response was increased in 4/6 participants on day 34 post-vaccination (Figure 3E). These results may suggest the presence of cross-reactive epitopes between SARS-CoV-2 and these five viruses.

Rapid ex-vivo expansion of spike-specific T cells from BNT162b2 vaccinated donors

We next investigated whether we could expand the S-specific T cells from BNT162b2 vaccinated donors, five months post-vaccination, using a rapid *ex-vivo* expansion protocol described in supplement 6 (see supplementary material). Briefly, PBMCs collected from four vaccinated donors were stimulated with the S peptide pools and then cultured in the presence of IL-4 and IL-7 for 11 days in the G-Rex 10 culture device. We first examined the S-specific T cell response in these four participants prior to expansion. T-spot results showed a positive S1-specific T cell response ranging from 10 to 46 SFCs in VAC-HD2, 3, and 4 (Figure 4A). However, VAC-HD 1 lacked detectable S1-specific T cells (SFCs=6, below the positive cutoff threshold) (Figure 4A). Interestingly, the T cells from VAC-HD2, 3, and 4 were expanded up to 9-fold ($136x10^6$ cells), 10.4-fold ($156x10^6$ cells), and 13.7-fold ($206x10^6$ cells), respectively eleven days post-stimulation (Figure 4B). The T cells from VAC-HD1 however failed to adequately expand likely due to the low frequency of the S1-specific T cells before expansion (1.8-fold; $26.5x10^6$ cells) (Figure 4B). These results indicate that the frequency of pre-existing S-specific T cells may play a major role in the expansion of such T cells.

We next evaluated the specificity of these expanded T cells by measuring IFN-γ secreting T cells in response to S1 stimulation using the T-spot assay, corresponding PBMCs collected 150 days post-vaccination (Pre-expansion PBMCs) were used as a control. Overall, expanded T cells demonstrated an S1-specific IFN-γ production which was significantly higher than the one detected in pre-expanded T cells for all participants (Figure 4A). Our data show that this T cell response varied between the four participants and positively correlated with the proliferative expansion fold (r=0.7263, R^2 = 0.5275) (Figure 4C). Significant increase in the number of IFN-γ SFC, after T cell expansion, was observed in the four participants (Figure 4A): VAC-HD1 (4-fold increase from 6-23 SFCs; *P=0.0109), VAC-HD2 (2-fold increase from 13-25 SFCs; *P=0.028), VAC-HD3 (13-fold increase from 10-129 SFCs; ***P=0.0001) and VAC-HD4 (41-fold increase from 47-1900 SFCs; ****P<0.0001) (Figure 4A).

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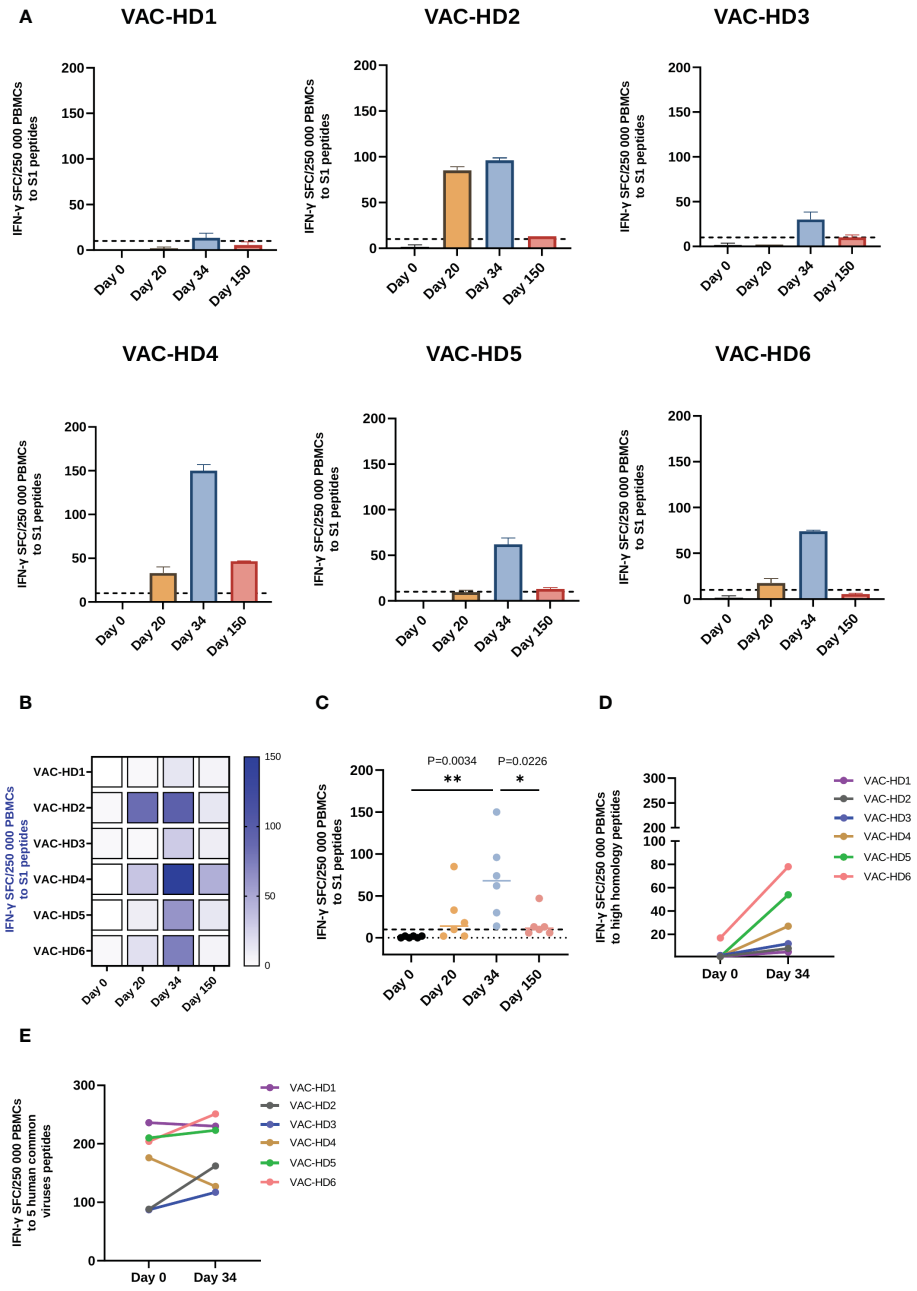

FIGURE 3
Spike-specific and spike cross-reactive T cell responses in BNT162b2 vaccinated participants. **(A)** T cell response to S1 peptide pools in six BNT162b2 vaccinated participant at the baseline, 20-, 34-, and 150-days post-vaccination. **(B)** Heat-map of S1- specific T cell responses in six BNT162b2 vaccinated participants over time **(C)** Dynamics of S1- specific T cell responses in six BNT162b2 vaccinated participants over time. Each symbol represents an individual participant with a line indicating the median of each time point. **(D)** T cell response to high homology peptide pools in BNT162b2 vaccinated participants at baseline (Day 0) and on day 34 post-vaccination (after the second dose) **(E)** T cell response to five human common viruses peptides (CMV, EBV, AdV 3 and 5 and BKV) in BNT162b2 vaccinated participants at baseline (Day 0) and on day 34 post-vaccination (after the second dose). Results are presented as the mean of the number of spots forming cells (SFCs) per 250 000 PBMCs subtracting the background (negative control) count. A positive response was defined as an SFCs of 10 or more. One-way ANOVA test was used, P value was considered statistically significant when *P ≤ 0.05. All samples were run in duplicates. The stars present the level of significance. *P < 0.05; **P < 0.01.

