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COVID-19 booster vaccination: Increasing immunity against life-threatening infection

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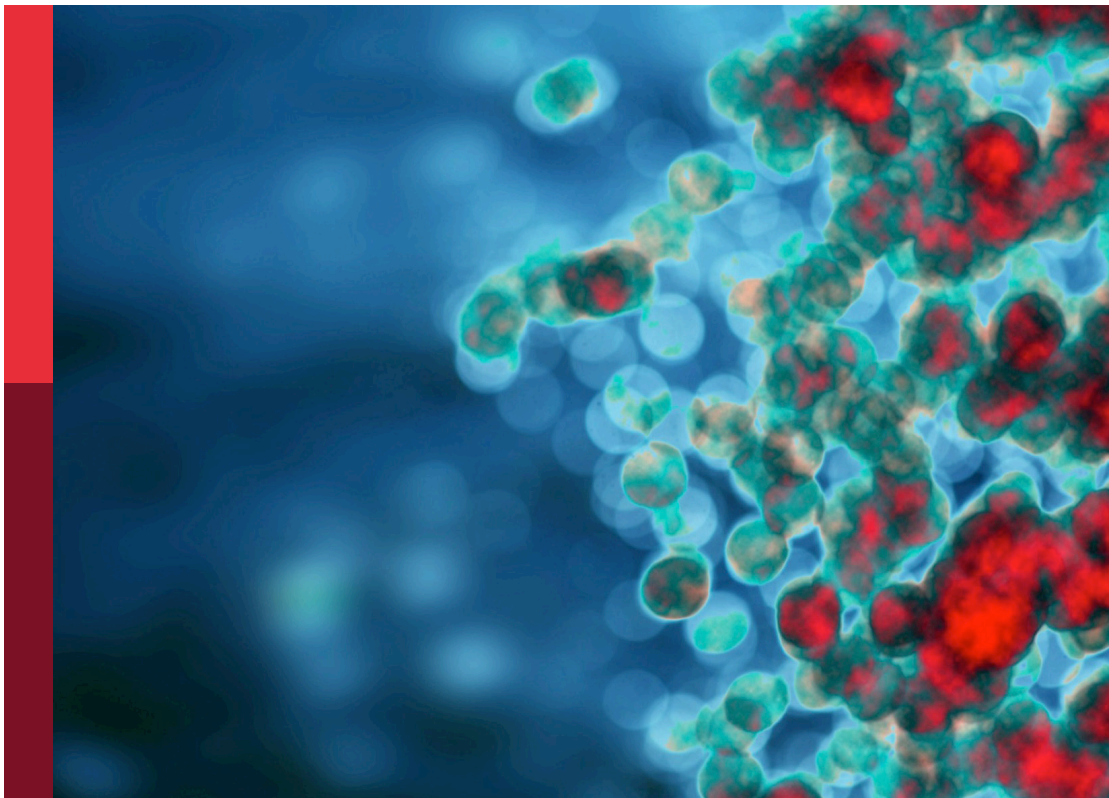

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COVID-19 booster vaccination: Increasing immunity against life-threatening infection

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Editorial: COVID-19 booster vaccination: increasing immunity against life-threatening infection

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Editorial on the Research Topic
COVID-19 booster vaccination: increasing immunity against life-threatening infection

The emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has caused the global transmission of Coronavirus disease 2019 (COVID-19) and continues to evolve. COVID-19 vaccines were rapidly developed within a year of the disease's emergence. In the early stages of the pandemic, COVID-19 vaccines were designed based on the related ancestral (wild-type) strain and were typically administered in two shots for full priming vaccination. They proved effective against severe infections but did not provide complete protection against symptomatic infections (1). Breakthrough infections commonly occur even after a full priming vaccination (1, 2). The main reasons for this are waning immunity (3) and the emergence of newly evolved variants of concern (VOCs), such as Delta (B.1.617.2) and Omicron (B.1.1.529), which have higher contagiousness and altered amino acid sequences that evade immunity (2–4). However, vaccines still offer protection against life-threatening infections and reduce the likelihood of long-term sequelae (long COVID-19) (1). Furthermore, high-risk groups, such as older adults (5), those with underlying medical conditions (6), obese individuals (7), immunocompromised individuals, solid organ transplant recipients (8), and oncology patients, including the recipients of immunotherapy or chemotherapy (9, 10), are at greater risk of life-threatening infection or mortality due to insufficient immune response compared to healthy individuals. Given the waning immunity and circulation of emerging VOCs, and the vulnerability of high-risk groups, it is evident that full priming vaccination may not provide sufficient protection against the widespread global spread of the disease.

During the crisis and with limited resources, booster vaccinations emerged as a potential strategy to tackle VOCs and served as a "makeshift" approach when reliable drugs and vaccines were not readily available. At the time, neither the second generation (e.g., bivalent) nor beyond (e.g., XBB monovalent) had been introduced. Boosters had the potential to significantly enhance immunity through an anamnestic response, addressing

the issue of waning immunity, restoring reduced effectiveness, and prolonging high levels of immunity. This approach aimed to reduce the viral load in breakthrough infections (2, 11), consequently reducing the likelihood of disease transmission. Immunity levels were closely associated with vaccine efficacy/effectiveness, particularly in protecting against life-threatening infections (12), making the maintenance of high immunity crucial during the crisis. Studies indicated that booster vaccinations reduced the rate of COVID-19 cases, severe illness, and mortality compared to those who received only the initial vaccination (13). Moreover, it was observed that the use of the inactivated platform with an "old-fashioned" adjuvant (aluminum-based) resulted in lower antibody levels compared to other platforms (14). In response, the adenoviral vector platform was considered a potential booster, demonstrating high efficacy against the Delta variant (15). Similarly, mRNA or protein subunit platforms have shown potential for enhancing immunity (16, 17), even in fractional dose vaccination (18). While the increased immunity from the ancestral strain vaccine remained effective against the Omicron variant, it was notably less effective than against the ancestral strain (19). Conversely, booster vaccine effectiveness was anticipated to be higher and more durable compared to relying solely on full priming vaccination, maintaining efficacy against VOCs (20). In particular, heterologous adenoviral vectors following mRNA booster vaccines have shown promise in reducing severe disease, even in immunocompromised and high-risk individuals and older adults (13, 21). Additionally, using the same platform (mRNA) with different vaccines provided better protection against symptomatic and severe infections than using the same vaccine (22). Several countries have endorsed booster vaccinations as an effective strategy to reinforce and sustain immunity against COVID-19. However, limited data are available to comprehensively explore the outcomes of COVID-19 booster vaccinations, including adverse events following immunization (AEFI). Therefore, this Research Topic aims to focus on the effects of COVID-19 booster vaccinations by examining evidence from animal models, clinical trials, real-world observations, and systematic reviews.

We have received a total of 41 manuscripts relevant to this Research Topic, of which 28 articles met the eligibility criteria for publication in three sections of Frontiers in Public Health/Infectious Diseases: Epidemiology and Prevention, Frontiers in Medicine/Infectious Diseases: Pathogenesis and Therapy, and Frontiers in Immunology/Vaccines and Molecular Therapeutics. These articles are of various types and include 21 original articles, 4 systematic reviews, 2 brief research reports, and 1 clinical trial article.

In preclinical studies using mouse models, researchers explored potential SARS-CoV-2 vaccine candidates. Qin et al. developed a universal mRNA vaccine platform containing Delta or Omicron variant spikes or a multi-T cell epitope (MTE). Vaccines with only the MTE protected mice from lethal Delta variant challenges. Combining spike-specific variants and the MTE showed promise as a universal SARS-CoV-2 vaccine. Zhou Y. et al. designed a live attenuated *Pseudomonas aeruginosa* bacterial vector expressing the SARS-CoV-2 RBD protein through a bacterial type III secretion system (injectisome) with candidate plasmids of wild-type, Delta, or Omicron BA.1. The complex bacterial nanomachine stimulated mucosal immunity via the intranasal route of administration, and vaccine safety was evaluated based on lung pathology. The results showed that the serum elicited good antibody and T-cell responses. Li et al. evaluated various adenoviral vector and/or mRNA platforms as prime-boost strategies in the intramuscular and/or intranasal route. The vectors were encoded with the wild-type or Beta variant spike gene; mRNAs were encoded with the wild-type or Omicron variant spike gene. This study assessed cellular immune responses, neutralizing different variants and subvariants. Interestingly, primary vaccination with an intranasal adenoviral vector encoding the Beta spike gene, followed by intramuscular mRNA encoding the Omicron spike gene, induced a broader spectrum and stronger IgA and neutralized against variants. This suggests that heterologous strategies between platforms, routes, and antigens could generate broad-spectrum immunity and enhance neutralizing capacity.

A brief research report from Wu et al. reported on the safety and immunogenicity of a full priming inactivated vaccine compared with a homologous prime-boost protein subunit vaccine in chronic hepatitis B patients. The results showed a safe and high seropositive rate. The seropositive rate was lower in patients with cirrhosis than in patients without cirrhosis. Another brief research report by Perico et al. assessed the humoral and cellular responses in healthcare workers, both naïve and convalescent subjects. This study was a long-term follow-up for 12 months, 9 months after the full priming, and followed by 3 months after the booster. Hybrid immunity resulted in significantly higher antibody levels than naïve individuals. The humoral response levels are linked to specific memory B cells. The study suggested that boosters may enhance the immune response, particularly in maintaining antibodies, especially in naïve subjects. The cohort studies focus on healthy participants, mainly on immunogenicity. Leung et al. conducted a clinical trial comparing humoral and cellular immunity, including tests specific for the Omicron variant of homologous inactivated prime-boost vaccination in healthy adolescents and healthy adults. The reactogenicity was mild. Immunogenicity outcomes in adolescents were higher than in adults, with neutralizing and cellular immunity potentially protective against the Omicron BA.1 subvariant. Hyun et al. compared the immunogenicity of Ad26.COV2.S or mRNA boosters in full priming of Ad26.COV2.S (single-shot). This study showed results for both humoral and cellular immunity. Neutralizing antibodies were enormously increased against wild-type but were lower in Omicron BA.5, and Omicron BA.1 elicited the lowest level compared with the others. IgG anti-RBD and specific interferon-γ were significantly increased after vaccination, except that the Ad26.COV2.S booster group did not increase the interferon-γ. This study suggested that the heterologous prime-boost adenoviral vector and mRNA platforms could increase immunity more than the homologous prime-boost adenoviral vector platform. Additional boosters may be helpful to increase immunity against the Omicron sub-variants. Lozano-Rodríguez et al. evaluated the overall immunological responses in naïve and convalescent participants vaccinated with heterologous prime-boost mRNAs (BNT162b2 and mRNA-1273) vaccination with long-term follow-up over 1 year. Humoral responses increased substantially but waned after 6 months, while T-cell responses remained stable. The

immune response in a convalescent group was higher than in naïve participants. However, immunity against the Omicron BA.1 sub-variant was lower than the wild-type in both groups. Huang et al. investigated T-cell responses from homologous inactivated prime-boost vaccination. The booster enhances and broadens T-cell responses against SARS-CoV-2 spike and non-spike antigens from wild-type, Delta, and Omicron BA.1. Hosseinian et al. assessed the antibody profile of participants who received mRNA boosters at 6-month follow-up. Neutralizing antibodies have reduced activity against Delta and substantially reduced activity against Omicron variants, particularly the BA.1 sub-variant, compared to the BA.2 sub-variant. Booster antibody levels remained significantly higher than pre-booster, even with waning antibody levels at 6 months. Severe systemic side effects were linked to higher antibody levels and could persist for several months. Antigen microarray characterization revealed little cross-reactivity between SARS-CoV-2 and other coronaviruses or influenza viruses. The study suggested that breakthrough infections may be driven by specific antigens from new variants rather than waning immunity. Dou et al. performed a pilot-scale single-cell sequencing analysis of the inactivated vaccine recipients. The inactivated vaccine promoted T cell proliferation, T cell receptor clone amplification, and diversity. This finding showed that the booster significantly enhanced $CD8^+$ mucosal-associated invariant T (MAIT) cell proliferation and differentiation, and KLRD1 gene expression in NK cells was significantly higher. This study suggests that an inactivated vaccine platform could stimulate an early adaptive T cell response against the virus.

Four cohort studies focus on the immunocompromised host. Collectively, these studies underscore the impact of booster strategies on enhancing immunity in immunocompromised individuals and highlight the complexities and challenges of achieving adequate responses in this population. Wang et al. conducted a clinical trial comparing the effects of shorter (3 months) and longer (5 months) intervals between the second dose and booster in people living with HIV. The longer-interval group had higher neutralizing antibody and seropositivity rates than the shorter-interval group. Interestingly, the longer-interval group showed prolonged immunity in both CD4 count subgroups (<200 and ≥ 200 cells μL^{-1}) and had a higher seropositivity rate than the shorter-interval group at 6-month follow-up. However, the neutralizing response to the Omicron BA.5.2 subvariant was inadequate in all groups, including the healthy control. The longer interval between doses was shown to be useful for boosting immunity. On the contrary, the interval period may increase the likelihood of severe infection if individuals become infected due to waning immunity, especially in immunocompromised subjects. Barkhordar et al. conducted a clinical trial assessing the homologous prime-boost vaccination of the SARS-CoV-2 RBD-Tetanus toxoid conjugated (RBD-TT-conjugated) vaccine in acute leukemia with allogeneic hematopoietic stem cell transplantation. AEFIs were mostly local reactions, with no serious AEFIs reported. The booster could substantially increase immunity compared to the pre-booster. The other two cohort publications also focused on immunocompromised patients. Gaete-Argel et al. compared mRNA booster responses in solid organ transplant recipients receiving full priming inactivated or mRNA vaccines. Boosters, whether homologous or heterologous, increased seropositivity rates against SARS-CoV-2 in wild-type and Omicron BA.1 recipients. However, some recipients did not respond to the booster, which is a common problem in the immunocompromised population. Bulnes-Ramos et al. reported that booster vaccination significantly increased immune responses in kidney transplant recipients (KTR), and these responses were positively correlated with thymosin-α1 levels.

The cross-sectional studies had different types of participants. Hossain et al. compared various types of vaccination among Bangladeshi migrant workers. The booster vaccination group, whether naïve or convalescent, exhibited significantly higher antibody levels compared to the non-booster group. As in other studies, the mRNA platform had higher antibody levels than other platforms. Al-Rifai et al. evaluated humoral and cellular immunity in various COVID-19 vaccine types and showed that boosters enhanced immunity compared to full priming vaccination alone. During the Omicron predominant wave, Yang et al. used data from a large hospital in Shanghai, China, during the Omicron BA.2 sub-variant wave and revealed that viral RNA was rapidly cleared in inactivated vaccine recipients, especially in booster recipients compared with unvaccinated individuals. The studies focus on immunocompromised host participants. Pérez-Flores et al. suggested that using mTOR inhibitors may enhance the capacity of the immune system to respond to the booster in kidney transplant recipients. Additional clinical trials are recommended to confirm this concept. Feng et al. focused on inflammatory bowel disease (IBD) patients in Shanghai, China, during the Omicron BA.2 and BA.2.2 sub-variants wave. The vaccination rate, including booster doses, in IBD patients was lower than in asymptomatic carriers and healthy individuals, with one-third of the unvaccinated citing fear of IBD exacerbation as the reason for refusing vaccination. However, reactogenicity was not significantly different between IBD and healthy individuals. Xu Y. et al. delineated vaccination status, reactogenicity, and perceptions among Chinese breast cancer survivors [Three studies focus on immunocompromised]. Unvaccinated individuals feared disease progression or interference with treatment, while vaccinated individuals were primarily concerned about infection or workplace requirements. Side effects were acceptable in the vaccinated group, suggesting the need to promote vaccination and raise awareness of vaccine safety among cancer patients to increase vaccination rates.

There are several population-based studies in this Research Topic. Montes-González et al. assessed nationwide surveillance in Mexico, focusing on the hybrid immunity against re-infection and severe disease during the Omicron-predominant circulation wave. This study suggested that hybrid immunity could significantly reduce the risk of re-infection and severe infection compared to unvaccinated convalescents. Moreover, the heterologous booster could reduce the risk of re-infection and severe infection compared to the homologous booster strategy. Zhou C. et al. comprehensively assessed the case fatality rate among booster recipients in 32 countries with multi-dimensional explanatory variables. Boosters were identified as a crucial factor in reducing the age-adjusted case fatality rate. The study also identified different risk factors at the country level. Matveeva and Shabalina analyzed data from 29

European countries and found that slower vaccination rates, including delayed booster administration, were associated with higher excess fatalities from COVID-19. Vaccine protection was highest during the Delta predominant circulation wave and decreased during the Omicron BA.1/BA.2 sub-variant predominant circulation wave. However, additional booster doses were found to be beneficial in preventing excess deaths during the Omicron wave.

Four systematic reviews are also included in this Research Topic, two of which focus on immunocompromised hosts. Sun et al. reviewed vaccine response and safety in cancer patients, indicating that vaccines are generally safe and well-tolerated, with mild reactogenicity. However, the seroconversion rate after the second dose is insufficient for all participants. Booster doses are critical to increasing seropositivity immunity, given the higher risk of severe infections in cancer patients. Martinelli et al. reviewed the fourth dose of COVID-19 vaccination (second booster) in immunocompromised recipients, including oncology patients, organ transplant recipients, CAR-T cell therapy, autoimmune disorders, and HIV infection subjects. This review focused on the humoral response, efficacy, and safety. The booster enhanced the humoral immune response. No serious AEFIs were reported. One study focused on older adults, with Xu K. et al. analyzing data from randomized control trials in this population (aged $\geq$60 years). Vaccination significantly reduced hospitalization, including ICU admission, and death in older adults. Booster doses notably elevated the geometric mean compared to full priming or partial vaccinations. Local reactions occurred more often than systemic reactions, and serious AEFIs were rare. The final study focused on adults: Xu J. et al. analyzed data from cohorts or randomized control trials that focused on booster vaccination. The booster, either homologous or heterologous, could enhance both humoral and cellular immune responses. Booster doses significantly reduced the risk of infection, including in severe conditions, in addition to ICU admission and death.

In summary, preclinical studies of novel vaccine candidates are promising. Multi-T cell epitope (MTE) stimulation could potentially prevent life-threatening disease, regardless of the antibodies presented. The live attenuated bacterial vector could be a possible future route to stimulate mucosal immunity, the first barrier before the viral invasion of the host, through needleless administration. Heterologous strategies with different antigens from VOCs could stimulate a broader spectrum against the virus. Most articles focused on immunogenicity from cross-sectional or longitudinal studies, which are easier to conduct and measure with straightforward outcomes. Continuous outcomes from immunological assessments require smaller sample sizes than dichotomous outcomes. Such immunological outcomes are likely to provide useful data for decision-making. All participants benefited from booster vaccination, which could substantially increase immunity and cross-reactivity to the VOCs. However, there remains controversy surrounding immunological assessments, particularly concerning "immunobridging," especially with new variants that reduce vaccine efficacy by evading immunity. The systematic reviews included here prove that booster vaccination enhances immunity and could protect booster vaccination recipients from life-threatening infections and fatalities more than non-booster vaccination recipients. Furthermore, big data analysis from European countries revealed that delaying booster vaccination was linked to higher excess deaths.

In conclusion, the current data support the benefits of booster vaccination over non-vaccination, particularly during a crisis. However, it's important to note that no intervention, including fully FDA-approved drugs or vaccines, is entirely risk-free. Any vaccination or treatment should be discussed with a healthcare professional to weigh the associated risks and benefits.

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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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COVID-19 vaccine response and safety in patients with cancer: An overview of systematic reviews

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Background: To date, the COVID-19 pandemic does not appear to be overcome with new variants continuously emerging. The vaccination against COVID-19 has been the trend, but there are multiple systematic reviews on COVID-19 vaccines in patients with cancer, resulting in redundant and sub-optimal systematic reviews. There are still some doubts about efficacy and safety of the COVID-19 vaccine in cancer patients.

Purpose: To identify, summarize and synthesize the available evidence of systematic reviews on response and COVID-19 vaccine safety in patients with cancer.

Methods: Multiple databases were searched from their inception to May 1, 2022 to fetch the relevant articles. Study quality was assessed by AMSTAR2. The protocol of this study was registered on PROSPERO (CRD42022327931).

Results: A total of 18 articles were finally included. The seroconversion rates after first dose were ranged from 37.30–54.20% in all cancers, 49.60–62.00% in solid cancers and 33.30–56.00% in hematological malignancies. The seroconversion rates after second dose were ranged from 65.30–87.70% in all cancers, 91.60–96.00% in solid cancers and 58.00–72.60% in hematological malignancies. Cancer types and types of therapy could influence vaccine response. COVID-19 vaccines were safe and well–tolerated.

Conclusions: This study suggests COVID-19 vaccine response is significantly lower in cancer patients. Number of received doses, cancer types and treatment strategies could influence response of COVID-19 vaccine in cancer patients. COVID-19 vaccines are safe and well–tolerated. Considering the emergence of several new variants of SARS-CoV-2 with potential influence on ongoing vaccination programs, there is a need for booster doses to increase the effectiveness of COVID-19 vaccines.

Systematic review registration: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42022327931, identifier CRD42022327931.

KEYWORDS

COVID-19, vaccine, cancer, response, safety

Introduction

Coronavirus disease 2019 (COVID-19), which is caused by SARS-CoV-2, has caused significant discomfort and death worldwide (1, 2). Globally, by 21 July 2022, more than 560 million COVID-19 cases were confirmed, and 6.37 million deaths were reported worldwide (3). There is substantial evidence that cancer patients are placed in a vulnerable state and the risk of death from COVID-19 is higher (4–6). To date, the COVID-19 pandemic does not appear to be overcome with the continuously emerging new variants (7, 8) and the burden of COVID-19 related morbidity and mortality in patients with cancer is still significant.

Despite established supportive therapies and new approved antiviral drugs, the COVID-19 vaccines emerged as the primary strategy fighting against COVID-19 pandemic. COVID-19 vaccines were rapidly developed with 172 vaccines in clinical development and 199 in pre-clinical stage at the time of writing (9). Several COVID-19 vaccines such as mRNA-1273 (Moderna) and BNT162b2 (Pfizer-BioNTech), displayed efficacy and safety in the large phase II and III clinical trials and obtained the emergency approval by the regulatory agencies (10, 11). As of April 8, 2022, several vaccines against COVID-19 assessed by WHO have met necessary criteria for efficacy and safety. There are some differences among these COVID-19 vaccines. At first, the vaccine types are different. ChAdOx1-S [recombinant] vaccine (AstraZeneca), Ad26.COV2.S vaccine (Johnson & Johnson) and Ad5-nCoV-S [recombinant] vaccine (CanSinoBio) are developed based on viral vector. NVX-CoV2373 (Novavax) is based on protein subunit. Vaccines based on mRNA include mRNA-1273 (Moderna) and BNT162b2 (Pfizer-BioNTech). COVID-19 vaccine BIBP (Sinopharm), CoronaVac (Sinovac) and BBV152 COVAXIN vaccine (Bharat Biotech) are based on inactivated viruses. Secondly, the recommended dosage and interval are various. In addition to CanSinoBio, Johnson & Johnson and Pfizer-BioNTech vaccines, the recommended dosage of other vaccines is two doses. The WHO Strategic Advisory Group of Experts on Immunization (SAGE) recommends the use of CanSinoBio vaccine as a single dose. A single dose regimen of Johnson & Johnson vaccine remains an acceptable option. However, WHO recommends all efforts should be taken to provide two doses of this vaccine. For persons aged 5 years and above, the recommended dosage of Pfizer-BioNTech vaccine is two doses, while for children aged 6 months to 4 years, the recommended schedule is three doses. The interval of majority vaccines between the first and second dose is 4–8 weeks. Furthermore, age group for vaccination is different. Most vaccines are authorized for use for individuals aged 18 years and above except for Moderna and Pfizer-BioNTech for those aged 6 months and above, Novavax for those aged 12 years and above. The detailed information about these COVID-19 vaccines were shown in Supplementary Table 1. Almost all COVID-19 vaccines have shown remarkable efficacy and safety in the general population and have decreased COVID-19 related-mortality and morbidity worldwide. To date, more than 12 billion vaccine doses have been administered (Figure 1) (12). These vulnerable populations should be prioritized for the vaccination against SARS-CoV-2 due to the higher rate of morbidity and mortality of COVID-19 in patients with cancer. However, the available data are limited among cancer patients because of their ineligibility in most clinical trials. Although vaccination against SARS-CoV-2 is recommended for cancer patients as long as no contraindications to any component of COVID-19 vaccines. Worryingly, the results of COVID-19 vaccination are considered insufficient in patients with cancer, especially when patients with hematological malignancies (HM) on anti-CD20 therapy (13).

It is significant to understand the efficacy and safety of COVID-19 vaccines in cancer patients because of the lack of effective treatments for COVID-19. Currently, the literature encompasses multiple systematic reviews on COVID-19 vaccines in patients with cancer, resulting in redundant and sub-optimal systematic reviews. Better understanding of overall efficacy and safety of COVID-19 vaccines in patients with cancer could protect the vulnerable populations. This overview of systematic reviews and meta-analyses aims to evaluate the current available evidence on the efficacy and safety of COVID-19 vaccines in cancer patients.

Methods

This study was conducted in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist (14). A protocol was registered with PROSPERO (CRD42022327931).

Search strategy

Multiple databases were searched from their inception to May 1, 2022 by two reviewers independently: PubMed, Cochrane Library, Web of Science, EMBASE, and CNKI databases. Keywords for searching included "COVID", "COVID-19", "SARS-CoV-2", "vaccine", "vaccination", "cancer", "neoplasms", etc., The detailed search strategy was listed in Supplementary Table 2. There was no restriction regarding the publication language.

Eligibility criteria

We aimed to identify all systematic reviews with or without meta-analyses that summarized and reported the COVID-19 vaccine response or safety in cancer patients. Only published systematic reviews with or without meta-analyses, which clearly

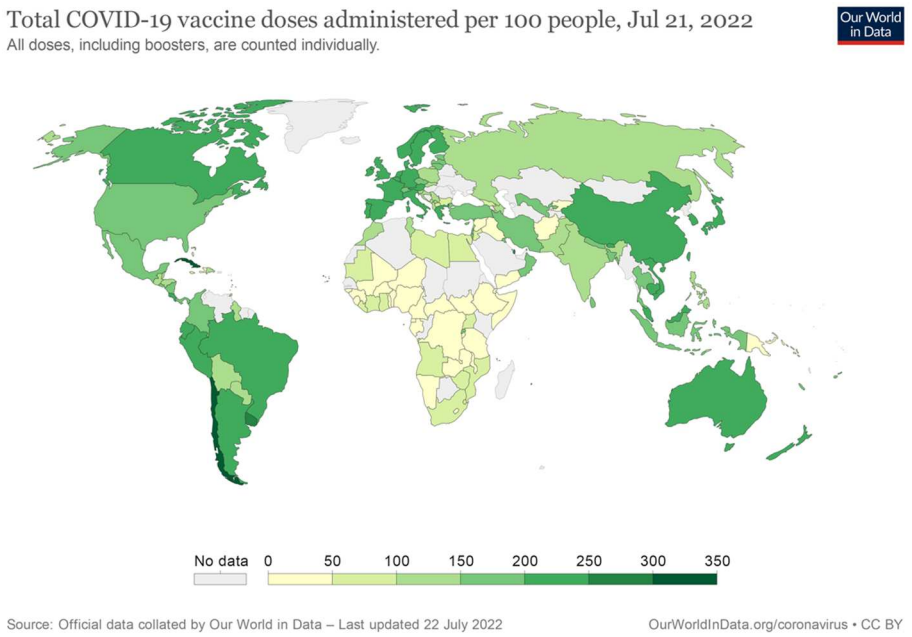

FIGURE 1
The distribution of total number of people who have been fully vaccinated against COVID-19.

identified the response of COVID-19 vaccines in patients with cancer, as compared to a non-cancer group (if any), and which investigated any adverse events (AEs) of COVID-19 vaccines in cancer patients were included. Systematic reviews and meta-analyses were eligible. The exclusion criteria were studies with different study designs such as case reports, editorial letters, narrative review articles, randomized controlled trials (RCTs), observational studies, opinion papers and animal studies.

Study selection and data extraction

According to the PRISMA guidelines, data extraction was performed and independently verified by two authors. Extraction data included: first author; publication year; search details; number of included studies; assessment of risk of bias and/or study quality; outcome investigated; COVID-19 vaccine types; cancer diagnoses; results. Discrepancies were resolved through discussion.

Study quality assessment

The methodological quality of included systematic reviews was evaluated by the Assessing the Methodological Quality of Systematic Reviews (AMSTAR 2) which consists of 16 items (15). Based on the weaknesses in critical domains, the AMSTAR 2 assessment could generate an overall quality rating. According to the quality rating confidence levels, quality of systematic reviews was rated as "high", "moderate", "low" and even "critically low". Two authors independently evaluated all systematic reviews to ensure interrater reliability. Disagreements among authors were solved by consensus with involvement of another author. The interrater reliability of quality assessment was assessed by Kappa coefficient.

Results

Search results

A total of 5,549 records were identified throughout database search. Records were 4,298 after the removal of the duplicate. By title/abstract screening, 188 articles were identified for full-text view. Finally, 18 systematic reviews (15 with meta-analyses and 3 without meta-analyses) were identified which fulfilled the eligibility criteria (16–33). The study flow was depicted according to the PRISMA guidelines in Figure 2.

Characteristics of the included studies

The details of the included studies were summarized in Supplementary Table 3. These systematic reviews were published between 2021 and 2022. The total number of original studies included in these systematic reviews ranged from 5 to 64. All systematic reviews included only observational studies,

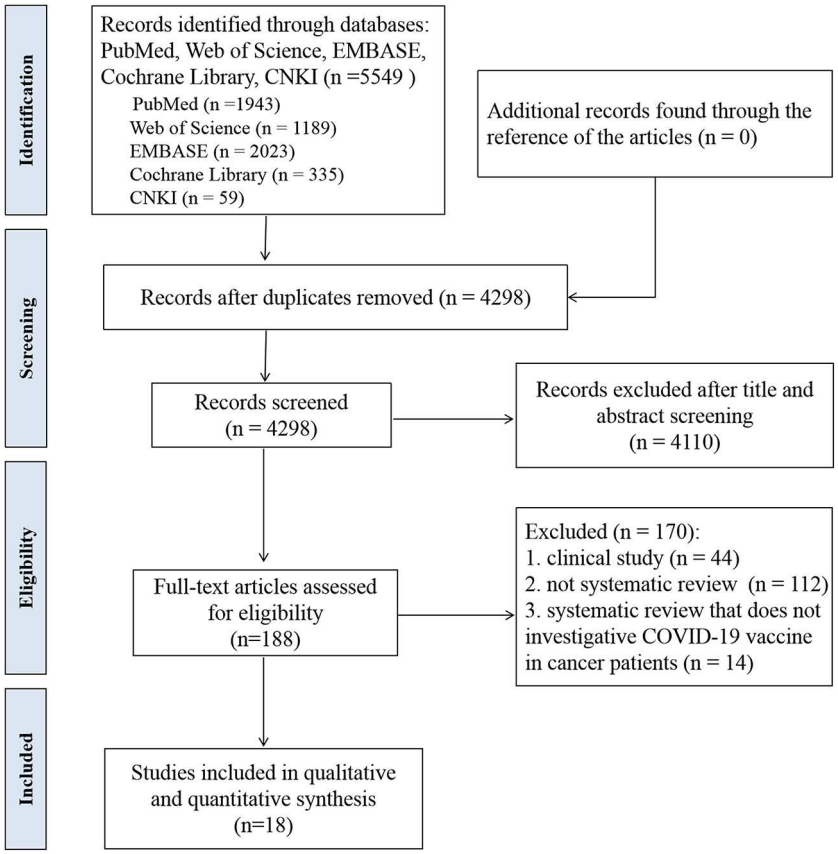

FIGURE 2
PRISMA flowchart of the identification and inclusion of systematic reviews.

without RCTs. The majority of systematic reviews (15/18, 83.30%) included a control group and three systematic reviews included only patients with cancer.

Quality assessment of included studies

Methodological quality of 18 eligible systematic reviews was evaluated by the AMSTAR 2. The results of AMSTAR 2 were summarized in Figure 3. The confidence levels of results of the included systematic reviews were moderate in three, low in four and critically low in eleven. The result of interrater reliability suggested good results (percentage agreement 90.28% and Cohens'k = 0.84). None of the systematic reviews met the criteria of Item 3 and Item 10. The failure to take these two items into account, no systematic review was rated with high confidence. The study selection and data extraction of systematic reviews were not carried out independently by two reviewers, resulting in one systematic review being rated with moderate confidence. Another systematic review was also rated as moderate with no discussion and explanation of the heterogeneity in the results and lack of study selection in duplicate. The majority of systematic reviews were lack of a study protocol which should be established prior to conduct of the systematic review. Some studies didn't take the risk of bias (RoB) into account. And some studies did not adequately investigate publication bias (small study bias) and did not discuss its possible impact on review results.

COVID-19 vaccine response

Overall, COVID-19 vaccine response was significantly lower in cancer patients. The number of received doses, cancer types and types of therapy could influence the response of COVID-19 vaccine in cancer patients.

Response after the first dose of COVID-19 vaccine

There were six systematic reviews with meta-analyses that assessed the seroconversion rate after first dose of COVID-19

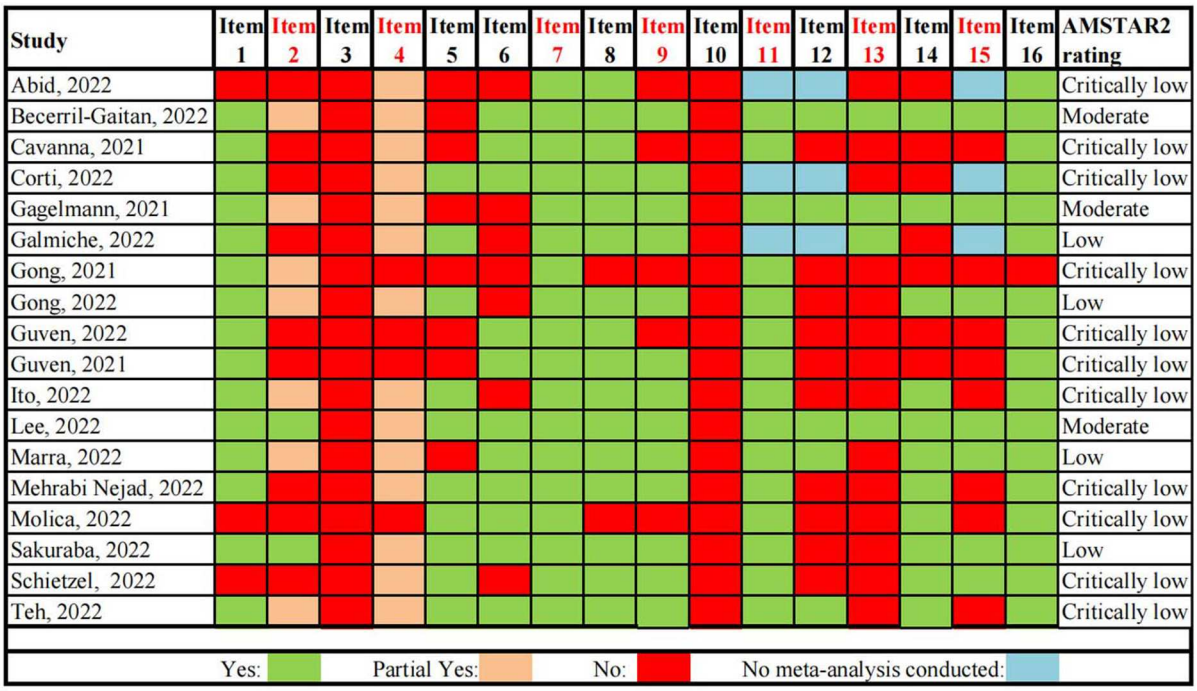

FIGURE 3
Quality assessment of included systematic reviews based on AMSTAR-2. Critical domains are highlighted in red.

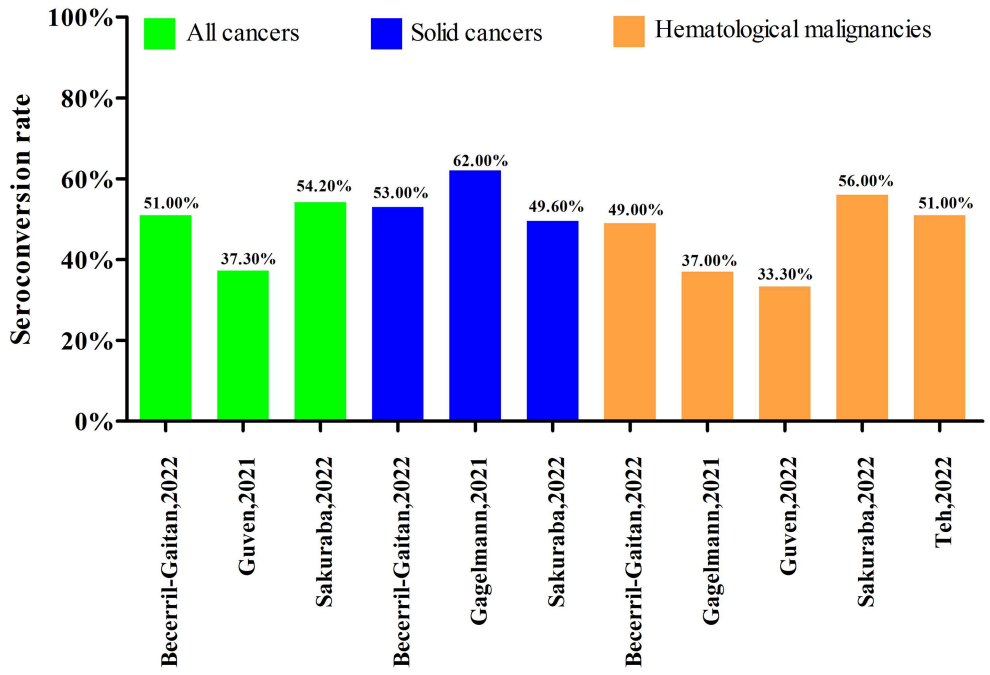

FIGURE 4
Seroconversion rate after the first dose of COVID-19 vaccine.

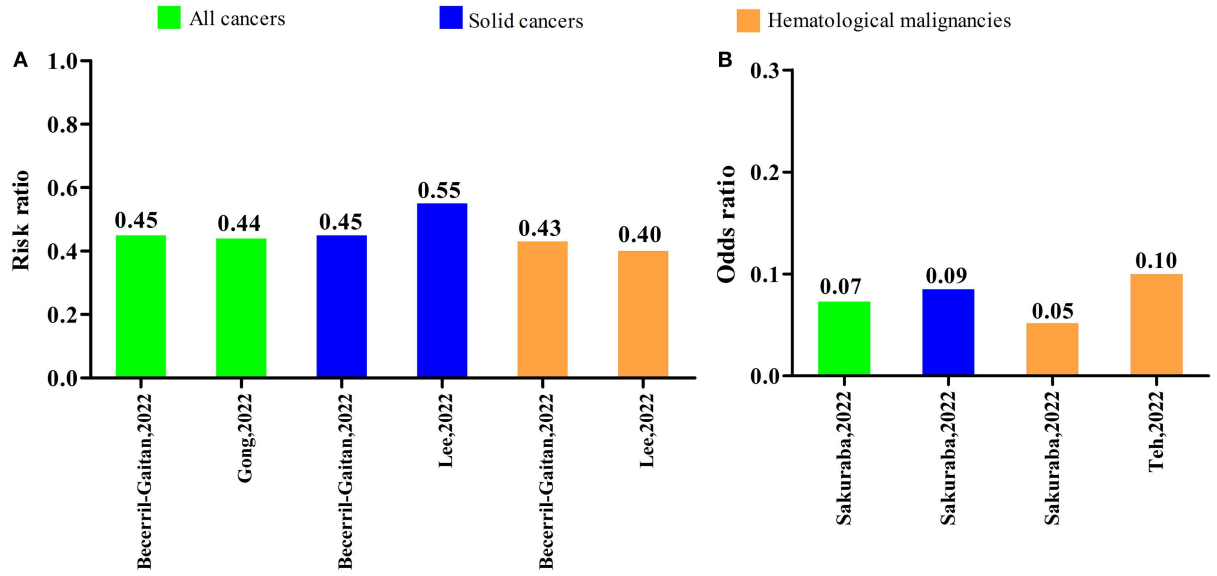

FIGURE 5
Serologic response after the first dose of COVID-19 vaccine compared to controls. **(A)** Risk ratio **(B)** Odds ratio.

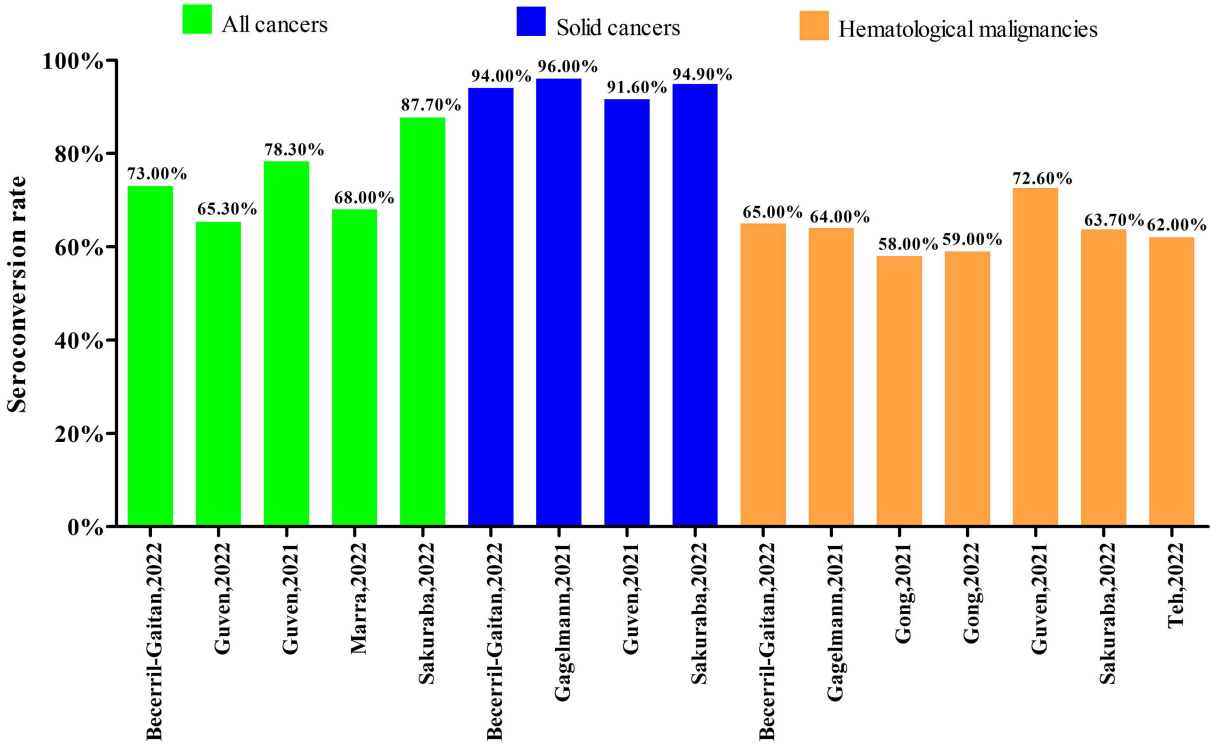

FIGURE 6
Seroconversion rate after the second dose of COVID-19 vaccine.

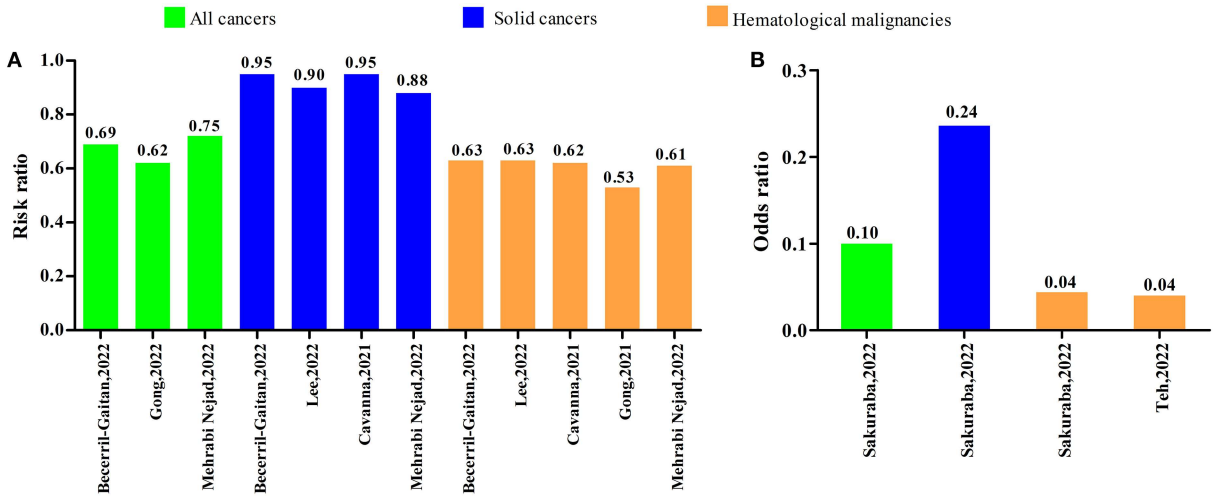

FIGURE 7
Serologic response after the second dose of COVID-19 vaccine compared to controls. **(A)** Risk ratio **(B)** Odds ratio.

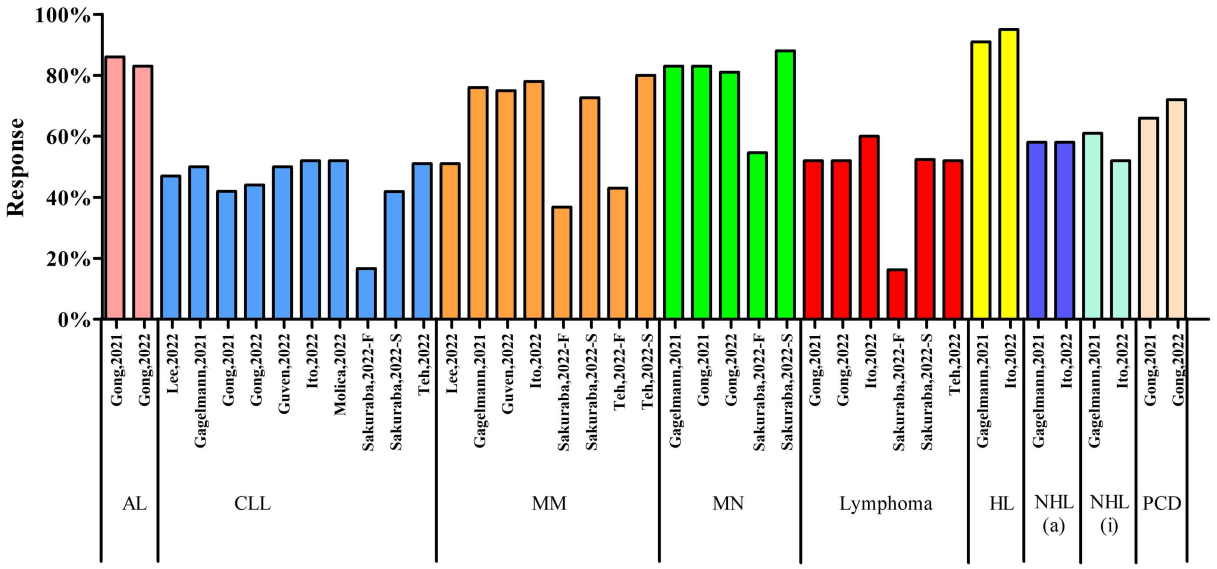

FIGURE 8
Subgroup analysis according to cancer types. AL, acute leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; MN, myeloproliferative neoplasms; HL, Hodgkin's lymphoma; NHL(a), aggressive non-Hodgkin's lymphoma; NHL(i), indolent non-Hodgkin's lymphoma; PCD, plasma cell dyscrasias.

vaccine in cancer patients. The studies by Becerril-Gaitan et al. (17), Gagelmann et al. (20), and Sakuraba et al. (31) reported outcomes both in solid cancers and hematological malignancies. The studies by Guven et al. (24) and Teh et al. (33) reported outcomes only in hematological malignancies. The study by Guven et al. (25) reported outcomes in all cancers without subgroup analysis by cancer type. After first dose of COVID-19 vaccine, the low seroconversion rates were consistent across all studies. As shown in Figure 4, the seroconversion rates after first dose of COVID-19 vaccine in cancer patients were, respectively 37.30, 51.00, and 54.20% in three systematic reviews with meta-analyses. The seroconversion rates were ranged from 49.60 to 62.00% in solid cancers and 33.30 to 56.00% in hematological malignancies. In addition, the seroconversion rate after first dose of COVID-19 vaccine in a systematic review without meta-analysis ranged widely from 11.00 to 87.50% in all cancers, 25.00 to 67.00% in solid cancers and 11.00 to 87.50% in hematological malignancies (19).

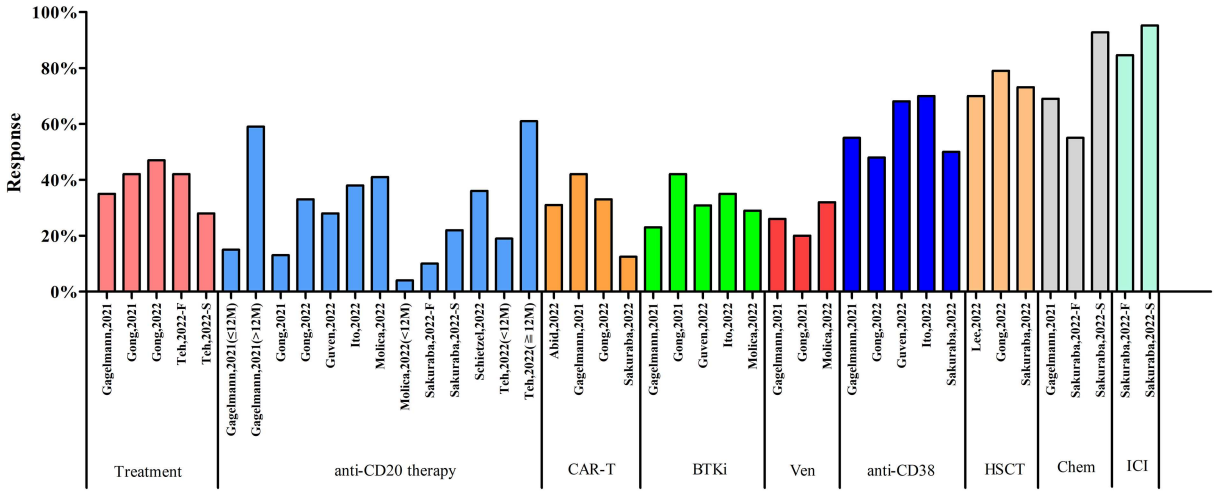

FIGURE 9
Subgroup analysis according to types of therapy. CAR-T, chimeric antigen receptor T-cell; BTKi, bruton tyrosine kinase inhibitor; Ven, venetoclax; HSCT, hematopoietic stem cell transplantation; Chem, chemotherapy; ICI, immune check-point inhibitors.

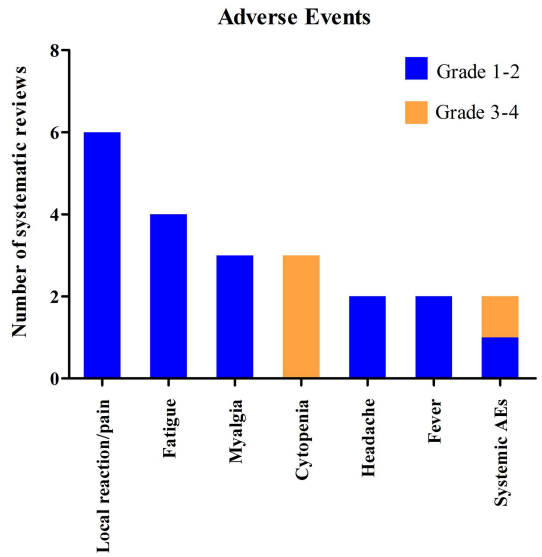

FIGURE 10
Systematic reviews included in the systematic review which investigated the adverse events of COVID-19 vaccines in cancer patients.

Five systematic reviews with meta-analyses reported on comparison of the serologic response after first COVID-19 vaccine dose to controls. Three systematic reviews with meta-analyses reported risk ratios (RRs) and two others reported odds ratios (ORs). The RRs were 0.44 [95% confidence interval (CI): 0.26–0.73] and 0.45 (95% CI: 0.35–0.58) in all cancers, 0.45 (95% CI: 0.37–0.55) and 0.55 (95% CI: 0.46–0.65) in solid cancers, 0.40 (95% CI: 0.32–0.50) and 0.43 (95% CI: 0.29–0.63) in hematological malignancies. The ORs were 0.07 (95% CI: 0.03–0.20) in all cancers, 0.09 (95% CI: 0.02–0.29) in solid cancers, 0.05 (95% CI: 0.01–0.33), and 0.10 (95% CI: 0.04–0.29) in hematological malignancies. These results were shown in Figure 5.

Response after the second dose of COVID-19 vaccine

Nine systematic reviews with meta-analyses focused on the seroconversion rate after the second dose of COVID-19 vaccine. Among them, five systematic reviews with meta-analyses reported the outcomes in all cancers, four in solid cancers and seven in hematological malignancies. As shown in Figure 6, the seroconversion rates after the second dose of COVID-19 vaccine were ranged from 65.30 to 87.70% in all cancers, 91.60 to 96.00% in solid cancers, and 58.00% to 72.60% in hematological malignancies. Two systematic reviews without meta-analyses also reported the data after second dose of COVID-19 vaccine. The systematic review by Corti et al. (19) reported that the seroconversion rate ranged from 7.30 to 100.00% in all cancers, from 47.50 to 100.00% for solid cancers and from 7.30 to 88.80% for hematological malignancies. The seroconversion rates among all cancers, solid cancers and hematological malignancies in the systematic review by Galmiche et al. (21) were ranged from 39.00 to 98.00%, 64.00 to 98.00%, and 39.00 to 86.00%, respectively.

Eight systematic reviews with meta-analyses investigated comparison of the serologic response after second COVID-19 vaccine dose to controls. Among the eight studies, six systematic reviews with meta-analyses reported RRs and the others reported ORs. As shown in Figure 7A, the RRs were ranged from 0.62 to 0.75 in all cancers, 0.88 to 0.95 in solid cancers, and 0.53 to 0.63 in hematological malignancies. The ORs were 0.10 (95% CI: 0.04–0.26) in all cancers, 0.24 (95% CI: 0.06–0.90) in solid cancers, 0.04 (95% CI: 0.01–0.16), and 0.04 (95% CI: 0.02–0.08) in hematological malignancies (Figure 7B).

Subgroup analysis according to cancer types

Nine systematic reviews with meta-analyses reported the subgroup analysis according to cancer types (Figure 8). The serologic response in acute leukemia (AL) reported in two systematic reviews were 86.00 and 83.00% (RR = 0.82), respectively. There were nine systematic reviews with meta-analyses reported the serologic response in chronic lymphocytic leukemia (CLL) and the outcomes were ranged from 16.70 to 52.00%. Among the nine articles, Sakuraba et al. reported both the results after first dose and second dose of COVID-19 vaccines (31). The vaccine response after the first dose was only 16.70% while the response was 41.90% after the second dose. Six systematic reviews with meta-analyses reported the vaccine response in multiple myeloma (MM). Two articles reported both the results after the first dose and second dose of COVID-19 vaccines. Sakuraba et al. reported that the response after the first dose and second dose of COVID-19 vaccine were 36.80% and 72.70%, respectively (31). Teh et al. reported the vaccine response was 43.00% after first dose and 80.00% after second dose (33). The results in the other four articles were ranged from 51.00 to 78.00%. Four systematic reviews with meta-analyses reported the vaccine response in myeloproliferative neoplasms (MN) and the results were ranged from 81.00 to 88.00%. Five articles focused on the lymphoma, the vaccine responses were over 50.00% (52.00 to 60.00%), except one first dose result was 16.30%. Gagelmann et al. (20) and Ito et al. (26) reported the vaccine response in Hodgkin's lymphoma, aggressive non-Hodgkin's lymphoma and indolent non-Hodgkin's lymphoma. The results were 91.00 and 95.00% (RR = 0.95) in Hodgkin's lymphoma, 58.00 and 58.00% (RR = 0.60) in aggressive non-Hodgkin's lymphoma, 61.00 and 52.00% (RR = 0.54) in indolent non-Hodgkin's lymphoma. Two articles reported the vaccine response in plasma cell dyscrasias and the results were 66.00% (RR = 0.73) and 72.00% (RR = 0.81). In addition, only one article reported the vaccine response in thoracic cancers, skin cancers, women's cancers, gastrointestinal cancers, urological cancers and brain cancers. The vaccine response in these solid cancers were ranged from 21.40 to 66.70% after first dose and 76.60 to 95.00% after second dose.

Subgroup analysis according to types of therapy

Ten systematic reviews with meta-analyses reported the influence of different treatments on COVID-19 vaccine response (Figure 9). Four articles reported the vaccine response in patients receiving active treatment. Teh, et al. reported the vaccine response was 28.00% after first dose and 42.00% after second dose (33). The vaccine response of other 3 articles were ranged from 35.00 to 47.00%. Nine systematic reviews with meta-analyses focused on the vaccine response in patients with anti-CD20 therapy. The response rates were ranged from 4.00 to 61.00%. Three articles reported vaccine response in patients receiving CD-20 antibody therapy within 12 months and seroconversion rates were 4.00, 15.00, and 19.00%, respectively. Two articles reported vaccine response in patients receiving CD-20 antibody therapy over 12 months with seroconversion rates were 59.00 and 61.00%. Sakuraba, et al. reported that seroconversion rate was 10.00% after first dose and 22.90% after second dose in patients receiving CD-20 antibody therapy (31). Four articles reported vaccine response in the patients receiving chimeric antigen receptor T-cell (CAR-T) therapy with seroconversion rates ranged from 12.50 to 42.00%. Five articles reported the influence of bruton tyrosine kinase inhibitor (BTKi) on COVID-19 vaccine response with seroconversion rates ranged from 23.00 to 42.00%. Several articles also reported the influence of other treatments on COVID-19 vaccine response, such as venetoclax (3 articles, 20.00 to 32.00%), anti-CD38 therapy (5 articles, 48.00 to 70.00%), hematopoietic stem cell transplantation (HSCT, 3 articles, 70.00 to 79.00%), chemotherapy (two articles; one article: first dose 55.00%, second dose 92.80%; another article: 69.00%), immune check-point inhibitors (one article; first dose 84.60%, second dose 95.20%), hormonal therapy (one article, 99.00%), and protease inhibitors (one article, 92.90%).

COVID-19 vaccine safety

The safety of COVID-19 vaccines in cancer patients was investigated in six systematic reviews. Overall, the COVID-19 vaccines were reported to be safe and well–tolerated in these systematic reviews. Although three systematic reviews reported grade 3 AEs, they included one same study with four patients developed grade 3–4 cytopenia (34). The most commonly reported adverse events were local reaction, fatigue, myalgia, headache, fever, and cytopenia (Figure 10). The systematic review by Abid, et al. reported that the grade 3 or 4 cytopenia was in ∼ 5% of entire cohort in one included study and lesser pain

in CAR-T recipients compared to controls in another included study (16). Three studies included in the systematic review by Cavanna, et al. performed a safety analysis and reported that COVID-19 vaccines were generally very safe, with mostly mild or moderate adverse reactions reported (18). The systematic review by Corti, et al. reported that any-grade AEs ranged from 9.70 to 87.00% after first dose and from 23.00 to 85.00% after second dose of COVID-19 vaccines (19). The most commonly any-grade AEs were fatigue (first dose: range from 4.20 to 47.60%; second dose: range from 3.00 to 23.40%) and local pain (first dose: range from 7.40 to 69.00%; second dose: range from 32.30 to 67.20%). The systematic review by Gagelmann, et al. reported that the most frequent systemic AEs were generalized muscle pain (4.00–30.00%) and weakness/ fatigue (6.00–30.00%) (20). The systematic review by Gong et al. (22) also reported that the COVID-19 vaccines were generally well–tolerated with the most commonly AEs being local reaction, fever, headache, fatigue and myalgia. The systematic review by Teh et al. indicated that at least 1 AE rate was 39.00% after first dose and 36.00% after second dose (33). Local and systemic AEs of COVID-19 vaccines were mild except for one study with grade 3 systemic AEs rate from 1.00 to 2.00%.

Discussion

COVID-19 vaccines have brought great hope to the whole world, although the end of the pandemic is still unclear. Patients with cancer are among the prioritized populations for the COVID-19 vaccination. However, data on the efficacy and safety of COVID-19 vaccines are limited in patients with cancer because of the exclusion of the vulnerable population from the clinical trials. Recently, there were several systematic reviews and meta-analyses on the COVID-19 vaccines in patients with cancer, resulting in redundant and sub-optimal systematic reviews. It is important to know the response and safety of COVID-19 vaccines in patients with cancer due to the lack of effective treatments for COVID-19. To our knowledge, this study is the first overview of systematic review and meta-analyses on response and safety of COVID-19 vaccines in cancer patients and provides a comprehensive summary of the currently available evidence on COVID-19 vaccine response and safety in patients with cancer. Although some overlaps existed among the included systematic reviews, these reviews still incorporated many different studies. The results demonstrated that cancer patients have a lower likelihood of COVID-19 vaccine response when compared with non-cancer controls. The responses of COVID-19 vaccine were especially lower in patients with hematologic malignancies which suggests an urgent need to improve the vaccination strategy in the vulnerable population.

The number of received doses could significantly influence the COVID-19 vaccine response. Patients with cancer have lower response rates after first dose of COVID-19 vaccine. Although the response rate was still lower than that of the controls, the rate was increased after second dose of COVID-19 vaccine, especially in patients with solid cancers (over 90.00% response rates). Cancer types could significantly influence the COVID-19 vaccine response. After second dose of COVID-19 vaccine, the patients with hematologic malignancies had a significantly lower response rate than those with solid cancers. We also performed subgroup analysis to investigate vaccine responses in different types of cancer. The subgroup analysis according to cancer types indicated that patients with CLL and non-Hodgkin's lymphoma have lower response rates compared to other types of cancers. In addition to the number of received dose and cancer types, types of therapy could also influence the response of COVID-19 vaccine. Patients with certain therapy have lower response rates of COVID-19 vaccine. Patients with active treatment have lower vaccine response rates. Additionally, anti-CD20, anti-CD38 therapy, BTKi, venetoclax and CAR-T therapy significantly decreased response rates. Furthermore, effect the of anti-CD20 therapy seemed to be long-lasting. Compare to patients received anti-CD20 therapy >12 months prior to vaccination, patientsreceived anti-CD20 antibody within 12 months prior to vaccination showed significantly reduced COVID-19 vaccine response. In contrast, therapies such as chemotherapy, hormonal therapy and immune check-point inhibitors had relatively higher response rates. Considering the limited studies on the influence of different therapies on COVID-19 vaccine responses among patients with cancer, further well-designed studies are warranted.

Due to properties of cancer and anti-cancer therapies, patients with cancer are immunocompromised and reported to have a greater COVID-19 related-mortality. The immune parameters such as B and T cell functions in patients with cancer might be changed by chemotherapy and patients with cancer have higher risks of various infections as well as reduced vaccine responses (35). Due to the lack of effective treatments of COVID-19, COVID-19 vaccine is vital for patients with cancer, although the vaccine response was lower. Patients with cancer should be encouraged to complete their COVID-19 vaccination schemes. Furthermore, several studies have indicated that there were some benefits with a booster vaccine dose after the completion of standard COVID-19 vaccination scheme among cancer patients. There was evidence that the vaccine efficacy against COVID-19 infection will decrease in time (36). Considering the emergence of several new variants of SARS-CoV-2 with potential influence on ongoing vaccination programs, there is a need for a booster dose to increase the effectiveness of COVID-19 vaccines (37). The booster doses of mRNA vaccines were reported to provide some protection against the omicron variant (38, 39) and severe COVID-19-related outcomes (40). Although several countries have removed the restrictions such as social distancing and use face masks for those individuals who have completed their COVID-19 vaccination schemes, these measures should not be applied to

patients with cancer in whom COVID-19 vaccine response is significantly lower than that of general population.

Among all the included systematic reviews, there were only six articles reported the safety of COVID-19 vaccine in cancer patients (16, 18–20, 22, 33). Overall, the COVID-19 vaccines were reported to be safe and well–tolerated in these systematic reviews. The most commonly AEs were local reaction, fatigue, myalgia, headache, fever and cytopenia. Due to the limited studies on safety of COVID-19 vaccines in cancer patients, further studies on this issue are needed.

This systematic review has several limitations. Firstly, all systematic reviews included only observational studies, without RCTs. Many factors might influence the response to the COVID-19 vaccines, such as age and comorbidities (41, 42). These factors might not have been controlled for between the cancer group and non-cancer control group. Secondly, after a stringent quality assessment of the methods, most of the included systematic reviews did't reach an acceptable quality level. Higher quality evidence is needed to substantiate these findings. In addition, systematic reviews included in this study predominantly reported mRNA vaccines, subgroup analysis according to types of vaccines was limited. Different types of vaccines might have different efficacy and adverse effects (43). Moreover, there are some overlaps among the included systematic reviews with a same primary study in more than one systematic review. This could result in an overestimation of evidence. Furthermore, the types of immunoassay used in the included systematic reviews were not standardized. Lastly, despite we performed subgroup analyses according to cancer types and types of therapy, the number of articles reporting these detailed data was limited.

Conclusions

In conclusion, the overview of systematic reviews demonstrates patients with cancer might have a lower COVID-19 vaccine response when compared with non-cancer controls, especially in patients with hematologic malignancies. The number of received doses, cancer types and types of therapy could influence the response of COVID-19 vaccine in patients with cancer. COVID-19 vaccines are reported to be safe and well–tolerated. There is a need for booster doses to increase the effectiveness of COVID-19 vaccines.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

HS and TH conceived and designed the study. HS and FB conducted the database search, extracted the data, and data analysis. LL and XZ extracted the data. HS wrote the manuscript. TH and JY revised it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.1072137/full#supplementary-material

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Evaluation of post-vaccination immunoglobulin G antibodies and T-cell immune response after inoculation with different types and doses of SARS-CoV-2 vaccines: A retrospective cohort study

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Introduction: The induction and speed of production of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) immune biomarkers may vary by type and number of inoculated vaccine doses. This study aimed to explore variations in SARS-CoV-2 anti-spike (anti-S), anti-nucleocapsid (anti-N), and neutralizing immunoglobulin G (IgG) antibodies, and T-cell response by type and number of SARS-CoV-2 vaccine doses received.

Methods: In a naturally exposed and SARS-CoV-2–vaccinated population, we quantified the anti-S, anti-N, and neutralizing IgG antibody concentration and assessed T-cell response. Data on socio-demographics, medical history, and history of SARS-CoV-2 infection and vaccination were collected. Furthermore, nasal swabs were collected to test for SARS-CoV-2 infection. Confounder-adjusted association between having equal or more than a median concentration of the three IgG antibodies and T-cell response by number and type of the inoculated vaccines was quantified.

Results: We surveyed 952 male participants with a mean age of 35.5 years ± 8.4 standard deviations. Of them, 52.6% were overweight/obese,

and 11.7% had at least one chronic comorbidity. Of the participants, 1.4, 0.9, 20.2, 75.2, and 2.2% were never vaccinated, primed with only one dose, primed with two doses, boosted with only one dose, and boosted with two doses, respectively. All were polymerase chain reaction-negative to SARS-CoV-2. BBIBP-CorV (Sinopharm) was the most commonly used vaccine (92.1%), followed by rAd26-S + rAd5-S (Sputnik V Gam-COVID-Vac) (1.5%) and BNT162b2 (Pfizer-BioNTech) (0.3%). Seropositivity to anti-S, anti-N, and neutralizing IgG antibodies was detected in 99.7, 99.9, and 99.3% of the study participants, respectively. The T-cell response was detected in 38.2% of 925 study participants. Every additional vaccine dose was significantly associated with increased odds of having ≥median concentration of anti-S [adjusted odds ratio (aOR), 1.34; 95% confidence interval (CI): 1.02–1.76], anti-N (aOR, 1.35; 95% CI: 1.03–1.75), neutralizing IgG antibodies (aOR, 1.29; 95% CI: 1.00–1.66), and a T-cell response (aOR, 1.48; 95% CI: 1.12–1.95). Compared with boosting with only one dose, boosting with two doses was significantly associated with increased odds of having ≥median concentration of anti-S (aOR, 13.8; 95% CI: 1.78–106.5), neutralizing IgG antibodies (aOR, 13.2; 95% CI: 1.71–101.9), and T-cell response (aOR, 7.22; 95% CI: 1.99–26.5) although not with anti-N (aOR, 0.41; 95% CI: 0.16–1.08). Compared with priming and subsequently boosting with BBIBP-CorV, all participants who were primed with BBIBP-CorV and subsequently boosted with BNT162b2 had ≥median concentration of anti-S and neutralizing IgG antibodies and 14.6-time increased odds of having a T-cell response (aOR, 14.63; 95% CI: 1.78–120.5). Compared with priming with two doses, boosting with the third dose was not associated, whereas boosting with two doses was significantly associated with having ≥median concentration of anti-S (aOR, 14.20; 95% CI: 1.85–109.4), neutralizing IgG (aOR, 13.6; 95% CI: 1.77–104.3), and T-cell response (aOR, 7.62; 95% CI: 2.09–27.8).

Conclusion: Achieving and maintaining a high blood concentration of protective immune biomarkers that predict vaccine effectiveness is very critical to limit transmission and contain outbreaks. In this study, boosting with only one dose or with only BBIBP-CorV after priming with BBIBP-CorV was insufficient, whereas boosting with two doses, particularly boosting with the mRNA-based vaccine, was shown to be associated with having a high concentration of anti-S, anti-N, and neutralizing IgG antibodies and producing an efficient T-cell response.

KEYWORDS

SARS-CoV-2, COVID-19, vaccination, coronavirus, vaccine

1. Introduction

Since the early 20th century, vaccines have proven to be effective tools for controlling and eliminating life-threatening infectious diseases. On 3 December 2022, more than 649.67 million cases and 6.64 million deaths have been reported, as the coronavirus disease 2019 (COVID-19) pandemic continues (1). In 185 countries, approximately 19.8 million lives were saved in the first year of COVID-19 vaccination. This estimate corresponds to a 63% reduction in COVID-19-related deaths in the absence of vaccines (2). Humoral and cellular immune responses are the main drivers of protection against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). While neutralizing antibodies (Nabs) established a clear role in protection against infection, particularly in the early post-vaccination period (3, 4), cellular immunity is also proven

to alleviate the disease severity and enhance recovery (5). The currently approved and available vaccines have different mechanisms of action in triggering the immune system to produce immune response biomarkers that predict vaccine effectiveness. Several studies have discussed the vaccine effect of various vaccine types on inducing immune responses to produce immune biomarkers and their durability (4–8). However, these studies were limited in examining the effect of whole inactivated vaccine, the effect of vaccine mixing between more than one type, and the change in immunity levels by the number and type of the received vaccine doses on different forms of immunity.

In the United Arab Emirates (UAE), which hosts the world's most fully vaccinated population (9), five types of vaccines were approved for emergency use to control the spread of the SARS-CoV-2 virus. These approved vaccines are BBIBP-CorV (commercial name: Covilo, Sinopharm's Beijing Institute of Biological Products), BNT162b2 (commercial name: Comirnaty, Pfizer-BioNTech), rAd26-S + rAd5-S (commercial name: Sputnik V, Gamaleya Research Institute of Epidemiology and Microbiology), ChAdOx1-S (commercial name: Vaxzevria, AstraZeneca-University of Oxford), and mRNA-1273 (commercial name: Spikevax, Moderna-NIAID) (10). Understanding the impact of different vaccine types and the number of vaccine doses in enhancing the immune response will help inform policymakers on future vaccination and immunization strategies. Therefore, this retrospective cohort study aimed to investigate the variation in the immune response to SARS-CoV-2 using the concentration of the anti-spike (anti-S), anti-nucleocapsid (anti-N), and neutralizing immunoglobulin G (IgG) antibodies and T-cell response among a cohort of participants who were previously seropositive to SARS-CoV-2 before the emergency use of vaccines in the Emirate of Abu Dhabi, UAE.

2. Materials and methods

2.1. Study population

The study included male participants who were naturally exposed to SARS-CoV-2 (tested seropositive to anti-S IgG and anti-N IgG antibodies) in a previously published cross-sectional study that covered 24 workstations for participants across the Emirate of Abu Dhabi. Following the random sampling approach, the cross-sectional study surveyed 4,855 male expatriate workers residing in 40 workstations across the Emirate of Abu Dhabi. Of the 3,585 seropositive workers, 952 workers were available between 3 October 2021 and 15 December 2021. The available workers were surveyed and retrospectively followed in this study. More details about that study are available elsewhere (11). Participants enrolled in the cross-sectional study were invited back and asked to re-consent to participate in the current study.

2.2. Survey data collection, nasal swab, and blood sampling

Participants available at the time of the survey were invited to participate in this study and provide blood samples. They filled in an online survey that collected data on their socio-demographics, existing chronic medical conditions, smoking status, body weight and height, and a history of testing positive for SARS-CoV-2 infection. Two whole blood samples (5–7 ml each) were collected from each participant in plain tubes and in a tube with an anticoagulant. Moreover, at the survey time, a nasopharyngeal swab was collected from each participant for SARS-CoV-2 testing using reverse transcription-polymerase chain reaction (RT-PCR). Collected blood samples in plain tubes were preserved at suitable conditions for serum separation and screening for three humoral SARS-CoV-2 IgG immune biomarkers [anti-spike (anti-S), anti-nucleocapsid (anti-N), and neutralizing IgG antibodies]. The other collected whole blood samples were screened for T-cell response. Malaffi ("my file" in Arabic), an Abu Dhabi-based central medical record database (12), was used to retrieve data on the history of vaccination against SARS-CoV-2, including the number and type of the vaccine doses received and the date of each dose, and the history of SARS-CoV-2 RT-PCR testing before blood collection. Any vaccination that occurred on or after the study of blood sampling was not counted.

2.3. Laboratory work: Immune biomarker testing

2.3.1. NAb immunoassays

Testing the collected blood samples for NAbs against the SARS-CoV-2 receptor-binding domain (RBD) was performed using the iFlash-2019-nCoV NAb kit, a one-step competitive chemiluminescence immunoassay (CLIA) on the iFlash 1800 analyzer (YHLO Biotech Co., Ltd., Shenzhen, China) according to the manufacturer's instructions.

2.3.2. Anti-S and anti-N IgG immunoassays

Two types of SARS-CoV-2 IgG antibodies were measured. The first assays released at the start of the pandemic were used. The first assay to measure the anti-N IgG antibodies was the SARS-CoV-2 nucleocapsid total antibodies, which we analyzed using the Roche Cobas 6000 platform (Roche Diagnostics International AG, Rotkreuz, Switzerland). This assay was CE marked/FDA EUA-approved. The second assay that quantified the anti-S IgG antibodies was the SARS-CoV-2 Trimeric S IgG using the DiaSorin LIAISON® (Saluggia, Italy) SARS-CoV-2 Trimeric S IgG. This assay is an indirect CLIA technology for the detection of IgG antibodies to SARS-CoV-2 Spikes 1 and 2 (S1 and S2) and RBD and is calibrated against the World Health Organization standard.

2.3.3. T-cell response

The interferon-gamma release assay (IGRA) (QuantiFERON^{TM}, Qiagen) was utilized as a marker for T-cell activation. The Qiagen QuantiFERON SARS-CoV-2 (QFN SARS-CoV-2) blood collection kit consists of two antigen tubes (long and short peptides), SARS-CoV-2 Ag1 and SARS-CoV-2 Ag2, which uses a combination of antigens specific to SARS-CoV-2 to stimulate lymphocytes in heparinized whole blood involved in cell-mediated immunity. Plasma from the stimulated samples was used for the detection of interferon-gamma (IFN-γ) using QuantiFERON ELISA. There is a null control to baseline circulating IFN-γ and a positive T-cell control tube using mitogen as the lymphocyte stimulant.

Table 1 presents the sensitivity, specificity, and lowest detection limit for the used laboratory assays.

2.3.4. SARS-CoV-2 RT-PCR testing

Viral RNA amplification was performed using the NeoPlex^{TM} COVID-19 detection kit. RT-PCR was used for the RNA detection targeting N gene, *ORF1a* PCRC (SolGent Co., Ltd. Daejeon, Korea).

2.4. Statistical analyses

Socio-demographics and other characteristics were described using frequency distributions and measures of central tendency. For continuous measures, means and standard deviations (SDs) were reported. The distribution of the measured three humoral immune biomarkers (anti-S, anti-N, and neutralizing IgG antibodies) was described using medians and the interquartile ranges. The normality assumption for the distribution values of the IgG antibodies was investigated using the Shapiro–Wilk test. Even after implementing several transforming strategies and investigating the normality assumption of residuals, the IgG antibody values violated the normality assumption. To be used as a binary outcome variable, each of the three measured IgG antibody biomarkers was subsequently categorized using the median value into two categories (<median or ≥median). Based on T-cell response, the participants were categorized into with responding or with nonresponding T-cell. Following the manufacturer's instructions, the responding T-cell was defined if the Ag1 or Ag2 antigen minus Nil (≤8.0 IU/ml) were ≥0.15 and ≥25% of Nil while the non-responding T-cell was defined if the Ag1 and Ag2 antigen minus Nil (≤8.0 IU/ml) were <0.15 or ≥0.15 and <25% of Nil and the Mitogen minus Nil is ≥0.50. The correlation between the level of each of the three measured SARS-CoV-2 IgG antibodies sero-biomarkers (<median vs. ≥median) and T-cell response (responding or non-responding) by the measured characteristics and history of vaccination was investigated. Chi-squared or Fisher's exact tests were used for categorical characteristics, and the two-sample non-parametric Mann–Whitney U-test was used for continuous characteristics. The distribution of, stratified by the type and number of vaccine doses received, anti-S, anti-N, and neutralizing IgG antibody concentration was plotted and presented in boxplots. The *P*-value for this comparison was elicited from the non-parametric independent samples of the Kruskal–Wallis test. The distribution of participants with T-cell response by type and number of vaccine doses received was plotted and presented in stacked bar graphs.

Univariate and multivariable binary logistic regression models were used to estimate the crude (OR) and adjusted odds ratio (aOR). The multivariable regression model was adjusted for age, body mass index (BMI), smoking, number of vaccine doses, type of vaccine (except for only-BBIBP-CorV-vaccinated), history of the previous SARS-CoV-2 infection, and time duration in days since the last vaccine dose received and blood collection. All vaccines received on the same date of or after blood collection were not considered. All statistical analyses were performed using SPSS IBM Statistics software (v26). *P*-values of <0.05 were considered statistically significant.

3. Results

3.1. Baseline characteristics

The enrolled 952 male participants were retrospectively followed up from the last vaccine dose received until blood collection for a mean time of 89.2 days ± 54.5 SD. The participants had a mean age of 35.5 years (±8.4 SD). The majority (92.5%) were of Asian nationalities, and 52.7% were with primary education or below. Of this population, 21.4% were current smokers, and 52.6% were overweight (40.9%) or obese (11.7%). Approximately, 11.0% of the participants reported having at least one chronic comorbidity. The most common comorbidities were high blood pressure (7.0%) followed by diabetes mellitus (4.1%) (Table 2). None of the participants reported having any immunodeficiency conditions or taking any immunosuppressive medications.

3.2. SARS-CoV-2 vaccination status

Before blood collection, the majority of the 952 workers were fully vaccinated and boosted with one vaccine dose (75.2%) or primed with two vaccine doses (20.2%). Only 2.2% were fully vaccinated and boosted with two additional vaccine doses. Few workers were never vaccinated or only partially vaccinated at the time of blood collection (1.4 and 0.9%, respectively). Overall, 77.5% of the participants were boosted with at least one dose. Regardless of the number of vaccine doses, 92.2, 1.5, 0.3, 4.0, and 0.7% of the participants were vaccinated with BBIBP-CorV only, rAd26-S + rAd5-S, BNT162b2 only, primed with BBIBP-CorV

TABLE 1 Sensitivity, specificity, and the lowest detection limit for the used laboratory assays.

Assay type	Sensitivity	Specificity	Lowest detection limit
LIAISON® SARS-CoV-2 TrimericS IgG	Days post-positive PCR ≥15 (94.5–99.6%)	99.7%	4.81 BAU/ml
Elecsys® Anti-SARS-CoV-2	Days post-positive PCR ≥14 days (97.0–100%)	99.80%	Qualitative test COI < 1.0–non-reactive COI ≥ 1.0 Reactive
iFlash-2019-nCoV Neutralization Antibody Test	95.4%–post-vaccination 90%–post-infection	98.0%	4 AU/ml
QuantiFERON SARS-CoV-2 (The QFN SARS-CoV-2 BCTs are for Research Use Only)	80.12%	92.99%	Qualitative test

and boosted with BNT162b2, and had mixed vaccine types, respectively. The mean duration since the last received vaccine dose and blood collection for immune biomarker measurement was 89.2 days (±54.5 SD). Only 2.0% were vaccinated within the past 14 days, 3.6% were vaccinated within 15–30 days, and the majority (92.8%) were vaccinated for more than 30 days before blood collection (Table 3).

3.3. Anti-S, anti-N, and neutralizing IgG antibody concentration

Seropositivity to anti-S, anti-N, and neutralizing IgG antibodies was detected in 99.7% (seropositive ≥33.8 BAU/ml), 99.9% (seropositive ≥1 COI), and 99.3% (seropositive ≥10 AU/ml) of the participants, respectively. The mean (±SD) and median concentrations of the anti-S, anti-N, and neutralizing IgG antibodies were 648.1 BAU/ml (±641.7) and ≥357.5 BAU/ml, 145.0 COI (±74.8) and ≥146.5 COI, and 363.1 AU/ml (±339.4) and ≥172.0 AU/ml, respectively. Participants who had ≥median concentration of any of the three IgG antibodies had significantly higher mean age (all $p < 0.05$). Of the participants who had ≥median concentration of anti-S, anti-N, and neutralizing IgG antibodies, 77.1, 75.2, and 75.4% never smoked tobacco, respectively. Of the participants who had at least one chronic comorbidity, 56.4, 39.6, and 54.5% had ≥median concentration of anti-S, anti-N, and neutralizing IgG antibodies, respectively (Table 2).

Most of the participants who had ≥median concentration of anti-S (≥357.5 BAU/ml), anti-N (≥146.5 COI), and neutralizing IgG antibodies (≥172.0 AU/ml) were boosted with at least one booster dose (79.3, 85.6, and 77.7%, respectively). Regardless of the number of the received vaccine doses, 47.3, 51.9, and 47.5% of the participants who were vaccinated with only the BBIBP-CorV vaccine had ≥median concentration of anti-S, anti-N, and neutralizing IgG antibodies, respectively. All of the 14 participants who were vaccinated with rAd26-S + rAd5-S only or BNT162b2 only ($n = 3$) had ≥median concentration of anti-S and neutralizing IgG antibodies although <median concentration of anti-N IgG antibodies (Table 3 and Figures 1A–C).

3.4. T-cell response

Of the 925 participants, 38.2% had a T-cell response. Participants with responding T-cells had a significantly higher mean age than those with non-responding T-cells (36.6 vs. 34.8 years, $p = 0.002$) (Table 2). Of the participants who were boosted with only one dose or with two doses, 38.0 and 84.2% had a T-cell response, respectively. Overall, of the 353 participants who had a T-cell response, 79.0% were boosted with at least one dose. Of the only-BBIBP-CorV-vaccinated participants, 36.4% had a T-cell response. Of the only rAd26-S + rAd5-S-vaccinated participants, 35.7% had a T-cell response. All of the three BNT162b2-vaccinated participants had responding T-cells. Of the participants who had a T-cell response, 59.3, 49.7, and 61.0% had ≥median concentration of anti-S, anti-N, and neutralizing IgG antibodies (Table 3 and Figures 2A, B).

3.5. History of SARS-CoV-2 infection and immune response

Compared with other participants, a statistically significantly higher proportion of individuals having ≥median concentration of anti-S (81.7% vs. 45.4%), anti-N (62.6% vs. 48.3%), and neutralizing IgG antibodies (80.9% vs. 45.7%) was noted in participants with a history of contracting COVID-19 during the past 12 months of blood collection (Table 2). In addition, participants with a history of SARS-CoV-2 infection had a significantly ($p < 0.001$) higher mean IgG antibody concentration than those with no infection in the past 12 months (Table 4). The T-cell response was not statistically significantly associated with a history of SARS-CoV-2 infection during the past 12 months ($p = 0.518$) (Table 5).

TABLE 2 Distribution of the study population by their measured socio-demographic and clinical characteristics and their correlation with the four-tested immune response biomarkers.

	N = 952 (valid %)	Anti-S IgG *N* = 952 (*n*, valid %)		*P*-value	Anti-N IgG *N* = 952 (*n*, valid %)		*P*-value	Neutralizing IgG *N* = 952 (*n*, valid %)		*P*-value	T-cell response *N* = 925 (*n*, valid %)		*P*-value
		Mean: 648.1 ± 641.7 SD Median = 357.5 stockticker BAU/ml (IQR: 173–930.5) Range: 25–2,080			Mean: 145.0 ± 74.8 SD Median = 146.5 COI (IQR: 92.0–205.5) Range: 0.0–320.0			Mean: 363.1 ± 339.4 SD Median = 172.0 AU/ml (IQR: 51–800) Range: 3–810			Yes 353 (38.2%)	No 572 (61.8%)	
		<357.5	≥357.5		<146.5	≥146.5		<172.0	≥172.0				
Age median, IQR–year (range, mean ± SD)	35.0, 29.0–41 (20–65, 35.5 ± 8.40)	34.0, 28.0–41.0 (34.7 ± 8.4)	36.0, 30.0–42.0 (36.3 ± 8.4)	0.003[1]	34.0, 28.0–41.0 (35.1 ± 8.5)	36.0, 30.0–42.0 (36.0 ± 8.3)	0.049[1]	34.0, 28.0–41.0 (34.8 ± 8.3)	36.0, 30.0–42.0 (36.2 ± 8.5)	0.018[1]	36.0, 30.0–42.0 (36.6 ± 8.7)	36.0, 29.5–42.0 (34.8 ± 8.2)	0.002[1]
Missing	*5*												
Nationality				<0.001			0.360			<0.001			0.017
Asian	881 (92.5)	424 (48.1)	457 (51.9)		441 (50.1)	440 (49.9)		423 (48.0)	458 (52.0)		335 (39.1)	523 (60.9)	
African	66 (6.9)	50 (75.8)	16 (21.2)		31 (47.0)	35 (53.0)		51 (77.3)	15 (22.7)		15 (23.8)	48 (76.2)	
Others	5 (0.5)	2 (40.0)	3 (60.0)		4 (80.0)	1 (20.0)		3 (60.0)	2 (40.0)		3 (75.0)	1 (25.0)	
Education													
Primary education and below[2]	502 (52.7)	–	–	–	–	–	–	–	–	–	–	–	–
Secondary education	352 (37.0)	–	–	–	–	–	–	–	–	–	–	–	–
University and postgraduate levels	93 (9.8)	–	–	–	–	–	–	–	–	–	–	–	–
Missing	*5*												
Tobacco smoking				0.030			0.507			0.258			0.780
Current smoker	203 (21.4)	116 (57.1)	87 (42.9)		106 (52.2)	97 (47.8)		112 (55.2)	91 (44.8)		76 (39.0)	119 (61.0)	
Ex-smoker	45 (4.7)	26 (57.8)	19 (42.2)		25 (55.6)	20 (44.4)		22 (48.9)	23 (51.1)		15 (33.3)	30 (66.7)	
Never-smoker	699 (73.4)	332 (47.5)	367 (52.5)		341 (48.8)	358 (51.2)		340 (48.6)	359 (51.4)		260 (38.1)	422 (61.9)	
Missing	*5*										*2*	1	
Received flu shot				0.082			0.559			0.082			0.027
Yes	3 (0.3)	0 (0.00)	3 (100)		2 (66.7)	1 (33.3)		0 (0.0)	3 (100)		348 (37.9)	571 (62.1)	
No	944 (99.2)	474 (50.2)	470 (49.8)		470 (49.8)	474 (50.2)		474 (50.2)	470 (49.8)		3 (100)	0 (0.0)	
Missing	*5*										*2*	*1*	

(Continued)

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TABLE 2 (Continued)

	N = 952 (valid %)	Anti-S IgG *N* = 952 (*n*, valid %)		*P*-value	Anti-N IgG *N* = 952 (*n*, valid %)		*P*-value	Neutralizing IgG *N* = 952 (*n*, valid %)		*P*-value	T-cell response *N* = 925 (*n*, valid %)		*P*-value
		Mean: 648.1 ± 641.7 SD Median = 357.5 stockticker BAU/ml (IQR: 173–930.5) Range: 25–2,080			Mean: 145.0 ± 74.8 SD Median = 146.5 COI (IQR: 92.0–205.5) Range: 0.0–320.0			Mean: 363.1 ± 339.4 SD Median = 172.0 AU/ml (IQR: 51–800) Range: 3–810			Yes 353 (38.2%)	No 572 (61.8%)	
		<357.5	≥357.5		<146.5	≥146.5		<172.0	≥172.0				
BMI, median, IQR (mean: 25.3 ± 3.8 SD)	25.2, 22.6–27.7	25.0, 22.3–27.7	25.4, 22.8–27.6	0.117	25.3, 22.6–27.9	25.2, 22.6–27.3	0.528	25.2, 22.6–27.8	25.2, 22.6–27.4	0.875	25.4 22.8–28.0	25.07 22.5–27.5	0.050[1]
Underweight	28 (3.2)	15 (53.6)	13 (46.4)	0.339	13 (46.4)	15 (53.6)	0.972	18 (64.3)	10 (35.7)	0.149	8 (28.6)	20 (71.4)	0.576
Normal weight	386 (44.2)	200 (51.8)	186 (48.2)		197 (51.0)	189 (49.0)		186 (48.2)	200 (51.8)		140 (37.3)	235 (62.7)	
Overweight	357 (40.9)	173 (48.5)	184 (51.5)		180 (50.4)	177 (49.6)		183 (51.3)	174 (48.7)		136 (39.0)	213 (61.0)	
Obese	102 (11.7)	43 (42.2)	59 (57.8)		52 (51.0)	50 (49.0)		43 (42.2)	59 (57.8)		41 (42.3)	56 (57.7)	
Missing	*5*										*2*	*1*	
BMI, median, IQR (mean: 25.3 ± 3.8 SD)	25.2, 22.6–27.7	25.0, 22.3–27.7	25.4, 22.8–27.6	0.117	25.3, 22.6–27.9	25.2, 22.6–27.3	0.528	25.2, 22.6–27.8	25.2, 22.6–27.4	0.875	25.4 22.8–28.0	25.07 22.5–27.5	0.050[1]
Underweight	28 (3.2)	15 (53.6)	13 (46.4)	0.339	13 (46.4)	15 (53.6)	0.972	18 (64.3)	10 (35.7)	0.149	8 (28.6)	20 (71.4)	0.576
Normal weight	386 (44.2)	200 (51.8)	186 (48.2)		197 (51.0)	189 (49.0)		186 (48.2)	200 (51.8)		140 (37.3)	235 (62.7)	
Overweight	357 (40.9)	173 (48.5)	184 (51.5)		180 (50.4)	177 (49.6)		183 (51.3)	174 (48.7)		136 (39.0)	213 (61.0)	
Obese	102 (11.7)	43 (42.2)	59 (57.8)		52 (51.0)	50 (49.0)		43 (42.2)	59 (57.8)		41 (42.3)	56 (57.7)	
Missing	*79*										*28*	*48*	
Chronic comorbidities				0.178			0.025			0.343			0.069
No	843 (89.3)	427 (50.7)	416 (49.3)		410 (48.6)	433 (51.4)		426 (50.5)	417 (49.5)		304 (37.1)	516 (62.9)	
Yes, at least one[3]	101 (10.7)	44 (43.6)	57 (56.4)		61 (60.4)	40 (39.6)		46 (45.5)	55 (54.5)		46 (46.5)	53 (53.5)	
Missing	*8*										*3*	*3*	
Tested PCR positive in the past 12 months				<0.001			0.004			<0.001			0.518
No	830 (87.8)	453 (54.6)	377 (45.4)		429 (51.7)	401 (48.3)		451 (54.3)	379 (45.7)		307 (37.9)	503 (62.1)	
Yes	115 (12.2)	21 (18.3)	94 (81.7)		43 (37.4)	72 (62.6)		22 (19.1)	93 (80.9)		46 (41.1)	66 (58.9)	
Missing	*7*											*3*	

[1] *P*-values extracted from the non-parametric Mann–Whitney U-test comparing distribution across groups.

[2] 99 with no education.

[3] 39 (4.1%), 66 (7.0%), 17 (1.8%), 2 (0.2%), 2 (0.2%), and 1 (0.1) with diabetes mellitus, high blood pressure, hyperlipidemia, heart problem, asthma/COPD disease, and cancer, respectively.

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TABLE 3 Distribution of the study population by their vaccination status and history of testing PCR-positive and their correlation with the four-tested immune response biomarkers.

	N = 952 (valid %)	Anti-S IgG *N* = 952		*P*-value	Anti-N IgG *N* = 952		*P*-value	Neutralizing IgG *N* = 952		*P*-value	T-cell response *N* = 925		*P*-value
		<357.5	≥357.5		<146.5	≥146.5		<172.0	≥172.0		Yes 353 (38.2%)	No 572 (61.8%)	
Vaccination against SARS-CoV-2				<0.001			<0.001			<0.001			0.001
Not vaccinated	13 (1.4)	9 (69.2)	4 (30.8)		9 (69.2)	4 (30.8)		9 (69.2)	4 (30.8)		4 (30.8)	9 (69.2)	
Only one dose	9 (0.9)	4 (44.4)	5 (55.6)		3 (33.3)	6 (66.7)		5 (55.6)	4 (44.4)		4 (44.4)	5 (55.6)	
Two doses	192 (20.2)	96 (50.0)	96 (50.0)		126 (65.6)	66 (34.4)		89 (46.4)	103 (53.6)		66 (35.5)	120 (64.5)	
One booster dose (three doses)	714 (75.2)	366 (51.3)	348 (48.7)		326 (45.7)	388 (54.3)		371 (52.0)	343 (48.0)		263 (37.8)	433 (62.1)	
Two booster dose (four doses)	21 (2.2)	1 (4.8)	20 (95.2)		12 (57.1)	9 (42.9)		1 (4.8)	20 (95.2)		16 (84.2)	3 (15.8)	
Missing	*3*											*2*	
Boosted vs. not boosted (n = 927)[1]				*1.00*			*<0.001*			*0.296*			*0.384*
Not boosted (two doses only) (mean duration: 159.6 ± 71.8 days)[2]	192 (20.7)	96 (50.0)	96 (50.0)		126 (65.6)	66 (34.4)		89 (46.4)	103 (53.6)		66 (35.5)	120 (64.5)	
Boosted (mean duration: 70.2 ± 24.8 days)[2]	735 (79.3)	367 (49.9)	368 (50.1)		338 (46.0)	397 (54.0)		372 (50.6)	363 (49.4)		279 (39.0)	436 (61.1)	
Vaccine type				<0.001			<0.001			<0.001			<0.001
BBIBP-CorV only (mean duration: 87.5 51.0 days)[2]	874 (92.2)	461 (52.7)	413 (47.3)		420 (48.1)	454 (51.9)		459 (52.5)	415 (47.5)		309 (36.4)	540 (63.6)	
rAd26-S + rAd5-S only (mean duration: 210 ± 13.0 days)[2]	14 (1.5)	0 (0.0)	14 (100)		14 (100)	0 (0.0)		0 (0.0)	14 (100)		5 (35.7)	9 (64.3)	
BNT162b2 only (mean duration: 106.7 ± 12.5 days)[2]	3 (0.3)	0 (0.0)	3 (100)		3 (100)	0 (0.0)		0 (0.0)	3 (100)		3 (100)	0 (0.0)	
Started BBIBP-CorV boosted with BNT162b2 (mean duration: 70.2 ± 69.2 days)[2]	38 (4.0)	5 (13.2)	33 (86.8)		24 (63.2)	14 (36.8)		5 (13.2)	33 (86.8)		27 (73.0)	10 (27.0)	

(Continued)

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TABLE 3 (Continued)

	N = 952 (valid %)	Anti-S IgG *N* = 952		*P*-value	Anti-N IgG *N* = 952		*P*-value	Neutralizing IgG *N* = 952		*P*-value	T-cell response *N* = 925		*P*-value
		<357.5	≥357.5		<146.5	≥146.5		<172.0	≥172.0		Yes 353 (38.2%)	No 572 (61.8%)	
Mixed vaccine type[3] (mean duration: 154.7 ± 64.4 days)[2]	7 (0.7)	1 (14.3)	6 (85.7)		6 (85.7)	1 (14.3)		2 (28.6)	5 (71.4)		5 (71.4)	2 (28.6)	
Not vaccinated (8) or the first dose was after blood collection (5)	13 (1.4)	9 (69.2)	4 (30.8)		9 (69.2)	4 (30.8)		9 (69.2)	4 (30.8)		4 (30.8)	9 (69.2)	
Missing	*3*											*2*	
Duration since last vaccine dose to blood collection, median (IQR), mean ± (SD)[4]	79.0 (56.0–96.0) 89.2 (±54.5) days			0.492		<0.001				0.873			<0.001
1–14 days	19 (2.0)	11 (57.9)	8 (42.1)		16 (84.2)	3 (15.8)		8 (42.1)	11 (57.9)		14 (77.8)	4 (22.2)	
15–30 days	34 (3.6)	14 (41.2)	20 (58.8)		16 (47.1)	18 (52.9)		16 (47.1)	18 (52.9)		16 (48.5)	17 (51.1)	
31–60 days	256 (27.0)	122 (47.7)	134 (52.3)		93 (36.3)	163 (63.7)		126 (49.2)	130 (50.8)		69 (28.0)	177 (72.0)	
61–295 days	624 (65.8)	319 (51.1)	305 (48.9)		339 (54.3)	285 (45.7)		315 (50.5)	309 (49.5)		248 (40.6)	363 (59.4)	
T-cell response (*n* = 925)				<0.001			0.975			<0.001			
Yes	353 (38.2%)	144 (40.7)	209 (59.3)		178 (50.3)	175 (49.7)		138 (39.0)	215 (61.0)		–	–	
No	572 (61.8%)	321 (56.1)	251 (43.9)		287 (50.2)	285 (49.8)		326 (57.0)	246 (43.0)		–	–	

[1]Excluding not vaccinated or received only one dose.
[2]Mean time duration post-last vaccine dose.
[3]Started with rAd26-S + rAd5-S and boosted with BNT162b2 or BBIBP-CorV (*n* = 5) or first dose was BBIBP-CorV and second dose BNT162b2 or rAd26-S + rAd5-S (*n* = 2).
[4]Included only received at least one vaccine dose.

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3.6. Having ≥median concentration of anti-S, anti-N, and neutralizing IgG antibodies by number and type of received vaccine doses at baseline

An increase in the number of received vaccine doses by one dose was significantly associated with increased odds of having, at the survey time, ≥median concentration of the anti-S [aOR, 1.34; 95% confidence interval (CI): 1.02–1.76], anti-N (aOR, 1.35; 95% CI: 1.03–1.75), and neutralizing IgG antibodies (aOR, 1.29; 95% CI: 1.00–1.66) and a T-cell response (aOR, 1.48; 95% CI: 1.12–1.95) (**Table 4**). Compared with participants who were primed with two doses, those boosted with only one more dose had significantly similar ($P > 0.05$) odds, whereas those who were boosted with two more doses had increased odds of having ≥median concentration of anti-S (aOR, 14.2; 95% CI: 1.85–109.4), neutralizing IgG (aOR, 13.6; 95% CI: 1.77–104.3), and T-cell response (aOR, 7.6; 95% CI: 2.09–27.8) (**Table 4**).

Within boosted with at least one dose, compared with boosting with only one dose, boosting with two doses was significantly associated with increased odds of having ≥median concentration of anti-S (aOR, 13.8; 95% CI: 1.78–106.5), neutralizing IgG antibodies (aOR, 13.2; 95% CI: 1.71–101.9), and T-cell response (aOR, 7.22; 95% CI: 1.99–26.5) although not with anti-N IgG antibody (aOR, 0.41; 95% CI: 0.16–1.08). Compared with priming and boosting with BBIBP-CorV, all of the 29 participants who were primed with BBIBP-CorV and boosted with BNT162b2 had ≥median concentration of anti-S and neutralizing IgG antibodies and 14.6-time increased odds of having a T-cell response (aOR, 14.63; 95% CI: 1.78–120.5). Every additional dose of the BBIBP-CorV vaccine was not significantly ($P > 0.05$) associated with any observed increased odds of having ≥median concentration of the measured three immunoglobulin types or with T-cell response. A similar finding was observed when comparing BBIBP-CorV boosted with BBIBP-CorV-non-boosted participants (**Table 6**).

4. Discussion

In a population with a history of natural exposure to SARS-CoV-2 before vaccination, we investigated the association between the number and type of inoculated SARS-CoV-2 vaccine doses and the concentration of the induced immune biomarkers (anti-S, anti-N, and neutralizing IgG antibodies and T-cell response). The antibody response was tested and detected in all participants; however, T-cell response was detected in only 38.2% of the participants. Having above the median concentration of the three measured IgG antibodies and the T-cell response was associated with being primed or boosted with mRNA-based vaccines and with inoculation with two but not one booster dose. The T-cell response was significantly associated with having above the median concentration of anti-S and neutralizing IgG antibodies. Furthermore, the T-cell response was significantly associated with increased odds of being boosted with two doses although not being boosted with only one dose of the SARS-CoV-2 vaccine. Among only-BBIBP-CorV–vaccinated participants, no difference in the measured four biomarkers was observed between boosted with only one BBIBP-CorV dose and with two BBIBP-CorV doses.

A significant mean age-related difference in all the studied immune biomarkers was observed. The study participants with a mean age of 36 years had a higher concentration (≥median) of IgG and NAbs as well as responding T-cells. It was unexpected that increasing age would be associated with greater SARS-CoV-2 IgG antibodies (13). However, this cohort only included individuals in the middle-aged working group (mean age: 35.5 ± 8.4 years). It has been reported that middle-aged adults in general have the most significant immune response (14). No significant difference in the concentration of the measured IgG antibodies or T-cell response by the BMI status of this population was noted. Although obesity has been established as a risk factor for mortality from COVID-19 (15), there is no established evidence of the association between the immune response to natural infection or vaccination and BMI. Never-smoking participants had a significantly higher anti-S IgG antibody concentration than those who currently smoke. The insufficient immune response among smokers is consistent with the established evidence that current smokers had an increased risk of severe COVID-19 disease (16).

In this study, participants who tested PCR-positive in the past 12 months had a higher concentration of the measured three IgG antibodies although a lower proportion of participants had detectable T-cell response than those with a negative PCR test. During the first few months, after SARS-CoV-2 infection, the T-cell response typically wanes at a slower rate than IgG antibodies (17–20). In this study, the Qiagen IGRA was utilized to study T-cell response. This assay measures IFNg release from activated T-cells upon stimulation and is a very general measure of T-cell function and may have lower sensitivity than other methods of assessing T-cell functions. Other studies assessing T-cell functions in vaccinated populations demonstrated approximately 100% of individuals have detectable responding T-cells 6 months after at least two vaccine doses; therefore, the rate of 38% in seropositive individuals at baseline and approximately all vaccinated individuals (79% with three doses) is remarkably low. Some of this may be related to the type of vaccine administered as most of our study population received the BBIBP-CorV vaccine. Typically, the presence of T-cells and antibodies is associated with the successful resolution of average cases of SARS-CoV-2; however, high heterogeneity has been observed in studies of adaptive immunity in patients with a variable magnitude of antibody responses to SARS-CoV-2, as well as in the magnitude of SARS-CoV-2–specific CD4+ and CD8+ T-cell responses (21, 22). We still have limited data on the correlation between antibody responses to natural infection or vaccination and T-cell responses measured using the QFN SARS assay. Nevertheless, data originating from one study suggest that

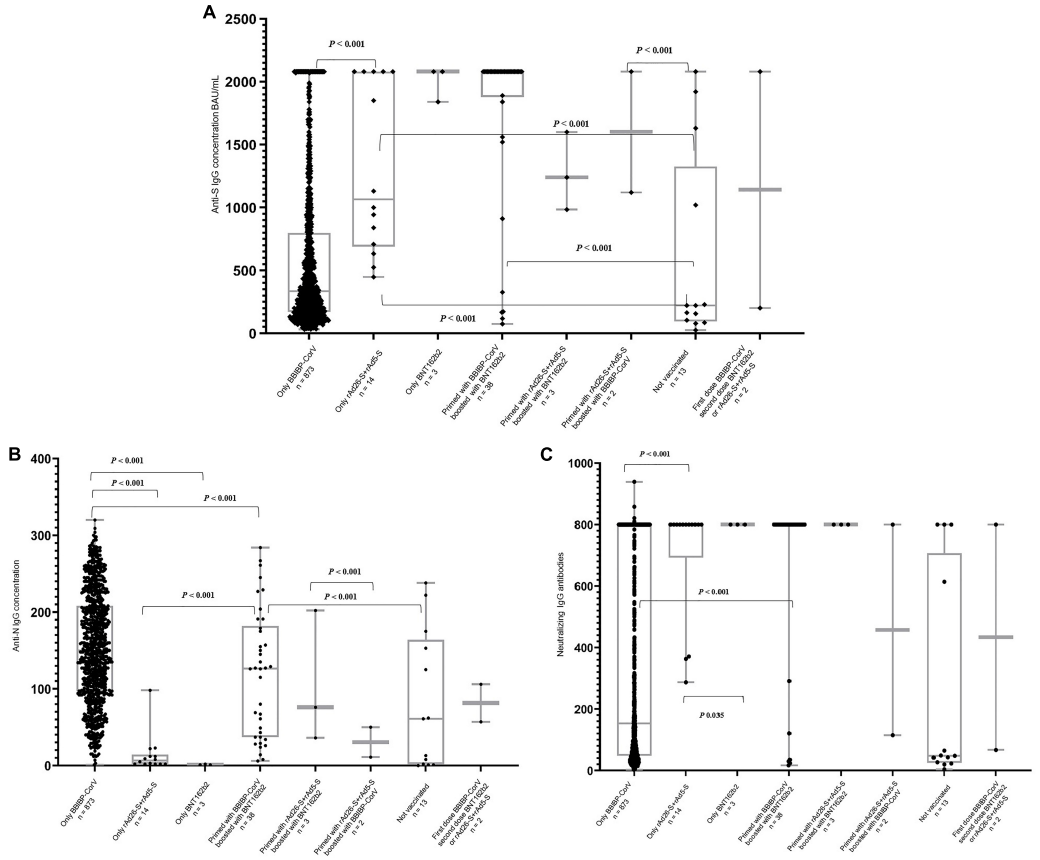

FIGURE 1
Distribution of the anti-S **(A)**, anti-N **(B)**, and neutralizing **(C)** IgG antibodies concentration by type of the received SARS-CoV-2 vaccines regardless of the total number of doses. *P*-values extracted from the Independent-Samples Kruskal–Wallis Test.

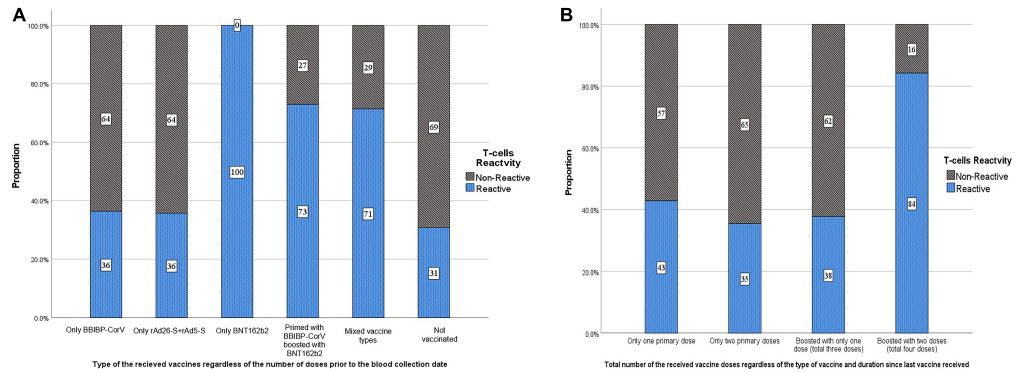

FIGURE 2
Distribution of the study participants by T-cells reactivity (response) according to the **(A)** type and **(B)** total number of the received SARS-CoV-2 vaccine doses.

in individuals vaccinated with mRNA vaccines, both humoral and cellular responses are detectable using the SARS-CoV-2 serological assay and the QFN SARS assay; however, the extent of correlation is inconclusive (23).

The post-vaccination SARS-CoV-2–induced immune response varied by the number and type of vaccine doses received. An increase in the number of vaccine doses by one dose was associated with increased odds of having more than the median concentration of the IgG antibodies and with producing responding T-cells, particularly among those who were boosted with two doses. This increased immune potency by increasing the number of vaccine doses supports

TABLE 4 Crude (OR) and adjusted odds ratio (aOR) of the association between every dose increase in number of anti-SARS-CoV-2 vaccine doses and boosting status with having ≥median concentration of anti-S IgG (≥357.5 BAU/ml), anti-N IgG (≥146.5 COI), and neutralizing IgG (≥172.0 AU/ml) antibodies and having a T-cell response.

	Anti-S IgG Abs (≥median vs. <median concentration)		Anti-N IgG Abs (≥median vs. <median concentration)		Neutralizing IgG Abs (≥median vs. <median concentration)		T-cell response (Yes vs. No)	
	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)
Additional one vaccine dose	1.22 (0.98–1.53)	1.34 (1.02–1.76)*	1.55 (1.22–1.97)***	1.35 (1.03–1.75)*	1.15 (0.92–1.44)	1.29 (1.00–1.66)*	1.26 (0.99–1.60)	1.48 (1.12–1.95)**
Booster status[1]								
Not boosted (primed with only two doses)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Boosted once (received three doses)	0.95 (0.69–130)	0.90 (0.65–1.25)	2.28 (1.64–2.18)***	2.17 (1.54–3.1)***	0.80 (0.59–1.10)	0.78 (0.56–1.09)	1.10 (0.80–1.55)	1.07 (0.76–1.51)
Boosted twice (received four doses)	20.0 (2.63–152.0)**	14.20 (1.85–109.4)*	1.43 (0.57–3.57)	1.27 (0.48–3.36)	17.30 (2.27–131.36)***	13.60 (1.77–104.3)*	9.70 (2.272–34.50)***	7.62 (2.09–27.87)**
Vaccine type–only vaccinated[2]								
BBIBP-CorV only	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Primed with BBIBP-CorV boosted with BNT162b2	7.39 (2.86–19.09)***	7.57 (2.61–21.94)***	0.54 (0.27–1.06)	0.48 (0.23–1.0)	7.32 (2.83–18.9)***	7.86 (2.71–22.83)***	4.72 (2.25–9.90)***	4.28 (1.93–9.50)***
rAd26-S + rAd5-S only	NA	NA	NA	NA	NA	NA	0.97 (0.32–2.92)	1.13 (0.36–3.60)
Others[3]	10.01 (1.27–79.8)*	9.95 (1.23–80.65)*	0.10 (0.01–0.81)*	0.14 (0.02–1.1)	4.41 (0.94–21.0)	4.14 (0.85–20.14)	7.00 (1.48–33.12)*	7.90 (1.63–38.6)*
Others[4]	25.74 (3.46–191.41)**	29.52 (3.9–223.9)***	0.04 (0.005–0.30)**	0.06 (0.01–0.48)**	25.73 (3.50–191.41)***	13.52 (3.06–59.2)***	2.07 (0.91–4.70)	2.46 (1.03–5.9)*

Adjusted odds ratio for age (continuous), BMI (continuous), type of vaccine, smoking status, chronic comorbidity, time duration since last vaccine dose, and history of previous infection (PCR+).

[1]Adjusted also for type of vaccine.

[2]Adjusted also for total number of vaccine doses.

[3]Three of them received only BNT162b2, and the rest received heterogeneous vaccine types.

[4]Three of them received only BNT162b2, and 14 received only rAd26-S + rAd5-S. The rest received heterogeneous vaccine types.

***$P < 0.001$, **$P = 0.002$, and *$P < 0.005$.

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TABLE 5 Correlation between history of SARS-CoV-2 infection during the past 12 months and anti-S, anti-N, and neutralizing IgG antibody concentration and T-cell response during follow-up.

Contracted COVID-19 in the past 12 months	Anti-S IgG AbsBAU/ml		Anti-N IgG AbsCOI		Neutralizing IgG AbsAU/ml		T-cell response		*P*-value
	Mean ± SD	Mean difference *P*-value	Mean difference	*P*-value	Mean difference	*P*-value	Yes	No	
No	594.4 ± 623.9	438.3, $P < 0.001$	144.8 ± 75.2	25.3, $P < 0.001$	330.4 ± 332.4	266.2, $P < 0.001$	37.9%	62.1%	0.518
Yes	1,032.7 ± 647.6		170.1 ± 68.01		596.3 ± 296.7		41.1%	58.9%	

the significance of boosting susceptible populations to avoid exposure and re-exposure, thereby expediting the process of pandemic containment.

Regarding the association between the type of the SARS-CoV-2 vaccine and the immune status, the results showed that the levels of the measured immune biomarkers varied according to the different types of studied vaccines, even after controlling for potential confounders, including the number of vaccine doses received, exposure to SARS-CoV-2 in the past 12 months, the time since the last exposure, age, and commodities. Populations who received only or boosted with an inactivated whole virus-based vaccine (BBIBP-CorV) were less likely to have a high anti-S or neutralizing IgG antibody concentration than those who received only or boosted with at least one dose of an mRNA-based (BNT162b2) or adenovirus-based (rAd26-S + rAd5-S) vaccine. This is also consistent with the findings of other studies, wherein a significant boost of anti-S IgG antibody after the second dose of the BNT162b2 vaccine was observed (13, 24). A previous study reported that the BNT162b2 vaccine is associated with producing a high peak of anti-S IgG responses (13). In fact, expediting the time in achieving high anti-S and neutralizing IgG antibody concentrations would play a significant role in protecting individuals and saving lives amid highly transmissible pandemics. This observed expedited immune response following mRNA-based vaccination compared with other vaccine types explains the reported high effectiveness of such vaccine types in preventing infection or disease progression in different population groups (25–28). Nonetheless, in the initial stages of the pandemic when no vaccines were available, there is no doubt that the emergency authorization and use of non-mRNA-based vaccines played a significant role in reducing the risk of viral transmission and alleviating the burden of the pandemic.

Although T-cells are generated following vaccination, they usually contract from the peak within 3 months (29). In this study, more participants with a T-cell response were within the first 2 weeks after their last vaccine dose, and significantly more participants who had no T-cell response were more than 1 month after their last vaccine dose. This seems to contradict what has been previously reported in studies where T-cell responses were better 6 months following vaccination (30), and T-cell responses decline at a slower rate than the antibody levels (31). However, investigating the variation in T-cell response according to the number and type of vaccine doses, populations boosted with one or two doses or those who received an inactivated whole virus vaccine type (BBIBP-CorV) and subsequently boosted with BNT162b2 vaccine were more likely to maintain T-cell response than their counterparts. It was previously reported that the IFNg-secreting SARS-CoV-2–specific T-cells were associated with a milder form of COVID-19

TABLE 6 Crude (OR) and adjusted odds ratio (aOR) of the association between every dose increase in the number of anti-SARS-CoV-2 vaccine doses and boosting status with having ≥median concentration of anti-S IgG (≥357.5 BAU/ml), anti-N IgG (≥146.5 COI), and neutralizing IgG (≥172.0 AU/ml) antibodies and having a T-cell response.

	Anti-S IgG Abs (≥median vs. <median concentration)		Anti-N IgG Abs (≥median vs. <median concentration)		Neutralizing IgG Abs (≥median vs. <median concentration)		T-cell response (Yes vs. No)	
	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)
Booster status								
Boosted once (received three doses)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Boosted twice (received four doses)	21.1 (2.82–158.0)**	13.8 (1.78–106.54)*	0.53 (0.26–1.51)	0.41 (0.16–1.08)	21.70 (2.90–162.53)**	13.18 (1.71–101.9)*	8.78 (2.53–30.42)***	7.22 (1.99–26.25)**
Vaccine type–only boosted[1]								
Primed and boosted with BBIBP-CorV (n = 704)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Primed with BBIBP-CorV boosted with BNT162b2	All the 29 were with ≥median concentration		0.50 (0.23–1.08)	–	All the 29 were with ≥median concentration		10.14 (3.48–29.56)***	14.63 (1.78–120.47)*
Others[3]	–	–	–		–	–		
Only-BBIBP-CorV–vaccinated								
Additional one vaccine dose	1.09 (0.80–1.55)	0.87 (0.57–1.33)	1.57 (1.15–2.16)**	1.03 (0.68–1.55)	0.97 (0.72–1.33)	0.75 (0.49–1.13)	1.14 (0.82–1.56)	1.24 (0.79–1.94)
Booster status								
Not boosted (primed with only two doses)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Boosted once (received three doses)	1.18 (0.94–1.67)	0.99 (0.61–1.59)	1.93 (1.36–2.73)***	1.24 (0.77–1.97)	0.97 (0.69–1.37)	0.71 (0.44–1.14)	1.23 (0.85–1.78)	1.35 (0.82–2.24)
Boosted twice (received four doses)	Only one case							

Adjusted odds ratio for age (continuous), BMI (continuous), type of vaccine (except for only-BBIBP-CorV–vaccinated), smoking status, chronic comorbidity, the time duration since the last vaccine dose, and history of previous infection (PCR+).

[1]Adjusted also for the total number of vaccine doses.

[3]Only one individual primed with rAd26-S + rAd5-S and boosted with one BBIBP-CorV dose.

***$P < 0.001$, **$P = 0.002$, and *$P < 0.005$.

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disease (32). SARS-CoV-2–specific T-cells are elicited during acute COVID-19, and T-cell memory pools durability sustain for up to 8 months. Following vaccination, a 10-fold increase in IFNg-secreting T-cells was observed. Data on SARS-CoV-2–specific CD4+ and CD8+ T-cells clearly demonstrate the generation of long-term immunological epitope-specific memory T-cell pools following mRNA COVID-19 vaccination (5). During vaccination with mRNA-based vaccine-induced T-cells, after the first vaccination, with peak responses after the second immunization, memory CD4+ T-cells were detectable in 85–100% of mRNA-based vaccine recipients at 6 months after immunization (31).

This study had some limitations. First, studying only previously naturally exposed middle-aged male participants without having a comparison group of never-naturally exposed and female gender would limit the generalizability and external validity of the present findings to the wider population. Second, the lack of baseline information prior to vaccination and during the follow-up on the concentration of the measured immune response biomarkers may limit the observed difference between the number/type of the received vaccines and the status of the measured immune biomarkers. Lastly, the small number of participants within a specific vaccine type or the number of vaccine dose groups also imposed a limitation on the present findings. Nevertheless, despite these limitations and the limitations of the retrospective nature of the study design (potential effect of unmeasured or uncontrolled confounding), the present study was unique in terms of studying several types of commonly authorized and used SARS-CoV-2 vaccines as well as the number of vaccine doses received by our studied population. Moreover, this study was unique in terms of investigating the post-vaccination immune response of three humoral immune biomarkers in the same population in addition to the T-cell response. Several studies have investigated the association between only one or two SARS-CoV-2 vaccines with only one or two immune response biomarkers (13, 33, 34).

5. Conclusion

Inducing humoral and T-cell response varies with the type and number of vaccine doses received as well as with mixing different types of vaccine platforms. To induce a high immune response and expedite achieving a high concentration of humoral immunity that plays a significant role in neutralizing viral particles, boosting a population's immunity with at least one booster dose is critical. Priming or boosting with mRNA-based vaccines was more potent for inducing high levels of humoral and T-cell response compared with other vaccine types. Present findings can inform policymakers and the public health system in designing future vaccination campaigns and allocating vaccination resources in the best way to achieve the accepted immune levels and protect populations.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the corresponding author. However, restrictions apply to the availability of the data. Data will be made available upon reasonable request and with permission of the ethical approval provider.

Ethics statement

This study was reviewed and approved by the UAE National COVID-19 Research Ethics Committee (reference number: DOH/CVDC/2021/856 and amendment number: DOH/CVDC/2021/1703). From each participant, consent to collect survey information, blood sample, and nasopharyngeal swab, was obtained. The participants provided their written informed consent to participate in this study.

Author contributions

RHA, SA, and LAW designed and conceived the study. RHA and LAA analyzed and interpreted the data. RA wrote the manuscript, and all co-authors provided input. All authors contributed to questionnaire development, and data collection, extracted the data from medical records, and coded the data, read, and approved the final manuscript.

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Conflict of interest

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

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Role of mTOR inhibitor in the cellular and humoral immune response to a booster dose of SARS-CoV-2 mRNA-1273 vaccine in kidney transplant recipients

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Background: Immunocompromised patients have an increased risk of developing severe COVID disease, as well as a tendency to suboptimal responses to vaccines. The objective of this study was to evaluate the specific cellular and humoral adaptive immune responses of a cohort of kidney transplant recipients (KTR) after 3 doses of mRNA-1273 vaccine and to determinate the main factors involved.

Methods: Prospective observational study in 221 KTR (149 non infected), 55 healthy volunteers (HV) and 23 dialysis patients (DP). We evaluated anti-spike (by quantitative chemiluminescence immunoassay) and anti-nucleocapsid IgG (ELISA), percentage of $TCD4^+$ and $TCD8^+$ lymphocytes producing IFNγ against S-protein by intracellular flow cytometry after Spike-specific 15-mer peptide stimulation and serum neutralizing activity (competitive ELISA) at baseline and after vaccination.

Results: Among COVID-19 *naïve* KTR, 54.2% developed cellular and humoral response after the third dose (vs 100% in DP and 91.7% in HV), 18% only showed cell-mediated response, 22.2% exclusively antibody response and 5.6% none. A correlation of neutralizing activity with both the IgG titer (r=0.485, p<0.001) and the percentage of S-protein–specific IFNγ–producing CD8-T cells (r=0.198, p=0.049) was observed. Factors related to the humoral response in naïve KTR were: lymphocytes count pre-vaccination >1000/mm^3 [4.68 (1.72-12.73, p=0.003], eGFR>30 mL/min [7.34(2.72-19.84), p<0.001], mTOR inhibitors [6.40 (1.37-29.86), p=0.018]. Infected KTR developed a stronger serologic response than naïve patients (96.8 vs 75.2%, p<0.001).

Conclusions: KTR presented poor cellular and humoral immune responses following vaccination with mRNA-1273. The immunosuppression degree and kidney function of these patients play an important role, but the only modifiable factor with a high impact on humoral immunogenicity after a booster dose was an immunosuppressive therapy including a mTOR inhibitor. Clinical trials are required to confirm these results.

KEYWORDS

kidney transplantation, SARS-CoV-2 vaccine, immune response, COVID-19, mTOR

Introduction

The coronavirus disease 2019 (COVID-19) pandemic has caused more than 6 million deaths worldwide (1), with immunocompromised individuals being particularly affected by severe conditions of this disease (2). Vaccination against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been revealed as the most effective measure to control this pandemic, leading to a marked reduction in infections, hospital admissions and mortality (3). Individuals with COVID-19 who have undergone solid organ transplant (SOT) experience higher mortality and prolonged viral shedding compared with the general population (4–7). However, recipients of SOT were excluded from the initial licensing trials of these vaccines. Kidney transplant recipients (KTR), who undergo pharmacological immunosuppression as basic therapy to prevent transplant rejection, are at risk of a defective response to vaccination, as already occurs with other vaccines (8). In contrast to immunocompetent participants in vaccine trials (9), a low proportion of SOT recipients mount a positive antibody response to the second dose of SARS-CoV-2 messenger RNA (mRNA) vaccines. Studies have reported varying results in antibody response rates of approximately 5%–50% after two doses of mRNA vaccine in KTR (10–15). Due to this low response, an additional primary shot (third dose of mRNA COVID-19 vaccine for those receiving BNT162b2 or a booster dose of mRNA-1273) was recommended. Several published studies have reported the humoral immunogenicity of a three-dose vaccination schedule, but only a few have assessed the contribution of the cellular arm to vaccine-mediated protection (16–18). These results would allow us to determine if this regimen is sufficient to achieve a generalized response in these patients and would help us discern what type of immunosuppressive agents could cause a greater increase in the vaccine response.