Phenotypic characterization of S-specific expanded T cells

To further analyze the phenotype of the expanded S-specific T cells, the distribution of activated and cytotoxic T cell subsets was assessed using flow cytometry following stimulation with the S1 antigen. We also compared the changes in the distributions of these T cell subsets before and after expansion. The gating strategy and raw data are presented in supplements 7 and 8 (see supplementary material). Overall, the frequencies of both pre-expanded S-specific activated $CD4^+$ ($CD4^+OX40^+CD69^+$) and $CD8^+$ ($CD8^+CD137^+CD69^+$) T cells were higher in VAC-HD3 and 4 compared to VAC-HD1 and 2

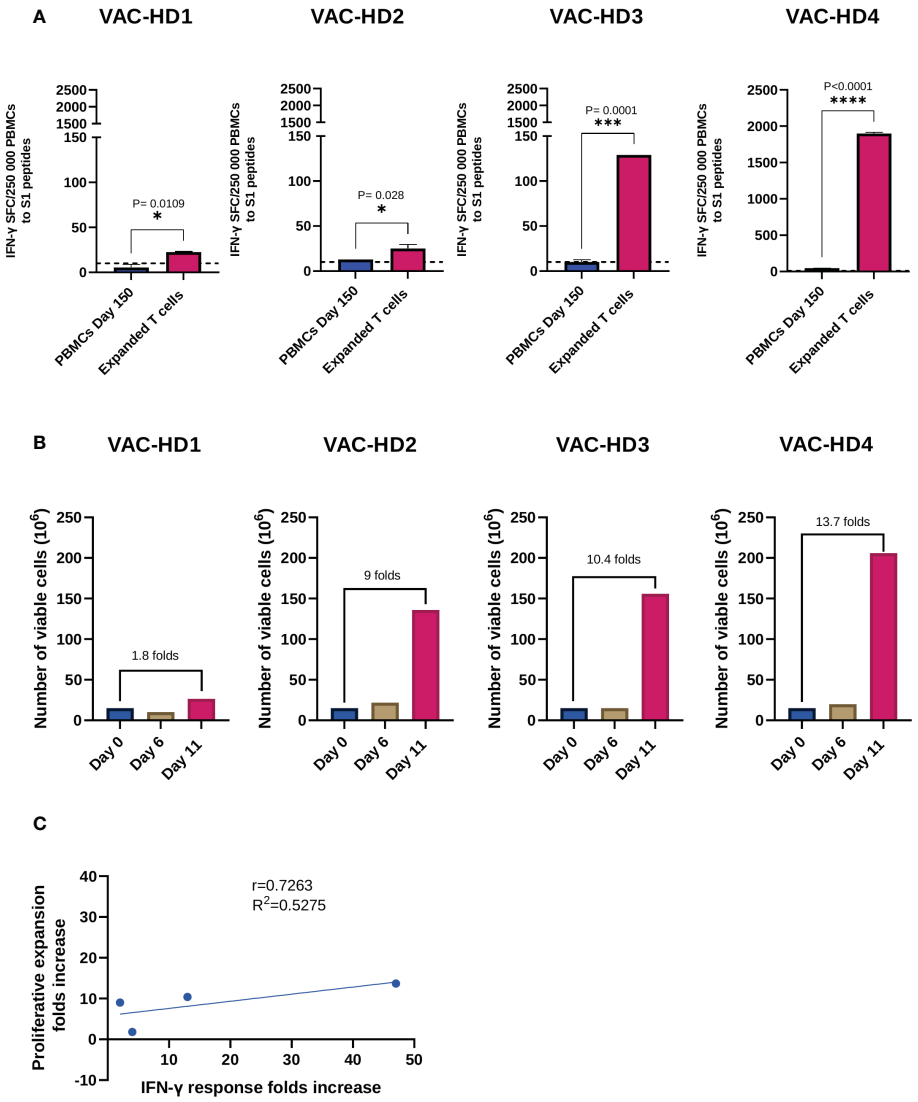

FIGURE 4
Expansion and functional characterization of expanded spike-specific T cells. **(A)** IFN-γ secretion by pre-expansion PBMCs collected 150 days post-vaccination and spike-specific expanded T cells following S1 peptide pools overnight stimulation. Results are presented as the mean of the number of spots forming cells (SFCs) per 250 000 PBMCs subtracting the background (negative control) count. A positive response was defined as an SFCs of 10 or more. Student t test was used, P value was considered statistically significant when *P ≤ 0.05. All samples were run in duplicates. **(B)** Viable cell counts, and fold expansion were assessed on days 0, 6, and 11 of expansion using trypan blue exclusion dye. **(C)** Correlation between IFN-γ response folds increase and the proliferative expansion folds increase between pre-expansion PBMCs and spike-specific expanded T cells in four BNT162b2 vaccinated participants. The dotted lines represent confidence intervals at 95%. The stars present the level of significance. *P < 0.05; **P < 0.01; ****P < 0.0001.

(Figure 5A). In this, the frequency of S-specific activated $CD4^+$ T cells was 0.93% and 0.73% in VAC-HD1 and 2 versus 5.15% and 5.39% in VAC-HD3 and 4 (Figure 5A). Whereas the frequency of S-specific activated $CD8^+$ T cells was 0.51% and 0.15% for VAC-HD1 and 2 versus 1.39% and 0.7% for VAC-HD3 and 4 (Figure 5A). These results indicate that the frequency of pre-existing S-specific T cells prior to expansion was higher in VAC-HD3 and 4 than in VAC-HD1 and 2 which can explain the fact that the expansion was more efficient for these two cases compared to others (Figure 4B). Interestingly, an increase in the frequencies of expanded $CD4^+$ and/or $CD8^+$ S-activated T cells was recorded in all participants when compared to the pre-expanded population (Figure 5A). We also showed that induction of the T cell activation markers against the S antigen was higher in the $CD4^+$ T cells for VAC-HD1 and VAC-HD3 (4.04% and 8.32% respectively) and in the $CD8^+$ T cells for VAC-HD 2 and VAC-HD4 (1.49% and 13.6% respectively) (Figure 5A).

We next examined the overall changes in $CD3^+$, $CD4^+$, and $CD8^+$ cytotoxic T cell frequencies within the S-specific expanded T cells compared to the pre-expanded T cells. Expanded T cells presented higher frequencies of $CD3^+CD107^+$, $CD4^+CD107^+$, and $CD8^+CD107^+$ cytotoxic T cells compared to the pre-expanded population in all four participants (Figure 5B). In this, the frequencies of $CD3^+CD107^+$ increased from 24.6-58.1% (Pre) to 49.1-85.7% (Post), $CD4^+CD107^+$ from 36.5-63% (Pre) to 76.1-91.1% (Post), and $CD8^+CD107^+$ from 19.9-58% (Pre) to 33.3-78.3% (Post) in all the four participants (Figure 5B). Interestingly, the

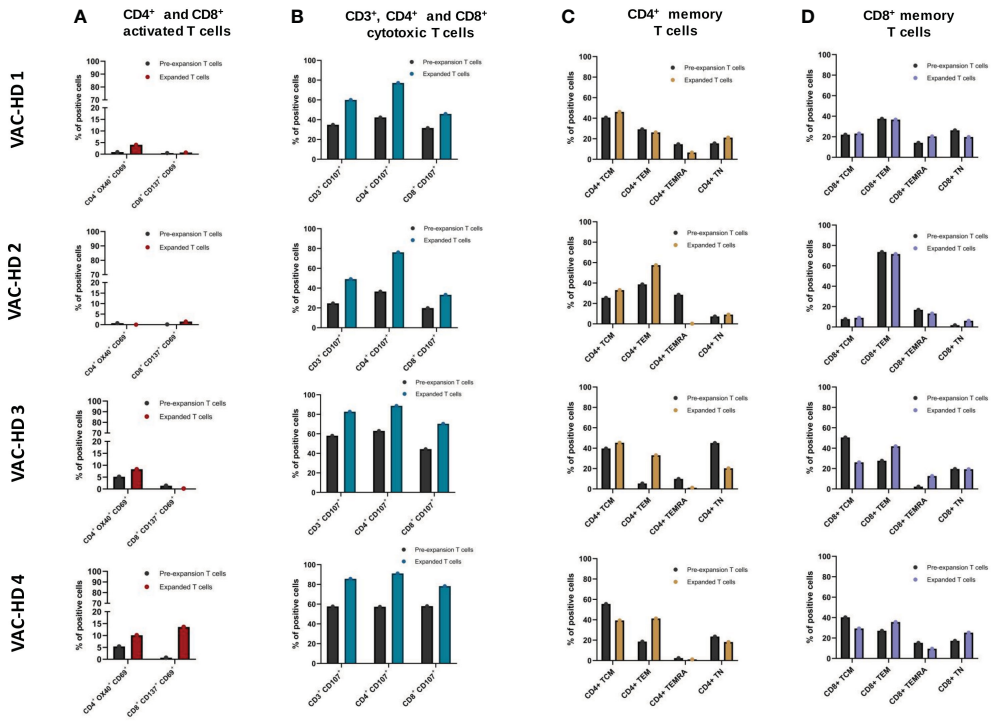

FIGURE 5
Phenotyping of the spike-specific expanded T cells. **(A)** Frequency of $CD4^+$ $OX40^+$ $CD69^+$ and $CD8^+$ $CD137^+$ $CD69^+$ activated T cells within pre-expansion and expanded T cells following S1 stimulation in four BNT162b2 vaccinated participants. **(B)** Frequency of $CD3^+$ $CD107^+$, $CD4^+$ $CD107^+$, and $CD8^+$ $CD107^+$ cytotoxic T cells within pre-expansion and expanded T cells following S1 stimulation in four BNT162b2 vaccinated participants. **(C)** Frequency of $CD4^+$ naïve ($CCR7^+CD45RA^+$), central memory ($CCR7^+CD45RA^-$), effector memory ($CCR7^-CD45RA^-$), and terminally differentiated memory ($CCR7^-CD45RA^+$) within pre-expansion and expanded T cells following S1 stimulation in four BNT162b2 vaccinated participants. **(D)** Frequency of $CD8^+$ naïve ($CCR7^+CD45RA^+$), central memory ($CCR7^+CD45RA^-$), effector memory ($CCR7^-CD45RA^-$), and terminally differentiated memory ($CCR7^-CD45RA^+$) within pre-expansion and expanded T cells following S1 stimulation in four BNT162b2 vaccinated participants.

cytotoxic T cell marker $CD107^+$ was higher in the $CD4^+$ expanded T cell population (76.1% to 91.1%) when compared to the $CD8^+$ counterparts (31.6% to 78.3%) in all four participants (Figure 5B).

In the next step, we examined the distribution of memory T cell subsets in the expanded T cell population. Based on phenotypic markers, T cell subsets can be classified into four subsets: naïve ($CCR7^+CD45RA^+$), central memory ($CCR7^+CD45RA^-$), effector memory ($CCR7^-CD45RA^-$), and terminally differentiated memory ($CCR7^-CD45RA^+$). The S-specific expanded $CD4^+$ and $CD8^+$ T cells were found to have different distributions of naïve (TN), effector (TEM), central memory (TCM), and terminally differentiated memory (TEMRA) phenotypes in all four participants (Figures 5C, D). For $CD4^+$ expanded T cells, the TCM subset was dominant in VAC-HD1 and 3 whereas the TEM subset was aberrant in VAC-HD2 and 4 (Figure 5C). However, for $CD8^+$ expanded T cells, the TEM subset was dominated in all four participants with a frequency ranging from 35.7 to 71.6% (Figure 5D). Interestingly, we demonstrated that VAC-HD1 and 2, who showed the highest frequencies of pre-expanded $CD4^+$ TEMRA (Figure 5C), had lower S-specific pre-expanded $CD4^+$ activated T cells ($CD4^+OX40^+CD69^+$) frequencies in the compared to VAC-HD3 and VAC-HD4 (Figure 5A). Moreover, the frequency of the $CD4^+$ TEMRA subset was greatly reduced after T cell expansion in all 4 participants (Figure 5C). Importantly, we showed that participants who had a $CD4^+$ TCM ($CCR7^+CD45RA^-$) dominance (VAC-HD1, 3 and 4) (Figure 5C) expressed high CD69 activation marker in the $CD4^+$ activated T cells among the expanded T cells (Figure 5A).

Discussion

Most of the investigations designed to assess the efficacy, immunogenicity, and protective immunity induced by the BNT162b2 vaccine were based on large cohort studies. However, despite their advantages, these studies are providing only a general observation of the vaccine-induced immune response without dissecting such immune responses that is laborious and expansive to investigate in large cohorts. In the present work, we dissected BNT162b2 vaccine-induced humoral and cellular responses in a small cohort study that includes recipients receiving two doses of this vaccine. Our results showed a broad range of variation in both humoral and cellular responses. This interpersonal variation was observed at three stages: the induction was detected on day 20, the peak response reached on day 34, and the response declined 150 days post-vaccination. The factors involved in the interindividual variation in the human immune response to BNT162b2 vaccine are still largely unknown. Previous reports suggest that the interindividual diversity of the human immune responses to common pathogens and vaccines is determined by intrinsic (age and gender), extrinsic (environment), and genetic factors such as genes coding for human leukocyte antigen

alleles, major histocompatibility complex molecules, Toll-like receptors and cytokines (36, 37). Recently, Ward et al. identified age and gender as important determinants of humoral response to BNT162b2 vaccine (38). They showed that antibody positivity was higher in females and the youngest age group (38). Although our study was carried out in a small cohort, we have also shown that females had stronger S1-specific T cells and antibody responses compared to male participants.