Abbreviations: ACE2, angiotensin-converting enzyme 2; BAU, binding antibody units; CI, confidence interval; COVID-19, coronavirus disease 2019; CNI, calcineurine inhibitors; DP, dialysis patients; eGFR, estimated glomerular filtration rate; HV, healthy volunteers; IFNy, Interferon gamma; IgG, immunoglobulin G; IQR, interquartile range; KTR, kidney transplant recipients; MPA, Mycophenolic acid; mRNA, messenger RNA; mTORi: mammalian Target Of Rapamycin inhibitors; OR, odds ratio; PBMC, peripheral blood mononuclear cells; RBD, receptor-binding domain; RT, room temperature; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation; SOT, solid organ transplant.

In this study, we assessed antibody and cellular response after the third dose of mRNA-1273 vaccine in a cohort of KTR. We focused on the analysis of the cellular response and the neutralization capacity of the patients' sera after the third dose.

Methods

Study design and sample collection

We performed a prospective study of a cohort of 221 KTR who received 3 doses of mRNA-1273 vaccine (Moderna-NIAID). Two cohorts with the same vaccination regimen, 55 healthy volunteers (HV) and 23 dialysis patients (DP), were also included as internal controls for the study. Patients and controls who became infected previously or during follow-up were excluded from the analysis of vaccine effectiveness.

We collected blood samples prior to vaccination (P0), 15 days (P1) and three months (P2) after the administration of the second dose, and 2 months after the third dose (P3).

The study was performed in accordance with the ethical standards as laid down in the Declarations of Helsinki and approved by the local ethics committee. Written informed consent was obtained from all subjects before the blood samples were taken.

Patients

All KTR followed up in outpatient Kidney Transplantation Department between March 1 and April 15, 2021 and wanted to be vaccinated were included. All patients received the same kind of vaccine, mRNA-1273: a first and second dose (100 μg each dose) between April 20 and Maye 30, 2021 and third vaccine dose (100 μg) between September 20, and October 30, 2021. Inclusion criteria were: (1) being >18 years old, (2) History of kidney transplant for at least 6 months, and (3) Approval of informed consent to the study. As exclusion criteria; (1) having a history of malignancy, (2) SOT different from kidney, (3) primary immunodeficiency disease, (4) having a previous history of allergy to any inactivated vaccine, and (4) having an unexplained 37.5°C fever or any symptoms of infection.

Controls

To characterize the impact of posttransplant immunosuppression on the ability of vaccination to elicit SARS-CoV-2–specific immunity,

we used a control group of 78 non-immunocompromised, 55 healthy volunteers (HV) and 23 dialysis patients (DP). HV were healthcare workers who received mRNA-1273 and wanted to participate in the study. DP group was made up of 12 patient on hemodialysis and 11 on peritoneal dialysis who also received Moderna-NIAID vaccine. We obtained samples from HV at equivalent time intervals under the same conditions as KTR. For DP, only the sample corresponding to the third dose (P3) was obtained.

For both patients and controls, meeting any of the exclusion criteria throughout the study implied the individual's exit from the study, as shown in the study flowchart (Figure 1).

SARS-CoV-2 serology

Sera were obtained and stored at −80°C until use. Quantitative SARS-CoV-2 anti-spike (S) IgG test (SARS-CoV-2 IgG II Quant; Abbott Diagnostics) was performed in the Abbott Architect device in accordance with the manufacturer's recommendations This antibody test is based on the principle of chemiluminescence microparticle immunoassay test. As the test gives data as AU/mL units, we applied a conversion factor in order to ease the comparison with other standardized serologic assessments, and quantitative results are given in BAU/mL (binding antibody units per mL, BAU/mL=AU/mL x 0.142). Samples with BAU/mL ≥7.1 are considered positive for SARS-CoV-2 IgG antibodies, the manufacturer-suggested thresholds (detection range, 2.8–16480 BAU/mL; positive agreement, 99.4%; negative agreement, 99.6%).

To determine which subjects had been infected prior to vaccination or in periods between sample collections, the presence of anti-Nucleoprotein (N) antibodies was tested by ELISA. Briefly, 96-well flat-bottom plates were coated with 2 µg/mL SARS-CoV-2 N-protein and 1:100 dilutions of the sera were incubated for 30 minutes at room temperature (RT), washed 5 times and detected with a goat anti-human IgG HRP-conjugated antibody (ThermoFisher Scientific). ELISA was developed with TMB and HCl and measured at 430 nm. To establish the cut-off of anti-N antibodies, we used the value of the mean plus twice the standard deviation (95% CI) of the absorbance value at 430nm of 8 pre-pandemic sera (PCR negative, anti-S IgG negative and with no COVID-19 compatible symptoms) per ELISA plate.

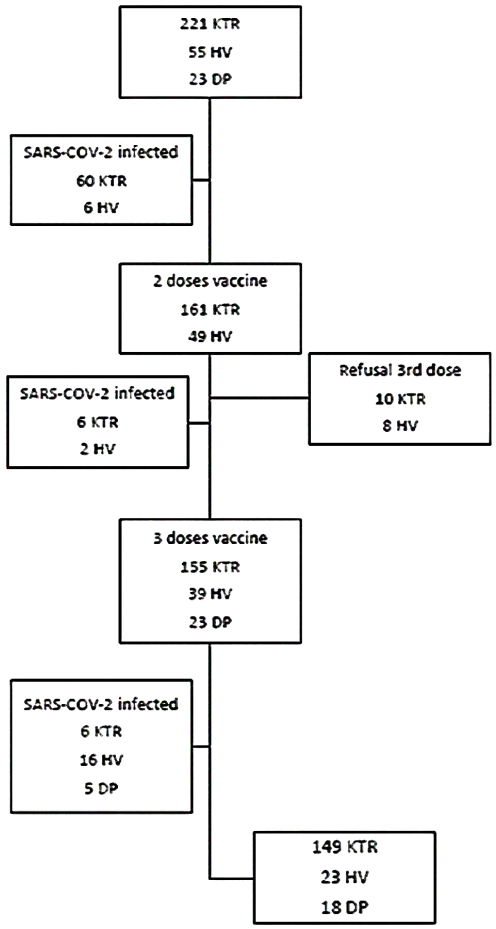

FIGURE 1
Study flowchart. Patients and controls who were infected or refused to the third dose were excluded from the analysis of vaccine effectiveness. KTR, Kidney transplant recipients; HV, Healthy volunteers; DP, Dialysis patients.

Cell-mediated immunity

Peripheral blood mononuclear cells (PBMC) were obtained by density gradient isolation with Lymphocyte Separation Medium reagent (Corning Life Sciences). Cells were maintained in RPMI 1640 (Corning Life Sciences) supplemented with 10% FBS (Gibco), 100 mg/mL streptomycin (Gibco), and 100U/mL penicillin (Gibco). Cells were stimulated with 15-mer overlapping peptide-pool covering immunodominant domain surface Spike-protein (PepTivator® SARS-CoV-2 Prot-S, Miltenyi Biotec) or with 10% DMSO for 6 hours at 37°C and 5% CO2 in the presence of Brefeldin A (Thermo-Scientific) during the last 4 hours of the assay. After stimulation, surface staining with anti-CD3-FITC (UCHT1), CD4-PE (OKT4) and CD8-PE/Cy7 (SK1) antibodies (Biolegend) was performed for 30 minutes at 4°C. After staining, they were fixed with 4% PFA for 30 min and permeabilized with 0.05% PBS Tween-20 (Thermo-Scientific) for 30 min at RT. Cells were stained with anti-IFNγ-APC antibody (B27) (Biolegend) for 30 min at RT. Staining was acquired on a FACSCalibur cytometer and analyzed with FlowJo v10 software (BD Life Sciences). Since the lower limit of detection for conventional flow cytometry techniques is ~0.02 to 0.05%, we set 0.05% as the lower limit for considering the cellular response positive for both T-CD4 and T-CD8.

ACE2-RBD neutralizing activity of the sera

To determine the ACE2-Spike (RBD) neutralizing activity of sera, we employed a competitive ELISA assay. 96 well-plate were coated with 2 µg/mL of recombinant RBD (Miltenyi Biotec) for 16h and incubated with 100 µL of the sera (1:25) for 1h at RT. After incubation, the plate was washed 5 times with 0.05% PBS-Tween and 0.5 µg/mL of recombinant ACE2-biotin (Miltenyi Biotec) was added at 37°C for 1 hour. After a washing step, a final incubation with 1µg/mL of streptavidin-HRP (Biolegend) was performed for 1 hour at 37°C. The interaction was retrieved with TMB and HCl (Thermo-Scientific) and absorbance is measured at 450 nm. To establish the maximum ACE2-RBD interaction,

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a pre-pandemic serum previously tested negative for anti-Spike antibody (0 BAU/mL) was used. From the maximum absorbance data, the decrease in signal of each serum with respect to the maximum was extrapolated to obtain the percentage neutralization data of each sample.

Statistical analysis

Quantitative data were reported as mean and standard deviation (SD) or the median with interquartile range (IQR). Qualitative variables were expressed as absolute and relative frequencies.

Categorical variables were compared using the $\chi 2$ test. The Student's *t* test or the Mann-Whitney U test was used for continuous variables. Repeated measures were compared with the Wilcoxon signed-rank test or the McNemar test, as appropriate. Correlations between continuous variables were evaluated with Spearman's rho. Logistic regression served for assessment of factors related to immune response. All factors showing a univariate association with a *p*-value<0.100 were entered in the final multivariate model. All calculations were performed using GraphPad Prism version 8.0 (GraphPad) and SPSS version 25.0 (IBM). $P < 0.05$ (2-sided) was considered statistically significant.

Results

Study population

Antibody response to the vaccine was determined in a total of 221 KTR and cellular immunity in 213 of them (viable T cells could not be obtained in 8 samples). Fifty-two patients (23.5%) had a history of prior COVID-19 diagnosed 12 months ago, 11 of them met criteria for severe COVID-19. Eight KTR became infected between the first and second dose of vaccine, six patients after the second dose and six after the third (9%), all of them with mild symptoms (Figure 1). The main clinical, analytic, and demographic characteristics of this cohort, *naïve* and infected KTR, are described in Table 1S. There were not significant differences in laboratory parameters during follow up in both groups (data not shown).

Non transplant control group made up of HV were younger than KTR [30(± 8) vs 57(± 15) years, p<0.001], while DP were similar in age [56(± 13) years]. The incidence of SARS-CoV-2 infection, assessed by PCR or positivity against N-protein in HV was 11% (6/55) at baseline, 4% (2/49) after the second dose and 34% (16/47) after the third dose and 5/23 in DP after the third dose (Figure 1). Patients and controls who were infected were also analyzed to see if there were differences between them, but they were excluded from the analysis of vaccine effectiveness.

SARS-CoV-2–specific cell-mediated immunity and correlation with total and neutralizing titers against the S-protein after the third vaccine dose

The proportion of positive S-protein–specific cell-mediated response after the third dose were lower in KTR compared to DP and HV ones: 59.3% of KTR showed reactive CD4-T cells vs 88.2% of HV and 100% of DP (p=0.008); 66% of KTR showed reactive CD8-T cells vs 100% in DP and 91.7% in HV (p=0.004). CD4 or CD8 reactivity was present in 76.7% of KTR vs 100% in DP and 91.7% in VH, p=0.033 (Figures 2A, B). There were not significant differences in the intensity of cellular response between groups (Figure 2C).

Analyzing humoral and cellular response in COVID-19 naïve KTR, 54.2% (78/144) had both responses, 18% (26/144) mounted cell-mediated responses without IgG response, 22.2% (32/144) only developed antibody response and 5.6% (8/144) did not develop any response.

In COVID-19 *naïve* KTR with positive cellular response, there was a significant correlation between the percentage of S-protein–specific IFNγ–producing CD8-T cells and total anti-S IgG titers after the third dose (P3: *r*=0.210, p=0.043) and between the percentage of S-protein–specific IFNγ–producing CD8-T cells and neutralizing titers against the S-protein (P3: *r*=0.307, p=0.004). No correlation was found between CD4-T cells and humoral response. In the univariate analysis we did not find any parameter that could predict the cellular response (Table 1).

SARS-CoV-2 IgG antibody response and serum ACE2-RBD neutralizing activity. Strong impact of immunosuppressive therapy

</u>Using the manufacturer-suggested thresholds, the rate of IgG seropositivity in COVID-19 *naïve* KTR was 44.1% (74/161) at 15 days (P1) and 58% (90/155) at 3 months (P2) after the second dose. This rate increased to 76.5% (114/149) at 2 months after the booster dose (P3). Significant differences in the humoral response were observed with the control group: 100% positivity in the three points in HV and 100% in DP after the third dose (p<0.001) (Figure 3A). Likewise, the serum anti-spiked IgG titers were higher in HV compared to KTR after the second (p<0.001) and the third dose (p<0.001) (Figure 3B). The evolution of IgG titers also differed between the control population and the KTRs (p<0.001). Some KTR showed a delay in antibody production as seen on (Supplementary Figure 1).

The factors associated with humoral immunogenicity in KTR are describe in Tables 1, 2.

Antibody titers were correlated to kidney function measured by estimated glomerular filtration rate (eGFR) (P1: *r*=0.333, P2: *r*=0.482, P3: *r*=0.550, p<0.001). KTR with better renal function pre-vaccination (eGFR>30 mL/min/1.73 m^2) achieved a higher humoral response rate than those with lower renal function after the second and third dose (P1: 48.3 vs 27.3%, p=0.032; P2: 63.5 vs 34.4%, p=0.003; P3: 84.5 vs 42.4%, p<0.001).

On the other hand, those patients with **lymphocyte count** greater than 1000/mm^3 were almost five times more likely to respond after the second [P2: OR 4.46 (1.64-12.13 CI), p=0.003] and third dose [P3: OR 4.68 (1.72-12.73 CI), p=0.003]. A correlation between lymphocyte count and antibody titers were also detected (P1: *r*=0.185, p=0.017; P2: *r*=0.263, p=0.001, P3: *r*=0.284, p<0.001).

Finally, the immunosuppressive therapy also had an influence on the antibody as can be seen in Tables 1, 2. Non-responders *after the second dose* were more frequently under MPA (P1: 82.9 vs 33.9%,

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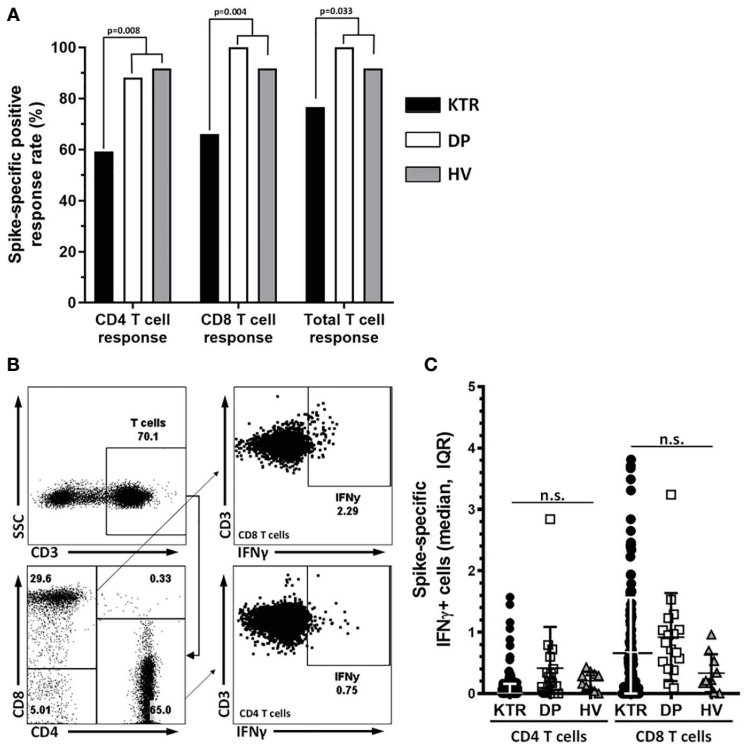

FIGURE 2
Cellular response rate after the third dose of vaccine. **(A)** Cellular response rate in CD4, CD8 and total T lymphocytes. A spike-specific response of IFNγ producing T-cells (%) >0.05 was consider as positive. The Y axis represents the response rate in CD4, CD8 and total T lymphocytes, for each subgroup of patients. **(B)** DotPlots show the gatting strategy to analyze the response to interferon gamma after stimulation of PBMC with the protein S peptide pool in CD8 and CD4 T cells. **(C)** Graph show Spike-specific IFNγ+ cells in CD4 and CD8 T cells median and IQR of KTR, DP and HV after three doses of vaccination. The median percentage of IFNγ-producing CD4-T cells were 0.17% (0-0.90 IQR) in KTR, 0.20% (0.07-0.50 IQR) in DP and 0.29% (0.09-0.35 IQR) in HV (p=0.820). In the case of CD8-T cell response the median percentages were: 0.41% (0-1.06 IQR) in KTR, 0.83% (0.46-1.13 IQR) in DP and 0.23% (0.17-0.65 IQR) in HV (p=0.054). n.s., non significant.

p<0.001; P2: 82.9 vs 50.4%, p=0.001; P3: 91.9 vs 77.5%, p=0.053) or had previously received thymoglobulin (P1: 52.3 vs 38.2%, p=0.082; P2: 71.9 vs 47.7%, p=0.003; P3: 74.3 vs 50.9%, p=0.016), whereas responders *after the booster dose* were more likely to receive mTOR inhibitor (mTORi) (P1: 76.7 vs 32.3%, p<0.001; P2: 81.4 vs 48.3%, p<0.001; P3: 94.6 vs 68.2%, p=0.001) (Figure 4A). This protective effect of mTORi was maintained regardless of combination of drugs (Table 1).

Moreover, differences are observed in the quantitative response in such a way that patients under MPA had lower IgG anti-spike titers than those without it in all points. In the case of use of mTORi, antibody titers were higher in patients who received it than the others (Figure 4B).

In the multiple logistic regression, MPA (p<0.001) and thymoglobulin (p=0.007) use were associated with lack of response to vaccine after second dose. However, the only immunosuppressor with significant association with the response after the third dose was mTORi (p=0.018), the most common drug among responders (Table 2).

Regarding serum neutralizing activity against the S-protein after the booster dose of vaccine, low percentage of neutralizing activity were found in KTR compared to those of DP and HV: 44.8 (16.9-71) vs 64.4 (52-93.6) vs 67.5% (42.7-79.1), respectively (p=0.009) (Figure 5A). Besides of correlation between neutralizing titers against the S-protein and T-cell response, as we described above, there were an association between anti-S neutralizing activity and total IgG titers (r=0.485, p<0.0001) (Figure 5B).

As with the IgG titer, we observed a relationship between neutralizing activity after the third dose, categorized according to p25 and p75, and mTORi; such that patients treated with this drug were more likely to develop greater neutralizing activity after vaccination (p=0.001). There were no differences in neutralizing activity when we analyzed MPA or thymoglobulin treatment (Figure 5C).

SARS-CoV-2 infected patients

Finally, we compared SARS-CoV-2–specific immunity elicited by mRNA-based vaccine between infected and non-infected patients. The rate of antibody response was higher in infected than COVID-19 *naïve* KTR; the seropositivity anti-S IgG was 96.8 vs 75.2% (p<0.001) and neutralizing activity, 98.2 vs 80.9% (p<0.001) (Figure 6B). Significant differences were also detected in the intensity of this humoral response between both groups. IgG titers were 5680.0 (3460.0-7524.7) versus 335.5 (7.1-1415.3) BAU/mL (p<0.001) (Figure 6A) and neutralizing activity in 78.9 vs 43.4% (p<0.001) in SARS-CoV-2 infected vs *naïve* KTR, respectively (Figure 6B).

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TABLE 1 Comparison of clinical characteristics between KTR who did or did not mount cellular and humoral response two months after 3 doses of mRNA-1273 (P3).

	SARS-CoV-2 specific humoral response			SARS-CoV-2 specific cellular response		
	Responders N=112	Non responders N=37	p	Responders N=109	Non responders N=35	p
Gender (male), N (%)	68 (60.7)	21 (56.8)	0.670	63 (57.8)	23 (65.7)	0.406
Age of recipient						
years (mean, SD)	58.6 (15.2)	62.0 (13.2)	0.670	59.0 (15.2)	60.8 (13.6)	0.698
>60 y, N (%)	56 (50.0)	25 (67.6)	0.063	58 (53.2)	20 (57.1)	0.685
Diabetes, N (%)	39 (34.9)	14 (37.8)	0.724	40 (36.7)	12 (34.2)	0.738
Time since transplantation						
years (median, IQR)	9.9 (5.6-16.6)	9.2 (3.6-13.7)	0.477	9.6 (5.3-16.7)	9.5 (4.8-12.4)	1
<5 years, N (%)	24 (21.4)	11 (29.7)	0.302	25 (22.9)	9 (25.7)	0.736
Previous Transplant, N (%)	14 (12.5)	4 (10.8)	0.895	10 (9.1)	7 (20)	0.053
Immunosuppressive drug, N (%)						
CNI	86 (76.8)	34 (91.9)	0.044	88 (80.7)	28 (80)	0.924
MPA	87 (77.6)	34 (91.9)	0.053	86 (78.9)	29 (85.3)	0.412
mTORi	35 (31.2)	2 (5.4)	0.002	27 (24.8)	10 (28.6)	0.654
Thymoglobulin	57 (50.9)	27 (73)	0.016	57 (52.2)	23 (65.7)	0.134
Immunosuppressive protocol, N (%)			0.004			0.715
MPA+CNI	73 (65.1)	35 (94.5)	0.001	80 (73.3)	25 (71.4)	0.716
MPA+mTORi	16 (14.2)	0 (0)	0.017	10 (9.1)	5 (14.2)	0.418
mTORi+CNI	23 (20.5)	2 (5.4)	0.046	19 (17.4)	5 (14.2)	0.804
eGFR (mL/min/1.73 m^2), median (IQR)	49.5 (38.1-71.4)	30.1 (21.3-41.4)	0.001	46.1 (30.0-64.4)	45.4 (36.3-63.0)	0.991
Stages CKD, N (%)			0.001			0.288
>60 mL/min/1.73 m^2	43 (38.4)	1 (2.7)		33 (30.3)	10 (28.6)	
30-60	55 (49.1)	17 (45.9)		48 (44.0)	20 (57.1)	
<30	14 (12.5)	19 (51.4)		28 (25.7)	5 (14.3)	
Cells count before vaccination, 1x10^3/mm^3, median (IQR)						
Lymphocyte	1.6 (1.1-2.0)	1.0 (0.7-1.6)	0.008	1.5 (1.0-1.8)	1.5 (1.1-2.3)	0.525
$CD4^+$ T cells	5.4 (3.9-7.7)	4.1 (2.7-6.6)	0.807	5.1 (3.5-8.9)	5.4 (3.5-7.6)	0.638
$CD8^+$ T cells	4.5 (3.0-7.4)	3.8 (1.8-7.6)	0.068	3.9 (2.6-6.9)	6.1 (3.6-7.1)	1
Lymphocyte						
>1x10^3/mm3, N (%)	92 (82.1)	20 (54.1)	0.001	79 (72.5)	29 (82.8)	0.129
Serum Immunoglobulins levels, mg/dL, median *(IQR)*						
IgG	1050 (877-1275)	939 (750-1125)	0.112 0.092	1020 (843-1245)	1020 (890-1130)	0.845
IgA	227(137-292)	148(111-281)	0.229	221(122-294)	165(131-257)	0.435
IgM	86(60-120)	72.5(35-116)		83(52-121)	79(48-105)	0.922

CNI, Calcineurin inhibitor; MPA, mycophenolic acid; mTORi, mammalian Target Of Rapamicin inhibitor; eGFR (CKD-EPI), estimated glomerular filtration rate; IQR, interquartile rate; SD, Standard deviation.

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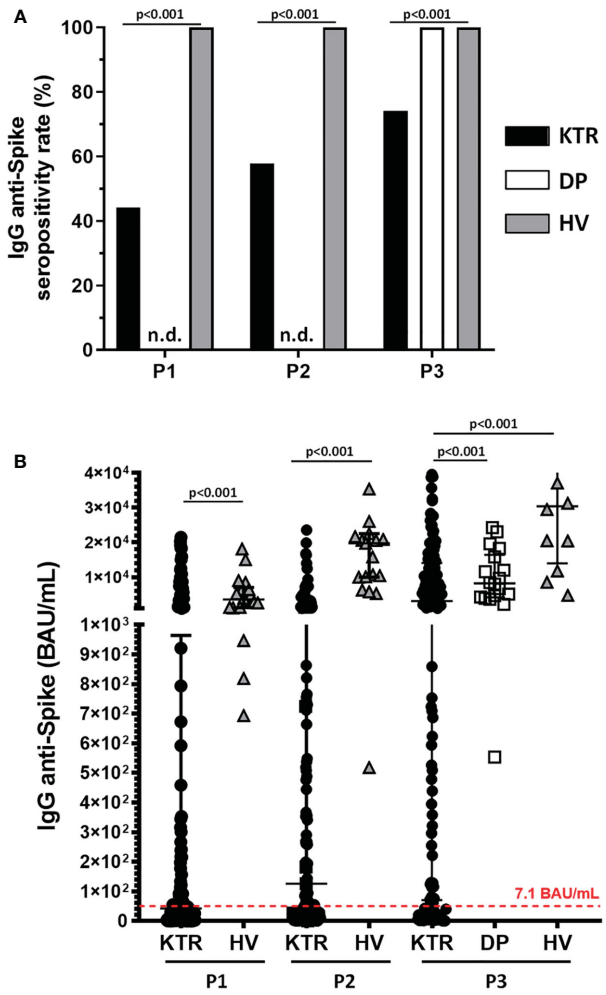

FIGURE 3
Kidney transplant recipients have lower humoral response rates and lower antibody titers after vaccination. **(A)** Seropositivity rate after vaccination in patients (KTR) and control groups (dialysis, PD; and healthy volunteers, HV) for each sample collection after vaccine administration. A threshold of >7.1 BAU/mL was used to consider seropositivity. **(B)** Antibody levels (BAU/mL) for each sampling and group after vaccine administration. Serum anti-spike IgG titers were higher in HV compared to KTR after second dose: 1782.8 (719.2-2519.2 IQR) vs 4.4 (0.3-135.6 IQR) BAU/mL at P1 (p<0.001), 394.3 (179.1-850.6 IQR) vs 18.3 (1.2-140.3 IQR) BAU/mL at P2 (p<0.001) and after the third dose: 335.5 (7.1-1415.3 IQR) BAU/mL in KTR, 908.9 (457.8-1872.8 IQR) BAU/mL in DP, 3058.9(1405.4-4366.1 IQR) BAU/mL in HV, p<0.001 Data were expressed as median and IQR, and p-values for each Mann-Whitney U test. n.d., non-determined.

Nevertheless, we did observe no differences in the cellular response among infected and *naïve* KTR: reactive CD4-T cells, 64.4 vs 58.3% (p=0.422); reactive CD8-T cells, 67.8 vs 64.6% (p=0.662) (Figure 6C).

Discussion

In this study, we analyzed SARS-CoV-2–specific cell-mediated and humoral immunities following two and booster doses of mRNA-1273 vaccine. KTR showed a marked reduction in the response rate, with a link between different types of immunosuppressive therapy.

As for cellular response, we did not find a correlation between the different clinical or treatment variants of the patients, although the transplanted cohort showed a lower response rate, both in CD8 and CD4 T cells, compared to control group. These results coincide with those of other studies in cohorts of patients with pharmacological immunosuppression, such as hematological cancer (19), as well as patients with various types of immunodeficiencies (20, 21). These studies also found no correlation between T-cell response and the clinical characteristics of the patients. This fact could be due to the high heterogeneity of the response against specific pools of the SARS-CoV-2 Spike protein (22).

In contrast to studies evaluating cellular response in PBMC pool or whole blood, our study has allowed independent study of CD4 and CD8 T response, identifying a higher response rate in CD8-T cells from patients undergoing KTR.

With regard to humoral response, KTR had a lower IgG response rate compared to the control group in each period, and a delay in the antibody production. In fact, some patients with low titers at day 15 after the administration of the second dose, raised antibody titers 3 months after this dose, in opposition to the downward curve observed in the general population (23).

We observed that the initial immunization schedule did not generate an adequate IgG response in KTR, and the third dose was not sufficient to rescue all non-responders, similar to other immunosuppressed populations (24). These patients could require several booster doses and seasonal vaccination patterns, as is already the case for other types of infectious diseases, like influenza vaccination (25).

Our data showed that reduced renal function decreased the likelihood of achieving seroprotection both after the second and third doses, as has been described in H1N1 vaccination (26). The mechanisms are still not very clear, since significant humoral response is observed in 100% of DP in this and other studies (27).

Also, we found an influence of lymphocyte count and lymphocyte depletion treatment, even when it was administrated several years earlier. Some studies have reported that lymphopenia is associated with infectious complications in cancer (28–30) and that there is an age-dependent decline in the capacity of the adult immune system to regenerate lymphocytes after thymoglobulin administration (31).

Interestingly, the prospective design and sample size and homogeneity of our cohort, which received the same vaccination type and schedule, also allowed us to identify relevant correlations with patient therapies. The immunosuppressive treatment had a different impact depending on whether we analyzed the response to second or booster dose. With the initial vaccination schedule (two doses), patients treated with MPA showed a pronounced decrease in IgG response compared to the rest of patients. Conversely, a higher probability of positive humoral response following the second dose was observed in those with mTORi in the univariate analysis. The relationship between mTORi and a better immune response were recently described by Netti et al (32). Nevertheless, this beneficial effect of mTORi after two doses of vaccine was no maintained in the

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TABLE 2 Factors related to antibody respond after second and third dose of SARS-CoV-2 mRNA-1273 vaccine in COVID-19 naïve KTR.

	UNIVARIATE ANALYSIS OR (95% ci), p	MULTIVARiate analysis OR (95% ci), p
P1 (N=161)		
Age >60 y	0.69 (0.36-1.32), 0.269	
Gender	1.02 (0.52-1.97), 0.953	
Diabetes	0.64 (0.31-1.30), 0.222	
Time since transplantation >5 y	0.60 (0.27-1.32), 0.203	
Previous transplant	1.55 (0.56-4.30), 0.390	
mTOR inhibitor	7.78 (3.37-17.97),<0.001	
MPA	0.06 (0.02-0.18), <0.001	0.05 (0.01-0.20), <0.001
CNI	0.32 (0.14-0.76), 0.008	0.17 (0.06-0.50), 0.001
eGFR pre-vaccination>30 ml/min/1.73 m^2	2.48 (1.06-5.81), 0.032	6.08 (1.84-20.06), 0.003
Lymphocyte count pre-vaccination >1x10^3/mm^3	2.50 (1.10-5.68), 0.025	3.47 (1.24-9.68), 0.017
Thymoglobulin	0.58 (0.29-1.14), 0.114	
P2 (N=155)		
Age >60 y	0.49 (0.26-0.91), 0.024	
Gender	1.22 (0.46-3.68), 0.521	
Diabetes	0.51 (0.26-0.98), 0.044	
Previous transplant	1.31 (0.50-4.18), 0.607	
Time since transplantation >5 y	0.50 (0.24-1.00), 0.050	
mTOR inhibitor	4.45 (1.93-10.24), <0.001	
MPA	0.22 (0.08-0.56), 0.001	0.10 (0.03-0.31), <0.001
CNI	0.58 (0.26-1.31), 0.191	
eGFR pre-vaccination>30 ml/min/1.73 m^2	3.33 (1.53-7.23), 0.002	4.78 (1.67-13.65), 0.003
Lymphocyte count pre-vaccination >10^3/mm^3	3.78 (1.78-8.02), <0.001	5.03 (1.93-13.10), 0.001
thymoglobulin	0.36 (0.18-0.72), 0.003	0.34 (0.15-0.74), 0.007
p3 (n=149)		
Age >60 y	0.48 (0.22-1.04), 0.063	
Gender	1.17 (0.55-2.50), 0.670	
Diabetes	0.86 (0.39-1.92), 0.724	
Previous transplant	1.08 (0.32-3.56), 0.895	
Time since transplantation >5 y	0.64 (0.27-1.48), 0.302	
mTOR inhibitor	7.95 (1.81-34.91), 0.002	6.40 (1.37-29.86), 0.018
MPA	0.30 (0.08-1.07), 0.053	
CNI	0.29 (0.08-1.02), 0.044	
eGFR pre-vaccination>30 mL/min/1.73m^2	7.38 (3.14-17.35), <0.001	7.34 (2.72-19.84), <0.001
Lymphocyte count pre-vaccination >10^3/mm^{+3}	4.11 (1.82-9.28), <0.001	4.68 (1.72-12.73), 0.003
Thymoglobulin	0.35 (0.15-0.84), 0.016	

P1: 15 days after second dose; P2: three months after second dose; P3: two months after third dose; MPA,: mycophenolic acid; CNI, calcineurin inhibitor; mTOR, mammalian Target Of Rapamycin; eGFR, estimated glomerular filtration rate.
Univariate and multivariate regression model (adjusted to age, gender and time since transplantation).

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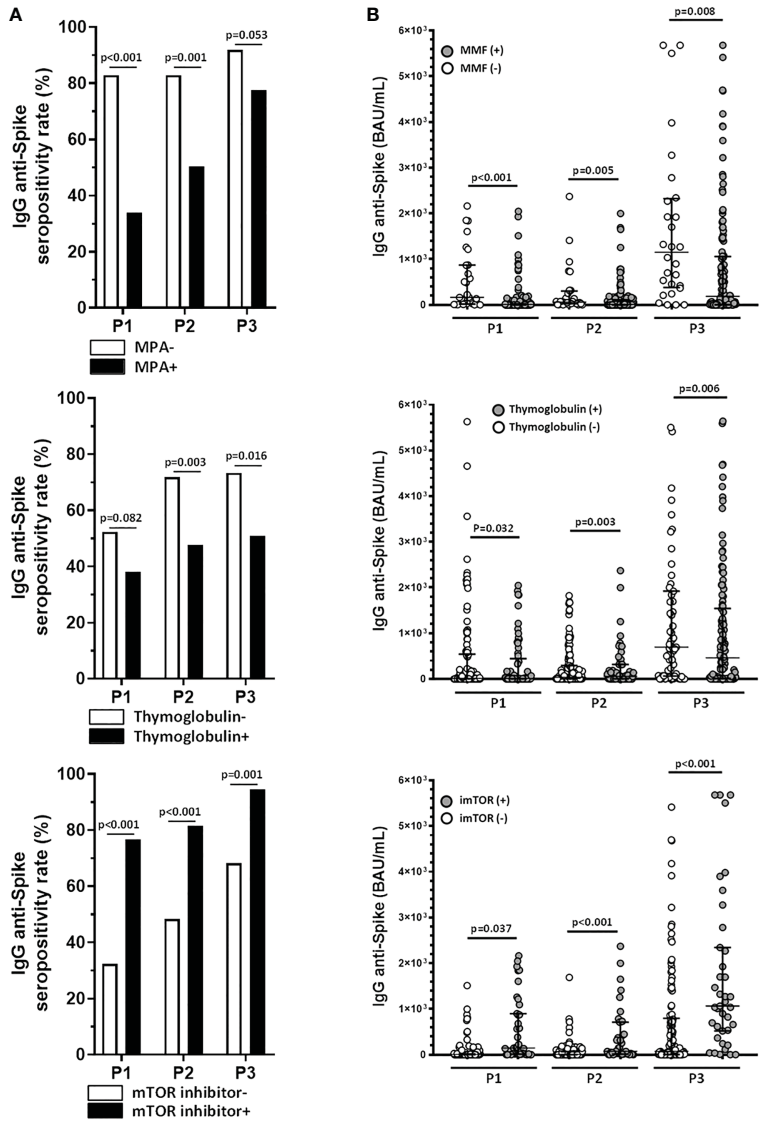

FIGURE 4
Treatment with mycophenolate and thymoglobulin reduces the efficacy of the humoral response in kidney transplant recipients, whereas therapy with mTOR inhibitors reverses the negative effect of immunosuppressive therapy in these patients. **(A)** Humoral response rates comparing mycophenolate (MPA), thymoglobulin and mTOR inhibitor therapies. **(B)** Levels of anti-Spike IgG antibodies comparing therapies with mycophenolate (MPA), thymoglobulin and mTOR inhibitors. Patients under MPA had lower IgG anti-spike titers than those without it in all points (P1: 2.6 (0.1-19.9) vs 149.4 (12.4-966.3) BAU/mL, p<0.001; P2: 7.1 (0.7-109.9) vs 50.1 (9.5-300.5) BAU/mL, p=0.005; P3: 104.2 (5.3-1051.5) vs 737.5 (213.1-2183.5) BAU/mL, p=0.008). The same behavior was performed in KTR who received thymoglobulin (P1: 4.5 (0.1-135.5) vs 13.8 (0.7-759.4) BAU/mL, p=0.032, P2: 5.8 (0.6-68.0) vs 46.9 (4.3-317.2) BAU/mL, p=0.003, P3: 56.7 (2.3-877.8) vs 1378.1 (53.0-1919.3) BAU/mL, p=0.006). In patients treated with mTORi, antibody titers were higher vs non-treated (P1: 162.7 (9.2-1093.4) vs 2.6 (0.1-20.3) BAU/mL, p<0.001; P2: 145.6 (12.6-709.4) vs 6.0 (0.7-70.4) BAU/mL, p<0.001; P3: 1036.2 (366.4-2270.0) vs 70.3 (3.4-761.7) BAU/mL, p<0.001). Graphs include data for each sampling after vaccination and p-values for each Mann-Whitney U test.

adjusted model in our cohort, findings also noted by Bae (33). Several authors found similar results with MPA and mTORi in different groups of patients who received two doses (33–37), even proposing the temporary suspension of treatment during the vaccination process (38).

But we go further and performed an analysis of immune response after the third or booster dose and different results were observed. There was no association between humoral response and MPA, and mTORi was the only treatment that showed an independent association with immunogenicity following the third dose. We hypothesized that the negative impact of MPA was diluted after booster dose, perhaps due to greater antigenic exposure, similar to what happened in the case of patients who have undergone COVID-19 in any of the study periods. The mTORi-treated patients with three doses of vaccine showed a quantitative and qualitative humoral immune response similar to controls, with high response rates.

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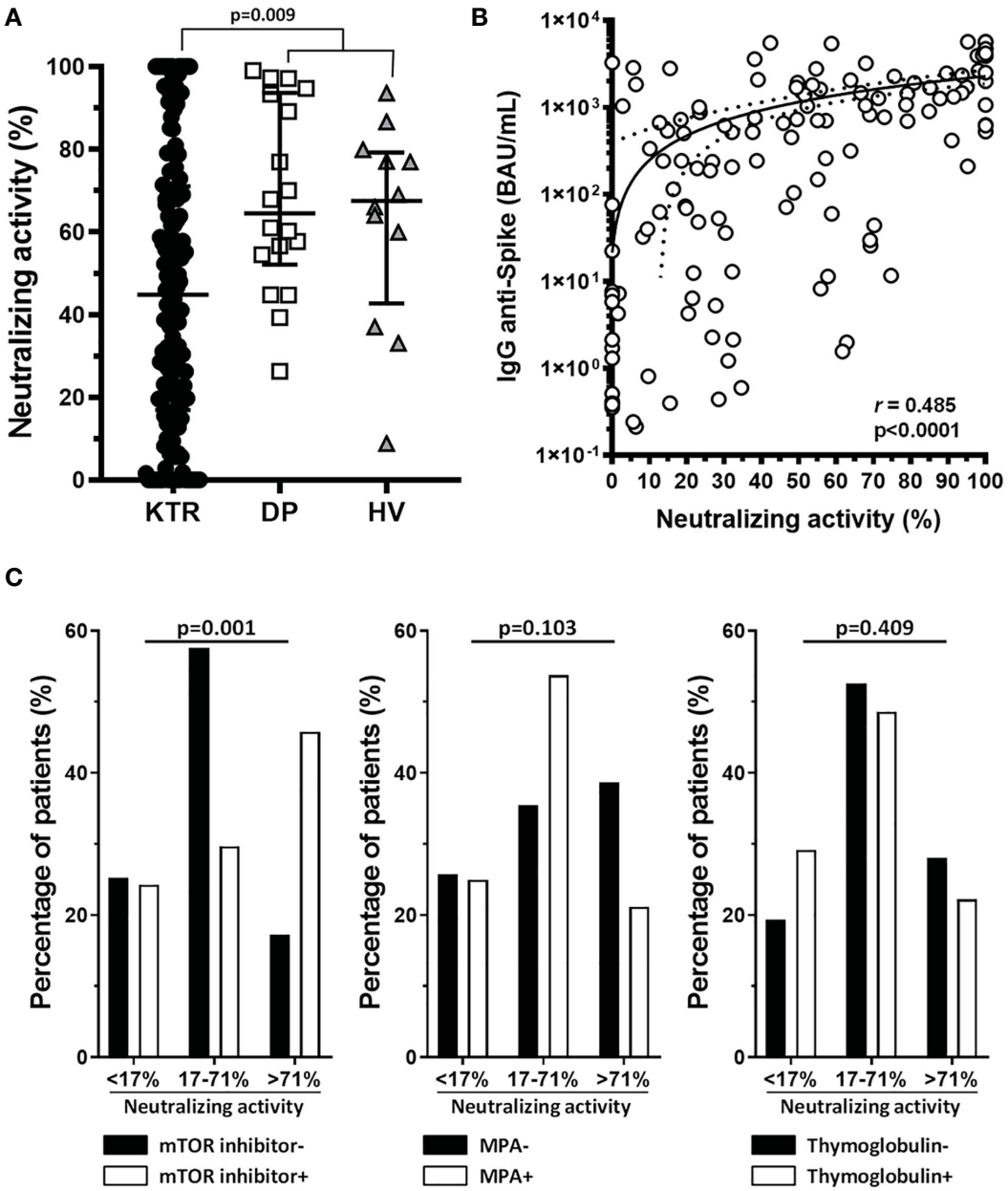

FIGURE 5
Sera from renal transplant recipient patients show lower RBD-ACE2 binding neutralizing activity than control groups, which correlates with antibody levels, and mTOR inhibitors treatment enhance neutralizing activity of sera of these patients. **(A)** Graph shows the neutralizing activity of patient sera at 1:25 dilution in patients (KTR) and control groups (DP and HV), with median and IQR, and p-value for Mann-Whitney U test. **(B)** Graph shows the correlation of neutralizing activity with antibody levels calculated with Spearman's Rho. **(C)** Graphs show the percentage of patients with a range of neutralizing activity (divided in three percentiles, according to p25 and p75, <17%, 17%-<71% and >71%) for mTOR inhibitor, MMF and thymoglobulin treatment after the third dose of vaccination.

Several studies found that mTORi can enhance the formation and differentiation of memory CD8 T cells in anti-tumor vaccines and in immunization against viruses and parasites (39–42). It has been suggested that mTOR blockade effectively potentiated antigen-specific T-cell and B-cell responses induced by HBV vaccines (43).

Finally, we found that neutralization capacity after the third dose is clearly linked to anti-S IgG antibody titers, as had already been described (44). This is especially relevant in KTR, as many of them generate response after vaccination, but with low titers, which may lead to an increased risk of infection and complications. As with antibody titers, treatments affected the neutralizing capacity of these sera. Patients treated with mTORi had a greater neutralization capacity, as they achieved higher IgG titers, suggesting a more efficient post-vaccination response. This data may lead to consider

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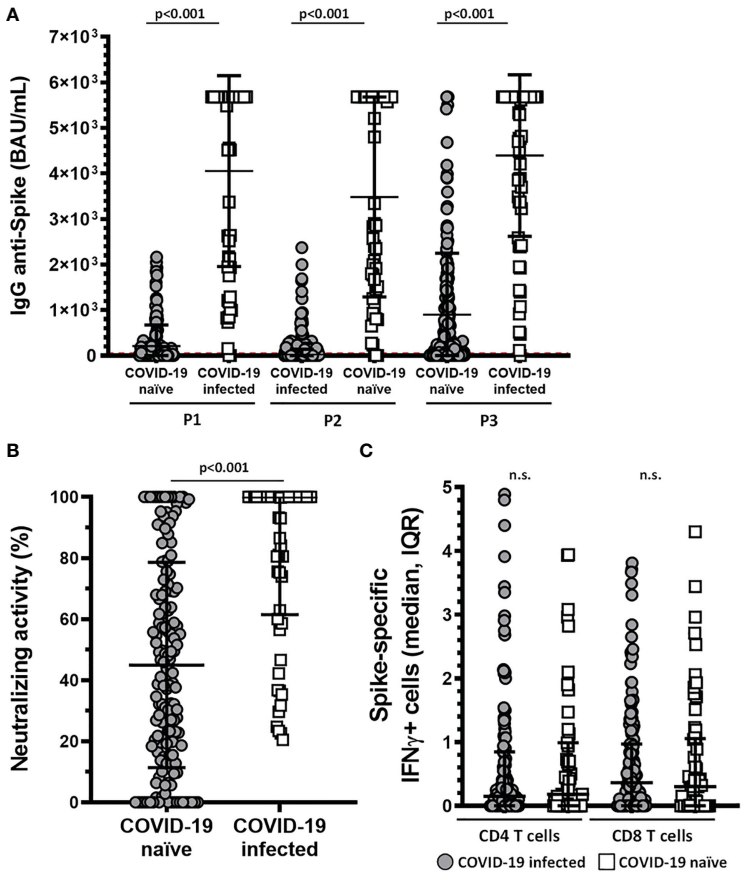

FIGURE 6
Patients affected by COVID-19 have higher levels of anti-Spike IgG antibodies and higher neutralizing activity. **(A)** Levels of anti-Spike IgG antibody (BAU/mL) in COVID-19 affected and unaffected patients for each of the post-vaccination samples (p<0.001). **(B)** Neutralizing activity of sera after the third dose in COVID-19 affected and unaffected patients. Neutralizing activity was 78.9 in SARS-CoV-2 infected vs 43.4% (p<0.001) in SARS-CoV-2 *naïve* KTR. Both graphs show median and IQR, an p-value for each Mann-Whitney U test. **(C)** Graph show IFNγ+ cells in COVID-19 naïve and COVID-19 infected individuals in CD8 and CD4 T cells. Non-significant differences were found between both groups. n.s., non significant.

the use of this therapy as an adjuvant for the response to new booster doses of mRNA vaccines.

Regarding the limitations of this study, all the assays were performed on the wildtype strain of SARS-CoV-2. In addition, the administration of a fourth dose to immunosuppressed patients has been standardized, so we have extended this study to verify the effect of the fourth dose.

In conclusion, this study shows that KTR have a lower response after to doses of mRNA-1273 vaccination, especially accentuated in those treated with MPA or thymoglobulin. Based on these observations, it can be assumed that COVID-19 still presents a major risk for vaccinated KTR. However, it is possible to rescue patients with the third dose and mTORi therapy could be a potential adjuvant therapy to improve the response to booster doses in this high-risk population.

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 Ethics Committee of Hospital Clínico San Carlos (June 28th 2021, 21/200-E). The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by IP-F, IJ, AA, AL-G, RG-G. NR, BR-C, MM and BP-J. The first draft of the manuscript was written by IP-F, IJ, AA, EN and AS-F and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1111569/full#supplementary-material

SUPPLEMENTARY FIGURE 1

Individual trends of KTR patients. Levels of anti-Spike IgG antibody (BAU/mL) in KTR (black lines and circles) and HV (blue lines and squares) for each of the post-vaccination samples. Part of the KTR showed a delay in the generation of Spike-specific IgG at P1 and a higher decrease of IgG levels at P2 compared to controls.

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Safety and immunogenicity of inactivated COVID-19 vaccine CoronaVac and the RBD-dimer–based COVID-19 vaccine ZF2001 in chronic hepatitis B patients

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Background and aims: Although COVID-19 vaccination is recommended for the patients with chronic liver disease, the clinical outcomes of COVID-19 vaccinated in patients with chronic hepatitis B (CHB) has not been well characterized. The study aimed to explore the safety and specific antibody responses following COVID-19 vaccination among CHB patients.

Methods: Patients with CHB were included. All patients were vaccinated with two doses of inactivated vaccine (CoronaVac) or three doses of adjuvanted protein subunit vaccine (ZF2001). The adverse events were recorded and neutralizing antibody (NAb) were determined 14 days following the whole-course vaccination.

Results: A total of 200 patients with CHB were included. Specific NAb against SARS-CoV-2 were positive in 170 (84.6%) patients. The median (IQR) concentrations of NAb were 16.32 (8.44–34.10) AU/ml. Comparison of immune responses between CoronaVac and ZF2001 vaccines showed no significant differences in neither the concentrations of NAb nor the seropositive rates (84.4 vs. 85.7%). Moreover, we observed lower immunogenicity in older patients and in patients with cirrhosis or underlying comorbidities. The incidences of adverse events were 37 (18.5%) with the most common adverse event as injection side pain [25 (12.5%)], followed by fatigue [15 (7.5%)]. There were no differences in the frequencies of adverse between CoronaVac and ZF2001 (19.3% vs. 17.6%). Almost all of the adverse reactions were mild and self-resolved within a few days after vaccination. Severe adverse events were not observed.

Conclusions: COVID-19 vaccines, CoronaVac and ZF2001 had a favorable safety profile and induced efficient immune response in patients with CHB.

KEYWORDS

COVID-19 vaccine, CHB patients, safety, immunogenicity, ZF2001, CoronaVac

Introduction

Following the pandemic of COVID-19, it is the most priority to control transmission of SARS-CoV-2 (1). Previous studies report that the patients with chronic liver disease, particularly cirrhosis presented worsened outcomes following COVID-19 infection (2, 3), presenting more disturbed liver abnormalities (4) and could lead to hepatitis B reactivation which can cause liver failure (5). Therefore, liver societies have recommended vaccination against COVID-19 for all patients with chronic liver diseases (6–8).

Although remarkable progress has been made in developing vaccines, only a few participants with pre-existing chronic liver diseases were included in clinical trials studying the safety and efficacy of COVID-19 vaccines. Recent studies have reported the responses of COVID-19 mRNA or inactivated vaccines in patients with nonalcoholic fatty liver disease (9), liver transplant recipients (10) and in chronic hepatitis B (CHB) patients (11). However, there is no detailed information available on the use of the SARS-CoV-2 adjuvanted protein subunit vaccine (ZF2001) (12) in CHB patients. In addition, the safety and effectiveness of ZF2001 vaccine remain to be clarified (13).

Given the large number of chronic hepatitis B patients in China (14), we aimed to explore the safety and immunogenicity of COVID-19 vaccines (CoronaVac and ZF2001) in CHB patients in this prospective study.

Methods

Study design

The study was performed at Fuyang People's Hospital in Anhui, China. Among 200 recruited CHB patients, 109 were vaccinated with inactivated virus vaccine against SAR-CoV-2 (CoronaVac) and 91 were vaccinated with adjuvanted protein subunit vaccine (ZF2001). The vaccination regimen for CoronaVac is two doses (3 ug) given intramuscularly with an interval of 3 weeks. The vaccination regimen for ZF2001 is a total of three doses (25 μg) given intramuscularly with an interval of >4weeks. The diagnostic criteria for CHB infection are: HBsAg or HBV DNA positive for at least 6 months. Exclusion criteria were: co-infection of HBV and HIV, HCV, HDV, EBV, or CMV, evidence of schistosomiasis or Wilson's disease, received antiviral therapy, alcohol liver disease (alcohol consumption ≥40 g/day for male and ≥20 g/day for female), or liver damage induced by other causes (non-alcoholic fatty liver, drugs, autoimmune hepatitis). All participants were over the age of 18 and had no known history of SARS-CoV-2 infection. Clinical data on anti-HBV therapy, HBV serological biomarkers, and liver function test results were extracted from electronic medical records prior to the first vaccination. Abnormal ALT test was defined as a value greater than the upper limit of normal (F: 40 U/L, M: 50 U/L). The presence or absence of liver cirrhosis is determined based on clinical evidence combined with liver imaging examinations. The current study is approved by the Ethics Committee of Fuyang People's Hospital. Under the guidance of professional physicians, adverse reactions after vaccination, including local (pain, swelling, induration) or systemic reactions (fever, fatigue, drowsiness, headache, dizziness, myalgia), were collected by filling out a standard questionnaire. The primary safety outcome was the overall incidence of adverse events within 7 days. The study was approved by the ethics committees of Fuyang People's Hospital.

Anti-SARS-CoV-2 NAb measurement

Plasma samples were collected 2 weeks following vaccination, and neutralizing antibodies (NAbs) were detected, using the SARS-CoV-2 Neutralizing Antibody Kit (CLIA) (China-based Maccura Biotechnology Co., Ltd). Concentrations equal to or higher than 6 AU/ml are considered positive immune responses, according to the Neutralizing Antibody Kit instructions.

Statistical analysis

Continuous variables were presented as mean ± standard deviation and categorical variables were presented as *n* (%). The concentrations of NAb were presented as median with interquartile range (IQR). GraphPad Prism v8.0 was used for statistical analysis. Two groups' continuous variables comparison were analyzed with Student *t*-test or Mann-Whitney U test. The categorical variables were analyzed with $\chi 2$ test. For all tests, a two tailed $p < 0.05$ was considered statistically significant.

Results

Participant's characteristics

Total of 200 patients with pre-existing CHB were eligible for analysis. The average age was 47.39 ± 13.60 years and 108 (54.0) were male. The mean BMI was 24.29 ± 2.62 kg/m (2). Among these CHB patients, 12 (6%) were diagnosed as CHB-related liver cirrhosis. Comorbidities were presented in 24 (12%) CHB patients with hypertension as the most prevalent condition (6%), followed by fatty liver (3.5%), diabetes (3%), hyperlipidemia (1%), coronary

TABLE 1 Baseline characteristics of study cohort.

Characteristics	Patients ($n = 200$)
Age, (mean ± SD), years	47.39 ± 13.60
Age groups, *n* (%)	
20–35, *n* (%)	43 (21.5)
35–50, *n* (%)	74 (37.0)
50–65, *n* (%)	57 (28.5)
65–80, *n* (%)	26 (13.0)
Gender	
Female, *n* (%)	92 (46.0)
Male, *n* (%)	108 (54.0)
Body mass index, mean ± SD, kg/m^2	24.29 ± 2.62
Cirrhosis, *n* (%)	12 (6.0)
Any comorbidity, *n* (%)	24 (12)
Hypertension, *n* (%),	12 (6.0)
Fatty liver, *n* (%)	7 (3.5)
Diabetes, *n* (%)	6 (3.0)
Hyperlipidemia, *n* (%)	2 (1.0)
Coronary artery disease, *n* (%)	2 (1.0)
Cerebrovascular disease, *n* (%)	2 (1.0)
Chronic kidney disease, *n* (%)	1 (0.5)
Others, *n* (%)	2 (1.0)

TABLE 2 Safety and immunogenicity of COVID-19 vaccination in patient with CHB.

Characteristics	Patients (n = 200)
Total reactions within 7 days after each injection	
Any, *n* (%)	37 (18.5)
Injection site adverse reactions	
Pain, *n* (%)	25 (12.5)
Induration, *n* (%)	4 (2.0)
Itch, *n* (%)	2 (1.0)
Systemic adverse reactions	
Fatigue, *n* (%)	15 (7.5)
Drowsiness, *n* (%)	5 (2.5)
Fever, *n* (%)	5 (2.5)
Nausea, *n* (%)	1 (0.5)
Abdominal bloating, *n* (%)	1 (0.5)
Antibody responses after whole-course vaccination	
Neutralizing antibody, median (IQR), AU/ml	16.32 (8.44–34.10)
Neutralizing antibody response, *n* (%)	170 (84.6)
IgM positive, *n* (%)	16 (8.0)
IgG positive, *n* (%)	173 (86.1)

artery disease (1%), cerebrovascular disease (1%), and chronic kidney disease (0.5%) (Table 1).

Vaccine safety

Among the 200 patients, 37 (18.5) patients reported adverse effects after the vaccination. Injection-site pain was the most frequent local adverse event (12.5%), followed by induration (2.0%) and itch (1.0%). The most common systemic adverse event was fatigue (7.5%), followed by drowsiness (2.5%), fever (2.5%), nausea (0.5%) and abdominal bloating (0.5%). Almost all of the adverse reactions were mild and self-resolved within a few days after vaccination. Serious side effects were not observed (Table 2). Importantly, the frequencies of adverse events from the CHB patients with cirrhosis or comorbidities were similar to the patients without them, suggesting the safety of COVID-19 vaccination in CHB patients regardless of cirrhosis or comorbidities (Supplementary Figures 2A, B). There were no differences in the frequencies of adverse events in CHB patients receiving different types of vaccines (19.3 vs. 17.6%) (Supplementary Figure 2C).

To further understand the safety of vaccines in CHB patients, we compared the biochemical characteristics prior to and post vaccination. Total bilirubin (TB), prothrombin time (PT), white blood cell (WBC) and hemoglobin levels were increased by 1.11, 1.03, 1.12 and 1.03 fold, respectively ($p < 0.05$) in trend after vaccination and albumin and platelet levels were decreased by 0.98 fold ($p < 0.05$) (Figure 1). Although these basal biochemical characteristics were changed, they were all within the normal range. Thus, we believe that COVID-19 vaccines appeared to be safe in CHB patients.

Neutralizing antibody (NAb) titers

The median (IQR) concentrations of NAb were 16.32 (8.44–34.10) AU/ml. The overall NAb response rates were 84.6%. Furthermore, IgM or IgG antibodies were present in 16 (8%) or 173 (86.1%) CHB patients 14 days post vaccination (Table 2). The concentrations of NAb were further stratified according to sex, age, and BMI, no significant differences were observed (Figure 2A). However, the positive rates of immune responses were significantly higher in younger CHB patients (<45 yrs) compared to older CHB (≥45 yrs) (91.6 vs. 79.0%, $p < 0.05$) (Figure 2B, middle panel). Comparison of immune responses between CoronaVac and ZF2001 vaccines showed no significant differences in neither the concentrations of NAb (Supplementary Figure 1A), nor the seropositive rates (84.4 vs. 85.7%) (Supplementary Figure 1B), suggesting that the immunogenicity was comparable between CoronaVac and ZF2001 vaccines.

Finally, to determine whether comorbidities affect the immune responses of COVID-19 vaccine, we compared the concentrations of NAb and seropositive rates in CHB patients with and without comorbidities. The concentrations NAb and seropositive rates were lower in the CHB patients with comorbidities compared to the CHB patients without comorbidities (Supplementary Figures 1C, D). In addition, among 30 CHB patients who had no response to vaccines, eight of them had CHB-related cirrhosis. Then we compared the immune responses in CHB patients with and without cirrhosis. The concentrations of NAb were dramatically low in patients with cirrhosis compared to patients without cirrhosis [5.18 (3.95–11.82) vs 17.04 (8.79–34.41)] (Supplementary Figure 1E) and seropositive rates were also lower in patients with cirrhosis compared to patients without cirrhosis (33.3 vs. 88.3%) (Supplementary Figure 1F).

Discussion

In our current study, 37 (18.5%) CHB patients reported adverse effects post the COVID-19 vaccination. The incidence of adverse effects in our study was similar to the adverse effects in volunteers (18.9%) post CoronaVac vaccination in Turkey (15), while it was lower than the overall incidence of adverse reactions (30.2%) in CHB patients after receiving inactivated vaccines (BBIBP-CorV, CoronaVac, or WIBP-CorV) (11), and lower than the incidence of adverse effects in NAFLD patients after BBIBP-CorV vaccination (29.4%) (9). There was only slight but not significant change of biochemical characteristics prior to and post vaccination. Taken together, our results support that CHB patients are safely vaccinated with COVID-19 vaccines.

To investigate the efficiency of two types of COVID-19 vaccine, CoronaVac and ZF2001 in CHB patients, NAbs were measured. Studies have showed that compared to the wild type, Omicron variant possesses comparable binding affinity to human ACE2 in comparison with the wild type SARS-CoV-2, and Delta variant possesses stronger binding affinity to human ACE2 than Omicron variant (16). Thus, neutralizing antibodies directed against both the original strains and the mutant strains of Wuhan. In several clinical trials and studies for CoronaVac and ZF2001, to evaluate the immunogenicity of the vaccine, the neutralizing antibodies were analyzed 14 days or 9–21 days after receiving the last dose of vaccination (17, 18). Thus, it is acceptable to

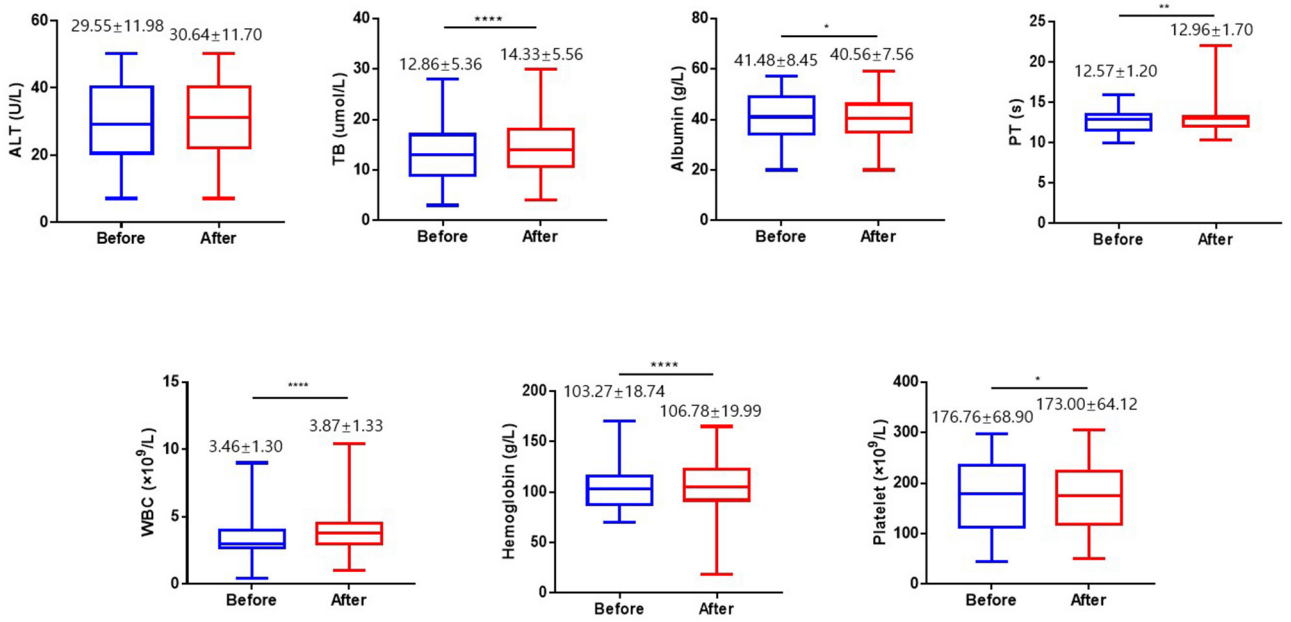

FIGURE 1
The biochemical characteristics before and after vaccination. The serum levels of alanine amino transferase (ALT), total bilirubin (TB), and albumin were compared before and after vaccination. The value of prothrombin time (PT), the levels of white blood cell, hemoglobin and platelet in the blood were compared before and after vaccination. ALT, alanine amino transferase; TB, total bilirubin; PT, prothrombin time; WBC, white blood cell. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

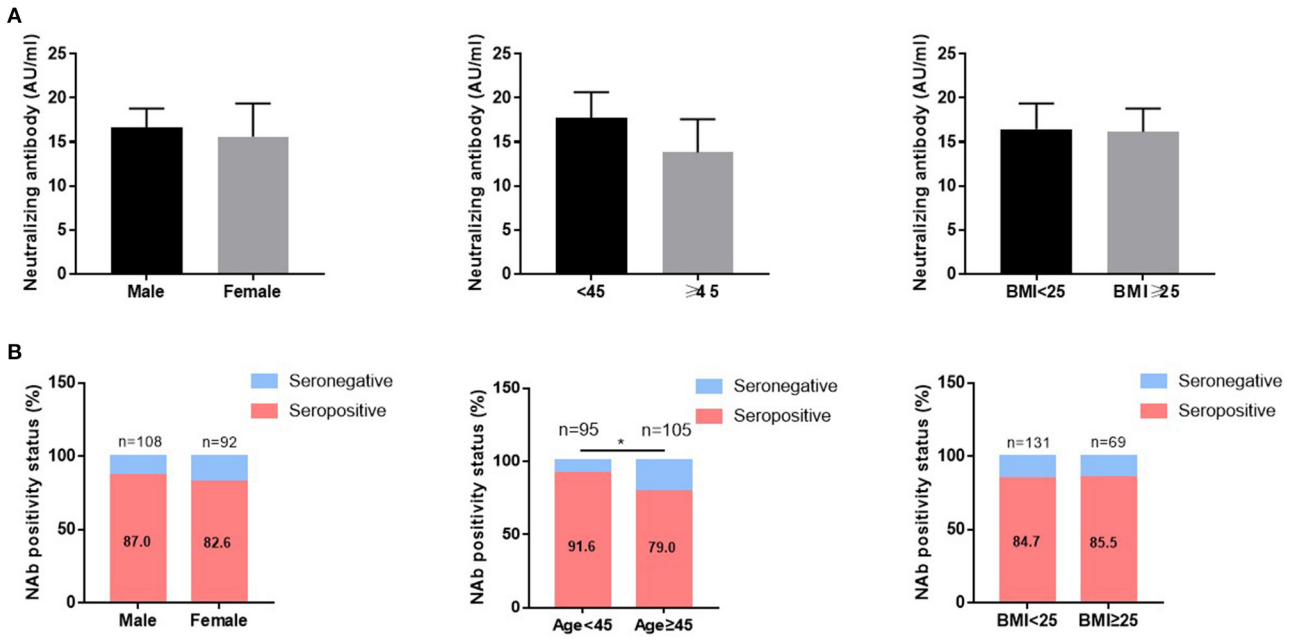

FIGURE 2
The influence of age, sex, and BMI on the immunogenicity of vaccines in CHB patients. The neutralizing antibody (NAb) concentrations **(A)** and NAb positive rates **(B)** were compared according to sex, age, and BMI in patients with chronic hepatitis B. ***$P < 0.0001$.

analyze neutralizing antibodies 14 days following the whole-course vaccination in our study. The positivity rate of NAb was 84.6% in our study, which is consistent with previous report in non-CHB cohort (11), suggesting that COVID-19 vaccinations are effective, regardless of CHB status. In addition, we found that older CHB patients exhibited weaker humoral immunity to vaccination than younger ones, suggesting that age is a contributing factor in determining host immunity. This is in line with other studies which also indicated that seropositivity decreased with increasing age (15, 19).

Cirrhosis contributes to deregulated immunity in the host (20). We revealed that the concentrations of NAb and seropositive rates were dramatically lower in patients with cirrhosis, compared to the patients without cirrhosis. These results were in line with previous study indicating that cirrhosis is associated with poor antibody response in patients with chronic liver diseases (21). Due to the limited number of patients with cirrhosis ($n = 12$), we cannot generally conclude that immunogenicity of COVID-19 vaccination was less effective in patients with cirrhosis. Further studies are needed in a larger group of patients.

In our study, 12% patients had underlying comorbidities. We observed lower efficiency of vaccine in patients with comorbidities, as evidenced by the decreased NAb concentrations and NAb seropositive rates in patients with comorbidities compared to patients without comorbidities. Unfortunately, due to the small number of patients we studied, it is difficult to find out which morbidities are responsible for the attenuated immune response. Previous studies have shown that the presence of underlying comorbidities, such as hypertension, diabetes, cardiovascular disease, and cerebrovascular disease are risk factors for COVID-19 infection (22) and lead to poor prognosis (23). Despite the possibility of reduced immune response, the benefits of the COVID-19 vaccination outweigh the risks. Our study support the recommendations that suggest COVID-19 vaccination for patients with comorbidities (24).

We realize that there are some limitations in the current study: the number of CHB patients, especially patients with CHB-related cirrhosis were relatively small, thus it is difficult to come to a convincing conclusion about the immunogenicity of COVID-19 vaccine in CHB patients with cirrhosis. Secondly, the clinical stages of HBV infection of CHB patients were not recorded. It will be interesting to analyze the antibody responses after COVID-19 vaccination in CHB patients with different immune phases. Thirdly, anti-viral therapy was not recorded in our study. In addition, we did not include normal people with negative hepatitis B, as control in this study. Further studies might be carried out to analyze the impacts of vaccination on antiviral therapy in CHB patients.

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 Medical Ethics Committee of Fuyang People's Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HW, BW, and XR designed, conceived the study, and revised the manuscript. SW, XL, and XF enrolled patients, acquired the data, and performed the experiments. XW and MF analyzed the data, contributed to producing the charts, and drafted the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1078666/full#supplementary-material

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Factors associated with the humoral response after three doses of COVID-19 vaccination in kidney transplant recipients

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Introduction: Kidney transplant recipients showed a weak humoral response to the mRNA COVID-19 vaccine despite receiving three cumulative doses of the vaccine. New approaches are still needed to raise protective immunity conferred by the vaccine administration within this group of high-risk patients.

Methods: To analyze the humoral response and identify any predictive factors within these patients, we designed a prospective monocentric longitudinal study of Kidney transplant recipients (KTR) who received three doses of mRNA-1273 COVID-19 vaccine. Specific antibody levels were measured by chemiluminescence. Parameters related to clinical status such as kidney function, immunosuppressive therapy, inflammatory status and thymic function were analyzed as potential predictors of the humoral response.

Results: Seventy-four KTR and sixteen healthy controls were included. One month after the administration of the third dose of the COVID-19 vaccine, 64.8% of KTR showed a positive humoral response. As predictive factors of seroconversion and specific antibody titer, we found that immunosuppressive therapy, worse kidney function, higher inflammatory status and age were related to a lower response in KTR while immune cell counts, thymosin-a1 plasma concentration and thymic output were related to a higher humoral response. Furthermore, baseline thymosin-a1 concentration was independently associated with the seroconversion after three vaccine doses.

Discussion: In addition to the immunosuppression therapy, condition of kidney function and age before vaccination, specific immune factors could also be relevant in light of optimization of the COVID-19 vaccination protocol in KTR. Therefore, thymosin-a1, an immunomodulatory hormone, deserves further research as a potential adjuvant for the next vaccine boosters.

KEYWORDS

COVID-19, kidney transplant, mRNA vaccine, relative telomere length, thymic function, thymosin-α1, sj/β-TREC ratio

1 Introduction

COVID-19 vaccination has been demonstrated as the best tool to control the SARS-CoV-2 epidemic and it is being administered worldwide since December 2020. Unfortunately, large series of patients including kidney transplant recipients (KTR) who have already received two doses of mRNA vaccines showed poor seroconversion rates in comparison to the general population related to their immunosuppressive treatment. Different studies have shown the seroprotection rates are under 20% after the first dose, and less than 50% after the second one, while in the general population, these values are close to 100% (1–3). Although an improvement in the humoral response has been observed after a third (booster) dose, the seroconversion rate varies from 55 to 67% which is still lower than the observed in the general population (4–6).

The distribution of immune cell populations impacts the outcome of immune responses to different viral infections and vaccination settings such as the CD4/CD8 T-cell ratio in HIV-infected subjects (7), or the total CD4+ and CD8+ T-cells producing IFN-γ or TNF-α in tuberculosis and herpes zoster vaccination (8, 9). In addition, delayed reconstituted T and B cells have been related to a higher risk of viral infection after stem cell transplantation (10). In this sense, the thymus plays a main role in the generation and maturation of T-cells, and in mediating innate and adaptive immunological responses both, by the thymic output of T-cells and by the secretion of several hormones, as thymosin-α1 (Tα1), with peripheral immunomodulatory properties (11, 12). Moreover, the thymic output impacts the homeostatic proliferation of peripheral T-cells and, hence, their relative telomere length. In addition, Tα1 plasma levels and T-cell peripheral proliferation have been related to a better immune restoration in immunodeficient patients and a better recovery in SARS-CoV-2 infected patients and other immunological contexts (13–15). We hypothesized that these thymic-related parameters (thymic output, hormone secretion and immune cells relative telomere length) might be associated with the lower response to COVID-19 vaccination in immunosuppressed patients, such as kidney transplant recipients. As far as we know, the potential role of such thymic-related parameters has not been yet explored in this setting, neither in the general population. We present herein data from six months' follow-up in a cohort of kidney transplant recipients receiving three doses of mRNA COVID-19 vaccine, including longitudinal data of humoral response and the analysis of potential predictive factors for both clinical and immunological, including distribution of main immune subsets and thymic function-related parameters.

2 Materials and methods

2.1 Study design and participants

We designed a prospective monocentric longitudinal study of kidney transplant recipients (KTR) receiving three doses of mRNA-1273 COVID-19 vaccine (100 μg per dose) and healthy controls receiving two doses of BNT162b2 vaccine (30 μg per dose). KTR from the Virgen del Rocio University Hospital (Seville, Spain), were recruited if they received kidney transplant more than one month before the beginning of the study, and were older than 18 years old and signed informed consent to participate. In our region, KTR received the first dose of vaccine in April 2021. Blood samples were collected up to one month before the first dose (T0), 3-4 weeks after the first dose (T1) (i.e. just before the administration of the second dose), one month after the second dose (T2), four months after the second dose (T3) and one month after receiving the third dose (T4) (Figure 1A). The procedures outlined in this study were approved by the local Ethic Committee for Clinical Research (Acta number: 02/2021) and were performed according to the Helsinki Declaration of the World Medical Association.

2.2 Specific IgG production anti-trimeric SARS-CoV-2 spike protein

IgG antibodies against the trimeric SARS-CoV-2 Spike protein were quantified in serum samples by chemiluminescence assay (LIAISON® SARS-CoV-2 TrimericS IgG, Diasorin S.p.A, Saluggia, Italy) and run on a DiaSorin LIAISON XL platform (DiaSorin, Stillwater, USA). According to the manufacturer's data, the sensitivity and specificity of this test were 98.7% and 99.5%, showing a good correlation with microneutralization test (PPA: 100%, NPA: 96.9%). Antibody concentration, expressed as BAU/mL, was automatically calculated by the analyzer from AU/mL by the following conversion formula: AU/mLx2.6=BAU/mL. A positive result was considered as ≥33.8 BAU/mL. The levels of IgG antibodies against the trimeric SARS-CoV-2 Spike protein were considered as a continuous variable, corresponding to the magnitude of the humoral response (titer values), but they were also transformed into a dichotomous variable, corresponding to the ability to response or seroconversion, defined as an antibody titter higher or equal to 33.8 BAU/mL (the established threshold for the used assay).

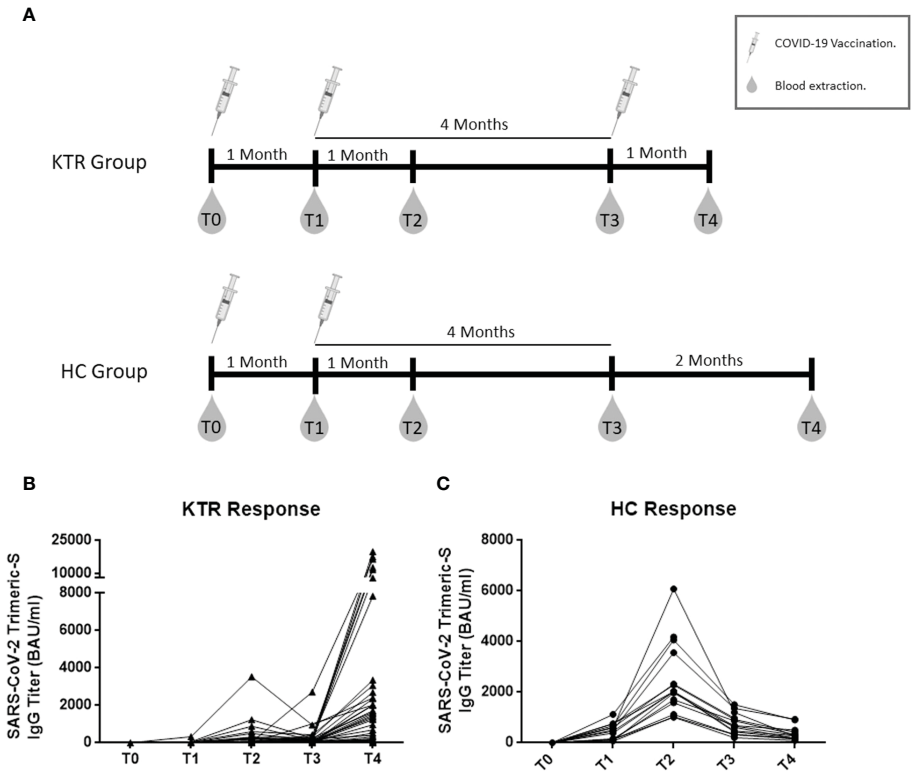

FIGURE 1
Follow-up of humoral response to COVID-19 vaccine, including design scheme of study design. Schematic study design and follow-up **(A)**, longitudinal follow-up of specific antibody IgG titers in 54 Kidney transplant recipients receiving three doses of vaccine **(B)** and in Healthy controls receiving two doses of vaccine **(C)**. KTR, kidney transplant recipients; HC, healthy controls; T0, baseline; T1, one month after the first dose and second dose administration; T2, one month after the second dose; T3, four months after the second dose and third dose administration in KTR group; T4, one month after the third dose. All comparison between time-points were statistically significant by Friedman test ($p<0.005$).

2.3 Immune cell populations

Cell counts and percentages of lymphocytes, monocytes, neutrophils, basophils, eosinophils and platelets were measured from fresh blood samples with an Epics XLMCL flow cytometer (Beckman-Coulter, Brea, California) by standard procedures at the Immunology Service of our Hospital.

2.4 Soluble biomarkers

Plasma and serum samples were collected and stored at -80°C until used. Biochemical and inflammation-related biomarkers were determined by standard procedures at Biochemistry Service of the Virgen del Rocío University Hospital, at T0, in serum or plasma samples when proceeded. Quantification of the homocysteine levels were determined by photometry, whereas high sensitivity C-reactive protein (hsCRP) and β2-microglobulin levels were determined by an immunoturbidimetric assay in Cobas 701 system (Roche Diagnostics, Mannheim, Germany). eGFR was calculated by CKD-EPI index. Tα1 was determined in baseline plasma samples using Human Thymosin Alpha 1 (Tα1) Elisa kit (competitive ELISA, MyBiosurce®).

2.5 Relative telomere length

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood using density gradient centrifugation. Then, PBMC were cryopreserved until DNA was extracted by using Omega BIO-TEK, E.Z.N.A blood DNA Mini Kit. The ratio between number of copies of the telomere sequence and the single copy gen Beta-globin was determined. Copy number quantifications were performed by qPCR following a standard protocol (16). For each reaction, 60 ng of DNA were used. Primers sequences (5´-3´) were: Telomere Forward (GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT) and Reverse (TCCCGACTATCCCTATCCCTATCCCTATCCCT ATCCCTA); Human Beta-Globin Forward (ACACAACTGTG TTCACTAGG) and Reverse (CAACTTCATCCACGTTCACC). The fluorescent reading for the copy number determination was performed in a Light-cycler 480 (Roche).

2.6 Thymic output quantification

Thymic output was calculated as the sj/β-TRECs ratio by Droplet digital PCR (ddPCR), in a single reaction, optimized from (17), and according to the manufacturer's recommendations, in a QX200 system (BIORAD). The primers and probes design were optimized

from (18). Each ddPCR reaction containing 150 ng of DNA from PBMC, 1x ddPCR Supermix no UTP for Probes (BIORAD), 250 nM of FAM labelled Beta Probe, 250 nM HEX labelled Delta Probe, 1 µM of 6 different Beta Forward primers, 1 µM of Beta reverse primer, 1 µM of Delta forward primer and 1 µM of Delta reverse Primers. The final volume of reaction was 20 µl. The results were analyzed by Quantasoft 1.7.1 Software

2.7 Statistical analysis

Statistical comparisons between groups were performed using non-parametric Mann-Whitney U-test for continuous variables or the chi-squared test for the categorical variables. For multiple longitudinal comparisons, the Friedman test applied. Correlations were explored by the non-parametric Spearman´s ρ coefficient. A p-value <0.05 was considered statistically significant. Multivariable linear or logistic regression were performed to determine independent factors affecting to antibody titers or seroconversion rates after vaccination, respectively. Those variables statistically significant in the previous bivariate analysis and those clinically or biologically relevant were included. Statistical analysis were performed using IBM SPSS v21.0 and graphics were generated with GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA).

3 Results

3.1 Characteristics of study subjects

Eighty-seven kidney transplant recipients (KTR) were initially recruited before receiving the first dose of the COVID-19 vaccine, but only 74 KTR were included for analysis and 54 of them remained at the last time-point (see Supplementary Figure 1). Demographical parameters, immunosuppression therapy, baseline kidney function and comorbidities of KTR before vaccination are detailed in Supplementary Table 1. Summing up, this cohort was composed of 31% females, median 59 years-old and the median period post-transplantation was 59 [14-129] months. Most of the KTR patients received corticosteroids (90.5%) (in that case, a fixed dose of 5 mg/day), tacrolimus (94.6%) and mycophenolate-mofetil (79.7%) as immunosuppressive therapy, and 10% received thymoglobulin induction. A reference group of 16 healthy controls (HC) was included (75% females, median 39 [30-45] years-old). No severe symptoms due to COVID-19 vaccine doses were reported.

3.2 Poor humoral response to the COVID-19 vaccine in KTR

Seroconversion rates (defined as ≥33.8 SARS-CoV-2 Trimeric-S IgG BAU/mL) were lower in KTR than HC group at T1 (12% vs. 100%, $p<0.001$) and T2 (44% vs. 100%, $p<0.001$) (Table 1). The KTR group achieved lower humoral titers than the HC group after the doses of vaccine ($p<0.001$ for all comparisons) (Table 1; Figure 1C).

Fifty-four patients remained at the follow-up after the administration of the third dose of mRNA vaccine for the analysis of the humoral response to the booster dose in those patients (Supplementary Figure 1). In these 54 KTR, we observed a significant increase in their antibody response, showing improved seroconversion rates (65% *vs.* 44%, $p=0.002$) and higher antibody levels than before the booster dose (1576 [313-3060] *vs.* 152 [112-279] BAU/mL, $p<0.001$). Figure 1B shows the full longitudinal analysis of the humoral response restricted to those 54 KTR.

3.3 A higher baseline thymic function improved the humoral response to COVID-19 vaccine in KTR

Seroconverted KTR patients after the third dose of vaccine showed higher baseline levels of Tα1 than non-responders (77.9 [62.1-96.8] vs. 69.5 [53.0-77.8], $p=0.04$), as well as higher baseline thymic output, measured as the sj/β-TRECs ratio (6.1 [3.7-10.8] vs. 2.2 [1.0-5.6], $p=0.018$) (Table 2; Figures 2A, B). In addition, sj/β-TRECs ratio correlated to a higher anti-trimeric SARS-CoV-2 S

TABLE 1 Humoral response to COVID-19 vaccination in KTR and HC group.

Humoral Response	KTR *	HC**	*p*-value
T1 seroconversion rate, n (%)	8 (12.1)	16 (100)	**<0.001**
T2 seroconversion rate, n (%)	27 (44.3)	15 (100)	**<0.001**
T3 seroconversion rate, n (%)	29 (47.5)	15 (100)	**<0.001**
T4 seroconversion rate, n (%)	35 (64.8)	15 (100)	NR***
T1 SARS-CoV-2 Trimeric-S IgG levels- BAU/mL	61 [37-109]	482 [129-711]	**<0.001**
T2 SARS-CoV-2 Trimeric-S IgG levels- BAU/mL	200 [44-573]	2020 [1580-3560]	**<0.001**
T3 SARS-CoV-2 Trimeric-S IgG levels- BAU/mL	140 [65-275]	683 [431-1020]	**<0.001**
T4 SARS-CoV-2 Trimeric-S IgG levels- BAU/mL	1576 [313-3060]	274 [176-458]	NR***

Categorical variables are expressed as n (%), and continuous variables as median [IQR]. Comparisons were tested by using Mann-Whitney U-test or χ^2 test when proceeded. *p*-values <0.05 were considered statistically significant and shown in bold. KTR, kidney transplant recipients; HC, healthy controls; T1 one month after the first dose and second dose administration; T2, one month after the second dose; T3, four months after the second dose and third dose administration in KTR group; T4, one month after the third dose. IQR, inter quartile range. *Number of KTR included on each analysis during the follow-up: T1 (n=74), T2 (n=61), T3 (n=61) and T4 (n=54). **One HC lost the follow-up after T1. ***NR, Not relevant because HC group did not receive the third dose of COVID-19 vaccine during the period of time of this study.

TABLE 2 Analysis of soluble markers in KTR depending of their humoral response to COVID-19 vaccination.

	T2 Response			T4 Response		
	Yes (n=27)	No (n=34)	*p*-value	yes (n=35)	no (n=19)	*p*-value
Baseline Demographical Parameters						
Female, n (%)	9 (33)	11 (32)	0.935	9 (25.7)	7 (36.8)	0.392
Age, years	53 [40-62]	62 [50-68]	**0.020**	59 [50-65]	63 [58-70]	*0.068*
Inflammation markers						
Ferritin, ng/mL	79 [45-143]	136 [74-276]	*0.072*	106 [50-179]	140 [82-280]	0.157
β2-microglobuline, mg/L	3.2 [2.5-5.4]	4.0 [3.2-5.9]	*0.057*	3.1[2.5-3.9]	4.6 [4.0-5.9]	**<0.001**
CRP, mg/L	1.1 [0.4-3.3]	1.5 [0.7-3.9]	0.405	1.1 [0.5-3.2]	1.0 [0.5-2.6]	0.465
Homocysteine, mg/L	2.6 [2.3-3.4]	3.3 [2.6-4.1]	*0.076*	2.6 [2.3-4.0]	3.4 [2.8-3.8]	0.211
Neutrophils, cells/μL	3.5 [2.7-4.4]	4.6 [3.4-5.8]	**0.035**	3.9 [3.1-5.2]	4.7 [3.1-5.6]	0.489
Neutrophils, %	55.7 [50.0-59.3]	61.4 [52.4-68.9]	**0.035**	56.6 [52.3-60.3]	65.4 [59.0-72.8]	**0.001**
Thymic Function Parameters						
Tα1, ng/mL	76.9 [61.1-88.8]	70.8 [61.0-92.5]	0.938	77.9 [62.1-96.8]	69.5 [53.0-77.8]	**0.040**
sj/β-TRECs Ratio	5.0 [3.2-11.5]	7.1 [2.0-12.2]	0.909	6.1 [3.7-10.8]	2.2 [1.0-5.6]	**0.018**
Relative Telomere Length	354 [162-572]	177 [103-489]	0.145	211 [104-560]	354 [128-471]	0.603
Baseline Kidney Function Parameters						
Creatinine, mg/dL	1.3 [1.0-1.9]	1.4 [1.1-1.9]	0.265	1.3 [1.1-1.6]	1.7 [1.2-2.2]	*0.082*
eGFR, mL/min	55.0 [39.0-77.2]	47.0 [29.5-63.0]	0.109	55.0 [39.0-71.0]	37.5 [27.0-54.2]	**0.045**
Proteinuria, mg/dL	265 [150-483]	217[135-437]	0.810	199 [106-362]	330 [198-584]	**0.025**
Immune cells populations						
Lymphocytes, cells/μL	1716 [1004-2193]	1731 [1405-2006]	0.936	1867 [1377-2269]	1134 [663-1668]	**0.012**
B-cells, cells/μL	107 [60-157]	52.0 [36.2-144.0]	*0.084*	90.0 [50.5-143.2]	42 [32-96]	**0.010**
T-CD3 cells, cell/μL	1300 [136-1895]	1439 [1157-1805]	0.847	1512 [1028-1907]	959 [431-1449]	**0.025**
T-CD4 cells, cells/μL	688 [348-1104]	666 [481-860]	0.445	767 [513-1068]	443 [184-667]	**0.010**
T-CD8 cells, cells/μL	558 [297-738]	693 [389-936]	0.274	610 [434.2-959.2]	415 [214-726]	0.131
NK cells, cells/μL	123 [50-256]	127 [56-276]	**0.041**	147 [79-289]	130 [80-211]	0.345

Categorical variables are expressed as n (%), and continuous variables as median [IQR]. Comparisons were tested by using Mann-Whitney U-test or χ^2 test when proceeded. *p*-values <0.05 were considered statistically significant and shown in bold, whereas *p*-values between 0.1 and 0.05 were shown in italics. T2: one month after second dose of COVID-19 vaccination; T4: one month after the third dose of COVID-19 vaccination; IQR, inter quartile range; eGFR, estimated glomerular filtration rate; CRP, C-reactive protein. eGFR was calculated by CKD-EPI index.

protein specific IgG production after vaccination (r=0.399, p=0.017). However, the baseline for the telomere relative length in circulating mononuclear cells did not associate with the seroconversion rate (Figure 2C) nor with antibody titers.

3.4 The immunosuppression therapy affected humoral response to the COVID-19 vaccine in KTR

We observed that 19/19 (100%) of KTR non-responders to the third dose were under mycophenolate mofetil as immunosuppressive therapy compared to 25/35 (71%) among responders (p=0.01). We could measure the pre-vaccination mycophenolate blood level in 38 patients and we found that, non-responders presented higher baseline blood levels of this immunosuppressive drug (4.6 [3.1-6.3] *vs.* 2.6 [0.8-3.1] p<0.001), and such levels correlated to a lower antibody production (r=-0.586, p<0.001) (Supplementary Tables 2, 3; Figure 2D). We also explored whether the combination of the immunosuppressive drugs could be affecting the humoral response after three doses of vaccination, by categorizing the KTR population into three groups: I) receiving corticosteroids, tacrolimus and mycophenolate-mofetil (n=37; 68.5%); II) receiving corticosteroids, tacrolimus and mTOR inhibitors (n=6; 11.1%); III) receiving corticosteroids and tacrolimus (n=4; 7.4%). We observed a better response, regarding the seroconversion rates (100 *vs.* 56.7; p=0.042) in patients who were treated with mTOR inhibitors in triple combined therapy instead of the mycophenolate-mofetil. No differences were found between these triple combinations and the dual therapy of corticosteroids plus tacrolimus (Supplementary Table 2).

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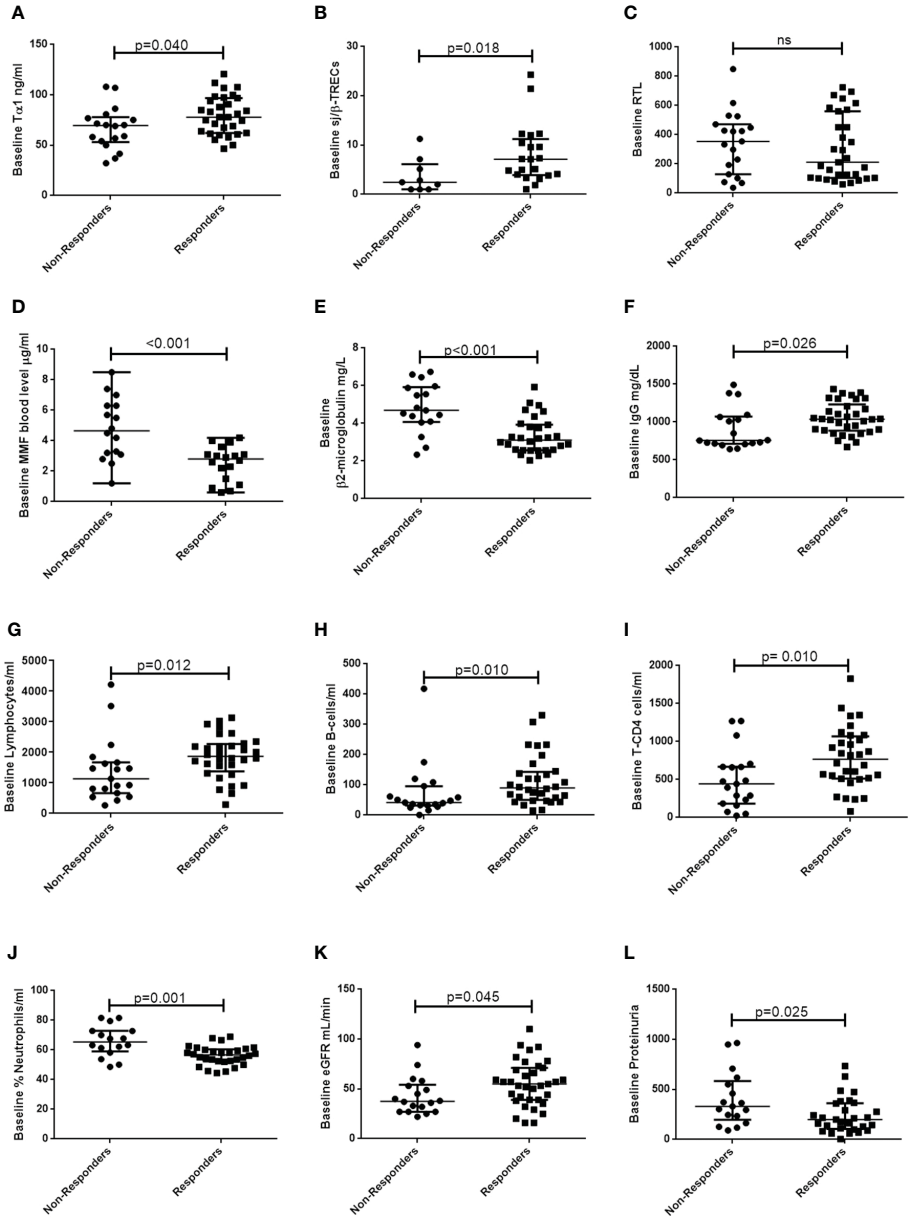

FIGURE 2
Baseline thymic function-related parameters, therapy-related factors, inflammatory-related markers, immune subsets and renal function-related factors in responders and non-responders KTR to the third dose of vaccine. Plots show comparisons of baseline levels of **(A)** Thymosin- α1 (Tα1), **(B)** sj/β TRECs ratio, **(C)** Relative Telomere length (RTL), **(D)** mycophenolate-mofetil (MMF) blood level, **(E)** β2-microglobulin, **(F)** IgG, **(G)** lymphocytes, **(H)** B-cells, **(I)** T-CD4, **(J)** Neutrophils, **(K)** eGFR and **(L)** proteinuria between responders and non-responders KTR to the third dose of vaccine. Groups were compared using Mann Whitney-*U* test, considering statistically significant a *p*-value <0.05. eGFR, estimated glomerular filtration rate, calculated by CKD-EPI index.

3.5 Inflammation, distribution of immune subsets and renal function also influenced the humoral response to COVID-19 vaccine in KTR

We obtained a data set comprised of the results from routine biochemical tests and hemograms which can provide insight into the immune response of patients to the vaccination. In addition to the demographical and immunosuppressive therapy related variables, we also analyzed inflammatory markers, kidney function markers and immune cell subsets. These variables are shown in Table 2, together with thymic function related parameters. The rest of the variables recorded from such routine tests can be found in the Supplementary Table 3. Patients that did not respond after three doses showed no differences in gender but tended to be older (p=0.068) and presented higher baseline levels of β2-microglobulin (p<0.001) (Table 2; Figure 2E). They showed also lower IgG levels (p=0.026) and lower counts of several immune cell populations, such as lymphocytes (p=0.012), B-cells (p=0.010), T-CD3 cells (p=0.025) and T CD4-cells (p=0.010) but higher frequency of neutrophils (p=0.001) (Table 2; Figures 2F–J). We also observed that seroconverted patients had higher eGFR and a lower proteinuria level (p=0.045 and p=0.025, respectively) (Table 2; Figure 2K, L). Correlations between all the continuous variables and the antibody titers after

full vaccination are shown in Supplementary Table 4. Regarding sex, no differences were found in median T4 antibody titer in women compared to men (313 [152-2380] vs. 1647 [644-4480], p=0.210). Regarding correlations, we found negative correlations between the specific antibody titer at T4 and age (r=-0.407, p=0.002, serum level of creatinine (r=-0.314, p=0.022), proteinuria (r=-0.418, p=0.003), ferritin (r=-0.329, p=0.017), β2-microglobulin (r=-0.490, p<0.001), neutrophils% (r=-0.459, p=0.001) and mycophenolate-mofetil blood level (r=-0.586, p<0.001), whereas positive correlations were observed between the specific antibody titers at T4 and T3 (r=0.796, p<0.001), baseline eGFR (r=0.388, p=0.004), B-cells/μL (r=0.410, p=0.003) and sj/β TRECs ratio (r=0.399, p=0.017).

3.6 Predictive factors of humoral response to COVID-19 vaccine in KTR

We performed a multivariable analysis including, besides sex and age, variables related to each of the following categories: demographical parameters, inflammatory-related markers, thymic function-related factors, immune cell subsets, kidney function parameters and immunosuppression therapy. Taking into account that, due to the sample size of our study, we were limited to include up to eight variables in a multivariable analysis, we selected at least one representative variable from each mentioned category, according also to its biological relevance in the vaccination response context. The best fitted model showed that baseline creatinine serum level (βI coeff: -7214, p=0.011), β2-microglobulin (βI coeff: 2035, p=0.003) and T3 antibody titer (βI coeff: 20.5, p=0.036) were independently associated with T4 antibody titers, whereas CD4 T-cells tended to (βI coeff: -4.8, p=0.093) (Table 3). Furthermore, thymosin-α1 levels were independently associated with seroconversion after the full vaccination challenge in KTR (OR: 1.1, p=0.037) whereas the blood concentration of mycophenolate-mofetil tended to (OR: 0.3; p=0.060).

4 Discussion

In our cohort of kidney transplant recipients (KTR), the humoral response against the COVID-19 vaccine was seriously impaired, even though a third dose/booster significantly improved the seroconversion rate and antibody titers. Not surprisingly, immunosuppressive therapy played a critical role in both, the capability of responding and the humoral magnitude achieved. Remarkably, the capability of responding was associated with baseline thymic-related factors, such as the concentration of thymosin-α1, while the magnitude of the response was associated with different factors, including age and baseline renal and inflammatory status.

The KTR cohort showed an inferior response to the COVID-19 vaccine in comparison to the HC group, after two doses and even after an additional third dose, in the case of KTR. Although both groups were not age and sex matched, and were vaccinated with different vaccine mRNA platforms/doses, this result is consistent with previous data on similar cohorts (4, 19, 20). In fact, the seroconversion rate in KTR against the three-dose protocol COVID-19 vaccine is usually around 40% lower than that of healthy subjects and significantly lower than rates obtained for vaccines against other pathogens, such as influenza or pneumococcus (21, 22).

Due to the risk of developing severe disease in this population (23) and since the antibody titer achieved after COVID-19 vaccination has been related to a lower risk of infection and a better outcome of infection (24), new strategies are urgently needed to improve antibody titer after COVID-19 vaccination, and the vaccination efficiency in this population. In this line, in a recently published clinical trial (25), three different vaccination strategies have

TABLE 3 Multivariable analysis of potential predictive factors (baseline determinations) in seroconversion and magnitude of humoral response to COVID-19 vaccine in KTR.

	T4 SARS-CoV-2 Trimeric-S IgG Levels (BAU/mL)			T4 Seroconversion	
	βI Coefficient (95% CI)	p-value		OR (95% CI)	p-value
Age (years)	-53.4 (-190, 83.8)	0.418	Age (years)	0.9 (0.7-1.1)	0.444
B-Cells (cells/mL)	7.6 (-20.5, 35.7)	0.573	Female (yes *vs.* no)	0.1 (0.01-10.6)	0.301
T-CD4 cells (Cells/mL)	-4.8 (-10.6, 0.9)	*0.093*	T-CD4 cells (Cells/mL)	1.0 (0.99-1.00)	0.972
Creatinine (mg/dL)	-7214 (-12495, -1934)	**0.011**	B-Cells (Cells/mL)	1.0 (0.9-1.0)	0.702
Mycophenolate-mofetil BL (mg/mL)	144 (-687, 975)	0.716	eGFR (mL/min)	0.9 (0.88-1.04)	0.336
Sj/β TRECs Ratio	26.3 (-233, 286)	0.831	Mycophenolate-mofetil BL (mg/mL)	0.3 (0.1-1.0)	*0.060*
β2-microglobulin (mg/mL)	2035 (806, 3463)	**0.003**	Tα1 (ng/mL)	1.1 (1.0-1.3)	**0.037**
T3 SARS-CoV-2 Trimeric-S IgG Levels (BAU/mL)	20.5 (1.6, 39.5)	**0.036**	Neutrophils (%)	0.9 (0.7-1.1)	0.327

Multiple linear regression (for the analysis of magnitude of response) and logistic (for seroconversion analysis) were applied. p-values <0.05 were considered statistically significant and shown in bold, whereas p-values between 0.1 and 0.05 were shown in italics. T4 one month after third dose of vaccine administration. T3 four months after the second dose and third dose administration. CI, Confidence interval; OR, Odds Ratio; BL, blood level; eGFR, glomerular filtration rate, was calculated by CKD-EPI index.

been tested in KTR without seroconversion after two or three doses of vaccination: a double dose of mRNA-1273, a heterologous vaccination (Ad26-COV2-S) and a transitory discontinuation of the mycophenolate mofetil administration as immunosuppressive therapy. Unfortunately, none of those strategies improved the vaccine response in comparison to the single dose of mRNA-1273, reinforcing the need for further research on potential modulators of vaccine response in this population. Moreover, in order to improve the protection against SARS-CoV-2 infection in KTR, the US and some European countries have approved the administration of SARS-CoV monoclonal antibodies to non-responder patients as a pre-exposure prophylaxis (26). So far, the advantage in KTR seems to be limited with the results differing according to the SARS-CoV-2 strain analyzed (27). Therefore, new approaches in this high-risk group of patients are still needed to improve the protection conferred by the vaccine administration.

In this study, we have analyzed immune-related factors potentially involved in the humoral response to vaccination. Interestingly, we found a strong positive correlation between the antibody titers after the two-doses schedule and the booster. Not surprisingly, we observed a poor response in those patients receiving mycophenolate-mofetil as immunosuppressive therapy. Moreover, age was related to lower antibody levels after three doses of vaccination. Both results are in accordance with those from a different KTR cohort receiving two doses of mRNA-1723 vaccine (26). Regarding the combined immunosuppressive therapy, we observed an improvement in the vaccination response when mTOR inhibitors were administered instead of mycophenolate-mofetil, similar to those data published in a KTR cohort receiving mycophenolate-mofetil in combination with tacrolimus (27). Currently, several trials intend to analyze the potential benefit of m-TOR inhibitors on the immune response. Our data suggest that such improvement could more probably reflect the negative effect of mycophenolate-mofetil than a beneficial effect of m-TOR inhibitors. However, the potential benefit of m-TOR inhibitors in the immune response cannot be discarded (28), since, as previously shown, mycophenolate substitution with mTOR inhibitor has a positive effect on virus clearance in kidney transplant recipients (29).

A better renal function and lower plasma levels of inflammation-related markers, such as neutrophils and B2-microglobulin, were associated with a better response in our KTR patients. B2-microglobulin is commonly elevated in KTR due to the immunosuppressive status and deteriorated kidney function (30). Furthermore, higher counts of immune cells, such as B-cells and T-CD4, as well as higher IgG levels, prior the immunization protocol favoured the response to the COVID-19 vaccine. The correct ratios of immune subsets are known to be critical during viral infections, such as the CD4/CD8 ratio in HIV-infection (7), or the total CD4+ and CD8+ T-cells producing IFN-γ or TNF-α as predictors of the immune response after vaccination against tuberculosis and herpes zoster (9).

In this line, we also explored the thymic function, which plays a main role in the maturation and functionality of T-cells and has been outlined in several contexts of limited immune responses, such as HIV-infection, as well as in aging (15, 31). As far as we know, the thymic function has not been yet explored in the context of the response to COVID-19 vaccine. We found a higher sj/β-TRECs ratio (which is the gold standard measure to asses thymic output) in seroconverted KTR after vaccination. Moreover, thymic function correlated with antibody titers in our group of patients. Globally, the KTR group showed a lower thymic output than the healthy group (data not shown). It is important to note that such difference could be easily explained by differences in sex and age between both groups. Nevertheless, thymic involution is suggested to be accelerated in kidney patients (32). Additionally, in our healthy group, no correlation was observed between the thymic output and the antibody titer after the COVID-19 vaccine (data not shown), but the limited availability of data from the thymic output in this group (n=11) precludes any conclusion about it. Furthermore, baseline thymosin-α1 (Tα1) plasma levels, an immunomodulatory hormone secreted by the thymus was positively associated with seroconversion. Tα1 has an immunologic-enhancing activity by the increase of CD4 and CD8 T-cell maturation and natural killer cell activation and is also considered as a humoral response enhancer (33). Furthermore, Tα1 has been related to a better recovery after SARS-CoV-2 infection and the efficacy of its administration for the treatment of COVID-19 is being explored (12, 13). This hormone has also been used in other infections, as in HIV-infection, with an immunomodulatory aim, and in HCV or pseudomonas infections with a therapeutic aim (34–36). Interestingly, Tα1 has also been used as an adjuvant in Influenza vaccine protocols (37, 38). Our results about the clinical benefit of Tα1 on COVID-19 vaccine response in KTR need to be confirmed in further studies. It would be important to evaluate the safety of Tα1 as a vaccination adjuvant in this clinical context since using immune stimulators in the context of transplanted patients could have negative consequences within the risk of organ rejection. However, Tα1 has been only administered to the solid organ transplant recipients for the treatment of serious complications such as cytomegalovirus infection and acute respiratory distress syndrome due to pneumonia (39, 40), showing a survival improvement without graft rejection.

We cannot conclude about the clinical protection achieved by the COVID-19 vaccine in our KTR cohort due to the low number of patients and the low rate of infection in our study period. However, from the five documented cases of SARS-CoV-2 infection after the administration of two doses of mRNA COVID-19 vaccine, three of them required hospital admission and one of those patients died due to a bilateral pneumonia. The other two patients presented mild symptoms. This is consistent with the rate of potentially seroprotected subjects in our cohort (50%), that is those patients reaching 260 BAU/mL, a threshold recently proposed for seroprotection (41), which is now being implemented by ours and others sanitary systems for clinical decisions.

We could not address the cellular response after vaccination in these patients, an important aspect to understand the full dynamics of the immune response after the immunization, and the role of thymic function in such response. Interestingly, an absence of correlation between SARS-COV-2 antibody titers and T cell response in kidney transplant recipients has been described (42).

Our study has additional limitations, such as a low sample size and a large number of variables analyzed which limits the statistical power to conclude about the immune predictors as well as about the role of the combinations of immunosuppressive drugs in the humoral response to COVID-19 vaccine in kidney transplant recipients. In addition, the thymic output data were obtained from PBMCs samples,

rather than the isolated T-cell compartment, comprising thymic emigrants, which would therefore have increased the sensitivity of quantification. However, our novel findings could help in the design of future immunization strategies aiming the improvement of the COVID-19 vaccine response in these immunosuppressed patients and suggest the possibility of the use of thymosin-α1 as a vaccination adjuvant to improve their response following the next boosters in this risk population.

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 Comité de Ética de la Investigación de los Hospitales Vírgen Macarena y Vírgen del Rocío de Sevilla. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Patient recruitment and clinical data collection (GB-B, AS-B, CG-C, MS-P, FG-R). Quantification of antibody titers (CL). Experiments of immune subsets (BS, IM-C, JL-R, MG-E). Experiments of biochemical and inflammatory markers (AA-R). Processing of samples and experiments of thymic function-related markers (AB-R, MP-B, IO-M, VG-R). Data base management and statistical analysis (AB-R, MP-B, YP). Manuscript preparation (AB-R, MP-B, YP). YP conceived the study, obtained funding and supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1099079/full#supplementary-material

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Delivery of spike-RBD by bacterial type three secretion system for SARS-CoV-2 vaccine development

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COVID-19 pandemic continues to spread throughout the world with an urgent demand for a safe and protective vaccine to effectuate herd protection and control the spread of SARS-CoV-2. Here, we report the development of a bacterial vector COVID-19 vaccine (aPA-RBD) that carries the gene for the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein. Live-attenuated strains of *Pseudomonas aeruginosa* (aPA) were constructed which express the recombinant RBD and effectively deliver RBD protein into various antigen presenting cells through bacterial type 3 secretion system (T3SS) *in vitro*. In mice, two-dose of intranasal aPA-RBD vaccinations elicited the development of RBD-specific serum IgG and IgM. Importantly, the sera from the immunized mice were able to neutralize host cell infections by SARS-CoV-2 pseudovirus as well as the authentic virus variants potently. T-cell responses of immunized mice were assessed by enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining (ICS) assays. aPA-RBD vaccinations can elicit RBD-specific $CD4^+$and $CD8^+$T cell responses. T3SS-based RBD intracellular delivery heightens the efficiency of antigen presentation and enables the aPA-RBD vaccine to elicit $CD8^+$T cell response. Thus, aPA vector has the potential as an inexpensive, readily manufactured, and respiratory tract vaccination route vaccine platform for other pathogens

KEYWORDS

SARS-CoV-2 vaccine, *Pseudomonas aeruginosa*, live-attenuated, type 3 secretion system (T3SS), anti-virus immunity

Introduction

To stop the ongoing COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), several vaccines have been developed through various platforms, among which live-attenuated bacteria has been gaining attention as a versatile tool. The live-attenuated bacterial vaccine stands out for its fast and low cost, suitable for mass production, promising to be leveraged for a rapid emergency response. Moreover, the bacterial vectors of the vaccine, exemplified by Bacillus Calmette-Guérin (BCG), could promote non-specific cross-protection against other bacterial and viral infections (1–4). BCG is a live attenuated *Mycobacterium bovis* vaccine that is widely used to prevent tuberculosis (TB) and was among the most broadly used vaccinations in the 20th century in neonatal and young children (5, 6). It could lead to long-term activation and reprogramming of innate immune cells, engaging pattern recognition receptors (PRRs) and trained innate immunity (7–9). To date, several bacterial vaccines have been studied or in clinical trial phase: *Bifidobacterium longum* DNA vaccine (bacTRL-Spike) from Canada, *Salmonella typhimurium* expressing spike protein (S.T. Ag-e.spike) and *Mycobacterium paragordonae* expressing receptor binding domain (Mpg-RBD-7) candidate vaccines from Korea, *Francisella tularensis* (rLVS *ΔcapB*) candidate vaccine co-expressing spike, nucleocapsid and membrane proteins from U.S.A. (10–12). However, currently available vaccines possess shortcomings, such as inefficient protein delivery capacity to antigen presenting cells, and thus trigger a weak cell-mediated immune response, especially memory T cell response (13, 14). The intracellular delivery of proteins is challenging, our work nonetheless suggests that a T3SS-based delivery system of live-attenuated *Pseudomonas aeruginosa* (aPA) serving as a platform could translocate the desired proteins and elicit immune memory response.

T3SS is a naturally occurring protein transport nanomachinery, highly conserved among Gram-negative bacteria. Expression of the machinery and its effectors is triggered upon contact with the host cells or induced by low calcium environment, such as in the presence of calcium chelator EGTA *in vitro*. Effectors are translocated through T3SS injectisome which is a syringe-like nanomachine that could puncture the host cell membrane and inject the effectors directly into host cytosol, making it a promising tool for protein delivery directly into the target cells (15). Furthermore, the ease of bacterial genetic and physiological manipulations also made them extremely attractive for used in vaccine applications. When proteins of interests are fused with the secretion signal of a T3SS effector ExoS (S_{54}) (16), and the strain *P. aeruginosa* was deleted of all its native T3SS effectors while maintaining a functional injectisome, the recombinant proteins can be efficiently injected into various cell lines such as A549, 5637, HL-60, mESCs and hESCs (17). Notably, the *P. aeruginosa* also naturally colonizes in the lungs, conferring a convenient intranasal route to stimulation of tissue-resident immunity (18, 19). Hence, due to its excellent delivery ability and possibility of eliciting both $CD4^+$ and $CD8^+$ immune response, we developed a series of T3SS-based aPA vaccines, in which the T3SS effectors, secretion repressor, and several acute virulence factors were deleted, as well as a gene essential for growth to confer the auxotrophic phenotype.

Herein, to investigate the possibility of developing intranasal administered COVID-19 vaccines using the RBD of SARS-CoV-2 spike protein along with the already informed aPA strains, we constructed an expression plasmid, in which the RBD was fused behind the N-terminal secretion signal of the T3SS effector ExoS. The aPA strains harboring the plasmid were able to inject the fusion protein into host cells in a T3SS-dependent manner. Upon nasal delivery, the vaccine strain triggered potent cellular and antibody responses. The data provide a reference for preparing other attenuated bacterial vaccines.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1, along with their description and sources. To ensure the stability of these genetically modified bacteria, a mandatory step in this study is that the bacteria need to be freshly streaked on selective plates from −80°C storage and the expression of the RBD was verified by western blotting before each experiment.

Immunization of mice

Specific pathogen-free (SPF) 7-9 weeks old female C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (licensed by Charles River). All mice used in this study are in good health and are not involved in other experimental procedure. Mice were housed with 5 companions per cage. All animals were allowed free access to water and standard chow diet and provided with a 12-hour light and dark cycle (temperature: 20-25°C).

To prepare inocula for infections, *P. aeruginosa* strains were grown in LB (Δ3, Δ5, Δ8) or LB with10 mM D-Glu (Δ6, Δ9) overnight, and then subcultured in fresh medium, grown at 37°C to an OD_{600} of 1.0. The cells were harvested by centrifugation and pellets washed twice were suspended in sterile 0.9% NaCl, adjusted to 5×10^8 CFU/ml. For vaccination, mice were immunized with indicated aPA strains [with a total volume of 20 μl (5×10^7 CFU bacteria) per mouse] *via* intranasal route at biweekly intervals. As an intranasal vaccination control, recombinant RBD protein (GenScript, Z03483) was diluted with PBS, and mixed with an equal volume of curdlan adjuvant (20 mg/mL) (24). Serum samples were collected after vaccination as indicated in figures legends.

Tissue bacterial loads

To assess bacterial loads in lungs and spleens, mice immunized with Δ5 and Δ6 were euthanized at indicated time points for each experiment. Tissues were extracted aseptically, homogenized in

TABLE 1 *P. aeruginosa* strains and plasmids used in this study.

Strain and plasmid	Description	Source
P. aeruginosa		
PAK-J	wild type *P. aeruginosa* strain with enhanced T3SS	(16)
Δ*exsA*	PAK-J deleted of *exsA*, which encoded the master activator of *P. aeruginosa* T3SS	(20)
Δ*popD*	PAK-J deleted of T3SS translocon pore formation gene *popD*, which is essential for the protein injection	(16)
Attenuated *P. aeruginosa* (aPA)		
Δ3	PAK-J deleted of *exoS*, *exoT*, and *exoY*	(16)
Δ5	Δ3 deleted of *ndk*, and *popN*	This study
Δ6	Δ5 deleted of *murI* (D-Glu auxotroph)	This study
Δ8	Δ5 deleted of *lasR-I*, *rhlR-I* and *xcpQ*	(21)
Δ9	Δ8 deleted of *murI* (D-Glu auxotroph)	(22)
Plasmids		
pExoS$_{54}$F	*Escherichia-Pseudomonas* shuttle expression plasmid, Cb^R	(23)
pS$_{54}$-RBD[wt]	pExoS54F fused with SARS-CoV-2 spike-RBD [YP_009724390.1 (R319-F541)] gene, Cb^R	This study
pS54-RBD[Delta]	Similar to the RBD[wt] sequence, except for two mutations (L452R, T478K)	This study
pS$_{54}$-RBD[BA.1]	Sequence containing 13 mutations (G339D, R346K, S371L, S373P, S375F, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H), compared to that of RBD[wt]	This study

sterile 0.9% NaCl and enumerated as colony forming units (CFU) by plating 10-fold serial dilutions on L-agar plates.

Indirect ELISA

All wells in 96-well plates were coated with the recombinant RBD protein (1 μg/ml) in 0.05 M carbonate-bicarbonate buffer at 4°C overnight, and blocked by PBST (PBS containing 0.05% Tween 20) supplemented with 5% skim milk at 37°C for 3 h. Serum samples were 10-fold serial dilution and added to each well, followed by incubation at 37°C for 1 h. After being washed with PBST for five times, plates were incubated with anti-mouse IgG/HRP (Promega, USA) and detected with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (ACMEC, China). Reactions were stopped with 1 M sulphuric acid, and the absorbance was measured at 450 nm in an ELISA reader (Varioskan Flash, USA). The endpoint titer was defined as the highest reciprocal dilution of serum to give an absorbance greater than 2.5-fold of the background values.

Pseudovirus neutralization assay

A lentivirus-based SARS-CoV-2 pseudovirus system [GenScript (Cat. No. SC2087A)] expressing a Spike protein on the surface (Accession number: YP_009724390.1) was generated according to the instruction manual. Briefly, neutralizing antibody activity is measured by assessing the inhibition of luciferase activity in HEK293 target cells expressing the ACE2 receptor, following preincubation of the pseudovirus with 5-fold serial dilutions of the serum specimen. Titers are reported as the highest reciprocal serum dilution at which the relative light units (RLUs) were reduced by greater than 50% compared with virus control wells.

Live SARS-CoV-2 neutralization assay

Δ6-RBD vaccines induced neutralizing activities against live SARS-CoV-2 WT or variants infection were detected using the plaque assay as described previously (25). The experiment was conducted in a BSL-3 laboratory at Shenzhen Center for Disease Control and Prevention. In brief, serum from each immunized mouse was diluted and mixed with the same volume of SARS-CoV-2 (100 PFU) and incubated at 37°C for 1 h. Thereafter, 200 μL of the virus-serum mixtures were transferred to pre-plated Vero E6 cells in 24-well plates. Inoculated cells were incubated at 37°C for two days. Then, Vero E6 cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The cells were incubated sequentially with primary antibody against the SARS-CoV-2 nucleocapsid (NP) (SinoBiological) overnight at 4°C, horse radish peroxidase (HRP)-conjugated secondary antibody (Abcam), and TMB substrate (KPL). The plaque reduction neutralizing antibody titer (PRNT$_{50}$) was defined as the minimal serum dilution that suppressed > 50% of viral plaques.

ELISPOT

To detect RBD-specific T lymphocyte response, an IFN-γ -based ELISPOT assay was performed. Mice spleens were collected and lymphocytes were isolated. 96-well plates were precoated with anti-mouse IFN-γ antibody overnight at 4°C and then blocked for 2 hours at room temperature. Different concentrations of the recombinant RBD protein were added to the well, and then lymphocytes were added to the plate (1.5×10^5/well). Cell Activation Cocktail (without Brefeldin A) [BioLegend (Cat. No. 423301)] was added as a positive control and cells stimulated with 0.9% NaCl were employed as a negative control. After 24 hours of incubation, the cells were removed, and IFN-γ was captured by biotinylated detection antibody, streptavidin-HRP conjugate and AEC substrate.

Flow cytometry

Approximately 1.5×10^6 cells were stained with antibodies and antibody application was followed by the recommendation. Mouse lymphocytes were stimulated with the peptide pool of SARS-CoV-2 RBD and incubated with monensin [BioLegend (Cat. No. 420701)] for 9 hours. Then, the cells were harvested. For surface staining, cells were stained with fluorescence-labeled mAbs of CD3-FITC (BioLegend, USA), CD4-APC-Cy7 (BioLegend, USA) and CD8-AF700 (BioLegend, USA). The cells were subsequently fixed and permeabilized in permeabilizing buffer (BD Biosciences, USA) and intracellularly stained with fluorescence-labeled mAbs of IFN-γ-BV605 (BioLegend, USA), IL-2-BV421 (BioLegend, USA) and IL-4-PE (BioLegend, USA). All stained cells were detected on BD LSRFortessa™ X-20.

Statistical analysis

Data are shown as mean ± SD. All calculations and statistical analyses were performed using GRAPHPAD PRISM 8.0.1 (GraphPad Software, USA) for Windows.

Results

Construction of aPA-RBD vaccine candidates

We generated a series of attenuated *P. aeruginosa* strains (aPA) by successive gene deletions of the intrinsic T3SS effectors and repressor, as well as several acute virulence factors and an essential gene (Table 1). These deletion mutations were constructed on genomic loci that are not be flanked by active mobile elements or gene duplications (15, 26, 27).Due to the deletion of a glutamate racemase gene *murI*, aPA strains (Δ6 and Δ9) acquired an auxotrophic phenotype and had to grow in the presence of exogenous D-glutamate (D-Glu) (Figure 1A) (17, 28). The spike-RBD (R319 to F541) of SARS-CoV-2 was fused behind the N-terminal 54 amino acids of ExoS (S_{54}) and expressed in *P. aeruginosa* on an expression plasmid pS_{54}-RBD (Figure 1B). Various aPA strains harboring the pS_{54}-RBD were subjected to T3SS induction by 5 mM EGTA for 3 h. As shown in Figure 1C, the S_{54}-RBD fusion protein was expressed in all of the aPA strains, but not in the T3SS-defective mutant strain (Δ*exsA*), indicating that the expression of S_{54}-RBD is dependent on T3SS activation. Moreover, similar to our previous observation (17), the D-Glu auxotrophic aPA strains produced more of the S_{54}-fusion proteins under the D-Glu depleted condition (Figure 1C). To further assess the capacity of aPA to deliver RBD into the human cells related to pulmonary infection, alveolar basal epithelial cell line A549, promyelocytic cell line HL-60, and monocytic cell line THP-1 were co-incubated with aPA strains of Δ3-RBD, Δ5-RBD, Δ6-RBD, Δ8-RBD or Δ9-RBD, individually, at MOI of 100 for 3 h. After removal of the bacterial cells, the human cells were examined for intracellular S_{54}-RBD proteins by western blotting. As the results shown in Figure 1D, the S_{54}-RBD could be translocated into human cells by aPA Δ5 and Δ6 the most efficiently. However, no translocated S_{54}-RBD was detected in the cells following co-culture with Δ*exsA* or the injection deficient mutant Δ*popD* strains (Figure 1D), although the fusion was produced by the Δ*popD* strain (Figure 1C). These results indicated that intracellular delivery of the S_{54}-RBD protein by aPA occurred in a T3SS-dependent manner, and aPA Δ5 and Δ6 exhibited high capability of antigen delivery.

Safety of aPA-RBD in mice

To evaluate the safety of aPA-RBD, we first measured mice survival after intranasal inoculation with strains Δ5-RBD and Δ6-RBD. Using this model of acute lung infection, the LD_{100} (the minimal lethal dose for 100% of mice) of wild-type (wt) PAK-J strain was 2×10^7 CFU. In contrast, the observed LD_{100} for the Δ5-RBD and Δ6-RBD strains were $> 1 \times 10^9$ CFU (Supplementary Figure S1). The survival rate of the Δ6-RBD group is obviously higher than that of the Δ5-RBD group. A 100% survival was observed in both Δ5-RBD and Δ6-RBD groups when the administration dose was 5×10^7 CFU (Figure 1E). Consistently, bacterial loads in lungs and spleens of Δ6-RBD were significantly lower than those inoculated with Δ5-RBD (Figure 1F). As no D-amino acids are available in mammals, the D-Glu auxotroph Δ6-RBD was eliminated within 72 hours after intranasal administration of 5×10^8 CFU bacterial cells, shorter persistence time than that of Δ5-RBD (Figure 1F).

Then, we also assessed the pathological manifestations of lungs in mice one week after vaccination. As shown in Figure 1G, compared to the saline group, the lung of the Δ5-RBD group exhibited a more severely distorted structure, with a larger number of inflammatory cells infiltration in the pulmonary interstitium. However, no obvious tissue damage was observed in the Δ6-RBD group. Collectively, these results suggest that the strain Δ6-RBD, absolutely requiring D-glutamate for growth and featured a stable auxotrophic phenotype, confers much lower virulence than that of strain Δ5-RBD. 16S rRNA gene sequencing analysis of intestinal and pulmonic samples of mice suggest that the

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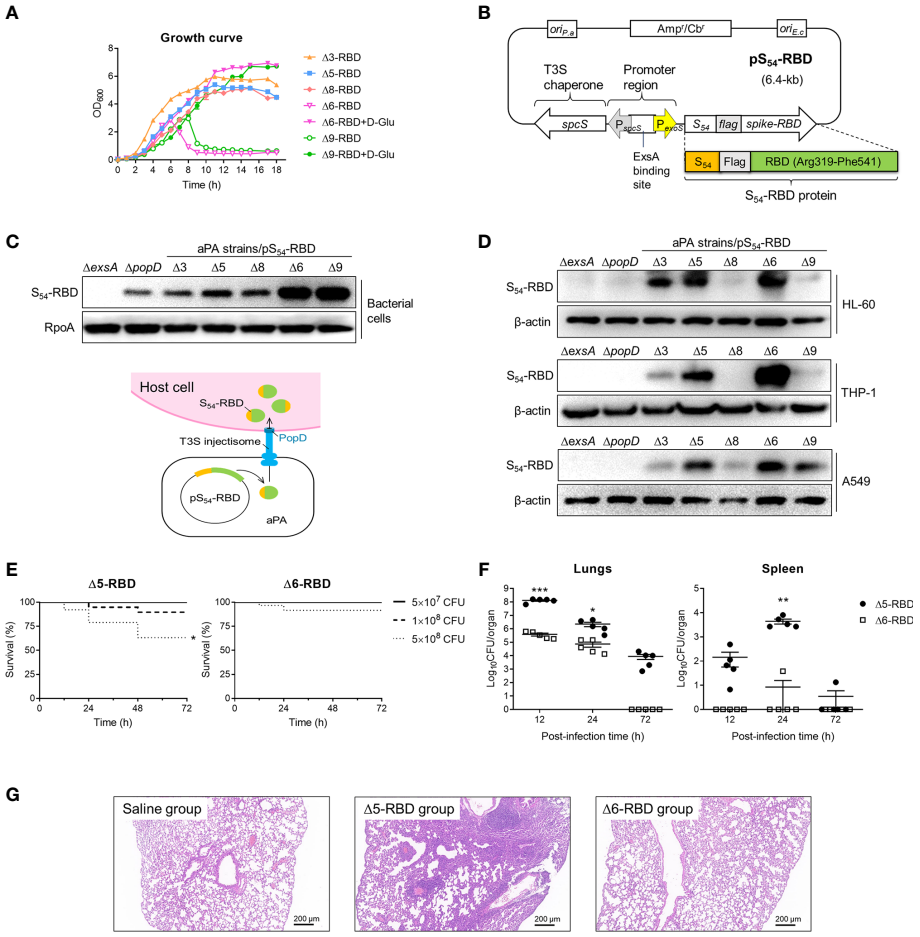

FIGURE 1
Construction and toxicity of candidate aPA-RBD vaccines against SARS-CoV-2. **(A)** Growth and viability of indicated attenuated *P. aeruginosa* (aPA) strains in LB liquid medium. D-Glu corresponds to 10 mM D-glutamate. **(B)** Expression vector of SARS-CoV-2 Spike-RBD fusing with the T3SS secretion signal S_{54} and a Flag tag on the N-terminal. ExsA is the master regulator for *P. aeruginosa* T3SS. **(C)** Identification of the fusion protein. Under 5 mM EGTA inducing conditions, aPA-RBD strains were examined for the ability to express the fusion protein by anti-Flag immunoblot of the bacterial pellets. Antibacterial RpoA (bacterial RNA polymerase subunit) immunoblot was used as the bacterial internal reference. **(D)** Ability of bacterially injection. Human alveolar basal epithelial cell line A549, promyelocytic cell line HL-60, and monocytic cell line THP-1 were cocultured with indicated *P. aeruginosa* strains for 3 hours at MOI of 100, lysed and examined for protein injection by anti-Flag immunoblot. β-actin was used as the internal reference of mammalian cells. **(E)** Mice survival after intranasal administration with different amounts of Δ5-RBD and Δ6-RBD strains. CFU, colony-forming unit. Survival curves are generated by the Log-rank (Mantel-Cox) test to determine the statistical significance; *, $P<0.05$. **(F)** Bacterial loads in lungs and spleen from mice after vaccination with Δ5-RBD or Δ6-RBD strain (5×10^8 CFU per mouse). Comparisons between Δ5-RBD and Δ6-RBD infected groups were performed by Student's *t*-test (unpaired, two-tail); Error bars represent SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **(G)** Hematoxylin and eosin **(HE)** staining of lung tissues collected at day 7 from immunized mouse. Scale bar = 200 μm.

diversities of microbiota in Δ5-RBD and Δ6-RBD groups were not significantly different from those of the blank and Δ6-vehicle groups when ignoring the differences exhibited within the groups (Supplementary Figure S2).

Generation of antibody-mediated immune responses

To assess the immunogenicity of aPA-RBD, we immunized each C57BL/6 mice with the Δ5-RBD, Δ6-RBD, and empty Δ6/pExoS$_{54}$F (Δ6 vehicle) by intranasal administration of 5×10^7 CFU. 10-μg of recombinant RBD protein with the adjuvant curdlan in PBS was administered intranasally as a comparison, while saline was given as sham control. We performed a two-dose regimen to assess the response dynamics (Figure 2A). Mice sera were collected one week after each immunization and measured for the humoral responses. The presence of RBD-specific IgG and IgM antibodies was evaluated by indirect ELISA using SARS-CoV-2 Spike-RBD recombinant protein as coating antigen. Sera obtained 7 days after the second dose of the candidate vaccines showed elevated IgG and IgM against the recombinant RBD (Figures 2B,C). By contrast, the sera from control mice treated with saline or empty aPA (Δ6 vehicle) showed only background-level antibody responses. Notably, the recombinant RBD protein with curdlan (RBD control) was more effective in inducing the production of RBD-specific antibodies, especially the group (RBD+adju IP) immunized through the intraperitoneal route (Figures 2B,C).

Then, to investigate the capability of Δ5-RBD and Δ6-RBD on inhibiting the infectivity of SARS-CoV-2 pseudovirus, a

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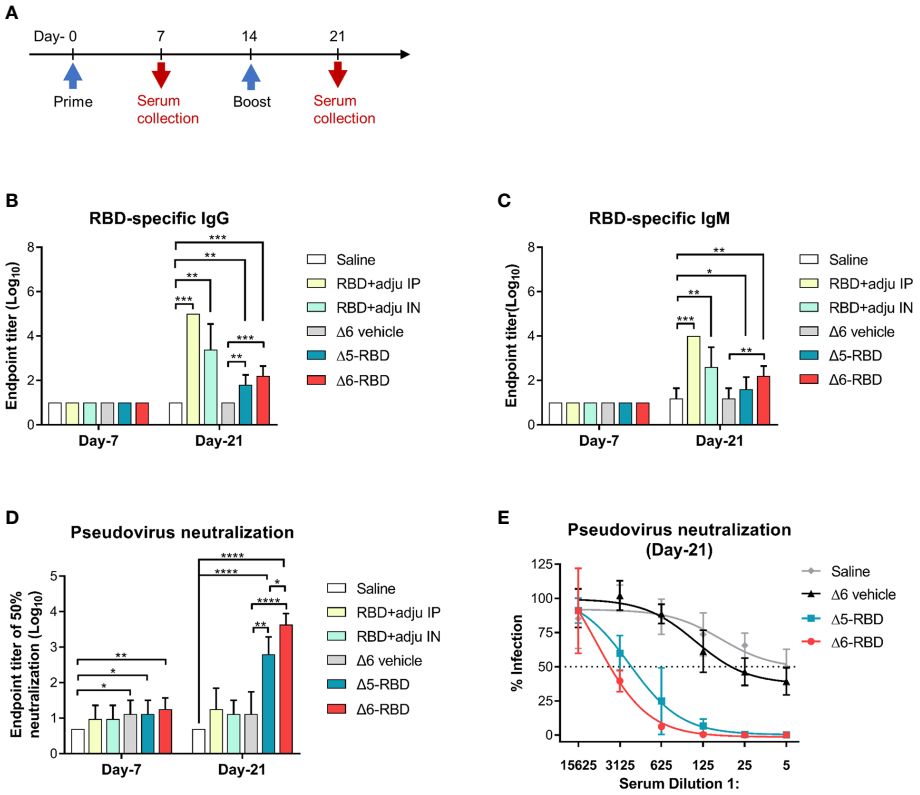

FIGURE 2
Characterization of aPA-RBD induced humoral immune response. **(A)** A prime-boost vaccination regimen was performed. Mice (n =5 per group) were immunized intranasally with 5 × 10 ^{7} CFU of the aPA-RBD vaccines (Δ5-RBD and Δ6-RBD) as well as the Δ6/pExoS$_{54}$F (Δ6 vehicle) at days 0 and 14. 10-μg of recombinant RBD protein, with curdlan as adjuvant, was given intranasally (RBD+adju IN) or intraperitoneally (RBD+adju IP) as the RBD controls. Saline was given as sham control. Sera were collected 7 and 21 days after first immunization and assessed for specific antibody against SARS-CoV-2 Spike-RBD. **(B, C)** Anti-RBD IgG and IgM titers. Comparisons were performed by Student's *t*-test (unpaired, two-tail); Error bars represent SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. **(D)** Neutralization potency of aPA-RBD. Five-fold serial dilutions of immune serum from immunized mice was assessed for neutralizing and inhibiting the infectivity of SARS-CoV-2 pseudovirus. Pseudovirus neutralization assay shows the 50% neutralization titer (NT$_{50}$). **(E)** Pseudovirus neutralization assay of Day-21 sera. The y-axis corresponds to observed percentage of pseudovirus infection in HEK293 cells that express human ACE2. The horizontal dashed line denotes 50% infection. The x-axis corresponds to reciprocal serum dilution.

neutralization assay was performed. Sera from both Δ5-RBD and Δ6-RBD groups resulted in a significant neutralization of the pseudovirus infectivity compared to those from the saline and Δ6 vehicle group. Furthermore, Δ6-RBD vaccination induced a higher neutralizing activity compared to the Δ5-RBD group on day 21 (Figure 2D), and the sera showed a strong 50% neutralization at a dilution > 1:3125 (Figure 2E). Interestingly, the groups immunized with intranasal and intraperitoneal inoculation of RBD protein plus adjuvant (RBD control) on day-21 showed low neutralizing activity (Figure 2D), although they elicit a high level of RBD-specific antibodies (Figures 2B,C), suggesting that Δ6-RBD group may have alternative ways enhancing the antibody-dependent neutralization (29).

Neutralizing activity against Live SARS-CoV-2 in the sera of two-dose Δ6-RBD vaccinated mice

To validate the neutralization results, we asked if aPA-RBD vaccinated sera also neutralize and inhibit the infectivity of authentic SARS-CoV-2 virus. A prime-boost regimen was performed as shown in Figure 3A. By the plaque reduction neutralization test (PRNT) assay, the Δ6-RBD group exhibited the average PRNT$_{50}$ (defined as the highest serum dilution that resulted in >50% reduction in the number of virus plaques) of 0.0005, and the Δ6 vehicle group showed average PRNT$_{50}$ of 0.04 (Figure 3B). The PRNT$_{50}$ value of Δ6-RBD group was 80-fold lower than that of Δ6 vehicle group, indicating that Δ6-RBD vaccinations can significantly induce humoral immune response that neutralize and inhibit the infection of Vero cells by SARS-CoV-2 Wuhan-Hu-1 (wild-type, WT). We further constructed the Δ6-RBD vaccines against SARS-CoV-2 delta and omicron BA.1 variants, respectively, and assessed the neutralizing activity against live SARS-CoV-2 delta, omicron BA.1 and BA.2 variants. The Δ6-RBD groups exhibited average PRNT$_{50}$ of 0.002 to 0.003, and the Δ6 vehicle groups showed average PRNT$_{50}$ of 0.01 to 0.04 (Figures 3C-E). Notably, the sera of the mice vaccinated with Δ6-RBD[BA.1] showed a cross-neutralizing activity against omicron BA.2 variant. Overall, these results demonstrate the neutralizing capacity of the Δ6-RBD vaccines against different SARS-CoV-2 variants.

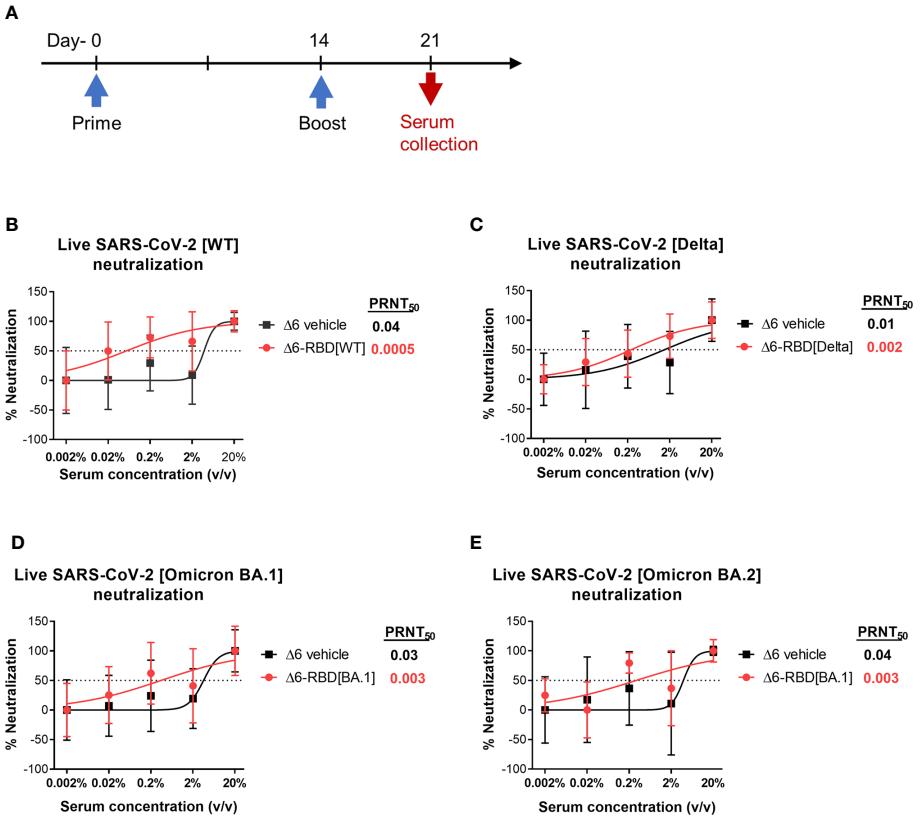

FIGURE 3
Assessment of aPA-RBD as vaccine against live SARS-CoV-2. **(A)** A prime-boost regimen was performed. Mice (n=6 per group) were vaccinated with Δ6-RBD[WT], Δ6-RBD[Delta] and Δ6-RBD[Omicron BA.1], and the sera were collected at 1 week after the second immunization. **(B, C)** Plaque reduction neutralization test (PRNT) assay of Δ6-RBD[WT] and Δ6-RBD[Delta] to wild-type virus (Wuhan-Hu-1) and Delta variant. Fifty percent of the plaque reduction neutralizing antibody ($PRNT_{50}$) titers against live SARS-CoV-2 were calculated as plaque reduction rate compared to Mpg (control) in Vero E6 cells. **(D, E)** PRNT50 of Δ6-RBD[Omicron BA.1] to Omicron BA.1 and Omicron BA.2. The dashed lines indicate the level of $PRNT_{50}$.

Activation of cell-mediated immunity

In patients, virus-specific $CD4^+$ and $CD8^+$ T cell responses are associated with milder disease, suggesting an involvement in protective immunity against COVID-19 (30, 31). Therefore, an ideal vaccine is expected to evoke both the humoral and cellular arms of the immune system (32).

To characterize the cellular immune responses, enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining (ICS) assays were performed. Groups of Δ6-RBD vaccinated mice were sacrificed one week after second immunization (Figure 4A). To evaluate RBD-specific responses, lymphocytes derived from spleens were stimulated *in vitro* with different concentrations of the recombinant RBD protein (Figure 4B). Resultantly, cells from mice vaccinated with Δ6-RBD and the recombinant RBD protein (RBD-IP) yielded higher amounts of IFN-γ compared to the saline, RBD-IN, and Δ6 vehicle ones. The spot forming cells (SFC) of the RBD-IP group in response to the enhanced stimulation were increased, whereas that of the Δ6-RBD group appears to be concentration-independent.

We next studied RBD-specific $CD4^+$ and $CD8^+$ T cells by flow cytometric analysis after ICS. The lymphocytes of the Δ6-RBD group were stimulated with a pool of synthetic peptides covering the RBD domain of SARS-CoV-2 spike protein *in vitro* and the peptide-specific IFN-γ and IL-2 responses were observed in $CD4^+$ and $CD8^+$ T cells, while IL-4-secreting cells were not detectable in any of the immunization groups (Figure 4C), confirming that RBD-specific memory T cells are Th1-oriented. Similar to the ELISPOT results, the saline and Δ6 vehicle groups were unable to induce IFN-γ-producing T cells (Figure 4C). These results indicate that intranasal Δ6-RBD vaccination is able to activate both $CD4^+$ and $CD8^+$ T cell responses, and lead to RBD-specific Th1 skewed memory T cells responses.

Discussion

The efficient delivery of SARS-CoV-2 spike RBD has been attributing to the bacterial T3SS of *P. aeruginosa*. The characteristic issue of proteins permeating cell membranes has been circumvented, and protein is directly delivered into target cells to stimulate an immune response, endowing the potential power of using bacterial T3SS-based antigendelivery as a vaccination method. However, the issues related to the detailed molecular mechanisms of proper folding and delivery of protein *via* the T3SS still need to be understood to broaden the application of

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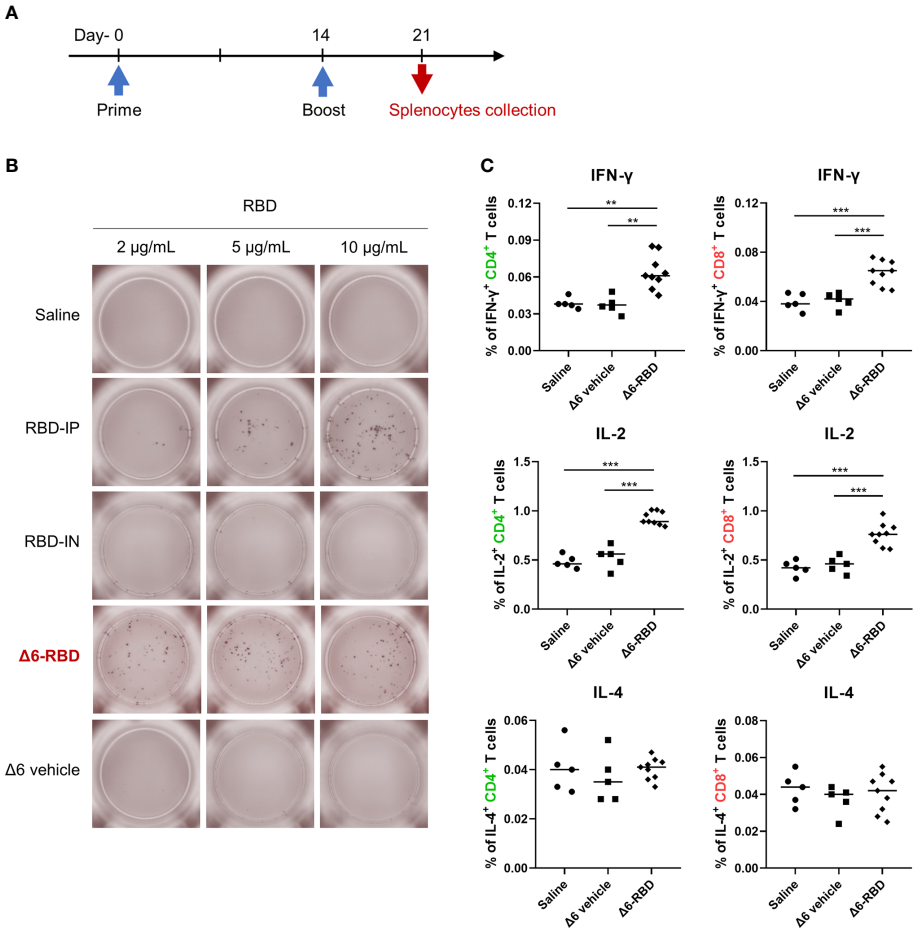

FIGURE 4
Induction of cellular responses by Δ6-RBD vaccination. **(A)** The vaccination regimen was performed. Mice were immunized at days 0 and 14, and spleens were isolated on day 21. The spleens soaked in 5 ml lymphocyte separation medium were dissociated by pressing it through the cell strainer (70 μm) using a syringe plunger. The splenocyte suspension was centrifuged at 800 x g for 30 min to obtain lymphocytes for cytokine detection. **(B)** ELISPOT analysis of lymphocytes. Cells were restimulated (20 hr) with recombinant RBD protein in the microwell of an ELISPOT plate that was precoated with the anti-mouse IFN-γ (5 μg/ml). Biotinylated anti-mouse IFN-γ (2 μg/ml) was used to detect the captured IFN-γ. Spots were visualized using Streptavidin-HRP enzyme and AEC substrate. **(C)** Cytokine profiling of lymphocytes by flow cytometry. Mice were immunized intranasally with 5 x 10^7 CFU of the Δ6 vehicle (n=5) and Δ6-RBD (n=9), and lymphocytes restimulated (9 hr) with a peptide pool consisting of 20-mers (10 μg/ml) spanning the SARS-CoV-2-S RBD were detected for the expression of IFN-γ, IL-2 and IL-4 in $CD4^+$ and $CD8^+$ cells. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

this method. Here, both the auxotrophic strains Δ6 and Δ9 express high level of RBD (Figure 1C), whereas the Δ9 strain hardly delivers the RBD into HL-60 and THP-1 cells (Figure 1D). In contrast, it was reported that the Δ9 expresses a high level of Cre recombinase, and is capable of high-efficiency protein delivery into HL-60 cell (17). This confirmed that the properties of the recombinant heterologous proteins affect the delivery efficiency, and it may exist the specific interplay between bacterial T3SS and host cells (33–35).

In addition to binding and directly interfering with viral entry, antibodies elicited by Δ5-RBD and Δ6-RBD may drive the neutralization of pseudovirus *via* their collaboration with the innate immune systems. As depicted in Figures 2B,C, adjuvants enhance the immune response to the two RBD-based subunit vaccines, especially of the group immunized *via* intraperitoneal injection. Intraperitoneal route could elicit a higher immunity and dissemination compared to the other ways such as oral gavage and aerosol (36, 37). Intriguingly, to the contrary of robust antibodies against RBD, groups vaccinated with RBD+adju IP and RBD+adju IN did not elicit a high neutralization titer against the SARS-CoV-2 spike protein to inhibit viral entry through the ACE2 receptor of HEK293 (Figures 2D,E), indicating that binding antibodies are not directly proportional to the neutralizing potency (38). Indeed, the accumulated interfering strength derives from the affinities of the binding antibody repertoire recognizing multiple epitopes of RBD, however, it displays a limited potency ceiling that can be surpassed by the assistance with innate immunity. One of the most striking illustrations is bacterial ghosts, an empty bacterial cell envelope retaining all the surface structural and antigenic components, which have an inherent immunogenicity and could function as both a vector and an adjuvant (39). Here, we postulate that the efficacy of Δ6-RBD may also be attributed to trained immunity. In particular, the complement system might enhance the neutralizing potency against SARS-CoV-2 (40, 41). To test this, we employed a pseudovirus assay for measuring antibody-mediated neutralization in

the presence and absence of fresh serum (Supplementary Figure S3). The normal mouse serum (NMS) was heated at 56°C for 30 min to inactivate the complement system. We found that the neutralization potency of the heat-inactivated mouse serum (HIMS) was decreased compared to those of NMS. However, addition of fresh serum (HIMS + FS) had recovered, at least partially, on the neutralization by NMS. These results demonstrate that complement is capable of augmenting the neutralization potency of antibodies *in vitro*, which agrees with prior studies with respiratory syncytial virus (42, 43). Furthermore, recent study provides compelling evidence that the complement C4 could seal a virus through capsid inactivation, indicating the role of complement to arouse the vigor of neutralization.

In general, the main concern of using bacterial vector-based vaccines is the absence of glycosylation modification occurring in eukaryotes, such as the glycosylated spike protein of SARS-CoV-2 (44, 45). However, a previous study showed that the non-glycosylated RBD bacterial vaccine can induce a significant antibody with a neutralizing capacity even compared to a vaccination with glycosylated RBD with alum (12). It suggests that the immune response by non-glycosylated RBD bacterial vaccine could compensate for the reduced humoral immune response caused by the lack of glycosylation, which appears to be consistent with our results, promising that a considerable variety of antigens could be targeted by the bacteria-based vaccines.

To further explore the mucosal immunity, we measured the RBD-specific mucosal secretory IgA (S-IgA) in the bronchoalveolar lavage fluid (BALF) of the mice intranasal immunized twice with Δ5-RBD and Δ6-RBD on day 0 and day 14. No anti-RBD IgA was detected on day 7, day 21 or day 35. A possible explanation is that the T3SS mediated intracellular delivery of antigen tends to elicit RBD-specific IgG rather than IgA response. Further studies are needed to understand the mechanism, which might provide clues to enhance the protection efficacy.

It is known that RBD is the major target for NAbs interfering with viral receptor binding. In this context, we focused on the systemic immunity induced by the aPA-based vaccine. Extended studies should be performed to further evaluate the mucosal immunity elicited by the aPA-based vaccine, and compare the protection efficacy with commercialized COVID-19 vaccines.

During our study, we monitored the stability of the aPA bacteria. The vaccine strains were eliminated within three days following vaccination in mice (Figure 1F). After immunization, we inoculated the remained bacteria in culture medium with or without D-glutamate, and found that the bacterium remained as auxotrophic. These results demonstrate the stability and safety of the aPA strains. In the future, the vaccine efficacy needs to be examined in a proper animal model. The main impediment of the mouse model is the lack of appropriate receptors for effectively binding the spike protein and initiating viral infection. Herein, the vaccine-challenge studies in other animal models could be conducted subsequently, such as Syrian hamsters, ferrets, and non-human-primates. They are new options to develop quantifiable clinical symptoms, especially weight loss, hematological changes, and lung pathology, akin to humans seriously ill with COVID-19 (11, 46).

In conclusion, we generated an aPA-based SARS-CoV-2 vaccine candidate that elicits efficient T cell responses after primer-boost immunization, and high titers of NAb that may cross-react with new circulating variants. These promising data support the efficacy of the T3SS-based *P. aeruginosa* delivery system, highlight the feasibility for the development of the live auxotrophic vaccine platform.

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 animal study was reviewed and approved by the institutional animal care and use committee of the College of Life Sciences of Nankai University (permit number NK-04-2012).

Author contributions

FB conceived the idea and ZY designed the experiments. YZ led the experiments and contributed to data analysis (with assistance from JQ). XS wrote the paper and all authors provided feedback. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1129705/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Mice survival after intranasal administration (1×10^9 CFU) with Δ5-RBD and Δ6-RBD (n = 5).

SUPPLEMENTARY FIGURE 2
Beta diversities analysis of bacteria in feces (A) and lungs(B) among five groups [negative(N), PBS as the blank(B), empty Δ6(E), Δ5-RBD(5) and Δ6-RBD(6)] determined at day 21 after the second intranasal immunization (n = 3), estimated from 16S rRNA amplicon sequencing. The negative group of the mice was directly exported from specific-pathogen free (SPF) unit of laboratory animal company. Color reflects differences from low (blue) to high (red).

SUPPLEMENTARY FIGURE 3
Neutralization assay of serum collected on day 21. SARS-CoV-2 spike (WT) Fluc-GFP pseudovirus (Cat. No. PSSW-HLGB001) was cocultured with normal mouse serum (NMS), heat-inactivated mouse serum (HIMS) or HIMS replenished with fresh serum (HIMS + FS) for 60 min prior to infection of HEK293T cells that express human ACE2. Forty-eight hours later, cells were analyzed by PerkinElmer EnSpire for GFP expression. HIMS, heat inactivated at 56°C for 30 min.

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Long-term adaptive response in COVID-19 vaccine recipients and the effect of a booster dose

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We examined the immune response in subjects previously infected with SARS-CoV2 and infection-naïve 9 months after primary 2-dose COVID-19 mRNA vaccination and 3 months after the booster dose in a longitudinal cohort of healthcare workers. Nine months after primary vaccination, previously infected subjects exhibited higher residual antibody levels, with significant neutralizing activity against distinct variants compared to infection-naïve subjects. The higher humoral response was associated with higher levels of receptor binding domain (RBD)-specific IgG^+ and IgA^+ memory B cells. The booster dose increased neither neutralizing activity, nor the B and T cell frequencies. Conversely, infection-naïve subjects needed the booster to achieve comparable levels of neutralizing antibodies as those found in previously infected subjects after primary vaccination. The neutralizing titer correlated with anti-RBD IFNγ producing T cells, in the face of sustained B cell response. Notably, pre-pandemic samples showed high Omicron cross-reactivity. These data show the importance of the booster dose in reinforcing immunological memory and increasing circulating antibodies in infection-naïve subjects.

KEYWORDS

COVID-19, SARS-CoV-2, Delta, Omicron, mRNA vaccine, neutralizing antibodies, T and B cells

Introduction

As of February 2023, the coronavirus disease 2019 (COVID-19) pandemic had resulted in over 670 million severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections and almost 6.8 million deaths worldwide (1). One key step in limiting the spread of SARS-CoV-2 and severe clinical outcomes in COVID-19 has been the development of effective and durable vaccine protection. Two mRNA vaccines that exhibited strong immunogenicity and efficacy were approved (2, 3) between December 2020 and February 2021.

Despite initial encouraging results in clinical trials, an increase in breakthrough SARS-CoV-2 infections over time in vaccinated individuals has raised concerns about the long-term efficacy of these vaccines in the real world, as well as their efficacy against new, emerging variants (4). Most of the available clinical and modeling studies suggested that increased breakthrough infections could be the result of a reduction in circulating antibody levels between 4 and 6 months after primary vaccination (5–8).

However, several groups have consistently documented that functional preservation of T cell responses following primary vaccination (9–12) could play an important role as a second-level defense against SARS-CoV-2. These results could explain the finding that a putative reduction in vaccine efficacy against infection with a SARS-CoV-2 variant did not result in a parallel decline in protection against severe disease, which was still apparent up to 9 months after primary vaccination (13, 14).

In mid-2021, the rapid emergence and spread of various SARS-CoV-2 variants with high infectivity and transmissibility, such as the Delta variant, which may elude vaccine-induced humoral immunity (15), prompted some countries to offer an additional booster dose to those who have received a primary vaccination (16). Despite the uncertainty, the booster dose was recommended to subjects at higher risk of developing severe COVID-19, such as the elderly and immunocompromised subjects, as well as to subjects at high risk of infection, such as healthcare workers (HCWs). The booster dose was recommended 4 to 6 months after the primary vaccination, to address potentially decreasing humoral immunity and to restore vaccine efficacy against infection with different emerging variants (17). Therefore, all of the studies that are currently available have investigated the immunological response to primary vaccination for up to 6 months, while the long-term response beyond this time point remained largely unexplored.

Here, using a cohort of healthcare workers (HCWs), we investigated the humoral and cellular response 9 months after primary BNT162b2 (Comirnaty, Pfizer-BioNTech) vaccination, with a special focus on the variants that have emerged most recently, Delta and Omicron. We also evaluated longitudinal immunological changes over a 3-month period following homologous booster dose administration in the same vaccine recipients.

Methods

Ethics statement

The ADAPTIVE study, involving human subjects, was reviewed and approved by the Ethical Committee of the Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani IRCCS (PARERE N. 444_2021). The study conforms to the principles of the Helsinki Declaration and written informed consent was obtained from all enrolled subjects. Study participation was voluntary. No potentially identifiable human images or data are presented in this study.

Enzyme-linked immunosorbent assay

Human IgG against the RBD of the spike protein of SARS-CoV-2 were measured using a quantitative ELISA (Proteintech, #KE30003). Briefly, serum samples diluted 1:200 were incubated on 96-microwell plates pre-coated with recombinant S-RBD recombinant protein. Captured anti-S-RBD human antibodies were detected using HRP-conjugated secondary antibodies against anti-human IgG. Averages of duplicate readings for each standard and sample were subtracted for the average zero standard absorbance. Data were obtained with a best-fit standard curve determined by regression analysis using four-parameter logistic curve fit (4-PL) and expressed as µg/mL. The threshold for sample positivity for anti-S-RBD antibodies was set by the manufacturer as > 0.625 µg/mL. To monitor SARS-CoV-2 infection during the study period, a quantitative ELISA was used to detect IgG against the SARS-CoV-2 nucleocapsid protein (Proteintech, #KE30001).

Cell culture and lentiviral neutralization assay

Vero E6 cells (ATCC, C1008; RRID: CVCL_0574) were cultured in Minimum Essential Medium Eagle EBSS with L-Glutamine (EMEM, Lonza, #BE12611F) supplemented with 1% non-essential amino acids, 10% heat inactivated fetal bovine serum (FBS; Life Technologies, #10270106) and 1% penicillin/streptomycin (P/S; Life Technologies, #15140122).

To potentiate lentivirus infection, we over-expressed in Vero E6 cells the main receptor involved in SARS-CoV-2 infection, angiotensin converting enzyme 2 (ACE2). Briefly, parental cells were transfected with replication incompetent, HIV-based, VSV-G pseudotyped ACE2 lentivirus (BPS bioscience, #79944). Specifically, 500,000 cells/well (6-well culture plate) were transduced with 10 M.O.I *per* cell of ACE2 lentivirus in the presence of 5 µg/mL of polybrene (Sigma Aldrich, #TR-1003). After 52 hours of transduction, the ACE2 overexpressing Vero E6 cells were harvested and seeded, 5,000 cells per well (96-well culture plate). The following day, cells were exposed to 2 M.O.I. *per* cell of an enhanced green fluorescent protein (eGFP) pseudotyped lentivirus expressing the spike protein of SARS-CoV-2 B.1.617.2 (Delta; BPS bioscience, #78216) or the spike protein of SARS-CoV-2 B.1.1.529.1 (Omicron; BPS bioscience, #78349) in the presence of 5 µg/mL polybrene overnight at 37°C, 5% CO_2. A bald lentiviral pseudovirion with eGFP reporter (BPS bioscience, #79987) was used at the same concentration as a negative control to confirm the spike-dependent pseudovirus infection.

To test the sera neutralizing activity, pseudotyped lentivirus were pre-incubated for 30 minutes with randomly selected sera from vaccinated individuals (1:200 dilution) before incubation with ACE2 overexpressing Vero E6 cells. After 24-hour incubation, infection medium was discarded and 500 µL of fresh EMEM medium was added to each well. After 48 hours, cells were fixed

and monitored under ApoTome Axio Imager Z2 (Carl Zeiss) to assess eGFP positivity. Before fixation nuclei were counterstained with Hoechst (NucBlue® Live ReadyProbes®; Thermo Fisher, Invitrogen, #R37605). At least 15 field *per* sample were acquired and the number of eGFP-positive Vero E6 cells counted (cells/field). The neutralizing activity was assessed as the ability of sera to reduce the number of infected cells and expressed as the percentage of reduction (%) in eGFP-positive cells exposed to lentiviral constructs pre-exposed to sera compared to the eGFP-positive cells exposed to lentiviral constructs alone.

B cell analysis

Peripheral blood mononuclear cells (PBMC) were isolated by gradient density centrifugation (Ficoll-Paque Plus, GE Healthcare, #17-1440-03). Frozen PBMC were thawed in complete RPMI medium (Thermo Fisher, #61870036) plus 5% human serum AB (Euroclone, #ECS0219D). B cells (8-10 million/each protein) specific for spike protein (Miltenyi, #130-1289-022), RBD protein (Miltenyi, #130-128-032) and for the spike B.1.1.529.1 – Omicron variant (Acro Biosystems, #SPN-C82Ee) were evaluated by double tetramer staining using specific B cell analysis kits (Miltenyi, #130-128-032 and #130-128-022), following the instructions (8-10 million PBMC/each protein). Data were acquired on FACS LSRFortessa X-20 (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (FlowJo LLC). Live singlets were gated based on 7AAD fluorescence and specific B cells detected using the double discrimination method gated on $CD3^-$ $CD14^-$ $7AAD^-$ and $CD19^+$ cells (CD3 PerCP-Vio700, #130-113-132; CD14 PerCP-Vio700, #130-113-151, Miltenyi). Cells incubated with streptavidin PE and PEVio770 alone were used as negative controls. Specific memory B cells were defined as $CD27^+$ $CD19^+$ on $tetramer^+$ B cells.

IFNγ enzyme-linked immunosorbent spot assay

PBMC ($1.5x10^6$/mL) were stimulated with 1 μg/mL of peptide pools covering complete SARS-CoV-2 spike protein (PepTivator SARS-CoV-2 Prot-S Complete, Miltenyi, #130-127-953), the receptor binding domain RBD 319-541 (JPT, #PM-WCPV-S-RBD-2), the spike protein of the BA.1 Omicron variant (SARS-CoV-2 Prot-S B.1.1.529/BA.1 Mutation Pool, Miltenyi, #130-129-928) in an IFNγ ELISPOT assay (TEMA Ricerca, #856.051.010). Cells incubated with dimethyl sulfoxide (DMSO, Sigma, #D2438) were used as negative controls, while cells stimulated with CEFX Ultra SuperStim pool (JPT, #PM-CEFX-1) were used as positive stimulation controls. Cells (300,000 PBMC/well) were stimulated for 20 hours at 37°C 5% CO_2 in three replicates and then the ELISPOT assay was carried out according to manufacturer's instructions. To quantify peptide-specific response, spots of the DMSO (usually less than 20) were subtracted from the peptide stimulation wells and the results expressed as spots/300,000 PBMC.

Compared to the activation-induced marker (AIM) assays (11, 12), in our hands the IFNγ ELISPOT assay provided more solid and wider differences between positive and negative controls and peptide stimulation wells. The negative control (DMSO) produced a very low number of spots while the spots in the positive control (CEFX) were consistently high in all subjects (over 200 spots/300,000 PBMC).

Statistical analysis

Data were reported as mean ± standard deviation or number (%). Differences between groups were evaluated by unpaired t-test or Fisher's exact test as appropriate. Continuous levels of cellular response against SARS-CoV-2 were expressed as median [interquartile range] and group comparisons were performed by non-parametric test Wilcoxon rank sum test. Correlations between continuous variables were evaluated with Pearson's index. Data were presented as box-and-whisker plots displaying the median, 25th and 75th percentiles of distribution and whiskers extend to the minimum and maximum values of the series. All analyses were carried out using SAS (version 9.4). All *p-values* were 2-sided.

For the analysis of the lentiviral infection assay, data were expressed as mean ± standard deviation and comparisons were made using ANOVA with corrected with Tukey *post hoc* test.

Results

This observational study included a total of 49 HCWs from the Mario Negri Institute's Clinical Research Centre. Baseline characteristics are reported in Figure 1A. On January 10, 2021, all HCWs received a primary vaccination with BNT162b2, according to the standard regimen of 2 doses administered 3 weeks apart. A baseline blood withdrawal was performed before vaccination (T1) and a subsequent withdrawal was scheduled 19 days after the second dose (T2) to obtain serum samples for antibody evaluation.

To evaluate the levels of neutralizing antibodies, we used an enzyme-linked immunosorbent assay (ELISA) pre-coated with recombinant receptor binding domain (RBD), the main region of the SARS-CoV-2 spike (S) protein involved in viral entrance into target cells (18, 19). As shown in Figure 1B, baseline evaluation (T1) identified anti-RBD antibody levels above the detection threshold in 17 HCWs (35%), who were categorized as previously infected. These subjects encountered SARS-CoV-2 synchronously during the peak of the first wave of the pandemic caused by Wuhan Hu-1 in March 2020 in northern Italy (20). When we analyzed the anamnestic questionnaires completed by previously infected volunteers, we found that COVID-19 mostly presented as a mild disease, with fever, muscle pain and fatigue the most commonly experienced symptoms and none of the infected subjects requiring hospitalization (Table S1). On the other hand, 32 HCWs (65%) tested negative for anti-S-RBD antibodies and were considered naïve to natural infection (Figure 1B). Mean antibody levels were 1.05 ± 0.44 and 0.08 ± 0.03 μg/mL in previously infected and

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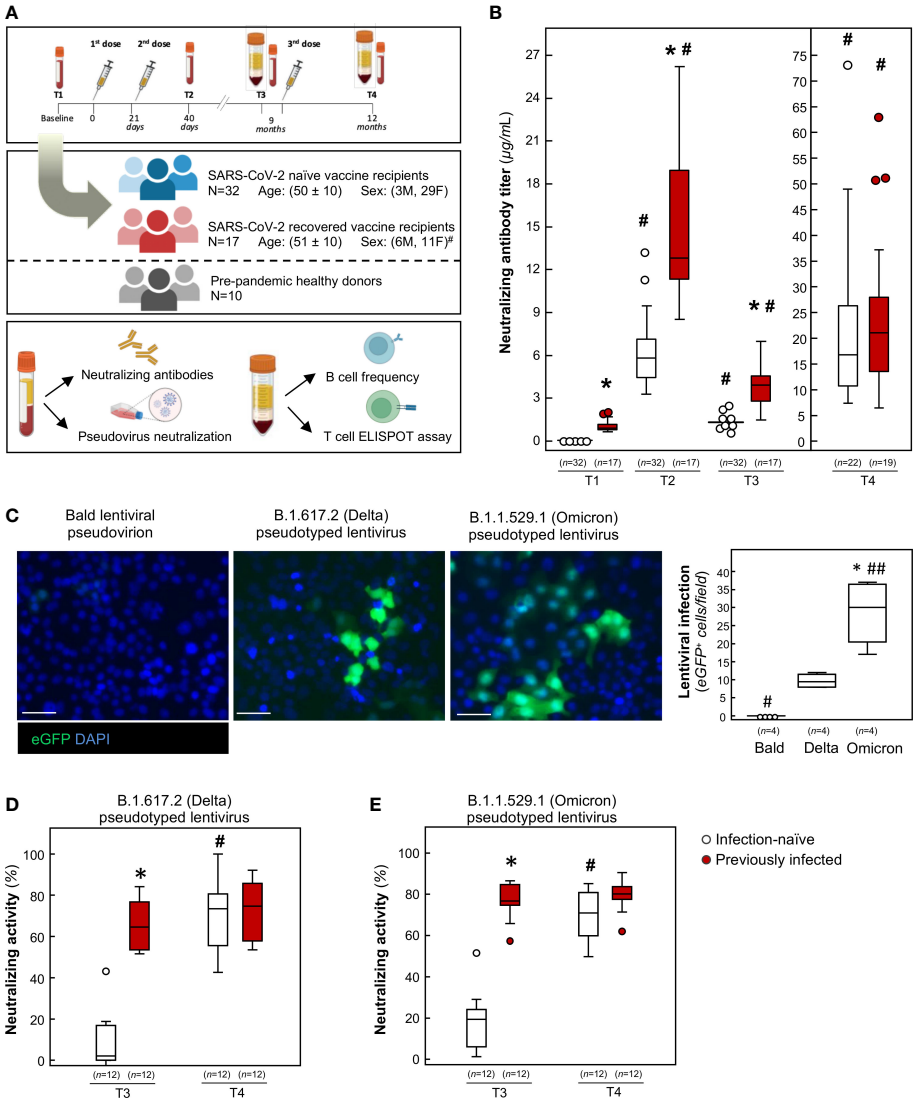

FIGURE 1
Humoral response and neutralizing activity of infection-naïve and previously infected vaccine recipients over time. **(A)** Schematic representation depicting the study design. Drawings were created using BioRender. #*p-value*=0.049 *vs* infection-naïve subjects. **(B)** Evaluation of neutralizing anti-RBD IgG in infection-naïve and previously infected vaccine recipients at baseline (T1), 19 days after primary vaccination (T2), 9 months after primary vaccination (T3), and 3 months after the booster dose (T4). **p-value*<0.0001 *vs* infection-naïve subjects; #*p-value*<0.0001 *vs* the respective T1. **(C)** Representative images and quantification of lentiviral construct infection in Vero E6 cells overexpressing human angiotensin converting enzyme 2 (ACE2). Nuclei are counterstained with Hoechst. Scale bar: 50 μm. **p-value*<0.001 *vs* Bald, #*p-value*<0.01; ##*p-value*<0.001 *vs* Delta. **(D, E)** Quantification of neutralizing activity of sera against **(D)** B.1.617.2 (Delta) and **(E)** B.1.1.529.1 (Omicron) at T3 and T4. **p-value*<0.0001 *vs* infection-naïve subjects; #*p-value*<0.0001 *vs* the respective T3. The sample size (*n*) for each panel is indicated in brackets.

infection-naïve individuals, respectively, at T1 (Figure 1B). Nineteen days after the primary vaccination (T2), all HCWs had mounted a robust neutralizing humoral response, although vaccine-evoked humoral response was significantly higher in previously infected subjects (Figure 1B). In line with other studies (21–27), our data indicate that response to primary vaccination is associated with a greater neutralizing antibody titer in individuals with a previous history of SARS-CoV-2 infection.

Following the approval of the booster dose by European regulatory agencies, all subjects received the BNT162b2 booster in November 2021. Before the booster dose, all available consenting HCWs underwent a blood withdrawal (T3) to obtain sera for antibody evaluation and peripheral blood mononuclear cells (PBMC) for T and B cell analysis. During the period between the primary vaccination and blood withdrawal before the booster dose (T3), no SARS-CoV-2 infections were reported in infection-naïve HCWs when they were tested using real-time reverse transcription polymerase chain reaction (28) or ELISA for anti-nucleocapsid antibodies. A blood sampling repeat was planned on February 2022, 3 months after the booster dose (T4). During the period between the booster dose and the blood withdrawal at T4, 3 infection-naïve HCWs were diagnosed with SARS-COV-2 using qRT-PCR tests and tested positive for anti-nucleocapsid antibodies. These 3 individuals were moved to the previously infected group in the analysis at T4.

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Analysis of anti-S-RBD antibody titer at 9 months (T3) revealed that neutralizing antibodies decreased substantially and to a similar extent in both groups (Figure 1B, decrease: 73.9 ± 9.9% *vs* 75.9 ± 7.0%, mean ± standard deviation), demonstrating that previous SARS-CoV-2 infection did not alter rates of antibody reduction over time. Despite this marked decline, both groups were still positive for anti-S-RBD IgG, and none of the subjects had dropped to subthreshold levels (Figure 1B). Our data extend the observations made by Goel and colleagues, who reported detectable neutralizing antibodies in most vaccine recipients for up to 6 months following primary vaccination (29, 30), even in the setting of a different mRNA vaccine (31). At T4, we found that the booster dose significantly restored neutralizing antibody levels, to a similar extent in both groups (Figure 1B).

When the study was completed (1 May 2022), 6 additional SARS-CoV-2 diagnoses were reported in the infection-naïve group after blood withdrawal at T4, and 2 were reported in previously infected subjects. Altogether, a total of 9 naïve subjects (28.1%) experienced SARS-CoV-2 infection after the booster dose, while only 2 infections (11.8%) were reported in previously infected subjects.

In order to assess whether the residual antibody levels detected at 9 months had a neutralizing effect on the most recent SARS-CoV-2 variants, we performed a lentiviral infection assay with an enhanced green fluorescent protein (eGFP) pseudotyped lentivirus that expressed the full length spike protein of either the B.1.617.2 (Delta) or B.1.1.529.1 (Omicron) lineages. As shown in Figure 1C, exposing the cells to the Delta lentivirus was associated with a lower frequency of eGFP expression compared to Omicron. No signal was observed when a bald lentiviral pseudovirion was used as a negative control (Figure 1C), confirming the spike-dependent lentiviral infection of target cells. In this setting, we tested the antibody-neutralizing activity by incubating lentiviral constructs with serum samples. As shown in Figure 1D, the residual antibody levels in previously infected HCWs retained potent neutralizing capacity at 9 months, as demonstrated by the ability of sera to halt the Delta pseudotyped lentivirus infection in target cells. Similarly, residual neutralizing antibody blocked Omicron lentiviral infection to a significant extent (Figure 1E). Our data extend the observations made by Luczkowiak and colleagues to Omicron. They had reported that COVID-19 patients who had recovered had strong neutralizing antibody titers against previous variants of concerns 8 months after primary vaccination (32). Given that several studies have reported that residual neutralization levels may still be sufficient to protect against symptomatic disease (33–35), our finding supports the hypothesis that previously infected vaccine recipients are protected at 9 months. In line with this hypothesis, a recent study documented that infection-acquired immunity, in combination with primary vaccination, conferred a high level of protection against SARS-CoV-2 more than 1 year after infection (36), even against the BA.5 variant that had recently emerged (37). This finding was also observed in high-risk populations (38). Conversely, sera from infection-naïve subjects collected 9 months after primary vaccination exhibited no neutralizing activity toward either the Delta or Omicron constructs (Figure 1D, E). Our findings are in line with data that show suboptimal post-vaccine immune responses in infection-naïve individuals (39, 40).

In previously infected subjects, the upsurge of neutralizing antibody levels following the booster dose was not paralleled by a comparable increase in neutralizing activity, which was only slightly enhanced (Figures 1D, E). In contrast, the vigorous upsurge in neutralizing antibody titer in infection-naïve individuals was associated with a significant increase in neutralizing activity against different variants (Figures 1D, E), suggesting that these subjects require a booster dose to achieve appropriate neutralizing activity against both SARS-CoV-2 variants (41). Our results are fully consistent with those from a different cohort which showed that, almost 9 months after primary vaccination, Delta neutralization was detected in only 19% of COVID-19 naïve subjects and 88% of subjects who had recovered from COVID-19 (42). As in our study, a booster dose was required to restore neutralizing activity against Delta in COVID-19 naïve vaccine recipients. These data are confirmed by real word data from two independent studies that show that vaccine efficacy against infection with the Delta variant was around 80% soon after primary vaccination (<120 days), while it decreased over time to 0-50% (>120 days) (43, 44). In both studies, vaccine efficacy against infection with the Delta variant was restored by the booster dose. Collectively, these data may suggest that immune protection against infection needs to be optimized through the booster dose for infection-naïve subjects.

We next investigated the B cell response to mRNA vaccination. Indeed, previously published studies have shown that mRNA vaccines generate functional memory B cells and that levels of these cells increase 3 months after primary vaccination (29) and persist until 6 months post-primary vaccination, despite the marked decrease in specific IgG neutralizing antibodies (45–48). Based on these findings, we sought to evaluate whether changes in SARS-CoV-2-specific B cell frequencies were responsible for the changes observed in the neutralizing activity. To this end, we used a double tetramer fluorescence activated cell sorting (FACS) staining approach to quantify memory B cells specific to the spike protein and the RBD of Wuhan Hu-1. To evaluate the magnitude of the specific B cells that recognize SARS-CoV-2, we also analyzed PBMCs from healthy donors, which had been collected before 2019. Representative flow cytometry pseudocolor plots of spike-specific B cells are shown in Figure 2A. Compared to pre-pandemic healthy donors, in vaccinated individuals at T3 and T4, circulating B cells specific for Wuhan Hu-1 spike and RBD were significantly higher (Figure 2B). No major differences were observed in the B cell frequency between previously infected and infection-naïve subjects (Figure 2B). When we further analyzed the specific $CD27^+$ memory B cell subsets, we found that Wuhan Hu-1 RBD-specific $CD27^+$ memory B cells and, in particular IgG^+CD27^+ and IgA^+CD27^+, but not IgM^+CD27^+, were significantly higher at 9 months in previously infected subjects compared to infection-naïve subjects (Figure 2C). Notably, anti-S-RBD IgG^+CD27^+ and IgA^+CD27^+ B cells significantly correlated with neutralizing antibody at T3 (Table S2). Although we did not investigate anti-S-RBD IgA levels, the finding that IgA^+CD27^+ RBD-specific memory B cells correlated positively with neutralizing antibody suggests that previously infected vaccine recipients have additional neutralizing protection, as IgA has been shown to mediate the early SARS-CoV-2

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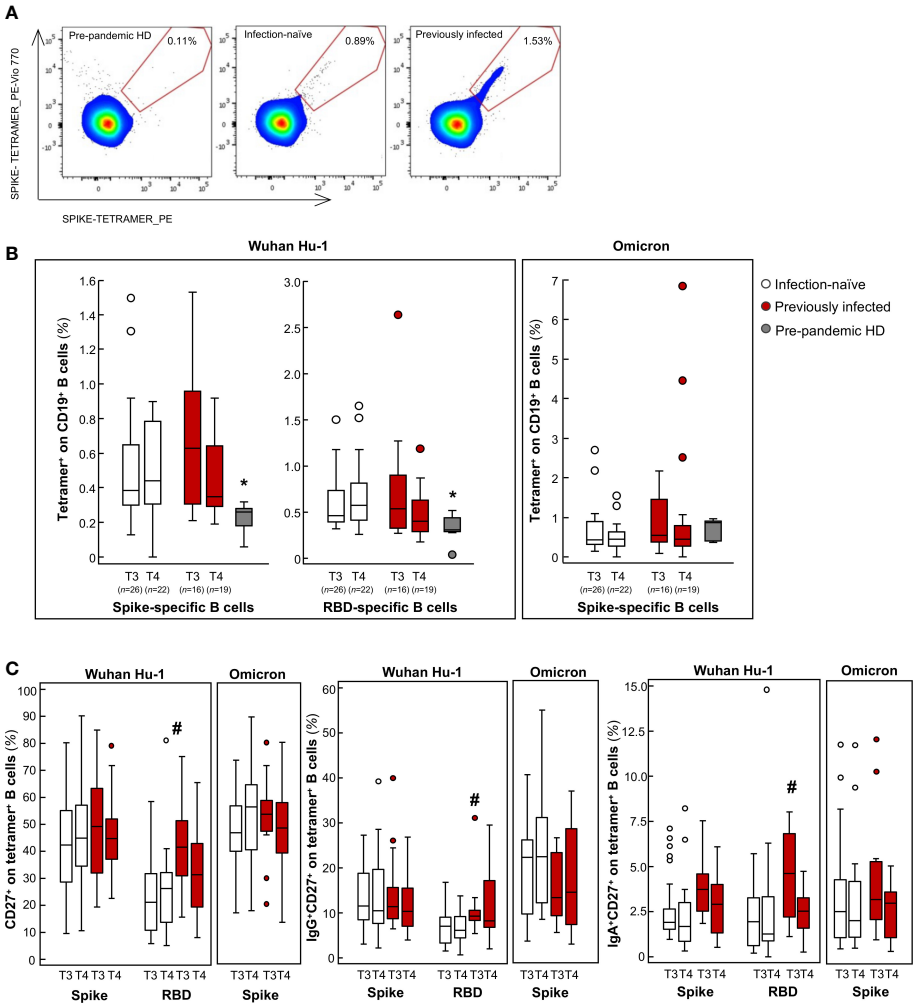

FIGURE 2
Analysis of B cell frequency in infection-naïve and previously infected vaccine recipients over time. **(A)** Representative flow cytometry pseudocolor plots of spike-specific B cells in pre-pandemic healthy donor (HD), in an infection-naïve and a previously infected subject. **(B, C)** Percentages of tetramer$^+$ on CD19$^+$ B cells **(B)** and the percentages of memory CD27$^+$, IgG$^+$CD27$^+$ and IgA$^+$CD27$^+$ B cells on tetramer$^+$ B cells **(C)** are shown for the spike and RBD protein of the Wuhan Hu-1 SARS-CoV-2 and in response to the B.1.1.529.1 (Omicron) spike protein in infection-naïve and previously infected subjects at 9 months after primary vaccination (T3) and 3 months after the booster dose (T4), as well as for pre-pandemic HD. *p-value<0.05 *vs* infection-naïve and previously infected subjects at T3 and T4; #p-value<0.05 *vs* infection-naïve subjects at T3. The sample size (n) for all B cell analyses is indicated in brackets in panel **(B)**.

neutralizing response (49). This hypothesis has been confirmed by recent data that showed that vaccination induced a minimal IgA response in individuals who had not been exposed to SARS-CoV-2, while IgA induction after vaccination was more efficient in patients with a COVID-19 history (50). A recent study also documented the critical role that IgA$^+$ B cell memory recall induced by vaccination plays in breakthrough infection (51). These data provide a novel insight into long-term immunity to SARS-CoV-2 in previously infected subjects who exhibited marked immunological imprinting from previous infection, shaping the long-term breadth and maturation of neutralizing activity, even in the absence of a booster dose. These data indicate that pre-vaccination immunological memory plays a major role in dictating better vaccination outcomes and may explain why fewer breakthrough infections were reported in our cohort of previously infected subjects, in line with real-world data (52). After the booster dose, we did not find any changes in either the frequency of B cells specific for the spike protein of Wuhan Hu-1 (Figure 2B) or in the number of IgG$^+$ and IgA$^+$ CD27$^+$ memory B cells (Figure 2C). These data indicate that the booster dose does not induce a major expansion of memory B cells, either in previously infected or in infection-naïve subjects. These findings apparently contrast with those reported recently by Goel and colleagues, who documented a significant expansion of the memory B cell repertoire following the booster dose (53). However, in their analysis the authors showed that the greatest expansion of spike-specific memory B cell was detected at 2 weeks post-booster, while it declined over the following months (53). Having investigated the B cell response 3 months after the booster dose, it is conceivable that we missed the early transient expansion of spike-specific B cells triggered by the antigenic stimulus of the booster dose. All these data suggest that, regardless of the rapid and transient immunogenic stimulus

provided by the booster dose, the B cell magnitude is durable and effective in producing a large amount of neutralizing antibodies when faced with additional antigen exposure (51).

To analyze the full spectrum of cellular immunity, we focused on T cells, given that their response to the spike protein is instrumental in the coordinated humoral response that follows primary mRNA vaccination (54). To assess the total effector T cell response, we performed an IFNγ ELISpot assay following stimulation with pooled overlapping 15-mer peptides spanning the full length and RBD of Wuhan Hu-1. In this setting, the specific IFNγ T cell responses against the spike and RBD were significantly higher in all vaccine recipients at 9 months (T3) compared to pre-pandemic healthy donors, with no difference between previously infected and infection-naïve subjects (Figure 3A). After the booster dose, no major differences were found between the vaccinated groups in terms of the magnitude of spike-specific T cell responses (Figure 3A). In a recent study, Naranbhai and colleagues reported that T cell reactivity to the SARS-CoV-2 spike protein was enhanced significantly after the booster dose, particularly in previously infected subjects (55). However, these data were obtained from samples collected soon after the booster dose, reflecting a transient response to the antigenic stimulus (55). The short-term nature of the acute T cell response following the booster dose was confirmed across different age groups (56–58).

In our study, we also found that the effector spike- and RBD-specific T cell response correlated significantly with neutralizing antibody titer in infection-naïve but not in previously infected subjects (Figure 3B). Our results are in line with three independent studies that showed that the SARS-CoV-2-specific T

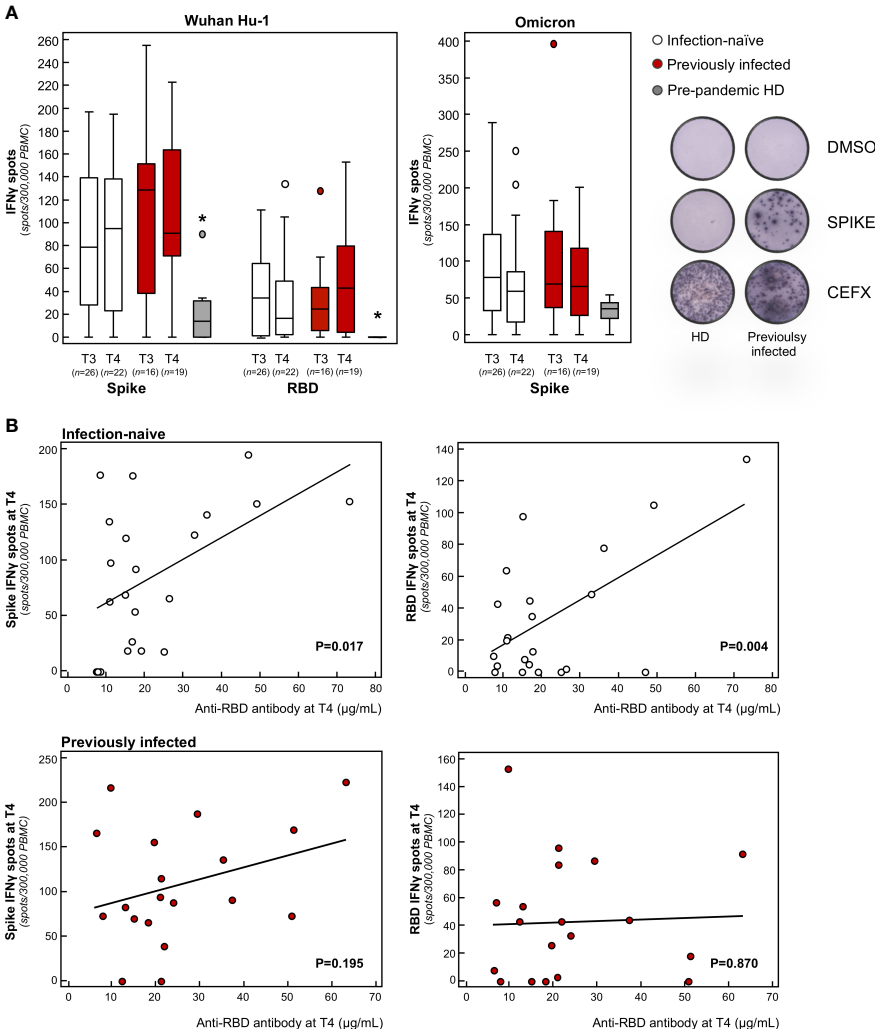

FIGURE 3
Analysis of effector T cell response in infection-naïve and previously infected vaccine recipients over time. **(A)** Frequency of IFNγ producing T cells in response to peptide pools of the spike and RBD protein of the Wuhan Hu-1 SARS-CoV-2 and in response to the B.1.1.529.1 (Omicron) spike protein in infection-naïve and previously infected subjects at 9 months after primary vaccination (T3) and 3 months after the booster dose (T4), as well as in pre-pandemic healthy donors (HD). Representative ELISPOT wells are shown on the right. Horizontal lines indicate median values; *p-value<0.005 *vs* of infection-naïve and previously infected subjects at T3 and T4. **(B)** Correlation of anti-RBD antibody levels with the frequency of spike-specific (left panels) or RBD-specific (right panels) IFNγ producing T cells at T4 in the two study groups. The sample size (n) for all T cell analyses is indicated in brackets in panel **(A)**.

cell response is required to induce long-term persistence of neutralizing antibodies during natural infection (59, 60), as well as in response to primary vaccination (61). On top of that, our study indicates that the additional antigenic challenge – through the booster dose – is essential for infection-naïve individuals to mount a coordinated T cell response, which sustains the neutralization breadth against SARS-CoV-2 variants. This finding is of particular clinical relevance considering the transient increase stimulation of T cells following the booster dose has been associated with enhanced affinity maturation of RBD-specific IgG in a cohort of adults above the age of 80 who were at risk of severe disease (56).

When we analyzed the B and T cell response against Omicron, we found that, after primary vaccination, subjects exhibited a B cell frequency and an IFNγ T cell response to the spike protein of Omicron that was comparable to the response to Wuhan Hu-1, with no additional changes following the booster dose (Figures 2B, 3A). Notably, the cellular responses against Omicron, but not Wuhan Hu-1, were also observed in pre-pandemic samples from healthy donors (Figures 2B, 3A). There was no difference in the extent of B and T cell responses between vaccinated individuals and healthy donors (Figures 2B, 3A). Our data are consistent with the presence of cross-reactive B cells against the non-RBD portions of Omicron in unvaccinated uninfected individuals (62) with no pre-existing B cell immunity against the spike protein of SARS-CoV-2 of the Wuhan Hu-1 variant (63). As for memory B cells, there were no differences in terms of either the frequency or phenotype of $CD27^+$ B cells between previously infected and infection-naïve subjects (Figure 2C). Omicron RBD-specific IgG^+CD27^+ B cell response positively correlated with neutralizing antibody levels in previously infected subjects (Table S2), which may explain why serum samples from these individuals strongly neutralized Omicron lentiviral infection 9 months after primary vaccination. Regarding T cells, to the best of our knowledge only two studies have investigated the response of IFNγ T cells to Omicron in pre-pandemic samples using the ELISPOT assay. In a pre-print study, Jergovic and colleagues found that T cell cross-reactivity to the spike protein of Wuhan Hu-1 was slightly higher than that of Omicron in samples from healthy adults collected prior the pandemic (64). Conversely, Naranbhai and colleagues found that the T cell response against the spike protein was low in 10 non-vaccinated and never-infected subjects, although effector T cell reactivity to the Omicron spike protein was higher than to the Wuhan Hu-1 spike protein (55), as in our experimental setting. A recent study suggested the existence of a unique insertion mutation in the Omicron spike protein that has a sequence that is identical to that of a coronavirus that causes the common cold (65), which may explain why T cells developed against common cold coronaviruses can cross-react with the SARS-CoV-2 spike protein (66–73). In light of these data and our present finding – of increased B and T cross-reactivity against Omicron in pre-pandemic samples from healthy donors – it is tempting to speculate that Omicron is acquiring mutations in the spike protein that are reminiscent, at least in part, of common cold coronaviruses, possibly explaining its increased infectivity but lower intrinsic virulence (74). At the time of writing, no study has addressed this issue and this topic is worth investigating further.

Discussion

Collectively, all these data converge to demonstrate that primary mRNA vaccination is a potent tool for inducing long-lasting protection against severe disease outcomes – as was recently shown in a clinical setting (75, 76), particularly for previously infected subjects (77, 78). The booster, on the other hand, may provide additional protection in infection-naïve subjects. However, when it comes to highly contagious variants, such as Omicron and its subvariant (79), the risk of breakthrough infections remains high even following a booster-induced upsurge of neutralizing IgG antibodies (80). Indeed, all the available data suggest that vaccine-induced protection against infection is limited to 4/6 months, although protection against severe COVID-19 and death remained high (81). However, additional booster doses in high-risk subjects, such as the elderly and immunocompromised patients, are required to maintain protection against mortality associated with highly infectious SARS-CoV-2 variants (82, 83). However, in the general healthy population, future vaccination strategies should focus on identifying tools for achieving sterilizing immunity, including those that stimulate mucosal immunity (84–86), in order to avoid the need for repeated booster administration to keep antibody levels high and prevent infection. Needless to say, the development of a universal vaccine against all coronavirus strains could be an additional tool for preventing the spread of highly contagious future variants. The mosaic RBD nanoparticle vaccine has been shown in animal models to protect against challenges from diverse coronaviruses (87).

Limitations of the study: due to the observational, prospective nature of this cohort study, the following caveats must be considered. No evidence of a temporal relationship between exposure and outcome could be provided, as exposure and outcome were assessed simultaneously. The sample size was limited by expediency, although it is completely in line with all of the most recent studies in the field designed for deep immunological analysis of vaccinated individuals. Additionally, we enrolled all available HCWs who were offered the vaccination at the start of the vaccination campaign in Italy. Our study population may therefore be affected by selection bias, which limits how far the results can be extended to the general population, including different age groups. In our study, previously infected subjects had mainly been infected with Wuhan Hu-1, preventing us from identifying how different viral variants encountered during natural infection may shape vaccine responses, with possible implications for future next-generation vaccines (24, 88–91).

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 Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani IRCCS (PARERE N. 444_2021). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: LP, FC, AB, GR. Methodology: LP, MT, FC, MM, AP, TP, ST, PT. Investigation: LP, MT, FC, MM, AP, TP, ST, PT. Supervision: AB, GR. Writing – original draft: LP, FC. Writing – review & editing: AB, GR. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1123158/full#supplementary-material

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Extending the dosing interval of COVID-19 vaccination leads to higher rates of seroconversion in people living with HIV

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Introduction: Vaccination against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is an effective way of protecting individuals from severe coronavirus disease 2019 (COVID-19). However, immune responses to vaccination vary considerably. This study dynamically assessed the neutralizing antibody (NAb) responses to the third dose of the inactivated COVID-19 vaccine administered to people living with human immunodeficiency virus (HIV; PLWH) with different inoculation intervals.

Methods: A total of 171 participants were recruited: 63 PLWH were placed in cohort 1 (with 3-month interval between the second and third doses), while 95 PLWH were placed in cohort 2 (with 5-month interval between the second and third doses); 13 individuals were enrolled as healthy controls (HCs). And risk factors associated with seroconversion failure after vaccination were identified via Cox regression analysis.

Results: At 6 months after the third vaccination, PLWH in cohort 2 had higher NAb levels (GMC: 64.59 vs 21.99, $P < 0.0001$) and seroconversion rate (68.42% vs 19.05%, $P < 0.0001$). A weaker neutralizing activity against the SARSCoV-2 Delta variant was observed (GMT: 3.38 and 3.63, $P < 0.01$) relative to the wildtype strain (GMT: 13.68 and 14.83) in both cohorts. None of the participants (including HCs or PLWH) could mount a NAb response against Omicron BA.5.2. In the risk model, independent risk factors for NAb seroconversion failure were the vaccination interval (hazed ration [HR]: 0.316, $P < 0.001$) and lymphocyte counts (HR: 0.409, $P < 0.001$). Additionally, PLWH who exhibited NAb seroconversion after vaccination had fewer initial COVID-19 symptoms when infected with Omicron.

Discussion: This study demonstrated that the third vaccination elicited better NAb responses in PLWH, when a longer interval was used between vaccinations. Since post-vaccination seroconversion reduced the number of symptoms induced by

Omicron, efforts to protect PLWH with risk factors for NAb seroconversion failure may be needed during future Omicron surges.

Clinical trial registration: https://beta.clinicaltrials.gov/study/NCT05075070, identifier NCT05075070.

KEYWORDS

inactivated COVID-19 vaccination, dosing interval, neutralizing antibody, seroconversion, people living with HIV

1 Introduction

Vaccination against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been proved to efficiently decrease the likelihood of severe coronavirus disease 2019 (COVID-19). To optimize the outcomes of vaccination, considerable efforts have been devoted to the development of various vaccine strategies, such as trialing different inoculation intervals and COVID-19 vaccine types, to determine which combinations elicit the best immune responses. Administering a second dose of COVID-19 vaccines and prolonging the inoculation interval have improved vaccine immunogenicity not only in healthy controls (HC) (1) but also in patients undergoing hemodialysis (2), or those with autoimmune rheumatic disease (3) and cancer (4). People living with human immunodeficiency virus (HIV; PLWH) are a group of patients with impaired immunity. These individuals were therefore regarded as a priority population for COVID-19 vaccination and were highly recommended to receive the third vaccine dose. Thus, the potential value of extending the inoculation interval between the second and third doses of the COVID-19 vaccine deserved further attention.

Although existing data demonstrate that COVID-19 vaccines could elicit neutralizing antibody (NAb) responses in PLWH, NAb could not fully account for the effectiveness of the vaccines. Furthermore, since SARS-CoV-2 can rapidly mutate and most COVID-19 vaccines were designed against the wild-type (WT) Wuhan strain, it is important to determine the NAb activity against SARS-CoV-2 variants. To date, studies performed in Canada (5), Spain (6), and China (7) suggested that two doses of COVID-19 vaccine induced adequate levels of NAb against the WT SARS-CoV-2 strain in PLWH. Moreover, a South African study by Khan et al. showed that inoculating PLWH with a single dose of the Ad26.CoV2.S vaccine induced a considerable NAb response against the Delta variant (8). Very recently, inoculation of PLWH with a third dose of an mRNA COVID-19 vaccine was shown to elicit a robust NAb response against the Omicron BA.1 variant (9, 10). The emergence of an increasing number of Omicron sub-lineages, which have a higher propensity for immune evasion compared with the Omicron variant BA.1, has been reported (11, 12). However, only a single cross-sectional study, has evaluated the NAb responses of PLWH against Omicron BA.4/5 variants (13). In addition, there is lack of evidence of NAb responses against Omicron sub-lineages in PLWH before and after the third vaccination.

This study aimed to reveal the influence of extending the inoculation interval on the humoral immunity induced by the third dose of the inactivated COVID-19 vaccine in PLWH with a CD4 count < 500 cells/μL. Of note, this work is a continuation of our previous research, which forms part of a long-term follow-up program (under review). In the present study, we measured the dynamic NAb responses of PLWH to different SARS-CoV-2 strains, including the Omicron BA.5.2 variant, which is one of the most prevalent strains in China. Moreover, potential factors linked to NAb seroconversion were identified. Collectively, our results will provide additional guidance for the COVID-19 vaccination of PLWH.

2 Materials and methods

2.1 Study population and design

This was an observational study that collected data from PLWH vaccinated with inactivated COVID-19 vaccines (called BBIBP-CorV, Beijing Institute of Biological Products) at Hangzhou Xixi Hospital. At enrollment, all PLWH received standardized antiretroviral therapy (ART), and had viral loads less than 50 copies/mL and $CD4^+$ T-cell counts less than 500 cells/μL. All PLWH were SARS-CoV-2-negative throughout the study period. A total of 158 PLWH, who had received two doses of the inactivated COVID-19 vaccine, were enrolled in this study. Based on their vaccination willingness, the 158 individuals were divided into two cohorts according to the interval between the second and the third doses of vaccine: 1) cohort 1, interval of 3 months; and 2) cohort 2, interval of 5 months. The original plan was to collect peripheral blood samples at three time points to evaluate the NAb responses: 1) prior to the third vaccination; 2) 1 month after the third vaccination; and 3) 6 months after the third vaccination. However, due to the COVID-19 pandemic and quarantine policies in China, the second time-point for collecting peripheral blood samples from cohort 1 was changed to 2 months after the third dose, while cohort 2 was sampled according to the original plan. In addition, 13 HCs were recruited; data from these individuals were collected 6 months after the third dose of vaccination. The study flowchart is shown in Figure 1. This study was reviewed and approved by the Clinical Research Ethics Committee of the Hangzhou Xixi Hospital (20210913121100011537 9) and was performed in accordance with the Declaration of Helsinki. This study was registered on *clinicaltrials.gov* (NCT05075070). The

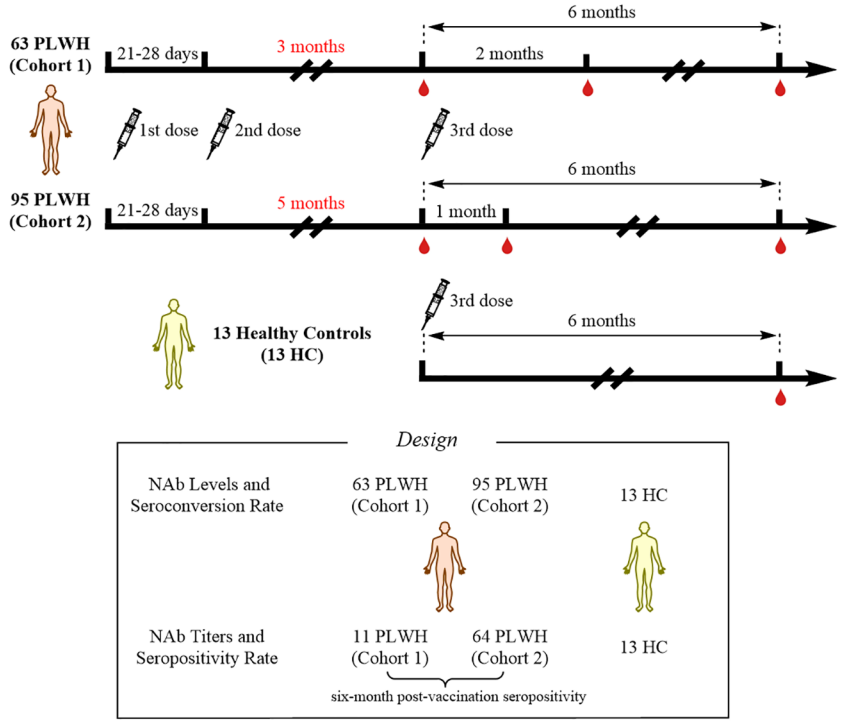

FIGURE 1
Study profile for the vaccination schedule and follow-up.

infection status and initial symptoms of all PLWH were collected using a questionnaire during additional follow-up.

2.2 SARS-CoV-2 NAb detection assay

The SARS-CoV-2 NAb assay (SHENZHEN YHLO BIOTECH CO., LTD, Shenzhen, China) is a paramagnetic particle chemiluminescent immunoassay designed for the qualitative detection of SARS-CoV-2 NAb in human serum samples using the automated iFlash immunoassay system (14). This assay is mainly used for the auxiliary evaluation of the efficacy of inactivated COVID-19 vaccines. The manufacturer has determined a cut-off value of 10 arbitrary units per milliliter (AU/mL) for NAb levels. Since the maximum measurable result is 800 AU/mL, anti-SARS-CoV-2 NAb levels above 800 AU/mL were assigned as a value of 800. Seroconversion was defined as a change in the status of NAb levels from negative to positive based on a 4-fold increase over baseline (15). At the same time, according to Ka-Shing Cheung et al. study, we also set a assay threshold of 15 AU/mL as the NAb seroconversion cutoff in the our study (16). The geometric mean concentration (GMC) of NAb was calculated with 95% confidence intervals (CI), according to WHO international standards (17).

2.3 Serum neutralization of live SARS-CoV-2 strains

Viral neutralization assays were performed in a BSL-3 laboratory. The sera from participants were heat-inactivated at 56°C for 30 min prior to use. The working stocks of live SARS-CoV-2 strains (the WT strain and the Delta B.1.617.2 and Omicron BA.5.2 variants) were obtained from sputum samples and propagated by infection of Vero E6 cells, as previously described (18). The serum neutralizing activity was measured using the micro-neutralization assay based on live SARS-CoV-2 virus, as previously described (19). Virus without serum and untreated E6 cells were used as positive and negative controls, respectively. Microplates were observed under the microscope for the presence of virus-induced cytopathic effects (CPE) on the cell monolayer on days 2 to 3. Serum neutralization titers were calculated on day 3 as the reciprocal of the serum dilution that resulted in a 100% reduction in the CPE (20). We calculated the geometric mean titer (GMT) of NAb with 95% CIs, according to the WHO international standard (17). A seropositivity threshold was defined as a GMT over 1:4 (21, 22). In addition, NAb titers < 4 were assigned as a titer value of 2 (23).

2.4 Outcomes of interest

The primary outcome was NAb seroconversion within 6 months after the third dose of inactivated COVID-19 vaccine in PLWH. The NAb level prior to the third vaccination was set as the baseline for this study. After the third vaccination, PLWH with a ≥ 4-fold increase in NAb levels relative to the baseline were defined as those who underwent NAb seroconversion, and were called seroconverters. PLWH who did not meet these standards were regarded as non-seroconverters. The secondary outcomes were the incidence of virologically confirmed SARS-CoV-2 infection and the number of initial symptoms post-infection among the vaccinated PLWH.

2.5 Statistical analysis

All statistical analysis was performed using the R statistical software version 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria) and IBM SPSS Statistic version 25.0 (IBM, Armonk, NY, USA). We assessed the statistical differences in NAb levels and titers elicited by different SARS-CoV-2 strains between the two cohorts using the Mann-Whitney *U*-test (for unpaired data) or the Wilcoxon rank-sum test (for paired data). Normally distributed continuous variables were presented as means with standard deviations (SD). The median (M) with interquartile range (IQR, 1st quartile–3rd quartile) were used to describe variables. For categorical variables, we reported the numbers and percentages of patients in each category. Proportions were compared using the Pearson's chi-squared test. All graphs were generated using GraphPad Prism version 8.0.2 (263) (GraphPad Software, San Diego, CA, USA).

A multivariable Cox regression model was applied to determine covariate association with NAb seroconversion. A hazard ratio (HR) greater or less than 1 was interpreted as an increased or decreased association with NAb seroconversion, respectively. We used the "Survival" and "Survminer" packages in R to analyze the NAb seroconversion data from PLWH. The predictive accuracy of the risk model was assessed using the receiver operating characteristic (ROC) curve, and the area under ROC curve (AUC) was plotted by the MedCalc software package version 18.2.1 (MedCalc Software, Ostend, Belgium) using sensitivity and specificity values (24). Kaplan-Meier curves were drawn for PLWH with or without NAb seroconversion and compared using log-rank tests. All reported levels of statistical significance were two-sided, and *P*-values < 0.05 were considered as a measure of statistical significance.

3 Results

3.1 Baseline characteristics of PLWH

In total, 158 PLWH received the third dose of inactivated COVID-19 vaccine, of which 63 PLWH were in cohort 1 (with a 3-month interval between the second and third doses) and 95 PLWH were in cohort 2 (with a 5-month interval between the second and third doses). The baseline characteristics mainly included age, sex, HIV transmission route, marital status, education, body mass index (BMI), time at initiation of ART, and $CD4^+$ T-cell counts. There were no obvious differences in these characteristics between the two cohorts at baseline (Table 1).

3.2 The NAb response to the third dose of vaccination in cohort 1

NAb levels and titers were characterized at three different time points in cohort 1. Compared with the baseline NAb levels (GMC = 9.79), the significant increase in the magnitude of the NAb response was observed at 2 months after the third dose of inactivated COVID-19 vaccine among the 63 PLWH of cohort 1 (GMC: 9.79 vs 55.38, $P < 0.0001$, Figure 2A; Table S1). At the 6 months post-vaccination time point, the GMC of NAb was lower that at the second time point (GMC: 55.38 vs 21.99; $P < 0.0001$, Table S1), but was still significantly higher than baseline (GMC: 9.79 vs 21.99; $P < 0.0001$, Table S1). Furthermore, a total of 12 PLWH in cohort 1 experienced NAb seroconversion (Figure 3A).

In addition, we assessed the NAb seroconversion rate and levels at 6-month post-third vaccination in PLWH with a CD4 count < 200 cells/μL (PLWH < 200) or ≥ 200 cells/μL (PLWH ≥ 200) subgroups to identify potential differences. The baseline characteristics of PLWH < 200 and PLWH ≥ 200 subgroups are showed in Table S2. As showed in Table 2, the NAb seroconversion rate (0.00% vs 27.27%) and levels (11.60 vs 24.95 AU/mL) in PLWH < 200 subgroup were significantly lower than those in PLWH ≥ 200 subgroup (Both $P < 0.05$, Table 2).

Next, we carried out live SARS-CoV-2 neutralization test in PLWH seroconverters. Except for one individual, who supplied insufficient blood sample volume, the remaining 11 PLWH were included in the next evaluation of neutralizing activity against different SARS-CoV-2 strains. Before the third vaccination, only three PLWH displayed the ability to neutralize live SARS-CoV-2 WT, and none of the 11 PLWH exhibited neutralization activity against live SARS-CoV-2 variants (Figure 2B; Table S1). Among the 11 PLWH, the detectable rate of neutralization activity was 11 of 11 (100%), 10 of 11 (90.91%), and 2 of 11 (18.18%); the GMTs were 38.48, 7.28, and 2.42 to WT, Delta, and Omicron strains 2 months after the third vaccination, respectively (Figure 2B; Table S1). The serum samples taken at the 6-month time point had lower neutralization activity to the three SARS-CoV-2 strains. At 6 months, the NAb GMTs against WT and Delta strains significantly decreased to 13.68 and 3.38, respectively (Figure 2B; Table S1), while none of the samples could neutralize the Omicron variant (Figure 2B; Table S1).

3.3 The NAb response to the third dose of vaccination in cohort 2

Among the 95 PLWH in cohort 2, the NAb responses to the third dose of inactivated COVID-19 vaccine were significantly elevated compared with those to the second dose, both at the 1-month and 6-month time points (both $P < 0.0001$, Figure 2C; Table S3). However, the NAb GMCs significantly dropped between the 1-month and 6-month time points (227.03 vs 64.59; $P < 0.0001$, Figure 2C; Table S3). In addition, 6 months after the third vaccination, 65/95 (68.42%) PLWH experienced NAb seroconversion (Figure 3A).

Then, the NAb seroconversion rate and levels at 6-month post-third vaccination were assessed in PLWH < 200 or PLWH ≥ 200 subgroups to identify potential differences. The baseline characteristics of PLWH < 200 and PLWH ≥ 200 subgroups are showed in Table S4. After subgroups analysis, there were no significant differences in the NAb seroconversion rate (76.00% vs 65.71%) and levels (44.80 vs 46.40 AU/mL) between PLWH < 200 and PLWH ≥ 200 subgroups (Both $P > 0.05$, Table 3).

TABLE 1 The comparison of baseline characteristics of participants between two cohorts.

Characteristics	Cohort 1 (n = 63)	Cohort 2 (n = 95)	*P* value
Age (years)			
<30	14 (22.22)	27 (28.42)	0.607
30-40	22 (34.92)	39 (41.05)	
41-50	12 (19.05)	12 (12.63)	
≥50	15 (23.81)	17 (17.90)	
Sex			
Male	61 (96.83)	92 (96.84)	1.000
Female	2 (3.17)	3 (3.16)	
Sexual transmission route			
Homosexual/bisexual	47 (74.60)	76 (80.00)	0.782
Heterosexual	15 (23.81)	18 (18.95)	
Others	1 (1.59)	1 (1.05)	
Marital status			
Married	21 (33.33)	28 (29.47)	0.876
Unmarried	37 (58.73)	59 (62.11)	
Divorced/widowed	5 (7.94)	8 (8.42)	
Education			
High school or lower	19 (30.16)	29 (30.53)	0.620
Junior college	18 (28.57)	21 (22.11)	
College or higher	26 (41.27)	45 (47.37)	
BMI	22.33 ± 3.36	21.80 ± 3.97	0.380
Time at initiation of treatment/years			
<2	13 (20.63)	17 (17.89)	0.224
2-5	15 (23.81)	35 (36.84)	
≥5	35 (55.56)	43 (45.26)	
$CD4^+$ T-cell counts (cells/μL)			
<200	19 (30.16)	25 (26.32)	0.690
200-350	23 (36.51)	40 (42.11)	
350-500	21 (33.33)	30 (31.57)	

BMI, body mass index.

Except for one PLWH, who supplied insufficient blood sample volumes, the remaining 64 PLWH seroconverters were selected to participate in the live SARS-CoV-2 neutralization test (Figure 2D; Table S3). At baseline, the sera from most PLWH could hardly neutralize live SARS-CoV-2 WT and the two variants. At 1 month post-vaccination, however, the sera of all PLWH (64/64, 100%) neutralized SARS-CoV-2 WT. Protection against the Delta variant was also built up in most PLWH (58/64, 90.63%). Nevertheless, the sera from only 14 PLWH (14/64, 21.88%) displayed neutralizing activity against the Omicron BA.5.2 variant. The NAb GMTs against the SARS-CoV-2 WT, Delta, and Omicron strains were 66.09, 10.83 and 2.60, respectively. An apparent drop in NAb GMTs was observed over time. At the 6-month time point, the NAb GMTs against SARS-CoV-2 WT and Delta strains dramatically decreased to 14.83 and 3.63, respectively, and none of the sera from cohort 2 participants were able to neutralize the Omicron BA.5.2 variant.

Besides, we further observed the differences between PLWH < 200 and PLWH ≥ 200 subgroups on the neutralizing activity against different SARS-CoV-2 strains among PLWH seroconverters in cohort 2. We found no evidence of a different titer of NAbs neutralization against SARS-CoV-2 WT, Delta, and Omicron strains between PLWH < 200 and PLWH ≥ 200 subgroups at one and six-month post-vaccination (Table S5).

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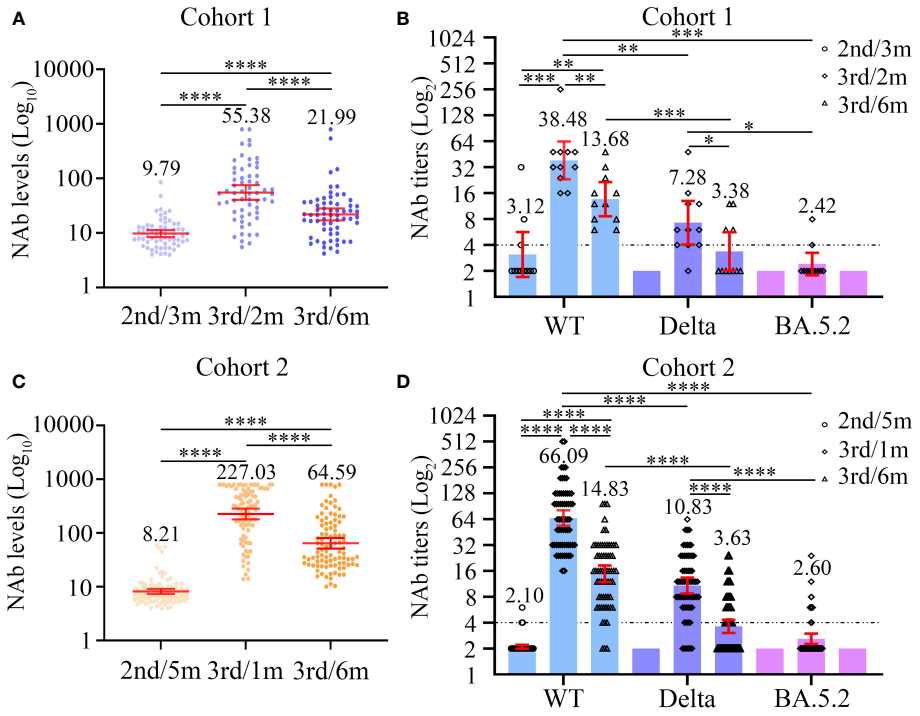

FIGURE 2
Dynamic changes in neutralizing antibody levels **(A, C)** and titers **(B, D)** to wild-type virus and the Delta (B.1.617.2) and Omicron (BA.5.2) variants before and after the third dose of COVID-19 vaccines among two cohorts. **(A, B)** The neutralizing antibody levels **(A)** and titers **(B)** at three different time points in cohort 1. **(C, D)** The neutralizing antibody levels **(C)** and titers **(D)** at three different time points in cohort 2. Wilcoxon matched-pairs signed-rank test with two-tailed p-value was used for comparison between groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

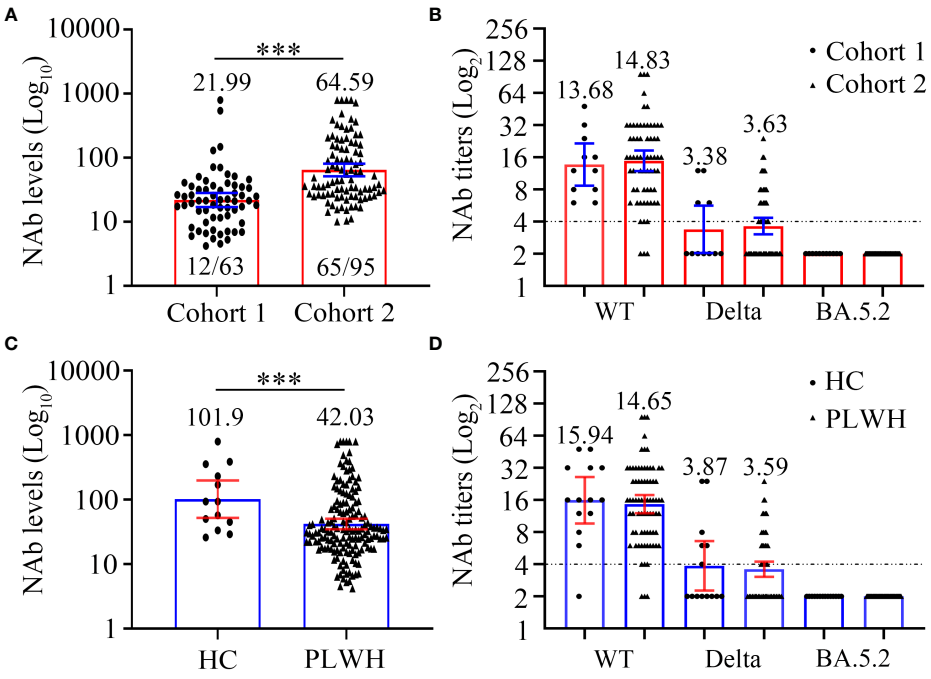

FIGURE 3
Comparison of SARS-CoV-2 neutralizing antibody levels **(A, C)** and titers **(B, D)** to wild-type virus and the Delta (B.1.617.2) and Omicron (BA.5.2) variants between two cohorts or PLWH and healthy controls at 6 months after the third vaccination. **(A, B)** The neutralizing antibody levels **(A)** and titers **(B)** of cohort 1 and cohort 2. **(C, D)** The neutralizing antibody levels **(C)** and titers **(D)** of PLWH and healthy controls. Mann-Whitney U test with two-tailed p-value was used for comparison between groups. ***$P < 0.001$.

TABLE 2 The comparison of NAb seroconversion rate and levels of participants between two subgroups in cohort 1.

Varibles	PLWH < 200 (n = 19)	PLWH ≥ 200 (n = 44)	*P* value
Seroconversion rate			
No (n,%)	19 (100)	32 (72.73)	0.029
Yes (n,%)	0 (0.00)	12 (27.27)	
NAb level	11.60 (6.40,25.80)	24.95 (16.60,39.45)	0.007

PLWH < 200: PLWH with a CD4 count < 200 cells/μL; PLWH ≥ 200: PLWH with a CD4 count ≥ 200 cells/μL.

TABLE 3 The comparison of NAb seroconversion rate and levels of participants between two subgroups in cohort 2.

Varibles	PLWH < 200 (n = 25)	PLWH ≥ 200 (n = 70)	*P* value
Seroconversion rate			
No (n,%)	6 (24.00)	24 (34.29)	0.322
Yes (n,%)	19 (76.00)	46 (65.71)	
NAb level	44.80 (26.25,191.50)	46.40 (26.75,144.50)	0.857

PLWH < 200: PLWH with a CD4 count < 200 cells/μL; PLWH ≥ 200: PLWH with a CD4 count ≥ 200 cells/μL.

3.4 Differences between the NAb responses of the two cohorts 6 months after the third vaccination

Firstly, we compared the NAb levels and seroconversion rate in the two cohorts 6 months after the third vaccination. As shown in Figure 3A and Table 4, the PLWH in cohort 2 (with a longer vaccination interval) had markedly higher seroconversion rate than those in cohort 1 (68.42% vs 19.05%, $P < 0.0001$). This trend was also observed with respect to the concentrations of SARS-CoV-2-specific NAbs in cohort 2 vs cohort 1 (GMC: 64.59 vs 21.99, $P < 0.0001$). Notably, both PLWH < 200 and PLWH ≥ 200 subgroups, the NAb levels and seroconversion rate in cohort 2 were significantly higher than those in cohort 1 ($P < 0.0001$, Table 4).

In addition to the levels of NAb, we also compared the neutralizing activity against different SARS-CoV-2 strains (Figure 3B; Table S6). Interestingly, regardless of the targeted SARS-CoV-2 strain (WT, Delta, or Omicron), the neutralizing activities were comparable between the PLWH seroconverters in the two cohorts. Specifically, the GMTs against the WT strain in cohort 1 and cohort 2 were 13.68 and 14.83, respectively, and a sharp reduction in the GMTs against the Delta strain was observed (GMT: 3.38 and 3.63, respectively, for cohorts 1 and 2). Furthermore, the data revealed that neither vaccination regiment was able to elicit NAbs against Omicron BA.5.2.

3.5 Differences between the NAb responses of PLWH and healthy controls 6 months after the third vaccination

The baseline characteristics of HC and PLWH, including age, sex, and BMI, are showed in Table S7. In the initial analysis, we compared the NAb levels 6 months after the third vaccination in 158 PLWH

TABLE 4 The differences on the neutralizing antibody levels between two cohorts at 6 months post-3rd vaccination.

Neutralizing Antibody	Cohort 1 (n = 63)	Cohort 2 (n = 95)	*P* value
Total seroconversion rate (n,%)	12/63 (19.05)	65/95 (68.42)	< 0.0001
Total GMC (95%CI)	21.99 (17.05,28.34)	64.59 (51.34,81.26)	< 0.0001
PLWH < 200 seroconversion rate (n,%)	0/19 (0.00)	19/25 (76.00)	< 0.0001
PLWH < 200 GMC (95%CI)	12.99 (8.87,19.01)	67.74 (41.38,110.90)	< 0.0001
PLWH ≥ 200 seroconversion rate (n,%)	12/44 (27.27)	46/70 (65.71)	< 0.0001
PLWH ≥ 200 GMC (95%CI)	27.60 (20.30,37.60)	63.50 (48.73,82.75)	< 0.0001

GMC, geometric mean concentration; CI, confidence interval; PLWH < 200: PLWH with a CD4 count < 200 cells/μL; PLWH ≥ 200: PLWH with a CD4 count ≥ 200 cells/μL.

and 13 HCs. As shown in Figure 3C and Table S8, PLWH had significantly lower NAb levels than controls (GMC: 42.03 vs 101.90, $P = 0.0056$). We then examined NAb titers in 75 PLWH seroconverters and 13 HCs with high NAb levels. The GMTs of serum NAb against WT strain were 15.94 and 14.65, while those against the Delta strain were 3.87 and 3.59 for the PLWH seroconverters and HCs, respectively (Figure 3D; Table S8). At 6 months post-vaccination, the sera of both PLWH and HCs were unable to neutralize the Omicron variant (Figure 3D; Table S8).

3.6 Factors associated with NAb seroconversion in PLWH

We next focused on identifying factors associated with NAb seroconversion. We used the univariate Cox regression analysis to calculate the HRs for 22 factors (Table S9). Our results showed that six variables, including age (HR = 2.057; 95%CI, 1.233–3.433; $P = 0.006$), sex (HR = 2.542; 95%CI, 1.022–6.325; $P = 0.045$), vaccination interval (HR = 0.336; 95%CI, 0.212–0.531; $P < 0.0001$), education (HR = 0.467; 95%CI, 0.282–0.775; $P = 0.003$), lymphocyte counts (HR = 0.593; 95%CI, 0.395–0.889; $P = 0.011$), and neutrophil-to-lymphocyte ratios (NLRs) (HR = 1.289; 95%CI, 1.069–1.553; $P = 0.008$) were independent significant predictors for NAb seroconversion (Table S9).

We then calculated the cut-off values of two continuous variables, which were significantly associated with the outcome of NAb seroconversion. The optimal cut-off value of lymphocyte counts was ≤ 1.59 cells/μL, with a sensitivity and specificity of 43.21% and 77.92%, respectively. The NLRs had 45.68% sensitivity and 64.94% specificity at an optimal cut-off value of > 1.96 (Table S10).

We next used the multivariable Cox regression analysis to screen out two variables, namely the vaccination interval (HR = 0.328; 95% CI, 0.204–0.528; $P < 0.0001$) and lymphocyte counts (HR = 0.497; 95% CI, 0.307–0.805; $P = 0.004$), which were significantly associated with NAb seroconversion (Table 5). The results were presented in the form of forest maps (Figure 4A). We then used ROC analysis to assess the ability of these two independent variables to predict the outcome of NAb seroconversion among PLWH. In the ROC analysis chart, the AUC values for the vaccination interval and lymphocyte counts were 0.737 (95%CI, 0.661–0.804; $P < 0.0001$) and 0.606 (95%CI, 0.525–0.682; $P = 0.0038$), respectively (Figure 4B; Table S11). We constructed a risk model with an AUC of 0.777 (95%CI, 0.704–0.840; $P < 0.0001$), based on the vaccination interval and lymphocyte counts (Figure 4B; Table S11). PLWH with a longer interval between doses (5 months) were more likely to experience NAb seroconversion than PLWH with a shorter interval (3 months) between doses (Figure 4C, $P < 0.0001$). Lower lymphocyte counts were associated with a significant decline in NAb seroconversion (Figure 4D, $P < 0.0001$).

In our risk model, the PLWH were divided into two groups according to risk (high vs low), and the grouping criterion was the median of the risk score. The subsequent survival analysis revealed that the status of NAb seroconversion between the two groups was significantly different (Figure 5A, $P < 0.0001$). The risk curve shows the relationship between NAb seroconversion and the risk of PLWH. Figure 5B shows the risk values of PLWH in the two groups. PLWH in the low-risk group had significantly higher NAb levels and seroconversion rate than those in the high-risk group (Figure 5C).

3.7 PLWH seroconverters have fewer SARS-CoV-2-induced symptoms

To prove the clinical relevance of the model, we recently conducted an additional follow-up to investigate the incidence

TABLE 5 Multivariate Cox regression analysis was used to analyze the factors of the outcome of NAb seroconversion among PLWH after the third vaccination.

Variables	β value	SE	Wald χ^2	P value	HR (95%CI)
Age (years)					
≥50 vs <50	0.417	0.329	1.609	0.205	1.517 (0.797,2.889)
Sex					
female vs male	0.781	0.497	2.476	0.116	2.185 (0.825,5.781)
Vaccination interval					
2 vs 1	-1.113	0.242	21.156	< 0.0001	0.328 (0.204,0.528)
Education			2.428	0.297	
2 vs 1	-0.322	0.327	0.969	0.325	0.725 (0.382,1.375)
3 vs 1	-0.475	0.306	2.408	0.121	0.622 (0.34,1.133)
Lymphocytes (cells/μL)					
>1.59 vs ≤1.59	-0.698	0.246	8.079	0.004	0.497 (0.307,0.805)
NLRs					
>1.96 vs ≤1.96	0.282	0.241	1.366	0.242	1.326 (0.826,2.129)

SE, standard error; HR, hazard ratio; NLRs, neutrophil/lymphocyte ratios.

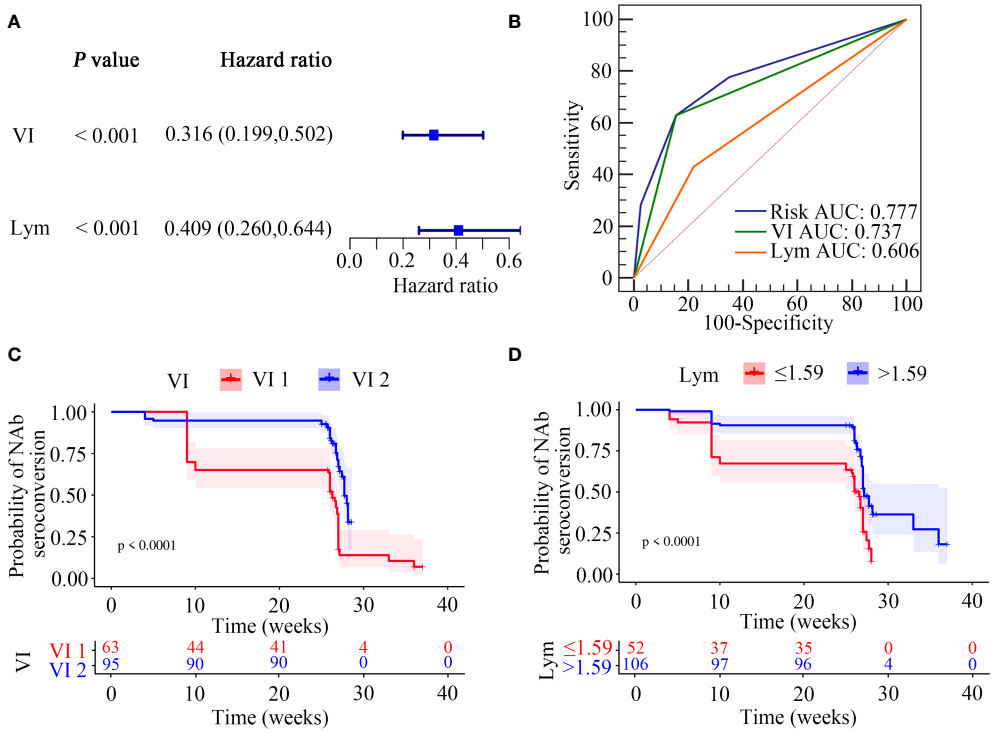

FIGURE 4
Screening of NAb seroconversion related factors. **(A)** Multivariate cox regression analysis ($P < 0.05$). **(B)** ROC demonstrated the predictive accuracy of the risk model was superior to other two clinical variables. **(C, D)** Kaplan-Meier survival function plot showed the effect of vaccination interval and lymphocyte counts on the outcome of NAb seroconversion.

rate and initial symptoms of SARS-CoV-2 (Omicron variant) breakthrough infection in the 158 vaccinated PLWH. Within 1 year after the third vaccination, a total of 108 PLWH tested positive for SARS-CoV-2 RNA and 24 PLWH had negative results. SARS-CoV-2 RNA test results could not be obtained for the remaining 26 PLWH, who were subsequently excluded form the analysis. Among the 132 PLWH, 60 experienced NAb seroconversion and 72 did not. As shown in Table S12, the rate of infection with SARS-CoV-2 Omicron variant was slightly higher in non-seroconverters compared to seroconverters; however, this did not reach statistical significance (80.00% vs 83.33%, $P > 0.05$).

Further analysis was focused on the 108 PLWH infected with the SARS-CoV-2 Omicron variant, including 48 seroconverters and 60 non-seroconverters. The baseline characteristics between the SARS-CoV-2-positive seroconverters and non-seroconverters were comparable (Table S13). Interestingly, the number of initial

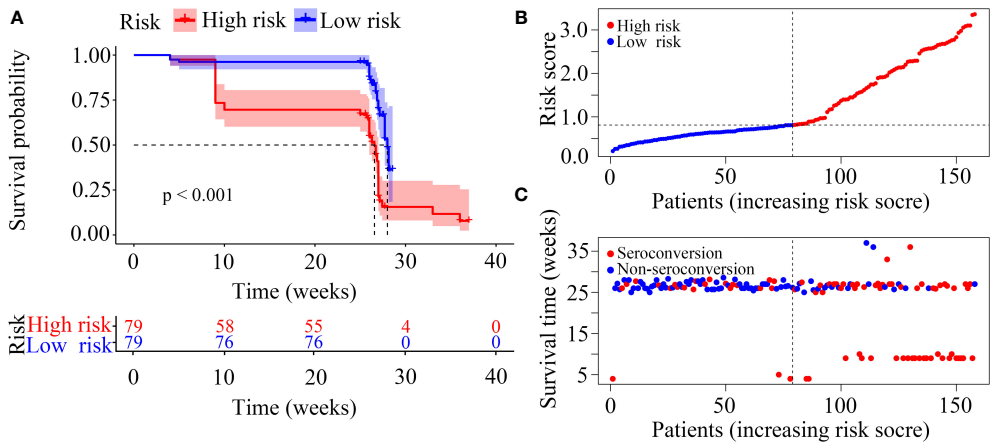

FIGURE 5
Construction of the risk score model with respect to PLWH without NAb seroconversion. **(A)** Kaplan-Meier curves of the overall survival of the two risk groups of PLWH with NAb seroconversion. **(B, C)** Risk score curves and scatter plots of the risk for PLWH without NAb seroconversion.

symptoms induced by the SARS-CoV-2 Omicron variant was significantly lower in seroconverters than in non-seroconverters (Table 6, $P = 0.027$); Table S14 lists the ten most common COVID-19-related symptoms experience by the 108 PLWH, including fever, cough, sore throat, muscle aches, nasal obstruction, runny nose, headache, loss of appetite, loss of taste and smell, and abdominal pain and diarrhea.

4 Discussion

There is insufficient evidence on the efficacy of three consecutive doses of inactivated COVID-19 vaccine against SARS-CoV-2 variants, especially in PLWH. This study dynamically evaluated the NAb response (NAb levels and titers at 6 months post-vaccination) in two PLWH cohorts with different vaccination intervals. We then built a risk model to predict the outcome of NAb seroconversion after vaccination and found that the risk of NAb seroconversion failure among PLWH was inversely proportional to lymphocyte counts and a vaccination interval. Once the COVID-19 restriction were eased in China, we were able to perform an additional follow-up, whereby we compare the frequency of SARS-CoV-2 infections and the number of COVID-19-related symptoms in the PLWH seroconverters and non-seroconverts after the third vaccination.

A substantial increase in NAb levels was observed in both cohorts after the third vaccination, which declined rapidly with time. The dynamics of NAb levels after the third dose of inactivated COVID-19 vaccine are in line with those elicited by other types of COVID-19 vaccines in immunocompromised populations worldwide (25, 26). 6 months after the third vaccination, nearly half of the PLWH (81/158) in our study failed to undergo NAb seroconversion. An interesting study showed that initially poor vaccine responders could generate 5.4-fold higher NAb levels following an additional vaccine dose (27). The above data imply that PLWH with a poor immune response to COVID-19 vaccination (28) should receive a booster vaccine dose.

Since the escape of SARS-CoV-2 variants from NAbs has been widely reported (29), it is necessary to evaluate the NAb responses against the Delta and Omicron variants. In the short-term follow-up period after the third vaccination, the serum samples of PLWH seroconverters from both two cohorts exhibited high neutralization activities against the WT and Delta strains, but not the Omicron variant. While the NAb levels of most PLWH were sufficiently high after the third dose of vaccination, only a minority PLWH (two participants in cohort 1 and 14 participants in cohort 2) developed effective antibody titers against the Omicron BA.5.2 variant over short-term follow-up. The main reason for this is likely that the current COVID-19 vaccine was designed to target the WT SARS-CoV-2 strain (30) and the Omicron variant differs more from the WT than the Delta variant (31). The results of a cross-sectional study showed that NAbs elicited in PLWH by the third vaccination were only mildly effective at inhibiting the replication of the BA.4/5 variant (13). By contrast, Vergori et al. observed that the third dose of the COVID-19 mRNA vaccine could effectively prevent infection of PLWH with SARS-CoV-2 strains (Wuhan-D614G and Omicron BA.1) within 2 weeks of vaccination (9). The weak neutralizing ability of NAbs against Omicron BA.5.2 observed in our study could be attributed to a longer interval for blood collection or the difference between COVID-19 vaccines. Another possible explanation is the differences in the immunogenicity of the BA.5.2 and BA.1 Omicron variants (10, 13). At 6 months after the third vaccination, the efficacy of the NAb response against the SARS-CoV-2 strains was inadequate in both cohorts; this was especially true for the Omicron BA.5.2 variant. A similar phenomenon was reported by Lapointe et al. and Zhan et al. (10, 13). Given that none of the PLWH exhibited neutralization activity against Omicron BA.5.2, we analyzed the sera from HCs to see whether the persistent immune dysregulation and chronic inflammatory status of PLWH (32) reduced their ability to mount a protective NAb response against Omicron (30). We found that HCs had significantly higher NAb levels than PLWH after vaccination, as previously reported (7, 13, 33). Interestingly, despite of higher NAb levels, the anti-Omicron-BA.5.2 NAb titers were undetectable in both PLWH and HCs. Indeed, the SARS-CoV-2 Omicron sub-variants are highly resistant to neutralization by NAbs induced by the COVID-19 vaccine, even in healthy participants (34). This could be attributed to the fact the Omicron variant undergoes large-scale mutations in the RBD region of the spike protein, which enable it to effectively evade the immune response (35, 36). This could also account for the occurrence of breakthrough infections involving the Omicron variant. Thus, the development of more effective Omicron-based vaccines is urgently needed.

Previous studies have indicated that a longer interval between the first and second doses of COVID-19 adenovirus vector and mRNA vaccine induced more immunogenic responses (37, 38). As a complement, we evaluated NAb responses 6 months after the third dose of inactivated COVID-19 vaccines in PLWH with different inoculation intervals. In accordance with the previous studies, we found that a longer interval between the second and third doses of COVID-19 vaccine was associated with markedly higher NAb levels (GMC: 64.59 vs 21.99 AU/mL) and seroconversion rates. The explanation could be extending the dosing interval may increase the B cells selection stringency and boost the formation of memory B cells, those would exhibit stronger

TABLE 6 The association between NAb seroconversion and the number of symptoms induced by SARS-CoV-2 Omicron variant infection in PLWH.

Number of Symptoms	Seroconverters (n = 48)	Non-seroconverters (n = 60)	*P* value
≤ 2 (n, %)	15 (31.25)	8 (13.33)	0.027
> 2 (n, %)	33 (68.75)	52 (86.67)	

antibody responses to the next-dose COVID-19 vaccine (39). Another study also pointed that a longer dosing interval may allow antibodies to mature for longer, and lead to enhanced immunogenicity and efficacy (40). These evidences supported the antibody responses in the longer interval group was better than those in the shorter interval group. The above data confirmed that an appropriate length of interval between COVID-19 vaccine doses should be part of an effective vaccination protocol (41). An optimal inoculation interval would reduce inoculation frequency and therefore limit costs, which is particularly important in the resource-poor areas with a shortage of vaccine supply.

Previous studies have shown that lower $CD4^+$ T cell count has been linked to lower serological responses among PLWH (42). Consistent with several prior studies (9, 43, 44), our study also indicated that PLWH < 200 subgroup in cohort 1 (3-month interval) showed a weaker humoral immune response to inactivated COVID-19 vaccination, comparing to PLWH CD4 ≥ 200 subgroup ($P < 0.05$). Interestingly, the gap in NAb levels and seroconversion rates disappeared as extending the dosing interval to 5-month in PLWH < 200 and PLWH ≥ 200 subgroups. The above data implied the appropriate dosing interval played a crucial role in PLWH with low CD4 count (< 200 cells/μL).

Furthermore, our study showed that an appropriate vaccination interval was as one predictor of the outcome of NAb seroconversion. Additionally, a decrease in the number of lymphocytes was also linked to the likelihood of NAb seroconversion failure in PLWH. Our results were similar to those of Zhang et al. (45), who found that healthy vaccine recipients with low lymphocyte counts failed to undergo NAb seroconversion. Our findings also supported the view that an additional vaccine dose may be necessary for PLWH with lower absolute lymphocyte count (46).

We further constructed a risk model to assess the serological status with respect to NAb seroconversion after vaccination. To the best of your knowledge, this is the first report of a model for predicting the outcome of NAb seroconversion in PLWH. We showed that the risk model had good predictive ability (AUC = 0.777) and maybe useful for providing tailored guidance on the vaccination of PLWH. To date, serologic status assessment has been shown to be vital in predicting the risk of SARS-CoV-2 infection (47, 48). Thus, we analyzed the association between the SARS-CoV-2 Omicron infection rates in PLWH and NAb seroconversion within a year after the third vaccination. We detected only a slight drop in the rate of infection in seroconverters (80.00%), compared with non-seroconverters (83.33%). The inability to efficiently mount a NAb response against the Omicron BA.5.2 variant after the third vaccination could explain this phenomenon.

Despite of the comparable rates of infection, we found that seroconverters displayed fewer initial symptoms on infection by the SARS-CoV-2 Omicron variant. Currently, the researcher are concerned about the clinical symptoms of vaccinated individuals with SARS-CoV-2 infection (49, 50). The Kohler et al. found the reduced occurrence of common COVID-19 symptoms (e.g., impaired olfaction/taste, limb/muscle pain, and chills) in vaccinated anti-SARS-CoV-2 seropositive individuals (51). Our study further showed the association between post-vaccination seroconversion and a reduced numbers of initial COVID-19 symptoms. We speculated that the reduction in the number of symptoms meant that individuals who achieved vaccine-associated seroconversion likely developed immunity against SARS-CoV-2 (50). In addition, some studies demonstrated that lower numbers of initial symptoms were associated with a lower risk of symptom persistence and less severe COVID-19 (52). The above evidence suggests that seroconverters were less likely to develop severe COVID-19; therefore, more research should focus on achieving adequate levels of anti-SARS-CoV-2 immunity in non-seroconverters.

The present study had some limitations. First, this was a single-center study with a small PLWH population. Second, further studies are needed to investigate long-term cellular immunity in addition to the humoral antibody response. Finally, additional work should focus on the evaluation of the risk of reinfection in vaccinated PLWH.

In conclusion, the third dose of the inactivated COVID-19 vaccine elicited a better NAb response in PLWH when the interval between the second and third doses was extended, especially for PLWH with a CD4 count < 200 cells/μL. Furthermore, our 6-month follow-up results showed that vaccinated PLWH mounted inadequate neutralizing responses to the Omicron variant. Lastly, the risk model highlighted that a longer interval between vaccinations and a high absolute lymphocyte count could increase the likelihood of post-vaccination NAb seroconversion. Collectively, our data will help optimize vaccination strategies in PLWH and highlight the need for developing more effective vaccines against the Omicron variant.

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 the Clinical Research Ethics Committee of the Hangzhou Xixi Hospital (ChiCTR2100051312). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JY and YZ contributed equally to this work. The study design was conducted by JY and YZ. The data analysis and manuscript writing were performed by YW and JL. The other authors mainly participated in data collection. All authors were involved in the

interpretation of the results and the statistical analyses. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1152695/full#supplementary-material

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A systematic review and meta-analysis of the effectiveness and safety of COVID-19 vaccination in older adults

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In the coronavirus disease 2019 (COVID-19) pandemic, vaccinations were essential in preventing COVID-19 infections and related mortality in older adults. The objectives of this study were to evaluate the effectiveness and safety of the COVID-19 vaccines in older adults. We systematically searched the electronic bibliographic databases of PubMed, Web of Science, Embase, Cochrane Library, ClinicalTrials.gov, Research Square, and OpenGrey, as well as other sources of gray literature, for studies published between January 1, 2020, and October 1, 2022. We retrieved 22 randomized controlled trials (RCTs), with a total of 3,404,696 older adults (aged over 60 years) participating, that were included in the meta-analysis. No significant publication bias was found. In the cumulative meta-analysis, we found that the COVID-19 vaccines were effective in preventing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection (OR = 0.38, 95% CI = 0.23–0.65, p = 0.0004) and in reducing the number of COVID-19-related deaths (OR = 0.16, 95% CI = 0.10–0.25, $p < 0.00001$) in elderly people. Antibody seroconversion (AS) and geometric mean titer (GMT) levels significantly increased in vaccinated older adults [OR = 24.42, 95% CI = 19.29–30.92; standardized mean difference (SMD) = 0.92, 95% CI = 0.64–1.20, respectively]. However, local and systemic adverse events after COVID-19 vaccine administration were found in older adults (OR = 2.57, 95% CI = 1.83–3.62, $p < 0.00001$). Although vaccination might induce certain adverse reactions in the elderly population, the available evidence showed that the COVID-19 vaccines are effective and tolerated, as shown by the decrease in COVID-19-related deaths in older adults. It needs to be made abundantly clear to elderly people that the advantages of vaccination far outweigh any potential risks. Therefore, COVID-19 vaccination should be considered as the recommended strategy for the control of this disease by preventing SARS-CoV-2 infection and related deaths in older adults. More RCTs are needed to increase the certainty of the evidence and to verify our conclusions.

Systematic review registration: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42022319698, identifier CRD42022319698.

KEYWORDS

COVID-19 vaccine, older adults, SARS-CoV-2, effectiveness, safety, meta-analysis

Introduction

The emergence and spread of coronavirus disease 2019 (COVID-19) brought about negative effects and unprecedented challenges that affected the physical and mental well-being of people worldwide (1). According to a World Health Organization (WHO) report, as of October 1, 2022, there have been more than 616.95 million cumulative cases of COVID-19 globally, including more than 6.5 million deaths (2). A meta-analysis showed that mortality increases from 9.5% in patients 60–69 years old up to 29.6% in those aged >80 years (3). In another study, adults aged 65 years and older were found to be 8.7 times more likely to require hospitalization for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, and have accounted for 22% of cases and up to 78% of COVID-19-related deaths (4). Due to the lower efficacy of treatment for severe COVID-19, older adults have poorer clinical outcomes, including a greater chance of 30-day hospitalization and mechanical ventilation, which also potentially leads to higher mortality rates (5).

As a result, there was a critical need to focus on the vaccination of older adults against SARS-CoV-2 infection and to lower their risk of severe disease and mortality. Although various vaccines and treatments have continuously emerged, these have remained unable to completely control the spread of the virus and eliminate infections. After COVID-19 infection, bodily injury becomes very serious, especially in elderly people (6–11). In the absence of definitive treatment, the development of vaccines against COVID-19 was perceived as an effective strategy to contain the spread of the pandemic.

As of October 1, 2022, there were 177 COVID-19 vaccines in clinical development, 199 in preclinical development, and 11 in phase 4 clinical trials (12). At this time, the WHO had approved 11 COVID-19 vaccines for emergency use listing (EUL), which are shown in Appendix 1 (13). More and more vaccines are now being approved for marketing and undergoing evaluation by the WHO EUL/PQ. The status of each of the 44 COVID-19 vaccines within the WHO EUL/PQ evaluation process is shown in Appendix 2 (14).

COVID-19 vaccines have become more readily available all around the world. Timely vaccination and high vaccination rates are necessary to effectively control diseases (15). Thus far, a total of 163.2 billion vaccine doses have been administered and 28 people per 100 population boosted worldwide, with China having the highest cumulative number of vaccine doses, followed by the United States (2).

Age is an important factor affecting the spread of and infection with COVID-19. Compared with young people, elderly people are more likely to be infected with COVID-19 and more likely to experience serious illness after infection, and their hospitalization rates and mortality rates after infection are higher than those of young people (8–10). Since older adults have the highest rates of COVID-19 mortality, many countries have invested more resources into finding better strategies to achieve and sustain higher vaccination coverage in the older adult population (16). However, there is still disbelief and hesitation about the effectiveness of COVID-19 vaccines. Vaccine hesitancy and rejection (VHR) is one of the top 10 threats to global health (17, 18). The effectiveness and safety of the COVID-19 vaccines against COVID-19 infection therefore need to be assessed in older people.

Clinical trials have shown COVID-19 vaccines to be immunogenic against SARS-CoV-2 infection and safe, with their efficacy ranging from 86% and 95% for the messenger RNA (mRNA) vaccines BNT162b2 (19) and mRNA-1273 (20), respectively, to 74% for the AZD1222 (ChAdOx1 nCoV-19) vaccine (21) in those aged 18 years and older. The other vaccines included in this review have been found to have intermediate efficacies of 67% for Ad26.COV2.S (22) and 78% for the inactivated SARS-CoV-2 vaccine (23) in adults (aged ≥18 years). Furthermore, the COVID-19 vaccines have also been shown to be immunogenic against SARS-CoV-2 infection and safe in older adults (aged ≥60 years), a result similar to that seen in young and middle-aged people (aged from 18 to 60 years) (19–23). In some randomized controlled trials (RCTs), all vaccine formulations have been well tolerated overall, and vaccine-related adverse events or outcomes after vaccination have been generally mild to moderate and transient in adults (19–24).

Although the current COVID-19 vaccination guidelines for older people differ by country, the WHO recommends vaccination with the mRNA, recombinant adenovirus vector, or inactivated coronavirus vaccines, among others, for all older people (25). Older people, as a specific population, are at very high risk of adverse outcomes from infectious diseases due to comorbidities associated with aging and their decreased immunological competence (immunosenescence) (26). Immunosenescence not only increases susceptibility to SARS-CoV-2 infection but also limits the effectiveness of the COVID-19 vaccines, which may lead to differences in vaccine effectiveness between younger (<55 years old) and older people. Vaccine formulations effective in younger people might not engender immunity in older populations (27). Furthermore, there have been concerns that the currently available COVID-19 vaccines may not be adequate to protect older people from COVID-19 infection. A number of studies have revealed that older people had a higher rate of vaccination hesitancy and distrust compared to the general population owing to uncertainties and fears associated with vaccine side effects (28–30). Consequently, it is vital to conduct a meta-analysis on the efficacy and safety of the COVID-19 vaccines in older people, which would provide additional scientific data that could be helpful in protecting older people, a vulnerable demographic during the COVID-19 pandemic.

Therefore, in this meta-analysis and systematic review, we aimed to summarize the overall effectiveness and safety of the COVID-19 vaccines against COVID-19 infection in older people in order to provide evidence for an improved vaccine strategy for this population.

Methods

Data sources and search strategy

PubMed, Web of Science, Embase, Cochrane Library, ClinicalTrials.gov, Research Square, and open gray and gray

literature were searched in the Chinese and English languages from January 1, 2020, to October 1, 2022. In addition, we also manually searched for articles that met the criteria. The search mesh terms included ("older adults" OR "old people" OR "old population" OR "the aged" OR "elder people" OR "the elderly" OR "older patients" OR "aging" OR "gerontology") AND ("COVID-19" OR "coronavirus" OR "SARS-CoV-2" OR "variant strain" OR "Delta variant" OR "B.1.617.2" OR "Omicron variant" OR "B.1.1.529") AND ("vaccine" OR "vaccination") AND ("randomized controlled trial" OR "controlled clinical trial" OR "randomized" OR "randomly" OR "trial"), as shown in Appendix 3. Zotero 6.0.4 (https://www.zotero.org/) was used to manage and screen records and to exclude duplicates. This meta-analysis and systematic review were performed in strict accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA; checklist provided in Appendix 4). This study was registered on PROSPERO (CRD42022319698).

Data selection criteria

This study included RCTs that evaluated the effectiveness and safety of COVID-19 vaccines in older adults (aged ≥60 years). Studies that met the following criteria were excluded: a) irrelevant to the subject of the meta-analysis (SARS-CoV-2 vaccination not involved); b) reviews, editorials, conference papers, case reports, or animal experiments; c) duplicate studies or studies with overlapping participants; d) unable to confirm diagnosis of COVID-19; and e) those with insufficient data to calculate the outcomes in terms of the effectiveness and safety of SARS-CoV-2 vaccines.

Data screening and extraction

The references of the retrieved studies were screened to further select relevant studies suitable for inclusion in this meta-analysis. Data extraction was performed by two independent investigators based on the inclusion and exclusion criteria. The collected documents were processed as references using the document management software Zotero. Any disagreements were discussed with a third investigator. The following materials were extracted from each article by two independent investigators: a) basic information on the studies, including the first author, publication year, and study design; b) characteristics of the study population, including sample sizes, age groups, gender groups, and setting or locations; c) types of SARS-CoV-2 vaccines and the number of doses administered; d) outcomes in terms of the effectiveness of the SARS-CoV-2 vaccines, including the following: number of laboratory-confirmed COVID-19 infections, hospitalizations for COVID-19, admissions to the intensive care unit (ICU) for COVID-19, COVID-19-related deaths, number and sample origin of antibody titer or seroconversion rates, and number of interferon gamma (IFN-γ)-positive T cells; and e) outcomes in terms of the safety of the SARS-CoV-2 vaccines.

Risk of bias and quality assessment

The Cochrane collaboration risk-of-bias tool RoB 2 IRPG beta v9 was used to assess all potential sources of bias in the included references (31), while the GRADE system was used to assess the quality of evidence for all systematic reviews (32, 33).