Consistent with previous reports (39–41), our results also showed that the dynamics of anti-S binding and neutralizing antibodies responses followed the same trend, where they were induced 20 days post-priming, significantly increased after boosting, then declined five months post-vaccination. The peak level of antibody response detected on day 34 after boosting was associated with an increase of 4-fold in total anti-S binding IgG levels but only a 2-fold increase in the neutralizing activity compared to day 20. Our result is in agreement with a recent study where they showed that the immune response induced at the time of peak response following BNT162b2 vaccination was characterized by a high ratio of non-neutralizing antibodies (42) that may confer protection against SARS-CoV-2 infection (43, 44). Interestingly, we were able to show that all six participants maintained the anti-S IgG binding antibody response five months post-vaccination, whereas only four of them maintained the neutralizing activity response.

Recent studies have demonstrated that neutralizing antibody titers are the most critical parameters for vaccine efficacy evaluation and prediction of SARS-CoV-2 protective immunity (5–7). Many efforts have been made to develop high throughput assays for neutralizing antibody detection that can surrogate the classical neutralizing cell-based assays that require specific laboratory facilities, skilled personnel, and a long (2-4 days) execution time (8, 9). We investigated in this study whether a novel simple automated assay (Jess) could surrogate the neutralization assay for estimation of neutralizing activity. Jess is a robust anti-SARS-CoV-2 binding antibodies surveillance test, which is simple, fully automated, rapid, and can be easily used in laboratories without the need for BSL3 facilities. Using this assay, we have shown that only anti-S IgG (antibodies against the whole spike antigen) had a strong positive significant correlation ($r=0.863$, $R^2 = 0.7448$, $P < 0.0001$) with the anti-S neutralizing activity compared to other SARS-CoV-2 spike domain-specific IgG (S1-RBD, S1, and S2). Our results are in agreement with two very recent studies that used a chemiluminescent immunoassay for the quantitative determination of SARS-CoV-2 IgG binding antibodies (45, 46). The first study reported a linear correlation between anti-S IgG and surrogate neutralizing antibody levels for wild-type SARS-CoV-2 and variants of concern (VOCs) in BNT162b2 vaccinated and recovered health care workers (45). the second study also showed a strong correlation ($R^2 = 0.72$) between the anti-S antibody IgG titers detected by a chemiluminescent immunoassay and surrogate neutralizing activity (46). Taken together, our results indicate that Jess provides a robust anti-S neutralizing activity surveillance/prediction test.

The characterization of the T cell response in BNT162b2 vaccinated individuals indicated that a single dose of vaccine was not able to induce spike-specific T cell response in 30% of the participants indicating the necessity of a booster dose for efficient and durable protection (47). Similar to this, our results showed that 33% of vaccinated participants lacked a detectable spike-specific T cell response after one dose of this vaccine. However, the second dose was able to induce the spike-specific T cell response in 100% of the participants with a median increase of 3-fold in the T cell response frequencies compared to that observed after priming. Importantly, we demonstrated that the booster dose was able to also activate and enhance T cell responses against other huCoVs suggesting the presence of cross-reactive epitopes between SARS-CoV-2 and other huCoVs. Our findings are supported by a recent study showing the presence of common epitopes between SARS-CoV-2 and huCoVs (48). We have also demonstrated that pre-existing memory T cells, naturally induced during past infections of our participants with the five human common viruses (CMV, EBV, Adv 3 and 5, BKV), were *in vivo* expanded following the BNT162b2 vaccination. This suggests the presence of cross-reactive epitopes between SARS-CoV-2 and these five viruses derived-antigens. Indeed, the sequence similarity between EBV and SARS-CoV-2 has been well established (49) however, no study related to sequence or epitope similarity for the other viruses have been investigated. Furthermore, our data showed the persistence of spike-specific T cell response five months post-vaccination in 67% of the participants.

BNT162b2 mRNA vaccine clinical trial showed 95% effectiveness in preventing SARS-CoV-2 infection (3). This trial predominantly excluded patients with immunocompromising conditions (50), which present 2% of the global population (51). Indeed, recent studies have demonstrated that a two doses vaccine regimen does not produce sufficient strong immune responses and protection in immunocompromised patients and elderly people (31–33). Considering the ineffectiveness of current SARS-CoV-2 antibody-based immunotherapy due to the development of novel mutations and the immune escape of the VOCs (52), other therapeutic options are warranted. Adoptive SARS-CoV-2 specific T cell therapy represents an attractive therapeutic option in which viral immune escape is likely to be avoided as the recognized T cell epitopes are well conserved among the emerging SARS-CoV-2 variants (25). Moreover, adoptive cellular therapy with *ex-vivo* expanded specific T cells against other viruses (CMV, EBV, Adv, HHV6, and BKV) has been demonstrated to have efficacy in combating severe viral diseases in patients with immunodeficiency (53). In addition, studies in animal models have also shown that adoptive therapy with $CD4^+$ and/or $CD8^+$ T cells can efficiently control respiratory infections including SARS-CoV-1, MERS, and influenza viruses (54–56). Here, we adapted a rapid *ex-vivo* T cell expansion protocol for spike-specific T cell expansion to be potentially used for adoptive-cell therapy in severe COVID-19, immunocompromised individuals, and other high-risk groups. In this protocol, we expanded spike-specific T cells from vaccinated donors isolated five months post-vaccination to avoid spike-specific T cells exhaustion during the e*x-vivo* expansion and to mimic the *in vivo* stimulation and boosting effect of the booster dose which is usually given between five to six months post-priming. Moreover, the long-term persistence of memory T cells following vaccination or viral infection has been well reported (57, 58). The response mediated by such memory T cells, upon re-exposure to the antigen, is more rapid and effective than the primary response (59). In this rapid protocol (11 days) we were able to expand T cells up to 11-folds in 3/4 participants. A minimum S-specific T cell number ≥10 SFCs was required to support T cell expansion. Moreover, participants who had a higher frequency of

pre-expanded S-specific activated $CD4^+$ ($CD4^+OX40^+CD69^+$) and $CD8^+$ ($CD8^+CD137^+CD69^+$) T cells tended to have a higher expansion rate of S-specific T cells. The specificity of expanded T cells was measured with a newly modified T-spot assay in which the high-homology peptide sequences cross-reactive with other coronaviruses were removed. Expanded T cells demonstrated a significant increase of S1 spike-specific IFN-γ producing cells compared to the pre-expanded T cells for all participants. Interestingly, these S-specific expanded T cells had higher frequencies of both activation and cytotoxic markers important for viral clearance after re-exposure (60). Adoptively transferring such expanded T cells may be used as an attractive approach to restore and/or boost the cytotoxic T cell response in severe COVID-19, immunocompromised patients and elderly people with impaired cytotoxic T cell response to SARS-CoV-2 (28, 31–33, 61). Interestingly, the cytotoxic T cell marker $CD107^+$ was higher in the $CD4^+$ expanded T cell population when compared to the $CD8^+$ counterparts in all four participants. This result indicates that $CD4^+$ cytotoxic T cells play a major role in the S-specific cell-mediated cytotoxic response following BNT162b2 immunization. In line with this, it has been reported that S-specific T cell response, elicited by SARS-CoV-2 infection, was dominated by the $CD4^+$ subset in COVID-19 patients (62, 63).