The Cochrane evaluation criteria include the following five aspects (31): randomization process, deviation from the intended intervention, missing outcome data, measurement of the outcome, and selection of reported results. Publication bias was visualized using funnel plots. Two reviewers (KX and ZW) independently assessed the risk of bias during the evaluation process. Any disagreements were resolved by negotiation or with the participation of a third reviewer (XM or JW). In accordance with the Cochrane Statement of Risk of Bias, the risk of bias for each study was rated as high, some concern, or low risk. Studies with a high overall risk of bias for any single outcome were excluded from the meta-analysis.

The quality of evidence according to the GRADE system was evaluated based on the following five aspects (32, 33): study design limitations, consistency between studies, directness (ability to generalize), precision of results (sufficient or precise data), and publication bias. In accordance with the scoring criteria of the GRADE system, the quality of evidence was classified into five levels: high, moderate, low, very low quality, or no evidence. Similarly, the GRADE system was used to classify the strength of recommendations into four levels: strong recommendation, weak recommendation, recommendation to use interventions only in research, or no recommendation (32, 33). Two reviewers (KX and ZW) independently assessed the studies on the GRADE system during the evaluation process. Any disagreements were resolved by negotiation or with the participation of a third reviewer (XM or JW). Studies with no evidence for any outcome were excluded from the meta-analysis.

Outcomes

The outcomes were the evaluation of the effectiveness and safety of SARS-CoV-2 vaccines in older adults, which included vaccine effectiveness (VE), vaccine immunogenicity, and vaccine safety (VS). VE was defined as the percentage of participants infected by SARS-CoV-2 in relation to the total vaccinated population, with the infected group after vaccination including symptomatic individuals, laboratory-confirmed asymptomatic infections (infection after vaccination), individuals admitted to the hospital or ICU for COVID-19 (hospitalized or admitted to ICU after vaccination), and COVID-19-related deaths (death after vaccination). The immunogenicity of the vaccines was characterized by antibody seroconversion (AS) rate and

geometric mean titer (GMT) of the relevant antibodies, which included neutralizing, anti-S (spike protein), and anti-RBD (spike protein receptor-binding domain) antibodies. VS was defined as the incidence of adverse events after the last vaccine dose had been administered, including total adverse events (AEs); solicited local adverse events (slAEs) such as pain, swelling, and redness; solicited systemic adverse events (ssAEs) such as fever, fatigue, and headache; and geriatric complications after vaccination.

Statistical analysis

Statistical analysis was performed using the Cochrane collaboration review management software (RevMan5.4). Binary variables representing the effectiveness and safety of the SARS-CoV-2 vaccines in comparison with a control group were expressed as odds ratios (ORs) and 95% confidence intervals (CIs), while continuous variables for the same measures in comparison with a control group were expressed in the form of standardized mean differences (SMDs) and 95% CIs. Heterogeneity was identified using the inconsistency (I^2) metric. Degrees of statistical heterogeneity were considered to be low (I^2 < 30%), moderate (I^2 = 30%–50%), or high (I^2 > 50%). The possible sources of heterogeneity were explored using sensitivity analysis. In cases where I^2 was <50%, which represents low-to-moderate heterogeneity and no statistical heterogeneity among the studies, a fixed effects model was used. Otherwise, a random effects model was used for analysis (I^2 ≥ 50%, which represents statistical heterogeneity among the studies). Publication bias was examined using Egger's regression test and a funnel plot visual test; this was measured only when a subgroup contained three or more studies. Values of $p < 0.05$ were considered to represent statistical significance.

Results

Systematic literature search

The PRISMA literature retrieval flowchart is shown in Figure 1. A total of 1,260 potentially relevant articles were identified up to October 1, 2022, from electronic databases, including 306 from PubMed, 107 from Embase, 77 from the Cochrane Library, 100 from Web of Science, 13 from ClinicalTrials.gov, 657 from Research Square, and 0 from OpenGrey or other sources of gray literature. After preliminary screening, 110 duplicate records were excluded. After reading the titles and abstracts, 1,028 publications were then excluded in accordance with the inclusion and exclusion criteria. Subsequently, after reading the abstract and full text of each publication in detail, another 100 records were excluded due to insufficient data, unavailability of the full text, or no confirmed diagnosis. Ultimately, 22 studies were included in this meta-analysis based on the inclusion criteria.

Basic characteristics

A total of 22 articles were included in the meta-analysis (21, 34–54), as shown in Table 1. From these publications, the relevant indicators were extracted, including information on the author, year

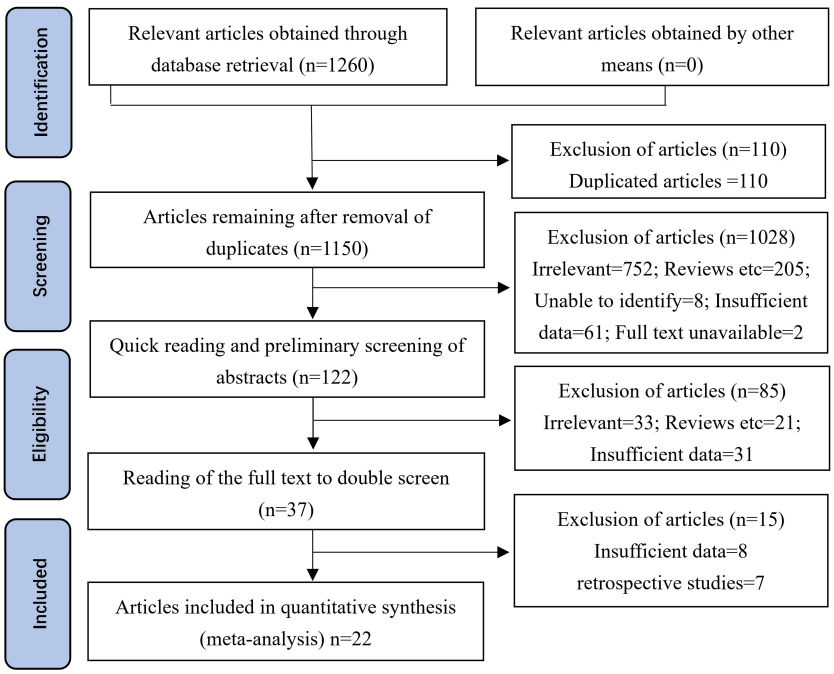

FIGURE 1
Methodological PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flowchart for the selection of the studies included in this meta-analysis. A total of 306 studies were obtained from PubMed, 107 from Embase, 77 from the Cochrane Library, 100 from the Web of Science, 13 from ClinicalTrials.gov, 657 from Research Square, and 0 from OpenGrey or other sources of gray literature.

of publication, number of participants, and vaccination efficacy and safety. Four articles reported data related to vaccination effectiveness (21, 35, 37, 54), 16 articles reported data related to antibody titer levels after vaccination (34, 36, 38–46, 48–51, 53), and 13 articles reported data related to the occurrence of vaccine-related adverse events in elderly people (21, 36, 38, 39, 41, 43, 45–49, 51, 52).

In addition to these, the same indicators (including author, year of publication, number of participants, and vaccine efficacy and safety) could be extracted from seven retrospective studies (55–61), shown in Appendix 5. Four articles reported on VE (58–61), while three articles reported antibody titer levels after vaccination (55–57) in the elderly. Six articles reported the effectiveness and safety of two vaccine doses (56–61), while only one article reported the effectiveness and safety of three vaccine doses (55) in elderly people. Five articles reported the effectiveness and safety of the nucleic acid vaccine (56, 58–61), while three articles reported the effectiveness and safety of the inactivated vaccine (55, 57, 58) in elderly people. Although vaccination was found to provide clear protection against SARS-CoV-2 infection in older adults, the results showed high and inexplicable heterogeneity in terms of both its effectiveness and its safety. Analyses of the data of these retrospective studies are presented in Appendix 5.

Finally, the same indicators (including author, year of publication, number of participants, and vaccination efficacy and safety) could be extracted from eight qualitative analysis articles (62–69), which are shown in Appendix 6. However, these studies could not be included in the meta-analysis due to insufficient data and/or descriptive explanations of vaccination efficacy and safety without the use of a parallel control, among other reasons. One article reported the VE (63), three articles reported antibody titer levels after vaccination (64, 65, 69), and six articles reported the occurrence of vaccine-related adverse events in the elderly (62, 64–68).

Quality assessment

The Cochrane Risk-of-Bias 2 tool (RoB 2 v9) was used to evaluate the quality of the individual studies included (Appendix 7). After evaluation, 19 articles were rated as low risk (21, 34, 36, 37, 39–45, 47–54), while the risk of bias for three articles was rated as "some concern" (35, 38, 46), as shown in Table 1 and Figure 2. Additionally, we used GRADEprofiler 3.6 to assess the quality of evidence for all systematic reviews. Of all the pieces of evidence included in the analysis, nine were characterized as high-quality evidence [VE, VEND (vaccine effectiveness by number of doses), VEVT (vaccine effectiveness by vaccine type), GMT, GMTAT (geometric mean titers by antibody type), GMTVT (geometric mean titers by vaccine type), AE, slAE, and ssAE], while one (AS) was characterized as evidence of moderate quality (Appendix 8). According to the quality evaluation using GRADE of the evidence on outcomes in terms of VE, immunogenicity, and VS, COVID-19 vaccination in older adults should be considered to be a strongly

TABLE 1 Basic features of the studies included in the meta-analysis.

Study	Study design	Vaccine name	Vaccine type	Number of doses	Research quantum (V/C)	Age (years)	Gender (M/F)	Outcome measures	RoB2
Poh et al. (34)	RCT	BNT162b2, mRNA-1273	NAV	Three doses	24/23 (BBB/BBM)	>60	22/25	GMT, GMTAT, GMTVT	L
Ioannou et al. (35)	RCT	BNT162b2, mRNA-1273, JNJ-78436735	VVV, NAV	Two doses	1,472,010/1,472,010	≥65	1,270,345/201,665	VE, VEVT, VEND	S
Formica et al. (36)	RCT	NVX-CoV2373	SV	Two doses	233/116	60-84	169/180	GMT, GMTAT, GMTVT, VS, slAE, ssAE	L
Sadoff et al. (37)	RCT	Ad26.COV2.S	VVV	One dose	6,403/6,340	≥60	NA	VE, VEVT, VEND	L
Lanini et al. (38)	RCT	GRAd-COV2	VVV	Two doses	45	65–85	NA	GMT, GMTAT, GMTVT, ssAE	S
Shu et al. (39)	RCT	V-01	SV	Two doses	360/80	60–83	262/178	AS, GMT, GMTAT, GMTVT, VS, slAE, ssAE	L
Alidjinou et al. (40)	RCT	BNT162b2	NAV	Two doses	47	77–90	17/30	GMT, GMTAT, GMTVT	L
Sadoff et al. (41)	RCT	Ad26.COV2.S	VVV	Two doses	320/88	65–88	190/211	GMT, GMTAT, GMTVT, VS, slAE, ssAE	L
Falsey et al. (21)	RCT	AZD1222	VVV	Two doses	4827/2411	≥65	NA	VE, VEVT, VEND, VS, slAE, ssAE	L

(Continued)

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TABLE 1 Continued

Study	Study design	Vaccine name	Vaccine type	Number of doses	Research quantum (V/C)	Age (years)	Gender (M/F)	Outcome measures	RoB2
Zakarya et al. (42)	RCT	QazCOVID-in vaccine	IV	Two doses	100	≥60	35/65	AS	L
Guo et al. (43)	RCT	Sinopharm	IV	Three doses	252/84	≥60	200/136	AS, VS, slAE, ssAE	L
Ramasamy et al. (44)	RCT	ChAdOx1 nCoV-19	VVV	Two doses	156/40	≥70	103/74	GMT, GMTAT, GMTVT	L
Richmond et al. (45)	RCT	SCB-2019	SV	Two doses	73/18	55–75	36/55	AS, VS, slAE, ssAE	L
Li et al. (46)	RCT	BNT162b1	NAV	Two doses	48/24	65–85	36/36	AS, VS, slAE, ssAE	S
Walsh et al. (47)	RCT	BNT162b1	NAV	Two doses	72/18	65–82	30/60	slAE, ssAE	L
Wu et al. (48)	RCT	CoronaVac	IV	Two doses	347/74	≥70	206/215	AS, GMT, GMTAT, GMTVT, VS, slAE, ssAE	L
Wynne et al. (49)	RCT	ReCOV	SV	Two doses	10/40	56-80	26/24	VS, slAE, ssAE	L
Sáez-Llorens et al. (50)	RCT	mRNA-LNP	NAV	Two doses	306	>60	164/142	AS, GMT, GMTAT, GMTVT, VS	L
Sadoff et al. (51)	RCT	Ad26.COV2.S	VVV	Two doses	24	≥65	NA	GMT, GMTAT, GMTVT	L
Tanishima et al. (52)	RCT	KD-414	IV	Three doses	90/14	≥65	53/51	VS, slAE, ssAE	L
Kundro et al. (53)	RCT	rAd26/BBIBP-CorV, rAd26/ChAdOx1, rAd26/rAd5	IV, VVV	Two doses	27/27/31	≥65	33/48	GMT, GMTAT, GMTVT	L
Song et al. (54)	RCT	BBIBP-CorV/CoronaVac	IV	Two doses	68/113	66.5–74	74/107	VE, VEVT, VEND	L

RCT, randomized controlled trial; IV, inactivated vaccine; SV, subunit vaccine; VVV, viral vector vaccine; NAV, nucleic acid vaccine; V/C, vaccine group/control or placebo control group; M/F, male/female; NA, not available; VE, vaccine effectiveness (infection, hospitalization or ICU admission, death after vaccination); VEND, vaccine effectiveness (by number of doses); VEVT, vaccine effectiveness (by vaccine type); AS, antibody seroconversion; GMT, geometric mean titer; GMTAT, geometric mean titer (by antibody type); GMTVT, geometric mean titer (by vaccine type); VS, vaccine safety; slAE, solicited local adverse event; ssAE, solicited systemic adverse event; H, high overall bias; L, low overall bias; S, overall bias rating of "some concern".

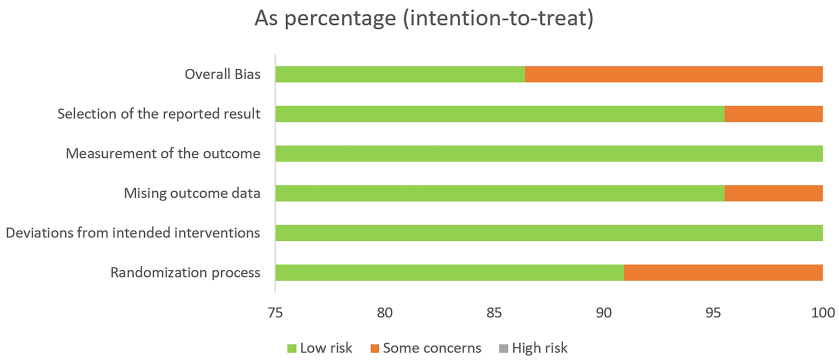

FIGURE 2
Risk of bias in the randomized controlled trials (RCTs) included, assessed using the Cochrane RoB2 tool.

recommended strategy for control of COVID-19 through prevention of SARS-CoV-2 infection and reduction of COVID-19-related deaths.

Heterogeneity and risk of bias

Prior to meta-analysis of the included articles, a heterogeneity test was performed for results in which there were two or more included papers. The results of the analysis showed that no study significantly interfered with the results of the meta-analysis. The risk of publication bias was evaluated through funnel plots produced using Revman5.3; evidence of significant publication bias can be ignored due to the good levels of symmetry observed in these funnel plots. The shapes of the funnel plots for VE, AS, GMT, and VS are shown in Figure 3, while those of the subgroups are shown in Appendix 9. Groups with heterogeneity scores over 50 ($I^2 > 50\%$) were examined using Egger's test; the results showed no evidence of publication bias ($p > 0.05$), except in the cases of GMT, anti-S of GMTAT, and nucleic acid vaccine of GMTVT groups ($p < 0.05$). Data from Egger's test are shown in Appendix 10.

Effectiveness of COVID-19 vaccines among older adults

Vaccine effectiveness

Four included studies contained data related to VE; these included a total of 1,711,591 and 1,709,676 participants in the vaccine and control groups, respectively. A random effects model was used for the meta-analysis due to the high heterogeneity ($p < 0.00001$, $I^2 = 95.4\%$) of the data (Figure 4). The meta-analysis on the effectiveness of the vaccine in this group of studies indicated an OR representing lower risk in the vaccine group compared to the control group (OR = 0.45, 95% CI = 0.28–0.70, $p = 0.0005$). The COVID-19 vaccines were shown to be more effective in preventing SARS-CoV-2 infection (OR = 0.38, 95% CI = 0.23–0.65, $p = 0.0004$) and in reducing COVID-19-related deaths (OR = 0.16; 95% CI = 0.10–0.25, $p < 0.00001$), but less effective in preventing hospitalization and ICU treatment (OR = 0.97, 95% CI = 0.71–1.33, $p = 0.85$) in elderly people. The subgroup analysis for each effectiveness indicator is shown in Appendix 11.

Subgroup analysis for vaccine effectiveness

The subgroup analysis for number of doses identified significant differences among four studies in the effects observed in experiments using one and two doses, which revealed that two vaccination doses had better effectiveness against SARS-CoV-2 infection compared to only one vaccination dose ($\chi^2 = 10.24$, $p = 0.001$, $I^2 = 90.2\%$) (Table 2 and Appendix 11). The outcomes demonstrated that the vaccinated group experienced better outcomes than the control group in both one-dose (OR = 0.81, 95% CI = 0.56–1.17, $p = 0.26$) and two-dose experiments (OR = 0.23, 95% CI = 0.11–0.45, $p < 0.0001$).

The subgroup analysis for vaccine type identified significant differences among four studies in the effects observed in experiments on the inactivated, nucleic acid, and viral vector

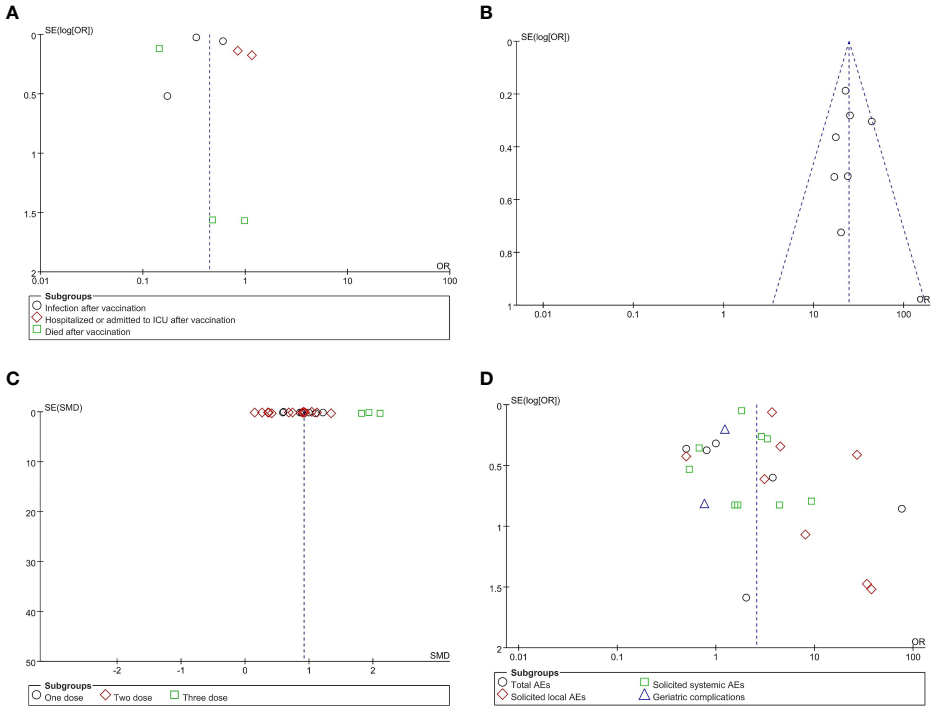

FIGURE 3
Funnel plots for publication bias. Publication bias in reports of vaccine effectiveness (VE) **(A)**, antibody seroconversion (AS) **(B)**, geometric mean titer (GMT) **(C)**, and vaccine safety (VS) **(D)**. *OR*, odds ratio; *SMD*, standardized mean difference.

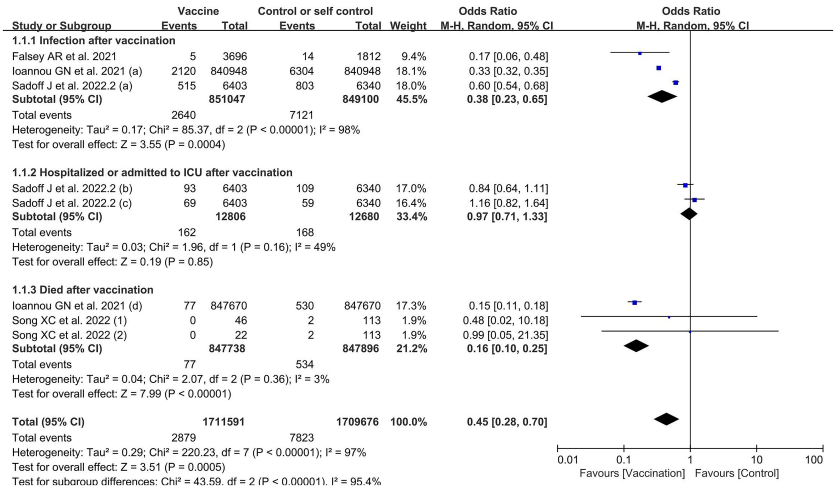

FIGURE 4
Forest plots of vaccine effectiveness by number of doses for the coronavirus disease 2019 (COVID-19) vaccine group compared with the control group. (a), infection after vaccination; (b), hospitalized after vaccination; (c), admitted to ICU after vaccination; (d), death after vaccination; *1*, one dose; *2*, two doses.

vaccine groups (Table 2 and Appendix 11). The subgroup of studies with the nucleic acid vaccine showed better effectiveness compared to studies with the viral vector and inactivated vaccines (χ^2 = 5.90, p = 0.05, I^2 = 66.1%). The outcomes demonstrated that the vaccinated group experienced better outcomes than the control group in the experiments with inactivated (OR = 0.69, 95% CI = 0.08–6.00, p = 0.74), nucleic acid (OR = 0.22, 95% CI = 0.10–0.50, p = 0.0003), and viral vector vaccines (OR = 0.69, 95% CI = 0.46–1.05, p = 0.08).

Immunogenicity of COVID-19 vaccines among older adults

Antibody seroconversion rate

Seven included studies presented data related to AS rate; these included 1,584 participants in vaccine groups (Figure 5). A fixed effects model was used for the meta-analysis due to the low heterogeneity (p = 0.51, I^2 = 0) of the data. The meta-analysis in the vaccine group found an OR indicating higher AS rates in the

TABLE 2 Subgroup analysis for vaccine effectiveness.

Study characteristics	Data			Test for heterogeneity			Test for effect		Subgroup	
	No. of studies	Vaccine	Control	I^2 (%)	Chi-squared test	p-value	OR (CI)	p-value	Statistic	p-value
No. doses										
One dose	4	19,255	19,133	80	15.24	0.002	0.81 (0.56–1.17)	0.26	10.24	0.001
Two doses	4	1,692,336	1,690,543	94	47.05	<0.00001	0.23 (0.11–0.45)	<0.00001		
Total	8	1,711,591	1,709,676	97	220.23	<0.00001	0.45 (0.28–0.70)	0.0005		
Vaccine type										
IV	2	68	226	0	0.11	0.74	0.69 (0.08–6.00)	0.74	5.90	0.05
NAV	2	1,688,618	1,688,618	98	45.14	<0.00001	0.22 (0.10–0.50)	0.0003		
VVV	4	22,905	20,832	86	21.78	<0.0001	0.69 (0.46–1.05)	0.08		
Total	8	1,711,591	1,709,676	97	220.23	<0.00001	0.45 (0.28–0.70)	0.0005		

IV, inactivated vaccine; VVV, viral vector vaccine; NAV, nucleic acid vaccine.

vaccinated groups compared with the control groups (OR = 24.42, 95%CI = 19.29–30.92, $p < 0.00001$).

Geometric mean titer

There were 11 included studies with data related to GMT; these included 2,312 and 1,072 participants in the vaccine and control groups, respectively (Figure 6). A random effects model was used for the meta-analysis due to the significant level of statistical heterogeneity ($p < 0.00001$, $I^2 = 91\% > 50\%$) among studies. The pooled effects of these studies (SMD = 0.92, 95% CI = 0.64–1.20, $Z = 6.41$, $p < 0.00001$) showed that antibody titer levels improved significantly in the vaccine group, with a large effect compared to the control group. In addition, the subgroup analysis for number of doses found significant differences among 11 studies in the effects of experiments in which one dose, two doses, and three doses were administered ($\chi^2 = 2.09$, $p = 0.35$, $I^2 = 4.3\%$). The three-dose subgroup showed better effectiveness than both the one-dose and two-dose subgroups. The outcomes demonstrated that the vaccine group experienced better outcomes than the control group in the experiments involving one dose (SMD = 0.84, 95% CI = 0.66–1.02, $Z = 8.99$, $p < 0.00001$), two doses (SMD = 0.73, 95% CI = 0.56–0.90, $Z = 8.42$, $p < 0.00001$), and three doses (SMD = 2.95, 95% CI = −0.65 to 6.55, $Z = 1.61$, $p = 0.11$).

Subgroup analysis for GMT

The subgroup analysis of GMT for different antibody types found no statistical differences among the subgroups of neutralizing, anti-S, and anti-RBD antibodies ($\chi^2 = 0.32$, $p = 0.85$, $I^2 = 0$) (Table 3 and Appendix 11). The outcomes demonstrated that the vaccine group experienced better outcomes than the control group in results pertaining to neutralizing antibodies (SMD = 0.82, 95% CI = 0.64–1.01, $Z = 8.73$, $p < 0.00001$), anti-S antibodies (SMD = 1.11, 95% CI = 0.08–2.15, $Z = 2.10$, $p = 0.004$), and anti-RBD antibodies (SMD = 0.88, 95% CI = 0.44–1.31, $Z = 3.94$, $p < 0.0001$).

Although the nucleic acid vaccine showed better effectiveness compared to the inactivated, subunit, and viral vector vaccines (Table 3 and Appendix 11), the subgroup analysis for vaccine type found no statistical differences among 11 studies in the effects of experiments involving subunit, nucleic acid, and viral vector vaccine subgroups ($\chi^2 = 4.28$, $p = 0.23$, $I^2 = 29.9\%$). The outcomes demonstrated that the vaccine group experienced better outcomes than the control group in the case of experiments involving inactivated vaccines (SMD = 0.76, 95% CI = 0.23–1.29, $Z = 2.82$, $p = 0.005$), subunit vaccines (SMD = 0.91, 95% CI = 0.77–1.04, $Z = 12.88$, $p < 0.00001$), nucleic acid vaccines (SMD = 1.57, 95% CI = 0.04–3.11, $Z = 2.01$, $p = 0.004$), and viral vector vaccines (SMD = 0.67, 95% CI = 0.46–0.88, $Z = 6.13$, $p < 0.00001$).

Safety of COVID-19 vaccines among older adults

Vaccine safety

There were 10 included studies with data related to vaccine-related adverse events; these included 14,297 and 6,290 participants in the vaccine and control groups, respectively (Figure 7). A random effects model was used for the meta-analysis due to the high heterogeneity ($p < 0.00001$, $I^2 = 89\%$) of the data. The meta-analysis found an OR reflecting higher odds of adverse events in the vaccine group compared to the control group (OR = 2.57, 95% CI = 1.83–3.62, $p < 0.00001$). In addition, the subgroup analysis for immune effect found significant differences among 10 studies in the effects of experiments examining total AEs, slAEs, ssAEs, and geriatric complications after vaccination ($\chi^2 = 14.22$, $p = 0.003$, $I^2 = 78.9\%$) (Figure 7). The outcomes demonstrated that the vaccine group experienced more AEs than the control group in the experiments on AEs (OR = 3.39, 95%CI = 1.01–11.40, $p = 0.05$), slAEs (OR = 6.45, 95%CI = 2.78–14.97, $p < 0.0001$), ssAEs (OR = 1.90, 95%CI = 1.24–2.92, $p = 0.003$), and geriatric complications (OR = 1.20; 95%CI = 0.82–1.76, $p = 0.36$).

Subgroup analysis for vaccine safety

A total of 10 studies included data related to the VS in terms of slAEs; these included 14,127 and 6,168 participants in the vaccine and control groups, respectively (Table 4 and Appendix 11). The random effects model was used for the meta-analysis due to the higher heterogeneity ($p < 0.00001$, $I^2 = 73\%$) of the data. The meta-analysis found an OR reflecting higher odds of slAEs in the vaccine group compared to the control group (OR = 3.82, 95% CI = 2.19–6.65, $p < 0.00001$). In addition, the subgroup analysis for immune effect found no statistical differences among the 10 studies in incidence of pain (OR = 5.04, 95% CI = 2.15–11.83, $p = 0.0002$), swelling (OR = 3.31, 95% CI = 0.89–12.28, $p = 0.07$), or redness (OR = 3.13, 95% CI = 0.90–10.94, $p = 0.07$), $\chi^2 = 0.51$, $p = 0.78$, $I^2 = 0$.

There were 12 included studies with data related to the VS in terms of ssAEs; these included 19,545 and 8,639 participants in the

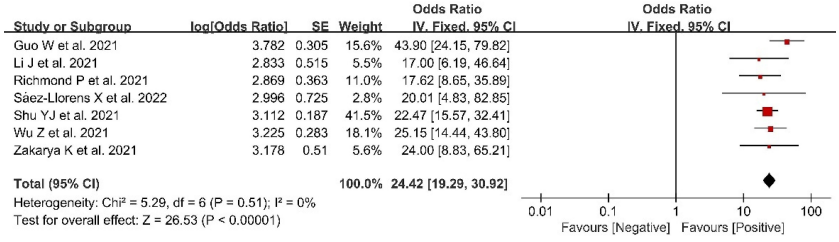

FIGURE 5
Forest plots of antibody seroconversion rate after coronavirus disease 2019 (COVID-19) vaccination.

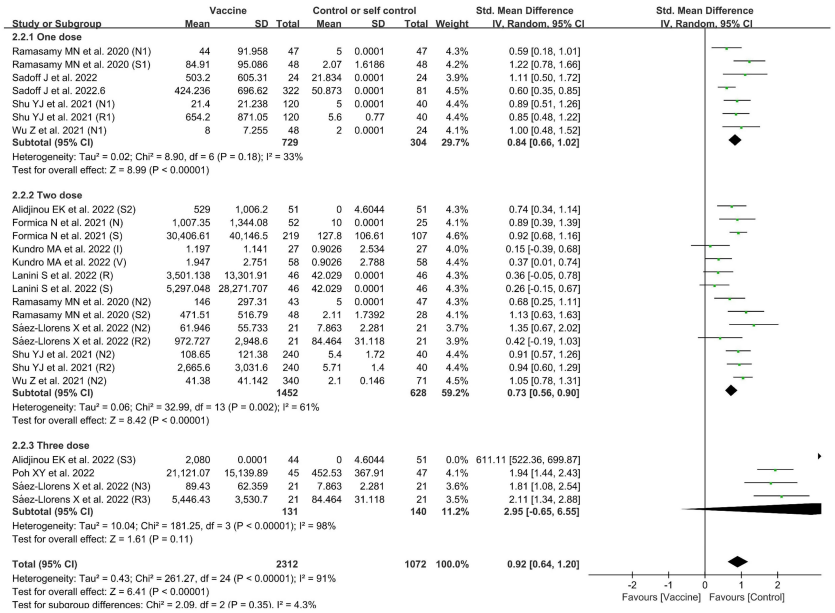

FIGURE 6
Forest plots of geometric mean titers (GMT) by number of doses for the coronavirus disease 2019 (COVID-19) vaccine group compared with the control group. *N*, neutralizing antibody; *S*, anti-S antibody; *R*, anti-RBD antibody; *1*, one dose; *2*, two doses; *3*, three doses; *I*, inactivated vaccine; *V*, viral vector vaccine.

TABLE 3 Subgroup analysis for geometric mean titers (GMT).

Study characteristics	Data			Test for heterogeneity			Test for effect		Subgroup	
	No. of studies	Vaccine	Control	I^2 (%)	Chi-squared test	*p*-value	SMD (CI)	*p*-value	Statistic	*p*-value
Antibody type										
Neutralizing antibody	13	1,363	526	60	30.21	0.003	0.82 (0.64–1.01)	<0.00001	0.32	0.85
Anti-S	7	501	378	97	210.49	<0.00001	1.11 (0.08–2.15)	0.04		
Anti-RBD	5	448	168	77	17.65	0.001	0.88 (0.44–1.31)	<0.0001		
Total	25	2,312	1,072	91	261.27	<0.00001	0.92 (0.64–1.20)	<0.00001		
Vaccine type										
IV	3	415	122	78	8.92	0.01	0.76 (0.23–1.29)	0.005	4.28	0.23
SV	6	991	292	0	0.15	1.00	0.91 (0.77–1.04)	<0.00001		
NAV	7	224	233	97	209.12	<0.00001	1.57 (0.04–3.11)	0.04		
VVV	9	682	425	60	19.87	0.01	0.67 (0.46–0.88)	<0.00001		
Total	25	2,312	1,072	91	261.27	<0.00001	0.92 (0.64–1.20)	<0.00001		

Anti-S, anti-spike protein antibody; Anti-RBD, anti-RBD (spike protein receptor-binding domain) antibody; GMT, geometric mean titers; IV, inactivated vaccine; SV, subunit vaccine; VVV, viral vector vaccine; NAV, nucleic acid vaccine.

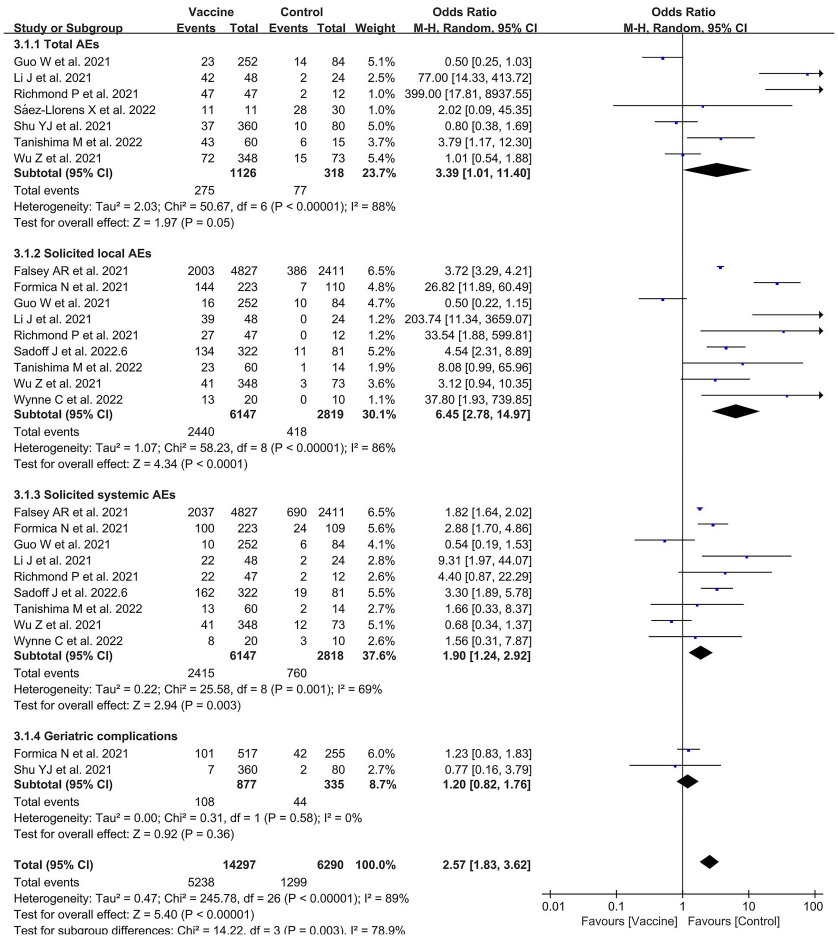

FIGURE 7
Forest plots of vaccine safety for the coronavirus disease 2019 (COVID-19) vaccine group compared with the control group.

vaccine and control groups, respectively (Table 4 and Appendix 11). A fixed effects model was used for the meta-analysis due to the lower heterogeneity (p = 0.05, I^2 = 31%) of the data. The meta-analysis found a higher OR in the vaccine group compared to the control group (OR = 1.91, 95%CI = 1.75–2.09, p < 0.00001). In addition, the subgroup analysis for immune effect found differences among the 10 studies in terms of the effects on fever (OR = 5.38, 95% CI = 2.79–10.37, p < 0.00001), fatigue (OR = 1.65, 95% CI = 1.46–1.86, p < 0.00001), and headache (OR = 2.12, 95% CI = 1.85–2.44, p < 0.00001), χ^2= 17.45, p = 0.0002, I^2 = 88.5%.

Discussion

In this study, we assessed the effectiveness of COVID-19 vaccines against COVID-19 infection and their safety among older people. In the analysis of VE, we found that any dose of the vaccine is protective in elderly people; however, administration of two doses is more effective than one dose. The nucleic acid vaccines are more effective than other types, inactivated and viral vector vaccines, with the inactivated vaccine being the least effective of these three types. These three types of COVID-19 vaccine were more effective in preventing SARS-CoV-2 infection and in reducing deaths after infection, but less effective in preventing hospitalization and ICU treatment. It was found that elderly people who received COVID-19 vaccines experienced better outcomes (or the same level of outcome) than those who did not receive a COVID-19 vaccine in all aspects. It can be considered that vaccination for COVID-19 is still the major strategy for the prevention of SARS-CoV-2 infection and reduction of the severity of illness after infection. In terms of immunogenicity, the meta-analysis revealed high AS rates [including those of neutralizing antibodies, spike-specific immunoglobulin G (IgG), and RBD-specific IgG] in the elderly population after vaccination. Levels of all three of these antibody types, i.e., neutralizing, anti-S, and anti-RBD antibodies, increased significantly after vaccination, but there was no significant difference between them. Any number of vaccine doses was able to induce the production of antibodies in elderly people; however, the more frequent the inoculation doses, the higher the antibody titer levels were. There was not much difference in antibody titer levels between one or two doses, but the antibody titer levels increased significantly after three doses. With an increase in number of inoculation doses, the immune effect also increased correspondingly (65). Booster doses helped to increase the antibody

TABLE 4 Subgroup analysis for vaccine safety.

Study characteristics	Data			Test for heterogeneity			Test for effect		Subgroup	
	No. of studies	Vaccine	Control	I^2 (%)	Chi-squared test	*p*-value	OR (CI)	*p*-value	Statistic	*p*-value
Solicited local AEs										
Pain	11	6,579	2,918	86	70.13	<0.00001	5.04 (2.15–11.83)	0.0002	0.51	0.78
Swelling	8	1,329	412	51	14.33	0.05	3.31 (0.89–12.28)	0.07		
Redness	10	6,219	2,838	61	22.89	0.006	3.13 (0.90–10.94)	0.07		
Total	29	14,127	6,168	73	101.89	<0.00001	3.82 (2.19–6.65)	<0.00001		
Solicited systemic AEs										
Fever	12	6,862	2,998	24	14.45	0.21	5.38 (2.79–10.37)	<0.00001	17.45	0.0002
Fatigue	10	6,327	2,833	42	15.40	0.08	1.65 (1.46–1.86)	<0.00001		
Headache	10	6,356	2,808	0	5.83	0.76	2.12 (1.85–2.44)	<0.00001		
Total	32	19,545	8,639	31	45.22	0.05	1.91 (1.75–2.09)	<0.00001		

AEs, adverse events.

titers and keep them stable. The standard fortification agent induces production of more antibody titers compared to the half-dose fortification agent and is more resistant to the delta variant than to the omicron variant (63).

Most of the approved COVID-19 vaccines, including the mRNA, recombinant adenovirus vector, inactivated coronavirus, and subunit vaccines, have been designed to elicit humoral and T-cell-mediated immune responses (70). In particular, COVID-19 vaccines can induce the production of anti-S, anti-RBD, and neutralizing antibodies against the spike protein of SARS-CoV-2 (71, 72), all of which bind to this spike protein and hinder its interaction with the angiotensin-converting enzyme 2 (ACE2) receptor (73). The viral entry of SARS-CoV-2 is facilitated by the interaction between its spike protein and the ACE2 receptor of the human host (74). Host protective antibodies induced by the COVID-19 vaccines hamper viral entry, the viral life cycle, and the pathogenicity of SARS-CoV-2. Although these antibody responses can be substantially boosted by two or three doses of the vaccines, the particular span of the duration of antibody responses remains unknown (75). The relevant findings of this meta-analysis once more suggests, in accordance with other reports on RCTs, that a second or booster dose of a vaccine triggers a considerable elevation in B-cell immune responses (57, 76). For this reason, the WHO recommends booster doses for priority vaccination groups, including elderly people, health workers, and other special groups (77, 78).

In this meta-analysis, it was found that, compared to the other types of vaccines, the nucleic acid vaccine produced the highest antibody titers in elderly people. This vaccine type induced the highest titer levels of spike-specific IgG, while the inactivated vaccine induced the lowest titer levels. Although the antibody titer levels of the elderly population after inoculation were lower than those of the younger population, the geometric mean ratios for antibodies were higher among the elderly population than among young people, which may have been a result of an insufficient number of studies involving the elderly population (64). In accordance with other reports, administration of the mRNA vaccine was associated with a significant increase in titer levels of neutralizing antibodies and in the antigen-specific production of IFN-γ, $CD4^+$, and $CD8^+$ T cells after the second vaccine dose (71, 72, 79). However, since only three articles involving this form of analysis were included, this conclusion may not be generalizable and may be limited to the vaccines included in the study. The analysis conducted in this study undoubtedly confirmed that the nucleic acid vaccine produced the best immune effect in the elderly population.

In the qualitative analysis, number of IFN-γ spot-forming cells (SFCs) and percentage of T-cell subsets were found to increase along with number of COVID-19 vaccine doses and time since inoculation among vaccinated elderly people (38, 44). The number of IFN-γ SFCs was slightly elevated on day 28 after the first dose of the viral vector vaccine compared to non-vaccination, but increased significantly after the second dose (44). After vaccination, the number of Th1 cells in the elderly population increased exponentially, while the number of Th2 cells fluctuated slightly with the increase in days since inoculation and number of doses (38, 66). Generally, the IFN response produced by alveolar macrophages, dendritic cells, natural killer cells, and inflammatory monocyte/macrophages is the primary

antiviral innate immune signaling pathway (80). However, the number of articles reporting on RCTs examining T-cell responses and cytokine production after COVID-19 vaccination was insufficient, and these measures were not included in the analysis of protective immunity in the current study. Additionally, a number of older adults with diabetes or high blood pressure showed little difference in titer levels of neutralizing antibodies after vaccination when compared with healthy adults (69). Overall, the outcomes analysis of the retrospective studies showed that vaccination was effective in providing protection from SARS-CoV-2 infection, as well as in increasing the antibody positive conversion rate after vaccination.

In terms of the VS analysis, we found that vaccination will, to some extent, cause certain adverse reactions. The incidence of lAEs was higher than that of sAEs, and vaccination was not statistically associated with complications of geriatric diseases. Pain and fever were the most common lAEs and sAEs. Comparison of the pain and fever responses showed that pain was more prevalent than fever in the inoculated population. The frequency of local and systemic adverse reactions was higher after the first dose than after the second dose. In the meta-analysis, local adverse reactions after vaccination were more prevalent than systemic adverse reactions. The main reason for this may be that different elderly individuals perceive adverse reactions differently, and some elderly people are more sensitive to perceptions of physical injury (63, 64). Therefore, the previous assumption that inoculated groups are more sensitive to perceptions of physical injury is clearer.

In addition, we also found that the incidence of serious adverse reactions was very low. Some of the studies also reported on cardiovascular and cerebrovascular diseases arising in elderly people after vaccination, including vascular embolism, arrhythmias, and nervous system bleeding (81). Although severe adverse events have been reported at a rate of around five cases per one million in all those administered vaccine doses, this is extremely rare and is a very low rate (81). The data in the articles that covered such adverse reactions showed that the nucleic acid vaccine, compared with viral vector vaccination, is less likely to trigger cardiovascular and cerebrovascular diseases (68). Such reports of adverse events due to administration of COVID-19 vaccines have created vaccine hesitancy among elderly populations in many parts of the world. Millions of doses of the COVID-19 vaccines have already been administered around the world, and the safety of the vaccines has been frequently stressed by the many health authorities monitoring VS (82). It needs to be made abundantly clear to the elderly that the advantages of vaccination, which is the best method of controlling COVID-19 by preventing severe illness and related deaths, far outweigh any potential risks.

The present meta-analysis and systematic review have several limitations. First, this research was limited to studies published in Chinese and English, and there were some shortcomings in the inclusion of research published in other languages. Second, due to insufficient data, we were not able to conduct a subgroup analysis for comorbidities in the elderly population, such as diabetes, hypertension, and cancer. Third, in the study of outcome indicators, due to a lack of or inadequacy of relevant data, some studies were not comprehensive. For example, the analysis of vaccine effectiveness was not comprehensive enough due to insufficient data on elderly people after vaccination. Fourth, there was a large degree of heterogeneity among the included studies regarding VE, GMT, and VS. The results of the subgroup analyses should be interpreted with caution due to the diversity of influencing factors. Finally, there were not enough data to analyze long-term adverse effects after vaccination, and only short-term adverse effects, including slAEs and ssAEs, were analyzed in the current study. Moreover, in cases where the author could not be contacted to obtain detailed data, we used image extraction methods for data presented in images. Although there was no qualitative impact on the outcome indices, there were still some limitations in terms of the fine-grained data. In addition, we did not find enough data for a subgroup analysis of all types of vaccines for the elderly population. Nevertheless, this research provides some degree of insight into the effectiveness and safety of vaccination in the elderly population.

Conclusions

This systematic review and meta-analysis have comprehensively synthesized the latest data on vaccine effectiveness, immunogenicity, and safety in older adults based on 22 RCTs. In the meta-analysis, we found that vaccination is more effective in preventing SARS-CoV-2 infection and in reducing the number of COVID-19-related deaths in elderly people. The effect of two doses s stronger than that of one dose. After vaccination, high AS rates are observed in the elderly population. With an increase in the number of inoculation doses received, antibody titer levels also increased among the older population, with the highest antibody titer levels in elderly people being induced by the nucleic acid vaccinein. Vaccination can produce certain adverse reactions in the elderly population, but their incidence is quite low. It needs to be made abundantly clear to elderly people that the advantages of vaccination, which is the best way to control COVID-19 by preventing severe illness and reducing related deaths, far outweigh any potential risks. However, more randomized clinical trials are needed to increase the certainty of the evidence and to draw more reliable conclusions.

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.

Author contributions

KX and ZW performed the literature search and data extraction, and drafted the manuscript. MQ and YG were responsible for the quality assessment. NL, WX, and YZ conducted the statistical analysis. JW performed the literature search and data extraction. XM was responsible for the design and conceived the original

idea. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1113156/full#supplementary-material

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Immunogenicity against wild-type and Omicron SARS-CoV-2 after a third dose of inactivated COVID-19 vaccine in healthy adolescents

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Introduction: Two doses of inactivated SARS-CoV-2 vaccine CoronaVac cannot elicit high efficacy against symptomatic COVID-19, especially against the Omicron variant, but that can be improved by a third dose in adults. The use of a third dose of CoronaVac in adolescents may be supported by immunobridging studies in the absence of efficacy data.

Methods: With an immunobridging design, our study (NCT04800133) tested the non-inferiority of the binding and neutralizing antibodies and T cell responses induced by a third dose of CoronaVac in healthy adolescents (N=94, median age 14.2 years, 56% male) compared to adults (N=153, median age 48.1 years, 44% male). Responses against wild-type (WT) and BA.1 SARS-CoV-2 were compared in adolescents. Safety and reactogenicity were also monitored.

Results: A homologous third dose of CoronaVac further enhanced antibody response in adolescents compared to just 2 doses. Adolescents mounted non-inferior antibody and T cell responses compared to adults. Although S IgG and neutralizing antibody responses to BA.1 were lower than to WT, they remained

detectable in 96% and 86% of adolescents. T cell responses to peptide pools spanning only the mutations of BA.1 S, N and M in adolescents were preserved, increased, and halved compared to WT respectively. No safety concerns were identified.

Discussion: The primary vaccination series of inactivated SARS-CoV-2 vaccines for adolescents should include 3 doses for improved humoral immunogenicity.

KEYWORDS

COVID-19, vaccine, CoronaVac, Omicron, adolescent

Highlights

1. A third dose of CoronaVac is needed for improved immunogenicity in healthy adolescents
2. Non-inferiority of antibody and T cell responses in adolescents versus adults
3. BA.1 S IgG and neutralizing antibodies were detectable in 96% and 86% adolescents after dose 3
4. T cell responses against BA.1 mutations in S, N and M were preserved, increased, and halved, respectively

Introduction

Inactivated vaccines against COVID-19 such as CoronaVac are widely used with more than 4 billion doses distributed worldwide because of simpler manufacturing requirements and greater vaccine stability during transport (1). Real-world vaccine effectiveness studies have also shown that 2 doses of inactivated vaccines could strongly protect against severe COVID-19 but less so against mild disease (2, 3). In comparison to the mRNA COVID-19 vaccines widely in use, 2 doses of inactivated COVID-19 vaccines elicit weaker neutralization responses yet higher T cell responses in adults (4), as well as in adolescents as we have shown (5). There is a growing consensus that the primary series of inactivated COVID-19 vaccines should include 3 doses, similar to other routinely used inactivated vaccines such as the inactivated polio vaccine. Homologous third dose of CoronaVac has been shown to improve vaccine effectiveness against mild and severe COVID-19 in adults (3, 6). However, as of September 2022, there is currently no published data on the paediatric use of 3 doses of CoronaVac.

Vaccine effectiveness, especially against mild disease, is susceptible to waning over time as well as to antigenically divergent variants of concern (3, 7). Neutralizing antibody escape by the newly emergent Omicron variant may account for high transmission in populations with high vaccine coverage (8, 9). On the other hand, T cell responses in adults are mostly (~80%) preserved against the Omicron variant as most immunodominant T cell epitopes are unaffected (10–12), which may explain the preservation of vaccine effectiveness against severe outcomes with Omicron variant (3). Data from the United Kingdom showed that in contrast to adults, adolescents are not at significantly lower risk of hospitalization due to Omicron relative to Delta (13), and paediatric COVID-19-associated hospitalizations increased rapidly during the Omicron outbreak in South Africa (14). In adults, a third dose of COVID-19 vaccine boosted neutralizing antibody and T cell response against the Omicron variant (11, 15), yet this remains unknown in adolescents.

To inform the paediatric use of CoronaVac, an inactivated COVID-19 vaccine, amid the spread of Omicron, we sought to determine the safety and immunogenicity of a third dose of CoronaVac in healthy adolescents. We adopted an immunobridging design, where adolescents were tested for whether various immunogenicity outcomes, including antibody binding and avidity, neutralizing and non-neutralizing antibody functions, and T cell responses against the wild-type (WT) virus (5), were non-inferior to those in adults. The goal is to support the extension of age group indication for the third dose of CoronaVac in the absence of efficacy data in adolescents, based on the established effectiveness of a homologous third dose of CoronaVac in adults (3, 6). In addition, immunogenicity against Omicron BA.1 was also assessed.

Methods

Study design

COVID-19 Vaccination in Adolescents and Children (COVAC; NCT04800133) is a non-randomized immunobridging study of BNT162b2 and CoronaVac in adolescents and children, as previously described (5, 16). The University of Hong Kong (HKU)/Hong Kong West Cluster Hospital Authority Institutional Review Board (UW21-157) authorized this study. ClinicalTrials.gov

Participants

The current analysis included adolescents aged 11-17 years and adults ≥18 years at the time of dose 1 who received 3 intramuscular

doses of CoronaVac. The exclusion were history of COVID-19, severe allergy, major neuropsychiatric issues, immune compromise conditions, blood transfusion within 60 days, significant bleeding tendency, and pregnancy or breastfeeding.

Procedures

Participants were recruited in Hong Kong from schools, media, or referral. Written informed consent was obtained from participants aged ≥18 years or above. Informed assent was obtained from underage participants and written consent was obtained from their parents or legally acceptable representatives. Vaccination consisted of three doses of 0.5 mL inactivated virus vaccine that contains 600SU of SARS-CoV-2 CZ02 strain whole virus antigen. Doses 1 and 2 were administered 28-35 days apart, while dose 3 was given ≥84 days after dose 1. The vaccination interval was chosen after the finding of limited durability of the 2-dose antibody response during an evolving pandemic and likely benefits of more persistent prime-boost interval (17). Blood was sampled on the day of dose 3 and 13-42 days following dose 3 to detect the expected peak antibody response and short-term cellular response after dose 3 (18).

Safety data collection

Participants were observed for 15 minutes after each vaccine injection. Prespecified adverse reactions (ARs) were recorded in an online or paper-based diary for 7 days after vaccine administration. Unsolicited adverse events were captured for 28 days after each vaccine dose. There will be ongoing surveillance for severe adverse events include hospitalizations, life-threatening complications, disabilities, deaths, birth defects in offspring, and breakthrough COVID-19 for 3 years. The study investigators determined whether there was causal relationship of the study vaccine with the reported adverse effects.

S-RBD IgG, N IgG and N-CTD IgG, surrogate virus neutralization test (sVNT) and plaque reduction neutralization test (PRNT)

Clotted blood and the serum from the participants was maintained at -80° C. Sera were inactivated at 56° C for 30 minutes before performance of the SARS-CoV-2 S receptor-binding domain (S-RBD) IgG, N and N-CTD IgG enzyme-linked immunosorbent assay (ELISA), sVNT (GenScript Inc, Piscataway, USA) and PRNT as previously described and validated according to the manufacturer's instructions (19–21). The cut-offs for ELISA-based tests were derived from mean of OD + 3SD of pre-pandemic samples. For sVNT, the cut-off was provided by the manufacturer. The cut-off for the PRNT was set at 1:10, which was the lowest dilution demonstrating inhibition to the virus.

In summary, S-RBD IgG ELISA plates were coated with 100 ng/well of purified recombinant S-RBD in PBS buffer overnight and 100 µL Chonblock Blocking/Sample Dilution (CBSD) ELISA buffer (Chondrex Inc, Redmond, USA) was added. This mixture remained at room temperature (RT) for 2 hours. Sera at 1:100 dilution in CBSD ELISA buffer were added to the wells at 37 for 2 hours. The wells were washed with PBS containing 0.1% Tween 20, followed by the addition of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (1:5,000) (GE Healthcare, Chicago, USA) for 1 hour at 37°C. These were washed with PBS containing 0.1% Tween 20 for five times, and then 100 µL HRP substrate (Ncm TMB One, New Cell & Molecular Biotech Co. Ltd, China) was added and kept for 15 minutes. This reaction was ceased with 50 µL 2 M H_2SO4. The OD of the mixture was analyzed in a Sunrise absorbance microplate reader (Tecan, Männedorf, Switzerland) at 450 nm wavelength. The background OD in the PBS-coated control wells with the sera was subtracted from each final OD reading. OD450 values below the cut-off of 0.5 were imputed as 0.25.

For N IgG and N-CTD IgG, the 96-well ELISA plates (Nunc MaxiSorp, Thermo Fisher Scientific) were coated with 125 ng (N) or 40.3 ng (N-CTD) purified recombinant protein in PBS buffer overnight. 100 µL Chonblock blocking/sample dilution ELISA buffer (Chondrex Inc, Redmon, US) was added to the plates, which were incubated for 1 hour at room temperature. Afterwards, the sera were diluted to 1:100 in Chonblock blocking/sample dilution ELISA buffer. Sera were added to the ELISA plates, which were incubated at 37 for 2 hours. Each well was washed with PBS containing 0.1% Tween 20 and incubated at 37 for 1 hour with anti-human IgG secondary antibody (1:2500, Thermo Fisher Scientific). The plates were washed five times with PBS containing 0.1% Tween 20, and 100 µL of HRP substrate (Ncm TMB One; New Cell and Molecular Biotech Co. Ltd, Suzhou, China) was added into each well. After a 15-minute incubation period, the reaction was stopped with 50 µL of 2M H_2SO_4 solution. OD450 was analyzed using an absorbance microplate reader.

10 µL of each sera was used for sVNT, with positive and negative controls prepared by dilution of 1:10 mixed with same volume of HRP-conjugated WT SARS-CoV-2 S-RBD (6 ng). The mixtures were incubated at 37 for 30 minutes, followed by the addition of 100 µL of sample to the microtitre plate wells coated with the recombinant angiotensin-converting enzyme-2 (ACE-2) receptor. The plates were sealed for 15 minutes at 37 and then washed with wash-solution and tapped dry. 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) was then added, followed by incubation for 15 minutes at RT in the dark. 50 µL of Stop Solution was added. The absorbance was recorded at 450 nm. The % inhibition was calculated using the formula: (1-sample OD value/negative control OD value) x100%. Inhibition % below 30%, which was the limit of quantification (LOQ), was imputed as 15%.

PRNT duplicates were performed in a biosafety level 3 facility. Serial serum dilutions at 1:10 to 1:320 were incubated with ~30 plaque-forming units of SARS-CoV-2 BetaCoV/Hong Kong/VM20001061/2020 virus (WT) or hCoV-19/Hong Kong/VM21044713_WHP5047-S5/2021 (Omicron BA.1) for 1 hour at 37 in culture plates (Techno Plastic Products AG, Trasadingen, Switzerland) (8). We added the virus-sera mixtures onto Vero-E6 TMPRSS2 cell monolayers, which were then placed in a 5% CO2 incubator for 1 hour at 37°C. After overlaying with 1% agarose in cell culture medium, these plates were incubated for 3 days while fixed and stained. The antibody titres were defined as the reciprocal of the highest dilution of serum resulting in a >=90% (PRNT90) or

>50% (PRNT50) reduction in the plaque numbers. Values above 1:320 were imputed as 1:640 and those below 10 were imputed as 5.

S IgG, avidity and FcγRIIIa-binding

We diluted the antigens for antibody detection in PBS and coated the plates (Nunc MaxiSorp, Thermofisher Scientific) with 250 ng/mL WT (AcroBiosystems) or Omicron BA.1 (AcroBiosystems) SARS-CoV-2 S protein for IgG and IgG avidity assessment and 500 ng/mL ancestral (Sinobiological) or Omicron BA.1 (AcroBiosystems) S for FcγRIIIa-binding detection. ORF8 protein of 300 ng/mL was coated at 37 for 2 hours. The plates were blocked with 1% FBS in PBS for 1 hour, followed by incubation with 1:100 HI sera diluted in 0.05% Tween-20/0.1% FBS in PBS for 2 hours for IgG detection, and 1:50 for 1 hour at 37 for FcγRIIIa-binding detection, prior to rinsing. For avidity, plates with 8M were washed with urea 3 times. IgG was measured after 2 hours of incubation period with anti-IgG-HRP (1:5000; G18-145, BD), HRP revealed with addition of stabilized hydrogen peroxide and tetramethylbenzidine (R&D systems) for 20 minutes. The reaction was terminated with 2N H2SO4, which was then analyzed at 450 nm wavelength with an absorbance microplate reader (Tecan Life Sciences). Similarly, FcγRIIIa-binding antibodies were assessed after incubation with biotinylated FcγRIIIa-V158 at 100 ng/mL for 1 hour at 37 after streptavidin-HRP (1:10000, Pierce).

T cell responses

Peripheral blood mononuclear cells (PBMCs) were extracted and maintained at -80° C. Thawed PBMCs were placed in 10% human AB serum supplemented RPMI medium for 2 hours. The PBMCs were stimulated with sterile ddH_2O or 1 μg/mL overlapping peptide pools representing the WT SARS-CoV-2 S, N and M proteins (Miltenyi Biotec, Bergisch Gladbach, Germany), or BA.1 S mutation pool and WT S reference pool (Miltenyi Biotec, Bergisch Gladbach, Germany), Omicron BA.1 N mutation pool, WT N reference pool, BA.1 M mutation pool and WT M reference pool (peptide sequences in Supplementary Table 6; synthesized by ChinaPeptides Co., Ltd) in 1 μg/mL anti-CD28 and anti-CD49d costimulatory antibodies (clones CD28.2 and 9F10, Biolegend, San Diego, USA) for 16 hours, followed by the addition of 10 μg/mL brefeldin A (Sigma, Kawasaki, Japan) (22). The PBMCs were then washed and stained for CD3 (HIT3a, 1:60), CD4 (OKT4, 1:60), CD8 (HIT8a, 1:60), IFN-γ (B27, 1:15), IL-2 (MQ1-17H12, 1:15) (Biolegend, San Diego, USA) and fixable viability dye (eBioscience, Santa Clara, USA, 1:60). Flow cytometry was performed by the LSR II (BD Biosciences, Franklin Lakes, USA). Flowjo v10 software (BD, Ashland, USA) was used to analyze the data. Calculation of measured IFN-γ^+ or IL-2^+ T cells were performed by deducting the background (sterile ddH2O) data, which are presented as the percentages of $CD4^+$ or $CD8^+$ T cells (23). T cell responses against the peptide pool was considered positive if the cytokine-expressing cell frequency was ≥0.005% and the stimulation index was >2. Negative values were imputed as 0.0025%. The total T cell responses against S, N and M peptide pools were summed and the cut-off of 0.01% was used.

Outcomes

For the current analysis, the primary immunogenicity outcomes were S-specific antibody markers, which included the S IgG and S-RBD IgG levels, sVNT %inhibition, 90% and 50% PRNT titres, S IgG avidity and FcγRIIIa-binding, and the total and separate S, N and M-specific IFN-γ^+ and IL-2^+ $CD4^+$ and $CD8^+$ T cell responses measured by flow cytometry 13-42 days after the third dose of CoronaVac. The primary reactogenicity outcomes were ARs and anti-pyretic use within 7 days after vaccine injection.

The secondary immunogenicity outcomes were N and N-CTD IgG levels, and antibody and T cell responses against Omicron BA.1. For safety, the secondary outcomes were AEs within 28 days post-vaccination and SAEs during the study period.

Statistical analyses

Sample size and power estimation

G*Power (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) and Sampsize (sampsize.sourceforge.net) were used for the power calcuation. For primary immunogenicity objectives, when comparing the peak geometric mean (GM) immunogenicity outcomes between adolescents and adults, 61 participants in each group would allow two-sided tests with α=0.05 and 99% power to detect a difference of 0.51 after natural logarithm transformation and the standard deviation (SD) of 0.65 within group on the natural logarithmic scale, with the Cohen's d value=0.78. Sample sizes were reduced when the feasibility for assays with higher technical requirements were limited, such as PRNT and assays which required greater volumes of blood, such as Omicron-specific tests, as participants with samples tested in earlier timepoints and earlier collection dates or higher blood volume collected chosen. For the proportion of participants with a positive result in immunogenicity outcomes or ARs, with the assumption of a prevalence of 80%, 62 participants would yield a 95% chance to detect the true value within 10% precision.

Analysis sets

The primary immunogenicity analysis was performed in healthy participants in the evaluable analysis population. This consisted of participants who were uninfected before and during the study period, which was based on clinical history, baseline S-RBD IgG negativity, and ORF8 IgG negativity, generally healthy status with no major protocol deviations, receipt of dose 3 ≥84 days after dose 1, had blood sampled days 13-42 post-dose 3, and had valid results for the relevant test (Protocol in Supplementary Materials). The expanded analysis population were more relaxed, which permitted inclusion of those who received dose 3 ≥56 days after dose 1 and had blood sampled days 6-56 post-dose 3 (Protocol in Supplementary Materials). Geometric mean ratios (GMRs) included two-sided 95% CI, corresponding to a one-sided 97.5% CI, which was used for testing non-inferiority at the 0.60 margin. This threshold promotes rapid delivery of study results that requires

a smaller sample size amid the evolving pandemic, a practice deemed allowable by the World Health Organization Expert Committee on Biological Standardization and adopted in another recent landmark COVID-19 vaccine study (24, 25). The inferiority analyses were confirmed in the expanded analysis population. Superiority was reached if the lower bound of the 95% CI for GMR was >1, or inferiority was declared if the upper bound of the 95% CI was <1. When both non-inferiority and inferiority were not met, the results were considered as inconclusive. Geometric mean fold rises (GMFR) were calculated for those who had valid results at both timepoints. When there were negative immunogenicity outcome data, values that were half the cut-off were imputed. Unpaired t test after natural logarithmic transformation was performed for comparisons of immunogenicity outcomes between groups. Proportions of positive or negative results were given in percentages with 95% Clopper-Pearson CI. The Fisher exact test was used for comparisons of proportions between groups.

Reactogenicity and safety outcomes were assessed in healthy, uninfected participants who had reported any safety or ARs post-dose 3 and before the study database was locked for this interim analysis in the adolescent group (the healthy safety population). In this primary reactogenicity analysis, the proportions of participants that had reported each of the ARs according to maximum severity and anti-pyretic use were shown as percentages with the 95% Clopper-Pearson CI. The incidences of AEs by severity and SAEs that were reported by the post-dose 3 study visit (28 days after dose 3) were presented as counts and events-per-participant.

Vaccine efficacy estimation

Vaccine efficacies (VEs) were estimated as a secondary objective by extrapolation according to the neutralizing titres, as previously established (5, 26). The mean neutralizing level (fold of convalescent) was based on the GMTs of $PRNT_{50}$ for SARS-CoV-2 WT or BA.1 in evaluable adolescents divided by that of 102 convalescent sera from patients aged ≥18 years on days 28-59 after the onset of illness (21, 27). The point estimates of VE were extracted from the best fit of the logistic model using the plot digitizer tool (https://automeris.io/WebPlotDigitizer/, version 4.5).

Results

Enrolment and study completion

Among 327 participants in the COVID-19 Vaccination in Adolescents and Children study (COVAC; NCT04800133) who received 2 doses of CoronaVac, 259 participants received a third dose of CoronaVac by January 31, 2022 (Supplementary Figure 1). Excluding participants who were infected during the study as determined by ORF8 serology assay or contributed no safety data and did not attend follow-up clinic, 94 adolescents aged 11-17 years and 153 adults aged 18 years or above were included in healthy safety analysis, with comparable demographic characteristics (Supplementary Table 1). Doses 1 and 2 were given 28-35 days apart while dose 3 was given at least 84 days after dose 1. Blood sampling was performed on the day of dose 3 and 13-42 days after dose 3. Primary immunogenicity analyses were performed in the evaluable analysis population which included participants with valid and timely immunogenicity results and no protocol deviations (adolescents N=60, adults N=119). Immunogenicity analyses were repeated in the expanded analysis population with relaxed vaccination and blood sampling intervals to further confirm the findings (adolescents N=82, adults N=149; see Methods). Protocol and Statistical Analysis Plan are available in Supplementary Materials.

Immunogenicity outcomes before and after the third dose in adolescents

We first assessed the durability of antibody responses against the WT virus after 2 doses of CoronaVac, including SARS-CoV-2 Spike receptor-binding domain (S-RBD) IgG by enzyme-linked immunosorbent assay (ELISA) and ACE2-blocking antibody by surrogate virus neutralization test (sVNT), as well as interferon-γ $(IFN\text{-}\gamma)^+$ and interleukin-2 $(IL\text{-}2)^+$ $CD4^+$ and $CD8^+$ T cells responses specific to WT SARS-CoV-2 S, Nucleocapsid (N), and Membrane (M) peptide pools by flow cytometry (see Methods). In evaluable adolescents with paired sera across all timepoints, S-RBD IgG and ACE2-blocking antibody declined significantly with geometric mean (GM) fold reduction of 1.60 and 2.12 fold respectively from post-dose 2 (mean 28 days after dose 2) to pre-dose 3 (Figure 1A). Total SNM-specific $IFN\text{-}\gamma^+$ and $IL\text{-}2^+$ $CD4^+$ and $IL\text{-}2^+$ $CD8^+$ T cells showed a reducing trend after 2 doses in evaluable adolescents, yet none of the paired analyses between the post-dose 2 and pre-dose 3 timepoints were significant, suggesting T cell responses were preserved (Figure 1B). Results for T cell responses to separate S, N and M peptide pools were presented in Supplementary Figure 2A-C.

At the post-dose 3 timepoint (mean 19 days after dose 3), evaluable adolescents were assessed for all primary humoral and cellular immunogenicity outcomes against the WT virus (see Methods). All adolescents had positive S-RBD IgG and S-RBD ACE2-blocking antibody post-dose 3 (Table 1). On plaque reduction neutralization test (PRNT), 100% and 78.3% adolescents were positive for 50% and 90% PRNT at a limit of detection of 1 in 10, and with GM 50% and 90% PRNT of 55.3 and 17.8 respectively. As CoronaVac is a whole-virion inactivated vaccine, N IgG and N-C terminal domain (N-CTD) IgG were also assessed with 98.3% seropositivity for both. SARS-CoV-2 S IgG, S IgG avidity and S IgG Fcγ receptor IIIa (FcγRIIIa)-binding testing were available in 56 evaluable adolescents, with S IgG and S IgG FcγRIIIa-binding detected in 98.2% tested evaluable

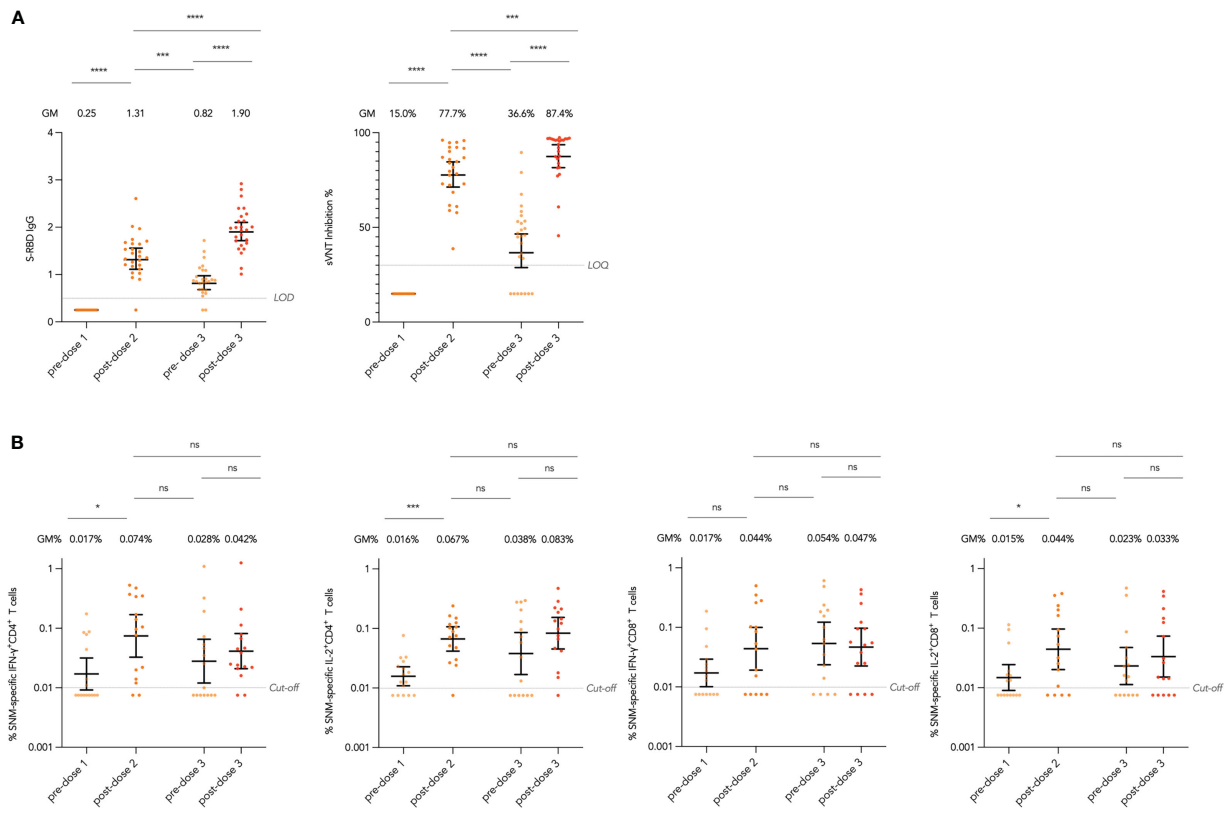

FIGURE 1
Longitudinal humoral and cellular immunogenicity in healthy evaluable adolescents receiving 3 doses of CoronaVac. **(A)** Longitudinal analysis of Spike receptor-binding domain (S-RBD) IgG OD450 values and surrogate virus neutralisation test (sVNT) inhibition % in evaluable adolescents. **(B)** Longitudinal analysis of total Spike (S), Nucleocapsid (N) and Membrane (M) protein-specific interferon-γ (IFN-γ)+ and interleukin-2 (IL-2)+ CD4+ and CD8+ T cells responses in evaluable adolescents. Geometric means (GM) are shown with centre lines and stated above each column, with corresponding 95% confidence intervals shown by error bars. Samples from the same participant were paired across timepoints and compared with paired t test after natural logarithmic transformation with p-values denoted (*, P<0.05; ***, P<0.001; ****, P<0.0001; ns, not significant). Limits of detection and quantification (LOD and LOQ) and cut-offs were drawn as grey lines.

adolescents, and GM S IgG avidity of 38.5%. Results in the expanded analysis population were similar (Supplementary Table 2). When compared to pre-dose 3 timepoint, evaluable adolescents showed significant GM fold rises in S-RBD IgG of 2.32 fold and sVNT inhibition of 2.39 fold (Figure 1A).

For cellular immunogenicity outcomes, among 58 evaluable adolescents tested, most participants tested positive for total WT SNM-specific IFN-γ^+ and IL-2^+ $CD4^+$ T cell responses (74.1% and 79.3% respectively) on flow cytometry at a cut-off of 0.01% (Table 2; see Methods). Yet, for IFN-γ^+ and IL-2^+ $CD8^+$ T cell responses, a lower but still high proportion of participants (62.1% and 65.5%) tested positive. When broken down into T cell responses against separate peptide pools, T cell responses appeared to be lowest for the M peptide pool, which elicited IFN-γ^+ and IL-2^+ $CD4^+$ T cells in 23.7% and 25.4% tested evaluable adolescents, and IFN-γ^+ and IL-2^+ $CD8^+$ T cells in 13.6% and 18.6% (Supplementary Table 3). Similar results were yielded in the expanded analysis population (Supplementary Table 4). When compared to pre-dose 3 timepoint, evaluable adolescents showed statistically insignificant increases in total SNM-specific IFN-γ^+ and IL-2^+ $CD4^+$ and IL-2^+ $CD8^+$ T cell responses after dose 3 (Figure 1B).

Non-inferiority hypothesis testing of immunogenicity outcomes between adolescents and adults

To support the use of CoronaVac in adolescents without the availability of efficacy data, we calculated the geometric mean ratios (GMRs) of various immunogenicity outcomes as a primary analysis (see Methods). Nine humoral immunogenicity outcomes assessed were all non-inferior in adolescents as the lower bounds of their two-sided 95% confidence intervals (CI) were at least 0.60 (Figure 2A), with 50% PRNT, S IgG avidity, N IgG and N-CTD IgG responses satisfying the criterion for superiority as well. These findings were confirmed by secondary analyses in the expanded analysis population (Supplementary Figure 3). On the other hand, total SNM-specific IL-2^+ $CD4^+$ and IFN-γ^+ and IL-2^+ $CD8^+$ T cells were non-inferior (Figure 2B), while it was inconclusive for total SNM-specific IFN-γ^+ $CD4^+$ T cells. When we considered separate S, N and M peptide pools-specific T cell responses, M-specific IL-2^+ $CD4^+$ T cell responses were inferior in evaluable adolescents (Supplementary Figure 4). Cellular immunogenicity outcomes were also confirmed in the expanded analysis population, yet total

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TABLE 1 Humoral immunogenicity outcomes against wild-type SARS-CoV-2 after the third dose of CoronaVac in evaluable analysis population.

	Adolescents 3 doses	Adults 3 doses
S IgG on ELISA		
N	56	49
GM OD450 value (95% CI)	0.93 (0.83-1.04)	0.91 (0.83-1.00)
% positive (>/=LOD at 0.3)	98.2%, *P*>0.9999	100%
S-RBD IgG on ELISA		
N	60	119
GM OD450 value (95% CI)	1.77 (1.68-1.87)	1.62 (1.53-1.73)
% positive (>/=LOD at 0.5)	100%, *P*>0.9999	99.2%
S-RBD ACE2-blocking antibody on sVNT		
N	60	119
GM % inhibition (95% CI)	84.9% (81.3-88.6%)	76.9% (71.8-82.3%)
% positive (>/=LOQ at 30%)	100%, *P*=0.30	96.6%
Neutralizing antibody on PRNT		
N	60	22
GM $PRNT_{90}$ (95% CI)	17.8 (13.7-23.3)	12.1 (8.68-16.8)
% positive (>/=LOD at 10)	78.3%, *P=0.57*	72.7%
GM $PRNT_{50}$ (95% CI)	55.3 (43.2-70.7)	32.1 (21.1-48.7)
% positive (>/=LOD at 10)	100%, *P=0.27*	95.5%
S IgG avidity on ELISA		
N	55	49
GM avidity index (95% CI)	38.5% (34.6-42.8)	32.1% (29.4-35.0)
S IgG FcγRIIIa-binding on ELISA		
N	56	49
GM OD450 value (95% CI)	1.41 (1.21-1.63)	1.47 (1.23-1.77)
% positive (>/=LOD at 0.28)	98.2%, *P*=0.60	95.9%
N IgG on ELISA		
N	60	22
GM OD450 value (95% CI)	2.82 (2.62-3.04)	2.08 (1.72-2.52)
% positive (>/=LOD at 0.88)	98.3%, *P*=0.47	95.5%
N-CTD IgG on ELISA		
N	60	22
GM OD450 value (95% CI)	2.97 (2.77-3.17)	1.96 (1.61-2.38)
% positive (>/=LOD at 1.34)	98.3%, *P*=0.17	90.9%

S, spike protein; ELISA, enzyme-linked immunosorbent assay; GM, geometric mean; OD, optical density; LOD, limit of detection; LOQ, limit of quantification; CI, confidence interval; RBD, receptor-binding domain; ACE-2, angiotensin-converting enzyme-2; sVNT, surrogate virus neutralization test; PRNT, plaque reduction neutralization test; $PRNT_{90}$, 90% plaque reduction neutralization titre; $PRNT_{50}$, 50% plaque reduction neutralization titre; FcγRIIIa, Fc gamma receptor III-a; N, nucleocapsid protein; CTD, C-terminal domain. P-values compare the proportion of positive responses between adolescents and adults by Fisher's exact test.

TABLE 2 Cellular immunogenicity outcomes against wild-type SARS-CoV-2 after the third dose of CoronaVac in evaluable analysis population.

	Adolescents 3 doses	Adults 3 doses
T cell responses		
Total SNM-specific T cell responses on flow cytometry		
N	58	118
GM % IFN-γ$^+$CD4$^+$ T cells (95% CI)	0.066% (0.041-0.106%)	0.063% (0.045-0.089%)
% positive (>/=cut-off at 0.01%)	74.1%, *P*=0.17	62.7%
GM % IL-2$^+$CD4$^+$ T cells (95% CI)	0.073% (0.049-0.109%)	0.070% (0.052-0.093%)
% positive (>/=cut-off at 0.01%)	79.3%, *P*=0.46	72.9%
GM % IFN-γ$^+$CD8$^+$ T cells (95% CI)	0.071% (0.040-0.125%)	0.051% (0.035-0.075%)
% positive (>/=cut-off at 0.01%)	62.1%, *P*=0.20	50.9%
GM % IL-2$^+$CD8$^+$ T cells (95% CI)	0.041% (0.027-0.063%)	0.034% (0.026-0.044%)
% positive (>/=cut-off at 0.01%)	65.5%, *P*=0.74	61.9%

S, Spike; N, Nucleocapsid; M, Membrane; GM, geometric mean; CI, confidence interval; IFN-γ, interferon-gamma; IL-2, interleukin-2. P-values compare the proportion of positive responses between adolescents and adults by Fisher's exact test.

WT SNM-specific IFN-γ$^+$ CD4$^+$ T cells also tested non-inferior (Supplementray Figure 5).

Humoral and cellular immunogenicity against Omicron in adolescents

As vaccine efficacy (VE) against SARS-CoV-2 infection may be susceptible to immune escape by novel variants, we included immunogenicity against variants of concern as a secondary objective. At the time of analysis, Omicron has emerged as the dominant variant worldwide and has amino acid substitutions predominantly in the S protein, although also some across the rest of the proteome. We investigated whether Omicron BA.1 could escape S IgG, neutralizing antibodies and T cells elicited by CoronaVac. For Omicron-specific binding antibody responses, we interrogated Omicron BA.1 S IgG binding, avidity, and FcγRIIIa-binding in subsets of adolescents and adults and compared these to the WT assay. As expected, S IgG was significantly reduced in BA.1 compared to WT in adolescents and adults (Figure 3A). S IgG avidity was reduced against BA.1 in adolescents as well, yet interestingly, S IgG FcγRIIIa-binding was not significantly reduced. In terms of neutralizing antibodies, GM 50% PRNT was reduced by 5.19 fold against BA.1, but neutralizing antibodies remained detectable in 86.2%.

To assess whether Omicron BA.1 mutations could lead to escape from T cell responses, we focused on BA.1-associated mutations and utilized S, N and M mutation pools which only contained peptides covering BA.1-associated mutations (37, 3 and 3 mutations in S, N and M respectively), and compared their T cell responses against those from WT reference peptide pools containing only the homologous WT peptides (Methods). As expected, no differences between WT and BA.1-S-specific T cells were found in both adolescents and adults (Figure 3B). Interestingly, BA.1-associated mutations in N increased IFN-γ$^+$ and IL-2$^+$ CD4$^+$ and CD8$^+$ T cell responses, differences which were significant in adolescents (Figure 3C). Meanwhile, T cell responses against BA.1 M mutation pool were reduced in comparison to WT reference pool, with the difference significant only for IL-2$^+$ CD8$^+$ T cells in adolescents, which had a 2.58-fold reduction (Figure 3D).

Reactogenicity and safety of the third dose of CoronaVac in adolescents

Among 94 adolescents in the healthy safety population, very common adverse reactions (ARs) included pain at the injection site (35.1% grade 1 and 8.5% grade 2) and fatigue (22.3% grade 1 and 4.3% grade 2) (Figure 4). Almost all ARs reported were of grades 1 and 2 severity; one grade 3 AR (diarrhoea) was reported. Only a single grade 1 adverse event (peripheral swelling) was reported within 28 days after vaccination in adolescents (Supplementary Table 5), and it was not considered to have been likely caused by vaccination. There were no serious adverse events reported in the follow-up period.

Estimation of VE based on neutralization titres against WT and BA.1 SARS-CoV-2 in adolescents

We extrapolated VE estimates against symptomatic COVID-19 from WT and BA.1 $PRNT_{50}$ results in evaluable adolescents as established by Khoury et al. (Methods) (21, 26, 27). The PRNT

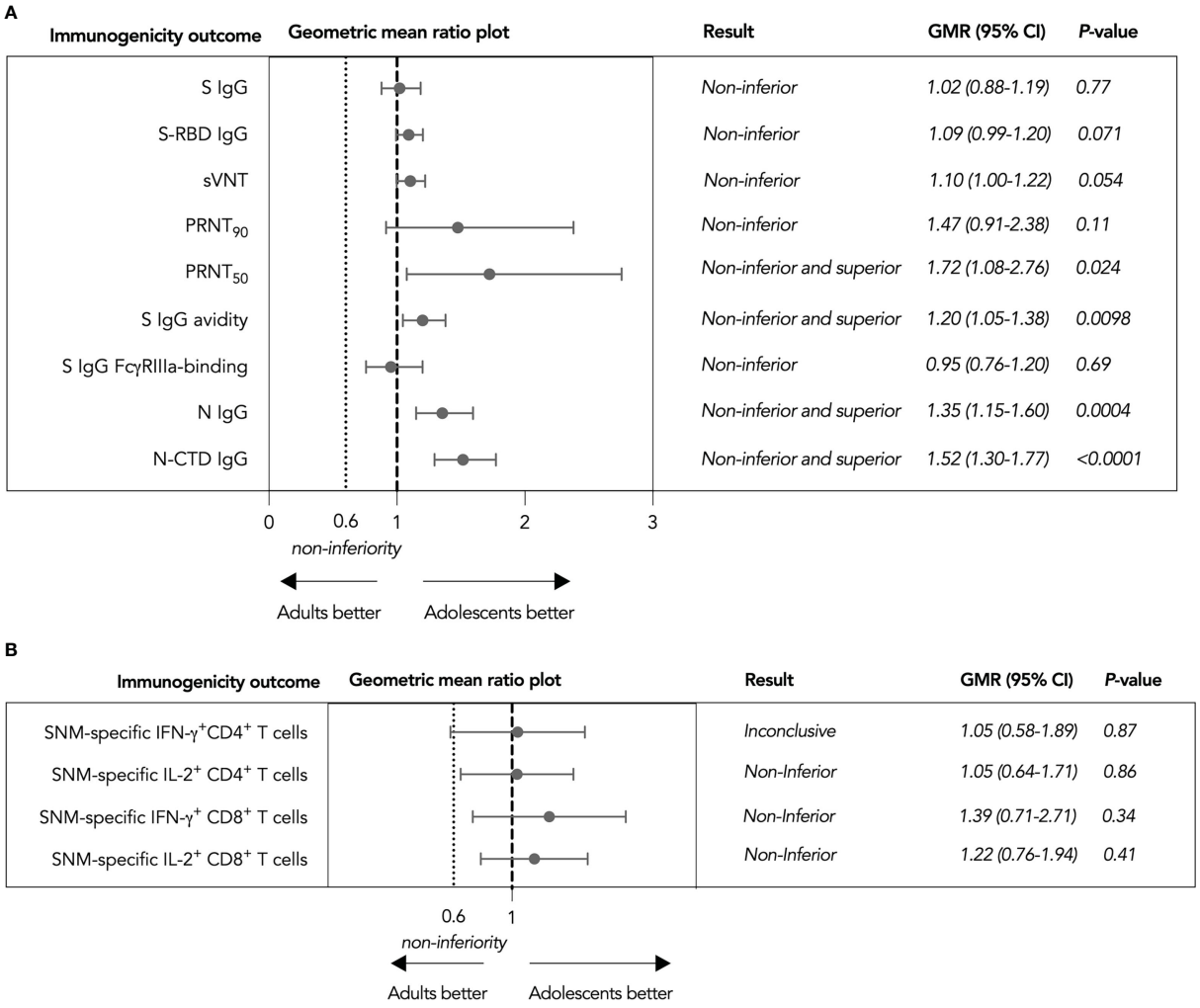

FIGURE 2
Non-inferiority hypothesis testing of humoral and cellular immunogenicity outcomes against wild-type SARS-CoV-2 after the third dose of CoronaVac in evaluable analysis population. **(A)** Non-inferiority testing of SARS-CoV-2 Spike (S) IgG, S-receptor binding domain (S-RBD) IgG, surrogate virus neutralization test (sVNT), plaque reduction neutralization test (PRNT), S IgG avidity, S IgG Fcγ receptor IIIa (FcγRIIIa)-binding, Nucleocapsid (N) IgG, and N-C-terminal domain (N-CTD) IgG **(B** Non-inferiority testing of total S, N and Membrane (M) protein-specific interferon-γ (IFN-γ)+ and interleukin-2 (IL-2)+ CD4+ and CD8+ T cells Geometric mean ratios (GMR) and two-tailed 95% confidence intervals (CI) were plotted.

results were normalized to 102 in-house convalescent sera collected on days 28-59 post-onset of illness in patients aged ≥18 years, and yielded mean neutralization levels against WT and BA.1 of 0.40 and 0.11, which extrapolated to 66% and 36% VE, respectively (Figure 5). These estimates will need to be validated in real-world effectiveness studies.

Discussion

This study is the first to assess the reactogenicity and immunogenicity of third dose of CoronaVac in healthy adolescents. We found a third dose of CoronaVac further boosted antibody responses after 2 doses in adolescents. Immunobridging analyses showed non-inferior and superior binding and neutralizing antibody responses as well as T cell responses in adolescents when benchmarked against adults. Mutations associated with Omicron BA.1 attenuated binding and neutralizing antibody responses in adolescents who received a third dose, yet binding and neutralizing antibodies remained detectable in most. Adolescents had divergent responses toward mutation pools of Omicron BA.1 S, N and M proteins. ARs were mild, and there were no safety issues observed.

Our finding in adolescents is comparable to that observed in healthy adults who had a further increase in antibody responses after a third dose of CoronaVac (15, 17, 28). As there is evidence of waning protection against symptomatic disease after 2 doses, a booster after the 2-dose primary series of mRNA and adenoviral vector vaccines has been authorized in many countries. For inactivated vaccines, Hong Kong and Singapore have both opined that 3 doses, rather than 2, should form the primary series due to more rapid waning of antibody responses and failure to seroconvert in a minority of healthy vaccinees (29–31). Yet, there have been no published immunogenicity and safety data to inform the use of a third dose in adolescents to date. Our findings of inadequate and

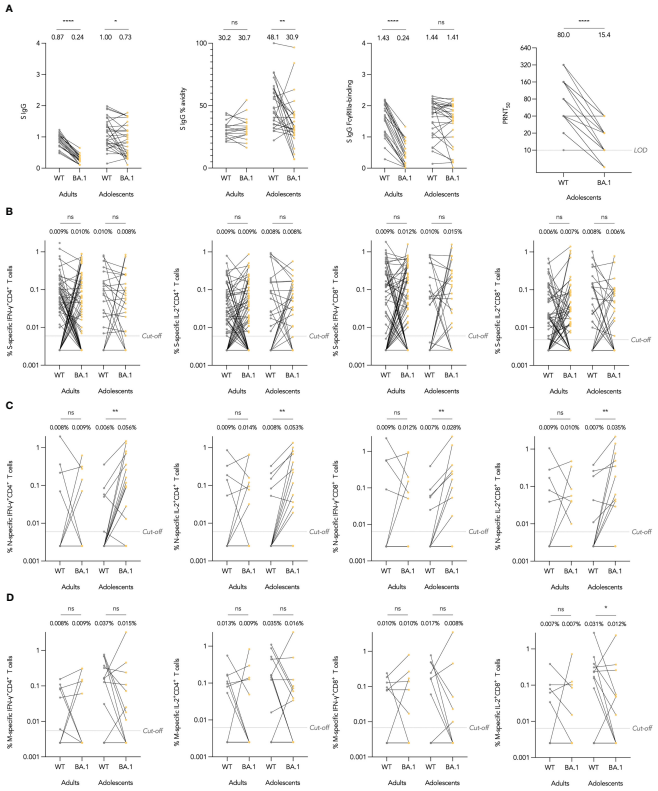

FIGURE 3
Omicron BA.1-specific humoral and cellular immunogenicity after the third dose of CoronaVac in healthy evaluable adolescents and adults. **(A)** Wild-type (WT) and BA.1 SARS-CoV-2 Spike (S) IgG OD450 values, S IgG avidity index, and S IgG Fcγ receptor IIIa (FcγRIIIa)-binding OD450 values, and 50% plaque reduction neutralization titers (PRNT). **(B–D)** Separate S, N and Membrane (M) protein WT reference pool and BA.1 mutation pool-specific interferon-γ (IFN-γ)+ and interleukin-2 (IL-2)+ CD4+ and CD8+ T cell frequencies. Samples from the same participant were paired between WT and BA.1 and compared with paired t test after natural logarithmic transformation with p-values denoted (*, P<0.05; **, P<0.01; ****, P<0.0001; ns, not significant).

rapidly waning antibody responses after 2 doses, and non-inferior antibody responses after a third dose in adolescents compared to adults, support the authorization of a homologous third dose in this age group.

In addition to antibody response, we also surveyed T cell responses, with no waning detected after 2 doses in adolescents, and they were unaltered by a third dose in adolescents. There is limited evidence in literature on waning of T cell responses after 2 doses of CoronaVac in adults. One study in Chile showed an age-dependent preservation of T cell responses with no decline in adults aged 18-59 years and a more significant decline in adults aged 60 years or above (15). In studies of natural infection, half-life of convalescent T cell responses was variably estimated to be 3-7 months (32–35). As our study includes a 3-year follow-up, we will investigate the longevity of T cell responses after a third dose in adolescents. On the other hand, when adolescents were compared against adults after the third dose in our study, T cell responses against SNM in total were non-inferior. Yet, T cell responses against M protein trended lower in adolescents, including IL-2^+ $CD4^+$ T cells which were statistically inferior. Our data hint at differences in targets of T cell reactivity in adolescents versus adults. Previously, our group also found IFN-γ^+ $CD4^+$ and $CD8^+$ T cell responses in children aged 1-13 years infected with SARS-CoV-2 appeared to favour non-structural proteins by flow cytometry, although individual proteins were not studied (36). In another study in the United Kingdom where an IFN-γ ELISpot assay was used, T cell responses in seropositive children aged 3-11 years were stronger to the S peptide pool than the combined NM peptide pool, while the responses appeared to be balanced in seropositive adults (37). These observations are possibly due to differential history of antigenic experience with common cold coronaviruses in different age groups, affecting cross-reactive T cell responses (38).

Omicron emerged in most parts of the world during the second year of COVID-19 vaccine rollout, and many studies in adults have pointed to dramatic escape of neutralizing antibodies (8, 9). Sixty-three percent adult vaccinees who received three doses of CoronaVac had detectable neutralizing antibodies against BA.1 in another study by our group (8). Using the same experimental platform, we found sera from a higher proportion (86%) of adolescents who received three doses of CoronaVac neutralized BA.1, suggesting adolescent vaccinees can make more cross-neutralizing antibodies. The neutralization data are in alignment with superior WT S IgG avidity observed in our study, and may lead to preserved VE against symptomatic disease with Omicron. As for T cells, we detected no difference in S-specific T cell response against WT and BA.1 mutated sequences, in agreement with

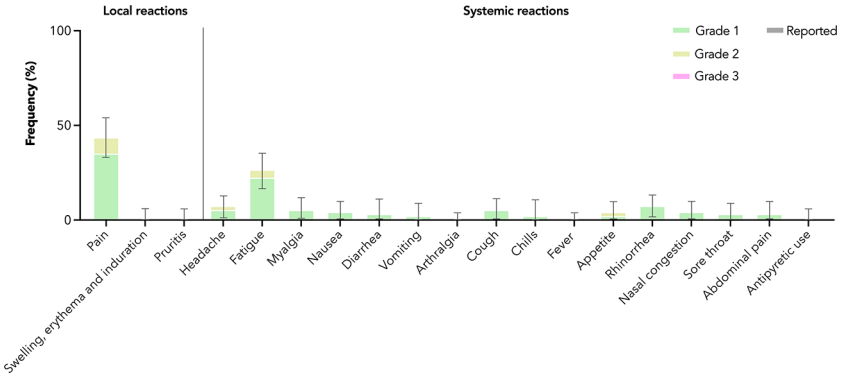

FIGURE 4
Adverse reactions in adolescents after the third dose of CoronaVac. Adverse reactions were reported by maximal severity (grade 1 – green, grade 2 – yellow, grade 3 – pink) within 7 days after vaccination. Antipyretic use was also captured (reported – grey). 95% confidence intervals are derived from the Clopper-Pearson method and marked by error bars.

previous studies (10, 11). Interestingly, we found a significant increase in both $CD4^+$ and $CD8^+$ T cell response against BA.1 mutations in N. It may be because 2 out of 3 mutations in N (31_33delERS, 203_204delRGinsKR) were at the fringes of the immunodominant antigenic regions of WT N protein (39, 40), and the mutations could have enhanced T cell reactivity (41). In contrast, our study revealed $CD4^+$ and $CD8^+$ T cell responses were both approximately halved against BA.1-associated mutations in M. Divergent changes in T cell responses towards BA.1-associated mutations in different SARS-CoV-2 proteins support that T cells exert very limited or absent selection pressure against SARS-CoV-2 (42). It is also noteworthy while our experimental design allowed us to zoom in on BA.1-associated mutations in each of S, N and M proteins. These changes in T cell response towards mutated sequences, albeit dramatic, should be considered in the context of the entire protein antigen, especially for N and M which contain only three small-scale mutations along the entire protein sequence. Overall, we do not expect a reduction of T cell response or any reduction in vaccine effectiveness against severe disease in vaccinees who received CoronaVac with Omicron BA.1. This conclusion is likely applicable towards other Omicron subvariants, which contain mostly point mutations only, supported by effectiveness data from Hong Kong's experience with BA.2 (3, 43).

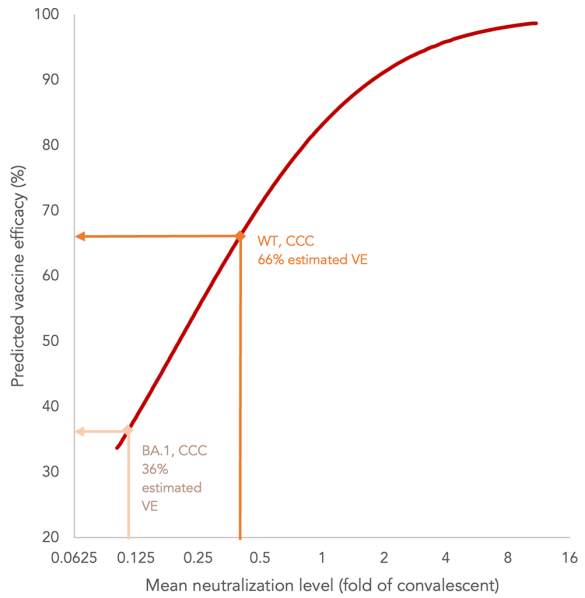

FIGURE 5
Estimation of vaccine efficacy (VE) of three doses of CoronaVac (CCC) based on neutralization titres against wild-type (WT) and BA.1 SARS-CoV-2 in adolescents.

Our study had several strengths and limitations. In addition to neutralizing antibodies, which is a well-established correlate of protection against symptomatic COVID-19 and the basis for other immunobridging studies (26, 44–47), we also studied binding antibodies and T cell responses which also play important roles in protection (48, 49). We were able to track both antibody and T cell responses in healthy adolescent vaccinees from pre-vaccine to post-dose 3, and excluded infection in our participants before or during the study with ORF8 serology at the last timepoint. This was possible also because Hong Kong maintained extremely low levels of local transmission of SARS-CoV-2 during the study period. Non-randomized study design may lead to bias. Sample sizes varied between immunogenicity outcomes as various humoral and cellular assays had different technical and blood volume requirements, and samples were prioritised based on whether the participant had the same test performed at an earlier timepoint, earlier date of sample collection, and sample volume available. We assayed T cell responses by peptide pool-stimulated intracellular IFN-γ and IL-2 cytokine staining, as IFN-γ is an important Th/c1 effector cytokine and $IL\text{-}2^+$ T cell populations are associated with long-term memory (36, 50, 51). We did not study other antiviral cytokines for polyfunctionality, nor memory and exhaustion markers. We estimated a VE against WT and BA.1 based on PRNT, though that will need to be validated in large-scale effectiveness studies. We only included uninfected adolescents aged 11-17 with good past health in the present analysis, so these findings may not be applicable to infected or younger children as well as paediatric patients with comorbidities. We did not investigate

heterologous vaccination or responses against other Omicron subvariants.

In conclusion, our findings support the authorization of a homologous third dose of CoronaVac in healthy adolescents for optimized antibody response. To determine whether a fourth dose of CoronaVac will be needed as a booster in this age group, we will further track the durability of immunogenicity after this third dose and hybrid immunity in this population.

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 The University of Hong Kong Institutional Review Board. Written informed consent to participate in this study was provided by the participants, their parents, or legally acceptable representatives.

Author contributions

YL conceptualized the study. YL, MP, WT, WL, DL, JR, and XW designed the study. YL led the acquisition of funding. YL, WT, and MP supervised the project. SMC, DL, XM, XW, SMSC, IYST, and JHL led the study administrative procedures. WW provided software support. SMC and WW contributed to recruitment of participants. YL and JR provided clinical assessments and follow-up. DL, SMC, JHL, JR, and YL collected safety data. SMSC, SC, KK, KC, JKL, LL, LT, NC, and MP developed and performed S-RBD IgG, N IgG, N-CTD IgG sVNT and neutralization antibody assays. CC and SV developed and performed the S IgG, IgG avidity, S IgG Fcγ receptor IIIa-binding and ORF8 antibody assays. MM provided and developed the specialised ORF8 protein. XW, XM, YZ, MW, WZ, and WT developed and performed the T cell assays. DL and JHL curated and analysed the data. DL, SMSC, YL, and MP performed the vaccine efficacy extrapolation. DL and JHL visualized the data. DL, XM, XW, SMSC, JR, CC, WW, JHL, and SMC validated the data. DL wrote the first draft supervised by YL, with input from JR, XM, XW, SMSC, and CC. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1106837/full#supplementary-material

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Humoral and cellular immunogenicity of homologous and heterologous booster vaccination in Ad26.COV2.S-primed individuals: Comparison by breakthrough infection

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Background: Whether or not a single-dose Ad26.COV2.S prime and boost vaccination induces sufficient immunity is unclear. Concerns about the increased risk of breakthrough infections in the Ad26.COV2.S-primed population have also been raised.

Methods: A prospective cohort study was conducted. Participants included healthy adults who were Ad26.COV2.S primed and scheduled to receive a booster vaccination with BNT162b2, mRNA-1273, or Ad26.COV2.S. The IgG anti-receptor binding domain (RBD) antibody titers, neutralizing antibody (NAb) titers (against wild type [WT] and Omicron [BA.1 and BA.5]), and Spike-specific interferon-γ responses of the participants were estimated at baseline, 3–4 weeks, 3 months, and 6 months after booster vaccination.

Results: A total of 89 participants were recruited (26 boosted with BNT162b2, 57 with mRNA-1273, and 7 with Ad26.COV2.S). The IgG anti-RBD antibody titers of all participants were significantly higher at 6 months post-vaccination than at baseline. The NAb titers against WT at 3 months post-vaccination were 359, 258, and 166 in the participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups, respectively. Compared with those against WT, the NAb titers against BA.1/BA.5 were lower by 23.9/10.9-, 16.6/7.4-, and 13.8/7.2-fold in the participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups, respectively, at 3 months post-vaccination. Notably, the NAb

titers against BA.1 were not boosted after Ad26.COV2.S vaccination. Breakthrough infections occurred in 53.8%, 62.5%, and 42.9% of the participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups, respectively. No significant difference in humoral and cellular immunity was found between individuals with and without SARS-CoV-2 breakthrough infections.

Conclusion: Booster vaccination elicited acceptable humoral and cellular immune responses in Ad26.COV2.S-primed individuals. However, the neutralizing activities against Omicron subvariants were negligible, and breakthrough infection rates were remarkably high at 3 months post-booster vaccination, irrespective of the vaccine type. A booster dose of a vaccine containing the Omicron variant antigen would be required.

KEYWORDS

SARS-CoV-2, COVID-19, vaccines, humoral immunity, cellular immunity, booster, breakthrough infection

1 Introduction

The coronavirus disease 2019 (COVID-19) pandemic has progressed since 2020. The humoral and cellular immunity elicited by vaccines is important to prevent disease transmission and progression (1–3). The spike (S) protein of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the target of most commercial vaccines because of its crucial role in disease transmission (4). Mutations in the S protein, along with immune waning in the vaccinated population, have resulted in the immune evasion of the virus and breakthrough infections in vaccinated populations (5). A single dose of Ad26.COV2.S (Johnson & Johnson–Janssen, adenovirus-vectored vaccine) is immunogenic and has shown acceptable efficacy in clinical trials (6). However, Ad26.COV2.S vaccination is currently considered only when messenger RNA (mRNA) vaccines (BNT162b2 [Pfizer–BioNTech] and mRNA-1273 [Moderna–NIAID]) or protein-conjugated vaccines (Nuvaxovid [Novavax]) are unavailable. In addition, mRNA vaccines are preferred over Ad26.COV2.S for booster shots (7). Therefore, heterologous booster vaccination is generally employed for the Ad26.COV2.S-primed population.

Recent studies on COVID-19 vaccine effectiveness have reported that homologous mRNA booster vaccination is the most effective strategy. However, heterologous booster vaccination, i.e., adenovirus-vectored vaccine priming followed by mRNA vaccine booster administration, also shows acceptable effectiveness even against the Omicron (B.1.1.529 [BA.1]) variant (8).

As of November 2022, approximately 630 million confirmed cases of COVID-19 were recorded worldwide. Many vaccines based on diverse platforms have been developed to mitigate the COVID-19 pandemic. As of September 2022, the cumulative number of administered COVID-19 vaccine doses was approximately 12.6 billion (9). Thus, the "hybrid immunity" or the immunity elicited against COVID-19 by vaccination and natural infection is necessary to evaluate (10). The vaccine immunity might be diverse depending on the vaccine platform, vaccination interval, and the existence of natural infection. Thus, this study aimed to investigate the short-term immunogenicity, longevity, and cross-reactive neutralizing activity of Ad26.COV2.S against Omicron subvariants. We conducted a prospective longitudinal cohort study up to 6 months after homologous and heterologous booster vaccination in Ad26.COV2.S-primed individuals. We also compared the humoral and cellular immune responses between individuals with and without SARS-CoV-2 breakthrough infections during the study period.

2 Methods

2.1 Study design and procedures

This prospective multicenter cohort study was conducted from October 2021 to June 2022 in three tertiary university hospitals (Korea University Guro Hospital, Ajou University Hospital, and Hanllym University Hospital) in South Korea. Eligible participants were healthy adults who had received a primary dose of Ad26.COV2.S at least 5 months prior and were scheduled to receive a booster vaccine. The eligible booster vaccines were BNT162b2 (30 μg), mRNA-1273 (50 μg), and Ad26.COV2.S (5×10^{10} virus particles). As for the Ad26.COV2.S-boosted group, considering the small number of participants, we did not limit the interval for the inclusion criteria between the primary and booster doses. Those who had prior SARS-CoV-2 infection or immunocompromising conditions were excluded from this study. Individuals with a positive anti-nucleocapsid (N) protein antibody at baseline were also excluded. The flowchart of the study is shown in Figure 1. Blood samples were collected at baseline (T0, day of the booster dose), 3–4 weeks post-booster dose (T1), 3 months post-

booster dose (T2), and 6 months post-booster dose (T3). The receptor binding domain (RBD) of SARS-CoV-2-specific immunoglobulin G (IgG) antibody and SARS-CoV-2-specific T-cell responses (against wild type [WT], Alpha [B.1.1.7], Beta [B.1.351], and Gamma [P.1]) were investigated at each time point. The neutralizing activities against WT and Omicron BA.1 were investigated at T0, T1, and T2. The neutralizing activities against Omicron BA.5 were investigated at T2. The neutralizing antibody (NAb) titers were measured in all participants from the BNT162b2- and Ad26.COV2.S-boosted groups and in 26 randomly selected participants from the mRNA-1273-boosted group (matched number to the BNT162b2-boosted group). The humoral and cellular immune responses of the participants from the three groups were compared at each time point. In addition, the peak post-booster immune status and immune responses derived from breakthrough infections were compared between SARS-CoV-2-uninfected and -infected individuals. We regarded the immune status at T1 as the peak post-booster immunity which was elicited by booster vaccination. To compare the peak post-booster immunity of the participants with respect to the breakthrough infections, 'SARS-CoV-2-infected participants' were defined as individuals with SARS-CoV-2 infection occurred between T1 and T2, while "SARS-CoV-2-uninfected participants' were determined as those without breakthrough infection or with SARS-CoV-2 infection identified at T3.

This study was approved by the ethics committees of Korea University Guro Hospital (2021GR0099), Ajou University Hospital (AJIRB-BMR-SMP-21-528), and Hanllym University Hospital (202111026) and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent was obtained from all participants.

2.2 Immunogenicity analysis

The Elecsys® Anti-SARS-CoV-2 S immunoassay (Roche) was performed to measure IgG anti-RBD antibodies using a Cobas 8000 (Roche Diagnostics, Basel, Switzerland) in accordance with the manufacturer's protocol. Anti-N antibodies were measured in each participant using a SARS-CoV-2 IgG assay (Abbott Laboratories, Chicago, IL, USA) to identify SARS-CoV-2 infection.

For the NAbs analysis, a plaque reduction neutralization test was performed using WT SARS-CoV-2 (βCoV/Korea/KCDC03/2020 NCCP No. 43326), Omicron BA.1 subvariant (GRA: B.1.1.529 NCCP No. 43408), and Omicron BA.5 subvariant (GRA: BA.5 NCCP No. 43426). Briefly, a mixture of serum dilution/virus (40 PFU/well) was incubated at 37°C for 2 h, added to the plate seeded with Vero E6 cells, incubated at 37°C for 1 h, and then added with 0.5% agarose (Lonza, Basel, Switzerland). After 2–3 days of incubation, the cells were fixed with 4% paraformaldehyde and stained to visualize plaques. A reduction in plaque count of 50% was then calculated for the median neutralizing titer (ND_{50}) using the Spearman–Karber formula, and $ND_{50} \geq 1:20$ was considered positive.

SARS-CoV-2-specific T-cell responses were evaluated using Covi-FERON FIA (SD Biosensor, Suwon, Korea), a fluorescence immunoassay (FIA) for detecting interferon-γ (IFN-γ) secreted by T cells in response to SARS-CoV-2-specific proteins, in accordance with the manufacturer's instructions. Whole blood was collected in heparinized tubes, which included Nil tubes (negative control), original S protein antigen tubes, variant S protein tubes, N protein tubes, and mitogen tubes (positive control). The original S protein antigen tube included antigens derived from the WT and Alpha variants (lineage B.1.1.7, 20I/501Y. V1) of SARS-CoV-2. The variant S protein tube contained antigens derived from the Beta (lineage B.1.351, 20H/501. V2) and Gamma (lineage P.1, 20 J/501Y. V3) variants of SARS-CoV-2. Blood samples were incubated at 37°C for 16–24 h and then centrifuged for 15 min at a relative centrifugal force of 2200–2300 gravity. After centrifugation, plasma was collected, and the amount of IFN-γ was measured using FIA. The cut-off value of IFN-γ was 0.25 IU/mL.

2.3 Statistical analysis

Humoral and cellular immune responses were compared among the three groups at each time point. The chi-square test or Fisher's exact test was used to compare categorical variables, and the Kruskal–Wallis test was used for continuous variables to compare the differences between the three groups. To analyze the longevity of humoral and cellular immune responses in SARS-CoV-2-naive participants, we excluded participants who had breakthrough infections at each time point. The geometric mean titer (GMT) with 95% confidence interval (CI) was calculated after logarithmic transformation of the antibody titers. The Wilcoxon signed-rank test was used to compare paired data, and the Mann–Whitney U test was used to compare unpaired data. Statistical analysis was performed using the Statistical Package for the Social Sciences version 20 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism software (version 9.0; GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

3 Results

3.1 Study participants

A total of 90 participants were recruited in this study: 26 boosted with BNT162b2, 57 with mRNA-1273, and 7 with Ad26.COV2.S. One participant from the mRNA-1273-boosted group had a positive result for N protein antibody at baseline and thus was excluded (Figure 1). The characteristics of the participants are listed in Table 1. The majority of the participants were male (90%) under the age of 40 years (median age, 34 years; interquartile range [IQR], 32–37). The median intervals between the primary and booster doses of the participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups were 27 weeks (IQR, 25–28), 25 weeks (IQR, 24–27), and 23 weeks (IQR, 15–24), respectively. During the study period, the rate of laboratory-confirmed breakthrough infections did not differ among the three groups (42.9–62.5%; Table 1). All breakthrough infections occurred 3–6 months after booster vaccination during follow-up. Five

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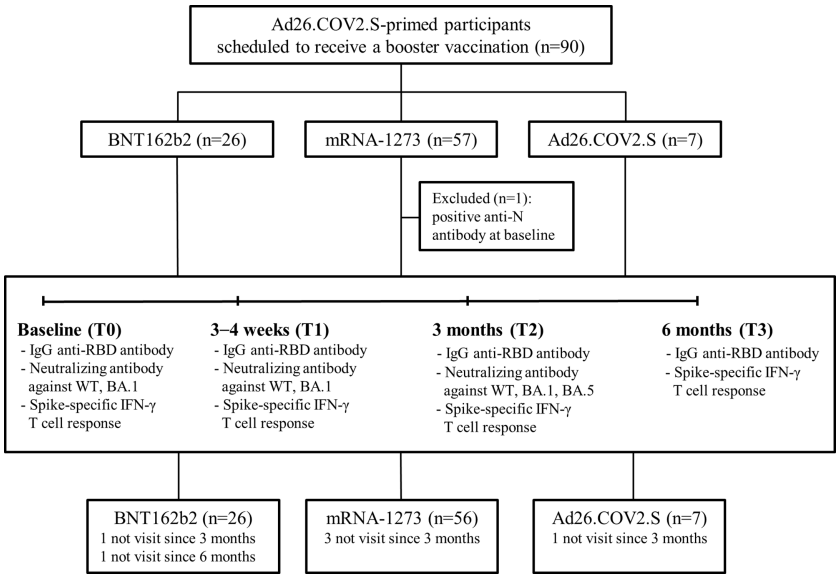

FIGURE 1
Study flowchart. Abbreviation: IgG, immunoglobulin G; RBD, receptor binding domain; WT, wild type.

participants, including one from the BNT162b2-, three from the mRNA-1273-, and one from the Ad26.COV2.S-boosted group, were lost to follow-up 3 months after vaccination. One participant from the BNT162b2-boosted group was lost to follow-up at 6 months post-vaccination (Figure 1).

3.2 Humoral immune response

Humoral immune responses after booster vaccination are shown in Figure 2 and Supplementary Figure 1. Humoral immune responses were compared among the three groups, excluding participants with breakthrough infections at each time point. At T0, the GMTs of IgG anti-RBD antibodies were lower in the participants from the BNT162b2-boosted group than in those from the mRNA-1273-boosted group (57 [95% CI, 41–79] vs. 113 [95% CI, 90–142], $P < 0.001$). The GMT of IgG anti-RBD antibodies was significantly increased at T1, and participants in all three groups maintained higher titers of IgG anti-RBD antibodies at T3 compared to T0 (Supplementary Figure 1A). However, the GMTs of IgG anti-RBD antibodies after booster vaccination were significantly lower in the participants from the Ad26.COV2.S-boosted group than in those from the mRNA vaccine-boosted groups at each time point (Figure 2A). No significant difference in the GMTs of IgG anti-RBD antibodies after booster vaccination was found between the participants from the BNT162b2- and

TABLE 1 Characteristics of study participants.

	BNT162b2 (N = 26)	mRNA-1273 (N = 56)	Ad26.COV2.S (N = 7)	*P*-value
Male, No. (%)	26 (100)	50 (89.3)	4 (57.1)	NA
Median age (IQR), years	36 (34–36)	34 (32–37)	35 (33–41)	0.110
Median interval between priming and booster dose (IQR), weeks	27 (25–28)	25 (24–27)	23 (15–24)	<0.001
Breakthrough infection, No. (%)	14 (53.8)	35 (62.5)	3 (42.9)	0.521
Male, No. (%)	14 (53.8)	30 (53.6)	3 (42.9)	NA
Median age (IQR), years	36 (34–38)[a]	33 (32–36)[b]	34 (33–34)[ab]	0.016
Timing of breakthrough infection				0.857
Confirmed at 3–4 weeks, No. (%)	0 (0)	0 (0)	0 (0)	
Confirmed at 3 months, No. (%)*	5 (19.2)	13 (23.2)	1 (14.3)	
Confirmed at 6 months, No. (%)†	9 (34.6)	22 (39.3)	2 (28.6)	

*Five participants (one from the BNT162b2-boosted group, three from the mRNA-1273-boosted group, and one from the Ad26.COV2.S-boosted group) were lost to follow-up.
†Six participants (two from the BNT162b2-boosted group, three from the mRNA-1273-boosted group, and one from the Ad26.COV2.S-boosted group) were lost to follow-up.
The values with different superscript letters in a column are significantly different ($P < 0.05$)
No, number; NA, not applicable; IQR, interquartile range.

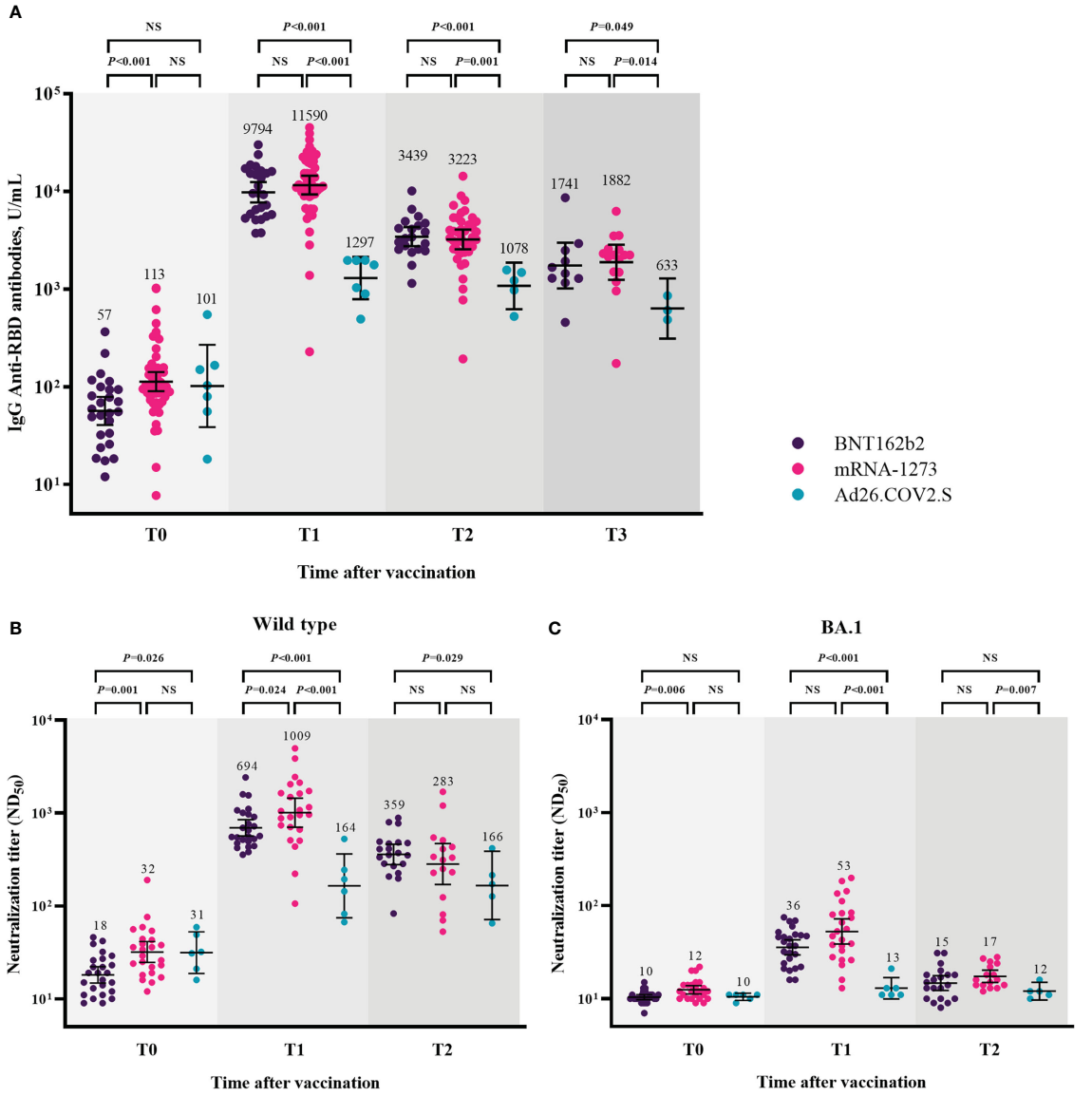

FIGURE 2
Humoral immune responses after booster vaccination. GMTs of IgG anti-RBD antibodies **(A)**, ND_{50} against wild type virus **(B)**, and ND_{50} against Omicron BA.1 **(C)**. Blood samples were collected at baseline (day of booster dose, T0), 3–4 weeks post-booster dose (T1), 3 months post-booster dose (T2), and 6 months post-booster dose (T3). The black bar represents GMT with 95% confidence intervals. Abbreviation: NS, not significant; IgG, immunoglobulin G; GMT, geometric mean titer; RBD, receptor binding domain; ND_{50}, 50% neutralization dose.

mRNA-1273-boosted groups at each time point. The GMTs of IgG anti-RBD antibodies and respective *P*-value among three groups were shown in the Supplementary Table 1.

The neutralizing activity against WT after booster vaccination is shown in Figure 2B. The GMTs of NAb against WT were significantly lower in the participants from the BNT162b2-boosted group than in those from the mRNA-1273- and Ad26.COV2.S-boosted groups at T0 (BNT162b2 group vs. mRNA-1273 group vs. Ad26.COV2.S group, 18 vs. 32 vs. 31, $P = 0.003$). At T1, the GMTs of NAb were 694, 1009, and 164 in the participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups, respectively ($P < 0.001$). At T2, the GMTs of NAb did not significantly differ between the participants from the mRNA vaccine-boosted groups (359 vs. 283, $P = 0.408$). The participants from the BNT162b2-boosted group had significantly higher NAb titers than those from the Ad26.COV2.S-boosted group (359 vs. 166, $P = 0.029$). The GMTs of NAb against BA.1 were negligibly low at T0 in all participants from the three groups (Figure 2C). Compared with the baseline (T0) levels, the neutralizing activities against BA.1 significantly increased in the participants from the BNT162b2- and mRNA-1273-boosted groups ($P < 0.001$) but not in those from the Ad26.COV2.S-boosted group at T1 and T2 (Supplementary Figure 1C). The GMTs of NAb against BA.1 were significantly lower in the participants from the Ad26.COV2.S-boosted group than in those from the mRNA-1273-boosted group at each time points after booster vaccination (Figure 2C). The GMTs of NAb against WT/BA.1 and respective *P*-value among three groups were shown in the Supplementary Table 1.

The cross-neutralizing activities against Omicron subvariants (BA.1 and BA.5) were assessed 3 months after booster vaccination (T2) (Figure 3). After excluding participants with breakthrough infections, 20, 16, and 5 participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups, respectively, were included in the analysis. Compared with the neutralizing activities against WT, those against BA.1 were lower by 23.9-fold (359 vs. 15, $P < 0.001$), 16.6-fold (283 vs. 17, $P < 0.001$), and 13.8-fold (166 vs. 12, $P = 0.063$) in the participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups, respectively. The neutralizing activities against BA.5 versus WT were lower by 10.9-fold (359 vs. 33, $P < 0.001$), 7.4-fold (283 vs. 38, $P < 0.001$), and 7.2-fold (166 vs. 23, $P = 0.063$) in the participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups, respectively. The GMTs of NAb against BA.5 were higher than those against BA.1 by 2.2-fold (33 vs. 15, $P < 0.001$), 2.2-fold (38 vs. 17, $P < 0.001$), and 1.9-fold (23 vs. 12, $P = 0.063$) in the participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups, respectively.

3.3 SARS-CoV-2-specific IFN-γ T cell response

The cellular immune response was assessed based on IFN-γ response against the S protein of SARS-CoV-2 by using a Covi-FERON ELISA kit (Figure 4). Positivity for IFN-γ response against the original S antigen (WT and Alpha) was observed in 81% (21/26), 93% (53/57), and 100% (7/7) of the participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups at T0, respectively. As for the variant S antigens (Beta and Gamma), positivity for IFN-γ response was observed in 77% (20/26), 81% (46/57), and 100% (7/7) of the participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups at T0, respectively. Compared with the baseline (T0) levels, the IFN-γ response against the original S antigen significantly increased at T1 and was sustained at T3 in the participants from the BNT162b2- and mRNA-1273-boosted groups but not in those from the Ad26.COV2.S-boosted group (Figure 4A). As for the IFN-γ response against variant S antigens, the significant increase in IFN-γ response was sustained at T2 and T3 in the participants from the BNT162b2- and mRNA-1273-boosted groups, respectively (Figure 4B). IFN-γ responses against original and variant S antigens did not differ between mRNA vaccine-boosted groups. However, mRNA-1273-boosted group showed significantly higher IFN-γ responses against original and variant S antigens compared to Ad26.COV2.S-boosted group at T1 and T2 (Supplementary Table 1).

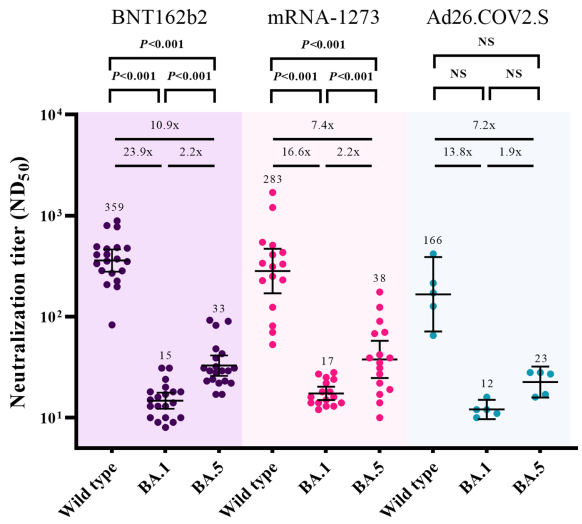

FIGURE 3
Comparisons of neutralizing activities against wild type, Omicron BA.1, and Omicron BA.5 viruses at 3 months after booster vaccination. The black bar represents GMT with 95% confidence intervals. NS, not significant; ND_{50}, 50% neutralization dose; GMT, geometric mean titer.

3.4 Comparison of immune responses between SARS-CoV-2-infected and -uninfected participants

At 6 months follow-up, SARS-CoV-2 breakthrough infections occurred in 53.8% (14/26), 62.5% (35/56), and 42.9% (3/7) of the participants from the BNT162b2-, mRNA-1273-, and Ad26.COV2.S-boosted groups, respectively. All cases of breakthrough infections were mild in severity and did not require hospitalization. Of the 52 cases of SARS-CoV-2 breakthrough infections, 19 and 33 cases were identified at T2 and T3, respectively (Table 1). The Ad26.COV2.S-boosted group had considerably fewer participants to compare the difference between SARS-CoV-2-infected and -uninfected groups. Thus, the comparison was conducted only among the participants from the mRNA vaccine-boosted groups. No laboratory-confirmed cases of COVID-19 were recorded at T1. The IgG anti-RBD antibody and IFN-γ responses against the original and variant S proteins at T1 were compared between 64 SARS-CoV-2-uninfected participants (33 without and 31 with SARS-CoV-2 breakthrough infections at T3) and 18 SARS-CoV-2-infected participants (SARS-CoV-2 breakthrough infections identified at T2). The neutralizing activities against WT and Omicron subvariants were compared between 36 SARS-CoV-2-uninfected participants (16 without and 20 with SARS-CoV-2 breakthrough infections identified at T3) and 13 SARS-CoV-2-infected participants. The neutralization assay was conducted only in selected participants from the mRNA-1273-boosted group age-matched with those from the BNT162b2-boosted group. Hence, a discrepancy in the number of participants was noted in some analyses. There was no difference in peak post-booster humoral immunity (IgG anti-RBD antibodies [10766 vs. 11812, $P = 0.702$], NAb against WT [817 vs. 881, $P = 0.885$], and BA.1 [43 vs. 44, $P = 0.969$]) and cellular immunity (original S antigen [2.20 vs. 1.77, $P = 0.810$] and variant S antigens [1.28 vs. 1.27, $P = 0.760$]) between the SARS-CoV-2-infected and -uninfected participants (Table 2; Figure 5 and Supplementary Figure 2).

After breakthrough infections, the SARS-CoV-2-infected participants had higher GMTs of IgG anti-RBD antibodies and NAb than the SARS-CoV-2-uninfected participants (Figures 5A, B). The GMTs of NAb against BA.5 also increased after the breakthrough infections. The neutralizing activities against BA.5

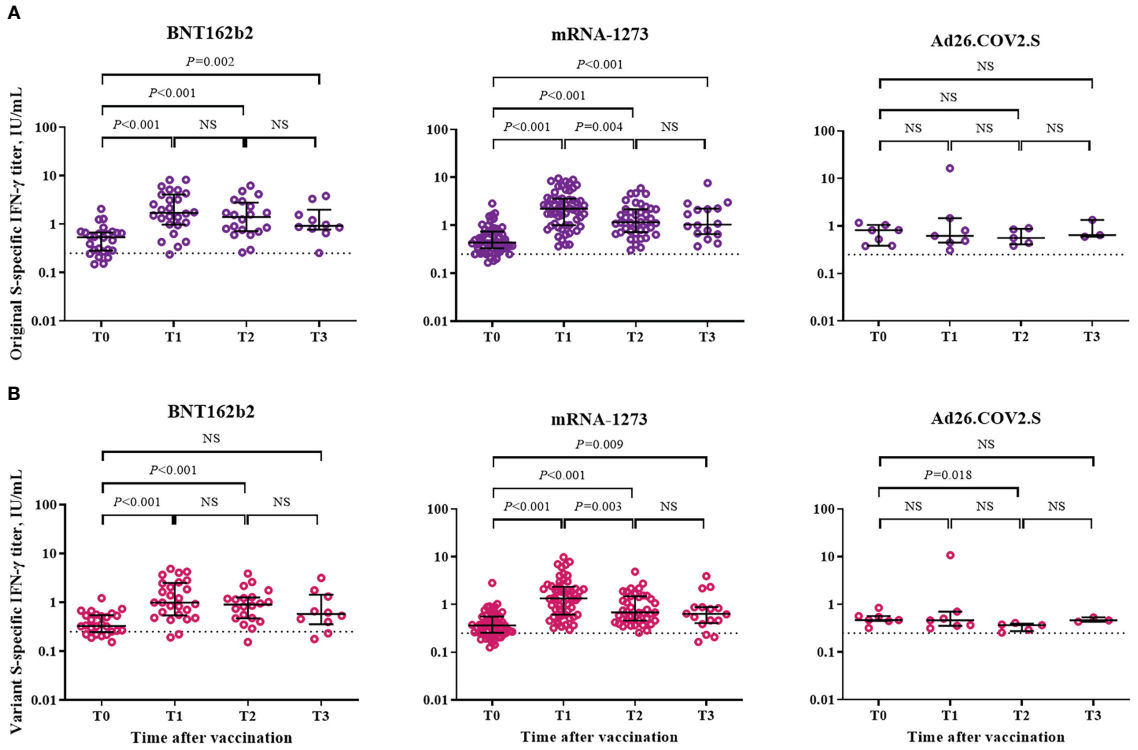

FIGURE 4
Cellular immune response after booster vaccination. **(A)** SARS-CoV-2 original spike protein-specific interferon-γ release assay. **(B)** SARS-CoV-2 variant spike protein-specific interferon-γ release assay. Blood samples were collected at baseline (day of booster dose, T0), 3–4 weeks post-booster dose (T1), 3 months post-booster dose (T2), and 6 months post-booster dose (T3). The black bar represents median with interquartile range. NS, not significant; S, spike; IFN- γ, interferon gamma.

were higher than those against BA.1 in the SARS-CoV-2-uninfected participants (35 vs. 16, $P < 0.001$) but not in the SARS-CoV-2-infected participants (186 vs. 204, $P = 0.724$; Figure 5B).

4 Discussion

The key findings of this study are as follows: (1) a heterologous booster dose of mRNA vaccine in the Ad26.COV2.S-primed population induced good and sustained humoral and cellular immune responses for up to 6 months; (2) despite booster vaccination, the neutralizing activities against Omicron subvariants were negligible in the Ad26.COV2.S-primed population; (3) breakthrough infections after booster vaccination elicited acceptable NAb against Omicron subvariants; and (4) no difference in baseline humoral and cellular immunities was noted between individuals with and without breakthrough infections.

TABLE 2 Comparisons of peak post-booster humoral and cellular immunities at 3–4 weeks after booster vaccination between SARS-CoV-2-uninfected and -infected participants*.

	SARS-CoV-2 uninfected	SARS-CoV-2 infected	*P*-value
IgG anti-RBD antibodies GMT (95% CIs), U/mL†	10766 (8895–13031)	11812 (8087–17251)	0.702
NAb against WT GMT (95% CIs)‡	817 (637–1048)	881 (596–1302)	0.885
NAb against BA.1 GMT (95% CIs)‡	43 (35–53)	44 (29–69)	0.969
IGRA against original spike median (IQR), IU/mL†§	2.20 (1.02–3.51)	1.77 (0.93–5.18)	0.810
IGRA against variant spike median (IQR), IU/mL†¶	1.28 (0.64–2.36)	1.27 (0.50–3.22)	0.760

*The Ad26.COV2.S-boosted group was excluded from analysis.
†The numbers of SARS-CoV-2-uninfected and-infected participants were 64 and 18, respectively.
‡The numbers of SARS-CoV-2-uninfected and-infected participants were 36 and 13, respectively. The reason for the decreased number of participants was that the neutralizing antibody assay was conducted in randomly selected participants from the mRNA-1273-boosted group.
§Original spike protein derived from wild type and Alpha variants of SARS-CoV-2.
¶Variant spike protein derived from the Beta and Gamma variants of SARS-CoV-2.
IgG, immunoglobulin G; RBD, receptor binding domain; GMT, geometric mean titer; WT, wild type; CI, confidence interval; NAb, neutralizing antibody; IGRA, interferon gamma release assay; IQR, interquartile range.

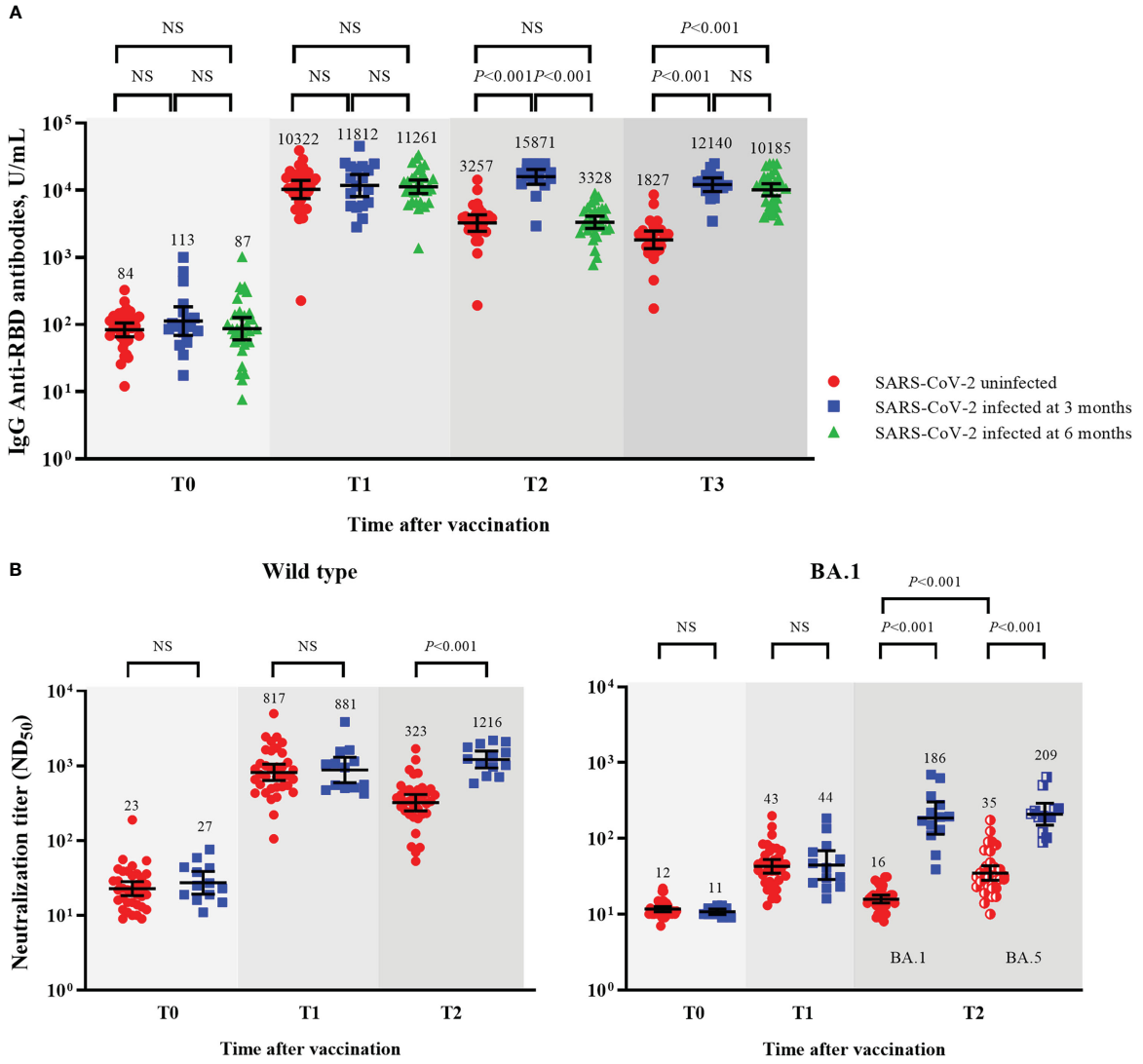

FIGURE 5
Comparisons of humoral immunity between SARS-CoV-2-uninfected and -infected participants among mRNA vaccine-boosted group. GMTs of IgG anti-RBD antibodies **(A)**, and ND_{50} against wild type and Omicron BA.1 viruses **(B)**. Blood samples were collected at baseline (day of booster dose, T0), 3–4 weeks post-booster dose (T1), 3 months post-booster dose (T2), and 6 months post-booster dose (T3). The black bar represents GMT with 95% confidence intervals. NS, not significant; IgG, immunoglobulin G; GMT, geometric mean titer; RBD, receptor binding domain; ND_{50}, 50% neutralization dose.

The Ad26.COV2.S vaccine showed acceptable efficacy and durable humoral/cellular immune responses in clinical trials (6). In the present study, homologous and heterologous booster vaccinations in the Ad26.COV2.S-primed participants were immunogenic. However, the quantities of IgG anti-RBD antibodies and NAbs were significantly higher in the heterologous booster groups than in the homologous booster group. In addition, T-cell immunity was enhanced for up to 6 months after heterologous booster vaccination but not after homologous booster vaccination. These results are consistent with those of previous studies (11–13). Repeated exposure to the adenovirus vector in homologous booster vaccination might result in diminished immune responses. In this aspect, heterologous Ad26.COV2.S-mRNA vaccine booster vaccination would be more immunogenic than homologous booster vaccination in the Ad26.COV2.S-primed population. Notably, the participants from the mRNA-1273-boosted group showed higher neutralizing activities (GMTs) against WT than those from the BNT162b2-boosted group at 3–4 weeks after booster vaccination (T1) (1009 vs. 694, $P = 0.024$). This result can be ascribed to the higher pre-booster titers of the participants from the mRNA-1273-boosted group than those of the participants from the BNT162b2-boosted group (32 vs. 18, $P = 0.001$). However, the difference between these participants became negligible 3 months after booster vaccination (T2).

As diverse SARS-CoV-2 variants of concern (VOCs) emerge and spread, cross-reactive neutralizing activities against VOCs are important to predict vaccine efficacy (14). Although the immune correlation of protection against SARS-CoV-2 infection is not well established, NAb titers may play a key role in protection against SARS-CoV-2 infection (15). A predictive model showed that a

normalized neutralization titer of 68 is associated with 50% protection against symptomatic SARS-CoV-2 infection (16). Another study on healthcare workers reported that NAb titers of 64–128 provide 94% protection against COVID-19 (17). In the present study, the cross-reactive neutralizing activities against Omicron subvariants were negligibly low at baseline (ND_{50}, 10–23). After heterologous booster vaccination, the neutralizing activities against Omicron BA.1 slightly increased at 3–4 weeks (T1), with titers of 58 and 36 in the mRNA-1273- and BNT162b2-boosted groups, respectively. In addition, NAb titers decayed to undetectable levels 3 months after booster vaccination (T2). Moreover, the NAb response against Omicron BA.1 was not enhanced after homologous booster vaccination with Ad26.COV2.S. This result suggests that the neutralizing activities against Omicron BA.1 elicited by a single dose of the booster vaccine could not provide sufficient protection against Omicron subvariants in the Ad26.COV2.S-primed population. Additional doses of booster or bivalent booster vaccinations should be considered for this population.

Omicron BA.5 is one of three lineages (BA.2.12.1, BA.4, and BA.5) derived from BA.2. Different from BA.2, Omicron BA.5 has additional mutations of L452R, F486V, and R493Q in the spike RBD (18). Recent studies have found low cross-reactive neutralization between Omicron BA.1 and BA.5 (19–21). Furthermore, the hybrid immunity elicited by Omicron BA.1 breakthrough infection can be evaded by Omicron BA.4/BA.5 because of the spike RBD mutations of L452Q, L452R, and F486V (20, 22, 23). In the present study, the neutralizing activities against BA.5 were low but still higher than those against BA.1 in the mRNA vaccine-boosted participants without breakthrough infections. In addition, after breakthrough infections, the neutralizing activities against BA.1 and BA.5 were enhanced in the heterologously boosted participants. Most cases of breakthrough infections in the present study developed in early 2022 when Omicron BA.1 was the predominant strain, explaining the remarkable cross-reactive neutralizing activities between Omicron subvariants BA.1 and BA.5, contrary to previous reports (19–24). Immune responses after vaccination and natural infection may vary depending on age, sex, race, vaccine type/dose, and SARS-CoV-2 strain (25, 26).

With respect to the breakthrough infection, there was no significant difference in the peak post-booster humoral and cellular immunity between SARS-CoV-2-infected and -uninfected participants. The frequency, intensity, and duration of viral exposure and the predominant strain of SARS-CoV-2 variants at the time of exposure may be important factors in determining the occurrence of breakthrough infections. Hybrid immunity elicited by natural infection and booster vaccination showed better neutralizing activity against WT and Omicron subvariants, but its longevity warrants further investigation.

This study has some limitations. First, most study participants were young and male. According to the policy of the Korean government, the Ad26.COV2.S vaccine was first administered in South Korea to military reservists aged 30–60 years, explaining why most of the participants were young men. As immune responses after vaccination can be diverse according to sex and age, the data in this study should be cautiously generalized. The immune responses in the elderly who are vulnerable to severe COVID-19 might be different from those in our study participants. Second, the cellular immune responses against SARS-CoV-2 Omicron subvariants were not evaluated. The cellular immune responses against Omicron differ from those against other variants. Third, the neutralization assay at 6 months post-booster vaccination was not conducted. Due to limitations in time and labor, neutralizing antibody tests could be only performed at limited points in time. Data on NAb titers against Omicron BA.1/BA.5 in SARS-CoV-2-infected and -uninfected participants at 6 months post-booster vaccination would be useful in establishing vaccination strategy. On the other hand, the strength of this study is that we conducted a serial estimation of humoral and cellular immune responses after booster vaccination in the Ad26.COV2.S-primed population for up to 6 months. In addition, a neutralization assay was performed against the Omicron subvariants.

In conclusion, heterologous booster vaccination is recommended for the Ad26.COV2.S-primed population. However, a single dose of heterologous mRNA vaccine booster was not sufficient to provide protection against Omicron subvariants in SARS-CoV-2 infection-naïve and Ad26.COV2.S-primed individuals. Therefore, additional booster vaccinations may be required.

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 The ethics committees of Korea University Guro Hospital The ethics committees of Ajou University Hospital The ethics committees of Hanllym University Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: HH, M-SP, and JYS. Data curation: HH, JH, YS, M-SP, and JYS. Formal analysis: HH, A-YJ, M-SP, and JYS. Funding acquisition: JYS. Investigation: HH, A-YJ, JH, YS, EN, JY, HS, JY, HP, JHS, JK, M-SP, and JYS. Methodology: HH, A-YJ, JN, M-SP, and JYS. Project administration: M-SP and JYS. Validation: HH, HC, JK, M-SP, and JYS. Visualization: HH and JYS. Writing - original draft: HH and JYS. Writing - review and editing: All authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1131229/full#supplementary-material

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Impact of inactivated vaccines on decrease of viral RNA levels in individuals with the SARS-CoV-2 Omicron (BA.2) variant: A retrospective cohort study in Shanghai, China

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Background: SARS-CoV-2 Omicron (BA.2) has stronger infectivity and more vaccine breakthrough capability than previous variants. Few studies have examined the impact of inactivated vaccines on the decrease of viral RNA levels in individuals with the Omicron variant, based on individuals' continuous daily cycle threshold (Ct) values and associated medical information from the infection to hospital discharge on a large population.

Methods: We extracted 39,811 individuals from 174,371 Omicron-infected individuals according to data inclusion and exclusion criteria. We performed the survival data analysis and Generalized Estimating Equation to calculate the adjusted relative risk (aRR) to assess the effect of inactivated vaccines on the decrease of viral RNA levels.

Results: Negative conversion was achieved in 54.7 and 94.3% of all infected individuals after one and 2 weeks, respectively. aRRs were shown weak effects on turning negative associated with vaccinations in asymptomatic infections and a little effect in mild diseases. Vaccinations had a protective effect on persistent positivity over 2 and 3 weeks. aRRs, attributed to full and booster vaccinations, were both around 0.7 and had no statistical significance in asymptomatic infections, but were both around 0.6 with statistical significance in mild diseases, respectively. Trends of viral RNA levels among vaccination groups were not significant in asymptomatic infections, but were significant between unvaccinated group and three vaccination groups in mild diseases.

Conclusion: Inactivated vaccines accelerate the decrease of viral RNA levels in asymptomatic and mild Omicron-infected individuals. Vaccinated individuals have lower viral RNA levels, faster negative conversion, and fewer persisting positive proportions than unvaccinated individuals. The effects are more evident and significant in mild diseases than in asymptomatic infections.

KEYWORDS

COVID-19, SARS-CoV-2, Omicron, inactivated vaccines, viral RNA levels

Introduction

Coronavirus disease 2019 (COVID-19) remains a severe threat to global public health at present. The current primary variant is SARS-CoV-2 Omicron, which has unprecedented mutations, higher infectivity, and greater vaccine breakthrough capabilities than previous variants (1–3). Individuals with Omicron mostly present with asymptomatic or mild symptoms (4). Due to the lack of apparent signs of upper respiratory infection (such as cough, sputum, and sneezing) (3), infected individuals were usually delayed in isolation, which led to more secondary transmissions.

Vaccines have been shown to not only control the spread of disease but also prevent severe illness in infected individuals with the Alpha, Beta, and Delta strains of COVID-19 (5–8). Vaccines were slightly less protective against Omicron strains than previous strains (9). Two-dose vaccine provided limited and short-lived protection against the Omicron virus (10), and the booster shot was more effective than two and one-dose vaccines (11). The duration of antibody titer levels and protective effects against the Omicron virus varied depending on the type of vaccine (12). However, most previous studies on the impact of vaccination were focused on mRNA vaccines and were primarily based on clinical or laboratory data from relatively small samples (12–14). Although some articles analyzed the inactivated vaccines, their primary focus was on the safety and immunogenicity of vaccines (15–20). The article (21) examined the effect of inactivated vaccination on a large population in Hong Kong, but it did not analyze individuals' continuous cycle threshold (Ct) values from infection to hospital discharge.

A recent Omicron BA.2 outbreak caused a COVID-19 pandemic in Shanghai, one of China's most populated and economically developed metropolises, with almost 25 million inhabitants (22). In just 5 months from January 1 to May 31, 2022, despite 75% population vaccine coverage and a certain degree of non-pharmaceutical interventions, the total number of Omicron BA.2 infections reached 626,000 in Shanghai. According to local policies, all infected individuals with positive nucleic acid tests were centralized and quarantined timely at the Fangcang Shelter Hospital (FSH) of the National Exhibition and Convention Center (NECC). The FSH is the largest shelter hospital during the Omicron BA.2 pandemic and is equipped to support non-severe patients in Shanghai. Totally 174,371 asymptomatic infections and mild diseases were isolated in the FSH from April 9 to May 24, 2022. To monitor disease progression and promote patient recovery, each infected individual in the FSH was screened daily by the nucleic acid test with pharyngeal swab specimens until discharge after negative conversion.

In this study, we mainly performed the survival data analysis and Generalized Estimating Equation (GEE) to estimate the decrease of viral RNA levels among four vaccine groups in asymptomatic and mild Omicron infections, for the purpose of providing the calibration of future pandemic control measures based on inactivated vaccines in China.

Materials and methods

Data

All data were obtained from the FSH. Primary data included the daily Ct values from admission with confirmed positive diagnosis to discharge after turning negative. Data for each patient were obtained from the electronic medical record system, including individual age, gender, marital status, comorbidities, symptomatic status, admission date, discharge date, infection date, ORF1ab value, N gene value, and location of first positive screening. The length of stay was calculated by subtracting the admission date from the discharge date.

Classification of vaccination status

Vaccination status was divided into four groups based on the vaccine doses received: unvaccinated, partially vaccinated, fully vaccinated, and booster. People who have received at least one vaccine dose but did not complete all doses prescribed by the vaccination protocol were defined as partially vaccinated. Those who had received all doses prescribed by the vaccination protocol were defined as fully vaccinated, while those who received a booster shot were regarded as the booster vaccination. Based on the immunization schedule, fully vaccinated was given 3 weeks after partial vaccinations, and booster vaccination was administered 6 months following full vaccinations.

According to Chinese epidemic prevention and control strategies (23) and Shanghai's requirement to promote free vaccination for the whole population (24, 25), vaccination time for residents in Shanghai was mostly concentrated. Individuals who administered partial vaccines were mainly focused between April and June 2021, the full vaccines were between May and July 2021, and boosters were between November 2021 and January 2022.

Definitions of infection, turning negative, and persisting positive

The pharyngeal swab specimens were tested for the ORF1ab and N genes of SARS-CoV- 2 with real-time RT-PCR using the SARS-CoV-2 Detection Kit (Easy Diagnosis Biomedicine Co., Ltd., Wuhan, China) with MA6000 Real-Time PCR Detection System (Molarray Bioscience Co., Ltd., Suzhou, China) by Shanghai Labway Clinical Laboratory. A Ct value (ORF1ab or N genes) below 35 indicated a positive for SARS-CoV-2 RNA (26). For infected individuals during those periods in Shanghai, the majority of positive samples were confirmed as BA.2 sub-lineages (22, 27, 28); no other variants were identified.

According to the Diagnosis and Treatment Guideline for COVID-19 (Ninth Edition) (26) by the National Health Commission of China, an asymptomatic infection was defined as a patient who did not have any of the following symptoms: fever (>37.5°C), chills, myalgia, fatigue, rhinorrhea, nasal congestion, hyposmia, hypogeusia, sore throat, dyspnea, cough, sputum production, hemoptysis, headache, dizziness, anorexia, nausea,

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TABLE 1 Characteristics of individuals infected with Omicron classified by type of vaccination.

Characteristics	Total	Classification of vaccination				*P* value
		Unvaccinated	Partially vaccinated	Fully vaccinated	Booster	
No. of cases (%)	39,811	10,515 (26.4%)	1,202 (3.0%)	11,504 (28.9%)	16,590 (41.7%)	
Age, years, Median (IQR[a])	42 (31–55)	46 (32–59)	33 (26–45)	36 (26–52)	46 (33–55)	<0.001[b]
≤18	1,662 (4.2)	593 (5.6)	70 (5.8)	967 (8.4)	32 (0.2)	<0.001[c]
18–30	8,281 (20.8)	1,697 (16.1)	434 (36.1)	3,073 (26.7)	3,077 (18.5)	
31–40	8,791 (22.0)	2,081 (19.8)	330 (27.5)	2,754 (23.9)	3,626 (21.9)	
41–50	6,962 (17.5)	1,671 (15.9)	155 (12.9)	1,598 (13.9)	3,538 (21.3)	
51–60	8,678 (21.8)	2,180 (20.7)	127 (10.6)	1,840 (16.0)	4,531 (27.3)	
61–70	4,842 (12.2)	1,979 (18.8)	76 (6.3)	1,146 (10.0)	1,641 (9.9)	
≥71	595 (1.5)	314 (3.0)	10 (0.8)	126 (1.1)	145 (0.9)	
Gender, *n* (%)						<0.001[c]
Female	16,339 (41.0)	4,341 (41.3)	386 (32.1)	4,830 (42.0)	6,782 (40.9)	
Male	23,472 (59.0)	6,174 (58.7)	816 (67.9)	6,674 (58.0)	9,808 (59.1)	
Marital status, *n* (%)						<0.001[c]
Married	23,244 (58.4)	5,808 (55.2)	499 (41.5)	6,012 (52.3)	10,925 (65.9)	
Unmarried	15,163 (38.1)	4,268 (40.6)	654 (54.4)	5,133 (44.6)	5,108 (30.8)	
Others	1,404 (3.5)	439 (4.2)	49 (4.1)	359 (3.1)	557 (3.4)	
Comorbidities, *n* (%)						
Hypertension	4,624 (8.4)	845 (8.0)	58 (4.8)	817 (7.1)	1,357 (8.2)	<0.001[c]
Diabetes	1,633 (3.0)	405 (3.9)	26 (2.2)	294 (2.6)	385 (2.3)	<0.001[c]
Length of stay, days, Median (IQR)	8 (7–11)	9 (7–11)	8 (7–10)	8 (7–10)	8 (7–10)	<0.001[b]
Time to negative conversion, days, Median (IQR)	6 (5–8)	7 (5–9)	6 (5–8)	6 (5–8)	6 (5–8)	<0.001[b]
Nadir Ct values, Median (IQR)						
ORF1ab gene	28.8 (25.7–32.0)	28.3 (25.2–31.5)	28.9 (26.0–32.2)	29.0 (25.8–32.1)	29.0 (25.9–32.1)	<0.001[b]
N gene	27.1 (24.0–30.1)	26.6 (23.5–29.6)	27.2 (24.2–30.2)	27.2 (24.1–30.2)	27.2 (24.3–30.2)	<0.001[b]
Location of first positive screening, *n* (%)						<0.001[c]
Community screening	15,543 (39.0)	3,281 (31.2)	489 (40.7)	5,214 (45.3)	6,659 (39.5)	
Companies/schools screening	5,870 (14.7)	593 (5.6)	175 (14.6)	1,762 (15.3)	3,340 (20.1)	
Active screening at nucleic acid sampling points	5,847 (14.7)	941 (8.9)	217 (18.1)	1,965 (17.1)	2,724 (16.4)	
Fever clinic screening	12,551 (31.5)	5,700 (54.2)	321 (26.7)	2,563 (22.3)	3,967 (23.9)	
Symptomatic status, *n* (%)						<0.001[c]
Asymptomatic infection	6,809 (17.1)	2,024 (19.2)	214 (17.8)	1,901 (16.5)	2,670 (16.1)	
Mild disease	33,002 (82.9)	8,491 (80.8)	988 (82.2)	9,603 (83.5)	13,920 (83.9)	

[a]IQR, interquartile range (P_{25}-P_{75}). [b]P-value calculated by Kruskal–Wallis test. [c]P-value calculated by Chi-squared test.

vomiting, abdominal pain, and diarrhea. The mild disease was defined as having any of the above symptoms, and additionally the symptoms are mild, the imaging examination showed no signs of pneumonia, and individuals had low oxygen saturation ($\leq$93%).

Turning negative was defined as a decrease in viral RNA levels and Ct values were more than 35 by two consecutive negative nucleic acid tests with sampling intervals more than 24 h. Persisting positive was defined as viral RNA levels not decreasing and Ct values were always >35 from admission to this nucleic acid test. Infected individuals were eligible for discharge if they had a normal body temperature for more than 3 days and significant improvement in their symptoms in addition to turning negative.

Data inclusion and exclusion criteria

We recruited all individuals with asymptomatic and mild symptoms of COVID-19. The first nucleic acid test after admission should be positive. Infected individuals should be isolated to the FSH on the day or the next day when they were confirmed positive (no more than 2 days between positive diagnosis and admission). Infected individuals with a Ct value of more than 35 on the day of admission, a hospital stay of fewer than 3 days, or no continuous nucleic acids after admission were all excluded. Individuals vaccinated with non-inactivated vaccines (e.g., adenovirus type 5 vector vaccines, recombinant protein subunit vaccines) were not included in the study. Missing values and outliers were omitted. The flow chart of study design was shown in Supplementary Figure 1.

Study design and statistical method

Since individuals in the FSH only received oral medications for their complications and were devoid of antiviral and immune-boosting drugs (which can affect nucleic acid conversion), it is reasonable to observe the impact of inactivated vaccines on the decrease of viral RNA levels in Omicron-infected individuals.

We treated the occurrence of individual's negative conversion as the binary dependent variable and used the length of stay as the survival time. We then used multivariate Cox regression, adjusted by other influential variables, to analyze adjusted relative risk (aRR) for the effect of four types of vaccinations on the decrease of viral RNA levels, respectively. We then treated whether the patient was persisting positive (1 indicates yes, 0 indicates not) at the 2- and 3-week as time points, respectively, adjusted by influential variables, to analyze the aRR for persisting positive associated with vaccinations through multivariable logistic regression.

We analyzed the trends of viral RNA decay among four vaccination groups through the GEE. In the model, continuous Ct values for O and N genes were considered as dependent variables, respectively. Type of vaccination was as the factor variable, influential variables (such as age, gender, hypertension, diabetes, marital status) as covariates, and id (a vector which identifies the clusters of individual repeated measurement) as the subject variable. The paired comparisons between groups were based on Fisher's Permutation test.

The *Chi*-square test was used to compare categorical variables. *Kruskal-Wallis H* test was used to compare the differences in age (years), duration (days), and Nadir Ct values among vaccination groups of unvaccinated, partial, full, and booster. All the analyses were performed in *R* software (version 4.1.2). The Cox regression was constructed with the "survival" and "survminer" packages, GEE with "geepack" package, Fisher's Permutation test with "EnvStats" package. The logistic regression was calculated using the "glm" function, $P < 0.05$ indicates a statistical significance.

Results

Characteristics of individuals with Omicron infection by vaccination type

A total of 39,811 individuals were enrolled in the study. 70.6% of them received full or booster vaccination, 3.0% were partially vaccinated, and 26.4% were unvaccinated (Table 1). Figure 1 depicts the trends in the number of infected individuals admitted to the FSH among four types of vaccination from April 9, 2022 to May 22, 2022.

Of all individuals, 17.1% were asymptomatic infections and 82.9% were mild diseases. 41.0% of the total were females and 59.0% were males. The average age was 42 years [Interquartile Range(IQR): 31–55]. There were 1,662 (4.2%) individuals aged $\leq$18 years, 32,712 (82.1%) aged 19 to 60 years, and 5,437 (13.7%) aged $\geq$60 years; 8.4 and 3.0% of all individuals had hypertension and diabetes, respectively.

The average length of hospital stay was 8 days [IQR: 7–11] and median time to negative conversion was 6 days [IQR: 5–8]. The ORF1ab gene averaged 28.8 [IQR: 25.7–32.0], slightly higher than the N gene average of 27.1 [IQR: 24.0–30.1]. Among locations of first positive screening, community screening had the highest proportion at 39.0%, followed by the fever clinic screening at 31.5%.

Relative risk for negative conversion associated with vaccinations

Figure 2A shows that 54.7% of infected individuals turned positive to negative after 1 week, and 94.3% turned positive to negative after 2 weeks (2 *vs. 1 week*, $P < 0.001$). 96.2% turned positive to negative after 3 weeks, and 96.4% turned positive to negative after 4 weeks. The change in the proportion of negative conversion become slow after 2 weeks. There were significant differences in negative conversion between 3-, 4-, and 1-week ($P < 0.001$), but they were not statistically significant between the 3 and 4 weeks ($P = 0.48$).

We further performed multivariate Cox regression, classified by symptomatic status and adjusted by age, gender, marital status, comorbidities, nadir Ct value, and location of first positive screening, to observe the aRR of turning positive to negative associated with vaccinations. Figure 3A indicates that in asymptomatic infections, whether turning negative within 1 or 2 weeks, aRRs related to partial and full vaccination were mainly around one and had no significance. aRRs associated with booster were slightly bigger than one and statistically significant

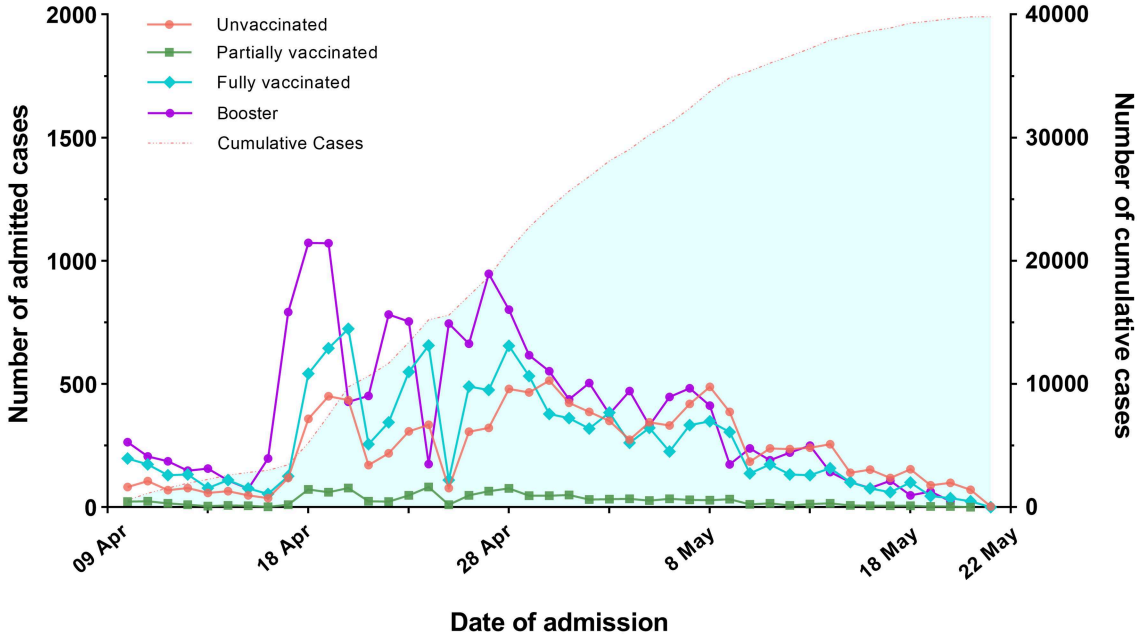

FIGURE 1
Trends in the number of individuals admitted to the FSH among four types of vaccinations.

($P < 0.001$). Moreover, we discovered that curves of the three vaccination groups were similar and close together for negative conversion within 1 and 2 weeks (Figures 4A, C).

For mild diseases (Figure 3B), vaccinated individuals were more likely to achieve negative conversion than unvaccinated individuals, regardless of whether negative conversion occurred within 1 or 2 weeks. The aRRs of negative conversion within 1 week were 1.05 (95% CI 1.00–1.09) and 1.07 (95% CI 1.03–1.11) in fully vaccinated and booster-vaccinated individuals, respectively. Within 2 weeks, the aRRs were 1.06 (95% CI 1.03–1.10) and 1.08 (95% CI 1.05–1.11). Figures 4B, D reveal that curves of booster, full, and partial vaccination are lower than the unvaccinated curve.

Relative risk for persistent positivity associated with vaccinations

Figure 2B reveals that among the infected individuals with persisting positive, 6.5% of them remained positive after 2 weeks. After 3 and 4 weeks, the proportions continued to decrease slowly. There were no significant differences between the proportions after 3 and 4 weeks ($P = 0.32$).

By treating whether the patient was persisting positive as the binary dependent variable, we used the multivariate logistic analysis, adjusted by age, gender, marital status, comorbidities, nadir Ct values, and locations of the first swab for diagnosis, to investigate the aRR of persistent positivity attributed to types of vaccinations grouped by symptomatic status. Figure 3A depicts that vaccination had a protective effect on persistent positivity over 2 and 3 weeks in asymptomatic infections. The aRRs for partial, full, and booster vaccinations were all less than one but not statistically different.

Figure 3B shows that vaccinated individuals are less likely to have persistent positivity than unvaccinated individuals with mild disease, regardless of whether the persistent positivity occurred over 2 or 3 weeks. Furthermore, fully vaccinated individuals are less likely to have persistent positivity than partially vaccinated individuals, and booster individuals are less likely than fully vaccinated individuals to develop persistent positivity. For full and booster vaccination, aRRs for persistent positivity over 2-week were 0.70 (95% CI 0.62–0.79) and 0.60 (95% CI 0.54–0.68), respectively ($P < 0.001$). aRRs for persistent positivity over 3-week were 0.72 (95% CI 0.62–0.84) and 0.59 (95% CI 0.51–0.68), respectively ($P < 0.001$).

Trends of viral RNA decay among four vaccination groups

Supplementary Table 1 shows that three types of vaccination all help to promote the decrease of viral RNA for both ORF1ab gene and N gene. Compared to unvaccinated, booster was the most effective, followed by fully and partially vaccinated. aRR values for the mild disease were generally higher than aRR values for asymptomatic patients. In asymptomatic infections, the aRRs for O genes were 1.24 (95% CI 1.14–1.34), 1.21 (95% CI 1.10–1.33) and 1.03 (95% CI 0.85–1.26). The aRRs for N genes were 1.26 (95% CI 1.21–1.31), 1.22 (95% CI 1.18–1.28) and 1.20 (95% CI 1.10–1.32). In mild diseases, the aRR for O genes were 1.29 (95% CI 1.18–1.42), 1.27 (95% CI 1.14–1.41), and 1.05 (95% CI 0.84–1.30). aRR of N

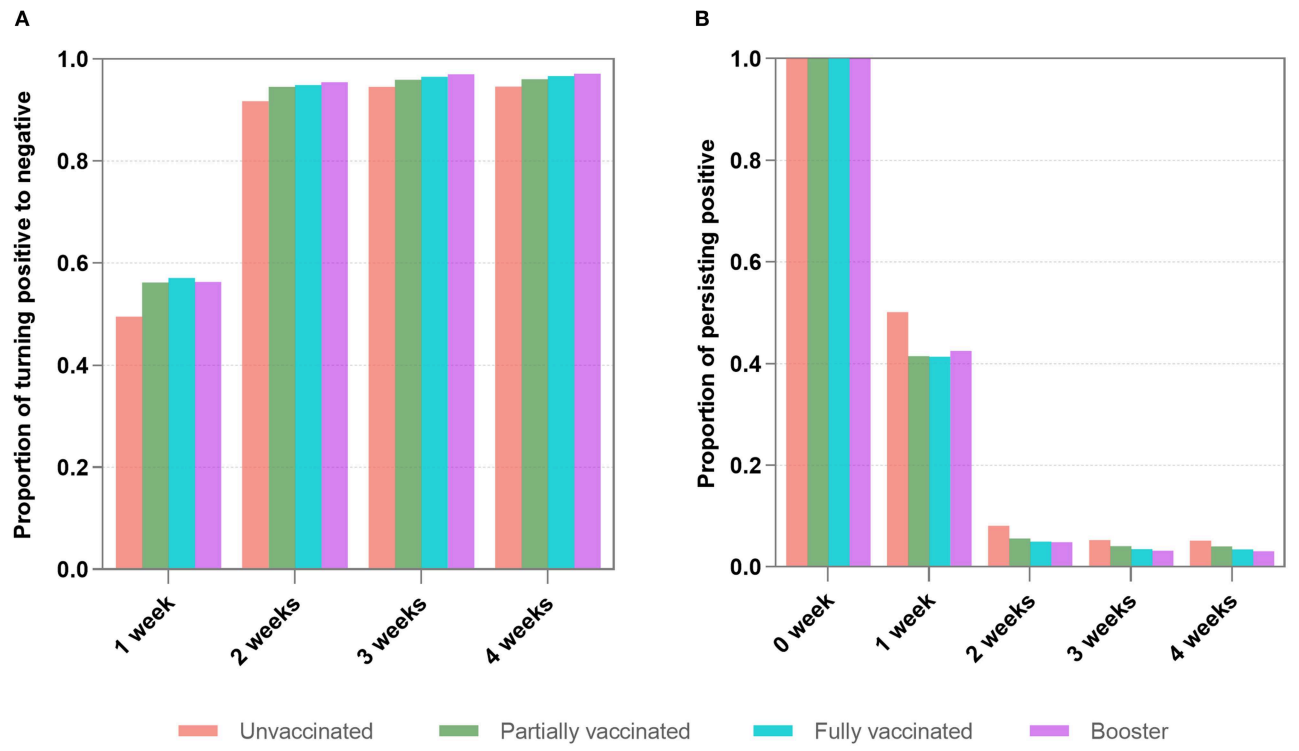

FIGURE 2
The proportions of turning positive to negative **(A)** and persisting positive **(B)** within 4 weeks. **(A)** Shows that 54.7% of infected individuals achieved negative conversion after 1 week, and 94.3% achieved negative conversion after 2 weeks (2 weeks *vs. 1 week*, $P < 0.001$). There were significant differences in negative changes between 3-, 4, and 1-week ($P < 0.001$), but they were not statistically significant between the three and 4 weeks ($P = 0.48$). **(B)** Reveals that 6.5% of infected individuals remained positive after 2 weeks. There were no significant differences between the proportions after three and 4 weeks ($P = 0.32$).

genes 1.33 (95% CI 1.28–1.39), 1.28 (95% CI 1.22–1.34), and 1.27 (95% CI 1.14–1.40), respectively.

Figures 5A, B show that, as viral RNA levels decreased, trends of Ct values (ORF1ab gene and N gene) of four vaccinations were almost the same in asymptomatic infections. In contrast, Figures 5C, D illustrate that in mild diseases, the Ct values of unvaccinated individuals were significantly lower than those of the other three vaccination groups ($P < 0.001$), while Ct values did not differ significantly between the three vaccination groups. *P* values of paired comparisons for ORF1ab gene and N gene were all more than 0.05.

Discussion

As far as we know, few studies examined the effect of inactivated vaccines on viral RNA levels using continuous Ct values on a large population. In this study, we extracted continuous daily Ct values and associated medical risk information of 39,811 individuals from 174,371 Omicron-infected individuals, and conducted a comprehensive analysis to explore the impact of inactivated vaccines on the decrease of viral RNA levels. We found that inactivated vaccines could facilitate the decrease of viral RNA levels in infected individuals to some extent. Vaccinated individuals had lower viral RNA levels and turned negative faster than unvaccinated individuals in mild diseases. Booster vaccination outperformed full and partial vaccination. The effects are more evident and significant in mild diseases than in asymptomatic infections.

Vaccination can stimulate humoral immunity and cellular immune response. Neutralizing antibodies induced by vaccination might bind to the viral particles rendering those particles non-infectious. Although the vaccine does not prevent infection, it makes the immune system respond more rapidly and effectively when exposed to the pathogen again, helping to decrease the viral RNA levels and clear the virus (29). Individuals with mild diseases may be more likely to produce neutralizing antibody responses that facilitate decreasing viral RNA levels, making vaccination more effective in mild diseases than in asymptomatic infections, which is consistent with a previous report that neutralizing antibody has a high correlation with COVID-19 severity (30). mRNA vaccines were reported to be less effective in controlling and eliminating the Omicron virus than delta virus. Booster vaccine outperformed the two-dose and one-dose vaccines (13, 14, 21, 31–33). Our findings on the efficacy of the inactivated vaccine are consistent with previous research.

The population in Shanghai was mainly vaccinated with domestically-produced inactivated vaccine, which had relatively lower effectiveness in preventing SARS-CoV-2 infections due to their lower antibody-neutralizing responses compared to mRNA vaccines (21, 34). However, we found that, although the inactivated vaccine had a weak effect on turning negative (aRR slightly larger than

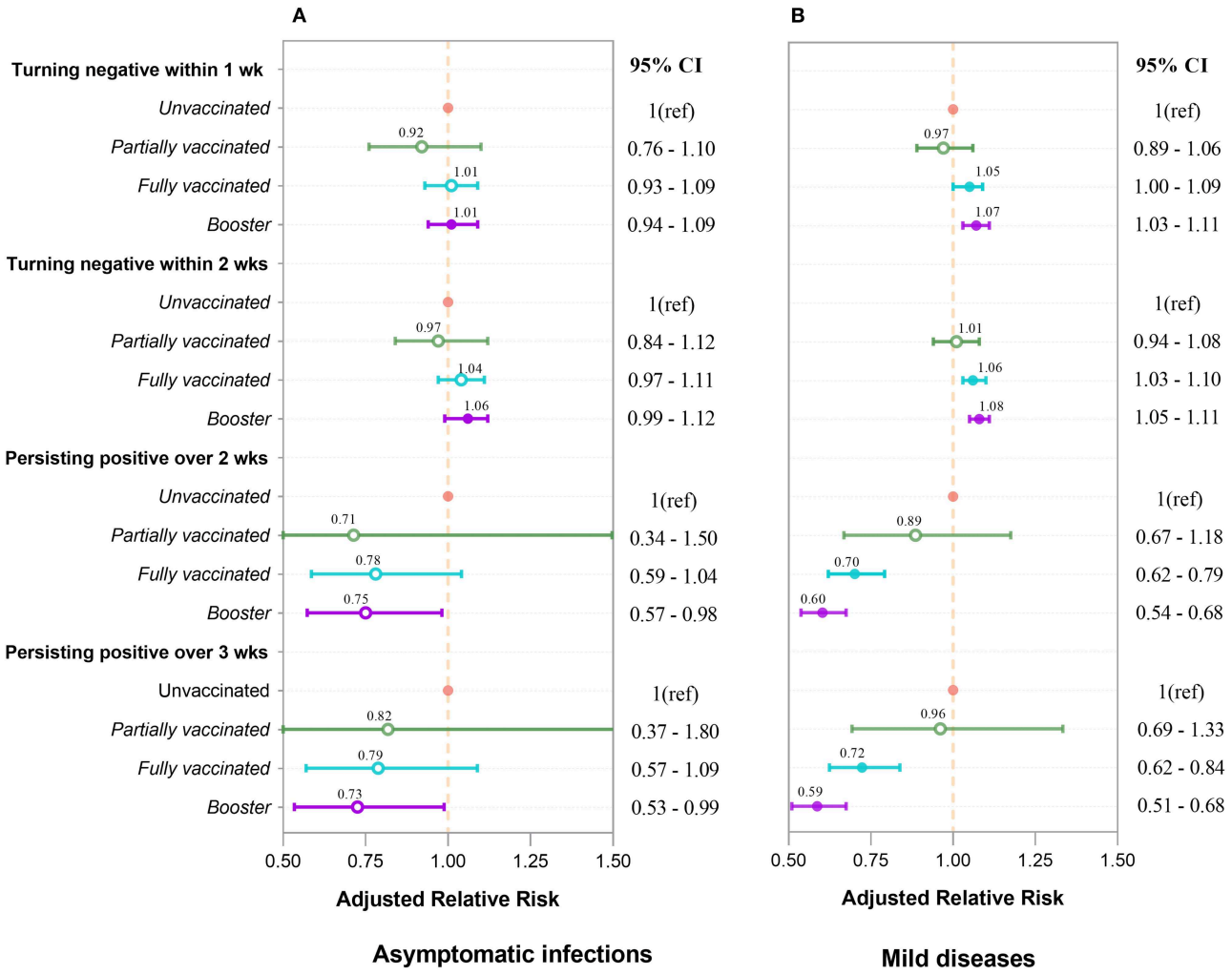

FIGURE 3
Adjusted relative risk for turning negative and persisting positive associated with four types of vaccination in asymptomatic infections **(A)** and mild diseases **(B)**. aRRs were analyzed by Cox regression and logistic regression. Inactivated vaccines accelerate negative conversion and prevent persisting positive. The booster's effect outperforms that of full vaccination, which outperforms that of partial vaccination. These effects are more pronounced and significant in mild diseases **(B)** than in asymptomatic infections **(A)**. Each point and interval in the figure represent the aRR and 95% confidence interval. Circles for points are filled if the value is significant ($P < 0.05$), and open otherwise.

1), it had a better effect on preventing the continuous positive. The booster had the lowest aRR of 0.59 (95% *CI*: 0.51–0.68).

As of April 18, 2022, 91.4% of the population in Shanghai were vaccinated full primary schedule of COVID-19 vaccine program, and 53.7% of them received a booster (35). We found that the full primary schedule in the FSH of NECC was 71.9% and the booster shot was only 42.7%, which were significantly lower ($P < 0.001$) than the total vaccination coverage rate in Shanghai. The fact that infected population usually had a lower vaccination rate and uninfected population had a higher vaccination rate may further suggest that vaccination could reduce the risk of Omicron infection.

The viral RNA level reflects the severity of individuals' infections. We found that Omicron-infected individuals were shown a consistent and rapid decay in viral RNA levels over time among four vaccination groups. Almost all of individuals' Ct values had exceeded 35 by about 1 week. The results are in line with previous researches (36, 37). We further discovered that trend lines of unvaccinated individuals were lower than those of vaccinated individuals for both ORF1ab gene and N gene (Figure 5). This tendency was still observed after 2 weeks: although four lines all went negative, the lines of unvaccinated individuals were slightly lower than those of the vaccinated individuals. These results further confirmed that vaccination decreased the viral RNA levels in individuals with Omicron infections (38).

In addition, we made a further analysis of the elderly (>60) and found that (Supplementary Table 2), compared to the whole population, the proportion of unvaccinated elderly people is higher (42.2%), which may be related to that some older people are inconvenient to move and cannot go to the vaccination site for vaccination. The proportions of older people with hypertension (24.4%) and diabetes (9.5%) were also higher. Meanwhile, the days of length of stay and days of negative conversion for older people were 9 (7–12) and 7 (5–9), respectively, which were higher than the

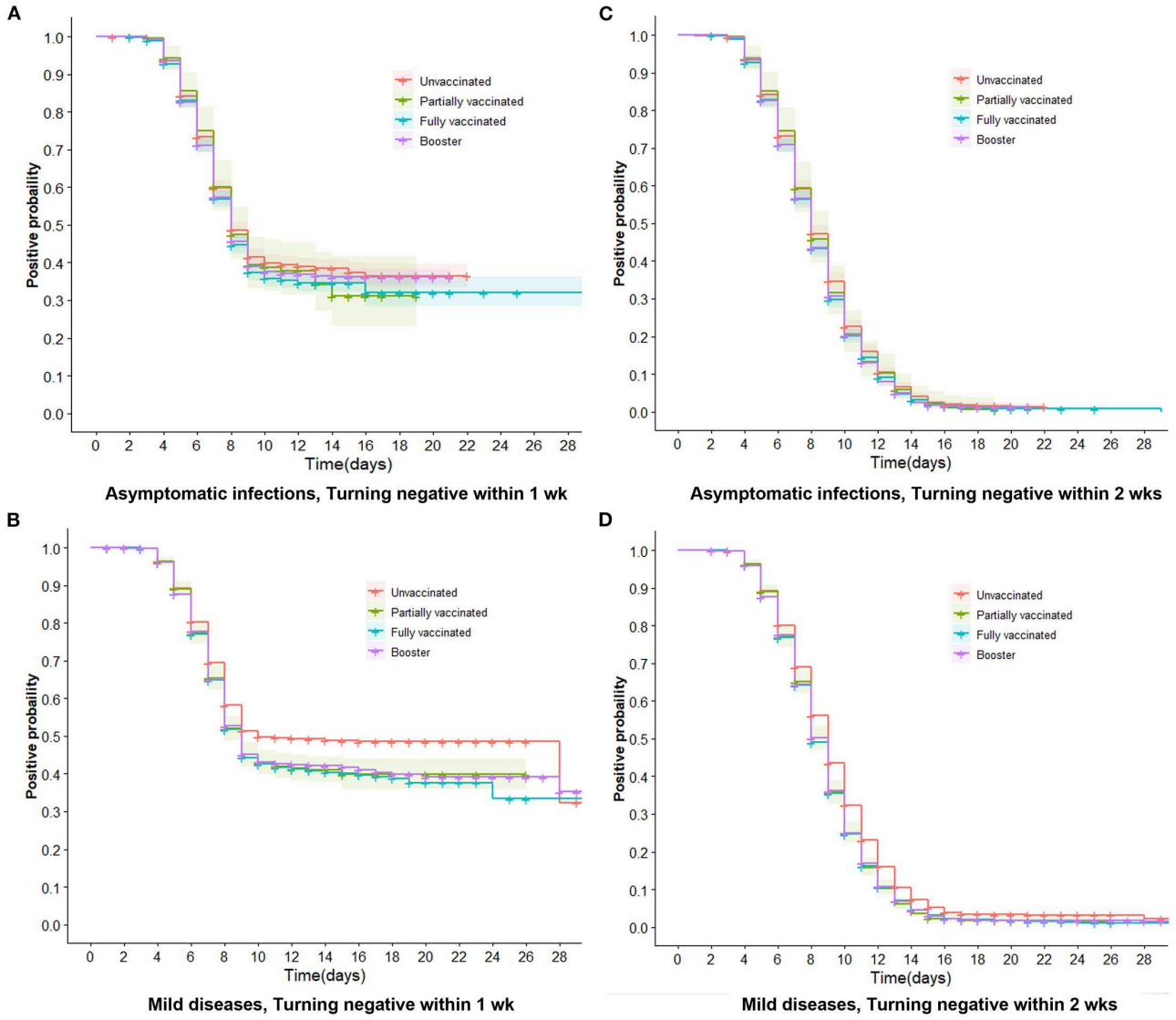

FIGURE 4
Trends of negative conversion within 1 week and 2 weeks **in** asymptomatic infections and mild diseases. Trends of negative conversion among four vaccine groups were analyzed by the Cox regression. Curves of four types of vaccinations are similar and close together for turning negative within 1 week and 2 weeks in asymptomatic infections **(A, C)**. The booster, full, and partial vaccination curves are lower than the unvaccinated curve **(B, D)**, indicating that vaccinated individuals have a faster decline in viral RNA levels than unvaccinated individuals ($P < 0.001$).

8 (7–11) and 6 (5–8) for the whole population. Nadir Ct values of ORF1ab gene and N gene were 27.4 (24.5–30.6) and 25.6 (22.8–28.8), lower than the whole population of 28.8 (25.7–32.0) and 25.6 (22.8–28.8), respectively. These may be associated with a lower immunity in the elderly than in other age groups, resulting in a higher viral load after infection, leading to longer days in hospital and days to negative conversion. Furthermore, we found that the effects of types of vaccines were largely consistent between the elderly and whole population. The effect was more pronounced in mild ($P < 0.001$) than in asymptomatic (ORF1ab gene, $P = 0.16$; N gene, $P = 0.06$), and the booster was more effective than the Fully vaccinated and unvaccinated.

Compared to other studies, this paper has some advantages. First, to control the epidemic as much as possible, Shanghai conducted continuous nucleic acid tests for the whole population. Once a positive case was screened, isolation was implemented immediately. Therefore, data on infections who were admitted to the FSH within 2 days can be obtained. Second, Ct levels, symptom information, and associated influential factors were collected for continuous daily monitoring after admission. Third, infected individuals in the FSH only received oral medications for their complications and were mostly without antiviral and immune-boosting drugs, so it is reasonable to observe the impact of inactivated vaccines on viral RNA levels in Omicron-infected individuals. Finally, to our knowledge, the number of Omicron infections in our study is the largest report to date.

Some limitations must be acknowledged. Firstly, although the FSH is the largest shelter hospital in Shanghai, these data are only

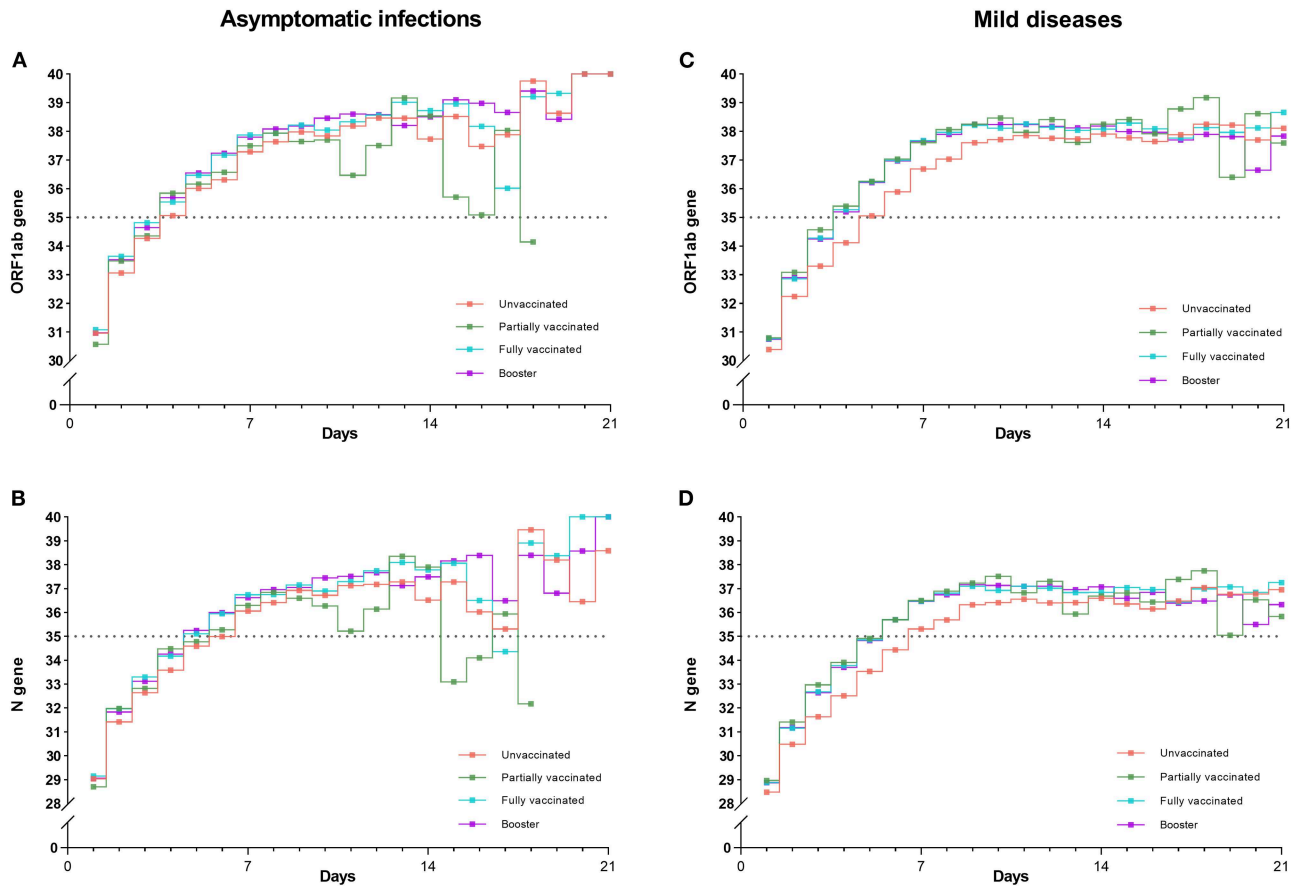

FIGURE 5
Trends of mean Ct values (ORF1ab and N genes) for four vaccinations in asymptomatic infections and mild diseases. Trends of Ct values for four vaccinations were almost the same in asymptomatic infections **(A, B)**. In contrast, the Ct values of the unvaccinated individuals were significantly lower than those of the other three vaccination groups in mild diseases **(C, D)** ($P < 0.001$).

from the FSH and not from all infected individuals in Shanghai, which may lead to selection bias and affect the interpretation of results. Secondly, all individuals admitted in this study were asymptomatic infections and mild diseases, and there were no infected individuals with severe infections. Thirdly, we lack the data on the duration time of latest vaccination to admission. Although we can estimate that the duration time after vaccination is approximately the same within different vaccination groups based on Shanghai's immunization strategies, this would affect the interpretation of results to some extent when comparing vaccine effects between different groups. Lastly, no CT images of the patient's chest or other laboratory tests are not included in the data.

Conclusions

Inactivated vaccinations accelerate the decrease of viral RNA levels in Omicron-infected individuals. Compared to unvaccinated individuals, vaccinated individuals have a lower viral RNA level, a faster negative conversion, and a smaller proportion of persisting positives. The impact of inactivated vaccines is more obvious and significant in mild diseases than in asymptomatic infections. This paper could provide the calibration of future pandemic control strategies based on inactivated vaccines in China.

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 Clinical Research and Ethics Committee of Tangdu Hospital of the Fourth Military Medical University (No. 202205-01). The patients/participants provided their written informed consent to participate in this study.

Author contributions

LS and WenzK conceived, designed, and supervised the study. PY, BD, and WenK co-prepared the first draft of the manuscript. PY analyzed the data and prepared the figures. XL, TW, RL, MP, YL, LW, YC, SY, MW, and HG provided critical revision of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1107343/full#supplementary-material

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Antibody response to different COVID-19 vaccines among the migrant workers of Bangladesh

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Background: Due to the ongoing COVID-19 pandemic, various host countries such as Singapore, imposed entry requirements for migrant workers including pre-departure COVID-19 seroconversion proof. To combat COVID-19 worldwide, several vaccines have acquired conditional approval. This study sought to assess antibody levels after immunization with different COVID-19 vaccines among the migrant workers of Bangladesh.

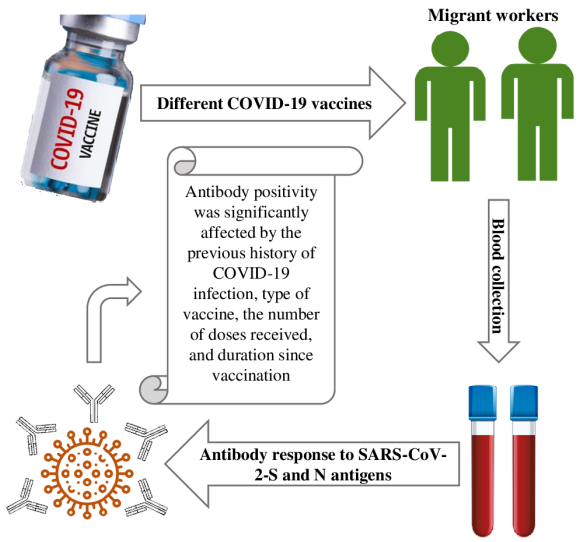
Methods: Venous blood samples were collected from migrant workers who were vaccinated with different COVID-19 vaccines (n=675). Antibodies to SARS-CoV-2 spike protein (S) and nucleocapsid protein (N) were determined using Roche Elecsys® Anti-SARS-CoV-2 S and N immunoassay, respectively.

Results: All participants receiving COVID-19 vaccines showed antibodies to S-protein, while 91.36% were positive for N-specific antibodies. The highest anti-S antibody titers were found among the workers who completed booster doses (13327 U/mL), received mRNA vaccines Moderna/Spikevax (9459 U/mL) or Pfizer-BioNTech/Comirnaty (9181 U/mL), and reported SARS-CoV-2 infection in the last six months (8849 U/mL). The median anti-S antibody titers in the first month since the last vaccination was 8184 U/mL, which declined to 5094 U/mL at the end of six months. A strong correlation of anti-S antibodies was found with past SARS-CoV-2 infection ($p < 0.001$) and the type of vaccines received ($p < 0.001$) in the workers.

Conclusion: Bangladeshi migrant workers receiving booster doses of vaccine, vaccinated with mRNA vaccines, and having past SARS-CoV-2 infection, mounted higher antibody responses. However, antibody levels waned with time. These findings suggest a need for further booster doses, preferably with mRNA vaccines for migrant workers before reaching host countries.

KEYWORDS

SARS-CoV-2, seroconversion, anti-spike-antibody, mRNA vaccines, booster dose, Bangladeshi migrant workers



GRAPHICAL ABSTRACT

Introduction

The first instance of COVID-19, a class of acute atypical respiratory illnesses in humans, was discovered in Wuhan, China, in December 2019 (1). The full extent of COVID-19 symptoms ranges from a benign, self-limiting respiratory condition to a merciless progressive pneumonia, multiple organ malfunction, and death (2–4). As per the World Health Organization (WHO), 753,823,259 cases including 6,814,976 deaths of COVID-19 were reported globally and in Bangladesh, there have been 2,037,578 confirmed cases of COVID-19 with 29,442 deaths, as of February 02, 2023 (5). To combat COVID-19 globally, several vaccines have acquired conditional approval (6). On January 27, 2021, COVID-19 vaccination began in Bangladesh with AstraZeneca (ChAdOx1-S/Covishield; manufactured by Serum Institute of India Pvt Ltd). To date, eight additional COVID-19 vaccines, i.e. Moderna/Spikevax (mRNA-1273), Gamaleya (Sputnik V), Pfizer-BioNTech/Comirnaty (BNT162b2), Sinopharm (BBIBP-CorV/Vero Cells), Johnson & Johnson/Janssen (Ad26.COV2.S), Oxford AstraZeneca: Vaxzevria, Sinovac (CoronaVac) and Novavax/COVOVAX (NVX-CoV2373) have received approval from the Government of Bangladesh (7). The Pfizer-BioNTech and Moderna vaccines use lipid nanoparticles to deliver spike-encoding mRNA. Adenovirus vector vaccine includes AstraZeneca, Gamaleya, and Johnson & Johnson/Janssen, while a protein subunit vaccine represents Novavax/COVOVAX. All these vaccines use the spike protein of the SARS-CoV-2 that first appeared in Wuhan, China, as the focal immunogen. To compare, the Sinopharm (BBIBP-CorV) and Sinovac (CoronaVac) are inactivated whole-virus vaccines that contain diverse viral proteins with possibilities of broadening immune protection beyond the spike-protein-specific immune response against the variants of concern (VOCs).

Currently 13 million Bangladeshis are engaged in various professions abroad. One of the key cornerstones of the Bangladeshi economy is the migrant labor force, which accounts for more than 12% of the total Gross Domestic Product (GDP) and 9% of all employment in Bangladesh (8–11). Almost all developed countries in the world implemented travel restrictions and border closures for migrant workers due to the coronavirus outbreak (12). Migrant workers have been caught between health and food crisis, the uncertainty of job retention, and have a continual desire to return to work for their livelihood (12). Restrictions on the entry of migrant workers has a significant negative impact on Singapore's construction, marine, and process (CMP) sectors as well as employment generation, remittance earning and economic growth in Bangladesh. The leading associations of the CMP sectors began an industry-led pilot program in June 2021 to address the labor shortfall and aid in industry recovery (13). The pilot program relied on testing the workers using a COVID-19 testing regime over a 14-day period at specific in-house quarantine facilities in their home countries before their travel to Singapore in order to ensure a consistent intake of migrant workers in a safe and secure manner (13). During the pilot program, workers underwent rapid antigen tests, COVID-19 RT-PCR and serology tests to determine their current or past infection and antibody response after vaccination to COVID-19 (14, 15).

To measure antibodies to a range of SARS-CoV-2 antigens, such as spike protein (S) and nucleocapsid protein (N), several serological tests have been developed (16, 17). The S protein of SARS-CoV-2 contains a receptor-binding domain (RBD), which binds to angiotensin-converting enzyme 2 (ACE2) receptor located on the surface of the host cell, facilitating the entry of virus into the cell. Thus, the S protein is a key target for virus inactivation and assessment of immune response after vaccination (18). Associated with the viral genome, the nucleocapsid (N) protein is generated in enormous amounts in the early stages of infection. There is no cross-reactivity seen with N-specific antibodies even with closely related

viruses (19). Both proteins are used as essential antigens in COVID-19 serology testing because of their strong immunogenicity (20).

The socioeconomic effects of COVID-19 on Bangladeshi migrant workers have been assessed in few studies (9, 21). Few other researchers have reported the immunological response to SARS-CoV-2 infection and the COVID-19 vaccination in Bangladeshi population (22–24). However, there is no report on seroconversion or post-vaccine COVID-19 antibody response in migrant workers. In the present study, we aimed to assess post-vaccination COVID-19 antibody response in Bangladeshi migrant workers to facilitate their migration to host countries. We also aimed to observe how vaccine types and previous SARS-CoV-2 infections influenced the antibody response. To achieve the objective, we evaluated SARS-CoV-2-S and SARS-CoV-2-N antibody responses in Singapore-outgoing Bangladeshi migrant workers.

Methods

Study design and population

We conducted a cross-sectional study from December 2021 to February 2022 among the legal migrant workers of different districts of Bangladesh, who were eligible to participate in the pre-departure pilot program conducted by an international health service provider assigned by the Singapore Government (Figure 1). Migrant workers who met the following inclusion criteria were chosen for participation in the pilot program: holding a Bureau of Manpower Employment and Training (BMET) emigration clearance card, valid Bangladeshi passports, and a previous COVID-19 vaccine certificate. Those who failed to meet the inclusion criteria were excluded from the study.

Study approval and ethical consideration

A written approval was obtained from the Ministry of Expatriates' Welfare and Overseas Employment (MoEWOE), Bangladesh to conduct this study. The Institutional Review Board of International Centre for Diarrhoeal Disease Research, Bangladesh reviewed and approved the study protocol under the protocol # PR-21093. The ethical principles adhered to the present Helsinki Declaration as well as the statutory needs of the country. Prior to enrolment, each participant provided written informed consent.

Enrolment of study participants and data collection

A Singapore outgoing migrant worker list was obtained from a Bangladesh Govt. authorized recruiting agency in Dhaka. The workers' list contained their date of birth, passport number, home district, mobile number, quarantine center's address, check-in date, and time. Migrant workers arrived at designated quarantine centers, which were located in several hotels in Dhaka, on the appointed date and time to participate in the host country's required 14 days pilot program. Using a structured questionnaire, we collected information related to the date of vaccination for different doses, the type of COVID-19 vaccine received (from the COVID-19 vaccine card) and the self-reported history of SARS-CoV-2 infection of each worker.

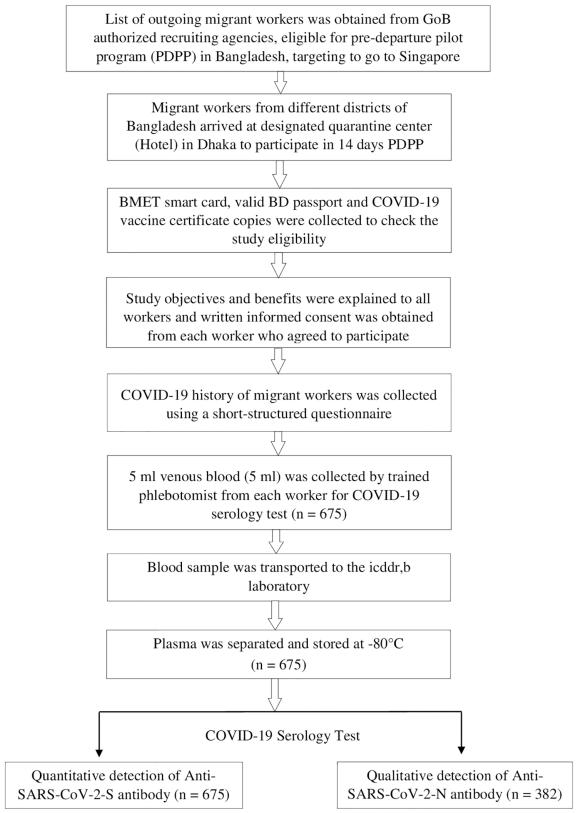


FIGURE 1
Flowchart showing enrollment, data and sample collection, and evaluation of COVID-19 post vaccine antibody response among the migrant workers, Bangladesh, 2021-22.

Blood sample collection

Single venous blood (~5 mL) was collected from participants (n=675) in anti-coagulant containing tubes (BD vacutainer® Sodium heparin). All collected samples were transported in a portable cool box to the icddr,b laboratory within three hours of collection for further processing and laboratory analysis.

Laboratory analysis

Plasma was separated from heparinized whole blood by centrifuging at 1900g at room temperature using a centrifuge (Eppendorf® 5702R, Hamburg, Germany). After separation, plasma samples were tested for antibodies to SARS-CoV-2-S (n=675) and SARS-CoV-2-N (n=382) antigens by electrochemiluminescence immunoassay (ECLIA) using Elecsys Anti-SARS-CoV-2 S and Elecsys Anti-SARS-CoV-2 test kit (Roche Diagnostics, Indiana, USA). Both assays were carried out using the Roche Cobas-e601

immunoassay analyzer (Roche Diagnostics GmbH, Mannheim) following the manufacturer's guidelines. In antibody detection, ECLIA can be compatible with the plaque reduction neutralization test (PRNT), microneutralization test (MNT), Pseudovirus Neutralization Assay (PNA) as revealed in the previous studies (24–26).

Anti–SARS–CoV–2-N antibody test

Elecsys Anti–SARS–CoV–2 is a qualitative immunoassay designed to detect antibodies (IgG and IgM) to the SARS–CoV–2 nucleocapsid (N) antigen in human serum/plasma. The software dictated the results automatically by comparing the electrochemiluminescence signal acquired from the reaction product of the sample with the signal of the previously calibrated cutoff value. The results were derived as sample/cutoff signal (COI) values and were qualitatively assessed as non-reactive (COI < 1.0; negative) or reactive (COI ≥ 1.0; positive). PreciControl Anti–SARS–CoV–2 method of Roche Diagnostics, USA was used for quality control.

Anti–SARS–CoV–2-S antibody test

Elecsys Anti–SARS–CoV–2 S is a quantitative immunoassay for the detection of antibodies to the receptor-binding domain (RBD) of the spike (S) antigen of SARS-CoV-2 in human serum or plasma. Anti –SARS–CoV–2 IgG, IgA and IgM antibodies in serum or plasma bind to specific recombinant antigens of SARS–CoV–2 S–RBD in a double-antigen sandwich assay format allowing quantitative determination of high-affinity antibodies through electrochemiluminescence technique. Results were obtained using a standard curve provided by the reagent barcode or e-barcode and a calibration curve that is instrument-specifically developed by two-point calibration. The analyte concentration of each sample was automatically computed by the analyzer in Units per milliliter (U/mL) and the numerical values were classified as "positive" (≥ 0.80 U/mL) and as "negative" (< 0.8 U/mL).

The WHO International Standard for anti-SARS-CoV-2 immunoglobulin (human), NIBSC code: 20/136, behaves identically to the internal Roche standard, with a correlation coefficient r = 0.9996 between Limit of Quantitation and 1000 BAU/mL (Binding Antibody Units (BAU)). Hence, the numeric results in U/mL of the Elecsys Anti SARS-CoV-2 S assay and BAU/mL are equivalent.

Statistical analysis

Stata 14.2 (StataCorp LLC, College Station, Texas, USA) and R Studio version 1.4.1106 were used for statistical analysis and graph preparation. To determine whether the data was normal, the Kolmogorov-Smirnov test and histograms with normal curves were used. The antibody level was expressed as the median and interquartile range (IQR) and visualized using Boxplots with jitter, whilst categorical data were expressed as proportions/percentages. The Mann-Whitney U test and Kruskal Wallis test were used to compare median antibody levels between groups and time intervals since the last vaccination. We also used the Poisson regression model with robust standard error to evaluate the effects of multiple factors on antibody response to COVID-19 vaccination. The model was adjusted for co-variates such as age, vaccine doses, vaccine types, past SARS-CoV-2 infection, time since last vaccination. Scatterplots were constructed between age and antibody titers. The correlation coefficient of immune response with age, SARS-CoV-2 infection, number of vaccine doses received, different vaccine types, and anti-N antibody response was calculated using Spearman's rank correlation. P-value < 0.05 represents statistical significance.

Results

Age, vaccination, and SARS-CoV-2 infection history of study participants

Table 1 shows the sex, age category, COVID-19 vaccination status, SARS-CoV-2 infection history of the migrant workers, and the time interval between the last dose of COVID-19 vaccination and SARS-CoV-2 antibody testing. Migrant workers included in the study were all male. The mean age of the workers was 32 years, and most were between 18-40 years. More than 86% of the workers received double doses of vaccines, 11.1% received a single dose, and only 2.4% received a booster dose. About one-third of the migrant workers were vaccinated with Sinopharm/BBIBP-CorV (33%), followed by Pfizer/Comirnaty (28%), Moderna/Spikevax (26%), AstraZeneca/Covishield (13%), and mixed vaccine doses (0.60%). The median time interval between the last dose of COVID-19 vaccination and blood sample collection ranged from 32 days (for workers receiving a single dose of vaccine) to 109 days (for workers receiving double doses of vaccine). About 14% of the migrant workers reported that they were infected with SARS-CoV-2 in the last six months.

Anti-S seroconversion status based on age, previous infection with SARS-CoV-2 and number of doses

All workers were positive for S- antibodies and the average (median) titer of anti-S antibody was 6437 U/mL (IQR: 9713 U/mL, Range: 28.77 – 100000 U/mL). There was no discernible difference in S-antibody titers between the workers in the two age groups (18-40 years vs 41-51 years). Previous infection with SARS-CoV-2 had profound effect on vaccine-induced S-antibody titers. The S-antibody titer in SARS-CoV-2 infected workers was significantly higher than that in uninfected workers was ($p < 0.001$) (Table 2). Again, the S-antibody titer was significantly higher in the workers who were anti-N antibody positive (5193 U/mL) compared to the workers who were anti-N antibody negative 2357 U/mL (Figure 2). Multivariate analysis also showed significantly higher antibody response in SARS-CoV-2 infected (3347 U/mL) and anti-N antibody positive workers (2375 U/mL) compared to uninfected and anti-N antibody negative workers (Table 3). Among the SARS-CoV-2 infected workers (self-reported; 14%), the highest antibody concentration (28563 U/mL) was detected in booster dose (3rd dose) recipients followed by workers receiving double doses (10416 U/mL) and single dose (6410 U/mL) of the vaccine. Among the uninfected workers (86%), the highest anti-S titer (13327 U/mL) was noted after receiving booster dose, followed by 8499 U/mL after

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TABLE 1 Age, sex, vaccination, and SARS-CoV-2 infection history of the migrant workers.

Characteristics	All workers (n=675)
Mean age (SD)	**32 (7.44)**
Age category (year)	n (%)
18-40	588 (87.11)
41-51	87 (12.89)
Sex	
Male	675 (100)
Different COVID-19 vaccines received by the workers	
Sinopharm (BBIBP-CorV)	224 (33.19)
Pfizer (Comirnaty)	188 (27.85)
Moderna (Spikevax)	174 (25.78)
AstraZeneca (Covishield)	85 (12.59)
Mixed vaccine (S/M/P) *	4 (0.59)
Number of vaccine doses received by the workers	
Single dose	75 (11.11)
Double dose	584 (86.52)
Booster dose	16 (2.37)
Past SARS-CoV-2 infection	
Yes	94 (13.93)
No	581 (86.07)
Frequency of reported past infection	
Once	94 (100)
Duration of reported past infection	Median (IQR)
Month	6 (16)
Time differences between last vaccination and blood collection for COVID-19 sero-survey (Day) collection for COVID-19 sero-survey (Day)	Median (IQR)
Overall	106 (101.50)
Single dose recipients	32 (208.50)
Double dose recipients	109 (92.30)
Booster recipients	90 (66.80)

*Spikevax plus Comirnaty and Comirnaty plus Covishield for double dose vaccine recipients; (Spikevax plus BBIBP-CorV and BBIBP-CorV) and (BBIBP-CorV plus BBIBP-CorV and Comirnaty) for booster dose vaccine recipients.

single dose and 5273 U/mL after double dose (Table 2). Among the infected workers who were vaccinated with SARS-CoV-2 S antigen-targeted vaccines, anti-S antibody concentration were significantly higher in participants receiving booster dose compared to participants receiving single and double doses. Again, double dose recipients showed significantly higher antibody response than single dose recipients. Similar response was observed for booster dose recipients in uninfected workers, however, double dose recipients showed lower response than single dose recipients (Figure 3). Significantly higher antibody titers in booster dose and double dose recipients compared to single dose recipients were also evident in multivariate Poisson regression analysis (Table 3).

Anti-S seroconversion status with different vaccine types

For the migrant workers who received double doses of COVID-19 vaccine, the highest anti-S antibody titer was found for Moderna/Spikevax (9459 U/mL) and Pfizer/Comirnaty (9181 U/mL) vaccines, followed by AstraZeneca/Covishield (5601 U/mL) and Sinopharm/BBIBP-CorV (1308 U/mL) vaccines. m-RNA base vaccines Moderna/Spikevax and Pfizer-BioNTech/Comirnaty elicited significantly higher anti-S antibody titers compared to vector-based Astrazeneca/Covishield and Sinopharm/BBIBP-CorV vaccines. Between Astrazeneca/Covishield and Sinopharm/BBIBP-CorV vaccinated participants, anti-S antibody concentration was significantly higher for the AstraZeneca/Covishield (Figure 4). Similarly, in multivariate analysis, migrant workers receiving AstraZeneca/Covishield vaccine showed significantly lower response compared to Moderna/Spikevax and Pfizer/Comirnaty vaccine recipients, but the response was significantly higher compared to Sinopharm/BBIBP-CorV vaccine recipients (Table 3). Among the workers who received booster doses, the highest anti-S antibody level (28563 U/mL) was found in Moderna/Spikevax vaccine recipients followed by 25498 U/mL in Pfizer/Comirnaty, 10023 U/mL in mixed vaccines, and 7551 U/mL in AstraZeneca/Covishield vaccine recipients (Supplementary Table 1).

Anti-S seropositivity at different time intervals since last vaccination

Among migrant workers receiving single dose of vaccine, anti-S antibody titer declined over time since last vaccination (from 9878 U/mL within one month to 7076 U/mL at six months intervals and 6500 U/mL after greater than six months). For the workers who were given double doses, the antibody titer was 8184 U/mL within one month of vaccination, significantly reduced to 5094 U/mL at six months interval and increased again at later time point (11861 U/mL). Among booster vaccine recipients, the antibody titer was increased from 7551 U/mL within one month of vaccination to 25120 U/mL at the six months interval (Figure 5).

Correlation of anti-S antibody with age, vaccine dose, anti-N antibody response, vaccine types, and SARS-CoV-2 infection

Anti-S titers were highly correlated with different types of vaccines received ($r = -0.441$, $p<0.001$), and moderately correlated with past SARS-CoV-2 infection ($r = 0.183$, $p<0.001$) and anti-N antibody response of the workers ($r = 0.108$, $p = 0.034$).

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TABLE 2 COVID-19 post vaccine Anti–SARS–CoV–2–S antibody response among the migrant workers, Bangladesh, 2021-22.

Variables	Anti–SARS–CoV–2–S antibody (N=675) [U/mL]			
Median (IQR)	Infected (n=94)		Non-infected (n=581)	
	8849 (13997)		6013 (9525)	
P value*	<0.001			
Age (year)	Median (IQR)	P value†	Median (IQR)	P value†
18-40	8849 (16750)	0.755	6209 (9671)	0.775
41-51	9296 (7702)		4850 (9315)	
Vaccine doses				
Single dose	6410 (10304)	0.006	8499 (7270)	0.001
Double dose	10416 (12042)		5273 (9492)	
Booster dose	28563 (73192)		13327 (15568)	

*P value is generated between SARS-CoV-2 infected and non-infected recipients by Mann Whitney U test.
†P value is generated by Kruskal Wallis test.

Anti-S antibodies did not show a significant correlation with age (r = 0.048, p = 0.212), and the number of vaccine doses received by the workers (r = 0.006, p = 0.875) (Figure 6).

Discussion

To our knowledge, this is the first scientific study assessing antibody response to different COVID-19 vaccines among Bangladeshi migrant workers. This study demonstrated higher anti-S antibody titers in workers receiving Moderna/Spikevax and Pfizer/Comirnaty vaccines compared to AstraZeneca/Covishield and Sinopharm/BBIBP-CorV vaccine recipients. In addition, higher anti-S antibody titers were observed in migrant workers who have received booster doses of vaccine as well as among those previously infected with SARS-CoV-2. Furthermore, antibody titers were found to decline after six months since the last vaccination.

Our result suggested that mRNA vaccines (Moderna/Spikevax and Pfizer/Comirnaty) elicited significantly higher anti-S antibody titers in Bangladeshi migrant workers compared to the viral vector-based vaccine (AstraZeneca/Covishield) and inactivated vaccine (Sinopharm/BBIBP-CorV). This result is consistent with findings from several immune response studies on COVID-19 vaccines in human populations (27–31). In a recently conducted community-based study in Bangladesh, mRNA vaccine recipients showed higher antibody response than adenovector and killed whole-virus vaccine recipients (24). The S protein of SARS-CoV-2 is used as the immunogen in the Pfizer/Comirnaty, Moderna/Spikevax, and AstraZeneca/Covishield vaccines to generate anti-S antibodies that block the S protein's binding to host cell (32–34). The AstraZeneca/Covishield vaccine uses S-protein coding DNA inserted in a chimpanzee adenovirus vector (35). Conversely, the Moderna/Spikevax and Pfizer/Comirnaty vaccines employ codon-optimized mRNA sequences supplied to the host cell *via* lipid nanoparticles (LNPs) that is directly translated into full-length S protein (36). Higher antibody response of mRNA vaccines compared to adenovector vaccines may be explained by quick mRNA transfer into the host cell by LNP, effective S protein synthesis, and stabilizing alterations blocking the structural change in the S protein (36–38). In the case of the whole cell Sinopharm/BBIBP-CorV vaccine, the concurrent decoupling of S1 and synthesis of the post-fusion S by inactivation and purification methods may lessen the antibody response (36). mRNA vaccines may provide superior protection in comparison to inactivated and vector-based vaccines through higher antibody response (35, 39).

Antibody titers were found to be higher in the workers receiving booster doses compared to double and single doses (p<0.001) vaccine recipient, irrespective of the previous history of SARS-

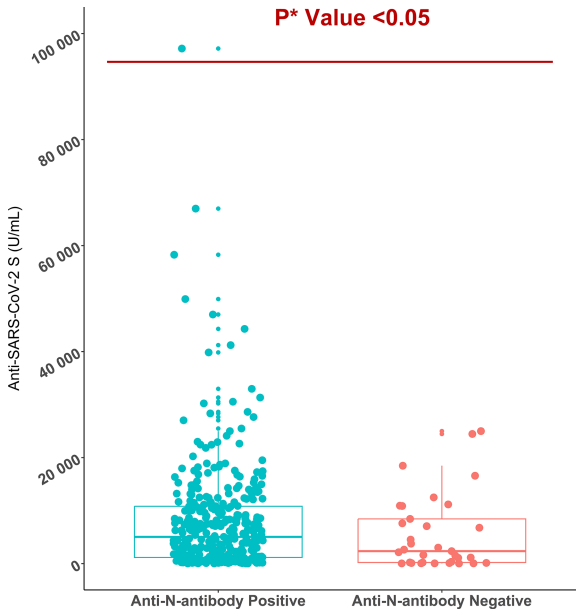

FIGURE 2
Anti-SARS-CoV-2 S antibody titers in anti-SARS-CoV-2 N antibody positive (n=349) and negative migrant workers (n=33). *P value is generated by Poisson regression model and the model was adjusted for age, vaccine types, and time since last vaccination.

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TABLE 3 Multivariate analysis for Anti-S antibody response to COVID-19 vaccines among the migrant workers, Bangladesh, 2021-22.

Variables	Coefficient	P-value*	95% CI
Vaccine Doses			
Single dose	Ref		
Double dose	1181.428	<0.001	(1153.132, 1209.723)
Booster dose	17839.58	<0.001	(17741.03, 17938.14)
Different COVID-19 Vaccines			
AstraZeneca (Covishield)	Ref		
Moderna (Spikevax)	5679.379	<0.001	(5648.231, 5710.527)
Pfizer (Comirnaty)	3557	<0.001	(3526.803, 3587.197)
Sinopharm (BBIBP-CorV)	-6234.395	<0.001	(-6261.249, -6207.542)
Past SARS-CoV-2 infection			
No	Ref		
Yes	3347.866	<0.001	(3321.804, 3373.928)
Anti-N Antibody			
Negative	Ref		
Positive	2375.98	<0.001	(2348.739, 2403.221)

*P value is generated by Poisson regression.

CoV-2 infection. Immunization with two doses of the vaccine also mounted a higher antibody response than a single dose in the workers with the exception of non-infected vaccine recipients. These findings were similar to previously studied vaccine dose-based antibody responses among healthcare workers (HCWs) and healthy individuals in Italy and India (40, 41). Ward H et al., also cited greater antibody response following vaccination in double-dose vaccine recipients than single-dose recipients among general populations in England (42). A study conducted among healthy individuals in the USA reported that all participants experienced significant elevation of measured antibodies following the second vaccination dosage, even those who had a weak or negative reaction to the first dose/shot of vaccine (43). A statistically significant relationship of antibody response was also found between single and double dose vaccine recipients in the Bangladeshi population (44). The lower anti-S antibody of non-infected double dose vaccine recipients than single dose recipients in our study may have resulted from longer time interval between last vaccination and sample collection (Table 1).

Age is one of the most crucial factors affecting the antibody response. Age-related declines in T-cell-derived antibody production and B-lymphocyte formation may result in a diminished antibody response to infectious pathogens or vaccinations (45). The post-vaccination antibody response was found to be inversely proportional to age in numerous studies conducted following immunizations against pneumococcus,

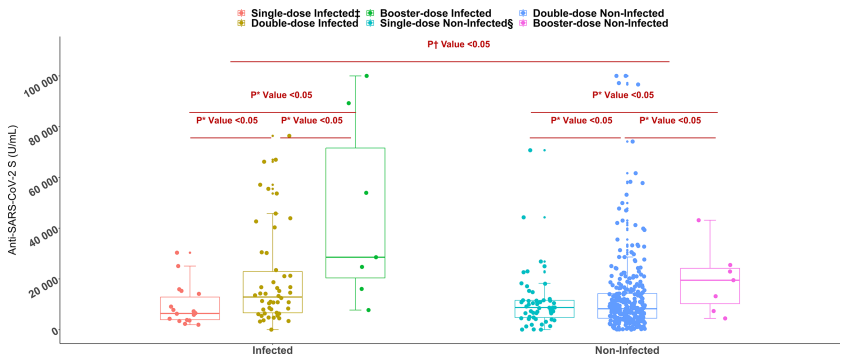

FIGURE 3
Anti-SARS-CoV-2 S antibody titers among single, double, and booster dose recipients of SARS-CoV-2 S antigen targeted vaccines (n= 447, Moderna/Spikevax, Pfizer/Comirnaty, AstraZeneca/Covishield). *P value is generated by Poisson regression model and the model was adjusted for age, vaccine types, and time since last vaccination. †P value is generated by Mann Whitney U test. ‡Infected = workers infected with SARS-CoV-2, §Non-Infected = workers who didn't infect with SARS-CoV-2.

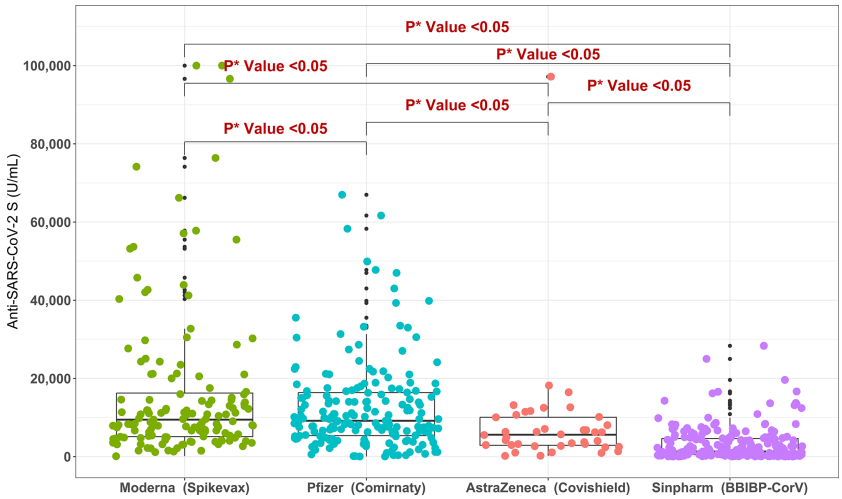

FIGURE 4
Anti-SARS-CoV-2 S antibody titers in migrant workers receiving double doses of different COVID-19 vaccine types. *P value is generated by Poisson regression model and the model was adjusted for age, past SARS-CoV-2 infection and time since last vaccination.

tetanus, hepatitis A, hepatitis B, influenza, tick-borne encephalitis (TBE), and SARS-CoV-2 (16, 46–48). However, age and antibody production did not have a significant relationship in our study. The non-significant relationship between age and antibody in our study is most likely due to narrow age range (18-51 years). In our study, we did not have elderly participants (> 60 years) because the study participants were working-age population departing Singapore. Observation of our study blends well with studies carried out in Turkey and Egypt (49, 50).

We found the highest anti-S antibody in workers who reported previous SARS-CoV-2 infection compared to the workers who did not report SARS-CoV-2 infection. A strong correlation between past SARS-CoV-2 infection and higher anti-S antibodies was also seen. Recently, in five major divisions of Bangladesh, persons with previous SARS-CoV-2 infection were shown to have higher post-vaccine immunological responses compared to non-infected individuals (24). In another study in the Bangladeshi population, after immunization, those with a record of SARS-CoV-2 infection had six times higher antibody titers than those without a history of infection (51). Healthcare workers in Italy also showed a 10 to 100-fold rise in anti-S antibody and neutralizing antibody titers who had already contracted SARS-CoV-2 (52). Collectively, these studies including ours strongly suggest the role of immunological memory after a natural infection or vaccination in generating rapid and high

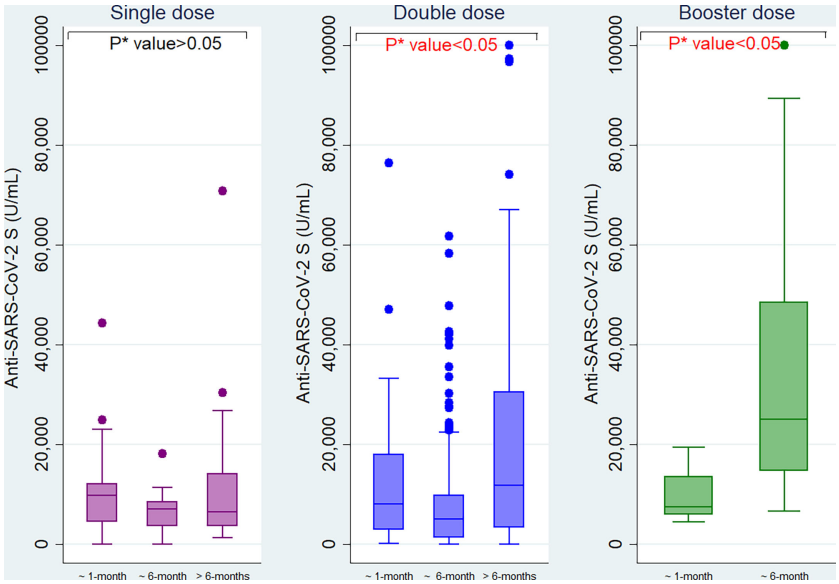

FIGURE 5
Anti–SARS–CoV–2 S antibody titers among the migrant workers at different time intervals since last vaccination. *P value is generated by Kruskal Wallis test.

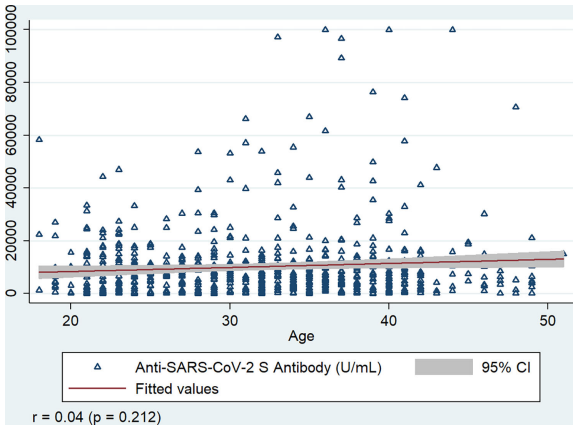

FIGURE 6
Correlation of Anti-SARS-CoV-2 S antibody with age of the workers, Bangladesh, 2021-22. r = Spearman's rho, P value is generated by Spearman's rank correlation.

response to subsequent exposure. Conversely, another Italian investigation found no correlation between the level of anti-S antibodies and a prior SARS-CoV-2 infection (53).

Our study demonstrated that antibody titers were significantly reduced at a six-month interval since the last COVID-19 vaccination among the workers. Consistent with this finding, community-based COVID-19 sero-epidemiological studies among healthy blood donors and healthcare workers in Hong Kong and South Korea revealed reduction of antibody levels over six months following Comirnaty and CoronaVac vaccination (54, 55). It was also reported that after vaccination with double doses of Comirnaty vaccine, antibody levels declined substantially at 6 months intervals since the last vaccination (56, 57). Several other studies have reported a waning of antibody response to different vaccines with time and highlighted the importance of providing booster doses (58–60). In our study, the rise in antibody titers after six months post-vaccination in double and booster dose recipients is likely due to a breakthrough infection of SARS-CoV-2 at that time.

Study limitations and way forward

Our study has several limitations. The major limitation of our study was that data and samples were collected at a single-time point, as the migrant workers participated in the study just before departure to the host country. Consequently, we were unable to assess the participants' long-term antibody response and safety profile. It is known that antibody level can vary based on sex (61, 62); our limitation was that we had only male participants as only male migrant workers went through the Singapore outgoing pilot program during the study period. Another limitation was the small sample size in some categories, e.g., the single and booster dose recipients of different COVID-19 vaccines; and infected people within different vaccine types. Moreover, the infection data of SARS-CoV-2 could not be confirmed by PCR.

Conclusion

A robust antibody response was observed among the migrant workers who reported past SARS-CoV-2 infection, were vaccinated with mRNA vaccines, and completed booster doses. However, antibody level significantly decreased over six months since the last vaccination, which warrants provision of further booster doses among the migrant workers, specially before departure. Regular monitoring of serological response is necessary for such programs to confirm the safety profile of the migrant workers. Moreover, this sero-monitoring initiative will help formulate appropriate policy regarding the migrant workers health and infection control during the ongoing and future pandemics by respective governments (source and host countries), local and international migrant-focused organizations, and Non-Governmental Organizations (NGOs).

Data availability statement

The data will be shared based on the demand or upon a reasonable request to the corresponding author from any reader.

Ethics statement

The studies involving human participants were reviewed and approved by International Centre for Diarrhoeal Disease Research, Bangladesh Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MH: Conceptualization, Methodology, Validation, Investigation, Data curation, Software, Formal analysis, Visualization, Writing – original draft, Review & editing. PS: Conceptualization, Methodology, Validation, Formal analysis, Visualization, Writing – original draft, Review & editing. RR: Conceptualization, Methodology, Validation, Writing – Review & editing, Supervision. MZR: Conceptualization, Methodology, Formal analysis, Data curation, Writing – Review & editing. RH: Conceptualization, Methodology, Formal analysis, Data curation, Writing – Review & editing. CS: Conceptualization, Methodology, Data curation, Writing – Review & editing, Visualization. MR: Conceptualization, Methodology, Formal analysis, Data curation, Writing – review & editing, Supervision. NA: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Review & editing, Visualization, Supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1128330/full#supplementary-material

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Impact of homologous and heterologous boosters in neutralizing antibodies titers against SARS-CoV-2 Omicron in solid-organ transplant recipients

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Introduction: Booster doses of SARS-CoV-2 vaccines improve seroconversion rates in solid organ transplant recipients (SOTRs) but the impact of homologous and heterologous booster doses in neutralizing antibody (NAb) titers and their ability to interfere with the variant of concern Omicron are not well studied.

Methods: We designed a prospective, open-label, observational clinical cohort study. 45 participants received two doses of BNT162b2 or CoronaVac (21-day or 28-day intervals, respectively) followed by a first and second booster with BNT162b2 (5-month apart each) and we analyzed the neutralizing antibody titers against SARSCoV-2 D614G (B.1 lineage) and Omicron (BA.1 lineage).

Results: Our results show that SOTRs receiving an initial two-dose scheme of CoronaVac or BNT162b2 generate lower NAbs titers against the ancestral variant of SARS-CoV-2 when compared with healthy controls. Although these NAb titers were further decreased against the SARS-CoV-2 Omicron, a single BNT162b2 booster in both groups was sufficient to increase NAb titers against the variant of concern. More importantly, this effect was only observed in those participants responding to the first two shots but not in those not responding to the initial vaccination scheme.

Discussion: The data provided here demonstrate the importance of monitoring antibody responses in immunocompromised subjects when planning booster vaccination programs in this risk group.

KEYWORDS

COVID-19, humoral response, neutralization, organ transplantation, vaccine

Introduction

Solid organ transplant recipients (SOTRs) are at increased risk for SARS-CoV-2 infection and remain at elevated mortality risk until the COVID-19 pandemic can be controlled (1). Different studies have shown low seroconversion rates of SOTRs that increased with one or two homologous booster doses of mRNA vaccines (2–6). However, studies analyzing and comparing neutralizing antibody (NAbs) titers elicited by different vaccine platforms or the impact of homologous versus heterologous boosters to neutralize the emerging variants of concern (VoCs), such as the Omicron variants in this group of severely immunosuppressed patients are limited (7–9).

To evaluate whether a fourth dose of the COVID-19 vaccine improves the neutralizing capacity in serum of SOTRs, we analyzed NAbs titers three months after an initial two-dose scheme of CoronaVac or BNT162b2; one month after 1st booster and 16 days after 2nd booster of BNT162b2 vaccine, in accordance with the booster vaccine policy implemented by the Chilean National Immunization Program (PNI), using an HIV-1-based SARS-CoV-2 pseudotype expressing the spike protein of the Omicron (BA.1 lineage) or SARS-CoV-2 D614G (B.1 lineage) (10–12).

Materials and methods

Study cohort

Healthcare workers without previously diagnosed SARS-CoV-2 infection and without the use of immunosuppressive drugs for any diagnostic from Clínica Santa María; Santiago, Chile and patients belonging to the transplant unit of the Clínica Santa Maria, Santiago, Chile were invited to participate in this study. Forty-five solid organ transplant recipients, 42.2% (19) women, with a mean age of 52 years (IQR 37-59) at the start of vaccination, were recruited. The transplanted organs were distributed as follows: 12 pulmonary (6 monopulmonary), 9 liver, 1 heart, 12 kidney and 11 kidney-pancreas transplants. More detailed information about the characteristics of the transplanted patients is shown in Table 1. Volunteers received the two-dose scheme of BNT162b2 (Pfizer-BioNTech) or CoronaVac (Sinovac Biotech), each dose being administered 21 or 28 days apart, respectively, according to the Chilean National Immunization Program (PNI). The participants subsequently received a first booster dose at day 148 (IQR 146-154) after the second initial dose and a second booster dose 153 (IQR 152-161) days after the first booster. All participants received both booster doses with BNT162b2 according to the regulations of the Ministry of Health. All participants were asked about their previous diagnosis of COVID-19 prior to every sample collection. If they had a record of a positive PCR, they were excluded from the analysis. The BNT162b2-vaccinated participants who tested positive for anti-N antibodies were discarded from the analysis.

All participants signed informed consent before any study procedure was undertaken and protocols were approved by the respective Ethics Committee at Clínica Santa María (No. 132604-21) and Facultad de Medicina at Universidad de Chile (No. 0361-2021). Serum samples were collected between June 2021 and February 2022.

Production of an HIV-1-based SARS-CoV-2-Spike pseudotyped virus

Pseudotyped viruses carrying SARS-CoV-2 Spike variants D614G (B.1 lineage) and Omicron (BA.1 lineage)) were produced as described in (11, 12). Briefly, HEK-293T cells were co-transfected with the HIV-1 proviral vector pNL4.3-ΔEnv-Luciferase and the corresponding pCDNA-SARS-CoV-2 Spike coding vectors using PEI. Spike codifying vectors were purchased from GenScript and designed to lack the last 19 amino acids of the C-terminal end (SΔ19) known to avoid retention at the endoplasmic reticulum. At 48 hours post-transfection, pseudotypes were recovered from the supernatant, cleared by centrifugation at 850g for 5 minutes at room temperature, diluted in 50% fetal bovine serum (Sigma-Aldrich), aliquoted and stored at -80°C until use. Pseudoviruses were quantified by HIV-1 Gag p24 Quantikine ELISA Kit (R&D Systems) following manufacturer's instructions.

Pseudotyped virus neutralization assay

Serum samples inactivated for 30 minutes at 56°C were 3-fold serially diluted (from 1:5 to 1:10935) in supplemented DMEM with 10% FBS. Samples were incubated with 3 ng of p24 HIV-1-based

TABLE 1 Characteristics of study subjects.

	Healthy volunteers	Solid organ transplant patients		
	sample after first 2 doses	sample after first 2 doses	sample after 1st booster	sample after 2° booster
n	50	45	19	20
Female N (%)	36 (72%)	19 (42.2%)	9 (47%)	8 (40%)
Age (IQR)	39.5 (30-51)	52 (37-59)	49 (37-60)	53 (39-64)
Months between transplant and start of vaccination (median -IQR))		24 (6.7 -47.9)	23.7 (6.5 - 48.0)	24 (6.9-48-4)
Days between vaccine dose and sampling (IQR)	99 (97-112)	90 (85-104)	36 (33-37)	16 (11-23)
Solid organ transplant (n)				
Lung (mono or bi)		12	5	8
Liver		9	4	3
Heart		1	0	0
Kidney-pancreas		11	6	6
Kidney		11	4	3
Type of Immunosuppression (n)				
Steroids		39	18	19
Calcineurin inhibitors		44	19	20
Purine metabolism inh		36	16	18
m-TOR		6	2	1

SARS-CoV-2 variant pseudotyped virus D614G (B.1 lineage) or Omicron (BA.1 lineage)) during 1 h at 37°C, and 1×10^4 HEK-ACE2 cells were added to each well. HEK293T cells incubated with the pseudotyped virus were used as a negative control. Cells were lysed 48 h later, and firefly luciferase activity was measured using the Luciferase Assay Reagent (Promega) in a Glomax 96 Microplate luminometer (Promega). Relative luminescence units (RLUs) of HEK293T cells transduced with the corresponding pseudovirus were averaged and considered as 100% neutralization while RLUs measured at the highest dilution of each sample were established as 0% neutralization. Thus, the percentage of neutralization of each one of the eight dilutions of a sample was calculated as the complement of the division between the corresponding RLUs and the RLUs obtained at the higher dilution after subtracting the background (HEK293T + pseudovirus). This calculation was done independently for each technical replica and for each spike variant. Relative pseudotyped virus neutralization titer 50 ($pVNT_{50}$) is defined as the dilution of the sample yielding a 50% diminution of firefly luciferase activity compared to the negative (HEK293T without pseudovirus) and positive controls (highest dilution of the sample). The $pVNT_{50}$ was calculated in GraphPad Prism v9.1.2 (La Jolla, California, USA) by modeling a four-parameter non-linear regression with variable slope constraining top values to 100 and bottom values to 0. Samples showing a $pVNT_{50}$ lower than the first dilution (1:10 for CoronaVac, 1:10 for BNT162b2) were considered as 10.

Anti-Spike RBD antibodies determination

Quantification of anti-Spike RBD and anti-N antibodies was performed as described in (13) by using the Electrochemiluminescent immunoassay (ECLIA) (Cobas, Roche). Values are reported as the analyte concentration of each sample in U/mL. Detection ranges for anti-Spike RBD were 0.4 to 2500 U/mL, where a detection <0.8 U/mL was interpreted as negative and ≥0.80 U/mL was interpreted as positive for anti-Spike RBD antibodies. Detection of anti-N antibodies with a cut-off index ≥ 1.0 was considered as positive and <1 negative (Roche Diagnostics GmbH. Elecsys Anti-SARS-CoV-2 assay method sheet. 2021-03; version 4.0). Analysis of IgG and IgM antibodies anti-Spike and anti-N was evaluated by using the OnSite COVID-19 IgG/IgM Rapid Test Kit (CTK, Biotech) using 10 µL of serum samples following manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software v9.1.2 (La Jolla, California, USA). Multiple group

comparisons for serum neutralization titers against a set of samples and the two SARS-CoV-2 pseudotyped viruses were applied using Kruskal-Wallis test with false discovery rate (FDR) method, and multiple testing correction was performed for each comparison using Benjamini-Hochberg (BH) procedure at a 5% FDR threshold. When indicated, factor change was calculated as the difference of geometric mean titer in the $pVNT_{50}$ or total anti-Spike IgG levels. The degree of correlation between neutralizing and total IgG antibodies from different groups was evaluated by computing the Spearman's ρ for every XY pair of values (13). A p-value ≤0.05 was considered statistically significant.

Results

In this work, we determined NAbs titers in a cohort of SOTRs inoculated with the two-dose regimen of the mRNA vaccine BNT162b2 or the inactivated virus vaccine CoronaVac and receiving two boosters of BNT162b2 five months apart (Figure 1). Serum samples from SOT recipients (N=45, Table 1) and healthy healthcare workers (N=50, Table 1) were used to determine the neutralizing antibody (NAb) titers measured as pseudotyped virus neutralization titer 50 ($pVNT_{50}$) as we have previously reported (10–12). Additionally, total IgG/IgM anti-Spike and anti-N antibodies were evaluated by a lateral flow rapid test and quantified by ELISA. Details regarding cohort demographics, methods and statistical analyses can be found in Table 1 and Supplementary Table 1.

The vaccination process in SOTRs began at a median of 24.7 months (IQR 7.6 - 48) after the transplant. All the patients were on immunosuppression (described in Table 1). Only individuals without a clinical history of SARS-CoV-2 infection and without a history of positive PCR testing were analyzed. The participants received an initial vaccination schedule with two doses of the BNT162b2 vaccine or two doses of CoronaVac administered 21 or 28 days apart, respectively, then they all received the first booster with BNT162b2 at day 148 (IQR 146-154) after the second initial dose and a second booster 153 (IQR 152-161) days later (Figure 1). The initial vaccine scheme utilized in each patient was determined according to the vaccine that was available in the vaccination center when the immunization campaign began. Only a statistically significant difference in age was identified between the groups that received BNT162b2 versus CoronaVac at the initial scheme, with a median of 42 years for BNT162b2 and 57 years for CoronaVac (p-value = 0.0004). There was no difference in distribution based on sex, transplanted organ type, or immunosuppressive treatment.

In a previous report, we showed that neutralization levels of antibodies elicited by CoronaVac and BNT162b2 against the SARS-CoV-2 Wuhan-Hu-1 Spike are not affected by the D614G mutation that originated the B.1 lineage (11). Thus, we decided to conduct our analysis using Spike D614G- and Omicron-pseudotyped viruses.

First, we compared NAb titers induced by the two-dose scheme of BNT162b2 or CoronaVac in SOTRs and healthy volunteers against the D614G pseudovirus at 90 days after the second dose (Figure 2A). Consistent with a low seroconversion rate (BNT162b2 = 50%, CoronaVac=28.6%), NAb titers measured as the geometric mean of the $pVNT_{50}$ were 29.1-fold and 8.8-fold lower for SOT recipients receiving BNT162b2 or CoronaVac, respectively, when compared with healthy controls whose seroconversion rate was 100%. Moreover, BNT162b2 elicited NAb titers that were 2.2-fold and 7.1-fold higher for SOT recipients and healthy controls, respectively, when compared with CoronaVac (Figure 2B). In the same line, anti-Spike RBD antibodies were 309.7-fold and 82.6-fold lower for BNT162b2- and CoronaVac-vaccinated SOTRs compared with healthy controls, respectively. Besides, as observed in the analysis of NAbs titers, anti-Spike RBD antibodies from healthy and SOT recipients vaccinated with BNT162b2 were 17.2-fold and 4.6-fold higher compared to CoronaVac (Supplementary Figures 1A, B). Interestingly, there was a strong correlation between NAbs measured with the HIV-1-based SARS-CoV-2-Spike pseudotyped virus and anti-Spike RBD antibodies from healthy- and SOTRs-BNT162b2 vaccinated groups (r=0.75 and r=0.7909, respectively) (Supplementary Figure 1C). However, the correlation was moderate in SOTRs receiving CoronaVac compared to the healthy control group (r=0.5633 versus r=0.7328), which further reinforces our conclusion that two doses of BNT162b2 elicit higher NAbs titers than CoronaVac in SOT patients (Supplementary Figure 1C).

Importantly, we assessed the seropositivity of the samples by a lateral flow rapid test kit to detect IgG anti-SARS-CoV-2 Spike

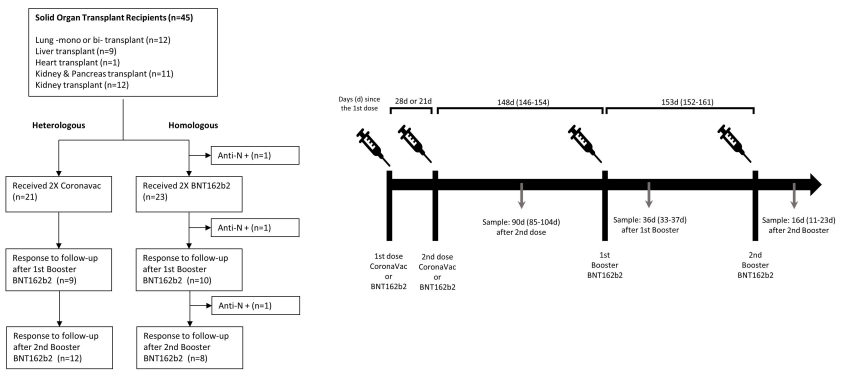

FIGURE 1
Study flow-chart and protocol of the observational clinical cohort study.

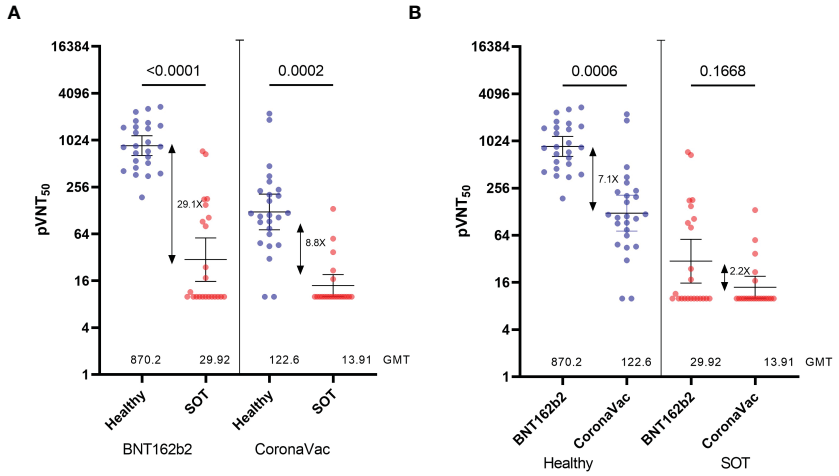

FIGURE 2
Neutralization titers of serum from Healthy and SOTRs 90 days after the two-dose BNT162b2 and CoronaVac vaccines. 50% pseudovirus neutralization titers ($pVNT_{50}$) of 50 healthy recipients (blue) of the CoronaVac (n=25) and BNT162b2 (n=25) vaccines and 44 SOTRs (red) of the CoronaVac (n=21) and BNT162b2 (n=23) against pseudotypes **(A, B)** ancestral reference strain (D614G). Statistical significance of the difference between the neutralization was calculated by the two-tailed Kruskal–Wallis test after adjustment for the false discovery rate. Two-tailed *P* values are reported. Geometric mean titers (GMTs) and 95% CIs are indicated. Factor changes are shown in brackets as the difference of the geometric mean titer in the $pVNT_{50}$. Graphs Y axes are presented in logarithmic (log_2) scale.

antibodies (14). Globally (SOTRs + healthy), subjects with $pVNT_{50}$ values near the detection limit ($log(pVNT_{50}<2.5)$) were tested negative regardless initial vaccination scheme (CoronaVac or BNT162b2), whereas subjects with a $log(pVNT_{50})>5$ were positive for anti-SARS-CoV-2 antibodies and predominantly BNT162b2-vaccinated (Supplementary Figure 2A). The analysis of IgG positivity by group (SOTRs vs healthy) showed that healthy subjects with low NAbs titers elicited by CoronaVac ($log(pVNT_{50}<5)$) were associated to a higher occurrence of negative results, whilst the mRNA vaccine induced high NAbs titers that were detected as positive by the lateral flow kit (Supplementary Figure 2B). Consistent with a low seroconversion rate, CoronaVac- and BNT162b2-SOTRs were mainly IgG negative with $pVNT_{50}$ values near the detection limit and presented a low occurrence of IgG positive results as well as NAbs titers that do not reach log ($pVNT_{50}$) values higher than 5 (Supplementary Figure 2C).

We then looked at the neutralizing ability of antibodies against the SARS-CoV-2 variant of concern Omicron (Figure 3A). As expected, we observed a 9.2-fold and 10.1-fold decrease in the NAb titers in the healthy group inoculated with BNT162b2 and CoronaVac, respectively, compared to NAb titers against the reference strain D614G (Figure 3A). While NAb titers from the SOT recipients group inoculated with CoronaVac were near the limit of detection ($pVNT_{50}$ <10), the low but detectable NAb titers against the D614G pseudovirus in the group of SOT recipients inoculated with BNT162b2 were decreased by 2.57-fold against Omicron (Figure 3B). These data show that a two-dose regimen of SARS-CoV-2 vaccines BNT162b2 and CoronaVac in SOT recipients elicits very low levels of NAbs titers, which are higher in those inoculated with the mRNA vaccine. However, these NAbs titers are not sufficient to neutralize the Omicron variant.

We then analyzed the impact of one and two BNT162b2 booster doses inoculated with a 5-month interval in those SOT recipients who do not drop out of the study (CoronaVac (n=21; 1st booster n=9, 2nd booster n=12) and BNT162b (n=23; 1st booster n=10; 2nd booster n=8)). We observed that while a single heterologous booster dose (2x CoronaVac + 1x BNT162b2) induced a 7.9-fold increase in NAb titers, participants who received a homologous booster (2x BNT162b2 + 1x BNT162b2) increased their NAbs titers against the D614G pseudovirus by 3.2-fold (Figure 3C). Different from what has been previously reported for healthy patients (15–18), we observed that a second booster with BNT162b2 (fourth dose) in both settings did not have major effects on NAb titers over the first booster in SOTRs (Figure 3C). Consistent with the analysis of NAbs, total anti-Spike IgG antibodies increased by 13.5-fold and 7.9-fold after a heterologous and homologous booster, respectively (Supplementary Figure 3), while no significant differences were observed after the second booster. Of note, we observed that 50% of the patients included in the follow-up did not respond to any of the boosters regardless of the initial vaccination scheme ($pVNT_{50}$ <10). Indeed, only 1 of the 6 (16.6%) SOTRs that did not seroconvert after the initial two-dose scheme turned positive for anti-Spike RBD antibodies after the first BNT162b2 booster. Moreover, 2 of the 7 (28.6%) CoronaVac- and 3 of the 6 (50%) BNT162b2-initially vaccinated SOTRs seroconverted solely after the second booster. In this manner, the cumulative percentage of seropositive SOTRs after the second booster dose was higher when receiving a homologous (75%) versus a heterologous (53%) vaccination scheme.

Finally, we evaluated whether NAbs induced by one or two booster doses were able to neutralize the Omicron variant. Interestingly, NAbs elicited by a heterologous booster in SOTRs neutralize the Omicron variant in a 5.9-fold increase (Figure 3D).

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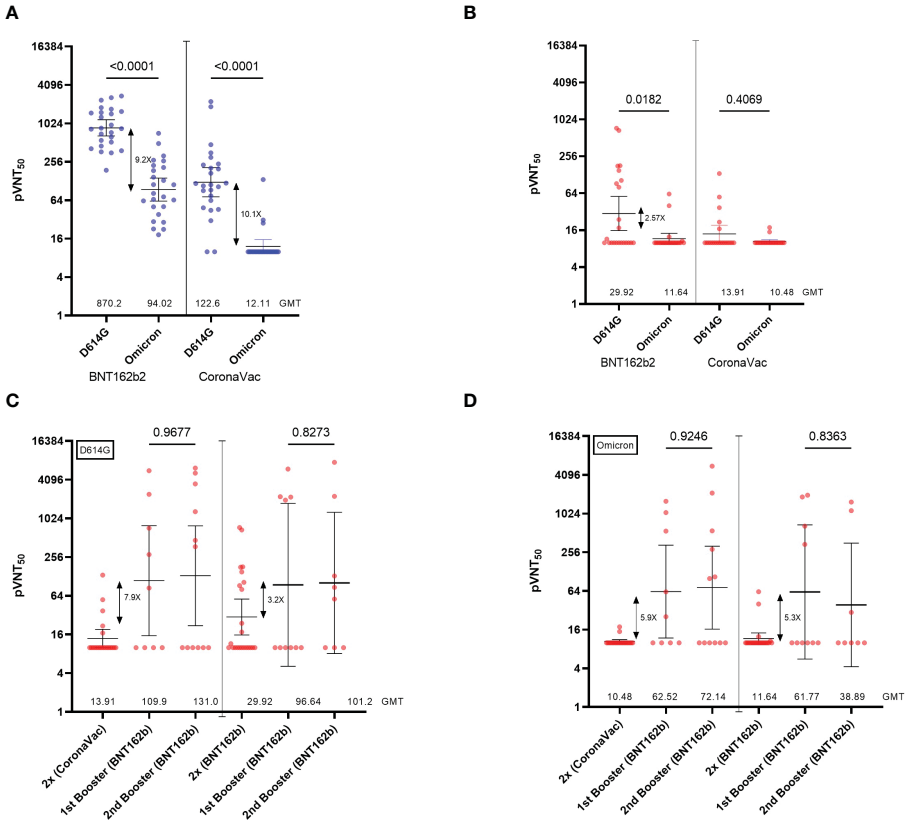

FIGURE 3
Neutralization titers of serum from SOTRs across the homologous and heterologous boosters. **(A)** 50% pseudovirus neutralization titers ($pVNT_{50}$) of 50 healthy recipients (blue) of the CoronaVac (n=25) and BNT162b2 (n=25) vaccines and **(B)** 44 SOT recipients (red) of the CoronaVac (n=21) and BNT162b2 (n=23) against pseudotypes ancestral reference strain (D614G) or Omicron (BA.1). **(C, D)** 50% pseudovirus neutralization titers ($pVNT_{50}$) of 44 SOT recipients of the CoronaVac (n=21; 1st booster n=9, 2nd booster n=12)) and BNT162b2 (n=23; 1st booster n=10; 2nd booster n=8)) against pseudotypes **(C)** ancestral reference strain (D614G) or **(D)** Omicron (BA.1). Geometric mean titers (GMTs) and 95% CIs are indicated. Factor changes are shown in brackets as the difference of the geometric mean titer in the $pVNT_{50}$. Statistical significance of the difference between the neutralization was calculated by the two-tailed Kruskal–Wallis test after adjustment for the false discovery rate. Two-tailed *P* values are reported. Graphs Y axes are presented in logarithmic (log_2) scale.