It has been well established that central memory (TCM) and effector memory (TEM) T cell subsets have distinct functions and migratory properties (64, 65). Therefore, we examined the distribution of these memory T cell subsets in our spike-specific expanded T cells. There was a variation in the distribution of the $CD4^+$ and $CD8^+$ memory T cell subsets among all four participants. For $CD4^+$ memory T cells, the TCM subset was dominant in 2/4 participants whereas the TEM subset was aberrant in the 2 remaining participants. $CD4^+$ TCM resides within the lymphoid organs and are known for their rapid proliferation and production of IL-2 and IL-10 upon restimulation (66). However, $CD4^+$ TEM reside in peripheral tissues and exhibit immediate cytokine secretion of IFN-γ and IL-4 upon restimulation (66). For $CD8^+$ T cells, the TEM subset was dominated in all four participants. $CD8^+$ TEM also resides in peripheral tissue and provides immediate protection following antigenic stimulation by the secretion of perforin (66). Another subset of memory T cells known as terminally differentiated memory (TEMRA) has been demonstrated to exhibit numerous characteristics of immuno-senescence such as defects in proliferation and effector functions (67). Both $CD4^+$ and $CD8^+$ TEMRA are known to accumulate in the aging process and pathological conditions such as arthritis rheumatoid and persistent viral infection (68–75). Therefore, we investigated this phenomenon in our expanded spike-specific T cells. Interestingly, we found that participants who showed the highest frequencies of $CD4^+$ TEMRA cells prior to expansion tended to have lower S-specific activated $CD4^+$ T cells and a lower fold increase of the S1-specific IFN-γ response of expanded spike-specific T cells compared to the pre-expanded population. These results suggest that this subset of $CD4^+$ TEMRA cells may contribute to the impairment in the S-specific $CD4^+$ T cells development and T cell expansion inefficacy. We have also demonstrated that the frequencies of $CD4^+$ TEMRA were greatly reduced after S-specific T cell expansion. On the other hand, immuno-senescence associated with defects in immune proliferation and effector functions has been shown to correlate with an increased susceptibility to viral infection and a decreased vaccine immunogenicity (76). Indeed, several studies suggested that influenza vaccine inefficacy in aged individuals can be mainly related the immune system immunosenescence (77, 78). Another recent study has also demonstrated that mRNA COVID-19 vaccine immunogenicity was negatively correlated with the accumulation of T cell expressing signs of immunosenescence (79).

CD69 is widely used as an activation marker for T cells and natural killer cells, however, the precise role of this marker in these immune cells is not yet well elucidated (80). Recent evidence suggests that the expression level of CD69 controls the migration and retention of $CD4^+$ memory T cells in their specific niches (81). Similarly, another study suggests that upregulation of CD69, after yellow fever vaccination, can promote T cell migration and retention in the lymph nodes, the home for TCM (80). Therefore, we hypothesized that an increase of the CD69 marker after BNT162b2 vaccination may control the homing and migration of S-specific $CD4^+$ TCM. Interestingly, we showed that participants who had a $CD4^+$ TCM dominance also expressed a high CD69 activation marker in the $CD4^+$ activated T cells among the expanded T cells. Further studies are required to clarify the role of CD69 in vaccine-induced $CD4^+$ memory T cell migration and homing.

In conclusion, this is the first pilot study that highlights the interpersonal heterogeneity of the humoral and cellular responses to the BNT162b2 vaccine. We have demonstrated for the first time a strong correlation between Jess and neutralization cell-based assay. we also validated the feasibility and efficacy of a rapid *ex-vivo* spike-specific T cell expansion protocol from BNT162b2 vaccinated individuals that can be used in the future to establish a biobank for adoptive transfer of allogeneic HLA-matched spike-specific T cells as therapeutic and/or prevention options in severe COVID-19, immunocompromised patients, and elderly people. The limitation of our study is the use of a small number of participants and further studies with larger sample sizes are needed to confirm our results.

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 authors.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB) committee of HMC (Project number MRC-01-21-113). The patients/participants provided their written informed consent to participate in this study.

Author contributions

SD, SM, MM, and VPI: Conceptualized the study, planned, and supervised the experiments. SM: performed experimental work,

analyzed data, and generated figures. SM and SD: designed and wrote the manuscript. NT, MM, VPI, MKS, FHA, QF, SH and FS helped in the experimental work. SD, MM, VPI, AR, HMY, FBA, ASO, AAH, MAM, MUA, AWA, SU, LJA, LA, MJ, MG, NA, HMHS, AMJ, MN, AZ, FA and SRA: Critical revision and editing of the scientific contents of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

SD, SM, MM, VPI, NT, FA, AR, SH, QF, AWA, FS, LA, MJ, MG, NA, MUA, SU, AMJ, MN, ASO, AAH, MAM, FBA, AZ, SRA, and HMHS were employed by Hamad Medical Corporation.

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

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The impact of circadian rhythm on Bacillus Calmette-Guérin vaccination effects on SARS-CoV-2 infections

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Background and objective: A recent study has suggested that circadian rhythm has an important impact on the immunological effects induced by Bacillus Calmette-Guérin (BCG) vaccination. The objective of this study was to evaluate whether the timing of BCG vaccination (morning or afternoon) affects its impact on severe acute respiratory syndrome–coronavirus-2 (SARS-CoV-2) infections and clinically relevant respiratory tract infections (RTIs).

Methods: This is a *post-hoc* analysis of the BCG-CORONA-ELDERLY (NCT04417335) multicenter, placebo-controlled trial, in which participants aged 60 years and older were randomly assigned to vaccination with BCG or placebo, and followed for 12 months. The primary endpoint was the cumulative incidence of SARS-CoV-2 infection. To assess the impact of circadian rhythm on the BCG effects, participants were divided into four groups: vaccinated with either BCG or placebo in the morning (between 9:00h and 11:30h) or in the afternoon (between 14:30h and 18:00h).

Results: The subdistribution hazard ratio of SARS-CoV-2 infection in the first six months after vaccination was 2.394 (95% confidence interval [CI], 0.856-6.696) for the morning BCG group and 0.284 (95% CI, 0.055-1.480) for the afternoon BCG group. When comparing those two groups, the interaction hazard ratio was 8.966 (95% CI, 1.366-58.836). In the period from six months until 12 months after vaccination cumulative incidences of SARS-CoV-2 infection were comparable, as well as cumulative incidences of clinically relevant RTI in both periods.

Conclusion: Although there was a difference in effect between morning and afternoon BCG vaccination, the vaccine did not protect against SARS-COV-2 infections and clinically relevant RTI's at either timepoint.