Similar results were obtained in those SOTRs that generated NAbs followed by a homologous booster showing a 5.3-fold increase. We also observed that a second booster with BNT162b2 (fourth dose) in both settings did not have major effects on NAb titers over the first booster in SOTRs against the Omicron variant (Figure 3D).

Discussion

In the present study, we show that SOTRs have weak neutralizing antibody responses against the SARS-CoV-2 B.1 and Omicron BA.1 variants even after two boosters with the BNT162b2 mRNA vaccine. Importantly, those SOTRs not responding to the first vaccination scheme do not show an increase in their NAb titers upon one and two boosters (19, 20). However, we detected an important increase in cumulative seroconversion rates, especially after the second booster under a homologous scheme. Limitations of this study include a low number of volunteers, that some volunteers failed to respond at follow-up, and the lack of data on B and T cell responses, which may provide antibody-independent protection.

Recently, a meta-analysis showed that booster vaccination enhances the immunogenicity of COVID-19 vaccines in SOTRs, however, a significant share of the recipients still has not built a detectable humoral immune response after the 3rd dose (21). Our results are also in line with studies in other groups at high risk of developing COVID-19 such as haemodialysis (HD) patients. Similar to SOTRs, HD patients present lower antibody titers and seroconversion rates after a two-dose BNT162b2 vaccination scheme compared to healthy controls, which can be significantly increased after a third BNT162b2 dose (22, 23). This confirms the urgent necessity of maintaining a booster dose in SOTRs at each 5-month interval and provides evidence that the use of the mRNA-based vaccines as boosters are sufficient to increase NAb titers able to neutralize the SARS-CoV-2 variant of concern Omicron. Interestingly, we showed that serum reactivity against RBD (ECLIA) as well as IgG positivity (OnSite) are associated to NAb titers predominantly when $pVNT_{50}$ values are medium-to-high according to our assay's range. Thus, the data provided here highlight the importance of monitoring antibody responses in immunocompromised subjects, which according to our results should be considered when planning vaccination programs in

these risk groups. While different strategies such as the use of monoclonal antibodies for early treatment or prophylaxis, convalescent plasma, drugs such as nirmatrelvir/ritonavir, molnupiravir and remdesivir, anti-inflammatory therapy, and virus specific T-cell therapy are being evaluated (24). we urgently need to find alternative approaches for this specific set of 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 authors.

Ethics statement

All participants signed informed consent before any study procedure was undertaken and protocols were approved by the respective Ethics Committee at Clínica Santa María (No. 132604-21) and Facultad de Medicina at Universidad de Chile (No. 0361-2021). The patients/participants provided their written informed consent to participate in this study.

Authors contributions

AG-A, CPC, FV-E, and RS-R designed the study. MAl and CPC provided clinical samples. AG-A, VS-A, LA-P and MAc performed neutralization assays. SB and AK provided reagents and plasmids. AG-A, DS and FV-E performed the statistical analysis. AG-A, FV-E, CPC, and RS-R wrote the manuscript. RS-R, FV-E and CPC acquired funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1135478/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Anti-Spike RBD antibodies of serum from Healthy and SOTRs 90 days after the two-dose BNT162b2 and CoronaVac vaccines. **(A, B)** Results of serological assay measuring serum reactivity to RBD expressed as U/ml (SARS-CoV-2 positive ≥0.80 U/ml) (Elecsys®, Roche Diagnostic GmbH)) from 41 healthy recipients (blue) of the CoronaVac (n=17) and BNT162b2 (n=24) vaccines and 44 SOTRs (red) of the CoronaVac (n=21) and BNT162b2 (n=23). Differences in the geometric means titers of anti-Spike RBD between CoronaVac and BNT162b2 vaccine are shown. Statistical analyses were performed with the two-tailed Kruskal–Wallis test after adjustment for the false discovery rate. **(C)** Correlation between NAbs measured as the $pVNT_{50}$ and anti-Spike RBD antibodies in U/mL for each group described in **(A)**. Spearman r and two-tailed p-value are reported in the legend. The dashed line shows the detection limit (0.8 U/mL) of the technique. Values are presented in log10 scale.

SUPPLEMENTARY FIGURE 2
Relation between IgG positivity and NAb titers against the ancestral reference strain (D614G) of serum from Healthy and SOTRs individuals. **(A)** Frequency of IgG positive and negative results evaluated by lateral flow rapid test kit as a function of the natural logarithm of the $pVNT_{50}$ value from CoronaVac- or BNT162b2- vaccinated healthy and SOTRs individuals included in this study (n=88). Distribution of IgG positive and negative results occurrence from CoronaVac- or BNT162b2- vaccinated healthy (n=40) **(B)** and SOTRs (n=44) **(C)** compared to NAb titers ($log(pVNT_{50})$) against the ancestral reference strain (D614G).

SUPPLEMENTARY FIGURE 3
Anti-Spike RBD antibodies of serum from SOT individuals across the homologous and heterologous boosters. Results of serological assay measuring serum reactivity to RBD expressed as U/ml (SARS-CoV-2 positive ≥0.80 U/ml) (Elecsys®, Roche Diagnostic GmbH)) from 44 SOTRs of the CoronaVac (n=21; 1st booster n=9, 2nd booster n=12)) and BNT162b2 (n=23; 1st booster n=10; 2nd booster n=8)). Geometric mean titers (GMTs) and 95% CIs are indicated. Factor changes are shown in brackets as the difference of the geometric mean titer. Statistical significance of the difference between the anti-Spike RBD titers was calculated by the two-tailed Kruskal–Wallis test after adjustment for the false discovery rate. Two-tailed P values are reported. Graphs Y axes are presented in logarithmic (log2) scale. The dashed line shows the detection limit (0.8 U/mL) of the technique.

SUPPLEMENTARY TABLE 1
Detailed information about the characteristics of the transplanted patients (SOTRs) and healthy controls (columns A-G). Results of neutralization assays

($pVNT_{50}$, confidence intervals and R^2), ELISA anti-Spike and anti-N as well as OnSite rapid test of serums at 90 days after the initial vaccination scheme (columns I-S), after the first booster (columns U-AF) and after the second booster (AH-AS) of each sample are shown. Excluded samples tested positive for anti-N antibodies are highlighted in yellow ("NA", non-applicable; "–", no data; ND, non-determined).

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Heterologous prime-boost immunisation with mRNA- and AdC68-based 2019-nCoV variant vaccines induces broad-spectrum immune responses in mice

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The ongoing evolution of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 or 2019-nCoV) variants has been associated with the transmission and pathogenicity of COVID-19. Therefore, exploring the optimal immunisation strategy to improve the broad-spectrum cross-protection ability of COVID-19 vaccines is of great significance. Herein, we assessed different heterologous prime-boost strategies with chimpanzee adenovirus vector-based COVID-19 vaccines plus Wuhan-Hu-1 (WH-1) strain (AdW) and Beta variant (AdB) and mRNA-based COVID-19 vaccines plus WH-1 strain (ARW) and Omicron (B.1.1.529) variant (ARO) in 6-week-old female BALB/c mice. AdW and AdB were administered intramuscularly or intranasally, while ARW and ARO were administered intramuscularly. Intranasal or intramuscular vaccination with AdB followed by ARO booster exhibited the highest levels of cross-reactive IgG, pseudovirus-neutralising antibody (PNAb) responses, and angiotensin-converting enzyme-2 (ACE2)-binding inhibition rates against different 2019-nCoV variants among all vaccination groups. Moreover, intranasal AdB vaccination followed by ARO induced higher levels of IgA and neutralising antibody responses against live 2019-nCoV than intramuscular AdB vaccination followed by ARO. A single dose of AdB administered intranasally or intramuscularly induced broader cross-NAb responses than AdW. Th1-biased cellular immune response was induced in all vaccination groups. Intramuscular vaccination-only groups exhibited higher levels of Th1 cytokines than intranasal vaccination-only and intranasal vaccination-containing groups. However, no

obvious differences were found in the levels of Th2 cytokines between the control and all vaccination groups. Our findings provide a basis for exploring vaccination strategies against different 2019-nCoV variants to achieve high broad-spectrum immune efficacy.

KEYWORDS

heterologous prime-boost, ChAdTS-S, ARCoV, intramuscular, intranasal, SARS-COV-2 variants, COVID-19

1 Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 or 2019-nCoV) is a highly mutable, enveloped, single-stranded RNA coronavirus of the betacoronavirus genus (1). According to the World Health Organization, the five variants of concern (VOCs), such as Omicron (e.g., BQ.1, BA.5, BA.2.75, BA.2, B.1.1.529 and BA.1), Gamma (P.1), Delta (B.1.617.2), Beta (B.1.351) and Alpha (B.1.1.7), have all caused COVID-19 pandemic waves at varying magnitudes (2–4). At present, the most prevalent global 2019-nCoV variant is the Omicron variant, with > 30 mutations in the spike protein and 15 point mutations in the spike receptor-binding domain (RBD) region compared to the Wuhan-Hu-1 (WH-1) strain (5). Omicron is more transmissible and can evade immunity more efficiently than the other VOCs, thereby increasing the risk of reinfection (6). Two doses of Pfizer/BNT162b2 mRNA vaccine elicited a 22-fold lower serum neutralising activity against Omicron (B.1.1.529) than against the D614G variants (7). Two doses of BNT162b2 or ChAdOx1 nCoV-19 had limited protection against symptomatic B.1.1.529 infection, with vaccine efficacy decreasing to <10% at 20 weeks or beyond (8). Thus, it is of utmost urgency to develop COVID-19 vaccines that are more effective against emerging variants.

Novel 2019-nCoV variant vaccines induce immune responses against different 2019-nCoV variants in mice (9–12). Although Omicron-specific vaccines induce high neutralising activity against Omicron, they are not effective against other variants (9, 10). COVID-19 vaccines expressing spike proteins from only Beta or Delta variants showed low immunogenicity, but induced relatively broad-spectrum neutralising antibodies against multiple variants (9, 10, 12). Moreover, the nucleocapsid (N) protein is highly conserved and immunogenic among 2019-nCoV variants and other coronaviruses. The plant-produced RBD and cocktail-based vaccine (RBD co-expressed with N protein) is highly effective against 2019-nCoV variants, such as Omicron and Delta variants, in mice (13). Higher systemic immune response and protective efficacy have been induced using a heterologous, rather than a homologous, prime-boost strategy (14, 15). We previously found that intranasal (in) or intramuscular (im) priming with ChAdTS-S and im boosting with ARCoV induced greater cellular and humoral immune responses than homologous vaccination with either vaccine (16). This vaccination strategy could potentially prevent immune escape of the current 2019-nCoV variants, especially Omicron strains. Thus, it is necessary to explore a new heterologous prime-boost strategy with different novel 2019-nCoV variant vaccines for improving broad-spectrum neutralising potency against the current and emerging variants.

Mucosal immune response (MIR) plays indispensable roles in fighting and preventing respiratory viral infections, and the high level of secreted IgA (SIgA) during MIR can significantly improve the neutralisation efficacy (17, 18). Polymeric secretory IgA (psIgA) antibodies against influenza A viruses of multiple hemagglutinin (HA) subtypes do not exhibit neutralising properties, but have broad cross-binding and protective capacities (19). Therefore, enhancing the vaccine-induced MIR might be crucial for preventing infection caused by different 2019-nCoV variants.

Herein, we explored the heterologous prime-boost strategies using chimpanzee adenovirus serotype 68 vector (AdC68)- and mRNA-based COVID-19 vaccines with different vaccination routes against different 2019-nCoV variants [prototype, Beta, and Omicron (B.1.1.529) strains]. Our study provides reference data for improving the spectrum and immunogenicity of multiple 2019-nCoV variant vaccines.

2 Materials and methods

2.1 Animals and vaccines

Six-week-old female BALB/c mice (pathogen-free) were obtained and maintained in the National Institutes for Food and Drug Control (NIFDC). We evaluated the chimpanzee adenovirus vector 2019-nCoV vaccine ChAdTS-S (5×10^{10} vp/0.5 mL, Walvax, Yunnan, China) was evaluated, which encoded the WH-1 strain spike protein and designated as AdW; chimpanzee adenovirus vector vaccine ChAdTS-SV2 (5×10^{10} vp/0.5 mL, Walvax), which encoded the Beta strain spike protein and designated as AdB; mRNA vaccine ARCoV (15 μg/0.5 mL, Abogen, Suzhou, China), which encoded the WH-1 strain spike RBD and designated as ARW; mRNA vaccine ARCoV-Omicron (15 μg/0.5 mL, Abogen) and designated as ARO, which encoded the Omicron variant B.1.1.529 with RBD mutations (20). The mice were randomly assigned to groups 1–6, 7–10, 11–13 and 14–15 (n = 5 per group) that received heterologous prime-boost, single doses, homologous prime-boost vaccinations, and phosphate-buffered saline (PBS, blank control), respectively (Figures 1A, B). Initial vaccination

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A

Groups No.	Group	Prime	Boost
1	in AdB>im ARO	in AdB	im ARO
2	im AdB>im ARO	im AdB	im ARO
3	in AdW>im ARO	in AdW	im ARO
4	im AdW>im ARO	im AdW	im ARO
5	in AdW>im ARW	in AdW	im ARW
6	im AdW>im ARW	im AdW	im ARW
7	in AdW	in AdW	-
8	im AdW	im AdW	-
9	in AdB	in AdB	-
10	im AdB	im AdB	-
11	im ARW>im ARW	im ARW	im ARW
12	im ARO>im ARO	im ARO	im ARO
13	im ARW>im ARO	im ARW	im ARO
14	in blank	in PBS	in PBS
15	im blank	im PBS	im PBS

B

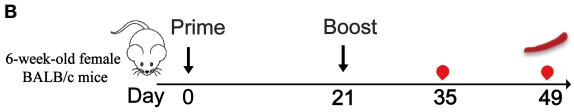

FIGURE 1
Overall scheme of the immunisation strategies and experimental timeline using female BALB/c mice. **(A)** Mice in 15 groups were immunised with four COVID-19 vaccines using different protocols. Dashes indicate no booster vaccination. **(B)** Immunisation and immunological characterisation scheme. ↓, vaccination; ●, bleeding; , spleen lymphocyte isolation. Im, intramuscular vaccination; in, intranasal vaccination.

was administered on day 0, and booster vaccination was administered on day 21. The inoculation was a fractional dose (one-fifth of the human dose), i.e., 1×10^{10} vp per mouse for AdW and AdB and 3 μg per mouse for ARW and ARO. Blood specimen was withdrawn from each mouse 35 and 49 days after the initial vaccination. This research was approved by the Institutional Animal Care and Use Committee of NIFDC, and was performed in compliance with the Committee guidelines.

2.2 Enzyme-linked immunosorbent assay

2019-nCoV spike protein-specific IgA and IgG titres were detected with ELISA. Briefly, 0.2 μg 2019-nCoV spike protein (Sino Biological, Beijing, China), and B.1.1.529 spike protein (Sino Biological) were coated overnight onto the Costar ELISA plates (Corning, NY, USA), respectively. The 2019-nCoV spike protein was fused with a polyhistidine tag at the C-terminus, while the B.1.1.529 spike protein was fused with the bacteriophage T4 fibritin and a polyhistidine tag at the C-terminus. After blocking with 0.05% Tween 20-containing PBS and 1% bovine serum albumin at 37°C for 1 h, the plates were rinsed 6 times with 0.05% Tween 20-containing PBS, the diluted sera were added to the wells by 4-fold serial dilutions. After washing 6 times with 0.05% Tween 20-containing PBS, the plates were exposed to horseradish peroxidase-conjugated goat anti-mouse IgA (1:10,000; Abcam, UK) or goat anti-mouse IgG (1:10,000; ZSGB-BIO, China) at 37°C for 1 h. TMB (3,3',5,5'-tetramethylbenzidine; Beyotime, China) was employed as a substrate to determine the antibody responses by measuring the absorbance at 450 and 630 nm. The end point titres were defined as the highest reciprocal serum dilution, which was 2.1-fold higher compared to the negative control.

2.3 Recombinant vesicular stomatitis virus-based pseudovirus neutralisation assay

Recombinant VSV-based pseudotyped 2019-nCoV was obtained from the Division of HIV/AIDS and Sex-Transmitted Virus Vaccines, NIFDC, including Delta (B.1.617.2) variant, Omicron (B.1.1.529) variant, Omicron (BA.4/5) variant and WH-1 strain. The assays were conducted according to a previous method (21). After inactivation at 56°C for 30 min, a 3-fold serial dilution of mouse serum was mixed with 650 median tissue culture infectious dose ($TCID_{50}$) of pseudoviruses, followed by incubation at 37°C for 60 min. Vero cells (2×10^5) were added, followed by incubation at 37°C and 5% CO_2 for 24 h. NAbs were detected by luciferase expression to determine the amount of pseudoviruses entering the target cells. A luciferase assay system (PerkinElmer, MA, USA) was used to detect the luciferase activity. We included a virus control containing both virus and cells and a negative control containing only cells. The half-maximal effective concentration was determined for each tested sample. For neutralising titre <30, the value was recorded as 30 for plotting.

2.4 Live SARS-CoV-2 neutralisation assay

The neutralising capacity of mouse sera was evaluated using a microneutralisation (MN) assay. Two-fold serial dilutions of heat-inactivated sera were exposed to 100 median cell culture infectious dose ($CCID_{50}$) of 2019-nCoV CAS-B001/2020 strain at 37°C for 2 h. Vero E6 cells (1.8×10^5) were added, followed by incubation at 37°C for 72 h. The MN antibody titres were calculated using the Spearman–Karber method to assess the serum dilution needed for 50% inhibitory action of the cytopathic activity, and the MN antibody titre of ≥ 4 was regarded as positive. Each dilution was

carried out in duplicate. The virus culture of 2019-nCoV and the MN assays was conducted in a biosafety level-3 facility at NIFDC.

2.5 ACE2-binding inhibition (neutralisation) ELISA

The V-PLEX COVID-19 ACE2 neutralisation kit (Panel 18 (ACE2) kit, K15570U; Panel 27 (ACE2) kit, K15609U-2; Meso Scale Discovery, Rockville, MD, USA) was employed to quantitatively analyze of antibody titres that blocked the binding of ACE2 to its cognate ligands (spike protein from BA.5, BA.4, BA.3, BA.2+L452R, BA.2+L452M, BA.2.12.1, BA.2, P.2, P.1, B.1.617.3, B.1.617.2, B.1.617.1, B.1.617, B.1.526.1, B.1.351, B.1.1.7 and WH-1 strains). The 96-well plate was pre-coated with the specific antigen on spots, and the bound antibodies in each sample (1:100 dilution) were analyzed by human ACE2 protein conjugated with the Meso Scale Discovery (MSD) SULFO-TAG using the MSD instrument. The ACE2-binding inhibition rate was calculated as: 1 - (average electrochemiluminescence signal value of sample - average electrochemiluminescence signal value of blank) × 100%.

2.6 IFN-γ ELISpot assay

Mice were euthanised and immersed in 75% ethanol. The spleen was collected and transferred into a 40-μm cell strainer. Then, 4–5 mL mouse lymphocyte separation medium (Dakewe, China) was added. After grinding with a 2-mL syringe piston, the spleen cell suspension was placed in a 15-mL centrifuge tube and added with 1 mL RPMI-1640 medium (Hyclone, Logan, UT, USA). After centrifugation (800 × *g*, 30 min), the mixture was classified into 4 layers from bottom to top: the cell fragment and erythrocyte layer, lymphocyte layer, fluid separation layer, and RPMI-1640 medium layer. The lymphocytes were transferred to a fresh tube, and then added with 10 mL RPMI-1640 medium. After centrifugation (250 × *g*, 10 min), lymphocytes were harvested and suspended in serum-free medium (Dakewe). A mouse IFN-γ ELISpot plus kit (Mabtech, Sweden) was used to detect IFN-γ-positive cells. Briefly, the plates were rinsed 4 times with 1× PBS (200 μL) and blocked with 10% FBS-containing RPMI-1640 medium at 24°C for 2 h. The freshly isolated lymphocytes (2.5×10^5) were incubated with a peptide pool (1 μg/mL per peptide, Genscript, Nanjing, China) obtained from a peptide scan (15-mers with 11-residue overlaps) of the whole spike glycoprotein of B.1.1.529, B.1.617.2 and 2019-nCoV at 37°C and 5% CO_2 and for 24 h. After incubation with anti-mouse IFN-γ antibody for 2 h, the plates were incubated again with streptavidin-horseradish peroxidase (1:1,000 dilution, Dakewe) for 1 h. TMB solution (100 μL) was added into each well and developed for 5 min until the appearance of different spots. The ImmunoSpot® S6 Universal instrument (Cellular Technology Limited, USA) was used to observe and count the spots.

2.7 Intracellular cytokine staining

Splenic lymphocytes were isolated and stimulated with 2 μg/mL of the spike protein peptide pool and brefeldin A (1:1,000 dilution, Biolegend, USA) at 37°C and 5% CO_2 for 6 h. After stimulation, the splenocytes were rinsed and stained with fixable viability stain and 780 the following antibodies: FITC rat anti-mouse CD8a antibody, BV510 rat anti-mouse CD4 antibody and BV421 hamster anti-mouse CD3e antibody (BD Biosciences, USA). The cells were rinsed twice with 1× PBS, fixed and permeabilised with Cytofix/Cytoperm (BD Biosciences). After rinsing with Perm/Wash buffer (BD Biosciences), the cells were stained with BB700 rat anti-mouse tumour necrosis factor (TNF), APC rat anti-mouse IL-10, PE-Cy7 rat anti-mouse IL-4, BV605 rat anti-mouse interleukin (IL)-2 and PE-conjugated rat anti-mouse IFN-γ (BD Biosciences). The cells were successively rinsed with Perm/Wash buffer, resuspended in 1× PBS, and determined using a FACS Lyric flow cytometric analyser (BD Biosciences). For each sample, 200,000 events were recorded, and data analysis was performed with FlowJo software (TreeStar, USA). $CD4^+$ and $CD8^+$ T cells were obtained by gating single cells (FSC-A versus FSC-H), lymphocytes (FSC-A versus SSC-A), and live $CD3^+$ T cells ($CD3^+$ versus $LD780^-$). All results are expressed as the percentage of cytokine+ cells in $CD4^+$ or $CD8^+$ T cells.

2.8 MSD profiling of Th1/Th2 cytokines

The supernatant was harvested from ELISpot plates, and the levels of IL-2, IL-4, IL-10 and TNF-α were measured using a V-PLEX Proinflammatory Panel 1 (mouse) Kit. Meanwhile, the levels of cytokines were detected using a MESO QuickPlex SQ 120. A standard curve was used to calculate the concentration of each cytokine.

2.9 Statistical analysis

GraphPad Prism v9 software was employed for all plotting and statistical tests. Data are shown as the geometric mean ± geometric standard deviation (except for ACE2-binding inhibition rates that are shown as mean ± standard deviation). Differences among multiple groups were compared by one-way ANOVA. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns, not significant.

3 Results

3.1 Intranasal or intramuscular administration of AdB followed by ARO immunisation induces strong humoral immune response against multiple 2019-nCoV variants in mice

The heterologous prime-boost designs are displayed in Figures 1A, B. To assess the humoral immune response, the spike-specific IgG titres in serum on day 35 after primary immunisation were detected by ELISA (Figure 2). The six heterologous prime-boost immunisation groups (in AdB > im ARO, im AdB > im ARO, in AdW > im ARO, im AdW > im

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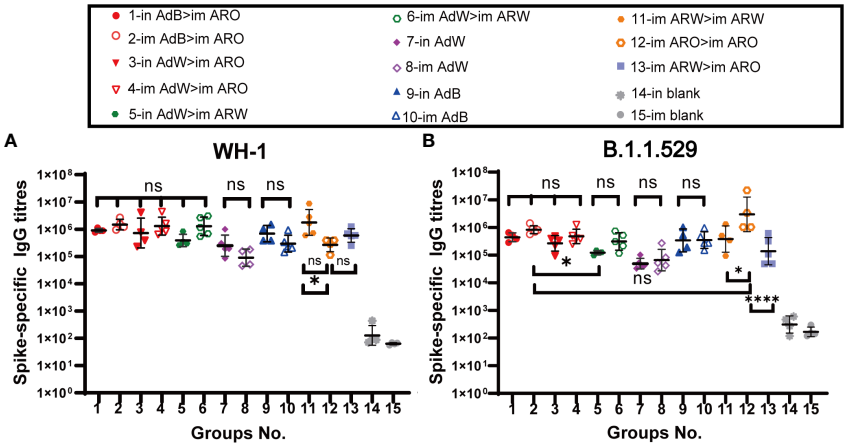

FIGURE 2
IgG responses induced by AdW, AdB, ARW, and ARO vaccines that were administered using various protocols. All titres were measured on day 35 after primary immunisation. Serum IgG titres against **(A)** WH-1 and **(B)** B.1.1.529 spike protein (n = 4–5 per group; each spot represents one sample). Bars represent the geometric mean ± geometric SD; *P < 0.05; ***P < 0.001; ns, P > 0.05.

ARO, in AdW > im ARW, and im AdW > im ARW) developed similar high spike-specific IgG antibody titres against the WH-1 strain (Figure 2A). However, no obvious differences were found among the six groups. The im AdB > im ARO, im AdW > im ARO, im AdW > im ARW and im ARW > im ARW groups developed the highest IgG geometric mean titres (GMTs) of 1,480,872, 1,299,190, 1,284,719 and 1,771,304, respectively. The IgG GMTs for single-dose vaccinated groups (in AdW, im AdW, in AdB and im AdB) were 248,649, 90,299, 689,171, and 295,854, respectively. AdB administered either intranasally or intramuscularly induced higher IgG GMTs than AdW. im ARW > im ARW had higher IgG titres than im ARO > im ARO and im ARW > im ARO, showing 5.6- and 2.0-fold increases, respectively.

Spike-specific IgG titres against the B.1.1.529 spike protein showed a downward trend compared with those against the WH-1 spike protein (Figure 2B), with the six heterologous prime-boost immunisation groups having IgG GMT values of 440,438, 829,265, 266,256, 475,462, 121,796 and 313,054, respectively, indicating a 1.1-, 0.8-, 1.7-, 1.7-, 2.2- and 3.1-fold decrease, respectively. The IgG GMTs for single-dose vaccinated groups against the B.1.1.529 spike protein (in AdW, im AdW, in AdB and im AdB) were 49,082, 66,044, 339,534 and 349,768, showing a 4.1-, 0.4-, and 1.0-fold decrease and a 0.2-fold increase, respectively, compared to those against the WH-1 spike protein. The highest IgG antibody titres were generated by im ARO > im ARO against the B.1.1.529 spike protein among all tested groups, with an IgG GMT of 2,982,389. The IgG GMT in the im ARO > im ARO group was remarkably increased compared to that in the im ARW > im ARW (P = 0.0287) and im ARW > im ARO (P<0.0001) groups, showing a 6.8- and 20.5-fold increase, respectively.

Serum neutralizing antibody titers against BA.4/5 variant, B.1.1.529 variant, B.1.617.2 variant, and WH-1 strain were evaluated with VSV-based pseudovirus assays on day 35 (Figures 3A–D) and 49 (Figures 4A–D) after primary immunisation. The six heterologous prime-boost immunisation groups produced relatively high NAb GMTs in response to the WH-1 strain on day 35 (Figure 3A), which were not significantly different. The in AdB > im ARO, im AdB > im ARO and im AdW > im ARW groups had the highest NAb titres against the WH-1 pseudovirus among all tested groups, with NAb GMTs of 2,387, 2,806 and 2,536, respectively. Higher NAb levels were induced by in AdB and im AdB compared to in AdW and im AdW, with NAb GMTs of 1,493, 725, 283 and 246, respectively. The NAb GMTs for im ARW > im ARW and im ARW > im ARO were 1,084 and 367, respectively. The im ARO > im ARO group had very low NAb levels against the WH-1 strain when compared to the im and in blank groups.

The neutralizing antibody titres against B.1.617.2 variant were comparable with those against WH-1 strain (Figure 3B) in all tested groups. The in AdB > im ARO, im AdB > im ARO, and im AdW > im ARW groups had the highest NAb titres against the B.1.617.2 pseudovirus among all tested groups, with NAb GMTs of 3,308, 5,653 and 2,966, respectively. The NAb GMTs for the in AdW, im AdW, in AdB, im AdB, im ARW > im ARW and im ARW > im ARO groups were 188, 156, 884, 650, 599, and 224 respectively. Of note, im ARO > im ARO induced few NAbs, similar to the im and in blank groups.

In response to the B.1.1.529 pseudovirus on day 35 (Figure 3C), AdB followed by ARO (in AdB > im ARO and im AdB > im ARO) induced high NAb titres that were comparable with those induced by im ARO > im ARO, showing NAb GMTs of 10,065, 12,152 and 106,635, respectively. The remaining four heterologous and two homologous prime-boost groups exhibited relatively low NAb titres, with GMTs of 2,237 in in AdW > im ARO, 1,915 in im AdW > im ARO, 785 in in AdW > im ARW, 473 in im AdW > im ARW, 186 in im ARW > im ARW, and 1,661 in im ARW > im ARO. Higher NAb levels were induced with in AdB and im AdB than with in AdW and im AdW, showing NAb GMTs of 723, 712, 64 and 30, respectively.

NAb titres against the BA.4/5 pseudovirus were lower than those against the WH-1 stain (Figure 3D). The in AdB > im ARO, im AdB > im ARO, im ARW > im ARW and im ARO > im ARO groups had the highest NAb responses, with NAb GMTs of 354,

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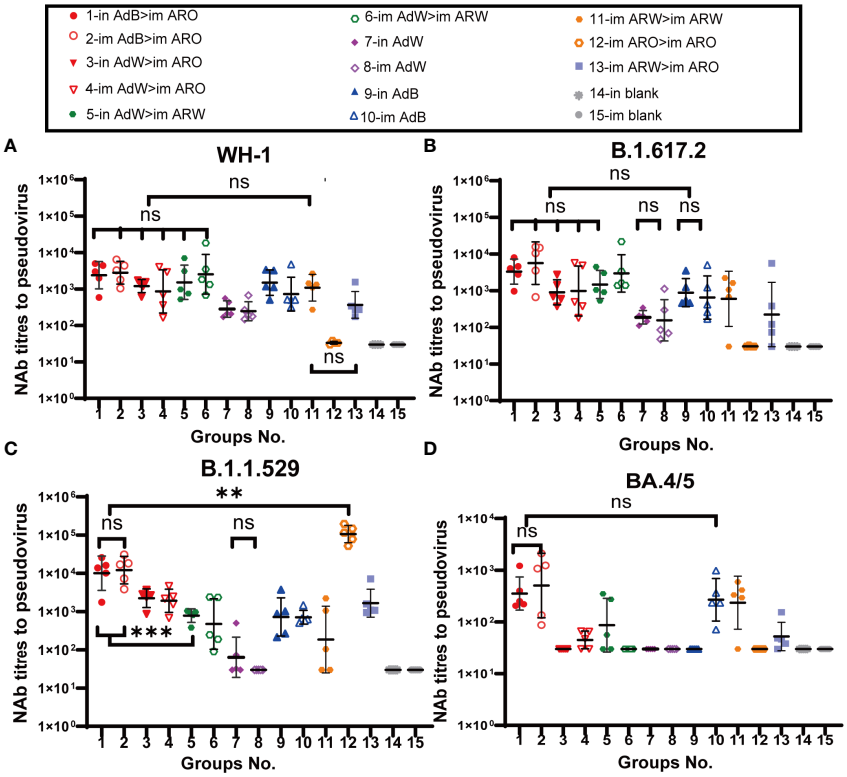

FIGURE 3
Pseudovirus-neutralising antibody (PNAb) titres were measured 35 days after primary immunisation. Serum NAb titres against **(A)** WH-1, **(B)** B.1.617.2, **(C)** B.1.1.529, and **(D)** BA.4/5 are expressed as 50% inhibitory dilutions (n = 4–5 per group; one spot represents one sample). Bars represent the geometric mean ± geometric SD; **P < 0.01; ***P < 0.001; ns, P > 0.05.

504, 268 and 235, respectively, which were 5.8-, 4.6-, 1.7- and 3.6-fold lower than those against WH-1. All remaining vaccination groups induced NAb GMTs comparable with those of the im and in blank groups.

Serum neutralizing antibody titers against the BA.4/5, B.1.1.529, B.1.617.2 and WH-1 pseudoviruses on day 49 (Figures 4A–D) after primary immunisation were comparable with those detected on day 35. AdB, followed by ARO groups, induced the broadest spectrum and highest NAb responses in all tested groups against the WH-1 strain, BA.4/5, B.1.1.529 and B.1.617.2 variants, with GMTs of 2,747, 421, 19,557 and 3,763 in in AdB > im ARO and 3,828, 2,951, 12,915 and 4,924 in im AdB > im ARO, respectively. Both im and in AdB induced higher broad-spectrum NAb responses against the four pseudoviruses than in AdW and im AdW. Moreover, im ARO > im ARO induced high NAb responses against its own B.1.1.529 pseudovirus but almost no NAbs against the other three pseudoviruses.

To further assess NAbs for live SARS-CoV-2, NAb titres in serum were detected using a virus-specific MN assay on day 49 (Figure 4E) after primary immunisation. The NAb GMTs of the in AdB > im ARO and im AdB > im ARO groups were 2,419 and 1,677, respectively.

Taken together, in AdB > im ARO and im AdB > im ARO induced systemic immune responses with the broadest spectrum, as indicated by the highest IgG and NAb GMTs against the WH-1 strain, BA.4/5, B.1.1.529 and B.1.617.2 variants.

3.2 Intranasal administration of AdB followed by ARO immunisation induces high mucosal immune response against WH-1 and B.1.1.529 strains in mice

Mucosal immunity is significantly associated with vaccine efficacy against COVID-19 at the early stages of infection. Mucosal immune response was evaluated by detecting spike protein-specific serum IgA titres with ELISA on day 35 after primary immunisation (Figures 5A, B). The results demonstrated that all five intranasal groups (in AdB > im ARO, in AdW > im ARO, in AdW > im ARW, in AdW, and in AdB) had high spike-specific IgA titres against the WH-1 strain on day 35 after primary immunisation, with GMTs of 1,939, 1,044, 648, 1,301 and 3,496, respectively (P>0.05; Figure 5A). The IgA GMTs of the eight intramuscular groups (im AdB > im ARO, im AdW > im ARO, im AdW > im ARW, im AdW, im AdB, im ARW > im ARW, im ARO > im ARO, and im ARW > im ARO) were 65, 46, 96, 14, 40, 194, 49 and 30, respectively. The IgA titres of vaccination groups were comparable with those of the in and im blank groups, indicating that no obvious mucosal immune response is induced by intramuscular vaccination.

Compared with spike-specific IgA titres against the WH-1 strain, higher spike-specific IgA titres were observed in all five intranasal groups (Figure 5B) against the B.1.1.529 strain, with IgA GMTs of 8,729, 1,128, 1,158, 1,991 and 7,576, respectively, showing a 3.5-, 0.1-, 0.8-, 0.5-, and 1.2-fold increase, respectively. The IgA GMTs of the eight intramuscular groups were 298, 170, 245, 151,

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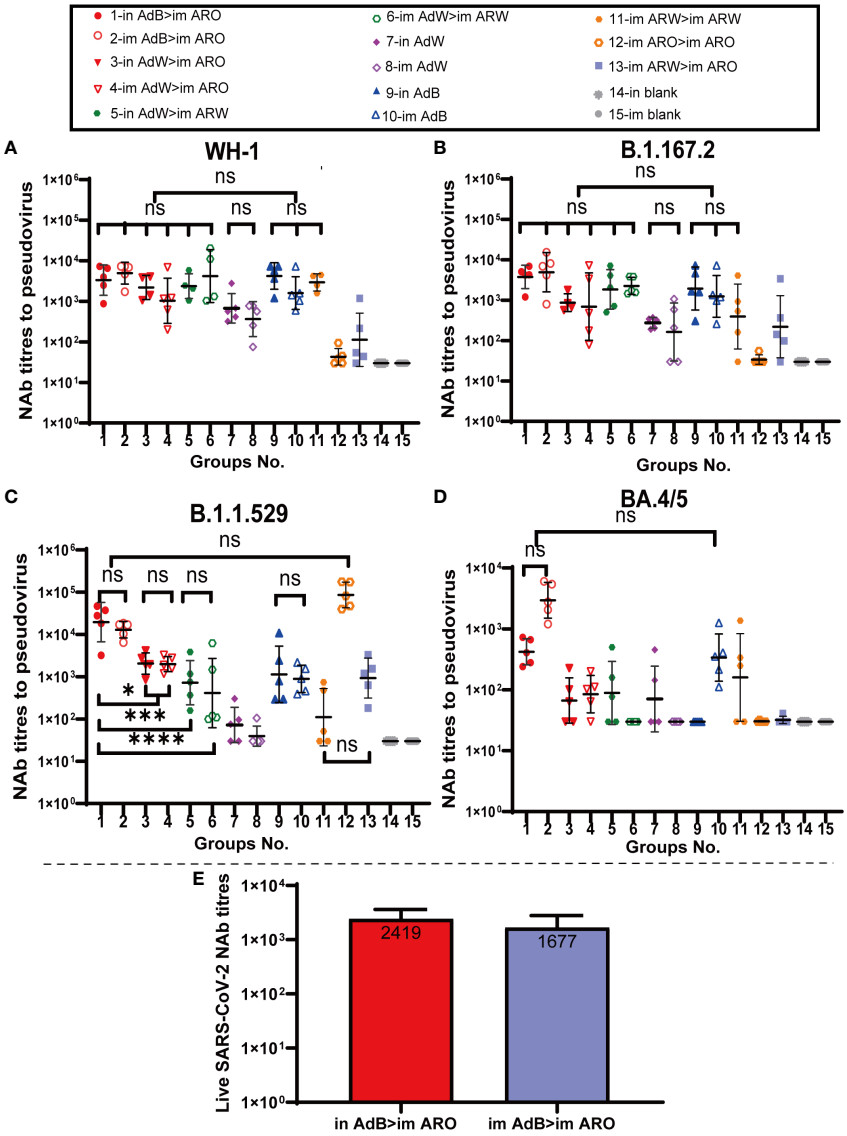

FIGURE 4
NAb titres were measured 49 days after primary immunisation. Serum PNAb titres against **(A)** WH-1, **(B)** B.1.617.2, **(C)** B.1.1.529, and **(D)** BA.4/5 are expressed as 50% inhibitory dilutions (n = 4–5 per group; one spot represents one sample). **(E)** SARS-CoV-2 NAb titration (n = 3 per group). Bars represent the geometric mean ± geometric SD; *P < 0.05; ***P < 0.001; ****P < 0.0001; ns, P > 0.05.

180, 230, 128 and 96, respectively. The IgA titres of vaccination groups were comparable with those of the in and im blank groups, suggesting that intramuscular vaccination may not trigger mucosal immune response.

3.3 Intranasal or intramuscular administration of AdB followed by ARO immunisation induces broad-spectrum neutralising activities against 2019-nCoV variants in mice

ACE2-binding inhibition (neutralising activity) rates (Figures 6, 7) in serum against spike proteins from the 2019-nCoV prototype and variants (BA.5, BA.4, BA.3, BA.2+L452R, BA.2+L452M, BA.2.12.1, BA.2, P.2, P.1, B.1.617.3, B.1.617.2, B.1.617.1, B.1.617, B.1.526.1, B.1.351 and B.1.1.7) were detected using ACE2-binding inhibition (neutralisation) ELISA on day 35 after primary immunisation to assess the broad-spectrum neutralising activity. The ACE2-binding inhibition rates induced in all tested groups were consistent with the NAb titres determined using the VSV pseudovirus assay. In the heterologous prime-boost immunisation groups, in AdB > im ARO and im AdB > im ARO generated high and broad neutralisation activity against the spike protein of all the strains tested, with arithmetic mean values (AMVs) of ACE2-binding inhibition rates of 48–93%. The in AdW > im ARO and im AdW > im ARO groups produced relatively low neutralising activity against the spike protein of all tested strains, with ACE2-

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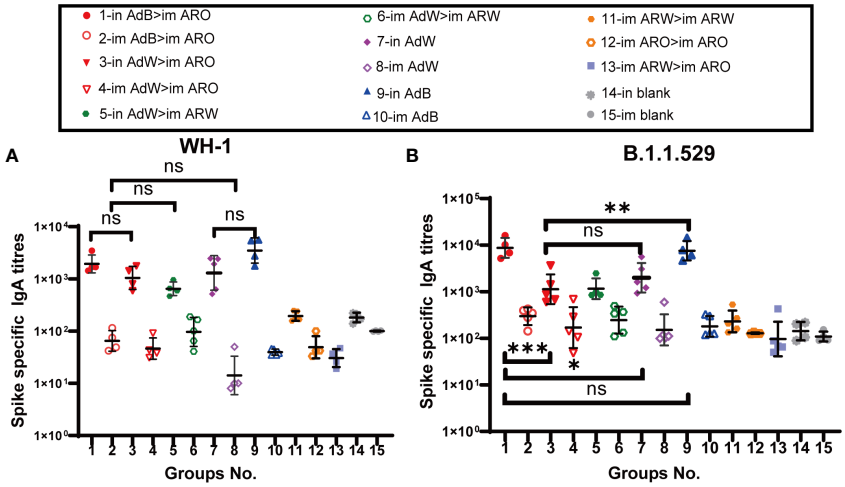

FIGURE 5
IgA responses induced by AdW, AdB, ARW, and ARO vaccines were administered using various protocols. All titres were measured on day 35 after primary immunisation. Serum IgA titres against **(A)** WH-1 and **(B)** B.1.1.529 spike protein (n = 4–5 per group; each spot represents one sample). Bars represent the geometric mean ± geometric SD; *P < 0.05; **P < 0.01; ***P < 0.001; ns, P > 0.05.

binding inhibition rate AMVs in the range of 18–74%. in AdW > im ARW and im AdW > im ARW produced high neutralising activities against the 2019-nCoV prototype and some variants (P.2, P.1, B.1.617.3, B.1.617.2, B.1.617.1, B.1.617, B.1.526.1, B.1.351 and B.1.1.7), with ACE2-binding inhibition rate AMVs in the range of 73–94%, but had low neutralising activity against Omicron lineages (BA.5, BA.4, BA.3, BA.2+L452R, BA.2+L452M, BA.2.12.1 and BA.2 variants), with ACE2-binding inhibition rate AMVs in the range of 7–42%. The im AdB > im ARO group possessed the highest and broadest neutralising activity, with inhibition rate AMVs of 54%, 55%, 69%, 56%, 66%, 67%, 67%, 92%, 88%, 88%, 81%, 89%, 89%, 82%, 93%, 85% and 90% against the BA.5, BA.4, BA.3, BA.2+L452R, BA.2+L452M, BA.2.12.1, BA.2, P.2, P.1, B.1.617.3, B.1.617.2, B.1.617.1, B.1.617, B.1.526.1, B.1.351 and B.1.1.7, respectively, which were as high and broad as those of the in AdB > im ARO group. The im and in AdB consistently induced higher ACE2-binding inhibition rates against the spike protein than in AdW and im AdW. Of all the strains tested, im ARW > im ARW induced higher ACE2-binding inhibition rates than im ARO > im ARO and im ARW > im ARO against 2019-nCoV prototype and some variants (B.1.1.7, B.1.351, B.1.526.1, B.1.617, B.1.617.1, B.1.617.2, B.1.617.3, P.1 and P.2), but lower rates against Omicron lineages (BA.5, BA.4, BA.3, BA.2+L452R, BA.2+L452M, BA.2.12.1 and BA.2 variants). The im ARO > im ARO group had low neutralising activities against the 2019-nCoV prototype and some variants (B.1.1.7, B.1.351, B.1.526.1, B.1.617, B.1.617.1, B.1.617.2, B.1.617.3, P.1 and P.2), with ACE2-binding inhibition rates equivalent to those of the in and im blank groups, but high rates against Omicron lineages (BA.5, BA.4, BA.3, BA.2+L452R, BA.2+L452M, BA.2.12.1 and BA.2 variants). The ACE2-binding inhibition rates on day 49 (Figures S1, S2) were consistent with those on day 35, which corresponded to NAb response levels on day 49 obtained using the VSV pseudovirus assay.

3.4 All AdW-, AdB-, ARW- and ARO-vaccinated groups exhibit strong cellular immunity against B.1.1.529, B.1.617.2 and WH-1 strains

Splenic lymphocytes were harvested on day 49 after primary immunisation and stimulated with the peptide pool that spans the spike proteins of the B.1.1.529, B.1.617.2 or WH-1 strain for 24 h, followed by IFN-γ ELISpot analysis (Figures 8A–C). The five AdB or AdW intramuscular-only vaccination groups (im AdB > im ARO, im AdW > im ARO, im AdW > im ARW, im AdW, and im AdB) had the highest T-cell responses against the WH-1 strain among all tested groups, with GMTs of spot forming units (SFUs) per 2.5 × 10^5 splenic lymphocytes of 268, 178, 136, 97 and 145, respectively. The intranasal vaccination and homologous prime-boost groups (in AdB > im ARO, in AdW > im ARO, in AdW > im ARW, in AdW, in AdB, im ARW > im ARW, im ARO > im ARO, and im ARW > im ARO) had relatively low T-cell responses, with GMTs of SFUs per 2.5 × 10^5 splenic lymphocytes of 47, 32, 38, 17, 33, 31, 25 and 39, respectively.

Similar results were observed against the B.1.617.2 (Figure 8B) and B.1.1.529 (Figure 8C) strains compared with the WH-1 strain. The GMTs of SFUs per 2.5 × 10^5 splenic lymphocytes of the five AdB or AdW intramuscular-only vaccination groups (im AdB > im ARO, im AdW > im ARO, im AdW > im ARW, im AdW and im AdB) against the B.1.617.2 strain were 288, 204, 143, 183 and 179, respectively, showing no significant differences. The GMTs of SFUs per 2.5 × 10^5 splenic lymphocytes of five AdB or AdW intramuscular-only vaccination groups (im AdB > im ARO, im AdW > im ARO, im AdW > im ARW, im AdW and im AdB) against the B.1.1.529 strain were 278, 205, 143, 168 and 165, respectively, showing no significant differences. The intranasal vaccination and homologous prime-boost groups had T-cell

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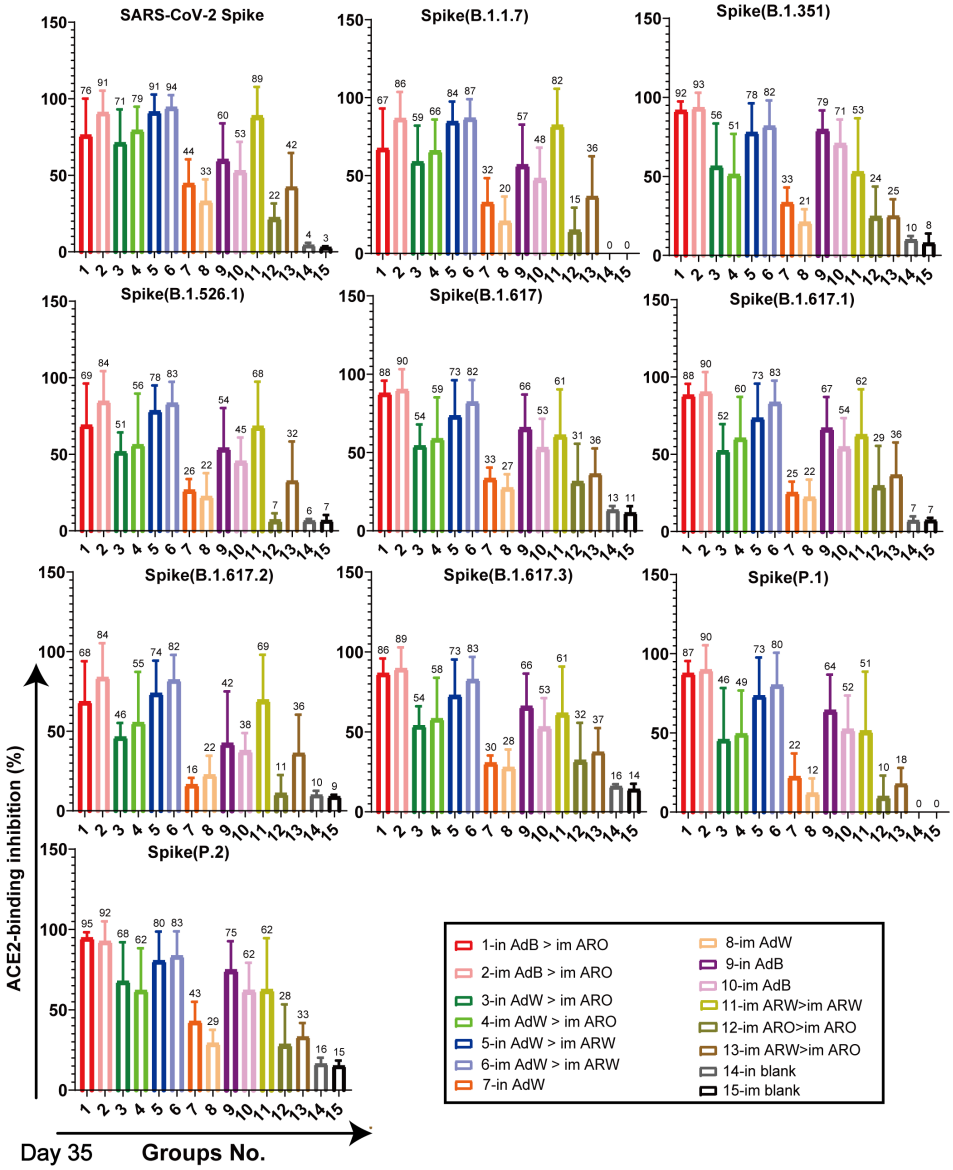

FIGURE 6
Neutralisation capacity of sera was observed by measuring the inhibition of binding between angiotensin-converting enzyme 2 (ACE2) and SARS-CoV-2 spike proteins on day 35 after primary immunisation. Spike proteins were from the SARS-CoV-2 prototype and B.1.1.7, B.1.351, B.1.526.1, B.1.617, B.1.617.1, B.1.617.2, B.1.617.3, P.1, and P.2 strains, respectively. Negative ACE2-binding inhibition rates are shown as zero (n = 5 per group). Bars represent the mean ± SD; numbers represent the mean of the corresponding group.

responses against the B.1.1.529, B.1.617.2 and WH-1 strains that were lower than those in the five AdB or AdW intramuscular-only vaccination groups.

3.5 Induction of skewed Th1 cell response in all AdW-, AdB-, ARW- and ARO-vaccinated groups

Next, we evaluated the Th1 skewing of T-cell responses specific to spike proteins. Splenic lymphocytes were harvested on day 49 after primary immunisation and stimulated with the peptide pool. Intracellular cytokine staining and MSD assays were performed to determine Th1-dominant T-cell responses against the different 2019-nCoV variants.

The intracellular cytokine staining results demonstrated that the ratios (%) of $CD4^+$ (Figure 9A) and $CD8^+$ T (Figure 9B) cells secreting Th1 typical cytokines (IL-2, TNF-α and IFN-γ) increased in all vaccination groups compared with the in and im blank groups, but the ratios (%) of $CD4^+$ and $CD8^+$ T cells secreting Th2 cytokines (IL-10 and IL-4) were not remarkably increased compared with the in and im blank groups. The proportions of IL-10 and IL-4 in $CD8^+$ and $CD4^+$ T cells are shown in Figures S3A, B. Overall, higher Th1 responses were found in the five AdB or AdW intramuscular-only vaccination groups compared with the other vaccination groups.

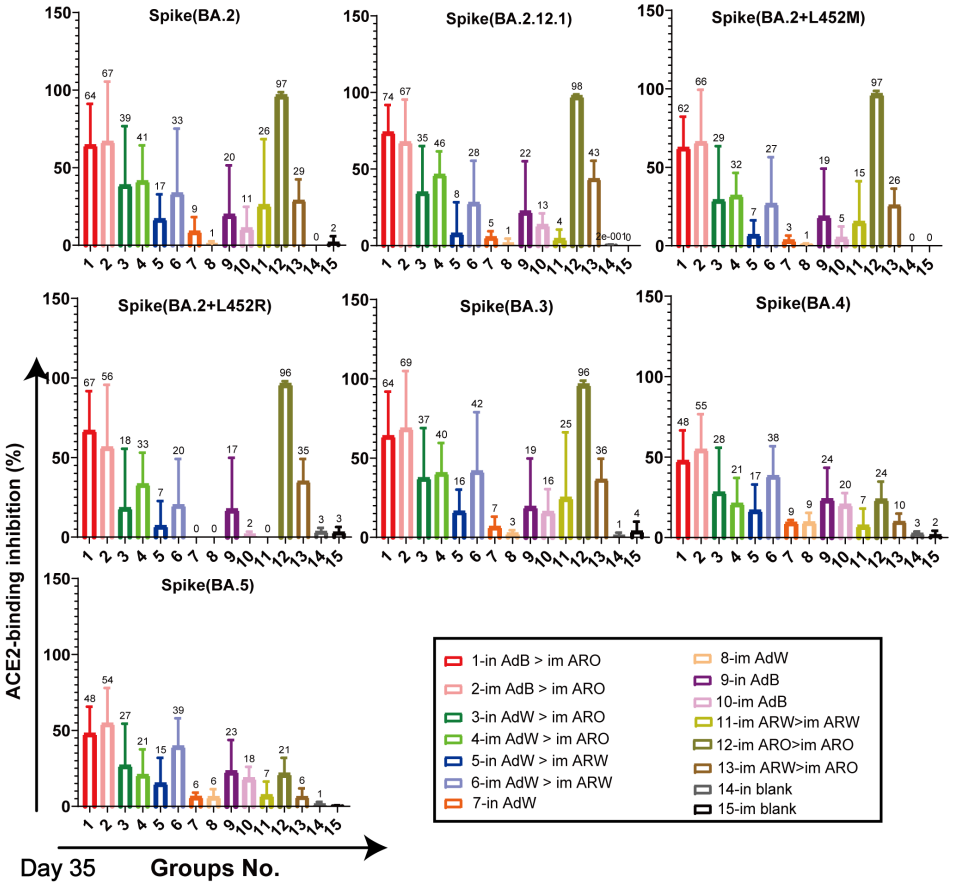

FIGURE 7
Neutralisation capacity of sera was observed by measuring the inhibition of binding between ACE2 and SARS-CoV-2 spike proteins on day 35 after primary immunisation. Spike proteins were from BA.2, BA.2.12.1, BA.2+L452M, BA.2+L452R, BA.3, BA.4, and BA.5 strains, respectively. Negative ACE2-binding inhibition rates are shown as zero (n = 5 per group). Bars represent the mean ± SD; numbers represent the mean of the corresponding group.

MSD cytokine profiling assays (Figures 10A–C) of TNF-α, IL-2, IL-4, and IL-10 were conducted to examine the functional preservation and polarisation of T cells specific to spike proteins against the B.1.1.529, B.1.617.2 and WH-1 strains. All vaccinated strategies elicited increased IL-2 and TNF-α concentrations, with higher levels in the five AdB or AdW intramuscular-only vaccination groups compared with the other groups, but lower concentrations of IL-10 and IL-4 that were comparable with the in and im blank groups (Figures S4A–C). These results suggest that the Th1-skewed, but not the Th2-skewed, response is considerably increased in all vaccination groups.

4 Discussion

The neutralisation and protective efficacy of different COVID-19 vaccines or monoclonal antibodies have markedly decreased with increasing VOCs (22–27). Different second-generation vaccines were developed to protect against multiple VOCs, but did not possess an ideal broad spectrum and cross-protection efficacy (28, 29). There is an urgent need to develop the optimal vaccination strategies for heterologous SARS-CoV2 variant-specific COVID-19 vaccines to improve their broad-spectrum protective efficacy against emerging 2019-nCoV variants. In this work, initial intramuscular vaccination with AdB followed by a booster vaccination with ARO exhibited the highest level of cross-reactive IgG, NAb responses, ACE2-binding inhibition rates, and Th1-based immune responses against the 2019-nCoV variants among all the strategies tested. However, this regimen did not induce local mucosal immune responses. Interestingly, intranasal administration of AdB followed by ARO not only induced systemic immune responses comparable to those induced by im AdB > im ARO, but also exhibited high levels of IgA and T-cell immune responses against multiple 2019-nCoV variants. Thus, in AdB > im ARO may be a promising immunisation strategy for enhancing the broad protection of chimpanzee adenovirus- and mRNA-based 2019-nCoV vaccines against VOCs.

Combining two different 2019-nCoV variant-specific vaccines was an effective strategy to increase the broad protection efficacy of chimpanzee adenovirus- and mRNA-based 2019-nCoV vaccines (11, 30, 31). Interestingly, we observed that heterologous prime-boost immunisation with AdB and ARO notably induced a broader and stronger systemic immune response than that induced by AdW and ARO or by AdW and ARW. Wang et al. (30) found that the

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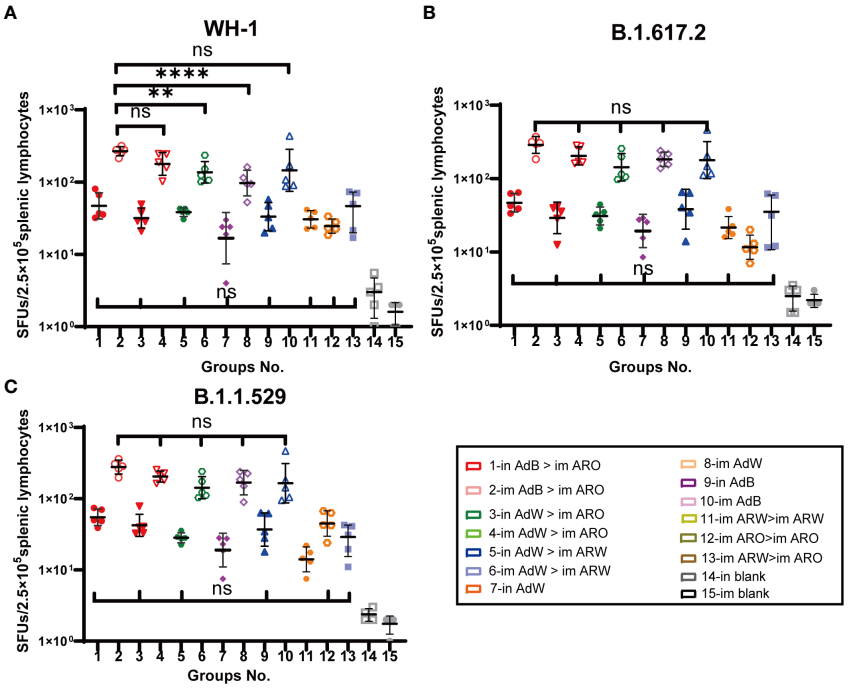

FIGURE 8
Cellular immune responses specific to SARS-CoV-2 spike proteins were measured 49 days after primary vaccination. Enzyme-linked immunospot (ELISpot) assays for IFN-γ after stimulation with the SARS-CoV-2 spike protein. Five mice per group were euthanised, and T-cell responses were measured. Lymphocytes were stimulated with **(A)** WH-1, **(B)** B.1.617.2, and **(C)** B.1.1.529 spike peptide pools spanning the entire spike protein sequence. Cells secreting IFN-γ were quantified using ELISpot assays (n = 5 per group; each point represents the mean number of spots from two wells per sample). Bars represent the geometric mean ± geometric SD; **P < 0.01; ****P < 0.0001; ns, P > 0.05.

heterologous Ad5-nCoV plus mRNA vaccine and homologous mRNA-Beta and mRNA-Omicron induced considerable cross-reactive neutralisation capacity against the prototype, Omicron, Delta and Beta variants in female C57BL/6 mice. Among these strategies, homologous prime-boost immunisation with mRNA-Beta and mRNA-Omicron induced the largest coverage of broad cross-neutralisation capacity, which was consistent with our results.

Numerous studies have indicated that 2019-nCoV Beta variant-specific COVID-19 vaccines induce broader spectrum immune responses against emerging 2019-nCoV variants than other variant-specific COVID-19 vaccines (9, 29). Our results demonstrated that single-dose immunisation with AdB could induce broader-spectrum NAb titres than AdW against different 2019-nCoV variants. Sun et al. (29) assessed the cross-reactive immune response capacity of recombinant COVID-19 protein vaccines that expressed spike protein RBD of the prototype and Alpha, Beta, Delta or Lambda variants in female C57BL/6 mice. The monovalent Beta-RBD vaccine generated higher and broader pseudovirus NAb responses against all five 2019-nCoV variants compared to the other tested vaccines. These findings are consistent with our previous results. The neutralisation site of most neutralising antibodies is in the RBD region of the spike protein (32). This observation may be attributed to the fact that B.1.351 strain (N501Y, E484K and K417T) has the same high-frequency mutations in RBD region as other 2019-nCoV variants such as N501Y of B.1.1.7, K417T, E484K and N501Y of P.1, E484A and N501Y of B.1.1.529, K417N, E484A and N501Y of BA.2, and K417N, E484A and N501Y of BA.4/BA.5, which are implicated in immune evasion and neutralising activity (2, 33, 34). The spike protein sequences containing these high-frequency mutation sites have been proposed as an effective strategy for designing universal COVID-19 vaccines (35).

Furthermore, we found that broader and more abundant cross-NAbs were generated by intranasal vaccination with AdB or AdW compared to intramuscular vaccination, probably due to the increased MIR induction with AdB or AdW *via* an intranasal route (18, 19, 36). Secretory IgA, as part of the MIR, plays an important role in preventing COVID-19 infection by limiting the virus at its point of entry in the upper respiratory tract (37–39). In addition, SIgA has non-specific neutralising properties (40), which may counteract the immune escape of emerging variants. However, we only evaluated IgA titres in the serum, which were inadequate to understand the role of SIgA after vaccination with AdB or AdW. Thus, the measurement of SIgA levels in bronchoalveolar lavage fluid is needed to characterize the MIR's protective capacity more precisely after intranasal vaccination with AdB or AdW (36).

T cells plays crucial roles in secreting specific antiviral cytokines as well as recognising and killing infected cells (41). Moreover, T cells are involved in the humoral immune responses against 2019-nCoV in mice (42). Tarke et al. (43) found that the memory T-cell responses induced by numerous COVID-19 vaccines (Ad26.COV.S, BNT162b2, mRNA-1273 and NVX-CoV2373) were preserved and could cross-recognise early 2019-nCoV variants. However, the proportions of memory B cells and neutralising antibodies were

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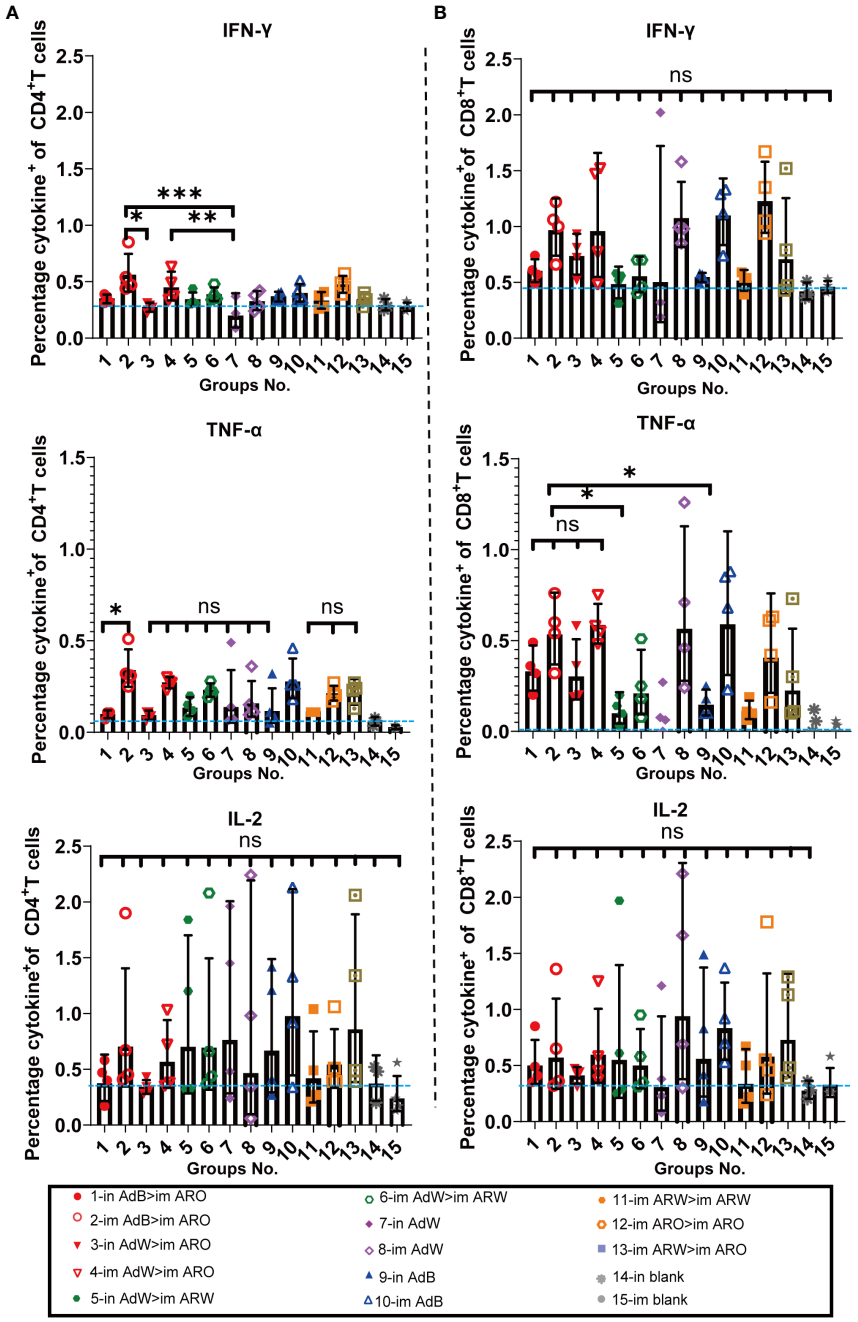

FIGURE 9
Th1/Th2 skewing was detected *via* intracellular cytokine staining on day 49 after primary immunisation. Percentage of spike protein-specific IFN-γ-, IL-2-, and TNF-α-positive memory **(A)** $CD4^+$ T and **(B)** $CD8^+$ T cells was measured on day 49 after primary immunisation (n = 4 per group; each point represents one sample). Bars represent the geometric mean ± geometric SD; *P < 0.05; **P < 0.01; ***P < 0.001; ns, P > 0.05. The blue dashed lines represent the blank value.

markedly reduced in response to emerging variants. Our results also indicated that T-cell immune responses induced by AdW, AdB, ARW and ARO vaccines could cross-recognise 2019-nCoV VOCs. Meanwhile, all vaccination groups induced a Th1-biased cellular immune response, with higher concentrations of Th1-secreted TNF-α, IL-2 and IFN-γ in the five intramuscular-only vaccination groups than those in the intranasal vaccination and homologous prime-boost groups. These findings were consistent with our previous results (44).

Nevertheless, this study has few limitations. First, the neutralisation titres of other live 2019-nCoV variants were not measured due to the limited resources. NAb exhibits an immune protective effect on symptomatic COVID-19 infection (45), and additional experiments are warranted to verify the efficacy of the in AdB > im AdO route after challenge of other live 2019-nCoV variants. Second, we did not analyse SIgA levels in nasopharyngeal and bronchoalveolar lavage fluid, which could be useful in preventing 2019-nCoV variant infection. Third, the functional preservation of most T-cell responses could act as a second-

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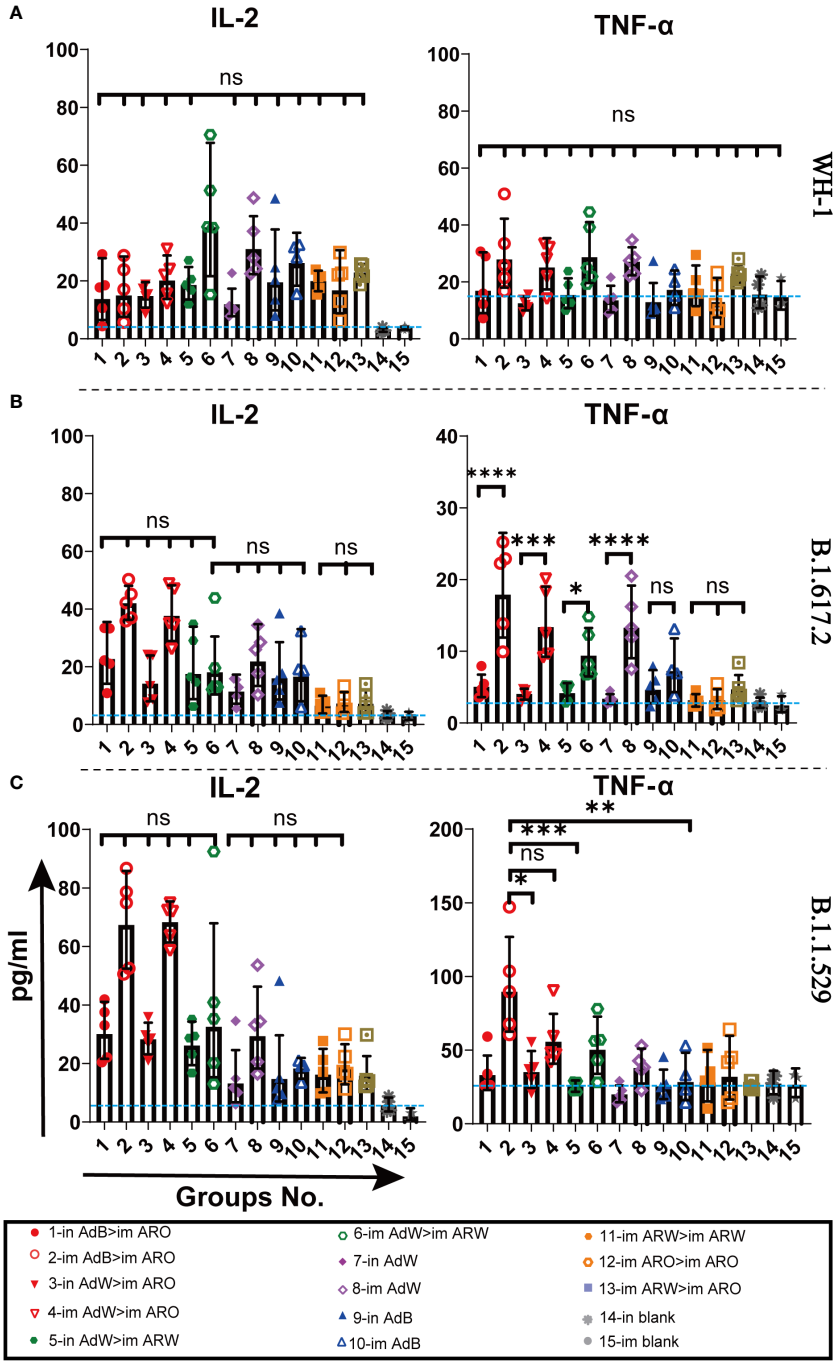

FIGURE 10
Th1/Th2 skewing in immunised mice was measured using Meso Scale Discovery (MSD) cytokine profiling. Lymphocytes were stimulated with **(A)** WH-1, **(B)** B.1.617.2, and **(C)** B.1.1.529 spike peptide pools spanning the entire spike protein sequence for 24 h. IL-2 and TNF-α levels in supernatants were measured (n = 5 per group; each point represents one sample). Bars represent the geometric mean ± geometric SD; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, P > 0.05. The blue dashed lines represent the blank value.

level defence against diverse variants. However, we did not further assess whether the T-cell responses induced by AdW, AdB, ARW and ARO can cross-recognise other emerging 2019-nCoV variants.

In conclusion, primary immunisation with intranasal AdB followed by intramuscular ARO can induce broader spectrum, stronger local and systemic mucosal immune responses against different 2019-nCoV variants, indicating a promising strategy against VOCs and possibly emerging new variants in the future. Our findings provide a scientific basis for further development of broad-spectrum vaccines and immunisation strategies.

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 Institutional Animal Care and Use Committee of the National Institutes for Food and Drug Control is affiliated with the National Institute for Food and Drug Control.

Author contributions

YL, JLi, and WH designed and supervised the study. XLi, JLiu, WL, ML, ZY, DZ, QP, YS, and LY performed the experiments. XLiu, JLiu, ZZ, ML, and QP analysed the data. YH, LS, HX, YW, GY, and XW provided administrative, technical, and material support. XLi, QP, JLiu and ML wrote the manuscript. WL and YL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1142394/full#supplementary-material

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Design and preclinical evaluation of a universal SARS-CoV-2 mRNA vaccine

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Because of the rapid mutations of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an effective vaccine against SARS-CoV-2 variants is needed to prevent coronavirus disease 2019 (COVID-19). T cells, in addition to neutralizing antibodies, are an important component of naturally acquired protective immunity, and a number of studies have shown that T cells induced by natural infection or vaccination contribute significantly to protection against several viral infections including SARS-CoV-2. However, it has never been tested whether a T cell-inducing vaccine can provide significant protection against SARS-CoV-2 infection in the absence of preexisting antibodies. In this study, we designed and evaluated lipid nanoparticle (LNP) formulated mRNA vaccines that induce only T cell responses or both T cell and neutralizing antibody responses by using two mRNAs. One mRNA encodes SARS-CoV-2 Omicron Spike protein in prefusion conformation for induction of neutralizing antibodies. The other mRNA encodes over one hundred T cell epitopes (multi-T cell epitope or MTE) derived from non-Spike but conserved regions of the SARS-CoV-2. We show immunization with MTE mRNA alone protected mice from lethal challenge with the SARS-CoV-2 Delta variant or a mouse-adapted virus MA30. Immunization with both mRNAs induced the best protection with the lowest viral titer in the lung. These results demonstrate that induction of T cell responses, in the absence of preexisting antibodies, is sufficient to confer protection against severe disease, and that a vaccine containing mRNAs encoding both the Spike and MTE could be further developed as a universal SARS-CoV-2 vaccine.

KEYWORDS

SARS-CoV-2, mRNA vaccine, neutralizing antibody, T cell response, multiple T cell epitopes (MTEs)

Introduction

The emergence of coronavirus disease 2019 (COVID-19) rapidly induced a global public health emergency. According to the World Health Organization, as of October 4, 2022, there have been more than 615 million confirmed cases worldwide and over 6 million confirmed deaths (1). In addition, almost one billion people in lower-income countries have not had access to life-saving vaccines and remain unvaccinated (2). COVID-19 continues to spread rapidly and evolve as the virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, changes over time. These changes may affect the pathological properties of the virus, such as its rate of infection and disease severity, as well as the performance of vaccines, therapeutics, diagnostic tools, or other public health and social measures (3). One of the challenges that threaten the performance and efficiency of vaccines is the emergence of novel viral variants, which are more contagious (4) and have the ability to infect a broader range of host species (5). Currently, there are two variants of concern (VOC), the Delta variant (6) and the Omicron variant, which includes BA.1, BA.2, BA.3, BA.4, BA.5, and descending lineages (7). A continuing concern is the ability of SARS-CoV-2 variants to emerge repeatedly with the ability to escape vaccine immunity (8, 9). Variant-updated vaccines and multiple rounds of immunization are essential to control viral spread. Many countries struggle with repeated waves of infection and do not have sufficiently effective vaccines against newly circulating viral variants. Therefore, further COVID-19 vaccine development is necessary.

The most common strategy in current vaccine platforms is the use of the Spike protein of the SARS-CoV-2 virus as the only antigen. These vaccines aim to induce anti-Spike neutralizing antibodies that specifically bind to the receptor binding domain (RBD) to block the entry of the virus into the host cell (10, 11). This strategy is effective with RNA vaccines showing up to 95% efficacy (10, 12). However, newly emerging VOCs threaten the efficacy of these vaccines because of mutations in Spike protein, allowing the virus to evade antibody-based immunity (13, 14). As a result, new strategies are needed to combat new VOCs.

Potent T cell responses are imperative to adaptive immunity (15). Moreover, conserved T cell responses can be particularly important when new viral variants evade the neutralizing antibodies (15). A number of clinical studies have shown that T cells induced by natural infection with SARS-CoV-2 or vaccination contribute significantly to the protective effect of COVID-19 (16, 17). Thus, if T cell epitopes are derived from conserved regions of the virus, T cell-inducing vaccines have the potential to be an alternative strategy for the development of a universal COVID-19 vaccine.

To address whether a broad COVID-19 vaccine could be achieved by unitizing T cell immunity that recognizes the conserved region of SARS-CoV-2, we designed a universal COVID-19 vaccine which is a lipid nanoparticle (LNP) formulated mRNA vaccine containing two mRNAs. One mRNA encodes SARS-CoV-2 Omicron S protein in prefusion confirmation for induction of neutralizing antibodies. The other mRNA encodes over one hundred T cell epitopes derived from non-Spike conserved regions of SARS-CoV-2. These multi-T cell epitopes (MTE) are conserved across all known SARS-CoV-2 variants, as well as other members of the coronavirus family. Our results show that immunization with MTE alone is sufficient to protect mice from lethal challenge in two mouse models. Immunization with both mRNAs induced the best protection with the lowest viral titer in the lung. Notably, these protections were achieved using 0.1 μg mRNA.

Materials and methods

DVS, OVS, and MTE mRNA and LNP formulation

Spike sequences in this study were derived from SARS-CoV-2 Wuhan strain, accession ID NC_045512 and modified for Delta variant Spike (DVS) and Omicron variant Spike (OVS) according to the Delta variant and Omicron variant mutation on the CDC website. Sequences of SARS-CoV-2 DVS, OVS, and MTE mRNA were codon optimized and inserted into PUC57, which contains a T7 promoter, 5'-UTR, 3'-UTR, and polyA tail (Figure 1A). MTE mRNA consists of hundreds of T cell epitopes derived from all genes except for the S gene, including structural protein E, M and N and other open reading frames encoding nonstructural proteins. T cell epitopes are based on published sequences (18–20) and predicted sequences using IEDB web server-based MHC T cell epitope identification tool as described previously (21, 22). Plasmid constructs PUC-DVS, PUC-MTE and PUC-OVS were synthesized by GenScript. DVS, OVS, and MTE mRNA were made with T7 polymerase *in vitro* transcription (IVT) using pseudo-UTP. The capping was done after the completion of IVT, the IVT product was purified first then subjected to capping reaction. We use the dual enzyme capping reaction, Vaccinia Capping System (NEB, Cat # M2080B-1ml) and MTE (NEB, M0266B-1ml).

Lipids used for LNP fabrication were ionizable lipid heptadecan-9-yl 8-[2-hydroxyethyl-(6-oxo-6-undecoxyhexyl) amino]octanoate (SM-102) purchased from Broadpharm (BP-25499). Helper lipid 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC,850365C-1g), cholesterol (700100P), and 1,2-dimyristoyl-snglycero- 3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DMG-PEG-2000, 880151P-1g), purchased from Avanti Polar Lipids. L002 is a candidate of ARV proprietary ionizable lipid.

The formulations were prepared by mixing lipids in an organic phase with an aqueous phase containing mRNA using a Nanoassemblr® Ignite microfluidic device (Precision NanoSystems). The molar percentage ratio for the constituent lipids is 50% for L002 or SM-102, 10% DSPC, 38.5% cholesterol, and 1.5% DMG-PEG. At a flow ratio of 1:3 organic: aqueous phases, the solutions were combined in the microfluidic device. The total combined flow rate was 12 mL/min per microfluidics chip. The LNP-mRNA mixture was dialyzed and concentrated by centrifugation.

For mRNA quantification post formulation, we applied RiboGreen assay. We followed the manufacturer's protocol (Thermo Fishers: Quant-iT™ RiboGreen™ RNA Reagent and

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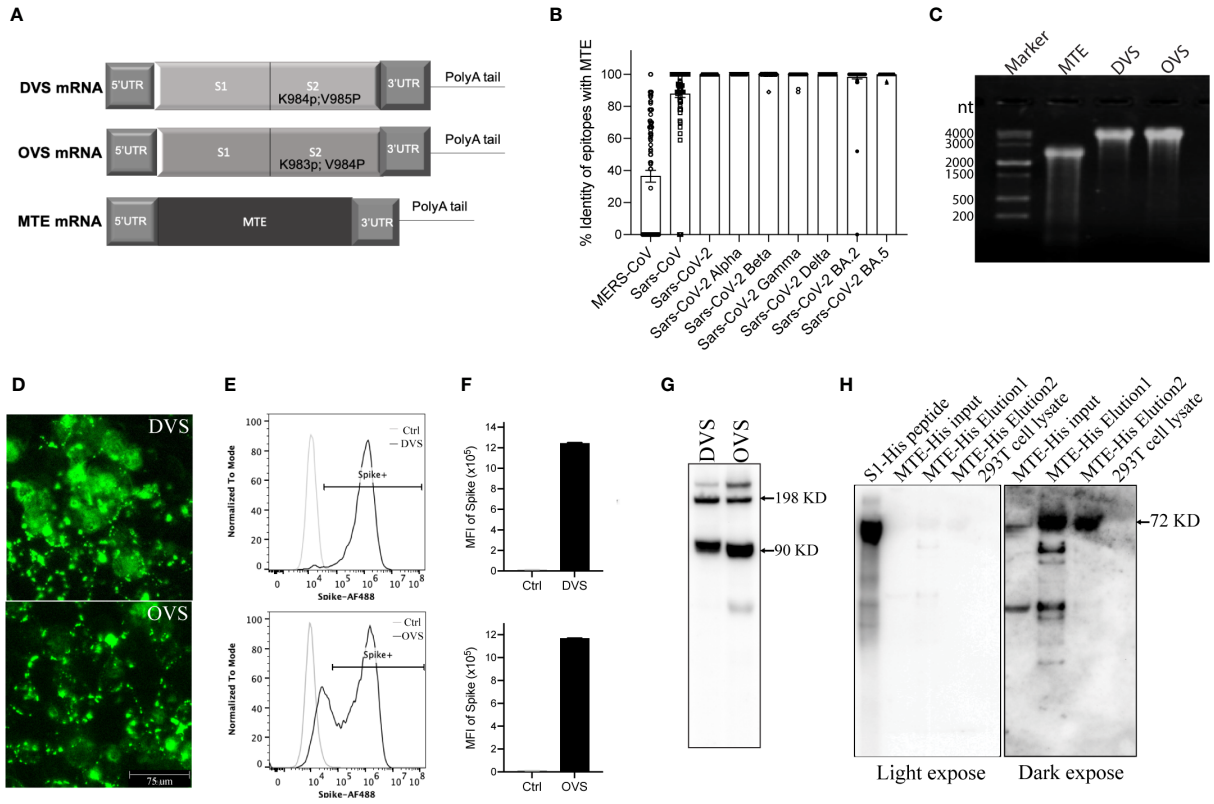

FIGURE 1
Design and validation *in vitro* of mRNA vaccine. **(A)** Schematic of SARS-CoV-2 Spike protein Delta (DVS) and Omicron (OVS) variants, as well as MTE mRNA. **(B)** Identity of epitopes of MTE with MERS-CoV, SARS-CoV and SARS-CoV-2 BA.2 variant. **(C)** MTE, DVS, and OVS mRNAs were synthesized with T7 RNA polymerase *in vitro* transcription and run on 0.8% MOPS agarose gel. **(D–G)** DVS, and OVS mRNA were synthesized with *in vitro* T7 transcription reactions and transfected into 293T cells with mRNA transfection kit. The expression level of DVS and OVS in 293T cells was detected by fluorescence microscopy **(D)** flow cytometry **(E, F)** and western blot **(G)**. **(H)** MTE-His mRNA was transfected into 293T cells and precipitated with His tag column. Eluted samples were detected by western blot.

Kit, Cat# R11490, R11491, T11493. Pub. No. MAN0002073). For mRNA quality control after formulation, we isolated mRNA from the LNPs and performed agarose gel electrophoresis.

Expression of DVS, OVS, and MTE mRNA *in vitro*

DVS, OVS, and MTE mRNA were transfected into 293T cells with MessengerMax (Invitrogen). After 48h, we evaluated the expression of the three mRNAs with flow cytometry (FC), immunofluorescence (IF), and western blot (WB). For IF, cells were incubated with rabbit anti-s1 antibody (Sino Biological) and AF488 conjugated anti-Rabbit secondary antibody (Abcam), and images were taken with the machine. For FC, cells were trypsinized and incubated with rabbit anti-s1antibody (Sino Biological) and AF488 conjugated anti-Rabbit secondary antibody (Abcam). Data was acquired with C6 (BD Biosciences) and analyzed with FlowJo (BD Biosciences). For WB, cells were harvested and denatured in lysis buffer. Samples were loaded and run in 4-12% gradient SDS-PAGE gel and transferred to the PVDF membrane. PVDF membrane was incubated with mouse anti-s2 monoclonal antibody (Thermofisher, Cat# MA5-35946) and HRP conjugated anti-mouse secondary antibody (Invitrogen, Cat#62-6520). Anti-beta-Actin HRP Antibody for protein loading control was purchased from Santa Cruz Biotechnology (sc-47778 HRP).

Mice and peptides

Six to eight-week-old female BALB/c mice were bred and maintained at an animal facility in Noble Life Sciences (Woodbine, MD). For immunogenicity studies, mice were immunized intramuscularly with formulated mRNA or PBS as indicated at Day 0 (prime) and Day21 (boost). Serum was collected after two weeks of prime and boost. Mice were euthanized at Day35, and spleens were collected for analysis of T cell immunity.

In the challenge study with SARS-CoV-2 Delta variant, K18-hACE2 transgenic mice were maintained at Bioqual Inc. (Rockville, MD) and immunized intramuscularly with formulated mRNA or PBS at Day 0 and 21. Serum was collected at Day 28 for antibody testing. At Day 35, mice were challenged intranasally with SARS-CoV-2 Delta variant at a dose of 5×10^3 the median tissue culture infectious dose ($TCID_{50}$) SARS-CoV-2 B.1.617.2 (Delta) (BEI Resources SARS-CoV-2, isolate hCoV-19/USA/MDHP05647/

2021, NR-55674). Mouse body weight and survival were recorded daily. At 4 days post of infection (DPI), 5 mice of each group were euthanized, and mouse tissues were collected and stored in Trizol or formalin.

In the challenge study with mouse adapted SARS-CoV-2 MA30, BALB/c mice were maintained at animal facility of Thomas Jefferson University (Philadelphia, PA) and immunized intramuscularly with formulated mRNA or PBS at Day 0 and 21. Serum was collected at Day 28 for antibody testing. At Day 35, mice were challenged intranasally with mouse adapted SARS-CoV-2 MA30 at a dose of 5 x 10^3 PFU in volume of 50 µL. Mouse body weight and survival were recorded post challenge. At four DPI, 5 mice of each group were euthanized, and mouse tissues were collected and stored in Trizol.

MTE overlapping peptides were synthesized by GenScript (Piscataway, NJ). Spike S1 overlapping peptides were purchased for JPT (Berlin, Germany).

Evaluation of antigen-specific T cell response by ELISPOT

Splenocytes from vaccinated mice were evaluated for antigen-specific IFN? by Enzyme-linked immunospot (ELISPOT). ELISPOT assays were performed as per ARV SOP. Briefly, a 96-well ELISPOT plate (Millipore, Cat#MSIPS4510) was coated with 10 µg/mL IFN? antibody (Biolegend, Cat# 517902, clone AN18) at 4°C overnight. Splenocytes were plated at 3x10^5 cells/well and co-cultured with either 0.5 µg/mL Spike peptides (JPT, Cat#PM-WCPV-S-1), 2 µg/mL human MTE overlapping peptides (synthesized by Genscript), concanavalin A (0.125 ug/mL) (Sigma, Cat#C5275-5MG), or medium alone in a total volume of 200 µL/well T cell media for 48h at 37°C in 5% CO2. The plates were incubated with detection antibodies, Biotin-IFN? (Biolegend, Cat# 505714, clone R46A2) and Streptavidin-HRP (Biolegend, Cat# 405210) at RT for 1-2 hours, respectively. The plates were developed with 50 µL/well AEC development solution (BDbiosciences, Cat#551015) for up to 30 min. Color development was stopped by washing under running tap water. After air-dried, colored spots were counted using an AID ELISPOT High-Resolution Reader System and AID ELISPOT Software version 3.5 (Autoimmun Diagnostika GmbH).

ELISA

The murine antibody response to the Spike was assessed by indirect ELISA. ELISA plates (Nunc MaxiSorp, Thermofisher, Cat#44-2404-21) were coated with 1 mg/mL recombinant Spike protein (Sino Biological Inc, Cat#40591-V08H) overnight and then blocked with 2% BSA in PBS. Serum samples were diluted by 200x, followed by a 1:5 serial dilution for up to 8 wells with 0.2% BSA in PBS. Samples were detected with 1:2000 goat anti-*mouse IgG-HRP* (Southern Biotech, Cat# 1031-05). The reaction was developed with TMB Substrate (Sigma, Cat#T0440-1001) and stopped with TMB Stop Solution (Invitrogen, Cat#SS04). Plates were read at OD450 using an Epoch ELISA reader (BioTek, Winooski, VT).

Lentivirus-based pseudovirus neutralization assay

The SARS-CoV-2 pseudoviruses, including the Delta variant and Omicron variant expressing a luciferase reporter gene, were purchased from Codex BioSolutions (Rockville, MD). HEK293T-hACE2 cells were seeded in 384-well tissue culture plates at a density of 7.5 × 10^3 cells per well overnight. Two-fold serial dilutions of heat-inactivated serum samples were prepared (17.5 µL) and mixed with 7.5 µL of pseudovirus. The mixture was incubated at 37°C for 1h before adding to HEK293T-hACE2 cells. After 48h, cells were lysed in Steady-Glo Luciferase Assay (Promega) according to the manufacturer's instructions. SARS-CoV-2 neutralization titres were defined as the sample dilution at which a 50% reduction in relative light units (IC_{50}) was observed relative to the average of the virus control wells.

Immunohistochemistry

Mouse lung tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of tissue with 5 µm in thickness were affixed to slides. Slides were stained with hematoxylin and eosin or Nucleocapsid antibody of SARS-CoV-2 (CST, Cat# 26369s) according to the standard program.

Statistical analysis

Statistical significance was calculated using Student's *t*-test with two-tailed analysis. A p-value less than 0,05 ($p < 0.05$) was considered statistically significant.

Results

mRNA design and expression *in vitro*

DVS mRNA encodes the full-length Spike protein from the SARS-CoV-2 Delta variant, in which we replaced amino acid lysine at 984 and valine at 985 with proline (SP2) for prefusion conformation (23) (Figure 1A). OVS mRNA encodes the full-length Spike protein from the SARS-CoV-2 Omicron variant, in which we replaced amino acid lysine at 983 and valine at 984 with proline (SP2) for prefusion conformation (Figure 1A). T cell epitopes are ~8-11 or ~13-25 amino acid long peptides respectively presented to $CD8^+$ and $CD4^+$ T-cells by MHC class I (MHC I) or MHC class II (MHC II) molecules. MTE mRNA encodes approximately one hundred T cell epitopes including both MHC I and MHC II from conserved sequences of SARS-CoV-2 Wuhan strain (Figure 1A). Some T cell epitopes strongly cross-react with mouse T cells, which has been experimentally

validated (data not shown). Thus, MTE vaccine is able to induce T-cell immune responses in mice. The sequence of each MTE epitope was compared and scored by percentage identity with those of Coronavirus including MERS-CoV, SARS-CoV and SARS-CoV-2 variants. T cell epitopes in MTE have 36.4% identity with MERS-CoV, 87.9% identity with SARS-CoV, 98.3% identity with SARS-CoV-2 omicron BA.2 variant, and more than 99.8% identity with SARS-CoV-2 Alpha variant, Beta variant, Gamma variant, Delta variant and Omicron BA.5 variant (Figure 1B). Further analysis found that there were over twenty epitopes in MERS-CoV with more than 70% identity (Supplementary Table 1). These data indicated sequences of T cell epitopes in MTE were highly conserved in SARS-CoV, SARS-CoV-2, as well as SARS-CoV-2 variants.

DVS, OVS, and MTE mRNAs were produced by IVT with T7 polymerase (Figure 1C). DVS and OVS mRNA were transfected in 293T cells and their expression was detected with the anti-Spike antibody. Immunofluorescent staining showed membrane localization of DVS and OVS protein (Figure 1D). Flow cytometry confirmed cell surface localization and high level of protein expression of DVS (MFI 1.24M) and OVS (MFI 1.17M) (Figures 1E, F). DVS and OVS proteins were also detected by Western blotting under denaturing conditions with mouse anti-Spike S2 monoclonal antibody. Two major bands were visible, corresponding to 90 KD S2 protein, and 198 KD full length of Spike protein (24) (Figure 1G).

Because there is no antibody available for MTE detection, in addition to MTE mRNA, MTE-His mRNA was parallelly constructed to tag MTE with six histidine at the C terminal. After transfection into 293 T cells, cells were lysed and MTE-His fusion protein was concentrated with His tag purification column kit (SigmaAldrich, Cat# H7787), followed by Western blotting under denaturing conditions. An expected size of 72KD major band and smaller molecular weight degraded products were observed in the first and second elution (E1 and E2) (Figure 1H), suggesting rapid degradation of MTE fusion proteins.

Immunogenicity in BALB/c mice

The strategy of our universal vaccines against SARS-CoV-2 is based on neutralizing antibodies (induced by Spike mRNA) and T cell responses (induced mainly by MTE mRNA). Next, we mixed either OVS mRNA or DVS mRNA with MTE mRNA at a ratio of 1:1 and formulated the RNA into SM102-based LNPs, referred to as LNP(SM102)-mRNA OVS/MTE and LNP(SM102)-mRNA DVS/MTE. We also prepared LNP(SM102)-mRNA DVS and LNP(SM102)-mRNA DVS/OVS/MTE as a control. BALB/c mice were immunized intramuscularly with 10 μg of LNP-mRNA vaccines at Day 0 (prime) and D21 (boost), sera were collected two weeks after prime and boost, and spleens were collected at D35 (Figure 2A). First, we measured Spike-specific IgG antibodies from serum after prime and boost by ELISA. The same pattern was observed in the generation of Spike-specific antibodies and there was no significant difference in Spike binding antibody IgG titres between DVS and DVS/MTE. The logarithm of IgG titres of Spike-specific antibodies were 4.93 (DVS) vs. 4.99 (DVS/MTE) after prime and 5.80 (DVS) vs. 5.80 (DVS/MTE) after boost (Figure 2B). The logarithm of IgG antibody titres in OVS/MTE (4.04) were slightly lower at D14 than DVS/MTE (5.0) and DVS/OVS/MTE (5.14) but were comparable with the other two groups at D35 (DVS/MTE vs. OVS/MTE vs. DVS/OVS/MTE were 5.8 vs. 5.7 vs. 5.7) (Figure 2B). It has been reported that the antigenicity of the Omicron Spike protein is different from previous variants (25). This could explain why the IgG titres were lower at D14 with the OVS/MTE vaccine.

Biases of either Th1 cells or Th2 cells prior to vaccine administration were also reported to be associated with immune disease (26). The percentage of IgG2a/IgG1 determined the Th1/Th2 ratio. The balance of Spike-specific IgG2a/IgG1 in the LNP-mRNA DVS/MTE group was 1.05. No Th1 or Th2 bias was detectable with our LNP-mRNA formulation (Figure 2C).

We then evaluated the inhibition of viral entry with mouse serum using Delta pseudovirus, Omicron pseudovirus, and BA.2 pseudovirus in 293T-hACE2 cells. As a control, DVS and DVS/MTE groups were evaluated using Delta pseudovirus. The logarithm of IC_{50} of the neutralizing antibody was comparable in mice immunized with LNP-mRNA DVS (3.28) or LNP-mRNA DVS/MTE (3.38). The titres were also similar to human COVID-19-convalescent serum (HCS) (3.35) (Figure 2D). As expected, the neutralizing antibody (the logarithm of IC_{50}) to Delta pseudovirus was detectable but lower in the OVS/MTE group (2.69), compared to DVS/MTE group (3.38), DVS/OVS/MTE group (3.53) and HCS (3.35) (Figure 2D). Reciprocally, the neutralizing antibody to Omicron pseudovirus were detectable but lower in the DVS/MTE group (2.43), compared to the OVS/MTE group (3.20), DVS/OVS/MTE group (3.44) or HCS (2.82) (Figure 2E). The neutralizing antibody to BA.2 pseudovirus was much lower in all groups. The logarithm of IC_{50} were 2.49 in the DVS/MTE, 2.51 in the OVS/MTE, 2.56 in the DVS/OVS/MTE group, and 2.72 in the HCS control (Figure 2F). Thus, the neutralizing antibody titres against BA.2 variant were lower than those against Delta and Omicron BA.1 variant, consistent with previous report (27). Therefore, the reduced neutralization of heterogeneous variants suggests the importance of developing an antibody-independent but universal SARS vaccine.

Next, we determined the T cell responses using ELISPOT assay with splenocytes stimulated with a Spike peptide pool or an MTE peptide pool. As a control, DVS and DVS/MTE groups were evaluated following stimulation with a Spike peptide pool. There were 1172 and 1272 spot-forming cells (SFC)/10^6 splenocytes in LNP-mRNA DVS and LNP-mRNA DVS/MTE immunized BALB/c mice, respectively. The results showed no significant difference in T cell responses between two groups (Figure 2G). The numbers of SFC/10^6 splenocytes in the DVS/MTE, OVS/MTE and DVS/OVS/MTE groups were, respectively, 1272, 1355, 1464 following stimulation with a Spike peptide pool (Figure 2G) and 891, 1742, 960 following stimulation with a MTE peptide pool (Figure 2H). The results showed that all the groups elicited strong Spike-specific T cell response or MTE-specific T cell response, respectively.

Together, these data indicated that integrating two mRNAs in one LNP formulation has similar immunogenicity to an LNP formulation utilizing one mRNA. In addition, DVS/MTE, OVS/

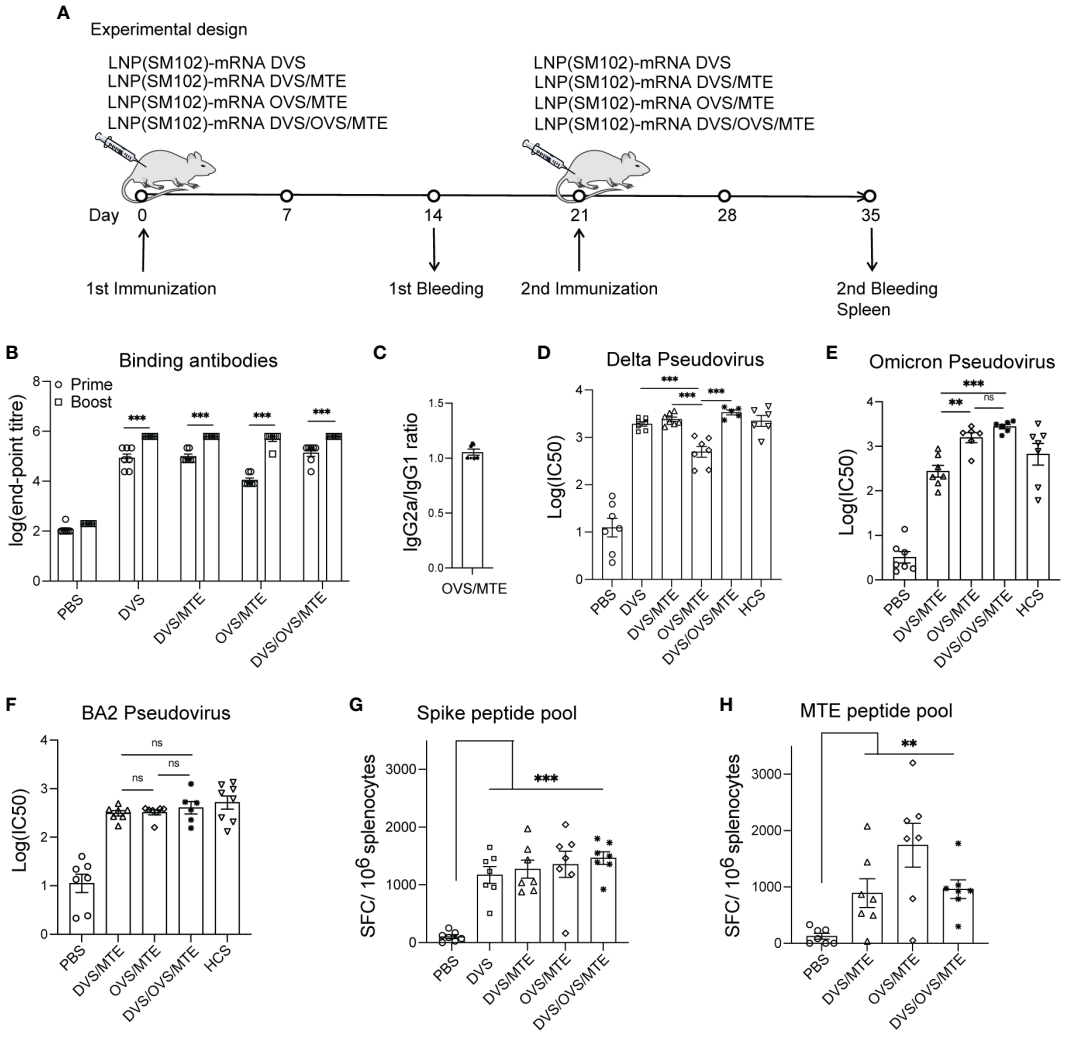

FIGURE 2
Universal vaccines elicits robust specific immunogenicity in BALB/c mice. **(A)** BALB/c mice were intramuscularly immunized on D0 and D21 with 10 μg of LNP(SM102) formulated mRNA vaccines (DVS or DVS/MTE or OVS/MTE or DVS/OVS/MTE). Mouse sera were collected on D14 (prime) and D35 (boost). Mouse spleens were collected on D35. **(B, C)** Spike-specific IgG **(B)** or subtype IgG1 and IgG2a **(C)** was detected by ELISA from serum samples. D-F, Neutralization assays were performed with different variants of SARS-CoV-2 pseudovirus, Delta variant **(D)**, Omicron variant **(E)**, and BA.2 variant **(F)**. **(G, H)** Splenocytes were isolated from mouse spleen and performed for ELISPOT assay with stimulation of a Spike peptide pool **(G)** or MTE peptide pool **(H)**. *P<0.05, **P<0.01, ***P<0.001; ns, presents not significant.

MTE, and DVS/OVS/MTE elicited a robust Spike-specific antibody response and strong Spike and MTE-specific T cell responses. We selected OVS/MTE for further evaluation.

LNP(L002)-formulated mRNA vaccine elicits robust immune responses at a low dose

Besides formulating mRNA into SM102-based LNPs, we also formulated them into a new L002 ionizable lipid-based LNPs. To determine a suitable dose for L002-formulated mRNA, BALB/c mice were immunized intramuscularly with different doses of LNP (L002)-mRNA DVS/MTE and LNP(SM102)-mRNA DVS/MTE, where each mRNA was 0.01 μg, 0.1μg, or 1 μg. Sera and spleen were collected at D35 (Figure 3A). Compared with LNP(SM102)-mRNA DVS/MTE mice, LNP(L002)-mRNA DVS/MTE mice exhibited higher neutralizing antibodies using the Delta pseudovirus. The logarithm of IC_{50} of neutralizing antibody was 1.62 vs. 2.99, 2.05 vs. 3.12, and 3.26 vs. 3.55 at 0.01 μg, 0.1 μg, and 1 μg of mRNA doses, respectively (Figure 3B). Notably, the logarithm of IC_{50} of neutralizing antibody at 0.01 μg mRNA with LNP(L002) was similar to 1 μg of LNP(L002)-mRNA and LNP(SM102)-mRNA (Figure 3B). The numbers of SFC/10^6 splenocytes in LNP(SM102)-mRNA DVS/MTE and LNP(L002)-mRNA DVS/MTE were 2 versus 269 at 0.01 μg administration groups, 20 versus 260 at 0.1 μg administration groups and 362 versus 272 at 1 μg administration groups (Figure 3C). Similar T cell responses were induced at 0.01 μg dose and 1 μg dose of LNP(L002)-mRNA DVS/MTE (Figure 3C). Thus, L002-based LNP-mRNA formulation induced stronger T and

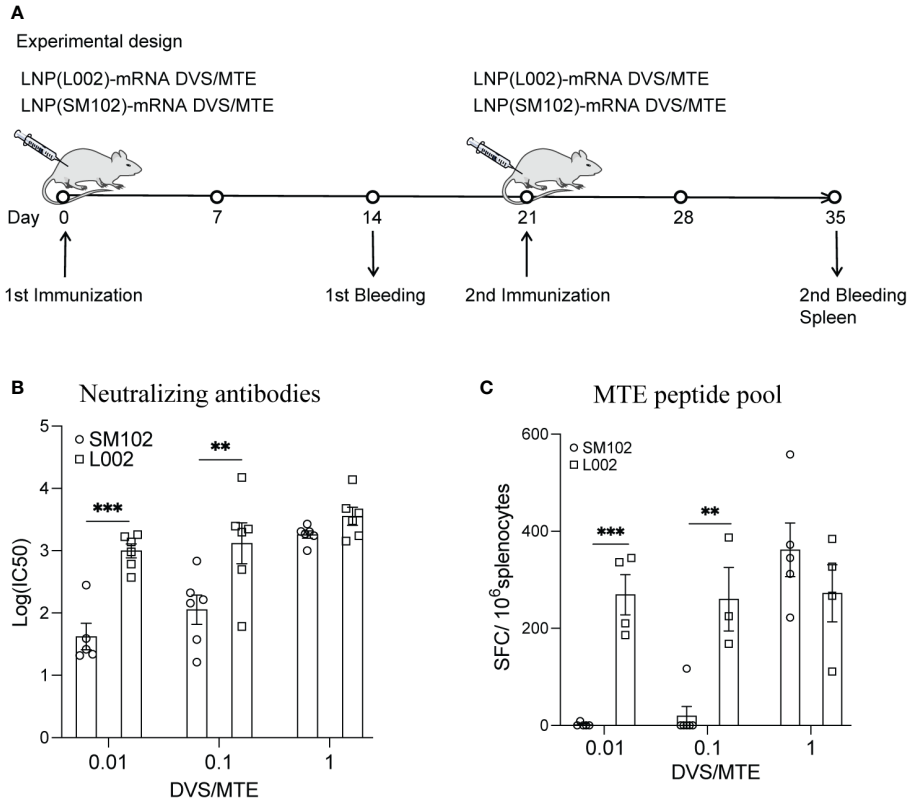

FIGURE 3
LNP(L002)-mRNA DVS/MTE vaccine elicit more robust specific immunogenicity than LNP(SM102)-mRNA DVS/MTE vaccine. **(A)** BALB/c Mice were immunized with 0.01, 0.1 or 1 μg of either LNP(L002)-mRNA DVS/MTE or LNP(SM102)-mRNA DVS/MTE vaccine on D0 and D21. Mouse sera were collected on D14 (prime) and D35 (boost). Mouse spleens were harvested on D35. **(B)** Serums from D35 were assessed for neutralizing antibodies against SARS-CoV-2 Delta pseudovirus. **(C)** Splenocytes from mouse spleen were isolated and performed for ELISPOT assay with stimulation of an MTE peptide pool. **P<0.01, ***P<0.001.

antibody responses than SM102-based LNP mRNA formulation. Therefore, we used a low dose (0.1 μg) of LNP(L002)-mRNA in the subsequent virus challenge studies.

LNP(L002)-formulated MTE mRNA vaccines protected mice from lethal infection of SARS-CoV-2 virus

To develop a universal COVID-19 vaccine, we propose the utilization of use two mRNAs, one encoding OVS and the other one encoding MTE. To determine if the MTE mRNA alone, and MTE plus OVS mRNAs, provide protection from SARS-CoV-2, two mouse models were used in this study, K18-hACE2 transgenic mice with SARS-Cov-2 Delta variant and BALB/c mice with mouse adapted SARS-CoV-2 MA30.

In the first model, K18-hACE2 transgenic mice were immunized intramuscularly with 0.1 μg of LNP(L002)-mRNA OVS/MTE, LNP(L002)-mRNA MTE, LNP(L002)-mRNA OVS, or PBS at D0 and D21 and challenged with a lethal dose of SARS-CoV-2 Delta variant at D35. The body weight and survival were recorded after the challenge. Tissues were collected at four days post infection (DPI) (Figure 4A). We observed a significant body weight loss in the PBS group (20% reduction at six DPI), but only a slight decrease at one DPI (5%) and recovery at four DPI in all the vaccinated groups (MTE, OVS, and OVS/MTE) (Figure 4B). At six DPI, all of the mice in the mock group but none in the vaccinated groups died (Figure 4C). We also used qPCR to quantify the SARS-CoV-2 Delta variant virus in 1 mg of lung mRNA. High copies of the SARS-CoV-2 viral RNA were detected in the PBS group ($1.4x10^7$), the viral RNA was below detection in the lungs from OVS/MTE group, and very low copies of the viral RNA were detected in the MTE group (3386) and OVS group (507) (Figure 4D). Lung histology also showed extensive neutrophil infiltration in the lungs of the PBS group but not of the MTE, OVS or OVS/MTE groups (Figure 4E). Similarly, a large number of viral particles were detected in the lungs of PBS group but few in MTE group and no particle in OVS or OVS/MTE group (Figure 4F).

In the second model, BALB/C mice were immunized intramuscularly with 0.1 μg of LNP(L002) formulated MTE, OVS, OVS/MTE or PBS at D0 and D21 and challenged with a lethal dose of mouse-adapted SARS-CoV-2 MA30 at D35. After challenge, mouse body weight and survival were recorded (Figure 5A). Similarly, mouse body weights were dramatically reduced in the PBS group (27% loss at six DPI). Although mice in MTE group also lost significant weight (19%) at five DPI but all mice recovered at twelve DPI (5% reduction). There was only a slight decrease in body weight (3%) at four DPI and recovered fully by ten DPI in the OVS

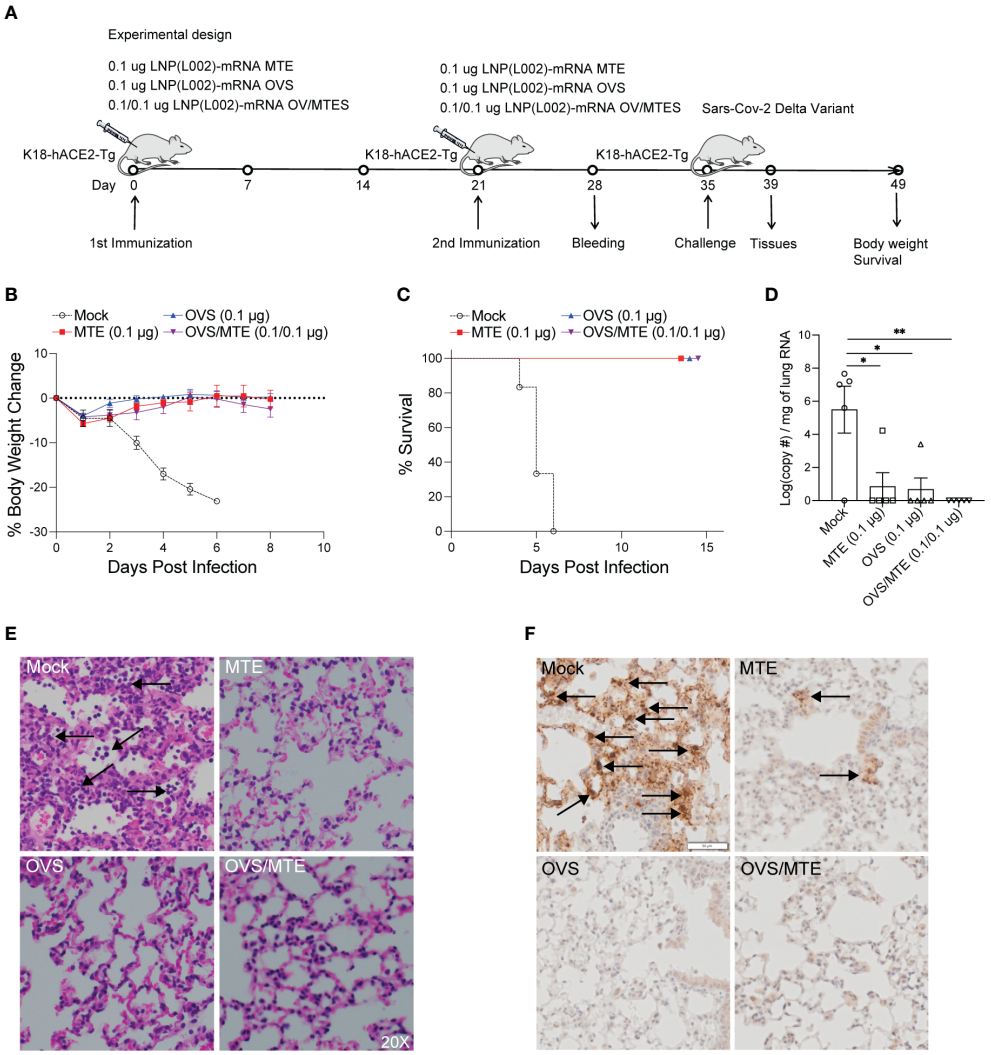

FIGURE 4
L002 formulated vaccines included LNP-mRNA OVS, LNP-mRNA MTE and LNP-mRNA OVS/MTE protected mice from SARS-CoV-2 Delta variant at a low dose. **(A)** K18-hACE2-Tg mice were immunized intramuscularly with a low dose (0.1 μg) of LNP(L002)-mRNA OVS, LNP(L002)-mRNA MTE, and LNP(L002)-mRNA OVS/MTE vaccines on D0 and D21 and challenged with a lethal dose of SARS-CoV-2 Delta variant on D35. Mouse body weight and survival were recorded after the challenge. Mouse tissues were collected 4 days after the challenge. **(B)** Mouse body weight after challenge. **(C)** Mouse survival curve after challenge. **(D)** Viral copy numbers were quantified in mouse lung tissue by qPCR. **(E)** Mouse lung was fixed and stained with H & E at 4 days after challenge. Neutrophil infiltration was marked with arrow. **(F)** Mouse lung was fixed and stained with Nucleocapsid antibody at 4 days after challenge. Arrow showed Viral particle. *P<0.05, **P<0.01.

and OVS/MTE groups (Figure 5B). At six DPI, all mice in PBS group and 1 mouse in MTE group died but none of OVS and OVS/MTE groups died (Figure 5C). MTE protection was weaker in this model (BALB/c background) compared to the first model (C57BL6/J), possibly due to differences in mouse strains and viruses used.

Together, these data clearly demonstrate that MTE or OVS alone can protect mice from lethal dose of SARS-CoV-2 but OVS/MTE provide the best protection.

Discussion

Using Spike as the only vaccine target has the disadvantage that as the virus evolves, the antibodies to any given variant have decreased neutralizing activity against newly emerging variants (28–31). Beginning in September 2020, SARS-CoV-2 has rapidly created multiple VOCs (Variants of Concern), mutating from the Alpha variant to the Delta variant, then to the Omicron variant, which gave rise to additional strains, most recently BA.4 and BA.5. As these variants emerged, the efficacy of the original vaccines decreased drastically (32, 33). A bivalent booster vaccine containing Spike mRNA from the 2019 Wuhan and Omicron strains was developed by Pfizer and Moderna (34). However, as the virus mutates, these bivalent vaccines will likely become ineffective again.

Other than neutralizing antibodies, the T cell epitopes are more conserved among the viral variants (35). Therefore, T cell epitopes could provide an immense advantage to the next generation of SARS-CoV-2 mRNA vaccines. It was found that T cell numbers are reduced in multiple immune tissues and organs of COVID-19 patients, and the magnitude of the cytotoxic T lymphocytes

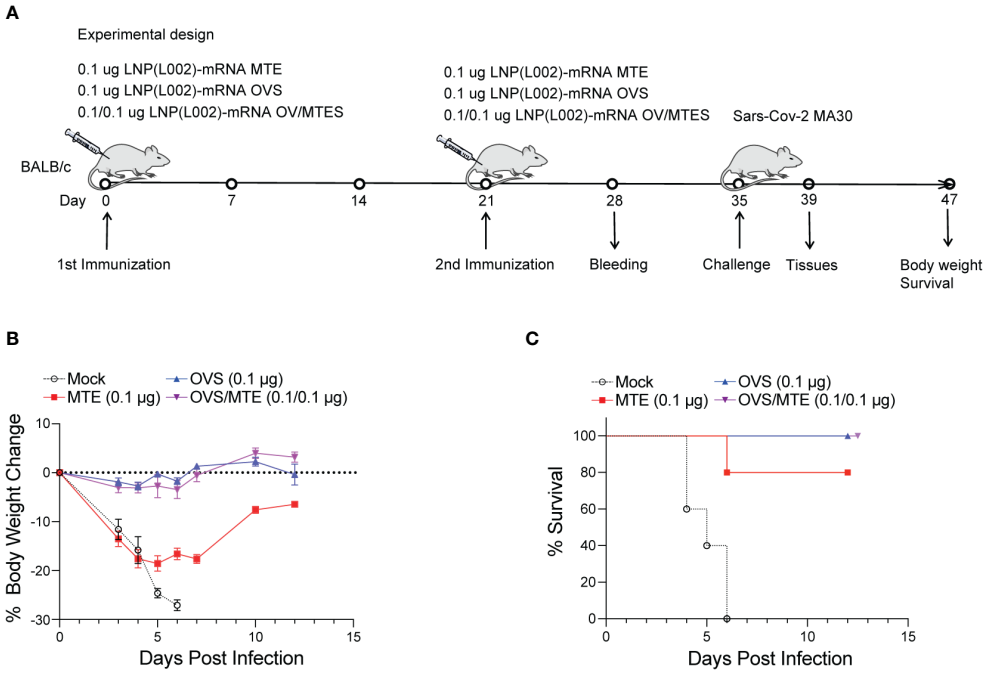

FIGURE 5
LNP(L002)-mRNA vaccines protected mice from mouse adapted SARS-CoV-2 MA30 at a low dose. **(A)** BALB/c mice were immunized intramuscularly with a low dose (0.1 ug) of LNP(L002)-mRNA OVS, LNP(L002)-mRNA MTE, and LNP(L002)-mRNA OVS/MTE vaccines on D0 and D21 and challenged with a lethal dose of SARS-CoV-2 MA30 on D35. Mouse body weight and survival were recorded after the challenge. Mouse tissues were collected 4 days after the challenge. **(B)** Mouse body weight after challenge. **(C)** Mouse survival curve after challenge.

(CTL) responses negatively correlated with the severity of the COVID-19 disease, suggesting that T cells are important for the recognition and clearance of infected cells (36). In addition, some studies have shown that virus-specific T cells can be maintained for 6 years after SARS-CoV infection (37), and are sufficient to prevent reinfection (38, 39).

MERS-CoV, SARS-CoV, and SARS-CoV-2 are coronaviruses that affect humans and may cause fatal infections (40). We compared the sequences of each epitope in MTE with MERS-CoV, SARS-CoV, SARS-CoV-2 and SARS-CoV-2 variants. T cell epitopes in MTE were 88% identical with SARS-CoV and over 94% identical with SARS-CoV-2 variants, while only 36% identical with MERS-CoV. These data showed that the sequence of MTE was highly conserved in SARS-CoV and SARS-CoV-2 variants. Although these three coronaviruses cause similar diseases and symptoms clinically, their genomic homology differs. SARS-CoV-2 have 80% genomic identity with SARS-CoV while it has only 50% identity with MERS-CoV (41). Despite the differences, we still found 24 epitopes in MTE with more than 70% similarity with MERS-CoV, which might confer some protection against MERS-CoV.

T cell immune response includes virus-specific $CD8^+$ and $CD4^+$ T cells, both of which play an important role in host defense against SARS-CoV-2 (42). Both the SARS-CoV-2 inactivated vaccine and the Spike mRNA COVID-19 vaccine are approved for the prevention of SARS-CoV-2 infection, and both induce comparable T cell responses. The Spike mRNA vaccine induces T cells targeting only the spike protein, whereas the inactivated vaccine targets not only the spike protein but also other viral proteins such as membrane and nucleoprotein, which are not often mutated like Spike protein. However, recently study has shown that, unlike mRNA vaccines, inactivated virus vaccines do not induce cytotoxic $CD8^+$ T cells as mRNA vaccines do (43). Therefore, in the present study, we designed an MTE mRNA encoding numerous T cell epitopes from the conserved region other than Spike to elicit a broad T cell response. In addition, one study showed that an mRNA vaccine encoding nucleocapsid (N) protein alone could confer protection against SARS-CoV-2 independent of neutralizing antibodies (44). However, this study used wildtype mice, which are not naturally susceptible to SARS-CoV-2 infection, and hamsters, which do not die from SARS-CoV-2 infection (44). In addition, N protein-based vaccine elicited a strong N-specific antibody response, which may contribute to virus control by other functions of antibody other than neutralizing the virus directly. To avoid these confounding issues, we used two models in our study, the hACE2-transgenic mouse model and mouse adapted SARS-CoV-2 in wild-type mice (45). Both are commonly used to assess the vaccine efficacy against SARS-CoV-2 infection as both models mimic human infection and cause dose-dependent respiratory symptoms and lethality. In our study, the MTE mRNA encodes over one hundred T cell epitopes from SARS-CoV-2 conserved regions, including 60 MHC I epitopes and over 40 MHC II epitopes (Supplementary Table 1). Our results show that this MTE vaccine activates $CD4^+$ T and $CD8^+$ T cell responses and protects the mice from lethal infection by SARS-CoV-2 and its variants. Thus, our result unequivocally demonstrates that vaccines based on multiple T-cell epitopes can protect mice from lethal challenge and perhaps severe disease in human by activating virus-specific T-cell responses.

Although T cells cannot prevent SARS-CoV-2 from entering host cells, our studies clearly show that a T cell-inducing vaccine, in the absence of pre-existing neutralizing antibodies, can offer adequate protection against a lethal dose of SARS-CoV-2 in two mouse models. This data provides strong support for the development of T cell-vaccines as a strategy to overcome the loss of efficacy against viral variants by antibody-based vaccines. We also found in this study, that a synergistic effect was achieved if the RNA vaccine induced both neutralizing antibody and T-cell responses, this result has an important implication for vaccine design not only for COVID-19, but also for other viral infections.

Another significant observation in our study is that, compare to lipid SM102 formulated vaccines, lipid L002 formulated vaccines elicits strong T cell and neutralizing antibody response at a low dose (0.1 μg). Our results show 0.1 μg LNP(L002)-mRNA OVS is sufficient to protect mice against SARS-CoV-2. A study evaluating various dose of mRNA-1273 vaccine in mice showed comparable Spike-specific binding antibodies and protection against SARS-CoV-2 induced by 1 μg dose but not 0.1 μg dose (46). It is reported that a high dose of LNP-mRNA-based SARS-CoV-2 vaccines in mice trigger inflammatory response and result in the observed side effects (47). A benefit from low dose administration of vaccine may minimize side effects.

In conclusion, our study shows that T cell-inducing vaccines may be an effective complement to antibody-inducing vaccines and that this strategy could be applied to universal vaccine development for SARS-CoV-2 as well as other viruses.

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

Individual animal studies were reviewed and approved by Nobel Life Sciences, Bioqual Inc. and Animal Facility of Thomas Jefferson University, respectively.

Author contributions

JQ and JJ helped conceive, design, perform, and interpret experiments. JX, LL, RM, SM, HJ and TZ provided help to JQ and JJ performed experiments. LL edited the manuscript. BM and CR-M provided technical help. LS provided SARS-CoV-2 MA30 virus, expertise, advice, and edited the manuscript. HZ and RX conceived, designed, and interpreted experiments, wrote the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

Author JQ, JJ, JX, LL, SM, RX and HZ are employed by ARV Technologies, Inc. Author RM, HJ and TZ were employed by ARV Technologies, Inc.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1126392/full#supplementary-material

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Humoral response after a fourth dose of SARS-CoV-2 vaccine in immunocompromised patients. Results of a systematic review

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Background and objective: The fourth dose the COVID-19 vaccine was first proposed to immunocompromised patients. The aim of the article is to systematically review the literature and report the humoral response and outcomes after the fourth dose administration in people with impaired immune system.

Methods: Published studies on the humoral response, efficacy and safety of the fourth dose of the COVID-19 vaccine were analyzed in various settings of immunocompromised patients. We conducted systematic searches of PubMed, Cochrane Library and WHO COVID-19 Research Database for series published through January 31, 2023, using the search terms "fourth dose" or "second booster" or "4th dose" and "Coronavirus" or "COVID-19" or "SARS-CoV-2." All articles were selected according to the PRISMA guidelines.

Results: A total of 24 articles including 2,838 patients were comprised in the systematic review. All the studies involved immunocompromised patients, including solid organ transplant recipients, patients with autoimmune rheumatic disease, patients with human immunodeficiency virus (HIV) and patients with blood cancers or diseases. Almost all patients received BNT162b2 or mRNA-1273 as fourth dose. All the studies demonstrated the increase of antibody titers after the fourth dose, both in patients who had a serological strong response and in those who had a weak response after the third dose. No serious adverse events after the 4th dose have been reported by 13 studies. COVID-19 infection after the fourth dose ranged from 0 to 21%.

Conclusion: The present review highlights the importance of the fourth dose of covid-19 vaccines for immunocompromised patients. Across the included studies, a fourth dose was associated with improved seroconversion and antibody titer levels. In particular, a fourth dose was associated with increasing immunogenicity in organ transplant recipients and patients with hematological cancers, with a very low rate of serious side effects.

KEYWORDS

immunocompromised, fourth dose, immune system, second booster, COVID-19, SARS-CoV-2

Introduction

The infectious disease caused by the novel Coronavirus SARS-CoV-2 (COVID-19) has been deemed one of the most critical global health emergencies in recent years and vaccine development has become crucial for limiting disease transmission, especially in fragile people and patients with impaired immune system (1). Worldwide, more than 5 billion people have

undergone at least one dose of the COVID-19 vaccine and ∼ 4.9 billions were fully vaccinated according to World Health Organization (WHO) (2). In Europe, the percentage of people who received a booster dose is 30.9% (2). In the USA, a third dose of COVID-19 vaccine has been administered to ∼ 33% of the population) (3).

The European Center for Disease Prevention and Control and European Medicine Agency recommend the administration of the fourth dose to people above 60 as well as vulnerable persons of any age, administered at least 4 months after the previous one, with a focus on people who have received a previous booster more than 6 months ago (4). In March 2022, the U.S. Food and Drug Administration allowed a fourth dose for immunocompromised people and anyone 50 years of age or older (5).

On the other hand, in Israel, administration of the fourth dose started from January 2022 for workers in health service and people over 60 years of age (6–8). Currently, a fourth dose has been granted for Israelis in immunocompromised groups.

Immunocompromised people represent ∼3% of the overall population, and deserve particular attention because of possible suppression or over-activation of the immune system attributable to the primary disease or concurrent treatment (9). In this group, SARS-CoV-2 infection and viral shedding is more severe and persistent, and the risk of death is higher (10). Given the reduced immune responses, immunodeficient patients are less prone to develop serious complications of COVID-19 and cytokine storm. However, they are more likely to develop opportunistic infections that can mimic the symptoms of SARS-CoV-2 infection (11). Therefore, a fourth dose has been proposed for immunocompromised patients, including organ transplant recipients (12–14), people on active treatment for solid tumor, people with hematologic malignancies, patients treated with chimeric antigen receptor (CAR)-T-cell therapy or hematopoietic stem cell transplant, patients with moderate or severe primary immunodeficiency (e.g., common variable immunodeficiency disease, severe combined immunodeficiency, DiGeorge syndrome, Wiskott-Aldrich syndrome), with advanced or untreated human immunodeficiency virus (HIV) infection (people with HIV and CD4 cell counts $<200/mm^3$, history of an AIDS-defining illness without immune reconstitution, or clinical manifestations of symptomatic HIV), on active treatment with high-dose corticosteroids (i.e., 20 or more mg of prednisone or equivalent per day when administered for 2 or more weeks), alkylating agents, antimetabolites, transplant-related immunosuppressive drugs, cancer chemotherapeutic agents classified as severely immunosuppressive, tumor necrosis factor (TNF) blockers, and other biologic agents that are immunosuppressive or immunomodulatory (15).

To date, no systematic reviews have been performed on the immunogenicity of a fourth dose of COVID-19 vaccines in immunocompromised cohorts. The aim of the article is to systematically review the literature and report the current use of the fourth dose in immunocompromised people, the categories of involved patients, and the results obtained till now.

Methods

Study design

This is a systematic review of literature that was completed in accordance with Preferred Reporting Project for Systematic Evaluation and Meta-Analysis (PRISMA) guidelines (16, 17).

Literature search strategy

A literature search for the studies published up to January 31, 2023 was conducted. No restrictions on language or period of publications were applied. Three different electronic databases (Medline, Cochrane Library and WHO COVID-19 Research Database, which also includes Embase, medRxiv and Scopus articles about COVID-19) were searched employing the keywords "COVID-19" OR "coronavirus" OR "SARS-CoV-2" AND "fourth dose" OR "4th dose" OR "second booster". Other relevant studies found in the references were also retrieved. The Boolean operator "AND" was used to combine parts of the subject terms and "OR" was used to expand the search. To increase the validity data, we removed non-peer-reviewed articles in the preprint database. Only the more informative publications would be chosen when there were similar studies carried out by the same authors and/or institutions.

Screening of articles for eligibility and data extraction

The articles identified from the databases and additional resources were screened for eligibility. First, the title and abstract were screened. The following inclusion criteria were used: (1) studies including men or non-pregnant women aged 18 and above, who had impaired immune system at the time of vaccination; (2) fourth dose of COVID-19 vaccination as the intervention measure; (3) randomized trials, observational studies, case series or retrospective studies including at least three patients. Studies were limited to human participants and of any follow-up duration and time points. The definition of immunocompromised patients was borrowed by the National Cancer Institute, identifying them as people with "reduced ability to fight infections and other diseases,... caused by certain diseases or conditions, such as AIDS, cancer, diabetes, malnutrition, and certain genetic disorders, … or by certain medicines or treatments, such as anticancer drugs, radiation therapy, and stem cell or organ transplant (18)."

Second, eligible studies that met the next circumstances were rejected: (1) medical news, popular science articles, non-medical papers, reviews, editorials, comments, basic research, conference abstracts; (2) in case of overlapping studies, the less informative was excluded.

Full articles were retrieved and read in the event of any doubt or uncertainty regarding the content relevance during the abstract screening. After a comprehensive list of abstracts was obtained, the articles were retrieved and reviewed in full text.

Two researchers (SM and DP) independently screened all studies and the results were collected and reviewed by a third researcher (PL). In the event of disagreement involving the study selection, the three reviewers collegially discussed to reach a consensus (PL). Two researchers (SM and DP) extracted data according to a predetermined proforma in Microsoft Excel Version 16.45. All key extracted data were reviewed and quality checked at the end of the data extraction phase by two researchers (SM and PL).

Data on study characteristics comprised setting, study design, sample size, dropout and non-response rates, and inclusion and exclusion criteria. Participant data comprised age, sex, and disease and treatment history, reason of impaired immune system or type of immunocompromising disease and immunosuppressive regimen. Intervention related data included vaccine type and brand, dosing schedule, number of participants receiving each type and brand of vaccine, and median or mean interval between doses. Outcome related data comprised assay type, antibody measured, method of measurement, intervals of sample collection, and number of measurements.

Data synthesis and quality assessment

Data retrieved was studied then synthesized using a descriptive method. The Risk of Bias In Non-randomized Studies of Interventions (ROBINS-I) tool was used to rate risk of bias for non-randomized included studies (19). This tool assesses seven domains: risk of bias from confounding, selection of participants, classification of interventions, deviations from intended interventions, missing data, measurement of outcomes, and selection of the reported results. The Cochrane Risk of Bias 2.0 tool was used for randomized trials (20). The tool is structured into five domains through which bias might be introduced into the result. These were identified based on both empirical evidence and theoretical considerations. Because the domains cover all types of bias that can affect results of randomized trials, each is mandatory, and no further domains should be added. The five domains for individually randomized trials (including cross-over trials) are: bias arising from the randomization process; bias due to deviations from intended interventions; bias due to missing outcome data; bias in measurement of the outcome; bias in selection of their ported result. A proposed judgment about the risk of bias arising from each domain is generated by an algorithm, based on answers to the signaling questions. Judgment can be "Low", or "High" risk of bias, or can express "Some concerns".

Two reviewers (SM and DP) independently judged these domains as having low, moderate, serious, or critical risk of bias, or no information. All discrepancies were resolved by the independent opinion of a third reviewer (PL). A study would be judged as having an overall low risk of bias if all the domains were judged as low risk. A study would be considered as having critical risk of bias if one domain was judged as high risk of bias.

The main results of this systematic review included the serological response after fourth dose vaccine in people with impaired immune system (primary endpoint). Furthermore, the safety and clinical effectiveness of the vaccine was evaluated as a secondary endpoint. The immunogenicity indicators included antibody titers, seroconversion rate, and the response of IgG or other specific antibodies to the receptor-binding domain. Indicators for evaluating safety included local adverse reactions and systemic adverse reactions. Data were reported as mean ± standard deviation or median (range), or number (%).

Results

The selection process of articles and inclusion in the systematic review was summarized in Figure 1, showing the PRISMA flow diagram. The initial search included a total of 5,690 articles. After removing the duplicates, 4,899 articles were screened for keywords and relevance for the title and abstract. The full-text versions of the publications were reviewed in case of uncertainty. Only those that fulfilled the inclusion criteria were included for eligibility assessment. The full-text of these studies were fully examined. A total of 24 articles including 2,838 patients published since January 2023 were comprised in the systematic review (21–44), consisting mainly in retrospective cohort studies, followed by research letters, prospective cohort studies and case series. All the studies involved immunocompromised patients, including solid organ transplant recipients, patients with autoimmune rheumatic disease, patients with HIV and patients with blood cancers or diseases. The majority of studies were carried out in Europe, United States and Israel.

Risk of bias

By using the Risk of Bias in Non-randomized Studies of Interventions (ROBINS-I), the risk of bias of the studies were summarized in Table 1 (19). In general, the individual studies had a low to moderate range of risk of bias due to adequate approach to the research question and findings, with presence of coherence among the sources of data collection and analysis.

Main findings

This systematic review reports the use of a fourth dose of vaccine against COVID-19 worldwide in patients with impaired immune system. The characteristics of the included studies are summarized in Table 2, where details of vaccine characteristics and developer information are reported. A total of 2,838 patients were included. Characteristics of included patients are reported in Table 3. The majority of included patients were >50 years old. In twenty studies, 100% of patients were receiving immunosuppressive or immunomodulatory treatment following solid organ transplantation or as a treatment for autoimmune disease or cancer. All studies reported the type of vaccine used. Almost all patients received BNT162b2 or mRNA-1273 as fourth dose (Table 2). The time frame between the third and fourth dose was reported by 19 studies (21–23, 26–31, 33–42), and ranged from 22 to 201 days. All studies but two reported the antibody IgG titer before the 4th dose, using different units of measurement, as reported in Table 4. Table 5 reports the type

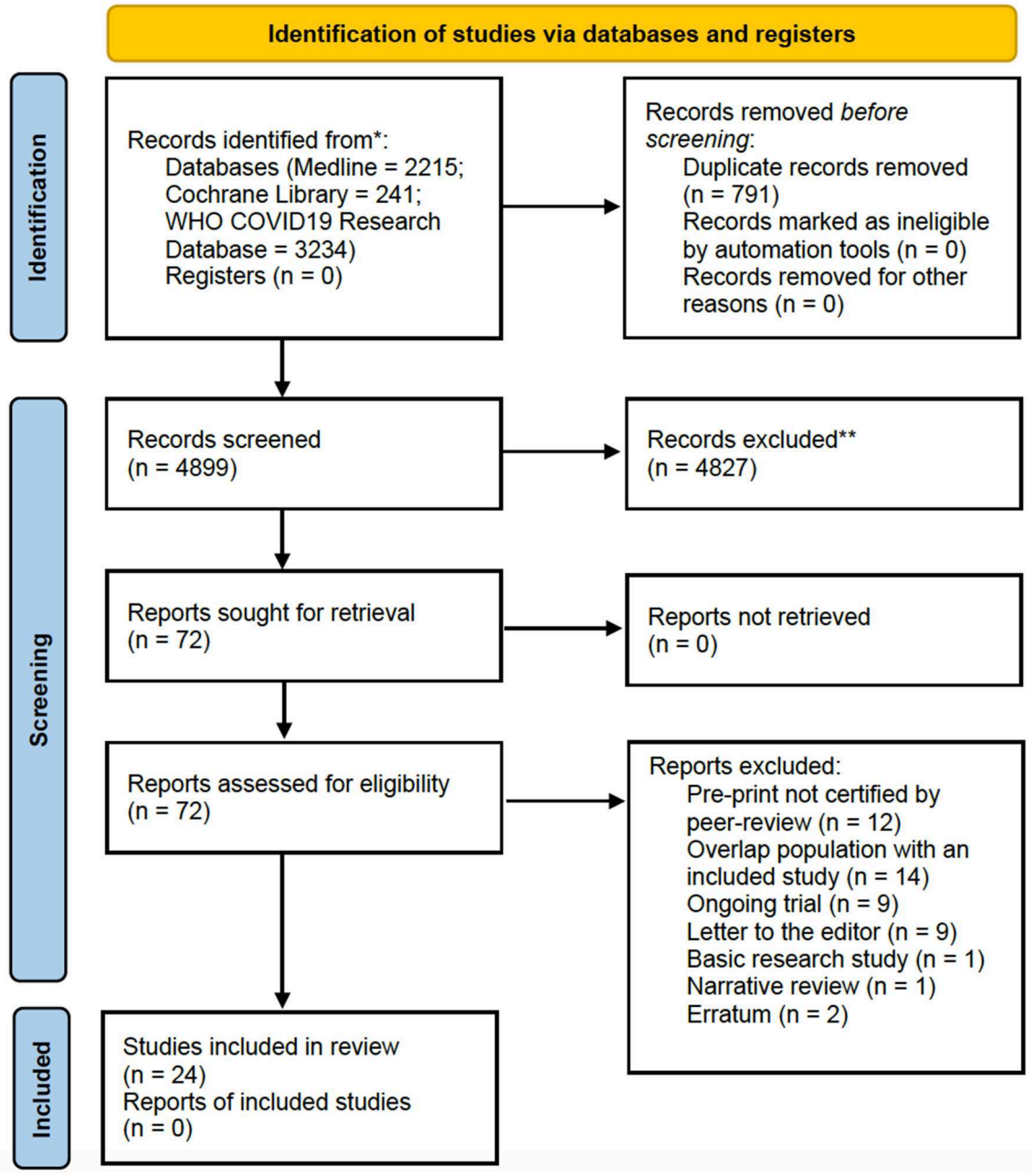

FIGURE 1
PRISMA 2020 flow diagram for the systematic review.

of antibodies measured, the units of measure and the used assays, which were heterogeneous among the studies. The timing of antibody measurement after the 4th dose was reported by 21 studies and ranged from 14 to 65 days. The values of antibody titers after the 4th dose were reported with different units of measurement by all studies except two. All these studies demonstrated the increase of antibody titers after the fourth dose, both in patients who had a serological strong response and in those who had a weak response after the third dose. One study demonstrated different serological responses according to the evidence of a prior infection with SARS-CoV-2 before the fourth dose (37), reporting higher antibodies levels in patients with history of coronavirus infection. Another study pointed out a weaker serological response in patients who remained seronegative after the third dose (30).

No serious adverse events after the 4th dose have been reported by 13 studies (21, 23, 26–28, 31, 33–35, 39–41, 44) (Table 6). COVID-19 infection after the fourth dose was reported by 10 authors (21, 23, 28, 29, 32, 34, 40–42, 44) and ranged from 0 to 21%. Overall, all the authors recommended the 4th dose of vaccine against COVID-19 in immunocompromised patients, except for Karaba and Thomson et al. (22, 37).

TABLE 1 Methodological quality evaluation of the included non-randomized studies according to ROBINS-1.

References	Bias due to confounding domains relevant to the setting of the study	Bias in selection of participants into the study	Bias in classification of interventions	Bias due to deviations from intended interventions	Bias due to missing data	Bias in measurement of outcomes	Bias in selection of the reported results
Caillard et al. (21)	PY	PN	PN	PN	PN	N	N
Karaba et al. (22)	PN	N	N	N	PN	PN	N
Kamar et al. (23)	PN	N	N	N	PN	N	N
Teles et al. (24)	PN	PN	N	N	N	N	N
Mitchell et al. (25)	PN	N	PN	N	N	N	N
Osmanodja et al. (26)	PN	N	N	N	PN	PN	N
Aikawa et al. (27)	PN	N	N	N	PN	N	N
Mrake et al. (28)	PN	N	Y	PN	Y	PN	PN
Ntanasis-Stathopoulos et al. (29)	PN	N	PY	PN	PY	PN	PN
Perrier et al. (30)	PN	N	Y	PN	Y	PN	PN
Assawasaksakul et al. (31)	PN	N	Y	PN	Y	PN	PN
Gössi et al. (32)	PN	N	Y	PN	PY	PN	PN
Harberts et al. (33)	PN	N	PY	PN	Y	PN	PN
Benjamini et al. (34)	PN	N	Y	PN	PY	PN	PN
Midtvedt et al. (35)	PN	PN	PY	PN	PY	PN	PN
Peled et al. (36)	PN	N	Y	PN	PY	PN	PN
Thomson et al. (37)	PN	PN	PY	PN	PY	PN	PN
Busà et al. (38)	PN	PN	PY	PN	PY	N	PN
Brandstetter et al. (39)	PN	N	Y	PN	Y	PN	PN
Hod et al. (40)	PN	N	Y	PN	Y	PN	PN
Bjorlykke et al. (41)	PN	N	Y	PN	Y	PN	PN
Lamacchia et al. (42)	PN	PN	PY	PN	PY	N	PN
Affeldt et al. (43)	PN	PN	PY	PN	PY	N	PN
Davidov et al. (44)	PN	N	Y	PN	Y	PN	PN

N, no; PN, probably no; PY, probably yes; Y, yes.

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TABLE 2 Design and characteristics of the included studies.

Author	Research type	Type of vaccine	Country	Number of included patients
Caillard	Retrospective study	BNT162b2 (34 patients) mRNA-1273 (58 patients)	France	92
Karaba	Prospective study	BNT162b2 (10 patients) mRNA-1273 (15 patients)	United States	25
Kamar	Retrospective study	BNT162b2	France	37
Teles	Prospective study	BNT162b2 (11 patients) mRNA-1273 (6 patients) Ad.26.CoV2.S (1 patient)	United States	18
Mitchell	Prospective study	BNT162b2 (46 patients) mRNA-1273 (74 patients) Ad.26.CoV2.S (8 patients)	United States	128
Osmanodja	Retrospective study	mRNA vaccines (217 patients) Others (33 patients)	Germany	250
Aikawa	Prospective study	BNT162b2 (164 patients)	Brazil	164
Mrak	Prospective study	BNT162b2 (29 patients), mRNA-1273 (8 patients)	Austria	37
Ntanasis-Stathopoulos	Prospective study	BNT162b2 (201 patients)	Greece	201
Perrier	Retrospective study	BNT162b2 (507 patients), not specified (16 patients)	France	523
Assawasaksakul	Prospective study	BNT162b2 (28 patients)	Thailand	28
Gössi	Retrospective study	BNT162b2, mRNA-1273	Switzerland	7
Harberts	Prospective study	BNT162b2, mRNA-1273	Germany	36
Benjamini	Prospective study	BNT162b2	Israel	67
Midtvedt	Prospective study	mRNA-1273	Norway	188
Peled	Prospective study	BNT162b2	Israel	90
Thomson	Prospective study	BNT162b2 (239 patients)	United Kingdom	239
Busà	Prospective study	BNT162b2, mRNA-1273	Italy	15
Brandstetter	Retrospective study	BNT162b2 (3 patients), mRNA-1273 (38 patients)	Austria	41
Hod	Prospective study	BNT162b2 (29 patients)	Israel	29
Bjorlykke	Prospective study	BNT162b2, mRNA-1273	Norway	536
Lamacchia	Prospective study	BNT162b2 (8 patients)	Italy	8
Affeldt	Prospective study	BNT162b2, mRNA-1273	Germany	29
Davidov	Retrospective study	BNT162b2 (50 patients)	Israel	50
Total				2,838

Discussion

Given the continuing COVID-19 emergency associated with the risk of the virus undergoing new mutations and in the view of the fact that a clear reduction in vaccine coverage is evident 4 months after the third dose, it is hypothesized that administration of a fourth dose of vaccine may protect against the risk of severe illness and mortality from coronavirus infection. However, specific considerations must be made for immunocompromised patients. At first, efficacy and safety data on booster doses are less because large trials have often excluded patients with cancer, organ transplant recipients, and those with rheumatological disorders although they constitute 3% of the population (45). On the other side, these patients experience more severe and persistent infection and viral shedding (46) and are at increased risk of death (47).

The present systematic review provides relevant evidence about the current role of the fourth dose of vaccine against COVID-19 in immunocompromised people. At first, several considerations emerge on the population of immunocompromised patients who received the fourth dose. The majority of them had history of solid organ transplant, necessitating long-term immunosuppressive therapy to prevent rejection. Only a few data concern patients with hematological cancers or autoimmune

TABLE 3 Characteristics of the included patients.

Author	Age, years Median (IQR)	Male sex	Disease causing impair immune response	On medication with immunosuppressive or immunomodulatory therapy (%)
Caillard	55.9 (47.1–64.2)	64 (69.5%)	Kidney transplant recipients	100%
Karaba	59 (45–66)	11 (44%)	Solid organ transplant recipients	100%
Kamar	NR	NR	Solid organ transplant recipients	NR
Teles	56 (52–66)**	5 (27.8%)	Autoimmune rheumatic disease	100%
Mitchell	## 63.5 (54.2–71.6) 62.3 (49.6–69.5) 58.4 (48.4–68)	58 (45.3%)	Solid organ transplant recipients	NR
Osmanodja	61 (51–70)	168 (67%)	Kidney transplant recipients	100%
Aikawa	55.7 (47.3–70.7)	25 (20%)	Autoimmune rheumatic disease	100%
Mrak	62.1 (14.0)*	11 (30.6%)	Autoimmune rheumatic disease	100%
Ntanasis-Stathopoulos	67 (15)	114 (56.7%)	Multiple myeloma	100%
Perrier	61.2 (50.9–69.3)#	550(66.7%)	Solid organ transplant recipients	100%
Assawasaksakul	## 39 (11.9)* 53.8 (9.3)*	7 (7%) #	Autoimmune rheumatic disease	97%
Gössi	58.5 (17–78)** #	28 (61%)	CAR-T-cell treated patients	100%
Harberts	61.0 (52.5–67.0)	23 (63.9%)	Liver transplant recipients	100%
Benjamini	71.46 (64.90–75.82)	47 (70.1%)	Chronic lymphocytic leukemia	47.8%
Midtvedt	60 ± 12*	109 (58%)	Kidney transplant recipients	100%
Peled	57.2 ± 13.8*	62 (68.9%)	Heart transplant recipients	100%
Thomson	## 61 (51–68)** 60 (49–67)**	149 (62.3%)	Kidney transplant recipients	100%
Busà	58 ± 13*	9 (60%)	Solid organ transplant recipients	100%
Brandstetter	## 66.8 (57.45–73.6) 67.75 (56.28–70.83)	26 (63%)	Kidney transplant recipients	100%
Hod	64.2 (54.3–70.4)	16 (55.2%)	Kidney transplant recipients	100%
Bjorlykke	59 (49–67)	229 (43%)	Immune-mediated inflammatory diseases	100%
Lamacchia	58.5 ± 8.9*	NR	HIV	100%
Affeldt	55 (20)	22 (61.1%)	Kidney transplant recipients	100%
Davidov	62.7 (53.1–70.6)	26 (52%)	Liver transplant recipient	100%

*mean (standard deviation).
**median (range).
#referred to the entire population of the study.
##data referring to different groups according to the sierological response before the 4th vaccine dose.
NR, not reported; HIV, human immunodeficiency virus.

rheumatological disease. Few data on HIV/AIDS patients and on patients with primary immunodeficiencies have been published till now.

It is clear that the availability of vaccines and the vaccination guidelines released from the single countries strongly influenced the use of the fourth dose. The inclusion of different ethnics groups better representing the global population may be limited according to the collected data. These findings generate ethical reflections in addition to scientific considerations.

Second, the types of vaccines used for the fourth dose were almost exclusively mRNA vaccines, produced by the two main companies, despite the large number of different types of vaccines available in the market (48). This observation may be explained because they have generally produced better antibody responses and are to some degree better available at least in the developed countries. Data about the other vaccine platforms should be accrued in the future.

The present review showed that the fourth dose was effective in increasing the antibody titer in immunocompromised patients. How the increase of the antibody titers impacted the rate and severity of COVID-19 infections was less clear, as the majority of studies did not follow the patients after the fourth dose to detect clinical infections, and follow-up data are scarcely reported. The COVID-19 infection rate after the fourth dose was reported only by 10 studies, with different follow-up times, and varied from 0% to 21%. Furthermore, the duration of further protection could not be

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TABLE 4 Immunological outcomes after 4th dose of vaccination.

Author	Median (IQR) delay between 3rd and 4th dose, days	Median (IQR) antibody IgG titer before the 4rd dose, BAU/mL	Median (IQR) antibody IgG titer after the 4th dose, BAU/mL	Ratio of the median antibody titer after/before the 4th dose	Median (IQR) timing of antibody IgG titer exam after the 4th dose, days
Caillard	68 (61–74.7)	16.4 (5.9–62.3)	145 (27.6–243)	8.8	29 (26–34)
Karaba	93 (28–134)	42.3 (4.9–134.2)	228.9 (115.4–655.8)	5.4	29 (17–38)
Kamar	65 (9)*	4 (1–9)** α	9.5 (1.7–658)** β	2.4	28
Teles	NR	<0.4–>2,500 γ	1,750 (26–2,500) γ	–	32 (28–34)
Mitchell	NR	207 (11.6–1,500) γ	2,132.5 (96.9–>2,500) γ	10.3	14–28***
Osmanodja	64 (55–84)	42% δ	74.2% δ	–	NR
Aikawa	90	29.5 (23.3–37.4) ε	215.8 (180.5–257.9) ε	7.3	30
Mrak	84	0.4 (0.4–8.1)	12.4 (0.4–197.3)	31	30
Ntanasis–Stathopoulos	180 (150–210)**	80 ± 3.5% ζ	96.1 ± 3.7% ζ	–	30
Perrier	201 (173–221)	η no = 174 (32.3%); weak = 103 (19.1%); strong = 261 (48.5%)	θ no = 56 (23.8%); weak = 26 (11.1%); strong = 153 (65.1%)	–	31.5**
Assawasaksakul	22	88 (49–155)	644 (398–1,041)	7	15
Gössi	NR	<12 (<12–>400)** ε	30.4 (<12–400)** ε	–	48–59***
Harberts	126.0(93.0–148.0)	134.6 ε	1,196.0 ε	9	NR
Benjamini	175 (174–175)	4.3 (0.1–117.65)	41.3 (0.4–1,185)	10	14
Midtvedt	18.0 (9.7–18.3) weeks	4.6 (2.5–32)	1,553 (356–3,703) in 79 patients with >200 BAU/ ml 53 (12–407) in 96 patients sero–negative before dose 4	–	3–4 weeks
Peled	173.4 ± 4.2*	12.5 ε	96.9 ε	7.8	16.1 ± 4*
Thomson	92–130***	3,791 (1,142–5,680) in patients with history of SARS-CoV-2 infection 295 (9.1–1,611) in patients without previous SARS-CoV-2 infection	3,993 (835–5,680) in patients with history of SARS-CoV-2 infection 437 (26–2,211) in patients without previous SARS-CoV-2 infection	–	23–66***
Busà	168.3 (116–246)**	330.2 (59.02–1,001)	1,020 (366.6–5,486)		65.33 (26–127**)
Brandstetter	26 (26–27)	NR	44.7 (17.9–111.6)		26 (26–27)
Hod	175 (164–176)	345 (124–956) 699 (244–2,008) η,	2,118 (761–5,900) 2,489 (1,098–5,640) η		29 (25–33)
Bjorlykke	84	5,087 (1,250–9,081)	6,192 (2,878–11,243)		2–4 weeks
Lamacchia	119 ± 2*	NR	NR		7, 30, 60 days
Affeldt	NR	134.4	NR		NR
Davidov	175 (164–176)	345 (124–955)	2,118 (761–5,900)		29 (25–33)

Continuous data are reported as median (IQR range).
BAU, basal antibody units; AU, antibody units; ELU, Elisa laboratory units.
*mean (standard deviation); **median (range); ***range.
$^{\alpha}$data referring to 5 patients seropositive before the 4th dose.
$^{\beta}$data referring to 31 patients seronegative before the 4th dose.
$^{\gamma}$reported in U/mL.
$^{\delta}$serological response rate.
$^{\varepsilon}$reported in AU/mL.
$^{\zeta}$median neutrilizing antibodies levels.
$^{\eta}$data referring to different groups according to the sierological response before the 4th vaccine dose.
$^{\theta}$235 patients had serological tests after the fourth dose.
NR, not reported.

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TABLE 5 Type of antibody measured, units of measure and assays.

Author	Antibodies measured	Unit of measure	Assay
Caillard	Anti-spike IgG	WHO BAU/mL	NR
Karaba	Antinucleocapsid antibody (anti-N), anti- RBD Protein, anti-S immunoglobulin IgG ACE2 neutralizing antibodies	WHO BAU/mL	Multiplex chemiluminescent Meso Scale Diagnostics (MSD, Rockville, MD) V-PLEX COVID-19 Respiratory Panel 3 Kit and ACE2 MSD V-PLEX SARS-CoV-2 ACE2 Panel 23 Kit
Kamar	Anti-spike total antibody concentration	WHO BAU/mL	Wantai enzyme-linked immunosorbent assay test
Teles	Anti-RBD Protein Ig	U/mL	Roche Elecsys immunoassay
Mitchell	Anti-RBD Protein Ig Anti-S1 domain of the spike protein	AU/mL	Roche Elecsys immunoassay and EUROIMMUN Anti-SARS-CoV-2 enzyme immunoassay
Osmanodja	Anti-spike protein S1 IgG Anti-RBD Protein Ig	Serological response rate	EUROIMMUN Medizinische Labordiagnostika AG and Roche Elecsys immunoassay
Aikawa	Total IgG against the SARS-CoV-2 S1 and S2 protein Circulating NAb	AU/mL	Chemiluminescent immunoassay on the ETI-MAX-3000, LIAISON SARS-CoV-2 S1/S2 IgG kit, DiaSorin SARS-CoV-2 sVNT kit GenScript
Mrak	Anti-RBD Protein Ig Anti-S1 domain of the spike protein	BAU/ml	Roche Elecsys immunoassay
Ntanasis-Stathopoulos	antibody-mediated reduction of SARS-CoV-2 RBD binding to the human host receptor angiotensin converting enzyme type 2	Serological response rate	cPass SARS-CoV-2 Nabs Detection Kit (GenScript, Piscataway, NJ)
Perrier	Anti-RBD Protein Ig	BAU/ml	Wantai SARS-CoV-2 Ab ELISA (Beijing Wantaï Biological PharmacyEnterprise), VIDAS SARS CoV-2 IgG II ELF Aassay (Biomérieux), Alinityi SARS-CoV-2 IgG II Quantassay (Abbott), Elecsysanti-SARS CoV-2 S assay (Roche Diagnostics) Atellica sCOVG IgG assay (Siemens Healthineers).
Assawasaksakul	Anti-RBD of the SARS-CoV-2 spike protein	AU/ml	SARS-CoV-2IgG II Quant assay (Abbott Diagnostics)
Gössi	Anti-SARS-CoV-2 IgG antibodies binding to S1 and S2 antigens	AU/ml	automated immunoassay analyzer Liaison® XL by DiaSorin, Saluggia, Italy
Harberts	Anti-RBD Protein Ig	AU/ml	Roche Elecsys immunoassay
Benjamini	(IgG), aimed at the SARS-CoV-2 S protein receptor–binding domain (RBD)	BAU/mL	SARS-CoV-2 IgG II Quant (AbbottLaboratories, Abbott Park, Illinois),
Midtvedt	Anti-SARS-CoV-2 (Wuhan) receptor-binding domain (RBD) binding-(x-axis) and neutralizing-antibodies	BAU/mL	NR
Peled	SARS-CoV-2 anti-RBD IgGantibodies; IgG against sublineage B.1 of the wild-type virus, the B.1.617.2(delta) variant and the B.1.1.529(omicron) variant	AU/ml	SARS-CoV-2 IgGIIQuant assay, Abbott, USA and live virus microneutralization assays
Thomson	Antibodies to nucleocapsid protein (anti-NP)	BAU/mL	Abbott Architect SARS-CoV-2 IgG 2 step chemiluminescent immunoassay (CMIA)
Busà	IgG antibodies against S1 and S2 fragments of the Spike protein	BAU/mL	Chemiluminescent immunoassay (CLIA) LIAISON® Trimeric SARS-CoV-2 S1/S2 IgG (DiaSorin S.p.A., Saluggia, VC, Italy)
Brandstetter	Anti-SARS-CoV-2 antibodies directed against the receptor binding domain of the S1 subunit of the spike (S) protein	BAU/mL	SARS-CoV-2 IgG II Quant assay (Abbott Ireland Diagnostics Division, Sligo, Ireland)
Hod	IgG antibodies to the RBD of the SARS-CoV-2 spike protein	AU/ml	SARS-CoV-2 IgGIIQuantassay, Abbott, USA and live virus microneutralization assays
Bjorlykke	IgG antibodies to the RBD of the SARS-CoV-2 spike protein	BAU/mL	In-house bead-based method validated against a micro-neutralization assay at the Department of Immunology at Oslo University Hospital

(Continued)

TABLE 5 (Continued)

Author	Antibodies measured	Unit of measure	Assay
Lamacchia	Evaluation of the SARS-CoV-2 spike protein antibodies, including the anti-spike-specific (in trimeric form) IgGs, anti-spike RBD-specific IgGs, anti-spike-specific IgMs, anti-nucleoprotein-specific IgGs, and neutralizing antibodies that block the binding of spike protein with the ACE2 receptor	BAU/ml	chemiluminescent immunoassay (CLIA) LIAISON® Trimeric SARS-CoV-2 S1/S2 IgG (DiaSorin S.p.A., Saluggia, VC, Italy), SARS-CoV-2 IgG II Quant (Abbott Rome, Italy), test for neutralizing antibodies that block the binding of spike protein with the ACE2 receptor (Dia.Pro Diagnostic Bioprobes, Milan, Italy)
Affeldt	IgG antibodies to the RBD of the SARS-CoV-2 spike protein, antibodies targeting additional regions of the spike protein, IgG against the S1 region	BAU/ml	Chemiluminescent microparticle immunoassay (CMIA) SARS-CoV-2 IgG II Quant by Abbott on the automated system Alinity I (Abbott, Abbott Park, IL, USA), (CLIA) LIAISON® SARS-CoV-2 TrimericS IgG assay by DiaSorin, Euroimmun anti-SARS-CoV-2-QuantiVac-ELISA (Enzyme-linked Immunosorbent Assay)
Davidov	Serum titers of IgG antibodies against the SARS-CoV-2 spike RBD	BAU/mL	SARS-CoV-2 IgGIIQuant assay, Abbott, USA and live virus microneutralization assays

BAU, basal antibody units; NR, not reported; NA, not assessed; WHO, World Health Organization; ACE, angiotensin-converting enzyme; Ab, antibody; RBD, Receptor Binding Domain; U, unit; AU, antibody units, ELU, Elisa laboratory units; Nab, neutralizing antibodies.

assessed by the review due to the lack of long-term follow-up after the fourth dose at the time that the articles were published.

It should be emphasized that the circulating viral strain(s) are very different from the Wuhan strain, on the basis of which current vaccines were synthesized, and that antibodies elicited by vaccination are unlikely to provide good protection from mutated strains. However, the protection that vaccines provide is not completely dependent on antibodies, but T-cells play a role, particularly in protecting against severe forms of the disease. In addition, T-cells are likely to be less affected by spike protein mutations, given the promiscuity of antigen recognition by the T-cell receptor. The effect of repeated vaccination on the receptors of the adaptive immune system (whether antibody, T-cell receptor, or B-cell receptor) has not yet been extensively evaluated.

However, overall the systematic review demonstrated a very low rate of early major side effects after the fourth dose in immunocompromised patients. No severe adverse events occurred in 13 studies.

Most of the authors of the included studies recommended the use of the fourth dose, while only Karaba et al. and Thomson et al. dissented (22, 37). Karaba et al. argued that additional dosing of the original vaccines in solid organ transplant recipients might not produce valid defense against infection in the form of neutralizing antibodies against the Omicron variant or new variants generated by Omicron (22). These authors recommend additive approaches, such as modulation of immunosuppressive regimens before booster doses of vaccine, vaccines with alternative antigenic sequences, or extensively neutralizing passive immunity products. Thomson and colleagues (37) asserted that repeated vaccinations do not adequately protect all transplant recipients, as there is a spectrum of immune responses in patients in relation to vaccination and infection and it will disadvantage many immunocompromised people if they are managed as a uniform cohort irrespective of underlying disease, treatment or infection status. They recommend developing a more personalized approach, starting with antibody screening to identify the vaccine non-responders who are likely to be the most immune suppressed and at risk of an adverse outcome with infection.

We also highlight a lack of homogeneity of fourth dose use in the included studies. Particularly, the delay between the third and the fourth dose varied from a minimum median value of 22 days to a maximum of 201 days (Table 4). Also timing of antibody dosage after the fourth dose to assess its efficacy on seroconversion varied, as the type of assays (Tables 4, 5).

Globally, even if the data of the literature should be implemented in the future, our results demonstrate that a fourth dose of mRNA vaccine is effective in increasing the antibody titer and associated with a very low rate of side effects in immunocompromised patients, and therefore in our opinion should be proposed to fragile patients (immunocompromised, elderly) (experts' recommendation). Among the international societies, the World Health Organization recommended a fourth dose for immunocompromised patients (49).

Limitations

This study has several limitations. Firstly, the included studies are mostly observational and small case series. Secondly, the definition of immunocompromised is not universally shared and varied between studies. We therefore specified in the Methods the definition of immunocompromised that we choose to select the articles. Third, we mainly analyzed the seroconversion rate, which is an indication of an immune response to a vaccine, but is not necessarily related to clinical effectiveness. Data are still few on clinical efficacy endpoints such as COVID-19 infection rates in vaccinated immunocompromised populations. It was not possible to perform a meta-analysis for the heterogeneity of the studies and data and because most of the studies lack a comparator. Finally, the definition of seroconversion and the type of immunoassay used were not standardized across the included studies. Even if vaccine type might influence seroconversion rates after COVID-19 vaccination, the studies included in this review predominantly used mRNA vaccines, limiting potential bias.

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TABLE 6 Clinical outcomes after 4th dose of vaccination.

Author	Side effects	COVID-19 infection	4th dose recommended (yes/no)
Caillard	0 (0%)	1 (1%)	Yes
Karaba	NR	NR	No
Kamar	0 (0%)	0 (0%)	Yes
Teles	NR	NR	Yes
Mitchell	NR	NR	Yes
Osmanodja	0 (0%)	NR	Yes
Aikawa	No serious adverse events	NR	Yes
Mrak	No serious adverse events	1 (2.7%)	Yes
Ntanasis-stathopoulos	NR	0 (0%)	Yes
Perrier	NR	NR	Yes
Assawasaksakul	No serious adverse events	NR	Yes
Gössi	NR	1 (14.2%)	Yes
Harberts	No serious adverse events	NR	Yes
Benjamini	No serious adverse events	14 (21%)	Yes
Midtvedt	No serious adverse events	NR	Yes
Peled	NR	NR	Yes
Thomson	NR	NR	No
Busà	NR	NR	Yes
Brandstetter	No serious adverse events	NR	Yes
Hod	No serious adverse events	9	Yes
Bjorlykke	No serious adverse events	35 (7% of 491)	Yes
Lamacchia	NR	2	Yes
Affeldt	NR	NR	Yes/No
Davidov	No serious adverse events	9 (18%)	Yes

NR, not reported.

Further issues

For long-term observations, there are relevant hesitations associated with the development of the virus and the specificities of new variants. The wide dissemination of new variants internationally suggests continued viral evolution with the emergence of future variants or sublines, as has been noted for some time. It has been shown in the literature that even a repetitive booster vaccination based on the Wuhan isolate has a limited ability to produce a durable humoral immune response toward a remote variant such as Omicron (50). This highlights the urgency of evaluating and adopting second-generation variant of SARS-CoV-2 vaccines. After all, a possible early availability of second-generation vaccines for the current SARS-CoV-2 variants might favor the administration of the aforementioned rather than the use of a fourth dose of first-generation vaccine. Therefore, further research is useful to study the long-term efficacy of vaccines and the influence of dose, seniority, and manufacturing process on protective efficacy (51).

Based on recent WHO guidance, we emphasize that future studies need to address other gaps in the evidence related to the need for additional booster doses, the duration of vaccine efficacy of inactivated, subunit, and viral vector vaccines over time, and according to disease outcome. Further data are required on the magnitude, extent, and duration of humoral and cell-mediated immune responses to variant (49).

Conclusion

Our findings highlight the importance of the fourth dose of COVID-19 vaccines for immunocompromised patients. Across the included studies, a fourth dose was associated with improved seroconversion and antibody titer levels. In particular, a fourth dose was associated with increasing immunogenicity in organ transplant recipients and patients with hematological cancers, with a very low rate of serious side effects.

Additional data are needed to define the long-term efficacy of the fourth dose and the influence of dose, age, immune disease and manufacturing process on the protective efficacy of different coronavirus variants.

Data availability statement

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

Author contributions

SM took care of the data synthesis, collection, and analysis. DP provided expert clinical advice on applied methodology, while PL provided expert clinical advice on community health. All authors critically corrected the manuscript for relevant scientific content and updated and approved the final version.

Conflict of interest

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

Publisher's note

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Protection of hybrid immunity against SARS-CoV-2 reinfection and severe COVID-19 during periods of Omicron variant predominance in Mexico

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Background: With the widespread transmission of the Omicron SARS-CoV-2 variant, reinfections have become increasingly common. Here, we explored the role of immunity, primary infection severity, and variant predominance in the risk of reinfection and severe COVID-19 during Omicron predominance in Mexico.

Methods: We analyzed reinfections in Mexico in individuals with a primary infection separated by at least 90days from reinfection using a national surveillance registry of SARS-CoV-2 cases from March 3rd, 2020, to August 13th, 2022. Immunity-generating events included primary infection, partial or complete vaccination, and booster vaccines. Reinfections were matched by age and sex with controls with primary SARS-CoV-2 infection and negative RT-PCR or antigen test at least 90days after primary infection to explore reinfection and severe disease risk factors. We also compared the protective efficacy of heterologous and homologous vaccine boosters against reinfection.

Results: We detected 231,202 SARS-CoV-2 reinfections in Mexico, most occurring in unvaccinated individuals (41.55%). Over 207,623 reinfections occurred during periods of Omicron (89.8%), BA.1 (36.74%), and BA.5 (33.67%) subvariant predominance and a case-fatality rate of 0.22%. Vaccination protected against reinfection, without significant influence of the order of immunity-generating events and provided >90% protection against severe reinfections. Heterologous booster schedules were associated with ~11% and~54% lower risk for reinfection and reinfection-associated severe COVID-19, respectively, modified by time-elapsed since the last immunity-generating event, when compared against complete primary schedules.

Conclusion: SARS-CoV-2 reinfections increased during Omicron predominance. Hybrid immunity provides protection against reinfection and associated severe COVID-19, with potential benefit from heterologous booster schedules.

KEYWORDS

reinfection, hybrid immunity, vaccination, vaccine boosters, SARS-CoV-2, heterologous booster, omicron variant (B.1.1.529)

Introduction

As the COVID-19 pandemic continues, a larger number of previously infected individuals have become reinfected with SARS-CoV-2 (1). Evidence of SARS-CoV-2 reinfection was first documented in August 2020 (2) and was initially considered a rare event (1, 3–7). Omicron SARS-CoV-2 variant and its subvariants have been shown to possess a higher capacity for immune escape (8, 9) and transmissibility (10), which resulted in the most significant increase of infection and reinfection rates as it became the dominant variant in circulation (11). With Omicron, approximately 41% of some countries' population are estimated to be at risk of SARS-CoV-2 reinfection (12). Although SARS-CoV-2 reinfections are described as less severe than primary infections (13), severe events continue to be reported despite increasing vaccination rates (3, 14); furthermore, some studies have reported no difference in severity between prior infections and reinfections (15, 16). For individuals who survive reinfections, all-cause mortality and hospitalization risk in the acute and post-acute phase has been reported, as well as a relationship between the frequency of COVID-19 reinfections and the prevalence of post-acute COVID-19 conditions (17).

Individuals with previous infection and at least one dose of a COVID-19 vaccine are benefitted from hybrid immunity, where natural immunity due to infection and vaccination-acquired immunity interact to enhance protection against reinfection and severe disease. As evidence supporting the protective role of hybrid immunity over either natural or vaccine-acquired immunity alone continues to arise (18–21), the complexity of this phenomenon becomes more evident. A series of different factors, such as the number of vaccine doses received, vaccine platform used, the severity of the first COVID-19 episode, SARS-CoV-2 variants, and subvariants responsible for both first infection and reinfection and time-dependent waning protection seem to have a significant effect on the level of protection conferred by hybrid immunity (22–28). Furthermore, since immune imprinting, a phenomenon in which B-cell immune response from first exposure to antigens related to an infectious agent (either through vaccination or previous infection) was demonstrated to condition the host response toward SARS-CoV-2 reinfections (10, 29, 30), studies have started to include the order in which immunity-generating events (vaccination and infection) occur as an additional factor to be considered in order to understand hybrid immunity better (31–33). Even though real-world evidence of hybrid-immunity protection continues to emerge, studies integrating the order of immunity-generating events are still scarce. Hybrid-immunity evidence is still missing in Latin American countries, which, due to their social determinants of health, continue to experience the COVID-19 pandemic differently from high-income countries (34). Here, we evaluated the role of primary infection and hybrid immunity on the risk of reinfection during periods of Omicron variant predominance in Mexico in individuals with a previous SARS-CoV-2 infection. We also evaluated the influence of the order of immunity-generating events, previous hospitalization, and SARS-CoV-2 variant predominance on the risk of reinfection and reinfection-associated severe outcomes using a nationwide COVID-19 registry.

Methods

Study population

We assessed cases of suspected SARS-CoV-2 reinfection using the SISVER registry, a daily updated nationwide surveillance system of suspected SARS-CoV-2 cases in Mexico (35, 36), managed by the General Directorate of Epidemiology of the Mexican Ministry of Health. Detailed sociodemographic and clinical information is ascertained, including details of SARS-CoV-2 infection and clinical course, as well as vaccination status, date, and vaccine applied. For this analysis, from March 3rd, 2020, to August 13th, 2022, we analyzed survivors of a first confirmed SARS-CoV-2 infection with suspected reinfection during the predominance of Omicron. Only suspected reinfections which were separated at least 90 days from primary infection and with either a positive or negative SARS-CoV-2 RT-PCR or antigen test were included. A flowchart of included and excluded subjects is presented in Figure 1.

Definition of immunity-generating events

An immunity-generating event was considered either a vaccination against COVID-19 (first, second dose, or booster shot) or a confirmed SARS-CoV-2 infection, either primary or reinfection. Reinfections were defined as a confirmed SARS-CoV-2 infection using RT-PCR or antigen test at least 90 days after confirmed primary SARS-CoV-2 infection (37). Nationally-available SARS-CoV-2 vaccines applied in Mexico during this period included BNT162b2, mRNA-1,273, Gam-COVID-Vac, Ad5-nCoV, Ad26.COV2.S, ChAdOx1-S, NVX-CoV2373, and CoronaVac (38). The vaccination schedule for most vaccines considered two doses, except for one-dose Ad5-nCoV and Ad26.COV2.S vaccines. Individuals were considered fully vaccinated if they had completed the vaccination schedule ≥14 days before the evaluated outcome (39, 40). Partially vaccinated individuals were considered if they had one out of a two-dose vaccine schedule or if the outcome happened <14 days in an otherwise completely vaccinated individual. COVID-19 vaccines were categorized according to their platform. Booster vaccination was considered if fully vaccinated individuals received an additional dose of a COVID-19 vaccine and at least 7 days had elapsed since vaccination (41), otherwise were reclassified as fully vaccinated. Booster schedules were categorized as homologous if booster shots were the same as the primary vaccination and heterologous if different.

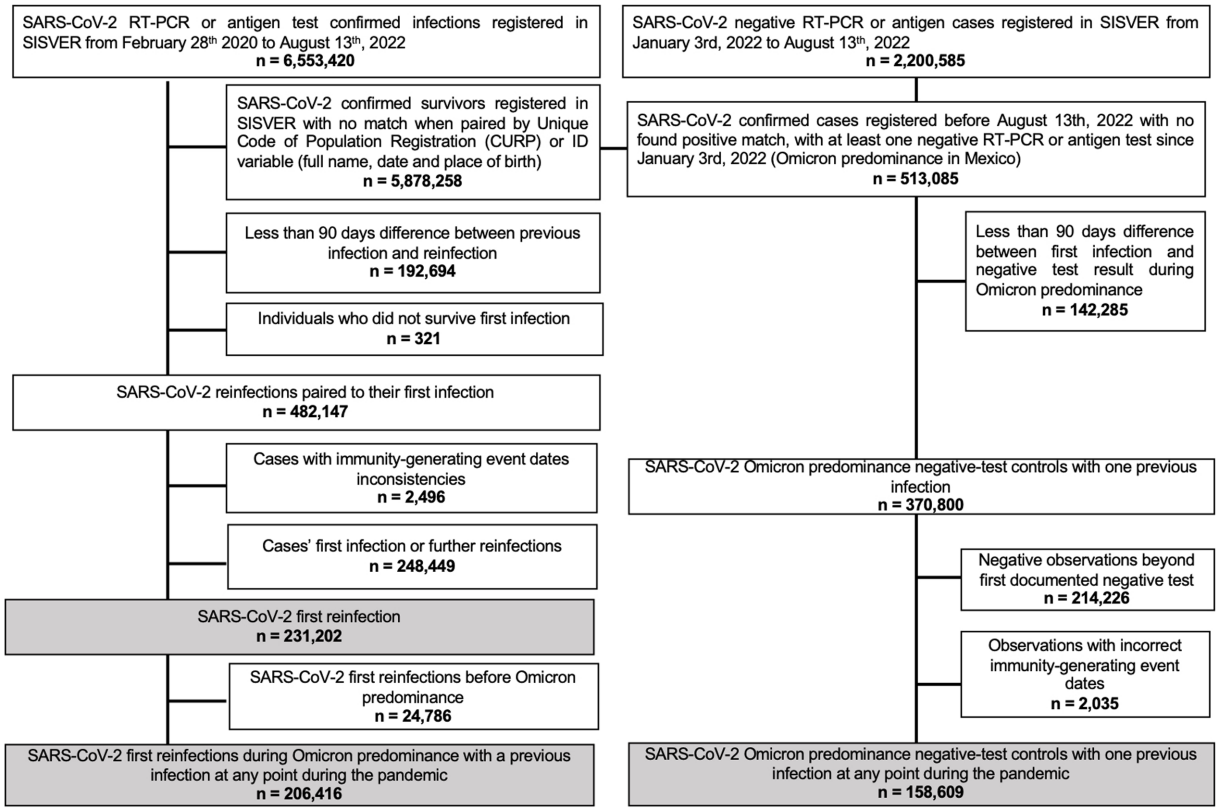

FIGURE 1
Flowchart of included and excluded subjects with SARS-CoV-2 documented reinfection or negative test as reported in SISVER from March 3rd, 2020, to August 13th, 2022.

Determinants of reinfection and severe COVID-19 risk

Previous evidence has shown varying degrees of protection for SARS-CoV-2 reinfection and severe COVID-19 (defined by hospitalization, ICU admission, intubation, or death) based on the variant responsible for primary and second infection (42, 43), time since vaccination or primary infection (24, 43), and antibody response (44–46). Therefore, we evaluated the following variables:

1. *Predominant SARS-CoV-2 variant for first infection* – A fraction of COVID-19 samples are sequenced by authorized national laboratories and submitted to the GISAID platform. Infection was assumed to be most likely caused by the predominant variant based on the date of symptom onset. Based on data submitted to GISAID (47), from March 3rd, 2020, until March 30th, 2021, the predominant SARS-CoV-2 variant was the ancestral strain, followed by the predominance of the B.1.1.519 variant until June 6th, 2021, the P.1 (Gamma) variant until July 4th, 2021, the B.1.617.2 (Delta) variant until January 2nd, 2022. B.1.1.529.1 (Omicron) BA.1 subvariant was considered from January 3rd, 2022, to April 24th, 2002, followed by Omicron subvariant BA.2 until June 19th, 2022.
2. *Predominant SARS-CoV-2 variant for reinfection* – Categorized based on the date of symptom onset for the reinfections, according to GISAID. Periods of Omicron subvariants BA.1 and BA.2 dominance were the same as previously described. Predominance of the BA.4 subvariant was considered from June 20th to July 3rd, 2022, followed by BA.5 subvariant predominance until August 13th, 2022.
3. *Previous COVID-19-related hospitalization* – Describes an individual who had been hospitalized and survived their primary SARS-CoV-2 infection.
4. *Order of immunity-generating events* – We developed an indicator variable that considered the order of immunity-generating events for a given individual, using as reference unvaccinated individuals with primary SARS-CoV-2 infection (Supplementary material). This variable considered the order of first infection, partial, complete, or booster vaccination before their second SARS-CoV-2 testing for suspected reinfection.
5. *Time elapsed since last immunity-generating events* – Defined as time elapsed in months since the last immunity-generating event, either vaccination or infection. Time was categorized based on whether individuals had ≥6 months since exposure, given previous evidence of vaccination or previous infection immunity waning over this period (48).

Statistical analyses

Epidemiology of SARS-CoV-2 reinfections in Mexico

We characterized all SARS-CoV-2 reinfections in Mexico to explore sociodemographic and clinical characteristics. Reinfection incidence and mortality were calculated over the number of

individuals who survived a confirmed primary SARS-CoV-2 infection. The number of confirmed reinfections was plotted over time and based on occurrence during periods of variant predominance in Mexico for the first and second SARS-CoV-2 infections using cross-tabulation matrix plots to visualize combinations of reinfections (Supplementary material).

Determinants of reinfection and reinfection-associated severe COVID-19 risk

We matched SARS-CoV-2 reinfections to individuals with a negative SARS-CoV-2 test after primary infection using propensity score matching for age and sex. Next, we fitted conditional logistic regression models, including matching weights to explore the role of previously defined determinants on the risk of reinfection. We also explored interaction effects between the order of immunity-generating events and time elapsed since last exposure to an immunity-generating or hospitalization during primary SARS-CoV-2 infection. For models on the risk of severe COVID-19 associated with reinfection, we only analyzed cases with confirmed reinfection and explored factors as described earlier.

Heterologous versus homologous vaccine boosting

Given the diversity of COVID-19 vaccination schedules applied in Mexico, we explored the risk of reinfection and severe COVID-19 associated with homologous and heterologous booster protocols compared to fully vaccinated individuals, exploring similar determinants as described above using conditional logistic regression.

Results

Epidemiology of SARS-CoV-2 reinfections in Mexico

We detected 231,202 confirmed reinfections over the study period, with most reinfections occurring during January and June 2022. A steady rate of reinfection-associated mortality was observed starting from March 2021 (Figure 2). Most SARS-CoV-2 reinfections occurred in women (60.12%) and individuals aged 31–40 years [30.26% and a median time between reinfections of 362 days (IQR 196–531 days); Supplementary material]. Reinfections occurred primarily in unvaccinated individuals (41.55%) or cases in which primary infection occurred before completing vaccination schedules or receiving a booster shot (41.0%). Reinfections in individuals with comorbid diabetes or obesity were low (5.97 and 8.34%). Most primary infections occurred during the predominance of ancestral strains (50.06%), followed by Delta (23.1%) and Omicron BA.1 (14.67%). Over 206,416 reinfections occurred during periods of predominance of the Omicron variant in Mexico (89.3%), primarily associated with the predominance of Omicron BA.1 (36.74%) and Omicron BA.5 (33.67%) subvariants. We identified 3,261 hospitalizations related to reinfections (1.41%), and 515 deaths, with a reinfection fatality rate of 0.22% (Supplementary Table S1). Overall, we identified a peak of reinfections with a stable mortality rate was observed since the start of Omicron predominance, mainly affecting incompletely or not vaccinated individuals.

Risk of SARS-CoV-2 reinfection with hybrid immunity

We paired 158,609 cases of confirmed reinfection with 158,609 controls with primary infection and a second negative SARS-CoV-2 test taken at least 90 days after primary infection (Supplementary material). Decreased risk of reinfection was associated with a primary infection during the predominance of most SARS-CoV-2 variants compared to the ancestral strain, with higher protection observed for the predominance of Gamma, Omicron BA.1, and BA.2 subvariants (Figure 3). Increased risk of reinfection was associated with the predominance of the Omicron BA.4 and BA.5 subvariants, compared to periods of BA.1 subvariant predominance. Hospitalization during the first SARS-CoV-2 infection was associated with a lower risk of reinfection, while ≥6 months since the last immunity-generating event was associated with a higher risk. The order of immunity-generating events did not significantly impact the risk of reinfection compared to unvaccinated individuals with primary infection; however, the lowest risk was observed in fully boosted

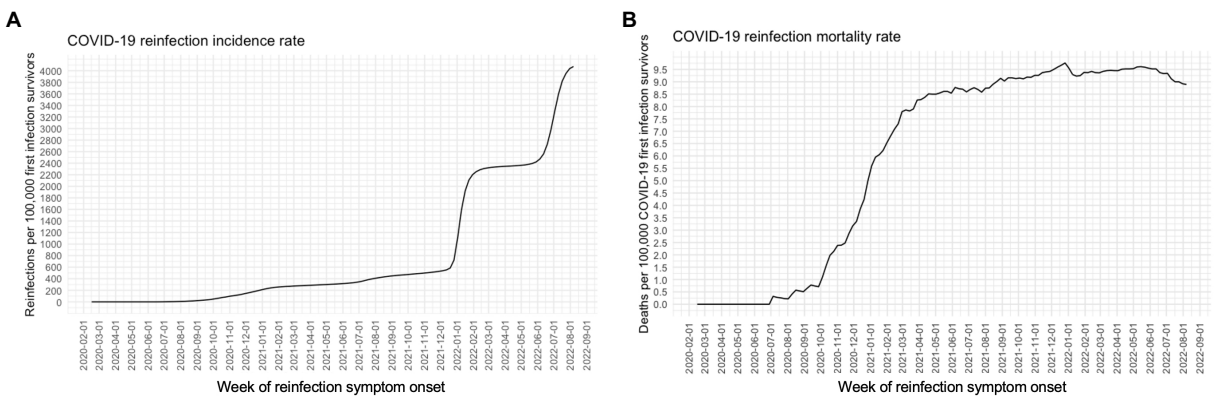

FIGURE 2
Incidence rates of SARS-CoV-2 cases associated with reinfections **(A)** and reinfection-associated deaths **(B)** per 100,000 first SARS-CoV-2 infection survivors in Mexico from March 3rd, 2020, until August 13th, 2022 (*n*=231,202).

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Variable		N	Odds ratio	p-value
Dominant variant FI	Previous variants	142093	Reference	
	B.1.1.519	27990	0.91 (0.88, 0.93)	<0.001
	Delta	80440	0.73 (0.72, 0.75)	<0.001
	Gamma	8814	0.89 (0.85, 0.94)	<0.001
	Omicron BA.1	57857	0.51 (0.50, 0.53)	<0.001
	Omicron BA.2	24	0.13 (0.05, 0.35)	<0.001
Dominant variant SI	Omicron BA.1	147369	Reference	
	Omicron BA.2	37689	0.62 (0.61, 0.64)	<0.001
	Omicron BA.4	38644	2.14 (2.09, 2.20)	<0.001
	Omicron BA.5	93516	2.55 (2.49, 2.60)	<0.001
Event order	First infection - Outcome	142194	Reference	
	First infection - Incomplete vaccination - Outcome	9813	0.90 (0.86, 0.94)	<0.001
	First infection - Complete vaccination - Outcome	86923	1.12 (1.10, 1.14)	<0.001
	First infection - Complete vaccination - Booster - Outcome	32563	0.92 (0.89, 0.95)	<0.001
	Incomplete vaccination - First infection - Outcome	2584	0.98 (0.90, 1.07)	0.690
	Complete vaccination - First infection - Outcome	20286	0.90 (0.87, 0.93)	<0.001
	Complete vaccination - First infection - Booster - Outcome	18471	0.90 (0.87, 0.93)	<0.001
	Complete vaccination - Booster - First infection - Outcome	4384	0.82 (0.77, 0.88)	<0.001
Previous hospitalization		317218	0.65 (0.63, 0.68)	<0.001
Six months or more since last immunizing event		317218	1.10 (1.08, 1.13)	<0.001

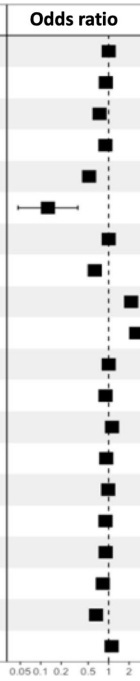

FIGURE 3
Conditional logistic regression model for risk of reinfection in subjects with confirmed reinfection (n=158,609) paired with subjects with first confirmed SARS-CoV-2 reinfection and a second negative test taken at least 90days after the first infection (n=158,609). Pairing was performed using propensity score matching for age and sex. FI, first infection; SI, second infection.

individuals prior to primary infection. A paradoxical increase in the risk of reinfection was observed in subjects fully vaccinated after primary infection; nevertheless, a higher risk in this category was observed for subjects with ≥6 months since the last immunity-generating event. When stratifying models according to variant predominance at the time of reinfection, no significant changes were observed for these associations (Supplementary material). Primary infection during the predominance of Omicron protected the best against reinfection by this same SARS-CoV-2 variant, with slight variation between subvariants and hybrid-immunity profiles.

Risk of severe COVID-19 associated with reinfections

We explored risk factors in subjects with confirmed reinfection and severe COVID-19 (n=2,078) paired 1:4 with subjects with reinfection without severe COVID-19 (n=8,312) using propensity score matching for age and sex. Regarding primary infection, a decreased risk for severe COVID-19 was observed during the period of Gamma predominance compared to that of the ancestral strain. An increased risk was associated with primary infection during Omicron BA.1 subvariant predominance. A progressively decreased risk of severe COVID-19 was observed during periods of Omicron BA.2, BA.4, and BA.5 predominance, compared to reinfection during the predominance of the BA.1 subvariant (Figure 4). Compared to unvaccinated individuals, all combinations of immunity-generating events that included at least one vaccine dose were associated with a >90% reduction in the risk of severe COVID-19, except for incomplete vaccination prior to primary infection, providing a ~87% reduction in risk. Having been hospitalized and survived the primary SARS-CoV-2 infection was associated with a higher risk of severe COVID-19 during reinfection, as well as having ≥6 months or more since the last immunity-generating event and having comorbid diabetes. Additional adjustment for age and sex to control for residual confounding did not modify these associations. Vaccination yielded the most considerable reduction in the risk of severe COVID-19 associated with reinfection, while previous hospitalization due to COVID-19, followed by diabetes, was related to a higher risk.

Homologous versus heterologous boosters and risk of reinfection and severe outcomes

The most frequently reported booster shot in subjects evaluated for reinfection was heterologous vaccination of BNT162b2 boosted with ChAdOx1-S (40.6%), followed by homologous vaccination with ChAdOx1-S (23.5%), homologous vaccination with BNT162b2 (5.3%), and heterologous vaccination of Ad5-nCoV boosted with mRNA-1,273 (4.8%, Supplementary material). We observed 102,634 reinfections in fully vaccinated or boosted individuals with primary infection (n=183,986, 55.8%), among which 67,880 occurred in fully vaccinated individuals without boosting (n=121,727, 55.8%) and 34,754 occurred in individuals with boosters (n=62,259, 55.8%). Risk factors for SARS-CoV-2 reinfection included older age and female sex, while a higher risk was observed for reinfections in periods of Omicron BA.4 and BA.5 predominance (Figure 5A). When compared against fully vaccinated individuals without boosting, heterologous boosters were associated with ~11% decreased risk of reinfection, with no significant

Variable		N	Odds ratio	p-value
Dominant variant FI	Previous variants	4843		Reference
	B.1.1.519	1049		0.66 (0.50, 0.85) 0.002
	Delta	2616		1.05 (0.85, 1.29) 0.662
	Gamma	274		0.57 (0.34, 0.94) 0.029
	Omicron BA.1	1608		1.56 (1.14, 2.15) 0.006
Dominant variant SI	Omicron BA.1	4317		Reference
	Omicron BA.2	826		0.54 (0.40, 0.72) <0.001
	Omicron BA.4	1526		0.31 (0.24, 0.40) <0.001
	Omicron BA.5	3721		0.31 (0.25, 0.38) <0.001
Event order	First infection - Outcome	2068		Reference
	First infection - Incomplete vaccination - Outcome	326		0.04 (0.02, 0.06) <0.001
	First infection - Complete vaccination - Outcome	4124		0.02 (0.02, 0.03) <0.001
	First infection - Complete vaccination - Booster - Outcome	1776		0.08 (0.06, 0.10) <0.001
	Incomplete vaccination - First infection - Outcome	87		0.13 (0.07, 0.24) <0.001
	Complete vaccination - First infection - Outcome	967		0.03 (0.02, 0.05) <0.001
	Complete vaccination - First infection - Booster - Outcome	847		0.06 (0.04, 0.08) <0.001
	Complete vaccination - Booster - First infection - Outcome	195		0.05 (0.03, 0.10) <0.001
Previous hospitalization		10390		12.81 (10.46, 15.68) <0.001
Six months or more since last immunizing event		10390		1.82 (1.52, 2.19) <0.001
Diabetes		10390		3.18 (2.56, 3.95) <0.001

FIGURE 4
Conditional logistic regression model for risk of severe COVID-19, defined as an event of hospitalization, the requirement for ICU admission, intubation, or death in subjects with confirmed SARS-CoV-2 reinfection and severe COVID-19 (*n*=2,078) compared to mild SARS-CoV-2 reinfections (*n*=8,312), paired using propensity score matching for age and sex. FI, first infection; SI, second infection.

A

Variable	OR[†]	95% CI[†]	p-value
Age	1	1.00, 1.00	<0.001
Sex			
Female	—	—	
Male	0.94	0.93, 0.96	<0.001
Dominant variant SI			
Omicron BA.1	—	—	
Omicron BA.2	0.51	0.49, 0.53	<0.001
Omicron BA.4	1.62	1.57, 1.67	<0.001
Omicron BA.5	1.93	1.88, 1.98	<0.001
Vaccination status			
Completely vaccinated	—	—	
Completely vaccinated and heterologous booster	0.89	0.87, 0.92	<0.001
Completely vaccinated and homologous booster	0.97	0.94, 1.01	0.168
Previous hospitalization	0.98	0.93, 1.03	0.391
Six months or more since last immunizing event	1.65	1.37, 2.00	<0.001
Vaccination status * Six months or more since last immunizing event			
Completely vaccinated and heterologous booster * Six months or more since last immunizing event	1.04	0.98, 1.11	0.164
Completely vaccinated and homologous booster * Six months or more since last immunizing event	0.94	0.86, 1.04	0.229

[†] OR = Odds Ratio, CI = Confidence Interval

B

Variable	OR[†]	95% CI[†]	p-value
Age	1.02	1.01, 1.02	<0.001
Sex			
Female	—	—	
Male	1.09	0.94, 1.25	0.245
Dominant variant SI			
Omicron BA.1	—	—	
Omicron BA.2	1.02	0.77, 1.32	>0.910
Omicron BA.4	0.69	0.54, 0.87	0.002
Omicron BA.5	0.63	0.52, 0.76	<0.001
Vaccination status			
Completely vaccinated	—	—	
Completely vaccinated and heterologous booster	2.51	2.01, 3.14	<0.001
Completely vaccinated and homologous booster	1.3	0.94, 1.77	0.106
Previous hospitalization	14.7	12.6, 17.2	<0.001
Six months or more since last immunizing event	1.65	1.37, 2.00	<0.001
Vaccination status * Six months or more since last immunizing event			
Completely vaccinated and heterologous booster * Six months or more since last immunizing event	0.46	0.31, 0.68	<0.001
Completely vaccinated and homologous booster * Six months or more since last immunizing event	0.73	0.37, 1.35	0.338

[†] OR = Odds Ratio, CI = Confidence Interval

FIGURE 5
Conditional logistic regression model for risk of reinfection **(A)** and severe COVID-19 **(B)** in subjects with complete vaccination protocol (*n*=121,727) with or without an additional booster shot (*n*=62,259) to evaluate the impact of heterologous or homologous boosting in subjects with first confirmed SARS-CoV-2 infection. SI, second infection.

difference from homologous boosters. Cases with ≥6 months since the last immunity-generating event had a higher risk of reinfection. Increased risk of reinfection-associated severe COVID-19 was observed for older age while infection during the predominance of Omicron BA.4 and BA.5 subvariants was associated with decreased risk (Figure 5B). Subjects with heterologous boosters and the last immunity-generating event having occurred at least 6 months before had ~54% lower risk of severe COVID-19 than completely vaccinated individuals with ≥6 months from exposure. Notably, no differences were observed for homologous boosters compared to subjects without boosting. Heterologous booster schedules were associated with a lower risk of both reinfection and severe reinfection even after 6 months or more from the last immunizing event, compared to complete primary and homologous booster schedules.

Discussion

Here, we conducted one of the largest evaluations of the risk of reinfection and severe COVID-19 by analyzing 231,202 individuals with primary SARS-CoV-2 infection in Mexico, with 89.3% reinfections occurring during the period of Omicron predominance. In this setting, we evaluated the role of hybrid immunity, the severity of primary infection, and the influence of time-waning immunity in the risk of reinfection and reinfection-related severe COVID-19 risk. As previously reported, most reinfections were associated with Omicron and its subvariants (28, 31, 49, 50), rapidly accelerating reinfection rates during BA.1 and BA.5 subvariant predominance and an intermediate plateau attributed to a stable primary infection-reinfection ratio. The predominance of Omicron subvariants did not modify reinfection-associated death rates. Vaccination protected against reinfection without significant influence from the order of immunity-generating events; nevertheless, the highest degree of protection was observed for fully vaccinated individuals boosted prior to their primary infection suggesting that, in fully vaccinated and boosted naïve individuals, primary infection is an effective additional immune booster against reinfection (51). Similar to previously reported evidence of antibody response to primary schedule vaccination with mRNA vaccines, after primary infection, one out of a two-dose SARS-CoV-2 vaccine schedule seemed to provide similar protection against reinfection as a complete two-dose schedule (33). Likewise, a complete schedule before primary infection would provide better protection against reinfection than an incomplete schedule, as previously suggested by serological studies for BNT162b2 mRNA vaccine (32). However, the protection provided by the different vaccine-primary infection profiles only differed modestly.

As for severe COVID-19 reinfections, primary infection with B.1.1.519 (52) or the Gamma variant yielded the highest protection among other variants and subvariants. Omicron BA.1 primary infection was related to a higher risk. Hospitalization during primary infection, 6 months or more since the last immunity-generating event, and comorbid diabetes conferred a higher risk of severe COVID-19 reinfection. Finally, we observed the superiority of heterologous boosters over complete vaccine schedules for protection against reinfection or severe COVID-19, confirming previous reports on the benefits of heterologous vaccination against COVID-19 (53, 54).

Considering that approximately ~50% of Mexican adults have antibodies against the Nucleocapsid protein of SARS-CoV-2, indicating exposure and likely a previous infection (55), and given the widespread circulation of Omicron, seroprevalence most likely increased along with the proportion of the population at risk of reinfection (56). The increased susceptibility to reinfection was demonstrated by the growing trends in the proportion of reinfections found in this study, representing as much as 12% of the weekly total COVID-19 cases (Supplementary material), which, interestingly, is a similar proportion to that reported in a Serbian study on reinfections after the advent of the Omicron variant (57). However, this proportion is likely higher due to limitations of case definitions and reporting. This proportion is expected to continue growing following the appearance of new variants. The contribution of COVID-19 vaccines to hybrid immunity wanes within a few months of vaccination, particularly in the context of each new variant of concern (58); however, effectiveness is still maintained for Omicron and its subvariants, particularly against severe COVID-19. Therefore, vaccines are observed to meet the strategic objectives highlighted in the WHO's SAGE Roadmap for prioritizing the uses of COVID-19 vaccines by preventing severe reinfections (59).

Among the strengths of our study, we highlight that it represents one of the largest reports of SARS-CoV-2 reinfections and risk factors in a country with high SARS-CoV-2 seroprevalence and broad vaccination coverage. Furthermore, given the diversity of the epidemiological situation in Mexico throughout each of the 32 states and the variety of vaccines employed, the use of an epidemiological surveillance dataset that concentrates information from a national level of not only confirmed cases but all suspected SARS-CoV-2 infections along with their laboratory test results allows for adequate assessment of reinfections and their associated outcomes, as well as the order of immunity-generating events, making it possible to address the heterogeneity and complexity of hybrid immunity. Finally, given the diversity of vaccines used in Mexico, we could also provide real-world evidence on the effectiveness of combinations of incomplete, complete, and booster vaccine schedules with primary infection and heterologous and homologous vaccine boosters in subjects with prior SARS-CoV-2 infection. Among the limitations to be acknowledged is the definition of reinfection. Recent evidence demonstrates that reinfections can occur within a shorter period and may incorrectly exclude some reinfections in our study (60).

Furthermore, when analyzing risk associated with SARS-CoV-2 variants, we assumed that predominant variants likely caused infections during each period; nevertheless, this type of inference has been used as an approach to variant analysis when individual-level genomic data is unavailable (10, 31, 48, 52). This approach limits our capacity to differentiate the effect of high community transmission pressure on reinfections from the ability of variants to evade hybrid immunity. However, the higher transmissibility of new SARS-CoV-2 variants is intimately related to their virulence and should therefore be considered a consequence of the latter. Finally, we did not perform individual analyses for each vaccine and booster combination, provided that not all combinations had a large enough number of outcomes to allow for adequate comparisons. The role of vaccination and boosters' interaction with predominant circulating variants remains an area of opportunity for future research. Given that most reinfections occurred during periods of Omicron predominance and that immunity-boosting by infection with Omicron seems to be low, real-world studies on hybrid immunity that consider the effects of immune imprinting from SARS-CoV-2 on protection against reinfection will be fundamental for determining the need of booster shots and its frequency in future vaccination waves.

Even though protection against reinfection conferred by boosters appears to decrease following the advent of new variants, protection against severe reinfection remains high. Hybrid-immunity studies should place the focus on severe disease and individuals prone to it—specifically, people at high risk for serious illness, older adults, individuals at high risk of exposure (61), immunocompromised individuals, and comorbid conditions (62) such as diabetes and hospitalization during first SARS-CoV-2 infection, both for which an association with severe reinfection was found in our study.

As of now, vaccination continues to be our most robust defense against COVID-19, regardless of previous exposure to SARS-CoV-2. Primary and booster vaccination should therefore be prioritized in those unvaccinated or those who have not received their first booster shot, providing additional protection against reinfection (31) and lowering the

risk of post-acute COVID-19 syndrome associated with reinfection; but mainly preventing severe outcomes, particularly for variants with increased transmission and associated immune evasion such as Omicron (63). As reinfections become more frequent (64), surveillance systems may benefit from the study of SARS-CoV-2 reinfections.

Research in context

Evidence before this study

We searched PubMed for the terms "SARS-CoV-2" AND "reinfection" AND "hybrid immunity" until November 20th, 2022, and identified a few population studies previously conducted in Israel, Sweden, Qatar, United States, and Canada which explored the risk of reinfection and the protective role of hybrid immunity in individuals with one, two, or three doses of COVID-19 vaccines, predominantly during periods of the predominance of Omicron BA.1 and BA.2 subvariants. Notably, no studies were conducted in any Latin American country or reported on the benefit of heterologous booster schemes or the order of immunity-generating events.

Added value of this study

We report the results of a nationwide study in Mexico of over 230,000 SARS-CoV-2 reinfections, with ~90% occurring during periods of Omicron predominance. We identified that vaccination provided additional benefits in reducing the risk of SARS-CoV-2 reinfection, with the highest benefit observed in individuals with complete vaccination and booster protocols prior to primary infection or with primary infection during periods of BA.1 and BA.2 subvariant predominance. Hybrid immunity also substantially reduces the risk of reinfection-associated severe COVID-19, with a > 90% reduction in risk compared to unvaccinated individuals with previous SARS-CoV-2 infection, regardless of the order of immunity-generating events. Finally, heterologous COVID-19 booster schedules were associated with ~11% and ~ 54% lower risk for reinfection and reinfection-associated severe COVID-19, respectively, modified by time-elapsed since the last immunity-generating event, when compared against complete primary schedules.

Implications of all the available evidence

Our results support that COVID-19 vaccination and boosters provide additional benefits to protect against SARS-CoV-2 reinfection and reinfection-associated severe COVID-19. Using heterologous boosters appears to provide additional protection in previously infected individuals. Such schemes may prove beneficial as newer and more transmissible variants emerge.

Data availability statement

The datasets presented in this article are not readily available because of privacy restrictions. Requests to access the datasets should be directed to the General Directorate of Epidemiology of Mexico (DGE).

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics and Research Committee at Instituto Nacional de Geriatría, project number DI-PI-005/2021. The ethics committee waived the requirement of written informed consent for participation.

Author contributions

JM-G and OB-C: research idea and study design and statistical analysis. JM-G, CZ-J, RG-V, GG-R, and HL-G: data acquisition. JM-G, OB-C, NA-V, CF-M, DR-G, and AV-V: data analysis/interpretation. JM-G, OB-C, NA-V, CF-M, DR-G, AV-V, and SV-F: manuscript drafting. OB-C: supervision or mentorship. All authors contributed important intellectual content during manuscript drafting or revision and accepted accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1146059/full#supplementary-material

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Non-spike and spike-specific memory T cell responses after the third dose of inactivated COVID-19 vaccine

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Background: During the COVID-19 epidemic, vaccination has become the most safe and effective way to prevent severe illness and death. Inactivated vaccines are the most widely used type of COVID-19 vaccines in the world. In contrast to spike-based mRNA/protein COVID-19 vaccines, inactivated vaccines generate antibodies and T cell responses against both spike and non-spike antigens. However, the knowledge of inactivated vaccines in inducing non-spike-specific T cell response is very limited.

Methods: In this study, eighteen healthcare volunteers received a homogenous booster (third) dose of the CoronaVac vaccine at least 6 months after the second dose. $CD4^+$ and $CD8^+$ T cell responses against a peptide pool from wild-type (WT) non-spike proteins and spike peptide pools from WT, Delta, and Omicron SARS-CoV-2 were examined before and 1-2 weeks after the booster dose.

Results: The booster dose elevated cytokine response in $CD4^+$ and $CD8^+$ T cells as well as expression of cytotoxic marker CD107a in $CD8^+$ T cells in response to non-spike and spike antigens. The frequencies of cytokine-secreting non-spike-specific $CD4^+$ and $CD8^+$ T cells correlated well with those of spike-specific from WT, Delta, and Omicron. Activation-induced markers (AIM) assay also revealed that booster vaccination elicited non-spike-specific $CD4^+$ and $CD8^+$ T cell responses. In addition, booster vaccination produced similar spike-specific AIM^+CD4^+ and AIM^+CD8^+ T cell responses to WT, Delta, and Omicron, indicting strong cross-reactivity of functional cellular response between WT and variants. Furthermore, booster vaccination induced effector memory phenotypes of spike-specific and non-spike-specific $CD4^+$ and $CD8^+$ T cells.

Conclusions: These data suggest that the booster dose of inactive vaccines broadens both non-spike-specific and spike-specific T cell responses against SARS-CoV-2.

KEYWORDS

COVID-19, inactivated vaccines, CoronaVac, T cell responses, spike, non-spike

Introduction

Inactivated viral vaccines have been successfully developed for decades around the world in immunization programs such as polio and influenza. Vaccines of this type are created by inactivating the whole pathogen through physical or chemical processes. The processing stops the pathogen's capacity to replicate in the vaccinated hosts, but the human immune system can still respond to the vaccines (1). As of 8 February 2023, the World Health Organization (WHO) has accepted three inactivated COVID-19 vaccines, including Covaxin (Bharat Biotech, India), Covilo (Sinopharm, China), CoronaVac (Sinovac, China), for emergency use. Covilo and CoronaVac vaccines have been approved by 93 and 56 countries, respectively (https://covid19.trackvaccines.org/agency/who/). They are also the most easily obtainable COVID-19 vaccines, particularly in developing countries. Covilo and CoronaVac vaccines account for more than half of the delivered doses in the world (2).

The SARS-CoV-2 Omicron variant (B.1.1.529), first reported in South Africa in November 2021 (3), has been the dominant variant globally. It has more than 30 mutations in the spike gene compared to the ancestral strain and is associated with elevated infectivity and immune evasion (4). There are also approximately 20 mutations in the conserved non-spike genome, which is less significant for infectivity and immune evasion (5). Fortunately, the Omicron variant results in lower hospitalization and death rates than the previous strains (6). Up to the present, the Omicron variant has many sub-lineages – BA.1, BA.1.1, BA.2, BA.3, BA.4, and BA.5, BA.4.6, BF.7, BQ.1, BQ.1.1, XBB.1, and XBB.1.5, which will continue to evolve in the near future. The SARS-CoV-2 Omicron variant can escape from humoral immunity due to the mutations in the receptor binding domain (RBD) of spike protein (S), which is essential for the binding for neutralization antibodies (7). However, escape from cell immunity of the Omicron variant has not been documented in the literature.

SARS-CoV-2 virus consists of four structural proteins including S, envelope (E), membrane (M) and nucleocapsid (N); and other non-structural proteins. It has been reported that strong $CD4^+$ and $CD8^+$ T cell memory responses against both spike and non-spike antigens were elicited in convalescent patients with SARS-CoV-2 infection (8). In COVID-19 convalescent patients, there was a significant correlation between the percentage of SARS-CoV-2 non-spike-specific $CD4^+$ T cells and anti-spike RBD IgG. Non-spike peptides stimulated cytokine secretion in $CD4^+$ T cells (9). Lang-Meli et al. demonstrated that $CD8^+$ T cells targeted both spike and non-spike epitopes, with non-spike-specific epitopes being dominant, in COVID-19 convalescent individuals (10). Naranbhai et al. showed that spike-specific $CD4^+$ T cell response was maintained against Omicron, while spike-specific $CD8^+$ T cell response was decreased (11). Therefore, $CD4^+$ and $CD8^+$ T cell responses against both non-spike and spike antigens play an essential role in SARS-CoV-2 infection.

Most existing studies of inactivated COVID-19 vaccines focus on humoral and cellular immune responses to spike antigens. The effect of inactivated COVID-19 vaccines on non-spike-specific T cell response is poorly understood. In the present study, peripheral blood mononuclear cells (PBMCs) were collected from individuals before and after the homogenous booster (third) dose of CoronaVac COVID-19 vaccine. We compared non-spike and spike-specific cellular responses of circulating $CD4^+$ and $CD8^+$ T cells.

Materials and methods

Human study subjects

This is an observational vaccine study conducted during China national COVID-19 vaccination campaign. We enrolled 18 volunteers from a cohort of health care workers at Children's Hospital of Zhejiang University School of Medicine. All of the volunteers enrolled in the study had body mass index in the normal range (18.5 to 24.9) and were in good physical status without significant co-morbidities. The volunteers had received two doses of SARS-CoV-2 inactivated vaccine CoronaVac between February 23, 2021 and May 28, 2021. Subjects were excluded from the study if they had a positive SAR-CoV-2 test at any time before the study period. The study protocol was approved (EC/IRB approval number: 2021029) by the ethics committee of the Children's Hospital of Zhejiang University School of Medicine (Hangzhou, China) and conducted according to the provisions detailed in Declaration of Helsinki. Written informed consent was acquired from each volunteer. A third dose of CoronaVac was administered to volunteers more than 6 months following the second dose between November 16, 2021 and January 18, 2022. Whole blood samples were collected from the volunteers before the booster dose and 1-2 weeks after.

Isolation of PBMCs

Peripheral blood samples were dispensed into heparin tubes and processed within 4 hours after collection. PBMCs were isolated from the whole blood *via* density gradient centrifugation using Lymphocyte Separation Medium (TBD, Tianjin, China) per the manufacturer's instructions. PBMCs were counted *via* trypan blue staining, cryopreserved in pre-cooled fetal bovine serum (FBS, Biological Industries, Kibbutz Beit-Haemek, Israel) with 10% DMSO (Sigma-Aldrich, Burlington, MA), and stored in liquid nitrogen until experiment.

Interferon-γ enzyme-linked immunospot (ELISpot) assays

ELISpot assay was performed using ELISpot Plus: Human IFN-γ (ALP) Kit (Mabtech, Sweden) according to the manufacturer's protocol. Before the assay, PBMCs were thawed and rested overnight at 37°C humidified incubator with 5% CO_2 in RPMI medium 1640 supplemented with 10% FBS. After preparation of ELISpot plates according to the protocol, 2×10^5 PBMCs were added to each well in duplicate. Cells were stimulated with spike peptide pools (15 mers with 11 amino acid overlap) from wild-type (WT),

Delta (B.1.617.2), and Omicron BA.1 (B.1.1.529) variant (GenScript, Piscataway, NJ) as well as a non-spike peptide pool containing N protein, M protein, and open reading frame proteins (ORF) of WT SARS-CoV-2 (Mabtech) at a final concentration of 2 μg/mL for 20 h at 37°C. No stimulation (DMSO only) and anti-CD3 stimulation were served as negative and positive control, respectively. Anti-CD28 (Mabtech) was added at a final concentration of 0.1 μg/mL to enhance antigen-specific responses. The plates were rinsed with Phosphate-buffered saline (PBS), incubated with Biotinylated mouse anti-human IFN-γ antibody (7-B6-1-biotin, 1 μg/mL) for 2 h, and rinsed with PBS again. The plates were then washed a second time and incubated for 1 h with Streptavidin-ALP (1:1000). After rinsing the plates, the cells were developed with 100 μL of BCIP/NBT-plus substrate solution until distinct spots were visible (usually 10-20 min). Color development was stopped by washing extensively in tap water. The plates were dried in a dim place for 2-3 days. ELISpot plates were counted *via* Mabtech ELISpot/FluoroSpot readers (Mabtech IRIS). The data were subtracted by the background value in the DMSO stimulation and were expressed as spot-forming unit (SFU)/10^6 PBMCs.

Flow cytometry-based assay for T cell response and cytokine-producing cells

PBMCs were thawed and rested overnight at 37°C humidified incubator with 5% CO_2 in RPMI medium 1640 supplemented with 10% FBS. A total of 1×10^6 PBMCs was stimulated with the spike peptide pools from WT, Delta (B.1.617.2), and Omicron BA.1 (B.1.1.529) as well as the non-spike peptide pool from wild-type SARS-CoV-2 for 20 h at a concentration of 2 μg/mL of final concentration. No stimulation (DMSO only) and anti-CD3 stimulation served as the negative and positive control, respectively. Anti-CD28 (BioLegend, San Diego, CA) and anti-CD49d (BioLegend) were added to all wells at a final concentration of 1 μg/mL to enhance antigen-specific responses and incubated for 20 h at 37°C. Subsequently, protein transport inhibitor GolgiPlug™ (containing Brefeldin A, BD Biosciences, NJ) and GolgiStop™ (containing Monensin, BD Biosciences) were added to cells at a dilution of 1:1000 and 1:1500, respectively. At the same time, BV421 anti-human CD107a (BioLegend) was added to cells. The incubation was continued for 4 h at 37°C.

For flow cytometry, cells were resuspended with cold flow cytometry buffer (PBS, 0.5% BSA) and stained with Fixable Viability Dye eFluor™ 780 (Thermo Fisher, Waltham, MA) for 30 min. After washing with flow cytometry buffer, cells were labelled with BV510 anti-human CD14 (BD Biosciences), FITC anti-human CD3 (Thermo Fisher), BV650 anti-human CD8 (BD Biosciences), AF700 anti-human CD4 (BioLegend), BV605 anti-human CD45RA (BioLegend), PerCP-cy5.5 anti-human CCR7 (BioLegend), PE anti-human CD137 (BioLegend), BV785 anti-human CD69 (BioLegend), and APC anti-human OX40 (BioLegend) or the isotype control at 4°C for 30 min. For intracellular staining, cells were incubated with Fixation/Perm working buffer (Thermo Fisher) for 30 min. The cells were then washed with flow cytometry buffer, resuspended in the perm diluent, and stained with BV711 anti-human IFN-γ (BioLegend) and PE-cy7 anti-human TNF-α (BioLegend) or the isotype control at 4°C for 30 min. After washing with perm diluent, PBMCs were resuspended with 300 μL flow cytometry buffer and examined *via* BD LSRFortessa™ flow cytometer. The data were analyzed using FlowJo V10 software.

Data analysis and statistics

Data are shown as mean ± standard error of the mean (SEM). Statistical analysis was performed using the GraphPad Prism V8.0 software (GraphPad, San Diego, CA). Data were tested for normality using the Anderson-Darling test, the D'Agostino-Pearson Omnibus normality test, the Shapiro-Wilk normality test, and the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefor corrected p value. To compare two groups, Wilcoxon matched-pairs signed rank test was performed for nonparametric data. Comparisons of multiple groups were performed *via* RM one-way ANOVA with Holm-Šídák's multiple comparisons test for parametric data or Friedman test with Dunn's multiple comparisons test for nonparametric data.

Results

Increased IFN-γ ELISpot response against spike and non-spike antigens after a third (booster) dose of CoronaVac

The characteristics of study participants and are shown in Table 1. To determine the T cell reactivity to non-spike and spike antigens induced by a third dose of CoronaVac, the number of IFN-γ-secreting cells in PBMCs obtained before and 1-2 weeks after the booster dose was examined *via* ELISpot assay (Figure 1A). The booster dose significantly increased the frequency of IFN-γ producing cells upon stimulation with the non-spike peptide pool derived from N, M, and ORF proteins of wild-type SARS-CoV-2 (Figure 1B). Similarly, the number of IFN-γ-secreting cells in PBMCs was elevated after the third dose in response to spike peptide pools from WT, Delta, and Omicron. However, no significant difference was observed among WT, Delta, and Omicron (Figure 1B).

TABLE 1 Characteristics of donor cohort.

	Vaccinees (n = 18)
Age, y	23-47 (Median = 31, IQR = 9)
Gender, %	
Male	33 (6/18)
Female	67 (12/18)
Duration of post-vaccination (days)	9-14 (Median = 10.5, IQR = 3)

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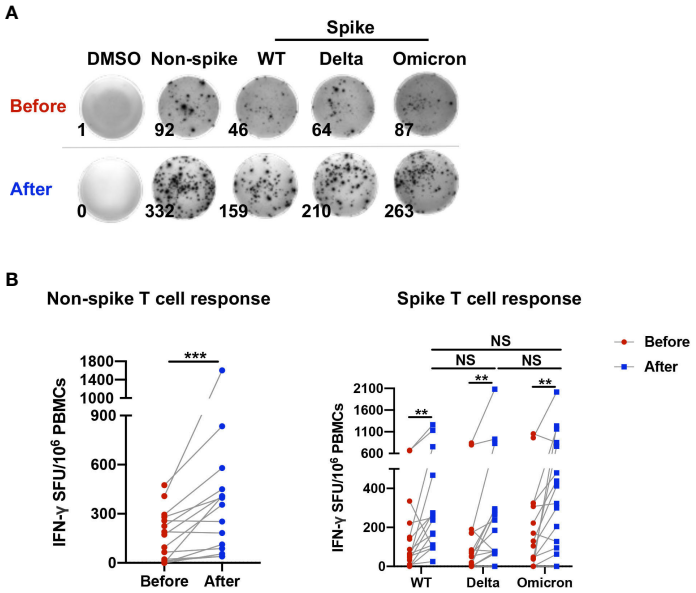

FIGURE 1
T cell reactivity to non-spike and spike antigens induced by a third dose of CoronaVac. PBMCs obtained before and 1-2 weeks after the third dose of CoronaVac were stimulated with spike peptide pools from WT, Delta, and Omicron as well as a non-spike peptide pool from WT SARS-CoV-2 for 20 h. ELISpot assay was performed on PBMCs. No stimulation (DMSO only) was served as negative control. **(A)** Representative photos of ELISpot wells were presented. **(B)** The number of IFN-γ-producing T cells was enumerated as the spot-forming units (SFU) per 10^6 PBMCs. Points and connecting lines represent raw data for a single participant. Data were normalized against the background value in the DMSO stimulation. Comparisons were performed *via* Wilcoxon matched-pairs signed rank test between two groups or Friedman test with Dunn's multiple comparisons test among multiple groups. n = 15. $^{**}p < 0.01$, $^{***}p < 0.001$. NS, not significant.

Elevated function of non-spike and spike-specific $CD4^+$ and $CD8^+$ T cells after the third dose

Induction of cytokine-secreting non-spike and spike-specific $CD4^+$ and $CD8^+$ T cells was analyzed by intracellular cytokine staining assay (Supplemental Figure 1). The mean percentage of TNF-α-producing non-spike-specific $CD4^+$ T cells was increased from 0.119% to 0.311% 1-2 weeks after the booster dose, while the proportion of IFN-γ-producing non-spike-specific $CD4^+$ T cells was elevated from 0.019% to 0.047%. The booster dose also induced spike-specific TNF-α^+CD4^+ T cell responses of 0.289%, 0.332%, and 0.338% against WT, Delta, and Omicron, respectively, as well as spike-specific IFN-γ^+CD4^+ T cell responses of 0.038%, 0.049%, 0.051%, respectively (Figure 2A). On the other hand, the percentage of TNF-α-producing non-spike-specific $CD8^+$ T cells was increased from 0.033% to 0.240%, while IFN-γ-producing non-spike-specific $CD8^+$ T cells was raised from 0.016% to 0.112%. Spike-specific TNF-α^+CD8^+ T cell responses induced by the booster dose had a mean of 0.134%, 0.243%, and 0.328% against WT, Delta, and Omicron, respectively, while spike-specific IFN-γ^+CD8^+ T cell responses had a mean of 0.091%, 0.106%, and 0.144%, respectively (Figure 2B). Overall, no significant difference was observed in cytokine-secreting spike-specific $CD4^+$ and $CD8^+$ T cells among WT, Delta, and Omicron after booster dose. The data variation among the three groups may be attributed to baseline levels before vaccination and small sample size. The responses at baseline may represent the durable T cell immunity from prior 2 doses of homogenous vaccination and/or prior seasonal coronavirus infection. It is worth noting that the frequencies of cytokine-secreting non-spike-specific $CD4^+$ and $CD8^+$ T cells (TNF-α^+IFN-γ^-, TNF-α^-IFN-γ^+, and TNF-α^+IFN-γ^+) correlated well with those of spike-specific of wild-type, Delta, Omicron (Figure 2C). The association between spike and non-spike T cell response within each individual was less robust for the CD8 compared with that of CD4. Similar phenomenon was also reported by Zuo et al. in participants at 6 months following SARS-CoV-2 infection (12). In summary, the booster dose induced TNF-α and IFN-γ production in $CD4^+$ and $CD8^+$ T cells in response to both non-spike and spike antigens.

In addition, the frequency of non-spike $CD107a^+CD8^+$ T cells, reflective of cytotoxic function, was elevated from 0.249% to 0.381% after the booster dose (Figure 2D). In the meantime, spike-specific $CD107a^+CD8^+$ T cell response stimulated by the booster dose had a mean of 0.450%, 0.338%, and 0.528% against WT, Delta, and Omicron, respectively. Again, no difference was shown in $CD107a^+CD8^+$ T cells among WT, Delta, and Omicron. This finding indicates that the booster dose promoted the cytotoxic function of $CD8^+$ T cells in response to both non-spike and spike antigens.

Enhanced expression of activation-induced markers (AIM) by non-spike and spike-specific $CD4^+$ and $CD8^+$ T cells after the third dose

To further capture the characteristics of viral-specific T cell response, AIM assay was adopted to study T cell response. The AIM

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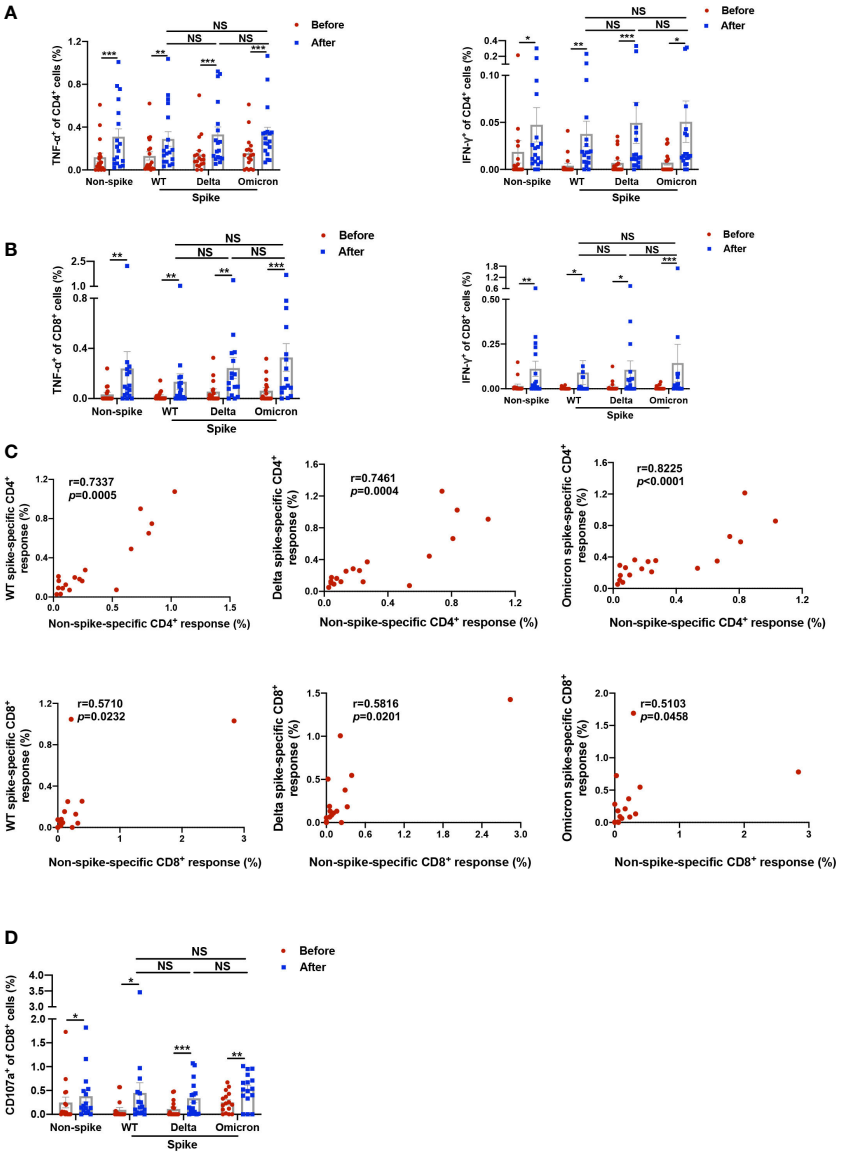

FIGURE 2
Functional analysis of non-spike and spike-specific $CD4^+$ and $CD8^+$ T cell responses. PBMCs obtained before and 1-2 weeks after the third dose of CoronaVac were stimulated with spike peptide pools from WT, Delta, and Omicron as well as a non-spike peptide pool from WT SARS-CoV-2 for 20 h. **(A–C)** TNF-α and IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in response to non-spike and spike peptides were detected by intracellular cytokine staining. **(A)** Data showed percentages of TNF-α and IFN-γ-producing $CD4^+$ T cells against non-spike and spike peptides. **(B)** Data showed percentages of TNF-α and IFN-γ-producing $CD8^+$ T cells against non-spike and spike peptides. **(C)** Cytokine-secreting $CD4^+$ and $CD8^+$ T cells against non-spike and spike peptides after the booster dose were calculated by aggregating amounts of TNF-α and IFN-γ-producing $CD4^+$/$CD8^+$ T cells, including TNF-α^+IFN-γ^-, TNF-α^+IFN-γ^+, and TNF-α^-FN-γ^+. Correlation analysis curve was plotted by the percentage of cytokine-secreting non-spike-specific $CD4^+$ and $CD8^+$ cells to their corresponding percentage of spike-specific $CD4^+$ and $CD8^+$ cells from WT, Delta, and Omicron. r, correlation coefficient. **(D)** Data showed percentages of $CD107a^+CD8^+$ T cells against non-spike and spike peptides determined by flow cytometry. Each point represents raw data for a single participant. Percentages show the percentage of T cells after subtracting the background value in a DMSO stimulation. Comparisons were performed *via* Wilcoxon matched-pairs signed rank test between two groups or Friedman test with Dunn's multiple comparisons test among multiple groups. Correlation analysis was performed *via* nonparametric spearman correlation. n = 16 - 18. *p < 0.05, **p < 0.01, ***p < 0.001. NS, not significant.

assay identifies early-responding T cells and is independent of cytokine production (8). It was reported that the percentage of AIM^+CD4^+ T cells did not have a strong correlation with the frequency of cytokine-producing $CD4^+$ T cells measured by intracellular cytokine staining or ELISpot (13). The expression of AIM on $CD4^+$ ($CD137^+OX40^+$) and $CD8^+$ ($CD137^+CD69^+$) cells was measured by flow cytometry (Figure 3A and Supplemental Figure 2). The booster dose increased the proportion of AIM^+CD4^+ cells from 0.057% to 0.280% in response to the non-spike peptide pool. When stimulated with spike peptide pools from WT, Delta, and Omicron, the percentages of AIM^+CD4^+ cells 1-2 weeks after the booster dose were elevated to 0.242%, 0.170%, and 0.244%, respectively, indicating substantial cross-reactivity of $CD4^+$ T cell responses between WT and variants (Figure 3B). The frequency of

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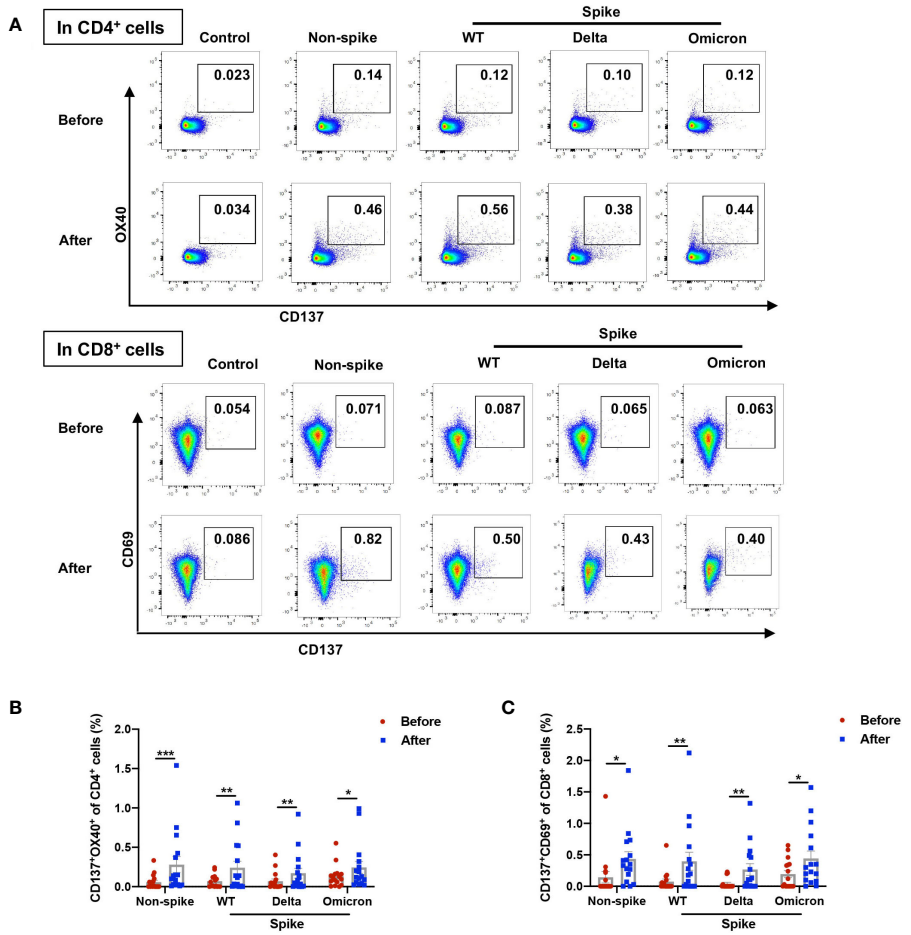

FIGURE 3
Non-spike and spike-specific AIM$^+$CD4$^+$ and AIM$^+$CD8$^+$ T cell responses after a third dose of CoronaVac. PBMCs obtained before and 1-2 weeks after the third dose of CoronaVac were stimulated with spike peptide pools from WT, Delta, and Omicron as well as a non-spike peptide pool from WT SARS-CoV-2 for 20 h. The expression of AIM on CD4$^+$ (CD137$^+$OX40$^+$) and CD8$^+$ (CD137$^+$CD69$^+$) T cells was measured by flow cytometry. **(A)** Representative flow cytometry plots showed the frequency of AIM$^+$CD4$^+$ and AIM$^+$CD8$^+$ T cells. **(B)** Percentages of AIM$^+$ (CD137$^+$OX40$^+$) cells among CD4$^+$ T cells responding to non-spike and spike peptides were displayed. **(C)** Percentages of AIM$^+$ (CD137$^+$CD69$^+$) cells among CD8$^+$ T cells responding to non-spike and spike peptides were displayed. Each point represents raw data for a single participant. Percentages represent the percentage of T cells after subtracting the background value in a DMSO stimulation. Comparisons were performed *via* Wilcoxon matched-pairs signed rank test between two groups or Friedman test with Dunn's multiple comparisons test among multiple groups. n = 16. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

AIM$^+$CD8$^+$ cells was augmented from 0.144% to 0.435% upon the stimulation with the non-spike peptide pool. The booster dose increased the frequency of AIM$^+$CD8$^+$ cells to 0.398%, 0.267%, and 0.442% against WT, Delta, and Omicron, respectively, which also indicates substantial cross-reactivity of CD8$^+$ T cell responses between WT and variants (Figure 3C).

Subset distribution of non-spike and spike-specific CD4$^+$ and CD8$^+$ T cells after a third dose of CoronaVac

To assess the differentiation status of AIM$^+$ T cells after the booster dose, the percentages of naive (CD45RA$^+$CCR7$^+$), central memory (T_{CM}, CD45RA$^-$CCR7$^+$), effector memory (T_{EM}, CD45RA$^-$CCR7$^-$), and terminally differentiated effector memory (T_{EMRA}, CD45RA$^+$CCR7$^-$) in the bulk and SARS-CoV-2 AIM$^+$ populations of CD4$^+$ and CD8$^+$ T cells were examined *via* flow cytometry (Supplemental Figure 2 for gating strategy). CD4$^+$ and CD8$^+$ T cells were enriched preferentially for the T_{EM} and naïve subsets in the bulk (Figures 4A, B). Both non-spike and spike-specific AIM$^+$CD4$^+$ T cells exhibited a significant increase in the T_{EM} population and a significant decrease in the naïve population compared with bulk CD4$^+$ cells (Figure 4A). Compared with the bulk CD8$^+$ cells, non-spike and spike-specific AIM$^+$CD8$^+$ had reduced expression of T_{EM} and elevated expression of T_{EM} re-expressing CD45RA (T_{EMRA}), the most differentiated subset of human CD8$^+$ T cells (Figure 4B). In contrast to CD8$^+$ T_{EM}, CD8$^+$ T_{EMRA} has elevated expression of perforin and granzyme B and is capable of killing specific target cells without prior activation (14). These results suggest that non-spike and spike-specific AIM$^+$CD4$^+$ and AIM$^+$CD8$^+$ T cells develop phenotypes of activation and functional capacity after the booster dose.

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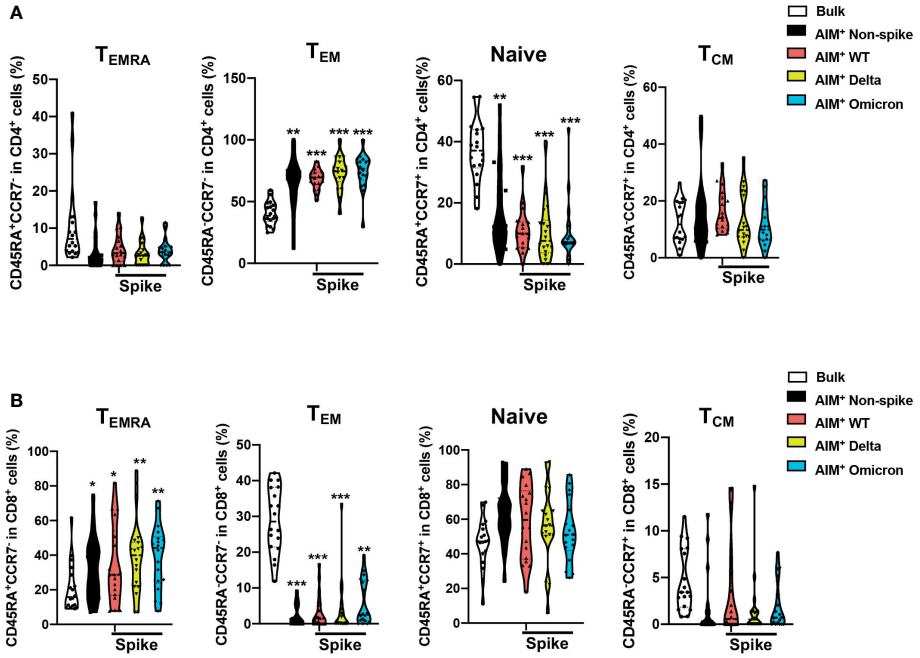

FIGURE 4
Distribution of memory T cell subsets among SARS-CoV-2–specific $CD4^+$ and $CD8^+$ T cells after a third dose of CoronaVac. The percentages of naive ($CD45RA^+CCR7^+$), central memory (T_{CM}, $CD45RA^-CCR7^+$), effector memory (T_{EM}, $CD45RA^-CCR7^-$), and terminally differentiated effector memory (T_{EMRA}, $CD45RA^+CCR7^-$) in the bulk and SARS-CoV-2 AIM^+ populations for $CD4^+$ **(A)** and $CD8^+$ **(B)** T cells were determined by flow cytometry. The percentages of T memory subsets in the AIM^+ populations were compared with those of bulk $CD4^+$ and $CD8^+$ cells. Comparisons were performed *via* RM one-way ANOVA with Holm-Šídák's multiple comparisons test for parametric data or Friedman test with Dunn's multiple comparisons test for nonparametric data. n = 18. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

COVID-19 vaccination induces neutralization antibodies, effector T cell responses, and memory T cell responses for viral antigens. In the present study, we conducted an examination of T cell responses after a homogenous booster (third) dose of an inactivated vaccine, CoronaVac. Our results showed that the booster dose induced cytokine production in $CD4^+$ and $CD8^+$ T cells as well as cytotoxicity of $CD8^+$ T cells after stimulation with non-spike or spike antigens. In addition, non-spike-specific cytokine responses in $CD4^+$ and $CD8^+$ T cells correlated well with the counterparts for spikes from WT, Delta, and Omicron. AIM^+CD4^+ and AIM^+CD8^+ T cell responses were elevated for non-spike and broadly conserved toward WT, Delta, and Omicron. In the meantime, booster vaccination expanded $CD4^+$ T_{EM} as well as $CD8^+$ T_{EMRA} subsets of non-spike and spike-specific T cells.

Several studies have been conducted to study spike-specific T cell response after booster dose of inactivated vaccine. Li et al. reported that the frequency of AIM^+ ($CD69^+$ $CD137^+$) spike-specific total T cells was significantly increased for both WT and Omicron after the third dose of CoronaVac, but the enhancement was diminished in the Omicron. Memory T cell subsets were examined in AIM^+ total T cells ($CD69^+CD137^+CD45RO^+CD45RA^-$), while no difference was observed in intergroup comparisons (15). Schultz et al. documented that a booster dose of CoronaVac elevated the percentage of AIM^+ ($OX40^+$ $CD137^+$) $CD4^+$ T cells against the Delta and Omicron, comparable to the WT strain (16). Chen et al. found that AIM^+CD4^+ and AIM^+CD8^+ T cell recognition against the Delta and Omicron was slightly reduced but largely maintained, when compared with the reference ancestral strain, after a booster dose of CoronaVac (17). Xiao et al. showed that spike-specific $CD8^+$ T cells for recognizing Delta and Omicron variants were lower than that of ancestral strain with booster dose of inactivated vaccine KCONVAC (18). The present study filled a knowledge gap by examining non-spike T cell response and memory cell subsets.

The present study used a peptide pool from WT non-spike proteins to stimulate $CD4^+$ and $CD8^+$ T cell responses. Tarke et al. has demonstrated a broad pattern of immunodominance for SARS-COV-2. Eight to nine proteins including non-structural protein (nsp) 3, nsp4, nsp12, open reading frame 3a (ORF3a), S, M, and N are required to induce 80% of CD4 and CD8 T cell responses (19). They also pointed out that S, N, and M may have immunodominance due to high abundance. The non-spike peptide pool in the present study was derived from 7 proteins (N, M, ORF1, nsp3, ORF-3a, ORF-7a, and ORF8) which has a significant overlap with the reported immunodominant proteins abovementioned. Although the non-spike peptide pool did not pinpoint the individual antigen of immunodominance, it serves to compare non-spike as a whole with spike in the present study. Similar non-spike peptide pool approach has also been reported by other labs (20).

Using intracellular cytokine staining assay, Rosa Duque et al. reported that two doses of BNT162b2 and CoronaVac generated similar spike-specific T cell responses to WT SARS-CoV-2, while non-spike T cell response was only detected in CoronaVac due to

lack of non-spike antigens in BNT162b2 (21). There are several differences between the aforementioned study and the present study. We examined non-spike and spike-specific memory T cell responses after the booster of CoronaVac. The methodologies for our study were more extensive and included ELISpot, intracellular cytokine staining, and AIM assay. In addition, we examined the T cell response not only against WT but also Delta and Omicron.

$CD8^+$ T cells directly kill virus-infected cells and generate proinflammatory cytokines and chemokines, which attract additional immune cells to sites of infection. $CD4^+$ T cells facilitate the expansion and function of both B cells and $CD8^+$ T cells. They also possess antiviral properties similar to $CD8^+$ T cells by producing proinflammatory cytokines and killing *via* direct cytolytic actions (22). Naranbhai et al. showed that T cell immunity to the Omicron variant was preserved in the majority of infected and vaccinated individuals (11). Redd et al. found that there was minimal crossover between mutations in the Omicron variant and viral epitopes recognized by $CD8^+$ T in COVID-19 convalescent patients indicating that cell responses should recognize the Omicron variant (23). In patients with multiple sclerosis receiving ocrelizumab, T cell responses were negligibly affected by the Omicron variant and may prevent the occurrence of severe COVID-19 (24). Gao et al. found that spike-specific $CD4^+$ and $CD8^+$ T cells responses induced by BNT162b2 vaccination or prior SARS-CoV2 infection were largely intact against Omicron B.1.1.529 (25). Keeton et al. discovered that the magnitude of spike-specific $CD4^+$ and $CD8^+$ T cell responses to Omicron B.1.1.529 was mostly sustained and similar to those of Beta and Delta variants in COVID-19 convalescent patients and participants with vaccination of Ad26.CoV2.S or BNT162b2 (26). In participants with Ad26.COV2.S or BNT162b2 vaccination, Liu et al. demonstrated that spike-specific $CD8^+$ and $CD4^+$ T cell responses were long-lasting and extensively cross-reactive for both the Delta and Omicron B.1.1.529 (27). In health care workers with vaccination of ChAdOx-1 S, Ad26.COV2.S, mRNA-1273, or BNT162b2, GeurtsvanKessel et al. revealed that there was no significant difference in spike-specific $CD4^+$ and $CD8^+$ T cell responses between WT and variants including Delta, Beta, and Omicron B.1.1.529 (28). In volunteers with diverse vaccination and SARS-CoV-2 infection backgrounds, De Marco et al. showed that approximately 87% of cellular immunity was maintained for S protein of the Omicron BA.1 (29). The present study also showed that the booster dose elicited cross-reactivity of functional $CD4^+$ and $CD8^+$ responses between WT and variants.

SARS-CoV-2 infection induces a wide spectrum of antigen-specific T cells. Fazolo et al. showed that pediatric COVID-19 patients had higher TNF^+CD8^+ T cell response for the M and N antigens compared with that of spike (30). They also found that N-specific TNF^+CD8^+ T cell response in pediatric COVID-19 patients was sustainable and had T_{EM} and T_{EMRA} phenotypes (31). Kundu et al. demonstrated that SARS-CoV-2 infection was lower in individuals with non-spike memory T cells reactive to ORF1 and N proteins (32). In convalescent COVID-19 patients, Ferretti et al. showed that the majority of epitopes recognized by CD8 T cells were localized in ORF1ab and the N protein (33). In convalescent individual following COVID-19, there was a broad and strong memory T cell response against spike, non-spike structural proteins, and non-structural proteins (8). The present study demonstrated that booster dose of CoronaVac elicited both non-spike and spike-specific $CD4^+$ and $CD8^+$ T cell responses. In addition, the induced spike-specific T cell response was cross-reactive toward WT, Delta, and Omicron. Therefore, second-generation mRNA vaccines targeting non-spike proteins might provide more protection against the Omicron variants.

The phenotypic features of SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cells after administration of inactivated vaccine are poorly understood. The present study demonstrated that the proportion of T_{EM} for both non-spike and spike-specific AIM^+CD4^+ T cells was elevated after the third dose of CoronaVac. In the meantime, the population of T_{EMRA} for AIM^+CD8^+ T cells was increased, regardless of the variant peptide analyzed. This finding is similar to previous reports examining T cell phenotypes after SARS-CoV-2 infection and mRNA vaccination. In studying memory response to SARS-CoV-2 infection, Dan et al. found that the majority of SARS-CoV-2-specific AIM^+CD4^+ T cells was T_{CM} and T_{EM} with small percentage of T_{EMRA}. However, the majority of SARS-CoV-2-specific AIM^+CD8^+ T cells was T_{EMRA} with small populations of T_{CM} and T_{EM} (34). In a study of infected and mRNA-vaccinated individuals, spike-specific AIM^+ T cells, irrespective of the variant peptide analyzed, showed enrichment for T_{CM} and T_{EM} subsets for $CD4^+$ T cells and T_{EMRA} subset for $CD8^+$ T cells (35). Rodda et al. reported that $CD4^+$ T_{EM} cells from individuals recovered from COVID-19 had the ability to proliferate and re-seed the memory pool upon antigen re-exposure, providing protection against re-infection (36). In 2009 H1N1 pandemic, $CD8^+$ T_{EMRA} cells were inversely correlated with symptom score and had cytotoxic potential (37). Our findings indicate that the booster dose of CoronaVac produces non-spike and spike-specific memory T cell responses that recognize variants and provide protection against infection.

Our study has several limitations. First, cellular immune response to non-spike peptide pool for Omicron was not examined in the study due to commercial unavailability. Ahmed et al. reported that 98% of 745 non-spike $CD8^+$ T cell epitopes and 95% of 373 non-spike $CD4^+$ T cell epitopes were unaffected by mutations in Omicron (B.1.1.529) (38). Therefore, we postulate that booster vaccination of inactivated COVID-19 vaccines would elicit similar non-spike-specific $CD4^+$ and $CD8^+$ T cell responses between wild-type and Omicron. Second, the sample size was small with 18 volunteers. A large sample size would provide higher statistical power in deciphering non-spike and spike-specific T cell responses. Third, the time point to assess immune response was conducted at 1-2 weeks after booster dose. Wang et al. demonstrated that non-spike and spike T cell responses as assayed by ELISPOT were readily detectable 6 months after the third dose of inactivated vaccine BBIBP-CorV (Covilo). A homologous booster 6 months after the third dose failed to further enhance T cell responses (39). Melo-González et al. reported that AIM^+CD4^+ T cell response remained stable after the third dose of CoronaVac, but showed a decline 4-6 months later (40). The durability of cellular immune response after booster dose of CoronaVac warrants further investigation.

Furthermore, this study was conducted in healthcare workers which skewed toward a healthy and young population.

Conclusions

The third dose of inactivated COVID-19 vaccine enhances cellular immune response against SARS-CoV-2. The booster dose elicits cytokine and AIM responses in $CD4^+$ and $CD8^+$ T cells towards non-spike and spike peptide antigens from SARS-CoV-2 of WT, Delta, and Omicron. Future COVID-19 vaccine formulations containing non-spike components may offer better coverage against variants.

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 Children's Hospital of Zhejiang University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RH: Conceptualization (equal); Data curation (lead); Formal analysis (equal); Funding acquisition (equal); Methodology (equal); Writing-original draft (lead); and Writing-review and editing (equal). LY: Conceptualization (equal); Data curation (lead); Formal analysis (equal); Methodology (equal); Writing-original draft (lead); and Writing-review and editing (equal). JW, JX, YZ, HM, RYZ, RXZ, and ZL: Data curation (equal); Formal analysis (equal); Methodology (equal); Validation (equal); and Writing-review and editing (equal). QS and JGX: Conceptualization (equal); Formal analysis (equal); Funding acquisition (equal); Supervision (equal); Writing-original draft (equal); and Writing-review and editing (equal). All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1139620/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Gating strategy for determining cytokine-producing cells among $CD4^+$ and $CD8^+$ T cells as well as cytotoxic $CD107^+CD8^+$ T cells.

SUPPLEMENTARY FIGURE 2
Gating strategy for determining AIM^+CD4^+ ($CD137^+OX40^+$) and AIM^+CD8^+ ($CD137^+CD69^+$) memory T cells as well as their subpopulations.

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COVID-19 vaccination status, side effects, and perceptions among breast cancer survivors: a cross-sectional study in China

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Introduction: Breast cancer is the most prevalent malignancy in patients with coronavirus disease 2019 (COVID-19). However, vaccination data of this population are limited.

Methods: A cross-sectional study of COVID-19 vaccination was conducted in China. Multivariate logistic regression models were used to assess factors associated with COVID-19 vaccination status.

Results: Of 2,904 participants, 50.2% were vaccinated with acceptable side effects. Most of the participants received inactivated virus vaccines. The most common reason for vaccination was "fear of infection" (56.2%) and "workplace/government requirement" (33.1%). While the most common reason for nonvaccination was "worry that vaccines cause breast cancer progression or interfere with treatment" (72.9%) and "have concerns about side effects or safety" (39.6%). Patients who were employed (odds ratio, OR=1.783, p=0.015), had stage I disease at diagnosis (OR=2.008, p=0.019), thought vaccines could provide protection (OR=1.774, p=0.007), thought COVID-19 vaccines were safe, very safe, not safe, and very unsafe (OR=2.074, p<0.001; OR=4.251, p<0.001; OR=2.075, p=0.011; OR=5.609, p=0.003, respectively) were more likely to receive vaccination. Patients who were 1–3years, 3–5years, and more than 5years after surgery (OR=0.277, p<0.001; OR=0.277, p<0.001, OR=0.282, p<0.001, respectively), had a history of food or drug allergies (OR=0.579, p=0.001), had recently undergone endocrine therapy (OR=0.531, p<0.001) were less likely to receive vaccination.

Conclusion: COVID-19 vaccination gap exists in breast cancer survivors, which could be filled by raising awareness and increasing confidence in vaccine safety during cancer treatment, particularly for the unemployed individuals.

KEYWORDS

COVID-19 pandemic, vaccine, side effects, breast cancer, SARS-CoV-2

Introduction

Coronavirus disease 2019 (COVID-19) is taking a huge toll on the people and healthcare system of China and the rest of the world (1). As of July 30, 2022, 229,510 confirmed cases and 5,526 deaths were reported in the Chinese mainland (2), and 557,917,904 confirmed cases and 6,358,899 deaths were reported globally (3).

Of specific interest are patients with breast cancer because of high prevalence, high mortality rate, (4–7) and potential immunosenescence to vaccination in this population (8–11). As the most common cancer and the fifth leading cause of cancer mortality worldwide, (12, 13) breast cancer is the most prevalent malignancy in the population diagnosed with COVID-19 (4). During the prevaccination phase from February 27, 2020 to November 30, 2020, the 28-day case fatality rate (CFR_{28}) of COVID-19 was 13.9% among patients with breast cancer (14).