KEYWORDS

COVID-19, respiratory tract infection, circadian rhythm, BCG, trained immunity, heterologous protection, SARS-CoV-2, circadian clock

Introduction

Trained immunity is an emerging concept which describes epigenetic, metabolic and functional reprogramming of innate immune cells that leads to an enhanced heterologous immune response to infections. Trained innate immune cells are characterized by an increased host defense function upon restimulation (higher cytokine production capacity, phagocytosis, intracellular pathogen killing). Bacillus Calmette-Guérin (BCG) is the vaccine against tuberculosis and has been shown to induce trained immunity and protect against heterologous infections (1). The magnitude of the heterologous responses induced by BCG is highly variable and dependent on many factors, including age, sex and environmental factors (2–6). A source of uncertainty is the duration of potential heterologous effects.

Recently, de Bree et al. have observed that the circadian rhythm may influence BCG-induced trained immunity (7). Three months after BCG vaccination, monocytes of individuals vaccinated between 8:00h and 9:00h produced higher cytokine levels upon ex-vivo stimulation compared to individuals vaccinated at 18:00h. These data support the large body of evidence suggesting that the innate immune system is under control of an intrinsic clock, similar to many functions in mammalian physiology. It has been hypothesized that endogenous oscillations of immune cells allow the host to anticipate variations and potential threats in the environment (8). For example, synchronizing the magnitude of the immune response against foodborne pathogens with the phase of feeding is much more energy efficient than synchronizing it with the phase of sleeping (9).

The protective effects of trained immunity and BCG have been shown against a variety of viral pathogens (10–13). Therefore, before specific SARS-CoV-2 vaccines were developed and became available, it was suggested BCG might provide some protection against Coronavirus disease 2019 (COVID-19). However, clinical trials on the effect of BCG on respiratory tract infections (RTIs) including COVID-19 produced mixed results. Two trials on BCG re-vaccination performed in Greek elderly populations resulted in lower numbers of RTIs of probable viral origin and COVID-19, respectively (14, 15). On the other hand, trials on BCG vaccination performed in Dutch elderly cohorts and in Dutch healthcare workers did not result in lower numbers of RTIs or COVID-19 (16, 17). Given the earlier report of a circadian rhythm effect on BCG immunological effects, with the strongest effect in the morning, we analyzed whether the time of the day of vaccination in one of these clinical trials (the BCG-CORONA-ELDERLY study) influenced the effect on susceptibility to infections. While the overall analysis in this trial found no significant effect of BCG vaccination on the incidence of RTI or COVID-19, one could envisage that an effect may be observed in the sub-group of volunteers vaccinated in the morning.

Methods

The present analysis is a sub-study of the BCG-CORONA-ELDERLY study (NCT04417335), a prospective, randomized, placebo-controlled trial in two tertiary centers in the Netherlands. For an extensive description of the protocol, methods and results, please refer to the protocol (18) and the clinical data reported by Moorlag et al. (16).

In short, 2014 immunocompetent individuals with a median age of 67 years (interquartile range 64–72 years) were included in the original trial. Participants were randomly assigned in a 1:1 ratio to receive either 0.1 mL of BCG (Danish strain 1331, SSI, Denmark) or 0.1 mL placebo (0.9% NaCl solution) *via* intradermal injection.The time slot of vaccination was randomly assigned. If participants were unable to attend at that time, they were allowed to reschedule their appointment. Subsequently, participants had to document clinical symptoms, COVID-19 testing, COVID-19 exposure, and visits to healthcare professionals in the 12 months following vaccination.

To analyze the effect of the circadian rhythm on the clinical outcomes, all participants were divided into four groups based on the time of vaccination (morning or afternoon) and based on the intervention (BCG or placebo). Participants in the morning were vaccinated between 9:00h and 11:30h and participants in the afternoon were vaccinated between 14:30h and 18:00h. Individuals vaccinated between 11:30h and 14:30h were not analyzed in the current study. We chose for those time intervals to obtain two groups of comparable size at the two ends of the vaccination time of the day. Furthermore, those intervals give a realistic reflection of common practice of vaccination. The time of randomization was regarded as the time of vaccination, since randomization was precisely registered in the database and randomization was usually not more than 5 minutes apart from the vaccination. Next to the full 12 months follow-up, we separately analyzed the periods from vaccination until six months and from six months until 12 months after vaccination to account for trained immunity effects that are much stronger in the initial months after vaccination. The primary outcome of our study was the cumulative incidence of SARS-CoV-2 infection, detected by a polymerase chain reaction (PCR) test and irrespective of symptoms. Secondary endpoint was the cumulative incidence of clinically relevant respiratory tract infection. In order to meet this criterion, participants had to have at least one respiratory symptom and one systemic symptom that required medical intervention within 5 days of the onset of symptoms. Medical interventions included the start or change in antibiotic, antiviral, corticosteroid or pulmonary treatment, or hospital admission.

The endpoints were analyzed using the Fine and Gray competing risks proportional hazards model. Time to event was defined as the dependent outcome, time of vaccination as the independent variable, and mortality as potential competing event. The model was adjusted for the participating hospital and statistically different baseline characteristics that were tested by analysis of variance and included age category, BMI and hypertension. The effect for both endpoints was reported as a hazard ratio with 95% confidence interval (CI). Participants who met one of the endpoints in the first six months after vaccination were removed from the analysis of the second part of the year.

First, we compared the group vaccinated with BCG in the morning with the group vaccinated with placebo in the morning, and the group vaccinated with BCG in the afternoon with the group

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vaccinated with placebo in the afternoon. Second, we compared the two BCG groups with each other. Data were analyzed using R version 4.1.1 (19).

Results

Patient characteristics are shown in Table 1. Among the volunteers participating in the study, 358 participants were vaccinated with BCG and 356 with placebo between 9:00h and 11:30h, and 320 participants were vaccinated with BCG and 309 with placebo between 14:30h and 18:00h. The median age of all groups was 67 years, although adults over the age of 80 were more prevalent in the morning groups compared to the afternoon groups ($p<0.01$). In the morning placebo group were more males than females, whereas in the afternoon placebo group more females were present. In the BCG groups the sex distribution was balanced. Participants vaccinated with BCG in the afternoon had a slightly higher BMI than the participants of the other groups ($p=0.04$). Hypertension was less prevalent amongst participants who got vaccinated with BCG in the morning compared to those who got vaccinated with placebo in the morning ($p=0.03$), and those who got vaccinated with BCG in the afternoon ($p=0.01$). The remaining baseline characteristics were comparable in all groups (Table 1).

In the first six months after vaccination, the cumulative incidence of SARS-CoV-2 infection was 0.014 (95% CI 0.005-0.031) in the placebo morning group and 0.034 (95% CI 0.018-0.056) in the BCG morning group (subdistribution hazard ratio [SDHR] 2.394, 95% CI 0.856-6.696) (Table 2A). In the afternoon results are in the opposite direction, but not statistically significant (SDHR 0.284, 95% CI 0.055-1.480). When comparing the BCG morning and afternoon group with each other, the interaction hazard ratio [IHR] is 8.966 (95% CI 1.366-58.836), indicating a difference in effect between the two timepoints. In the second part of

TABLE 1 Baseline characteristics of study participants.

Variable	BCG in the morning (N=358)	Placebo in the morning (N=356)	BCG in the afternoon (N=320)	Placebo in the after-noon (N=309)	p-value BCG morning – BCG after-noon	p-value BCG morning – placebo morning	p-value BCG afternoon – placebo after-noon
Median age (IQR) – yr	67 (64-72)	67 (64-72)	67 (64-70)	67 (63-70)	0.047	0.545	0.720
Age category – no. (%)							
60 – 69 yr	221 (61.7)	230 (64.6)	225 (70.3)	211 (68.3)	0.019	0.426	0.581
70 – 79	109 (30.4)	99 (27.8)	86 (26.9)	90 (29.1)	0.305	0.437	0.530
80+	28 (7.8)	27 (7.6)	9 (2.8)	8 (2.6)	0.004	0.906	0.863
Sex – no. (%)							
Male sex	176 (49.2)	193 (54.2)	163 (50.9)	139 (45.0)	0.644	0.177	0.135
Female sex	182 (50.8)	163 (45.8)	157 (49.1)	170 (55.0)			
Median BMI (IQR) – kg/m^2	24.8 (22.9-27.4)	24.8 (23.1-27.4)	25.7 (23.5-28.1)	25.1 (23.3-28.1)	0.015	0.827	0.537
Comorbidities							
Cardiovascular disease	65 (18.2)	73 (20.5)	51 (15.9)	52 (16.8)	0.444	0.427	0.763
Hypertension	89 (24.9)	115 (32.3)	110 (34.4)	89 (29.0)	0.007	0.028	0.133
Diabetes	29 (8.1)	20 (5.6)	21 (6.6)	20 (6.5)	0.444	0.190	0.964
Asthma	17 (4.7)	19 (5.3)	17 (5.3)	20 (6.5)	0.737	0.719	0.537
Other pulmonary disease	9 (2.5)	12 (3.4)	9 (2.8)	8 (2.6)	0.809	0.498	0.863
Renal disease	3 (0.8)	9 (2.5)	8 (2.5)	8 (2.6)	0.087	0.079	0.944
Allergic rhinitis	81 (22.6)	92 (25.8)	70 (21.9)	75 (24.3)	0.815	0.316	0.476
Use of any medication – no. (%)	246 (68.7)	250 (70.2)	220 (68.8)	219 (70.9)	0.992	0.661	0.562

(Continued)

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TABLE 1 Continued

Variable	BCG in the morning (N=358)	Placebo in the morning (N=356)	BCG in the afternoon (N=320)	Placebo in the after-noon (N=309)	p-value BCG morning – BCG after-noon	p-value BCG morning – placebo morning	p-value BCG afternoon – placebo after-noon
Median number of daily used medication (IQR)	1.0 (0.0-3.0)	2.0 (0.0-3.0)	1.0 (0.0-3.0)	2.0 (0.0-4.0)	0.524	0.154	0.488
Never smoked	124 (34.6)	137 (38.5)	102 (31.9)	108 (35.0)	0.446	0.286	0.413
Past smoking	219 (61.2)	199 (55.9)	199 (62.2)	185 (59.9)	0.786	0.153	0.551
Current smoking	13 (3.6)	18 (5.1)	19 (5.9)	15 (4.9)	0.158	0.350	0.548
Second hand smoke	2 (0.6)	1 (0.3)	0	1 (0.3)	0.181	0.566	0.309
BCG vaccination history – no. (%)							
Unknown	49 (13.7)	64 (18.0)	64 (20.0)	67 (21.7)	0.028	0.116	0.732
No	204 (57.0)	196 (55.1)	160 (50.0)	156 (50.5)	0.069	0.604	0.903
Yes	105 (29.3)	94 (26.4)	95 (29.7)	86 (27.8)	0.919	0.383	0.607
Median years since BCG vaccination (IQR)	50 (45-56)	48 (42.3-55)	49 (44-53)	49 (44.5-58)	0.325	0.146	0.365
SARS-CoV-2 vaccination history – no. (%)							
Vaccinated	358 (100)	355 (99.7)	318 (99.4)	307 (99.4)	0.134	0.156	0.972
Pfizer/BioNTech	252 (70.4)	248 (69.7)	217 (67.8)	196 (63.4)	0.468	0.832	0.247
AstraZeneca	99 (27.7)	100 (28.1)	95 (29.7)	106 (34.3)	0.559	0.897	0.215
Moderna	6 (1.7)	6 (1.7)	3 (0.9)	4 (1.3)	0.402	0.992	0.670
Janssen	1 (0.3)	1 (0.3)	2 (0.6)	1 (0.3)	0.498	0.997	0.583
Median days until first SARS-CoV-2 vaccination (IQR)	346 (332.8-358)	347 (333.8-358)	349 (332-358)	350 (335-359)	0.288	0.286	0.292
Other vaccines – no. (%)							
Live vaccines in the past year	3 (0.8)	2 (0.6)	1 (0.3)	2 (0.6)	0.372	0.658	0.524
Non-live vaccines in the past year	251 (70.1)	242 (68.0)	226 (70.6)	221 (71.5)	0.884	0.537	0.804

BCG, Bacillus Calmette-Guérin; CI, confidence interval; BMI, body mass index.

the year, cumulative incidences were more comparable with SDHRs of 0.745 (95% CI 0.437-1.600) and 1.460 (95% CI 0.505-4.223) for the morning and the afternoon group, respectively (Table 2B). The IHR of the two BCG groups is 0.530 (95% CI 0.149-1.881). The analysis of the full 12 months follow-up is in line with the aforementioned and did not reveal any statistically significant differences in the cumulative incidence of SARS-CoV-2 infection (Table 2C).

Due to the interventions of the COVID-19 pandemic, such as quarantine, isolation, and social distancing, the number of clinically relevant RTIs was much lower than SARS-CoV-2 infections. The SDHR was comparable in all time periods (Table 2A–C).

In conclusion, neither participants vaccinated with BCG in the morning nor in the afternoon were protected against respiratory infections including SARS-CoV-2.

Discussion

The results of the present study show that the time of day of BCG vaccination did not affect the susceptibility to respiratory infections. We observed some differences in the cumulative incidence of SARS-CoV-2 infections, especially in the first six months after vaccination, but the number of events was too low and consequently confidence intervals were too wide to draw any conclusion. Notably, the direction of the effects was even in the opposite direction of our initial hypothesis that BCG vaccination offers better protection in the morning. It is important mentioning that the initial trial was not powered nor designed to analyze the effect of circadian rhythm. The most likely explanation for our findings is that BCG vaccination simply has no effect on the protection against RTIs and SARS-CoV-2 infections in this study.

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TABLE 2A Effect of morning and afternoon BCG vaccination compared to placebo in the first six months after vaccination.

Subgroup	Intervention	Follow-up time (years)	Events (n)	Cumulative incidence	SDHR	IHR
SARS-CoV-2 infections						
Morning	Placebo	168.5	5	0.014 (0.005-0.031)	[ref]	–
	BCG	171.3	12	0.034 (0.018-0.056)	2.394 (0.856-6.696)	8.966 (1.366-58.836)
Afternoon	Placebo	149.7	7	0.023 (0.010-0.045)	[ref]	–
	BCG	153.4	2	0.006 (0.001-0.021)	0.284 (0.055-1.480)	[ref]
Clinically relevant RTIs						
Morning	Placebo	173.9	3	0.009 (0.002-0.023)	[ref]	–
	BCG	174.6	4	0.011 (0.004-0.027)	1.510 (0.367-6.207)	0.351 (0.025-4.978)
Afternoon	Placebo	150.4	1	0.003 (0.000-0.017)	[ref]	–
	BCG	155.2	4	0.013 (0.004-0.030)	4.916 (0.569-42.461)	[ref]

BCG, Bacillus Calmette-Guérin; RTI, Respiratory tract infection; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; CI, confidence interval; SDHR, subdistribution hazard ratio; IHR, interaction hazard ratio.
Cumulative incidences and hazard ratios are reported with 95% confidence interval.

TABLE 2B Effect of morning and afternoon BCG vaccination compared to placebo in the period from six months to 12 months after vaccination.

Subgroup	Intervention	Follow-up time (years)	Events (n)	Cumulative incidence	SDHR	IHR
Clinically relevant RTIs						
Morning	Placebo	171.0	4	0.011 (0.004-0.028)	[ref]	–
	BCG	172.4	7	0.020 (0.009-0.039)	1.748 (0.524-5.828)	2.260 (0.376-13.571)
Afternoon	Placebo	144.7	5	0.017 (0.006-0.037)	[ref]	–
	BCG	150.8	4	0.013 (0.004-0.031)	0.805 (0.199-3.249)	[ref]
SARS-CoV-2 infections						
Morning	Placebo	165.8	16	0.046 (0.027-0.072)	[ref]	–
	BCG	167.1	12	0.035 (0.019-0.058)	0.745 (0.437-1.600)	0.530 (0.149-1.881)
Afternoon	Placebo	141.9	6	0.021 (0.009-0.042)	[ref]	–
	BCG	149.5	9	0.029 (0.014-0.052)	1.460 (0.505-4.223)	[ref]

BCG, Bacillus Calmette-Guérin; RTI, Respiratory tract infection; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; CI, confidence interval; SDHR, subdistribution hazard ratio; IHR, interaction hazard ratio.
Cumulative incidences and hazard ratios are reported with 95% confidence interval.

TABLE 2C Effect of morning and afternoon BCG vaccination compared to placebo in the full 12 months follow-up.

Subgroup	Intervention	Follow-up time (years)	Events (n)	Cumulative incidence	SDHR	IHR
Clinically relevant RTIs						
Morning	Placebo	348.6	7	0.020 (0.009-0.039)	[ref]	–
	BCG	351.2	11	0.031 (0.016-0.053)	1.616 (0.646-4.046)	1.218 (0.295-5.037)
Afternoon	Placebo	299.4	6	0.020 (0.008-0.041)	[ref]	–
	BCG	310.4	8	0.026 (0.012-0.048)	1.417 (0.468-4.296)	[ref]
SARS-CoV-2 infections						
Morning	Placebo	344.0	21	0.060 (0.038-0.088)	[ref]	–
	BCG	309.8	24	0.067 (0.044-0.097)	1.160 (0.641-2.098)	1.422 (0.527-3.832)

(Continued)

TABLE 2C Continued

Subgroup	Intervention	Follow-up time (years)	Events (n)	Cumulative incidence	SDHR	IHR
Afternoon	Placebo	296.4	13	0.043 (0.024-0.071)	[ref]	–
	BCG	309.8	11	0.035 (0.019-0.060)	0.837 (0.368-1.902)	[ref]

BCG, Bacillus Calmette-Guérin; RTI, Respiratory tract infection; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; CI, confidence interval; SDHR, subdistribution hazard ratio; IHR, interaction hazard ratio.
Cumulative incidences and hazard ratios are reported with 95% confidence interval.

A protective effect has previously been demonstrated in several smaller studies (14, 20–22), but pathophysiological differences between SARS-CoV-2 infections and other RTIs (such as influenza) may account for these differential effects of BCG (23). Another explanation why our results contradict those from de Bree et al. may be that in their experiments the time period between vaccination and blood collection was just three months, and that the morning group was vaccinated between 8:00 and 9:00 and the afternoon group at 18:00 (7).

We have chosen for greater intervals of vaccination in this study to give a better reflection of common practice of vaccination and to guarantee a certain number of events per group. Furthermore, the median age of the individuals in the study from de Bree et al. was 26 years, while in contrast, the median age in our cohort was 67. Ageing has been associated with both changes in circadian-influenced biological processes and innate immune responses (20). Lastly, de Bree et al. observed the trained immunity effects upon stimulation with bacterial stimuli, whereas SARS-CoV-2 and the most common RTIs are viral infections. One could also speculate whether the difference in cytokine response between the morning and the afternoon is too small to affect clinical outcomes. *In-vitro* effects often do not correspond with clinical significance and generally need to be interpreted with care (21).

A protective effect has previously been demonstrated in several (smaller) studies (14, 20–22), but pathophysiological differences between SARS-CoV-2 infections and other RTIs (such as influenza) may account for these differential effects of BCG (23). Our study is the first to evaluate the clinical impact of circadian rhythm on BCG effects in humans. Research on the impact of the circadian clock on other vaccines is also limited to experimental data and focuses on adaptive immune responses. Clinical trials are generally lacking. However, studies on influenza, hepatitis A and SARS-CoV-2 found either no effect of circadian rhythm on antibody titers or mostly better efficacy when administrating vaccines in the morning (22–24).

On the cellular level, virtually all cell lines of the innate and the adaptive immune system have been associated with circadian variations (25). This is also the case for myeloid cells, natural killer cells and innate lymphoid cells, all of which are involved in the induction of trained immunity. The same effects have been demonstrated for metabolic processes and the expression of pathogen recognition receptors, such as TLR-9 (26, 27). Both mechanisms also play a role in the induction of innate immune responses and have been identified to be under control of a molecular clock. It has been suggested that fluctuating cytokine and cortisol levels may be the underlying mechanism for the effect of circadian rhythm on vaccine immunogenicity, as they are both known to be potent regulators of immune functions (28, 29). Taken together, these formed the basis of our hypothesis that the circadian clock also affects trained immunity (7).

Although we did not detect that BCG vaccination, either in the morning or the afternoon, offers beter protection than placebo against RTIs or COVID-19, we believe that further unravelling the mechanisms of clock-controlled immunomodulation has the potential to enhance both the immunogenicity of vaccines and the efficacy of immunotherapies. Therefore, further research is needed to identify potential clinical implications of the previously found *in vitro* effects on immune response.

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 Arnhem-Nijmegen Ethical Committee (# NL73430.091.20). The patients/participants provided their written informed consent to participate in this study.

Author contributions

KF and ET wrote the manuscript. KF, ET, and CvW analyzed the data and performed the statistical analysis. ET and SM conducted the study. MB, RvC, JtO, CvW, MN, JvdM, and JH conceptualized the study and participated in writing the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MN is scientific founder of TTxD and Lemba has received scientific support from GSK, Ono Pharma, and TTxD.

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.

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