Periodic vaccination is expected to be an effective solution. It was reported that vaccinated patients diagnosed with breast cancer achieved an improved CFR_{28} and reduced COVID-19 severity compared with unvaccinated controls (14). The National Comprehensive Cancer Network (NCCN) recommended patients with breast cancer receive COVID-19 vaccination as soon as possible. Patients with breast cancer under active treatment or not were prioritized for a third dose of mRNA vaccines within 1 year of the initial vaccine administration (15). However, safety reports and acceptance of COVID-19 vaccines in patients with breast cancer were limited, resulting in vaccine hesitancy and policy delay.

In this population-based survey study, we investigated the vaccination status, side effects, and perceptions among breast cancer survivors during the COVID-19 pandemic. To our knowledge, this is the largest cross-sectional study on COVID-19 vaccination in the breast cancer population. The findings of this study would help recognize the current COVID-19 vaccination status in the breast cancer population, and provide evidence for customizing strategies to promote vaccination globally.

Methods

Study population

Data were collected through a nonprobability online survey between May 22 and July 9, 2022.

We recruited patients who were older than 18 years, pathologically diagnosed with breast cancer, and underwent breast surgery at Peking Union Medical College Hospital (PUMCH), Beijing, China between 2010 and 2022. Participants who did not reside in the Chinese mainland (e.g., Hong Kong Special Administrative Region, Macao Special Administrative Region, and Taiwan Province), and those with documented severe cognitive impairment were excluded. Patients were quota-sampled to match the respective population (Chinese breast cancer population) distributions for age (by both incidence and prevalence) and years after surgery. The survey was conducted using a self-administered questionnaire *via* a web-based investigation platform Wenjuanxing.[1] Potential participants can fill in the survey after receiving an invitation to participate *via* the telephone or WeChat (a free social communication application with more than 1.2 billion active users in China). The questionnaire consists of 37 questions on sociodemographic characteristics, health and disease status, COVID-19 pandemic, and vaccination (Supplementary file 1). A pilot study had been conducted before the formal initiation of the study. The questionnaire's content was refined based on feedback from 30 participants, with an average time of 5.8 min taken to complete the questionnaire. The response rate was not available, neither were the characteristics of the nonresponders because of the recruitment methods. Information confidentiality was guaranteed to each participant. Data were accessed and analyzed by members of the research team.

Variables

The survey assessed numerous sociodemographic variables of the participants, including educational attainment, monthly household income, administrative regions, rurality, work status, and having children under 18 years of age. Furthermore, the questionnaire variables related to health and disease status were assessed, including self-perceived health, recent breast cancer-related treatment, time of surgery, history of food or drug allergies, and history of other vaccine allergies. The questionnaire submission time was automatically recorded by the platform, and the time after surgery was obtained by calculating the period between the questionnaire submission time and the time of surgery. Participants were asked to voluntarily give their identification numbers registered at PUMCH to minimize the time required to complete the questionnaire and improve accuracy. Variables, including age, gender, and time of surgery were attained and validated using the identification number by referring to the hospital information system (HIS) of PUMCH. Additionally, the participants' clinical stage at diagnosis, histology, histological grade, and molecular subtype were determined by referring to the participants' pathological reports of surgical specimens from HIS in accordance with the Chinese Society of Clinical Oncology and NCCN guidelines (16, 17). Ki67 values of 20% and more were considered high.

Variables related to the COVID-19 pandemic and vaccination were assessed, including history of COVID-19 infection and vaccination status. Furthermore, participants were asked whether they were worried about COVID-19 infection. They were also asked whether they had a former experience in consulting healthcare workers about COVID-19 vaccination, and, if any, whether the questions were answered. Participants who had not been vaccinated were asked to provide reasons for nonvaccination. Other reasons, apart from the choice options, were allowed. Participants who had been vaccinated were asked about the time, type, and side effects of each dose, as well as the main reason for and the main concern before vaccination. Participants were asked to check their vaccine records before filling in the questionnaire to ensure accuracy of the self-reported information. Additionally, participants were asked whether they believed vaccines could prevent COVID-19 and to what extent did they believe the COVID-19 vaccines are safe. Finally, fully or partially immunized participants were asked whether, if possible, they were willing to receive another dose of COVID-19 vaccine. Participants who answered no were asked to provide reasons.

1 https://www.wjx.cn

Statistical methods

Data cleaning was performed using Microsoft Excel 2016 version 15.27 (Microsoft Corporation, Redmond, WA, USA) and R software version 4.1.1 (R Foundation for Statistical Computing, Vienna, Austria) (18). Descriptive statistics were performed to summarize participants' characteristics using IBM SPSS Statistics version 26 (IBM Corporation, Armonk, NY, USA) (19). Continuous variables were described using median and interquartile range (IQR) after performing the Shapiro–Wilk test, showing skewness distribution, or using mean and standard deviation given symmetric distribution. Variables were compared among different subgroups using a *t*-test, one-way analysis of variance, or Wilcoxon rank-sum test when appropriate. Categorical variables were reported as percentages, and variables were compared among different subgroups using Pearson's chi-squared test. Or Fisher's exact test was performed when one or more of the cell counts in an R × C table was <5.

Univariate and multivariate binary logistic regression analyses were performed to explore potential and independent variables associated with vaccination status using IBM SPSS Statistics. Vaccinated participants ($Y = 1$) were a combination of 1,459 participants, who had been administered with one, two, or three doses of vaccines. While nonvaccinated participants ($Y = 0$) were 1,445 participants. The variables included in logistic regression analyses were chosen based on previous studies and *a priori* discussion by the research team (20–22). For the multivariate logistic regression analyses, forward stepwise (likelihood ratio) selection was used to eliminate variables with a value of $p \geq 0.05$ to arrive at the final model. The goodness of fit for the multivariable logistic model with procession was tested using the Hosmer–Lemeshow test. Missing indicators were used to represent missing values in the model. The odds ratio (OR), 95% confidence intervals (CI), and value of p were calculated. A two-sided value of $p < 0.05$ was considered statistically significant.

Results

Participant characteristics

A total of 2,915 participants completed the questionnaire. Among them, six who did not reside in the Chinese mainland were excluded, together with five duplicates. Therefore, the final analysis included 2,904 participants. All participants were female. Some characteristics, such as regional distributions, differed, whereas age distribution was comparable with the Chinese breast cancer population, and years after surgery were balanced (Supplementary file 2).

Participants' age ranged from 25 to 95 years (median = 51, IQR = 14). More than half of the participants (51.3%) had a bachelor's degree or higher, 61.2% reported a monthly household income of more than 5,000 yuan *per capita*, 37.2% had children under 18 years of age, 43.3% were employed, and 27.1% had lived with breast cancer for more than 5 years. No participants had metastatic disease at diagnosis, 56.2% had invasive ductal carcinoma, and 54.3% had luminal subtypes. Furthermore, 98.2% thought their health status was general or good, and 76.2% recently underwent breast cancer-related treatments, including 28.4% underwent chemotherapy, radiotherapy, or targeted therapy (Table 1).

COVID-19 vaccination status and underlying reasons

Of the 2,904 survey participants, 99.5% had no history of COVID-19 infection, though 70.1% were worried about infection. A total of 15 participants had a history of COVID-19 infection, of them seven participants had not been vaccinated. The COVID-19 vaccination coverage rate was 50.2%. Reasons for nonvaccination are shown in Figure 1. The most common reason was "worry that vaccines cause breast cancer progression or interfere with treatment," accounting for 72.9%, followed by "have concerns about side effects or safety," accounting for 39.6% of nonvaccinated participants. The most common main reason for vaccination was "fear of infection," accounting for 56.2%, followed by "workplace/government requirement," accounting for 33.1% of the vaccinated participants (Figure 2A). Furthermore, for vaccinated participants, "the vaccine could cause breast cancer progression" represented the second leading main concern before vaccination (35.3%), following "other side effects" (54.7%; Figure 2B).

In total, 1.8% (52/2,904) of the participants received one dose, 23.7% (687/2,904) received two doses, and 24.8% (720/2,904) received three doses (Figure 2C). Inactivated virus vaccines, including BBIBP-CorV (Sinopharm's Beijing Institute of Biological Products), CoronaVac (Sinovac Biotech), KCONVAC (Shenzhen Kangtai Biological Products), and WIBP-CorV (Sinopharm's Wuhan Institute of Biological Products), were used in 94.8, 95.3, and 90.4% of the first, second, and third dose of vaccines, respectively. CoronaVac was the most popular type, accounting for more than half of each dose. By contrast, mRNA vaccine (mRNA-1,273 (Moderna-NIAID)), viral vector-based vaccines (Ad26.COV2.S (Janssen), AD5-nCoV (CanSinoBio)), and protein subunit vaccine (ZF2001 (Anhui Zhifei Longcom)) were used on a relatively small scale (Figures 2D–F).

Factors cross-sectionally associated with vaccination status

The survey participants were divided into two groups: the vaccinated group [1,459 cases (50.2%)] and the unvaccinated group [1,445 cases (49.8%)]. Table 1 shows the differences in the basic characteristics between the two groups. In the univariate model (Table 2), the vaccination status was significantly associated with monthly household income, work status, self-perceived health, recent breast cancer-related treatment, time after surgery, history of food or drug allergies, history of vaccine allergies, stage at diagnosis, former experience in consulting healthcare workers, and perceptions of vaccine protection or safety. However, age, educational attainment, administrative regions, rurality, having children under 18 years of age, histology, histological grade, molecular subtype of breast cancer, and history and worries about infection were not significantly associated with the vaccination status.

In the multivariable model (Table 2), the value of p for the Hosmer–Lemeshow test was 0.866, suggesting an acceptable fit. Self-perceived health, monthly household income, history of vaccine allergies, and former experience in consulting healthcare workers turned out to not significantly associate with the vaccination status. Employment was closely associated with vaccination status, compared with unemployment (OR = 1.783, 95%

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TABLE 1 Basic characteristics of breast cancer survivors.

	Total sample (*N*=2,904) *n* (col%)	Vaccinated participants (*n*=1,459) *n* (col%)	Not vaccinated participants (*n*=1,445) *n* (col%)	Value of *p*
Sociodemographic variables				
Age in years				
25–39	312 (10.7)	162 (11.1)	150 (10.4)	0.202
40–49	836 (28.8)	439 (30.1)	397 (27.5)	
50–59	900 (31.0)	450 (30.8)	450 (31.1)	
60–69	457 (15.7)	229 (15.7)	228 (15.8)	
70–79	131 (4.5)	57 (3.9)	74 (5.1)	
80+	36 (1.2)	13 (0.9)	23 (1.6)	
Missing*	232 (8.0)	109 (7.5)	123 (8.5)	
Educational attainment				
Undergraduate	1,162 (40.0)	599 (41.1)	563 (39.0)	0.038
Postgraduate	327 (11.3)	180 (12.3)	147 (10.2)	
High school and below	1,415 (48.7)	680 (46.6)	735 (50.9)	
Monthly household income *per capita*, yuan				
2,000–5,000	947 (32.6)	446 (30.6)	501 (34.7)	0.065
<2000	181 (6.2)	88 (6.0)	93 (6.4)	
5,000-10,000	973 (33.5)	497 (34.1)	476 (32.9)	
>10,000	803 (27.7)	428 (29.3)	375 (26.0)	
Administrative regions				
East	242 (8.3)	122 (8.4)	120 (8.3)	0.448
North	2,246 (77.3)	1,119 (76.7)	1,127 (78.0)	
Northeast	230 (7.9)	123 (8.4)	107 (7.4)	
Central	77 (2.7)	40 (2.7)	37 (2.6)	
South	25 (0.9)	17 (1.2)	8 (0.6)	
Southwest	21 (0.7)	8 (0.5)	13 (0.9)	
Northwest	63 (2.2)	30 (2.1)	33 (2.3)	
Living area				
Urban	2,709 (93.3)	1,360 (93.2)	1,349 (93.4)	0.023
Rural	195 (6.7)	99 (6.8)	96 (6.6)	
Work status				
Unemployed	307 (10.6)	149 (10.2)	158 (10.9)	<0.001
Employed	1,257 (43.3)	707 (48.5)	550 (38.1)	
Retired	1,337 (46.0)	601 (41.2)	736 (50.9)	
Student	3 (0.1)	2 (0.1)	1 (0.1)	
Have children under age 18				
No	1824 (62.8)	891 (61.1)	933 (64.6)	0.051
Yes	1,080 (37.2)	568 (38.9)	512 (35.4)	
Health and disease status				
Self-perceived health				
Bad	53 (1.8)	35 (2.4)	18 (1.2)	0.008
General	702 (24.2)	327 (22.4)	375 (26.0)	
Good	2,149 (74.0)	1,097 (75.2)	1,052 (72.8)	

(Continued)

TABLE 1 (Continued)

	Total sample (*N*=2,904) *n* (col%)	Vaccinated participants (*n*=1,459) *n* (col%)	Not vaccinated participants (*n*=1,445) *n* (col%)	Value of *p*
Recent breast cancer-related treatment				
Cytotoxic therapy**	826 (28.4)	495 (33.9)	331 (22.9)	<0.001
Endocrine therapy	1,298 (44.7)	541 (37.1)	757 (52.4)	
Traditional Chinese medicine	90 (3.1)	38 (2.6)	52 (3.6)	
No treatment	662 (22.8)	372 (25.5)	290 (20.1)	
Missing*	28 (1.0)	13 (0.9)	15 (1.0)	
Time after surgery				
<1 year	585 (20.1)	426 (29.2)	159 (11.0)	<0.001
1–3 years	916 (31.5)	379 (26.0)	537 (37.2)	
3–5 years	583 (20.1)	256 (17.5)	327 (22.6)	
>=5 years	787 (27.1)	375 (25.7)	412 (28.5)	
Missing*	33 (1.1)	23 (1.6)	10 (0.7)	
History of food or drug allergies		23		
No	2,260 (77.8)	1,214 (83.2)	1,046 (72.4)	<0.001
Yes	644 (22.2)	245 (16.8)	399 (27.6)	
History of other vaccine allergies				
No	2,769 (95.4)	1,425 (97.7)	1,344 (93.0)	<0.001
Yes	135 (4.6)	34 (2.3)	101 (7.0)	
Stage at diagnosis				
0	165 (5.7)	74 (5.1)	91 (6.3)	<0.001
I	589 (20.3)	426 (29.2)	163 (11.3)	
II	662 (22.8)	317 (21.7)	345 (23.9)	
III	515 (17.7)	224 (15.4)	291 (20.1)	
IV	0	0	0	
Missing*	973 (33.5)	418 (28.6)	555 (38.4)	
Histology				
Carcinoma *in situ*	246 (8.5)	132 (9.0)	114 (7.9)	0.665
Invasive ductal carcinoma	1,633 (56.2)	830 (56.9)	803 (55.6)	
Invasive lobular carcinoma	74 (2.5)	42 (2.9)	32 (2.2)	
Others	170 (5.9)	88 (6.0)	82 (5.7)	
Missing*	781 (26.9)	367 (25.2)	414 (28.7)	
Histological grade				
G1	213 (7.3)	103 (7.1)	110 (7.6)	0.648
G2	1,041 (35.8)	535 (36.7)	506 (35.0)	
G3	590 (20.3)	307 (21.0)	283 (19.6)	
Missing*	1,060 (36.5)	514 (35.2)	546 (37.8)	
Molecular subtype				
Luminal A	491 (16.9)	254 (17.4)	237 (16.4)	0.685
Luminal B	1,085 (37.4)	528 (36.2)	557 (38.5)	
HER2 over-expression subtype	153 (5.3)	77 (5.3)	76 (5.3)	
Basal-like	183 (6.3)	94 (6.4)	89 (6.2)	

(Continued)

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TABLE 1 (Continued)

	Total sample (*N*=2,904) *n* (col%)	Vaccinated participants (*n*=1,459) *n* (col%)	Not vaccinated participants (*n*=1,445) *n* (col%)	Value of *p*
Missing*	992 (34.2)	506 (34.7)	486 (33.6)	
Variables related to COVID-19				
History of COVID-19 infection				
No	2,889 (99.5)	1,451 (99.5)	1,438 (99.5)	0.987
Yes, no symptoms	2 (0.1)	1 (0.1)	1 (0.1)	
Yes, mild symptoms	6 (0.2)	3 (0.2)	3 (0.2)	
Yes, severe symptoms	7 (0.2)	4 (0.3)	3 (0.2)	
Worried about infection				
No	867 (29.9)	422 (28.9)	445 (30.8)	0.270
Yes	2037 (70.1)	1,037 (71.1)	1,000 (69.2)	
Have you consulted healthcare workers about COVID-19 vaccines?				
No	1,123 (38.7)	571 (39.1)	552 (38.2)	0.046
Yes, my questions were answered.	1,263 (43.5)	653 (44.8)	610 (42.2)	
Yes, my questions were not answered.	518 (17.8)	235 (16.1)	283 (19.6)	
Think vaccines can provide protection				
No	557 (19.2)	231 (15.8)	326 (22.6)	<0.001
Yes	2,347 (80.8)	1,228 (84.2)	1,119 (77.4)	
Perceptions on vaccine safety				
General	1,206 (41.5)	493 (33.8)	713 (49.3)	<0.001
Safe	1,114 (38.4)	605 (41.5)	509 (35.2)	
Very safe	250 (8.6)	168 (11.5)	82 (5.7)	
Not safe	249 (8.6)	127 (8.7)	122 (8.4)	
Very unsafe	85 (2.9)	66 (4.5)	19 (1.3)	

Values in red indicates these are statistically significant.
*Missing values were not included for statistical analysis.
**Chemotherapy/radiotherapy/targeted therapy, with/without endocrine therapy or traditional Chinese medicine.

CI, 1.118–2.842, $p = 0.015$). The vaccination rate decreased for participants who had recently undergone endocrine therapy compared with those receiving cytotoxic therapy (OR = 0.531, 95% CI, 0.376–0.749, $p < 0.001$). Compared with less than 1 year after surgery, 1–3 years, 3–5 years, and more than 5 years significantly decreased the rate of vaccination (OR = 0.277, 95% CI, 0.176–0.436, $p < 0.001$; OR = 0.277, 95% CI, 0.170–0.451, $p < 0.001$, OR = 0.282, 95% CI, 0.179–0.443, $p < 0.001$). Participants with stage I disease at diagnosis were more likely to be vaccinated (OR = 2.008, 95% CI, 1.124–3.590, $p = 0.019$). Additionally, history of food or drug allergies significantly decreased the rate of vaccination (OR = 0.579, 95% CI, 0.417–0.804, $p = 0.001$).

As for perceptions, participants who thought vaccines could provide protection were more likely to be vaccinated than those who did not (OR = 1.774, 95% CI, 1.170–2.690, $p = 0.007$). Finally, participants who thought COVID-19 vaccines were safe (OR = 2.074, 95% CI, 1.513–2.843, $p < 0.001$), very safe (OR = 4.251, 95% CI, 2.452–7.369, $p < 0.001$), not safe (OR = 2.075, 95% CI, 1.185–3.635, $p = 0.011$), and very unsafe (OR = 5.609, 95% CI, 1.807–17.407, $p = 0.003$) showed higher vaccination rates than those who held general ideas (between safe and not safe).

Side effects reported for different types of COVID-19 vaccines

The side effect rates for each vaccine dose are illustrated in Figure 3. Of the 1,459 vaccinated participants, 186 (12.7%) reported side effects after the first dose, including 99 cases (6.8%) of fatigue, 66 cases (4.5%) of muscle pain, and 38 (2.6%) cases of allergic reaction. The most common side effect for the second dose was fatigue, accounting for 10.9% of 1,407 participants, while muscle pain (73/720, 10.1%) was the most common side effect for the third dose. Notably, breast discomfort, described as breast itching, tenderness, swelling, or pain, was reported by 0.3–0.6% of the participants. The side effect rates among different types of COVID-19 vaccines were significantly different for first and the third dose ($p = 0.007$ and 0.019, respectively), whereas no difference was observed for the second dose ($p = 0.169$,

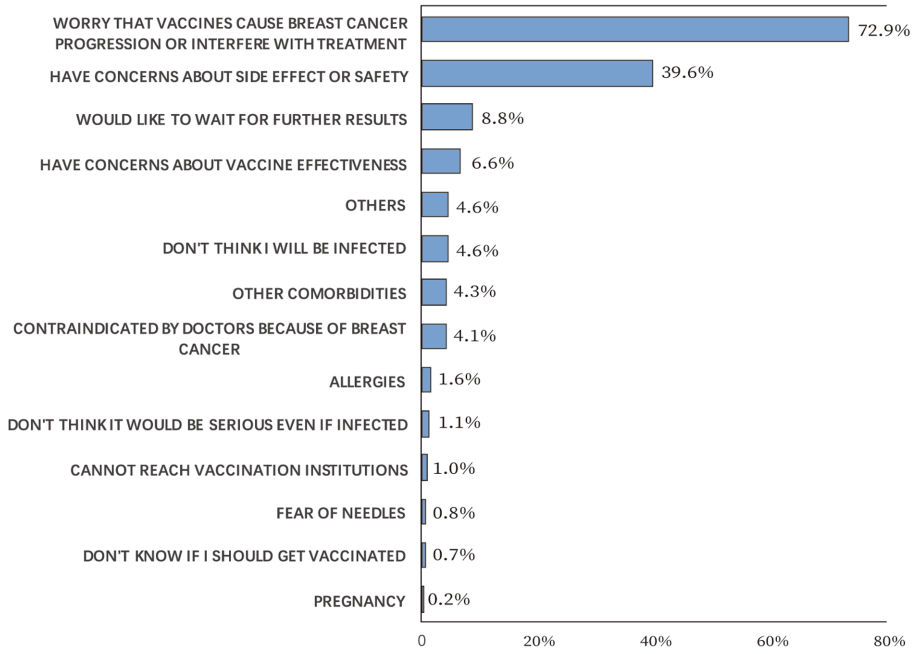

FIGURE 1
Reasons for non-vaccination.

Table 3). Pearson's chi-squared test showed that the side effect rate was significantly increased if any previous COVID-19 vaccine dose led to side effects ($p < 0.05$).

Willingness to receive another dose of COVID-19 vaccine

Participants' willingness to receive another dose of COVID-19 vaccine was explored among the vaccinated cases. Of the 1,459 participants, 639 (43.8%) would accept another vaccine dose. Participants' reasons for not taking another COVID-19 vaccine dose are illustrated in Figure 4. The most common reason was "have concerns about side effects or safety" (74.8%), followed by "the current vaccine is enough to provide protection" (9.3%). Only 4.8% of the vaccinated participants worried that vaccines would cause breast cancer progression or interfere with treatment, and 3.0% of the vaccinated participants thought there was no use to take the next dose. According to Pearson's chi-squared test, participants' willingness to receive another vaccine dose was significantly decreased if they experienced COVID-19 vaccine side effects ($p < 0.05$).

Discussion

In the Chinese population-based survey study, we used a quota-sampled method to recruit a total of 2,904 patients with breast cancer who had undergone breast surgery at PUMCH. COVID-19 vaccination coverage rates, side effects, concerns and perceptions were assessed, along with other relevant variables. People who were administered with the complete protocol, first dose, and booster dose in the Chinese mainland accounted for 89.7, 92.1, and 71.7% of the total population, respectively (23). By contrast, our results revealed relatively lower rates of complete-protocol administration (24.8%) and first-dose administration (50.2%) among breast cancer survivors in the Chinese mainland. The finding underscores the importance of promoting COVID-19 vaccination among patients with breast cancer. More importantly, we sought to find reasons underlying the vaccination rate gap between breast cancer survivors and the general population, and customize strategies to improve the vaccination rate in cancer population.

A major concern for COVID-19 vaccination is safety. Our results indicated that more than half of the vaccinated cases had concerns about side effects, which accounted for nonvaccination in 39.6% of the unvaccinated cases. What's more, 74.8% of the vaccinated patients did not want to receive another dose of COVID-19 vaccine mainly because of safety concerns. This is consistent with results from the general population and other population groups (24–27). According to a survey study in Poland, 49.2% of the participants refused to receive a booster dose because of side effects experienced after previous doses, and 22.4% because of safety uncertainties (28). In addition, a recent study among university students in Egypt revealed that the main reason for vaccine hesitation was being afraid of serious side effects (29).

In reality, the safety profiles of COVID-19 vaccines reported by our study are largely acceptable. The side effect rates are comparable to those of inactivated virus vaccines in the general population (30, 31), and noticeably better than those of mRNA vaccines in cancer patients (9, 32, 33). A cohort study of 160 breast cancer patients in Iran who received BBIBP-CorV showed that the most common local and systemic side-effects were injection site pain and fever, accounting for 22.3 and 24.3% of the patients, respectively (34). While our results showed that the most common local and systemic side-effects were local pain and

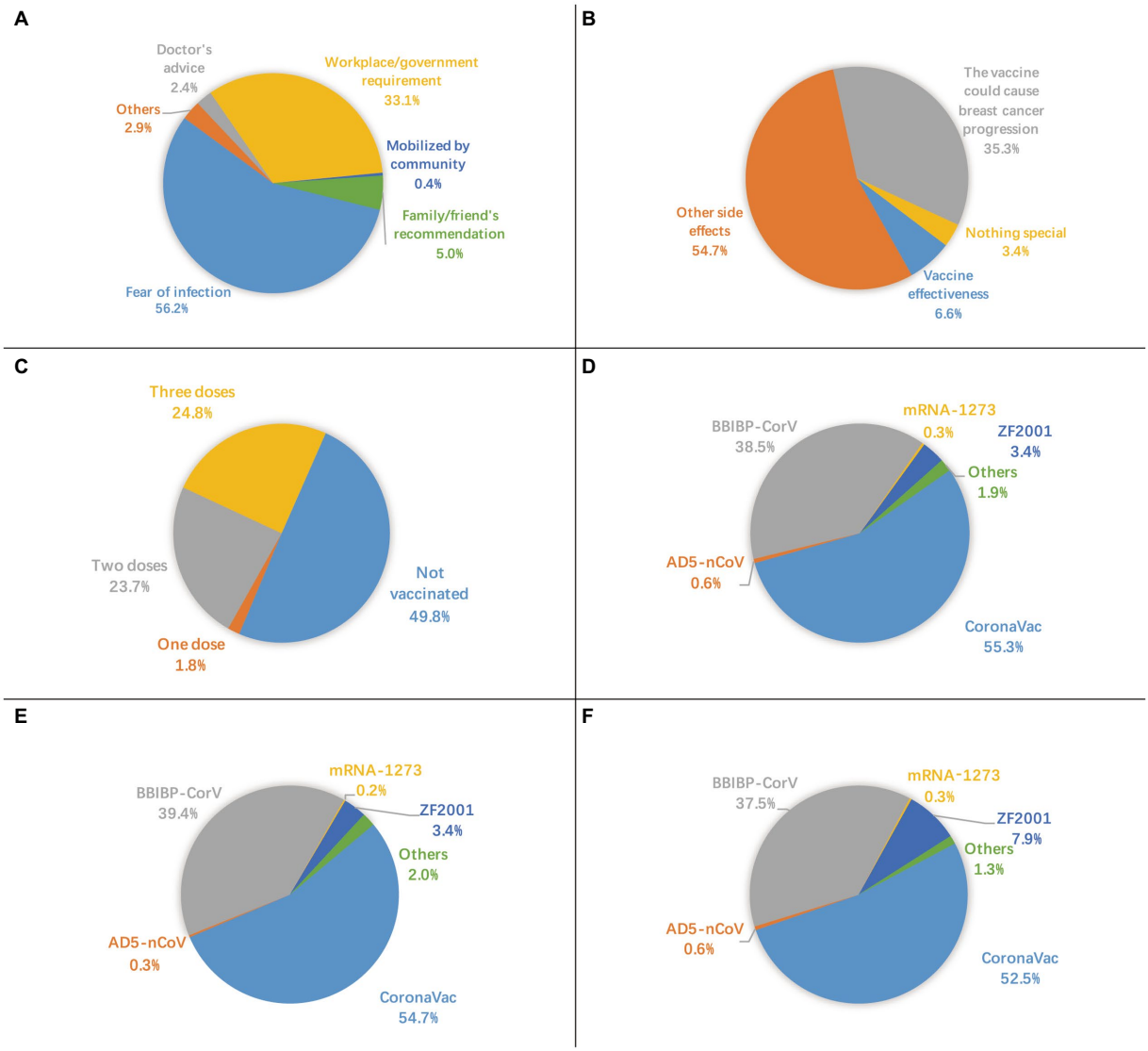

FIGURE 2
(A) main reason for vaccination, **(B)** main concern before vaccination, **C** status of vaccination, **D** type of first vaccine dose, **E** type of second vaccine dose, **F** type of third vaccine dose.

fatigue, accounting for 2.7–4.6% and 6.8–10.9% of the patients, respectively. Because many clinical trials on COVID-19 vaccines excluded patients with malignancies, the report of our findings would help reduce vaccine hesitation.

Meanwhile, disease-related concerns cannot be overestimated in vaccination behaviors. 72.9% of the participants did not receive COVID-19 vaccines because they worried that vaccines would cause breast cancer progression or interfere with treatment, and 35.3% of the vaccinated cases were primarily concerned that vaccines would cause breast cancer progression. Although long-term follow-ups remain unavailable, results from our study indicate low rates (0.3–0.6%) of breast discomfort following vaccination. Besides, axillary lymphadenopathy, which could be a clinical manifestation of ipsilateral breast cancer progression, was more commonly reported in cases who received mRNA vaccines (0.1–16%) (35), and most inactivated virus vaccines did not document axillary lymphadenopathy as a solicited adverse event (36–38).

In our study, recent breast cancer-related treatment, time after surgery, and stage at diagnosis were found to be independently related to vaccination status. We found that patients who recently underwent endocrine therapy were less likely to take COVID-19 vaccines. And patients who were less than 1 year after surgery or at stage I were more likely to receive vaccination, probably because there was no ongoing adjuvant treatment. Some participants reported that doctors asked them to wait for 6 months to 3 years after systematic therapies before vaccines. As far as we know, this criterion was extensively used in China in 2020 and early 2021, when COVID-19 vaccines initially came to market with limited safety results in cancer population (30, 36). In late 2021, the vaccination criterion became obscure following more experience gained in breast cancer patients (39). However, it is of note that the inconsistency of contraindications would cause confusion and vaccine hesitancy, and 8.8% of the participants were not vaccinated because they would like to wait for further results. Because

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TABLE 2 Univariate and multivariate logistic regression of characteristics for association with vaccination status.

	Univariate logistic regression analysis	95% CI for OR			Multivariate logistic regression analysis***	95% CI for OR		
	OR	Lower	Upper	Value of *p*	OR	Lower	Upper	Value of *p*
Sociodemographic variables								
Age in years								
25–39	Ref.							
40–49	1.024	0.789	1.328	0.859				
50–59	0.926	0.715	1.198	0.558				
60–69	0.930	0.697	1.240	0.621				
70–79	0.713	0.472	1.074	0.107				
80+	0.523	0.249	1.056	0.076				
Missing*								
Educational attainment								
Undergraduate	Ref.							
Postgraduate	1.151	0.900	1.474	0.264				
High school and below	0.870	0.744	1.016	0.078				
Monthly household income *per capita*, yuan								
2,000–5,000	Ref.							
<2000	1.063	0.773	1.461	0.707				
5,000-10,000	1.173	0.981	1.403	0.081				
>10,000	1.282	1.062	1.548	0.010				
Administrative regions								
East	Ref.							
North	0.977	0.749	1.274	0.861				
Northeast	1.131	0.788	1.624	0.505				
Central	1.063	0.636	1.780	0.815				
South	2.090	0.894	5.291	0.100				
Southwest	0.605	0.232	1.489	0.283				
Northwest	0.894	0.512	1.558	0.693				
Living area								
Urban	Ref.							
Rural	1.023	0.765	1.369	0.879				
Work status								
Unemployed	Ref.				Ref.			
Employed	1.363	1.062	1.751	0.015	1.783	1.118	2.842	0.015
Retired	0.866	0.675	1.110	0.256	1.049	0.661	1.666	0.839
Student	2.121	0.201	45.916	0.541	390185542.547	0.000	.	1.000
Have children under age 18								
No	Ref.							
Yes	0.861	0.740	1.001	0.051				
Health and disease status								
Self-perceived health								
Bad	Ref.							

(Continued)

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TABLE 2 (Continued)

	Univariate logistic regression analysis	95% CI for OR			Multivariate logistic regression analysis***	95% CI for OR		
	OR	Lower	Upper	Value of *p*	OR	Lower	Upper	Value of *p*
General	0.448	0.244	0.797	0.007				
Good	0.536	0.296	0.940	0.034				
Recent breast cancer-related treatment								
Cytotoxic therapy**	Ref.				Ref.			
Endocrine therapy	0.478	0.400	0.570	<0.001	0.531	0.376	0.749	<0.001
Traditional Chinese medicine	0.489	0.313	0.757	0.001	0.932	0.389	2.233	0.875
No treatment	0.858	0.697	1.055	0.147	1.124	0.745	1.693	0.578
Missing*								
Time after surgery								
<1 year	Ref.				Ref.			
1–3 years	0.263	0.210	0.329	<0.001	0.277	0.176	0.436	<0.001
3–5 years	0.292	0.228	0.373	<0.001	0.277	0.170	0.451	<0.001
> = 5 years	0.340	0.270	0.427	<0.001	0.282	0.179	0.443	<0.001
Missing*								
History of food or drug allergies								
No	Ref.				Ref.			
Yes	0.529	0.442	0.632	<0.001	0.579	0.417	0.804	0.001
History of other vaccine allergies								
No	Ref.							
Yes	0.317	0.211	0.467	<0.001				
Stage at diagnosis								
0	Ref.				Ref.			
I	3.214	2.255	4.598	<0.001	2.008	1.124	3.590	0.019
II	1.130	0.803	1.594	0.485	1.062	0.637	1.772	0.817
III	0.947	0.666	1.349	0.760	0.801	0.472	1.360	0.411
IV								
Missing*								
Histology								
Carcinoma *in situ*	Ref.							
Invasive ductal carcinoma	0.893	0.682	1.167	0.408				
Invasive lobular carcinoma	1.134	0.673	1.923	0.639				
Others	0.927	0.626	1.371	0.704				
Missing*								
Histological grade								
G1	Ref.							
G2	1.129	0.841	1.518	0.420				
G3	1.159	0.847	1.586	0.358				
Missing*								

(Continued)

TABLE 2 (Continued)

	Univariate logistic regression analysis	95% CI for OR			Multivariate logistic regression analysis***	95% CI for OR		
	OR	Lower	Upper	Value of *p*	OR	Lower	Upper	Value of *p*
Molecular subtype								
Luminal A	Ref.							
Luminal B	0.884	0.714	1.095	0.259				
HER2 over-expression subtype	0.945	0.657	1.360	0.762				
Basal-like	0.985	0.702	1.385	0.933				
Missing*								
Variables related to COVID-19								
History of COVID-19 infection								
No	Ref.							
Yes, no symptoms	0.991	0.039	25.078	0.995				
Yes, mild symptoms	0.991	0.183	5.363	0.991				
Yes, severe symptoms	1.321	0.291	6.719	0.716				
Worried about infection								
No	Ref.							
Yes	1.094	0.933	1.282	0.270				
Have you consulted healthcare workers about COVID-19 vaccines?								
No	Ref.							
Yes, my questions were answered.	1.035	0.881	1.215	0.676				
Yes, my questions were not answered.	0.803	0.651	0.989	0.039				
Think vaccines can provide protection								
No	Ref.				Ref.			
Yes	1.549	1.285	1.867	<0.001	1.774	1.170	2.690	0.007
Perceptions on vaccine safety								
General	Ref.				Ref.			
Safe	1.719	1.459	2.027	<0.001	2.074	1.513	2.843	<0.001
Very safe	2.963	2.229	3.967	<0.001	4.251	2.452	7.369	<0.001
Not safe	1.506	1.145	1.980	0.003	2.075	1.185	3.635	0.011
Very unsafe	5.024	3.038	8.699	<0.001	5.609	1.807	17.407	0.003

Values in red indicates these are statistically significant
OR, odds ratio; CI, confidence interval.
*Missing values were not included for statistical analysis.
**Chemotherapy/radiotherapy/targeted therapy, with/without endocrine therapy or traditional Chinese medicine.
***Intercept = 0.15 (p = 0.722); Cox & Snell R Square = 0.192; Nagelkerke R Square = 0.256.

fragmented reports and biased information could foster vaccine hesitancy (40), it is imperative for the government and health institutions to launch educational campaigns to provide breast cancer survivors with adequate information on the precautions, indications, contraindications, and potential side effects of COVID-19 vaccines.

Efficacy (protection) is a driving force for vaccination. Compared with the unvaccinated group, a significantly larger proportion of the vaccinated group thought vaccines could provide protection (77.4 vs. 84.2%). The rates are in parallel with those of the general population (29, 41). Over half of the participants got vaccinated because of "fear of infection," and nearly 10% of the vaccinated participants did not

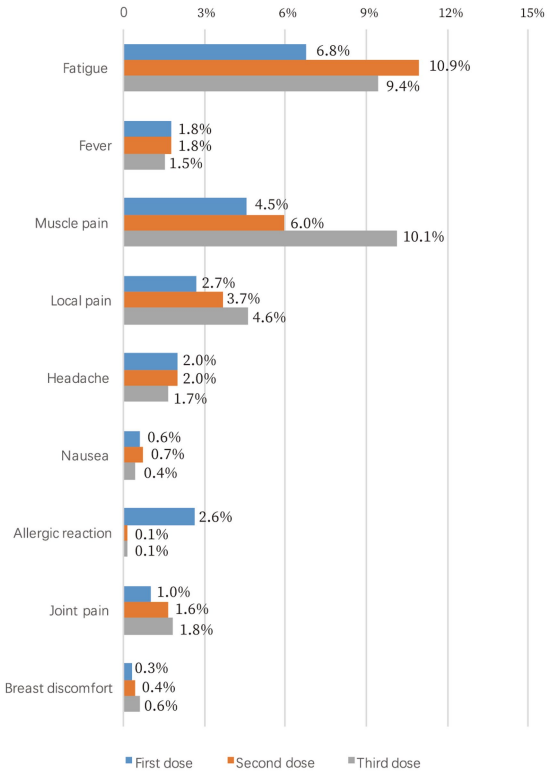

FIGURE 3
Side effects of COVID-19 vaccines.

TABLE 3 COVID-19 vaccine type and side effect rate.

	Total sample *N* (col%)	Sample with side effect *n* (col%)	*Value of p***
Type of the first dose	**1,459**	**186 (12.7)**	
BBIBP-CorV	561 (38.5)	78 (13.9)	0.007
CoronaVac	807 (55.3)	99 (12.3)	
WIBP-CorV	13 (0.9)	1 (7.7)	
AD5-nCoV	9 (0.6)	5 (55.6)	
ZF2001	50 (3.4)	1 (2.0)	
KCONVAC	2 (0.1)	0 (0.0)	
mRNA-1,273	5 (0.3)	0 (0.0)	
Ad26.COV2.S	1 (0.1)	0 (0.0)	
Sorry, I do not remember*	11 (0.8)	2 (18.2)	
Type of the second dose	**1,407**	**207 (14.7)**	
BBIBP-CorV	554 (39.4)	77 (13.9)	0.169
CoronaVac	770 (54.7)	121 (15.7)	
WIBP-CorV	14 (1.0)	4 (28.6)	
AD5-nCoV	4 (0.3)	1 (25.0)	
ZF2001	48 (3.4)	3 (6.3)	
KCONVAC	3 (0.2)	0 (0.0)	
mRNA-1,273	3 (0.2)	1 (33.3)	
Sorry, I do not remember*	11 (0.8)	0 (0.0)	
Type of the third dise	**720**	**101 (14.0)**	
BBIBP-CorV	270 (37.5)	38 (14.1)	0.019
CoronaVac	378 (52.5)	50 (13.2)	
WIBP-CorV	2 (0.3)	1 (50.0)	
AD5-nCoV	4 (0.6)	3 (75.0)	
ZF2001	57 (7.9)	8 (14.0)	
KCONVAC	1 (0.1)	0 (0.0)	
mRNA-1,273	2 (0.3)	0 (0.0)	
IMBCAMS	1 (0.1)	1 (100.0)	
Sorry, I do not remember*	6 (0.8)	0 (0.0)	

*Not included for statistical analysis.
**Results from Fisher's exact test.
Bold values are the sums for each dose

want to receive the next dose because they believed the current vaccine was enough to provide protection. However, unlike healthy individuals, the low seropositive rate of vaccine-induced antibodies in patients with malignancies indicates a lack of virus-neutralizing activity and justifies the use of booster doses (10, 42, 43). A better understanding of their vulnerability to COVID-19 and potential immunosenescence to vaccination would help facilitate periodic vaccination in patients with breast cancer. To evaluate the efficacy of COVID-19 boosters in patients with breast cancer, our research team is currently investigating the immunogenicity and immune response following COVID-19 vaccines in breast cancer cohorts.

To accelerate COVID-19 vaccination and tackle healthcare inequities, the Chinese government has implemented a series of robust measures. Resources from around the nation were galvanized for vaccine development and adequate domestic production capacity (44). As of July 30, 2022, more than 3.4 billion doses of COVID-19 vaccines had been administered in China (45). Till now, seven types of domestically developed vaccines have been offered free of charge to the public, including five inactivated virus vaccines (IMBCAMS, KCONVAC, BBIBP-CorV, CoronaVac, WIBP-CorV), one protein subunit vaccine (ZF2001), and one adenovirus vaccine (AD5-nCoV) (46). Results from our study show that inactivated virus vaccines led the Chinese COVID-19 vaccine market in patients with breast cancer. Additionally, the local governments have undertaken plenty of measures to stimulate vaccination, including setting up temporary inoculation points and extending the service hours of inoculation sites. Vaccines were offered door-to-door for certain works and for those with poor spatial accessibility or mobility. The study shows that administrative regions, household income, and having children under 18 years of age were comparable between the vaccinated and unvaccinated groups. Only 1.0% of the participants did not receive vaccination because of difficulties in reaching vaccination institutions. Of note, work status was significantly associated with vaccination status in the univariate and multivariate analyses. In fact,

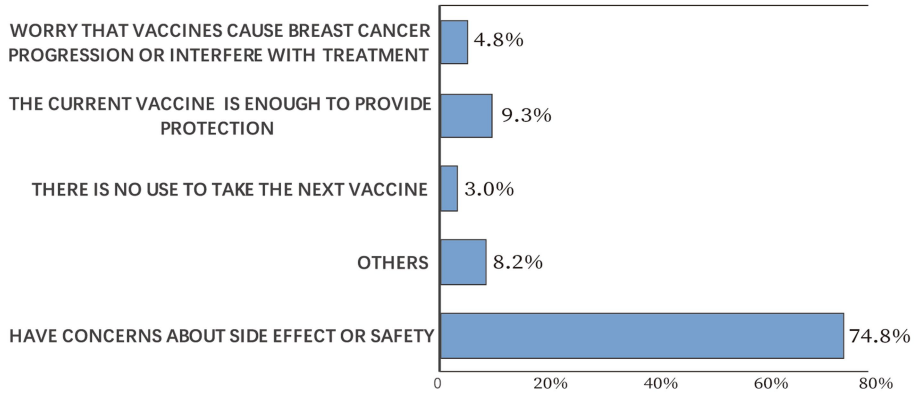

FIGURE 4
Participants' reasons for not taking another COVID-19 vaccine dose.

approximately one-third of the participants reported receiving vaccination mainly because of workplace or government requirement. In this context, future vaccination promotion should particularly target at the unemployed.

This study has strengths and limitations. The cross-sectional survey design enabled a swift collection of valuable, real-world data on the ever-evolving COVID-19 pandemic. Our strengths are the large sample size and representativeness of the sample. Importantly, the quota-sampled approach achieved expected distributions with respect to age and years after surgery. However, because of the single-center design, the study failed to achieve equalized distributions of certain sociodemographic variables, such as educational attainment, administrative regions, and living area, even though these variables were not associated with vaccination status according to the results from univariate and multivariate analyses. Moreover, this study managed to assess valuable pathological records and clinical stage in around 60–80% of the participants. Also, the questionnaire was piloted, enabling its capacity to cover appropriate questions. For example, breast discomfort was not *a priori* defined as one of the multiple choices of side effects, but it was decided to be an independent choice after discussion by specialists accessing the pilot results. Consequently, the survey could, to a large extent, avoid misleading and underreporting. We provided valuable records of the side effects of COVID-19 vaccines. However, we did not collect data on the time and severity of side effects. These and other unmeasured variables (e.g., chronic disease history) could cause residual confounding or bias, which might have skewed our results. Finally, though we applied multiple methods to avoid inaccuracy of self-reported information (e.g., information attainment and validation with HIS, asking participants to check their vaccine records), the use of an online questionnaire might have an influence on information validity.

In conclusion, this study suggests an overall need for vaccination promotion among Chinese breast cancer patients. Vaccination could be promoted by stressing the importance of periodic vaccination in cancer patients, and increasing confidence in vaccine safety during breast cancer treatment. Efforts should be particularly focused on the unemployed individuals.

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 Peking Union Medical College Hospital. The informed consent of the survey was implied by completing the online survey.

Author contributions

QS and YoL: conceptualization and supervision. LL and YX: investigation and data cleaning. LL: methodology, visualization, and writing. LL, YX, XL, HL, YS, YLiu, CC, HZ, ZW, XF, ML, YW, GL, YuL, and YQ: data collection. QS and YoL: funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1119163/full#supplementary-material

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The role of booster vaccination in decreasing COVID-19 age-adjusted case fatality rate: Evidence from 32 countries

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Background: The global COVID-19 pandemic is still ongoing, and cross-country and cross-period variation in COVID-19 age-adjusted case fatality rates (CFRs) has not been clarified. Here, we aimed to identify the country-specific effects of booster vaccination and other features that may affect heterogeneity in age-adjusted CFRs with a worldwide scope, and to predict the benefit of increasing booster vaccination rate on future CFR.

Method: Cross-temporal and cross-country variations in CFR were identified in 32 countries using the latest available database, with multi-feature (vaccination coverage, demographic characteristics, disease burden, behavioral risks, environmental risks, health services and trust) using Extreme Gradient Boosting (XGBoost) algorithm and SHapley Additive exPlanations (SHAP). After that, country-specific risk features that affect age-adjusted CFRs were identified. The benefit of booster on age-adjusted CFR was simulated by increasing booster vaccination by 1–30% in each country.

Results: Overall COVID-19 age-adjusted CFRs across 32 countries ranged from 110 deaths per 100,000 cases to 5,112 deaths per 100,000 cases from February 4, 2020 to Jan 31, 2022, which were divided into countries with age-adjusted CFRs higher than the crude CFRs and countries with age-adjusted CFRs lower than the crude CFRs (n=9 and n=23) when compared with the crude CFR. The effect of booster vaccination on age-adjusted CFRs becomes more important from Alpha to Omicron period (importance scores: 0.03–0.23). The Omicron period model showed that the key risk factors for countries with higher age-adjusted CFR than crude CFR are low GDP *per capita* and low booster vaccination rates, while the key risk factors for countries with higher age-adjusted CFR than crude CFR were high dietary risks and low physical activity. Increasing booster vaccination rates by 7% would reduce CFRs in all countries with age-adjusted CFRs higher than the crude CFRs.

Conclusion: Booster vaccination still plays an important role in reducing age-adjusted CFRs, while there are multidimensional concurrent risk factors and precise joint intervention strategies and preparations based on country-specific risks are also essential.

KEYWORDS

COVID-19, vaccination, age-adjusted case fatality rate, XGBoost (Extreme Gradient Boosting), SHapley Additive exPlanations

Introduction

The COVID-19 pandemic has triggered a public health and economic crisis the like of which has not been seen for generations (1, 2). With the gradual reduction of COVID-19 restriction policies, the long-term epidemiological trend of COVID-19 is unpredictable. The risk of death from COVID-19 varies between countries, and case fatality rate (CFR) is an important indicator used to assess it. It is widely considered that the COVID-19 CFRs are affected by multidimensional factors, such as the SARS-CoV-2 variant infected (3), vaccination coverage (4), population age structure (5, 6), health service (7), disease burden (8, 9), environment (10), and so on (11). Of these, as the COVID-19 CFR is strongly associated with age, considering age structure when comparing CFR differences across countries is more helpful in minimizing potential bias (6). To best of our knowledge, studies have been conducted using age-adjusted CFRs for comparison across at most seven countries to illustrate the significant effect of confounding by the age distribution of the cases when using crude CFRs for country comparisons, however, cross-country and cross-period differences in risk factors for age-adjusted CFR have not been identified (12, 13). Therefore, it is essential to adjust COVID-19 CFRs according to patient age structure in the widest possible number of countries and to compare and clarify possible risk factors for age-adjusted CFRs with a global perspective, which could provide an updated and practical reference for future pandemic control.

Since December 2021, the global COVID-19 vaccination program has been in place and, due to declining antibody levels, booster doses of COVID-19 vaccine have subsequently been offered to eligible individuals (14). However, the limited and unbalanced medical resources result in the global inequity of both vaccination rate and further recovery rate. As of January 2023, more than 60% of the population worldwide has received at least one dose of COVID-19 vaccine, while in low-income countries, only 26% have received at least one dose (15), which possibly leads to an unbalanced protection ability and heavy burden in the overall health systems. Furthermore, the dominant strains in each period of the COVID-19 pandemic have different characteristics. For example, relative to the original variant, the Alpha strain has around 43–90% greater transmissibility along with a 42–82% higher risk of death (16), and the Delta strain has a transmission rate still faster (17), with concomitant greater risk of hospitalization and death (18, 19). The emergence of the Omicron variant brought the COVID-19 epidemic into a new pattern (20). Omicron's immune escape properties make it more contagious than previous strains, but it appears to also be milder, usually causing less severe disease (21). Thus, analysis of risk factors for COVID-19 CFR based on international inequalities in boosters and the complexity of SARS-CoV-2 variants has greater research implications.

The complex factors involved in real-world health emergencies are more effectively analyzed with fast-evolving machine learning algorithms, such as Extreme Gradient Boosting (XGBoost) algorithm. XGBoost is a decision tree-based gradient boosting ensemble machine learning algorithm with improved performance based on other tree-based models such as Random Forest and Gradient Boosting Decision Tree (GBDT), which is well suited for solving classification and regression problems (22). It features several advantages that allow it to be effectively adapted to real-world studies: (1) the objective function can be customized and we choose the most appropriate loss function based on the distribution of the outcome variables; (2) it handles missing data by assigning it to a default direction and finding the best imputation value, which means it is more suitable for dealing with real-world data with limited matching; (3) it penalizes more complex models by LASSO and Ridge regularization to improve the generalization of the model; (4) it can detect and learn from non-linear data patterns, making it easier to identify the non-linear effects of features (23). SHapley Additive exPlanations (SHAP) is a well-established method for interpreting machine learning models (24). On the one hand, SHAP values can clarify the importance of each feature in the model, and on the other hand, SHAP values can break down a prediction to show how each feature affects the prediction. XGboost algorithm with SHAP explanation allows us to identify what factors are driving each country's risk and enabled countries to directly address those risk factors with targeted interventions (25).

Here, we analyze COVID-19 age-adjusted CFRs across countries using the latest available database, as well as crude CFRs. The main aim is to identify the effects of vaccination coverage (e.g., booster vaccination) and other features in six dimensions that may affect heterogeneity in age-adjusted CFRs, including demographic characteristics, disease burden, behavioral risk factors, environmental risk factors, health services and trust levels, using machine learning approaches. Then, to further identify country-specific risk features that affect age-adjusted CFRs. Finally, we predicted the reduction in CFR by country with increased vaccination rates to assess the future health benefits of vaccination in each country.

Materials and methods

Data collection

COVID-19 crude CFR and age-adjusted CFR

Global daily confirmed infections and deaths of COVID-19 by age over the period of 4 February 2020 to 2 February 2022 (the latest database update time) were extracted from the COVerAGE-DB database, which contains the widest range of COVID-19 case and death data by age group for countries worldwide (26). The COVerAGE-DB database contains data for 108 countries, and after we filtered the countries for which both case and death data are available and the countries for which time series containing four time periods are available, there are 32 countries throughout the original, Alpha, Delta and Omicron periods, including Argentina, Australia, Austria, Belgium, Bulgaria, Chile, Colombia, Czechia, Denmark, Finland, France, Germany, Greece, Indonesia, Isreal, Italy, Jamaica, Japan, New Zealand, Nigeria, Peru, Philippines, Portugal, Slovakia, Slovenia, Somalia, South Korea, Spain, Sweden, Switzerland, Togo, and United State America.

Weekly crude CFRs were calculated from the number of new deaths and new cases per week. Weekly age adjusted CFRs were calculated by the direct method (13). The population structure for each country was calculated using World Bank population data for 2020, and the WHO World Standard Population was used as the standard population structure (27). In addition, before building the models, the outliers in CFRs were removed based on the interquartile range.

Vaccination data

Daily vaccination data from January 28, 2020 to January 31, 2022 in 32 countries were extracted from Our World in Data (OWID) and pre-processed by linear interpolation (28). The OWID COVID-19 vaccination dataset is the largest publicly aggregated global dataset on administered vaccinations by country and up-to-date in real time. The advantage of this database is that it differentiates the number of vaccine shots, including share of the population completed the initial vaccination protocol (2 doses for most vaccines, 1 or 3 for a few manufacturers) and share of the population receiving booster doses (doses administered beyond those prescribed by the original vaccination protocol - for example, a third dose of Pfizer/BioNTech vaccine, or a second dose of Johnson & Johnson vaccine) (28). Furthermore, considering that the protection offered by the COVID-19 vaccine drops sharply after 6 months (29–31), we calculated four categories of vaccination status: (1) the proportion of the population having completed the initial vaccination protocol within 6 months (fully vaccinated), (2) the proportion of the population having received a booster within 6 months (booster given), (3) the cumulative proportion of the population having completed the initial vaccination protocol, (4) the cumulative proportion of the population having received a booster.

SARS-CoV-2 lineage data

SARS-CoV-2 lineage data were obtained from the China National Center for Bioinformation (CNCB), which integrated global SARS-CoV-2 sequences from the Global Initiative on Sharing All Influenza Data (GISAID), NCBI GenBank, National Genomics Data Center (NGDC), National Microbiology Data Center (NMDC), and China National GeneBank (CNGB) and identified variants among those sequences (32). We identified those variant types that accounted for >70% of all detected sequences on a global scale for each day in the study period, and considered variants meeting that criterion as having been world-dominating. We defined periods of main VOCs dominance. The starting time of a VOC is based on the World Health Organisation's definition of the start time of each VOC (33). The ending time was set as the next VOC occurring in no more than 10% of the countries. The COVID-19 pandemic was thus divided into four periods: the ancestral variant dominance period (original period, 28 January to 17 December 2020), Alpha variant dominance period (Alpha period, 18 December 2020 to 6 April 2021), Delta variant dominance period (Delta period, 11 May to 21 November 2021), and Omicron variant dominance period (Omicron period, 26 November 2021 to 31 January 2022; Figure 1).

Multi-dimensional explanatory variables

We included six dimensions of features including demographic characteristics, disease burden, behavioral risk factors, environmental risk factors, level of national health services and level of trust to comprehensively assess risk factors for COVID-19 age-adjusted CFR. Demographic characteristics include gender ratio (34), average years of schooling (35), and GDP *per capita* (36). Disease burden include the top three causes of death globally: cardiovascular diseases (CVD), stroke, and chronic obstructive pulmonary disease (COPD); comorbidity which are known to affect the outcome of COVID-19: cancers, diabetes, chronic kidney disease (CKD), and hypertension; upper and lower respiratory infections (URI and LRI), and tuberculosis (TB), which affect lung function; as well as mental disorders, acquired immunodeficiency syndrome (37), and the overall prevalence of noncommunicable diseases (NCD) (38). Behavioral risk factors include overweight (39), low physical activity, smoking, and dietary risks (37). Environmental risk factors include PM2.5 pollution (37), tree density (40), average temperature (41), and population density (42). The level of health services is indicated by the healthcare access and quality (HAQ) index (43), International Health Regulations (IHR) core capacity scores (44), health expenditure (45), number of hospitals (46), and hospital beds *per capita* (47). The trust indices include the level of people' trust in the national government, media, and science during the pandemic (48). Data sources and detailed descriptions are detailed in Supplementary Table 1.

XGBoost model

Model building

We used XGBoost to capture the non-linear associations between COVID-19 age-adjusted CFRs and vaccination rates as well as multiple dimensional features to build explanatory and predictive models. XGBoost is a decision-tree-based ensemble machine learning algorithm that uses a gradient boosting framework (23). It produces a robust, more accurate prediction model in the form of an ensemble of weak prediction models and introduces a penalty term for model complexity to provide better performance. The objective function of the XGBoost algorithm is as follows:

$$Obj(\theta) = L(\theta) + \Omega(\theta) = \sum_i L\left(\hat{y}_i, y_i\right) + \sum_k \Omega\left(f_k\right), f_k \in F$$

Where L is the training loss function. $L\left(\hat{y}_i, y_i\right)$ corresponds to the training loss function for each sample, where y_i denotes the true value of the i sample and $\hat{y}_i$ denotes the estimated value of the i sample. Ω is regularization function that measures the complexity of the model, where k is the number of trees, F is the set of all possible regression trees.

Feature selection

We used a recursive feature elimination (RFE) algorithm to filter main features with the aim of retaining as few features as possible while still capturing the variation in age-adjusted CFRs (49). The RFE strategy uses all features to train a supervised model, then evaluates the features according to their importance in the model. In each iteration, only one feature with minimal model importance is eliminated, and the model fit in each iteration is compared by RMSE; ultimately, features from the better-fitting model are selected.

Hyperparameter tuning

The best combination of hyperparameter values was selected using a fivefold cross-validation grid search. The tuned parameters consisted of learning rate (from 0.05 to 0.2 with an interval of 0.05) and the maximum depth of the tree (from 4 to 10 with an interval of 1). The objective function was set as "reg:tweedie," as our dependent variable of interest was zero-inflated right-skewed data. The training

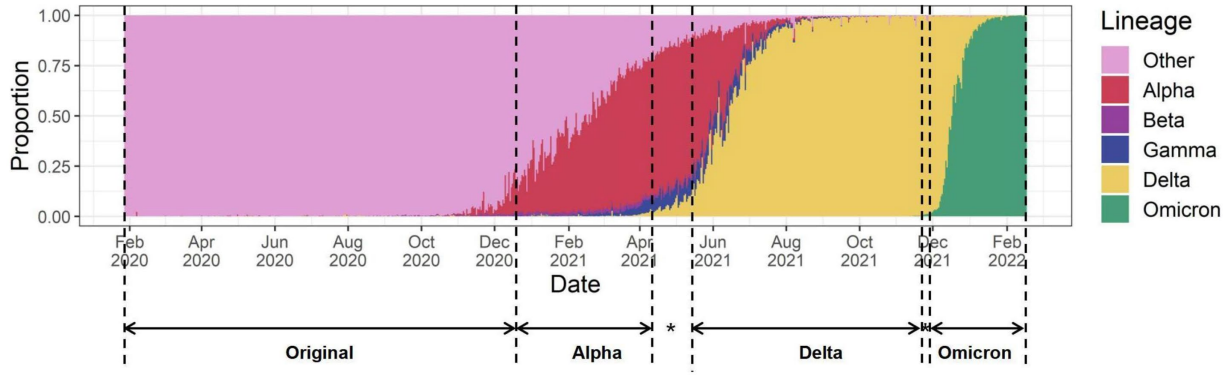

FIGURE 1
The four periods of the pandemic defined by the dominance of the different VOC strains. * A period of multiple variants mixing, with the next VOC already occurring in more than 10% of countries.

process was stopped when the performance of the validation dataset did not improve after further training iterations. The dataset was split into three parts: 60% for training, 20% for validation, and 20% for testing. The accuracy of the model was evaluated in terms of R^2 and RMSE.

Model interpretation

We adopted the SHAP framework to rank features according to their importance and explain how booster vaccination and other key features affect the age-adjusted CFR. SHAP is a game theoretic approach that can explain the output of the XGBoost model. It connects the optimal credit allocation with a local explanation using the classical Shapley values from game theory and their associated extensions (24). The variability of the predictions is assigned to the available features, allowing evaluation of the contribution of each feature to each prediction point. SHAP provides valuable insights into a model's behavior by overcoming the main drawback of inconsistency in classical global feature importance measures, minimizes the possibility of underestimating the importance of a feature with a certain attribution value, shows consistency and accuracy in its importance ordering, and interpreting the model's global behavior while retaining local faithfulness. The overall importance of a feature was scored as the mean absolute value of all SHAP values for that feature, and we considered features scoring 0.1 or higher as important. The relationship between age-adjusted CFR and each key feature was examined *via* partial dependence plots, with adjustment for all other confoundings.

Prediction

We predicted the change in CFR for scenarios when booster vaccination was increased by 1–30% in each country. The approach is to determine the model parameters from the training and validation sets and then predict the CFRs when booster vaccination rates increase by 1–30% for each country respectively, holding all other variables constant. The principle of increasing booster vaccination is based on the actual full and booster vaccination rate in each country, so we predicted the CFRs of increasing booster vaccination rates within the range of a country's booster vaccination rate not exceeding the cumulative proportion of the population fully vaccinated.

Statistical analysis

Continuous data are presented as a mean with standard deviation (SD) where normally distributed and as a median with the 25th and 75th percentiles where non-normally distributed. Univariate analyses relating CFRs and multi-dimensional explanatory variables were assessed with Spearman's rank correlation.

Analyses were performed in the R 4.1.1 and Python 3.8 environments.

Results

Temporal and regional heterogeneity of age-adjusted CFRs

COVID-19 age-adjusted CFRs were available in 32 countries throughout the pandemic from February 4, 2020 to January 31, 2022. The crude CFRs in these countries ranged from 63 to 5,886 deaths per 100,000 people, and the age-adjusted CFRs still varied significantly across the country, ranging from 110 to 5,112 deaths per 100,000 people. The age-adjusted CFRs for the 32 countries during the original, Alpha, Delta, and Omicron periods were 1.25, 1.19, 1.37, and 0.16% respectively, showing a significant decrease for the Omicron period. According to the age-adjusted CFRs, the 32 countries were grouped into two groups: (1) Countries with higher age-adjusted CFRs than crude CFRs ($n = 9$, median age-adjusted and crude CFRs: 0.013 and 0.010), mainly in Asia and Africa; and (2) Countries with lower age-adjusted CFRs than crude CFRs ($n = 23$, median age-adjusted and crude CFRs: 0.003 and 0.007), mostly in Europe (Figure 2A). The countries with the highest age-adjusted CFR in group 1 are Indonesia, Colombia and Jamaica, and in group 2, they are Somalia, Peru and Bulgaria. The median cumulative full and booster vaccination rates for countries with higher age-adjusted CFRs than crude CFRs were 51.08 and

6.49%, respectively, while the median cumulative full and booster vaccination rates for countries with lower age-adjusted CFRs than crude CFRs were 73.83 and 39.7%, respectively. Univariate analyses revealed that age-adjusted CFRs were relatively strong associated with both the cumulative proportion of the population having completed the initial vaccination protocol and that having received a booster ($r = -0.625$, $p = 0.0001$; and $r = -0.514$, $p = 0.0030$, respectively) (Figure 2B). The higher the vaccination rate, the lower the CFRs, however, some countries with high vaccination rates, such as Peru, Chile and Colombia, still have relatively high CFRs.

The determinants of age-adjusted CFRs over the pandemic

Most cross-country variation in age-adjusted CFRs in the Alpha, Delta, and Omicron periods could be well explained by the SHAP-interpreted XGboost model (R^2: 0.78, 0.88, 0.79, respectively) (Figure 3A). The XGboost-SHAP model showed that important determinants [importance score (IS) ≥ 0.10] in all three periods included HAQ index (IS: 0.36, 1.33 and 0.31 in the Alpha, Delta, and Omicron periods, respectively), GDP *per capita* (IS:0.19, 0.13, and 0.91) and vaccination (IS of fully vaccinated: 0.19, 0.49, and 0.28; IS of boost given: 0.03, 0.08, and 0.23), in addition to CKD (IS: 0.10), smoking (IS: 0.13) in the Alpha period, tree coverage (IS: 0.14), NCD (IS: 0.17), URI (IS: 0.17) in the Delta period, and dietary risks (IS: 0.17) in the Omicron period. Figure 3B showed that high booster vaccination rates and high GDP and HAQ indices are protective against age-adjusted CFR and that high dietary risks would be a risk for age-adjusted CFR in the Omicron period. Comparing the important determinants of CFR over the three periods shows that completing the initial vaccination protocol is more important in the Delta period (IS: 0.49), while the protective effects of booster vaccination increasingly become more important from Alpha to Omicron period (IS: 0.23; Figure 3B). Various underlying disease burdens were identified as important risk factors for CFR, such as chronic kidney disease (CKD) (IS: 0.10 in both Alpha and Delta period), and NCD (IS: 0.17 in the Delta period), but the risk posed by these underlying diseases was reduced for the Omicron period. In addition, high levels of trust in government, journalists and science are also protective factors for COVID-19 deaths in almost all periods. The importance of dietary risk on age-adjusted CFR is revealed in the Omicron period (IS: 0.17).

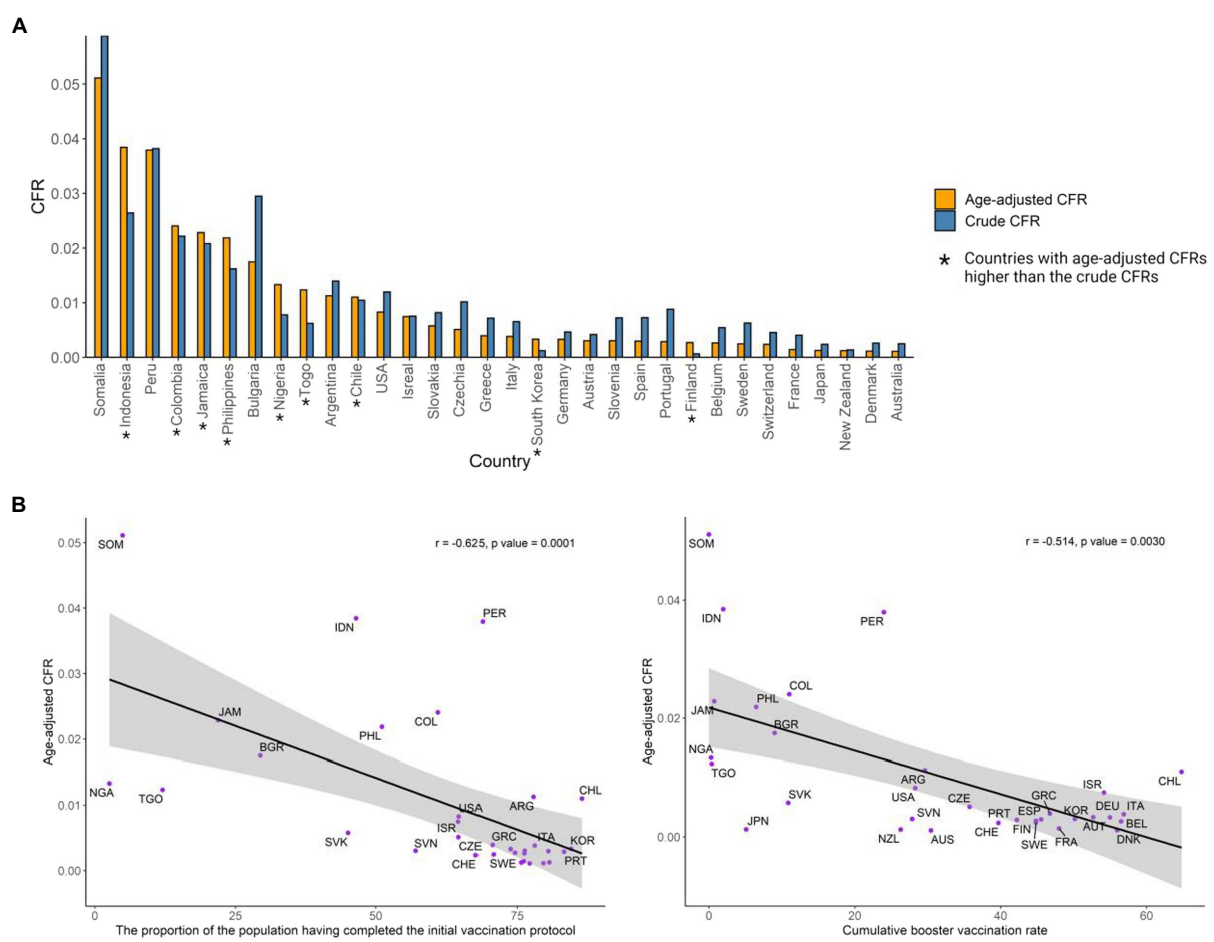

FIGURE 2
(A) Crude CFRs and age adjusted CFRs in the 32 countries. **(B)** The correlation between age-adjusted CFRs and vaccination coverage (fully vaccinated and booster given).

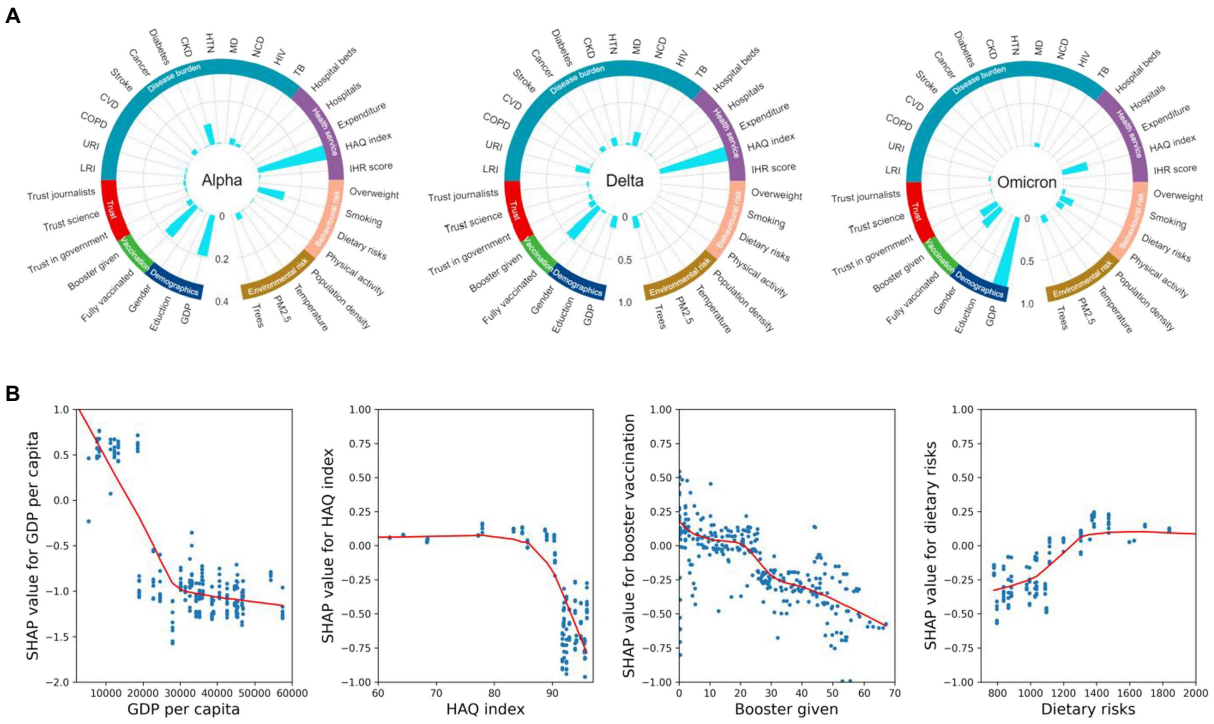

FIGURE 3
(A) Relative importance scores for each feature affecting age-adjusted CFR in each period model, obtained by taking the absolute mean of the SHAP values. The 35 features represent seven distinct dimensions: vaccination coverage, demographic factors, disease burden, behavioral risk factors, environmental risk factors, health services, and trust levels. **(B)** SHAP dependence plots for GDP *per capita*, HAQ index, booster vaccination rate, and dietary risks in the XGBoost models. SHAP values above zero represent an increased risk of higher COVID-19 age-adjusted CFR. LRI, lower respiratory infections; URI, upper respiratory infections; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular diseases; CKD, chronic kidney disease; HTN, hypertension; MD, mental disorders; NCD, noncommunicable diseases; HIV, HIV infection; TB, tuberculosis.

PM2.5 as a risk factor in all periods became more important in the Omicron period (IS: 0.09).

Assessment of country-specific risks for age adjusted CFR in the omicron period

The determinants, including GDP *per capita*, HAQ index, vaccination coverage (population receiving booster doses and fully vaccinated) and behavioral risk factors (dietary risks, low physical activity, and smoking), disease burden (NCD and LRI), as well as PM2.5 and trust science, contribute to the COVID-19 age-adjusted CFR in each country during the Omicron period as shown in Figure 4. Countries are sorted from left to right in descending order of risk of death from COVID-19. The key risk factors for countries with higher age-adjusted CFR than crude CFR are low GDP *per capita* and low booster vaccination rates, while the key risk factors for countries with higher age-adjusted CFR than crude CFR were high dietary risks and low physical activity. Moreover, most countries were already protected by booster vaccination, but there were still seven countries (Sweden, Bulgaria, Jamaica, Indonesia, Somalia, Togo, Nigeria) with an increased risk of death from COVID-19 due to low booster vaccination rate. These countries have more concurrent risk factors, with all seven at risk of high NCD burden, six of the seven at risk of low HAQ index, and five of the seven at risk of low GDP *per capita*. Furthermore, high dietary risk and low physical activity also increased the risk of death from COVID-19 in 23 countries, with it being the main risk in 12 of these countries. The 11 of these 12 countries that are high-income countries already have a booster vaccination rate of 41.3%. CFRs are adversely affected by the burden of NCD to some degree in 65.6% of countries in this study, but of these only Portugal and Sweden have the main risk from NCD. In addition, high PM2.5 and low trust in science are the key risk factor in South Korea and Isreal, and Switzerland, respectively.

Future benefits of increasing booster vaccination vary by country

Countries show varying degrees of reduction in age-adjusted CFRs when simulating 1–30% increase in booster vaccination (Supplementary Figure 2). Countries with age-adjusted CFRs higher than crude CFRs showed a reduction in CFRs when simulated booster vaccination rates were increased by 1–7%, and in addition, 11 of the countries with age-adjusted CFRs higher than crude CFRs (48%) also showed a reduction in CFRs. Furthermore, increasing booster vaccination for just up to 3% of the population would reduce CFR in 15 countries. These countries include five countries (Nigeria, Togo, Indonesia, Sweden, and Jamaica) with pre-existing low booster vaccination rates as a risk factor. However, Bulgaria, as a country where low vaccination rates are also a risk factor, would need to increase vaccination rates by 9% and above to bring down its

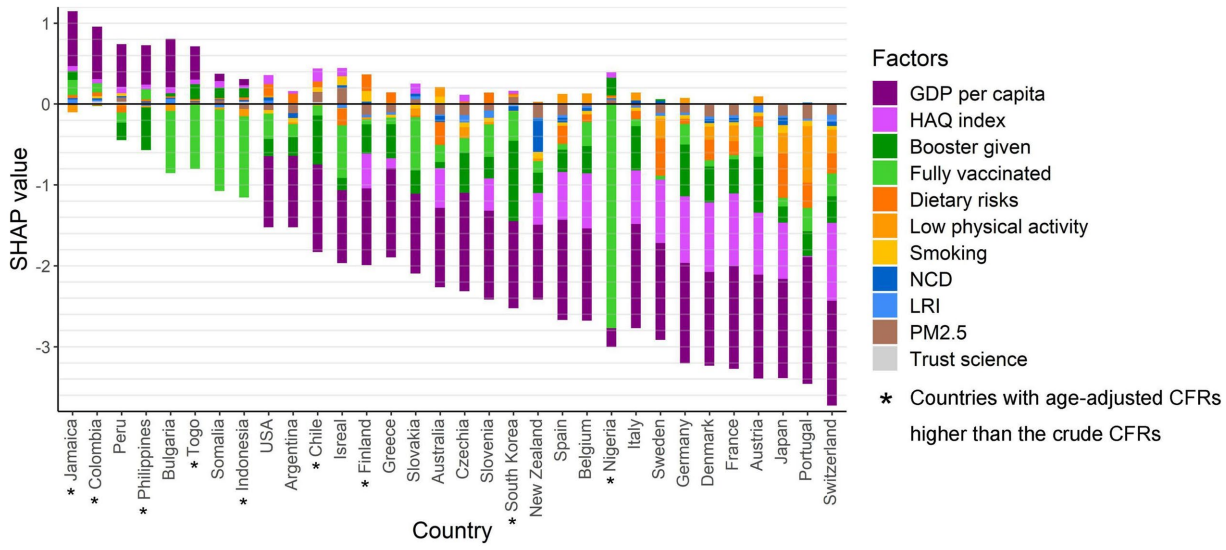

FIGURE 4
The protection and risk contributions of the determinants of the COVID-19 age-adjusted CFR for each country in the Omicron period. SHAP values above 0 are regarded as risk effects and below 0 as protective effects.

CFR. Moreover, in Czechia, Australia and Portugal, the CFRs did not show a decrease until booster vaccination rates increased by more than 12%. Furthermore, in Austria, Belgium, Chile, Germany, Denmark, Italy, and Slovakia, the increase in booster vaccination did not significantly reduce the CFRs, where the average booster vaccination rate has reached 51.9%.

Discussion

This is the first study to comprehensively identify risk factors affecting COVID-19 age-adjusted CFRs at the country level, particularly to assess and predict the effect of booster vaccination in the COVID-19 pandemic. Our models fit well allowing for a real-world assessment of the risk of COVID-19 death and the health benefits of vaccination in each country to more rationally guide vaccine distribution. We draw two conclusions from this study. First, booster vaccinations showed stronger importance in the Omicron period as previous vaccine effectiveness waned, while the importance of other factors such as disease burden and behavioral risk factors for CFR changed during the pandemic. Our study confirms the importance of vaccination, especially booster doses, in reducing the risk of death in Omicron pandemics. Patients during the Omicron period also benefited from the strong protection against severe disease and death still afforded by the COVID-19 vaccine (50). In the stage dominated by the "Stealth" Omicron, during which strict prevention policies are challenged by insidious transmission and the number of infections has become difficult to control, improving vaccination coverage is a cost-effective approach for reducing severe health outcomes and relieving pressure on the healthcare system. On the issue of vaccine allocation, as advocated by Jeremy Bentham's Utilitarianism, a rule for society should be established that has the best outcome for the greatest amount of people in society, in the sense that a cost-effective vaccine allocation scheme should be developed in a global perspective that reduces the risk of death for the greatest proportion of people worldwide. Our study simulated the reduction in CFR after increasing vaccination by country and found that the health benefits of increasing vaccination varied by country, for example, countries such as Togo, Isreal, and Nigeria showed significant reductions in CFR with only a small increase in vaccination. The WHO has worked to this end by convening COVAX (51), a ground-breaking global collaboration aimed at accelerating the development and production of and equitable access to the COVID-19 vaccine, ensuring that every country has access to the vaccine and is able to promote vaccination to protect their whole population, starting with the most vulnerable. On the other hand, GDP *per capita* and HAQ index have been important determinants of age-adjusted CFR during the different variant-dominated periods of the pandemic. The HAQ index reflects the accessibility and quality of health care for individuals. The accessibility and quality of healthcare in a country are important when responding to a pandemic; moreover, regional inequities in access and quality may lead to greater regional disparities in the burden of infectious diseases in the future. Adjusting investments to improve access and quality across healthcare needs will not only benefit routine care, but also improve overall health coverage in preparation for the next pandemic (43). For instance, our study presented that there were several countries (e.g., Sweden, Bulgaria, Jamaica, Indonesia, Somalia, Togo, Nigeria) have low GDP and HAQ indices, as well as low boost vaccination coverage, which contribute to their high risk of death.

The second major conclusion of this study is that CFRs are also affected by a broad range of concurrent risks, such as high dietary risk, low physical activity, high disease burden, and high PM2.5. Consequently, we believe that a joint intervention would be an effective measure for reducing CFRs in this class of countries. In the short term, in addition to vaccination, a promising area for

interventionists to work on is raising the level of national trust. Our findings support previous research that trust in government and science can increase risk perceptions of COVID-19 among the population, promote cooperation with outbreak prevention and control efforts, and more quickly control the number of cases and deaths (52). Pandemics have always posed a challenge to trust between the public and the government, and maintaining and rebuilding trust during a crisis is crucial to maintaining political participation and social cohesion (53). In the long term, behavioral factors such as smoking, diet, and nutrition, along with environmental factors such as PM2.5, are all risk factors that can be changed through health education and policy development, and are areas in which advanced preparation is needed in order to mitigate the effects of future epidemics. In high-income countries, dietary risks are revealed. Dietary risk is the intake of too much or too little of certain foods or nutrients. As studies have shown, a healthy dietary pattern is associated with lower risk and severity of COVID-19 (54). Therefore, improving the dietary health of the population or correcting micronutrient deficiencies in people already diagnosed with COVID-19 infection may help to reduce the risk of death (55). Moreover, regulating taxes on tobacco, tightening restrictions on smoking places, and setting a legal age for smoking would contribute to reducing the potential harm from smoking at a national level. In addition, environmental factors are of increasing concern to epidemiologists, and our research suggests that PM2.5 have some impact on severe health outcomes in COVID-19. It has also been suggested that PM2.5 may potentially serve as a carrier for the virus (56). Therefore, an improved environment with less air pollution would benefit both patients with COVID-19 and healthy populations. Consequently, we believe that a joint intervention would be an effective measure for reducing CFRs in the countries.

There are several limitations in our analysis. First, the study design is a country-level ecological analysis based on retrospective data, and care should be taken regarding ecological fallacies in the interpretation and generalization of the results. Second, our data were sourced from multiple publicly available data sources, and after comparing them we selected the more credible sources and also applied outlier treatment, but the credibility of our analysis relies greatly on the quality of the data. Third, COVID-19 cases and deaths are from national self-reported data and do not consider excess deaths from COVID-19. Fourth, we predicted the future CFR only when increasing booster vaccination rates in each country, keeping other factors constant, considering that only vaccination rates are relatively changeable in the short term among the factors that affect CFR. Fifth, some of the potential factors affecting CFR were not available in this study, such as vaccine type and ethnicity, and in addition there may be incorrect estimates based on missing values due to the missing values in the data.

In conclusion, the cross-temporal and cross-country variation in COVID-19 age-adjusted CFRs illustrates the importance of conducting further research on risk assessment. The future health benefits of increased vaccination are country-specific due to differences in risk factors of CFR by country. Booster vaccination still plays an important role in reducing age-adjusted CFRs, while there are multidimensional concurrent risk factors and precise joint intervention strategies and preparations based on country-specific risks are also essential. Our study reminds policy makers to consider risk factors holistically and assess whether their countries can rebuild policy trust, face the challenges of vaccine hesitancy, revitalize primary healthcare, and strengthen behavioral and environmental risk management and investment in the post-COVID era.

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.

Author contributions

CuZ: data collection, conceptualization, investigation, data analysis, and writing original draft and revision. ÅW: conceptualization, supervision, and investigation. ChZ, JM, and ZL: data analysis. KD and JP: revision. WL: conceptualization and supervision. JG: data collection, conceptualization, supervision, investigation, data analysis, and writing original draft and revision. LX: conceptualization, supervision, and funding acquisition, writing, and revision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1150095/full#supplementary-material

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Three doses of a recombinant conjugated SARS-CoV-2 vaccine early after allogeneic hematopoietic stem cell transplantation: predicting indicators of a high serologic response—a prospective, single-arm study

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Background: Allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients must be vaccinated against SARS-CoV-2 as quickly as possible after transplantation. The difficulty in obtaining recommended SARS-CoV-2 vaccines for allo-HSCT recipients motivated us to utilize an accessible and affordable SARS-CoV-2 vaccine with a recombinant receptor-binding domain (RBD)–tetanus toxoid (TT)-conjugated platform shortly after allo-HSCT in the developing country of Iran.

Methods: This prospective, single-arm study aimed to investigate immunogenicity and its predictors following a three-dose SARS-CoV-2 RBD–TT-conjugated vaccine regimen administered at 4-week (± 1-week) intervals in patients within 3–12 months post allo-HSCT. An immune status ratio (ISR) was measured at baseline and 4 weeks (± 1 week) after each vaccine dose using a semiquantitative immunoassay. Using the median ISR as a cut-off point for immune response intensity, we performed a logistic regression analysis to determine the predictive impact of several baseline factors on the intensity of the serologic response following the third vaccination dose.

Results: Thirty-six allo-HSCT recipients, with a mean age of 42.42 years and a median time of 133 days between hematopoietic stem cell transplant (allo-HSCT)

and the start of vaccination, were analyzed. Our findings, using the generalized estimating equation (GEE) model, indicated that, compared with the baseline ISR of 1.55 [95% confidence interval (CI) 0.94 to 2.17], the ISR increased significantly during the three-dose SARS-CoV-2 vaccination regimen. The ISR reached 2.32 (95% CI 1.84 to 2.79; p = 0.010) after the second dose and 3.87 (95% CI 3.25 to 4.48; p = 0.001) after the third dose of vaccine, reflecting 69.44% and 91.66% seropositivity, respectively. In a multivariate logistic regression analysis, the female sex of the donor [odds ratio (OR) 8.67; p = 0.028] and a higher level donor ISR at allo-HSCT (OR 3.56; p = 0.050) were the two positive predictors of strong immune response following the third vaccine dose. No serious adverse events (i.e., grades 3 and 4) were observed following the vaccination regimen.

Conclusions: We concluded that early vaccination of allo-HSCT recipients with a three-dose RBD–TT-conjugated SARS-CoV-2 vaccine is safe and could improve the early post-allo-HSCT immune response. We further believe that the pre-allo-HSCT SARS-CoV-2 immunization of donors may enhance post-allo-HSCT seroconversion in allo-HSCT recipients who receive the entire course of the SARS-CoV-2 vaccine during the first year after allo-HSCT.

KEYWORDS

hematopoietic stem cell transplantation, SARS-CoV-2, RBD subunit vaccine, conjugate vaccine, humoral response, T-cell response, immunogenicity predictors

1 Background

The novel coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus (SARS-CoV-2), has generated a severe medical crisis. Immunodeficiency after allogeneic hematopoietic stem cell transplant (allo-HSCT) increases the susceptibility of the recipient to the most severe SARS-CoV-2 infection and a greater fatality rate than the general population (1, 2). The timely vaccination of hematopoietic stem cell transplant (allo-HSCT) patients can boost immunity, decreasing the morbidity and mortality associated with COVID-19.

Although immune responses to vaccination are frequently restricted and uncertain in the initial phases of allo-HSCT (3, 4), some professional bodies, notably the European Society for Blood and Marrow Transplantation (EBMT), advocate prophylactic vaccination as quickly as 3 months after the transplant to provide initial immune protection (5, 6). The most widely used vaccination platforms in allo-HSCT recipients were mRNA vaccines, such as BNT162b2 from Pfizer-BioNTech and mRNA-1273 from Moderna, and adenoviral vector vaccines, such as Ad26.COV2.S from Johnson & Johnson and ChAdOx1-S from AstraZeneca (7, 8). In Iran, we followed the EBMT recommendation for post-allo-HSCT SARS-CoV-2 vaccination; however, owing to limited access to mRNA-based platforms, we mainly utilized available vaccines, including inactivated platforms (e.g., the Sinopharm vaccine), for allo-HSCT recipients.

Recent investigations, however, have demonstrated that many allo-HSCT patients, particularly those vaccinated soon after allo-HSCT, reacted poorly to two doses of the mRNA vaccine (7, 8). The serologic response was 32% [95% confidence interval (CI) 15%–50%] for patients vaccinated 6 months post transplantation (9, 10), 50% (95% CI 42% to 61%) for patients vaccinated between 6 and 12 months post transplantation (11–13), and 87.9% (95% CI 72% to 95%) for patients vaccinated after 1 year following allo-HSCT (12–14). However, further research showed that giving the third dose of the SARS-CoV-2 vaccine markedly improved the serological response after allo-HSCT (15–18).

The protein subunit platform, based on SARS-CoV-2 protein components, such as the spike protein (S1) and receptor-binding domain (RBD), is a different vaccination technology that has demonstrated advantages in terms of tolerability, efficacy, and cost (19). According to published data, RBD-based SARS-CoV-2 vaccines, such as Abdala, Zhifei, and Noora, have shown promising results in healthy people (20–22). Furthermore, as demonstrated in preclinical investigations, humoral and cellular immune responses were strengthened by coupling RBD with the tetanus toxoid (TT) (23).

Soberana 2, also called PastoCovac, is the first SARS-CoV-2 vaccination using RBD conjugated to TT, manufactured in collaboration between the Cuban Finlay Institute and the Iranian Pasteur Institute. Soberana 2 (PastoCovac) has been certified for emergency use in adults and children aged more than 2 years in Cuba and Iran. This platform is simple to construct and offers benefits in terms of storage and transportation. The safety and immunogenicity of Soberana 2 have previously been studied in dedicated phase 1, 2, and 3 clinical studies (24–26). In a recently

published study with autologous HSCT patients, we demonstrated that two doses of the novel RBD–TT-conjugated SARS-CoV-2 vaccine (PastoCovac) given soon after autologous transplants were safe and significantly enhanced the serologic response to a level comparable to the mRNA-based platform, although less than that of the healthy controls (27).

The difficulty in obtaining recommended SARS-CoV-2 vaccines for allo-HSCT recipients, such as the mRNA- or adenoviral vector-based platforms, as well as the necessity for timely immunization of allo-HSCT recipients, prompted us to explore the use of an accessible and affordable (RBD–TT-conjugated) SARS-CoV-2 vaccine early after allo-HSCT. We also examined how the characteristics of the patients and donors and their immunological status against SARS-CoV-2 at the time of allo-HSCT influenced subsequent serologic responses to early post-allo-HSCT vaccination.

2 Methods

2.1 Study design, registry, and ethical approval

This prospective and single-group clinical trial assessed the immunogenicity and safety of three RBD–TT conjugated SARS-CoV-2 vaccine doses in adult acute leukemia patients who underwent allo-HSCT at the Hematology, Oncology and Stem Cell Transplantation Research Center (HORCSCT) of Tehran University, Tehran, Iran. The study was registered on ClinicalTrial.gov (as NCT05185817) and the Iranian Registry of Clinical Trials (as IRCT20140818018842N22). Recruitment for the trial began in January 2022.

The trial was conducted under the Helsinki Declaration and Good Clinical Practice and was certified by the Ethics Committee of Tehran University's Hematology, Oncology and Stem Cell Transplantation Research Center (IR.TUMS.HORCSCT.REC.1400.021). Each recipient provided written informed consent for the PastoCovac vaccine (Pasteur Institute, Tehran, Iran) to be administered, blood samples to be collected, and results to be published.

2.2 Inclusion criteria

The research included all adult patients with acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL) who had received allo-HSCT within the previous 3–12 months, were older than 18 years, had achieved complete engraftment, and had no documented history of SARS-CoV-2 infection after allo-HSCT.

2.3 Exclusion criteria

Having grade 3 or 4 acute graft-versus-host disease (GvHD) or severe extensive chronic GvHD, taking more than 0.5 mg/kg of prednisolone per day, suffering from severe thrombocytopenia or a coagulation disorder, having a history of an allergic reaction to the vaccine's active ingredients, being unable to provide consent forms, continuing post-allo-HSCT infection, graft rejection, or experiencing a relapse of the underlying disease were all among the exclusion criteria.

2.4 Procedures and data collection

The research selection flowchart is provided in Figure 1. Starting in January 2022, 75 recipients of allo-HSCT were enrolled. A total of 52 people satisfied the eligibility criteria and stayed in the study for post-allo-HSCT vaccination. Acute GvHD, COVID-19 infection, and refusal to volunteer were responsible for most study exclusions. The study ultimately comprised 36 patients who received the three-dose RBD–TT conjugated SARS-CoV-2 vaccine and were given the available serologic tests at four time points: at baseline and after the first, second, and third doses.

Medical personnel administered the vaccination, comprising 0.5 mL of vaccine injected intramuscularly into the deltoid area. We developed an electronic case report form (CRF) using our institution's web-based software to collect research information, including patients' and donors' characteristics, concurrent medications, lymphocyte subpopulation count, and SARS-CoV-2 anti-S1 titers. To evaluate the safety profiles of a new RBD–TT-conjugated SARS-CoV-2 vaccine, we employed active surveillance

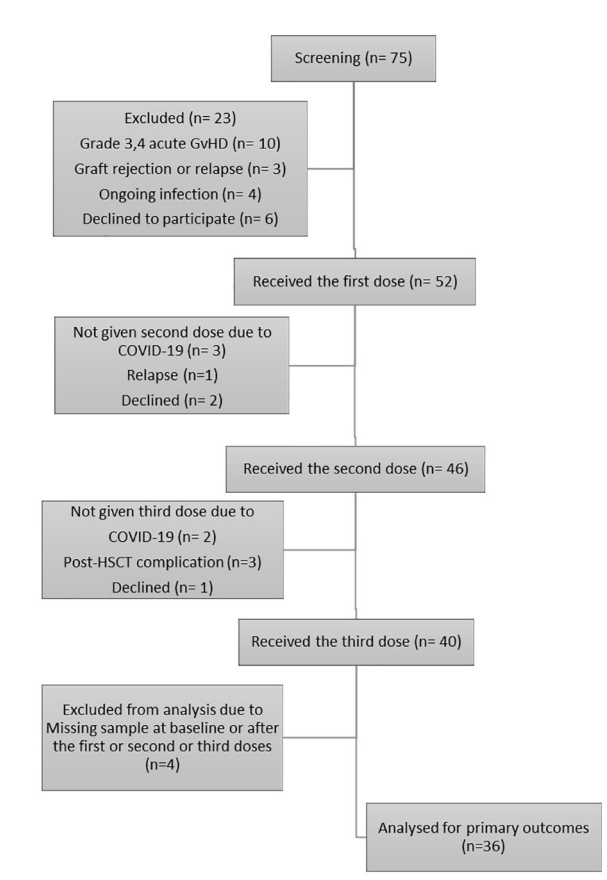

FIGURE 1
Flowchart of the single-arm study. The chart depicts the subjects screened before the study, those recruited for vaccination, and the processes for selecting or excluding patients.

systems to report any vaccination-related adverse events through daily telenursing calls, which have a higher accuracy than passive monitoring in specific population subgroups (28, 29).

2.5 Outcome

The main objectives of our study included the following outcomes:

1. The anti-SARS-CoV-2 spike protein (anti-S) serologic response at 4 weeks (± 7 days) after the third dose of vaccine, defined as an increase in the immune status ratio (ISR) above the cut-off point for a positive result in the semiquantitative test.
2. The predicting factors of a strong immune response following the third vaccination dose, using the median level of ISR as a cut-off point (30).
3. The vaccination's safety and tolerability up to 1 week after each dosage.

2.6 Anti-SARS-CoV-2 antibody evaluation

We used the ChemoBind SARS-CoV-2 Neutralizing Antibody Test Kit (ChemoBind, Tehran, Iran) to measure total antibodies against the receptor-binding domain (RBD) spike protein of SARS-CoV-2 using a semiquantitative immunoassay. Based on the instructions from the manufacturer, an immunoglobulin G (IgG) immune status ratio (ISR) of less than 0.8 is negative, and an IgG ISR greater than 1.1 is positive; ratios between these values are ambiguous and need to be repeated.

All allo-HSCT recipients had their anti-S antibody levels (as ISR) assessed before vaccination and 4 weeks (± 1 week) after receiving the first, second, and third doses of the vaccine. We also measured the pre-allo-HSCT ISR for patients and donors to evaluate the potential predictive impact of the pre-allo-HSCT immune status of patients and donors against SARS-CoV-2 on post-allo-HSCT vaccine-induced antibody production.

2.7 Flow cytometry

Peripheral blood samples were collected for all recipients to assess the absolute count and percentage of specific lymphocyte subpopulations at the first (i.e., baseline) and third vaccination doses. The immunophenotype of natural killer (NK), T, and B cells was determined by a 10-color multiparameter flow cytometric analysis of blood samples. The blood samples were collected into ethylenediaminetetraacetic acid (EDTA) tubes and incubated with the following recombinant monoclonal antibodies: anti-CD16 (REA423), anti-CD56 (REA196), anti-CD3 (REA613), anti-CD4 (REA623), anti-CD8 (REA734), anti-CD19 (REA675), and anti-CD45 (REA747). Based on antigen density and brightness, one seven-color panel was designed in pairing markers and fluorochromes: CD16-FITC, CD56-PE, CD3-VioBlue, CD4-PerCP-Vio700, CD8-PEVio770, CD19-APC, and CD45-VioGreen. The experimental controls were unstained, stained with one dye, and fluorescence minus one control.

2.8 Safety assessments

Using active surveillance, we reported any reactogenicity adverse effects (AEs), including specific local (pain and swelling at the injection site) or systemic (fever, lethargy, headache, diarrhea, vomiting, and muscle pain) AEs were reported *via* daily telenursing calls for up to 7 days following each vaccination dose. All reactogenicity events were classified as none/mild (grades 0 or 1), moderate (grade 2), severe (grade 3), or life-threatening/death (grades 4 or 5) using the Common Terminology Criteria for Adverse Events (CTCAE) (31). Across the follow-up period, all immunized patients were monitored weekly through phone calls or clinical appointments to identify any occurrences of new or worsening GVHD, a diagnosis of COVID-19, a relapse of underlying disease, or cytopenia until 20 December 2022.

2.9 Statistical analysis

The generalized estimating equation (GEE) model was used for assessing the dynamics of the serologic response following each vaccine dose overall and based on the main variables. The predictive impact of confounding factors on the GEE model was then determined by univariate and multivariable analysis.

Using the median ISR as a cut-off point for immune response intensity, we performed a logistic regression analysis to determine the predictive impact of several baseline factors on the intensity of the serologic response following the third vaccination dose. Factors correlated with a vigorous immunological response in the univariate analysis ($p \leq 0.20$) were then entered into the multivariable model with stepwise forward selection.

The Shapiro–Wilk test was used for assessing the normal distribution of quantitative variables. All tests were two-way, and a p-value of less than 0.05 was considered statistically significant. GraphPad Prism version 8 was used to create the graphs (GraphPad Software Inc., San Diego, CA, USA). All statistical analyses were performed using IBM SPSS Statistics, version 23.0 (IBM Corporation, Armonk, NY, USA).

3 Results

3.1 Patient characteristics

The study included 36 allo-HSCT individuals who received three PastoCovac doses and four serologic tests of blood samples with which to assess the trial's main end points (Figure 1). The study comprised 15 females (41.7%) and 21 males (58.3%), with a mean age of 42.42 years (SD 15.84 years), as shown in Table 1. Regarding participants' primary diseases, 27 patients with AML

TABLE 1 Baseline characteristics and lymphocyte subpopulations by the strength of immune response after the three doses of receptor-binding domain (RBD)–tetanus toxoid (TT)-conjugated SARS-CoV-2 vaccine in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients.

Baseline characteristics		Total	Strength of immune response#		
			Moderate immune response	Strong immune response	*p*-value
Total, *N*		36	18	18	
Patient's sex, *n* (%)	Female	15 (41.7)	8 (53.3)	7 (46.7)	0.790
	Male	21 (58.3)	10 (47.6)	11 (52.4)	
Donor's sex, *n* (%)	Female	18 (50)	4 (22.2)	14 (77.8)	0.004
	Male	18 (50)	14 (77.8)	4 (22.2)	
Primary disease, *n* (%)	AML	27 (75)	13 (48.1)	14 (51.9)	0.791
	ALL	9 (25)	5 (55.6)	4 (44.4)	
Patient's age in years (median ± IQR)		42.41 ± 11.4	45.6 ± 11.42	39.16 ± 11.66	0.111
Donor's age in years (median ± IQR)		43.94 ± 11.55	47.61 ± 12.75	40.27 ± 9.15	0.044
Using cyclosporine ≥ 25mg/day at the time of vaccination, *n* (%)		26 (72.2)	12 (46.2)	14 (53.8)	0.463
Using prednisolone ≥ 5mg/day at the time of vaccination, *n* (%)		9 (25)	6 (66.7)	3 (33.3)	0.255
Having GvHD at the time of vaccination¥		15 (41.7)	8 (53.3)	7 (46.7)	0.739
Patient's pre-allo-HSCT PCR-positive COVID-19 status, *n* (%)		15 (41.7)	4 (26.7)	11 (73.3)	0.020
Patient's pre-allo-HSCT SARS-CoV-2 vaccination status, *n* (%)		13 (36.11)	8 (61.5)	5 (38.5)	0.486
Donor's pre-allo-HSCT SARS-CoV-2 vaccination status, *n* (%)		24 (66.7)	11 (45.8)	13 (54.2)	0.360
Patient's ISR pre-allo-HSCT, mean ± SD		1.19 ± 0.73	1.25 ± 0.88	1.13 ± 0.55	0.860
Donor's ISR pre-allo-HSCT, mean ± SD		1.94 ± 0.95	1.49 ± 0.65	2.4 ± 1.00	0.005
Median (IQR) time between allo-HSCT and vaccination in days		133 (107.5 - 228)	130.5 (99 - 202)	133 (115 -231)	0.521
Lymphocyte subpopulations, mean ± SD					
At the first vaccine dose (baseline)	$CD4^+$ cells	319.00 ± 208.99	215.79 ± 98.80	399.28 ± 237.87	0.091
	$CD8^+$ cells	788.41 ± 461.51	743.62 ± 525.86	823.25 ± 417.13	0.220
	$CD4^+/CD8^+$ ratio	0.46 ± 0.25	0.40 ± 0.25	0.50 ± 0.26	0.280
	$CD19^+$ cells	113.77 ± 101.00	74.39 ± 70.02	144.40 ± 122.16	0.090
	$CD16^+$ 56^+ (NK cells)	139.34 ± 91.69	112.70 ± 52.59	160.06 ± 110.34	0.357
At the third vaccine dose	$CD4^+$ cells	389.95 ± 193.51	319.45 ± 148.28	448.69 ± 210.58	0.073
	$CD8^+$ cells	1082.48 ± 768.64	1207.78 ± 904.20	978.06 ± 642.96	0.708
	$CD4^+/CD8^+$ ratio	0.47 ± 0.25	0.37 ± 0.25	0.55 ± 0.23	0.018
	$CD19^+$ cells	180.82 ± 161.94	122.46 ± 125.24	229.45 ± 175.89	0.044
	$CD16^+$ 56^+ (NK cells)	192.95 ± 218.80	147.60 ± 106.81	230.74 ± 278.19	0.929

#Defined based on the median level of ISR after the third vaccine dose.
¥ Including grades 1 or 2 acute GvHD or mild to moderate chronic GvHD.
ISR, immune status ratio; allo-HSCT, allogeneic hematopoietic stem cell transplant; AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; GvHD, graft-versus-host disease; NK, natural killer.
The median time between allo-HSCT and the start of vaccination was 133 days (interquartile range 107.5 - 228 days).

(75%) and nine with ALL (25%) were included in the trial. All recipients were given the same myeloablative conditioning regimen of busulfan and cyclophosphamide (Bu/Cy) and the same graft source of peripheral blood stem cells.

Before allo-HSCT, 13 (36.11%) patients and 24 (66.7%) donors had been fully vaccinated against SARS-CoV-2. Information regarding patients' and donors' SARS-CoV-2 vaccination history and ISR serologic test results at the time of allo-HSCT is shown in Table 1. Information on the use of immunosuppression drugs and the grade and severity of GvHD before the first vaccine dose is also given in Table 1. At the time of immunization, 26 patients (72.2%) were receiving calcineurin inhibitors (cyclosporine ≥ 25 mg/day) and nine (25%) were also receiving prednisolone ≥ 5 mg/day but < 0.5 mg/kg/day. Fifteen (41.7%) patients were shown to have grades 1 or 2 acute GvHD, or mild or limited chronic GvHD at the time of vaccination (patients with high-grade acute GvHD or severe chronic GvHD were excluded). The median time between allo-HSCT and the start of vaccination was 133 days (interquartile range 107.5 - 228 days).

3.2 Serological outcomes

From January to October 2022, 146 blood samples were obtained from the 36 patients in the study cohort and tested for antibodies against SARS-CoV-2. A scatterplot of the SARS-CoV-2 IgG ISRs during the trial was created (Figure 2A).

The mean ISR was 1.55 (95% CI 0.94 to 2.17) at baseline (before the first dose). This markedly increased to 2.32 (95% CI 1.84 to 2.79; $p = 0.010$) and 3.87 (95% CI 3.25 to 4.48; $p = 0.001$) after the second and third doses of vaccine, respectively. Out of 36 patients, 10 (27.17%) had a baseline ISR over the threshold for a positive result in the semiquantitative test. After doses two and three, the proportion of seropositive tests increased to 69.44% and 91.66%, respectively.

As depicted in Table 2, taking the pre-vaccination ISRs as the reference group in the multivariable GEE model, the ISR increased dramatically across the second ($p = 0.041$) and third ($p < 0.001$) vaccine doses, regardless of any confounding factors. The donor's sex ($\beta = -1.33$; $p = 0.010$) was the independent factor associated with ISR during the vaccination.

Given that the ISR values after the third vaccine dose mostly exceeded the cut-off value for the positive result in the semiquantitative test, it was possible to distinguish between patients with moderate and strong serologic responses based on the median level of the ISR after the third vaccine dose (30). Table 1 presents the strength of the immune responses (divided into strong or moderate immune response) to the entire course of RBD–TT-conjugated SARS-CoV-2 vaccine following allo-HSCT according to the baseline characteristics of the patients and donors at the time of allo-HSCT and also based on the post-allo-HSCT immune cells' reconstitution at the time of first and third vaccine doses.

As depicted in Table 1, the strong serologic response following the three doses of the PastoCovac vaccine was more common in recipients who received their allo-HSCT from female donors than those who received their allo-HSCT from male donors (77.8%; $p = 0.004$), and in those with a history of pre-allo-HSCT PCR-positive COVID-19 (73.3%; $p = 0.020$). The median age of donors was lower (i.e., 40.27 vs. 47.61 years; $p = 0.044$) and the mean ISR of donors before allo-HSCT was higher (i.e., 2.4 vs. 1.49; $p = 0.005$) in the strong serologic response group than in the moderate response group. Regarding the correlation of post-allo-HSCT immune cell reconstitution with the strength of the serologic response, as depicted in Table 1, the mean counts of $CD19^+$ cells and mean $CD4^+/CD8^+$ ratio at the third vaccine dose were significantly higher in patients with a strong serologic response. The mean counts of $CD4^+$ cells, $CD8^+$ cells, and $CD19^+$ cells at the third vaccine dose in the allo-HSCT recipients with strong as compared with moderate serologic responses are shown in Figure 3.

Univariate and multivariate logistic regression analyses were performed to determine the predictive indicators of a strong immune response following the third vaccine dose. In the multivariate analysis, the female sex of the donor [odds ratio (OR) 8.67; $p = 0.028$] and a higher donor ISR before allo-HSCT (OR 3.56; $p = 0.050$) remained the two independent predictors of a

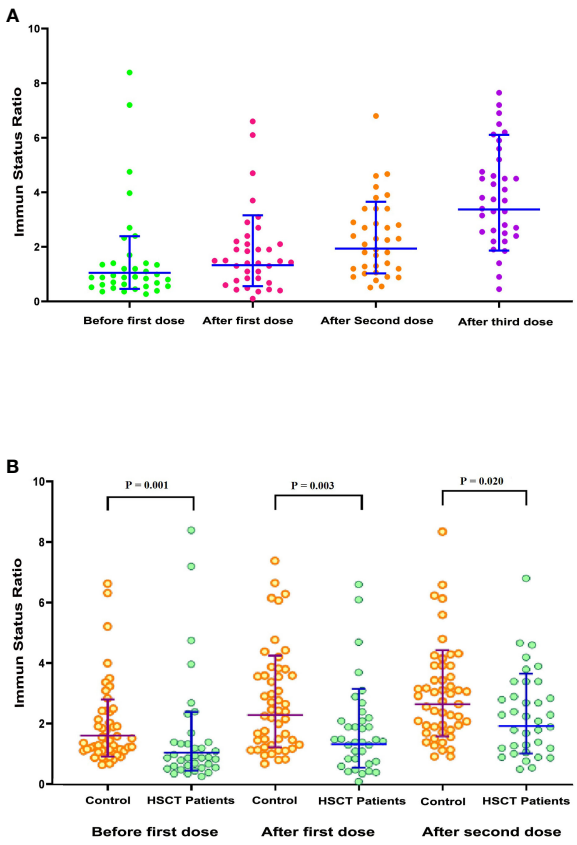

FIGURE 2
Scatterplot of SARS-CoV-2 immune status ratio (ISR). **(A)** Scatterplot of SARS-CoV-2 ISR in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients ($n = 36$) during the predefined samples of before the first dose, after the first dose, after the second dose, and after the third dose. **(B)** Comparison scatterplots of SARS-CoV-2 ISR in allo-HSCT patients ($n = 36$) and healthy participants ($n = 50$) during the planned samples before the first dose, after the first dose, and after the second dose.

TABLE 2 Univariate and multivariable generalized estimating equation (GEE) model to assess the dynamics of the immune status ratio (ISR) during the three doses of the receptor-binding domain (RBD)–tetanus toxoid (TT)-conjugated SARS-CoV-2 vaccination regimen in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients, adjusted for confounding factors.

		Univariate		Multivariable	
		β (95% CI)	*p*-value	β (95% CI)	*p*-value
Vaccine doses	Before the first dose	1			
	After the first dose	0.26 (−0.20 to 0.73)	0.260	0.32 (−0.23 to 0.87)	
	After the second dose	0.76 (0.19 to 1.34)	0.010	0.64 (0.02 to 1.26)	0.041
	After the third dose	2.31 (1.62 to 3.00)	< 0.001	2.36 (1.55 to 3.18)	< 0.001
Patient's sex	Female	1			
	Male	1.15 (0.49 to 1.81)	0.001	0.64 (0.01 to 1.28)	0.051
Donor's sex	Female	1			
	Male	−1.68 (−2.52 to −0.83)	< 0.001	−1.33 (−2.33 to −0.32)	0.010
Donor's age (years)		−0.04 (−0.07 to −0.003)	0.040	−0.14 (−0.42 to 0.02)	0.350
Patient's ISR pre-allo-HSCT status		−0.31 (−0.81 to 0.18)	0.210	−0.12 (−0.52 to 0.29)	0.572
Donor's ISR pre-allo-HSCT status		0.37 (−0.05 to 0.78)	0.080	−0.26 (−0.77 to 0.24)	0.313
Patient's pre-allo-HSCT PCR-positive COVID-19 status	No	1			
	Yes	0.88 (0.09 to 1.66)	0.030	0.65 (−2.33 to 1.58)	0.172

ISR, immune status ratio; allo-HSCT, allogeneic hematopoietic stem cell transplant.

strong immune response following the third dose of vaccine (Table 3).

We lacked a parallel control group of healthy participants. However, to compare the serologic response of healthy individuals with this vaccine platform, we used the results of 50 healthy volunteers (22 females and 28 males) with a mean age of 37.92 years (SD 12.62 years) who had received two doses of the RBD–TT-conjugated SARS-CoV-2 vaccine as part of a phase 3 trial at the Pasteur Institute of Iran (IRCT20210303050558N1). For healthy participants, the serologic response was also measured by semiquantitative immunoassay at baseline and 4 weeks (± 1 week) after each vaccine dose. A scatterplot of ISR values at baseline and following the two doses of RBD–TT-conjugated SARS-CoV-2 vaccine for allo-HSCT patients and healthy participants was created (Figure 2B). In healthy participants, consistent with allo-HSCT patients, the ISR increased significantly following two vaccination doses ($p < 0.001$). However, the ISR was considerably greater in healthy participants than in allo-HSCT recipients at all three available time points.

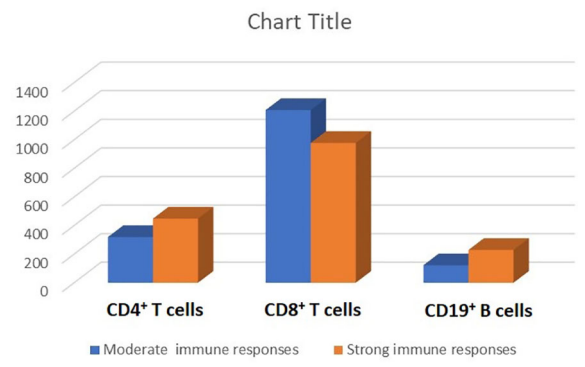

FIGURE 3
Lymphocyte subpopulations (the mean counts of $CD4^+$, $CD8^+$, and $CD19^+$ cells) stratified by the strength of serologic response (i.e., strong vs. moderate) after three doses of the receptor-binding domain (RBD)–tetanus toxoid (TT)-conjugated SARS-CoV-2 vaccine in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients.

3.3 Safety

Figure 4 provides data about vaccine-related side effects. According to the CTCAE, no participant experienced an AE of grade 3 or 4. After the third dose, AEs occurred more frequently than after the second and first doses. Pain or tenderness at the injection site was the most prevalent AE, occurring in 44.5% of participants after the third dose and 22.2% of participants after the first vaccine dose. Fatigue was the second most frequent AE, seen in 27.7% of participants after the third vaccine dose and 11.1% of participants after the first vaccine dose.

During the study period, over a median follow-up period of 242 days (range 162–309 days) from the beginning of vaccination, one patient died after the first dose because of a relapse of their underlying disease, three patients were excluded after the second dose because of worsening of GvHD, and five patients with documented COVID-19 after the first or second doses were also excluded (Figure 1). Over a median follow-up period of 174.5 days (range 106–251 days) from the end of vaccination until the last contact, four PCR-documented COVID-19 infections were reported; these occurred in fully vaccinated patients who

TABLE 3 Univariate and multivariate logistic regression analysis to determine the predictive factors of a strong serologic response following three doses of the receptor-binding domain (RBD)–tetanus toxoid (TT)-conjugated SARS-CoV-2 vaccine in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients.

Effect	Univariate		Multivariate	
	Unadjusted OR (95% CI)	P Value	Adjusted OR (95% CI)	P Value
Patient's Age	0.95 (0.89 - 1.01)	0.103	0.94 (0.85 – 1.04)	0.277
Donor's Age	0.94 (0.88 - 1.00)	0.064	0.95 (0.86 – 1.04)	0.273
Donor's Sex (Female vs. Male)	12.25 (2.54 - 58.96)	0.002	8.67 (1.25 -59.9)	0.028
Donor's ISR Pre-allo-HSCT	3.34 (1.38 - 8.07)	0.007	3.56 (0.98 - 12.89)	0.050
Patient's Pre-allo-HSCT PCR-positive COVID-19	5.50 (1.27- 23.69)	0.022	3.09 (0.35 - 27.05)	0.264
$CD4^+$ cells count at vaccination	1.005 (1.00 -1.01)	0.025	1.006 (0.99 – 1.01)	0.185
$CD19^+$ cells count at vaccination	1.008 (0.99 -1.01)	0.063	0.99 (0.98 – 1.01)	0.947

OR, odds ratio; CI, confidence interval; ISR, Immune status ratio; allo-HSCT, allogeneic hematopoietic stem cell transplant.

presented with mild respiratory symptoms, and no hospitalizations were required.

4 Discussion

The impossibility of easy access to the SARS-CoV-2 mRNA- or adenoviral vector-based platforms and the need for timely immunization of allo-HSCT recipients led us to, for the first time, use an accessible and affordable RBD–TT conjugated SARS-CoV-2 vaccine soon after allo-HSCT. Active surveillance showed that the RBD–TT-conjugated vaccine was generally well tolerated in allo-HSCT recipients. Most of the adverse effects were minor and temporary, that is, equivalent to those reported in the general population for this platform (25, 26) and in allo-HSCT recipients who received mRNA-based platforms (7, 8).

Furthermore, as illustrated in Figure 2A, the serologic response increased considerably following the three doses of the RBD–TT-conjugated SARS-CoV-2 vaccine in allo-HSCT patients over 3 months post allo-HSCT and without ongoing high-grade GvHD, which is similar to the results reported with mRNA-based platforms (13–15). However, the value of ISR was less than healthy individuals at each available time point (Figure 2B). After three doses of the SARS-CoV-2 vaccine, the immune response reached 91.66% at a median duration of 199 days between allo-HSCT and the third vaccine dose. Similarly, Kimura et al. (32) and Watanabe et al. (33) reported that 89.1% and 95% of allo-HSCT patients achieved seroconversion after the third dose of the mRNA-based vaccination, respectively, albeit with a median interval of more than 1 year between allo-HSCT and the third vaccine dose.

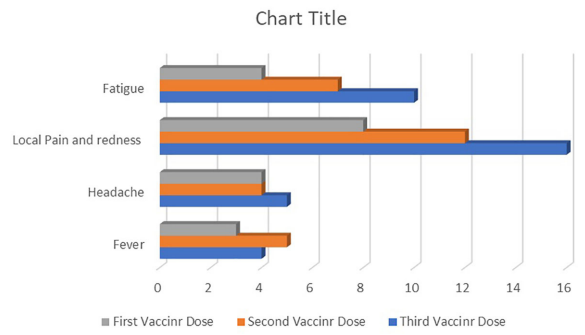

FIGURE 4
The frequency of reactogenic adverse events after the first, second, and third doses of receptor-binding domain (RBD)–tetanus toxoid (TT)-conjugated SARS-CoV-2 vaccine in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients.

Therefore, despite early post-allo-HSCT vaccination, the acceptable seroconversion rate may be partly attributed to the chemical engineering of the vaccine we used. After allo-HSCT, protein-conjugated antigens were found to be more immunogenic than unconjugated ones (34). Notably, TT-conjugated platforms significantly impact early immunogenicity following allo-HSCT (35). The conjugation of RBD to TT has also been shown to promote immune responses to SARS-CoV-2 (23).

Considering the predictors of immune response to the three doses of RBD–TT-conjugated SARS-CoV-2 vaccine early after allo-HSCT, a female donor was a positive predictor for dynamic changes in the ISR during vaccination (Table 2) and a strong immune response after the three doses (Table 3). A higher level of donor immunity at allo-HSCT was the other predictive factor for a strong immune response after the entire course of post-allo-HSCT vaccines (Table 3).

In our study, a stronger immune response after vaccination was not affected by the age of the patients or donors, the length of time between allo-HSCT and vaccination, the presence of GvHD, or the use of immunosuppressive drugs. In contrast, some studies have reported the adverse effects of immunosuppressant medications (33, 36), GvHD (14, 36), and the limited time interval between allo-HSCT and vaccination (15, 33, 36) on the immune response to mRNA-based vaccination in allo-HSCT patients. This disparity may be partly explained by the fact that our patients were vaccinated between 3 and 12 months after allo-HSCT and were reasonably homogeneous in regard to their baseline characteristics. Regarding the optimum time frame for post-allo-HSCT SARS-CoV-2 vaccination, the European and US transplant guidelines advise starting vaccination 3 months after

transplantation (5, 6), despite conflicting findings about the impact of the period between allo-HSCT and vaccination on the vaccine-induced immune response (8).

The potential predictive effect of patients' and donors' immune conditions at the time of allo-HSCT on antibody production following post-allo-HSCT vaccination was particularly interesting. We found that, as evaluated by ISR shortly before harvesting, donor immunity enhanced the immunological response to SARS-CoV-2 vaccination soon after allo-HSCT. Leclerc et al. proposed that the adoptive transfer of memory cells against SARS-CoV-2 from the vaccinated donors to the recipient induces noticeably higher anti-spike receptor-binding domain [RBD] IgG (IgG (S-RBD)) production after post-allo-HSCT vaccination (37).

Furthermore, our findings revealed that 10 out of 36 allo-HSCT patients (27.78%) exhibited positive ISR before post-allo-HSCT vaccination. Regarding this, Jullien et al. (38) demonstrated the persistence of anti-SARS-CoV-2 antibodies for up to 9 months after allo-HSCT in patients immunized before allo-HSCT. As a result, as the limited published evidence implies, vaccinating donors and recipients against SARS-CoV-2 before allo-HSCT might increase the immune response to prompt post-allo-HSCT SARS-CoV-2 revaccination (37–40).

Similar to the results of mRNA-based vaccines in allo-HSCT patients (33, 36, 41, 42), we found that $CD4^+$ T-cell count and $CD4^+/CD8^+$ ratio after the first and third vaccines were correlated with a serologic response after the third dose. The $CD4^+/CD8^+$ ratio and $CD19^+$ B-cell counts were also associated with the strength of immunological response following the third dose (Table 1). Similarly, Clémenceau et al. (43) and Ram et al. (44) suggest that innate immune response is crucial to the immunogenicity of the COVID-19 vaccine, especially in patients vaccinated in the first year following transplantation.

Our findings revealed a positive prognostic effect of donor sex on the immunological response to SARS-CoV-2 vaccination following allo-HSCT. Recent studies in the general population (45, 46) or allo-HSCT patients (9) have demonstrated an association between the female sex and a higher immune response to the SARS-CoV-2 vaccine, which may be reflected in the more robust T-cell activation in females than in males during COVID-19 infection or vaccination (46). To our knowledge, the sex-related SARS-CoV-2 immunity in allo-HSCT patients has only been examined for the recipient's sex. However, the post-allo-HSCT immune response largely depends on the donor's origin.

The following observations should be made on the limits and merits of the study. Our investigation faced constraints owing to its small sample size and single-center design. We could not accurately measure the concentration of anti-S antibodies using the semiquantitative method. We could not form a healthy control group because most of the healthy population had already received the SARS-CoV-2 vaccine. We could not assess cellular immunity as were unable to access the functional assay kit for SARS-CoV-2-specific T-cell responses.

Regarding the study's strengths, our study was conducted on a homogeneous cohort of adult acute leukemia patients who underwent allo-HSCT with the same myeloablative conditioning regimen and graft source. In a constrained window of between 3 and 12 months after transplantation, all patients received three doses of an innovative and widely available RBD–TT-conjugated SARS-CoV-2 vaccine at 4-week intervals. All patients were monitored for reactogenic and non-reactogenic adverse effects regularly and actively.

4.1 Conclusion

In conclusion, we found that three doses of the novel RBD–TT-conjugated SARS-CoV-2 vaccine are safe, highly immunogenic, and affordable for allo-HSCT patients. Our findings indicate that allo-HSCT recipients, particularly in endemic regions, should be offered a full course of COVID-19 vaccination starting 3 months after allo-HSCT, assuming that they do not have high-grade acute GVHD. This suggestion is in line with European and US transplant guidelines. However, following the immunization of allo-HSCT patients, active surveillance is necessary. We further believe that pre-allo-HSCT SARS-CoV-2 immunization in donors may enhance subsequent post-allo-HSCT seroconversion in patients who receive the entire course of the SARS-CoV-2 vaccination during the first year post-allo-HSCT.

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 Ethics Committee of Tehran University's Hematology, Oncology and Stem Cell Transplantation Research Center (IR.TUMS.HORCSCT.REC.1400.021). The patients/participants provided their written informed consent to participate in this study.

Author contributions

MV and MaB contributed to the conception and design of the study. LSA organized the database. FA performed the statistical analysis. MaB wrote the first and final draft of the manuscript. BC, MA, and MRS performed the laboratory evaluation. FA carried out the review and edited the manuscript. TB and MaB contributed to clinical management. AB contributed to the vaccine platform design. 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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mRNA-1273 boost after BNT162b2 vaccination generates comparable SARS-CoV-2-specific functional responses in naïve and COVID-19-recovered individuals

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Introduction: COVID-19 vaccines based on mRNA have represented a revolution in the biomedical research field. The initial two-dose vaccination schedule generates potent humoral and cellular responses, with a massive protective effect against severe COVID-19 and death. Months after this vaccination, levels of antibodies against SARS-CoV-2 waned, and this promoted the recommendation of a third vaccination dose.

Methods: We have performed an integral and longitudinal study of the immunological responses triggered by the booster mRNA-1273 vaccination, in a cohort of health workers previously vaccinated with two doses of the BNT162b2 vaccine at University Hospital La Paz located in Madrid, Spain. Circulating humoral responses and SARS-CoV-2-specific cellular reactions, after *ex vivo* restimulation of both T and B cells (cytokines production, proliferation, class switching), have been analyzed. Importantly, all along these studies, the analyses have been performed comparing naïve and subjects recovered from COVID-19, addressing the influence of a previous infection by

SARS-CoV-2. Furthermore, as the injection of the third vaccination dose was contemporary to the rise of the Omicron BA.1 variant of concern, T- and B-cell-mediated cellular responses have been comparatively analyzed in response to this variant.

Results: All these analyses indicated that differential responses to vaccination due to a previous SARS-CoV-2 infection were balanced following the boost. The increase in circulating humoral responses due to this booster dropped after 6 months, whereas T-cell-mediated responses were more stable along the time. Finally, all the analyzed immunological features were dampened in response to the Omicron variant of concern, particularly late after the booster vaccination.

Conclusion: This work represents a follow-up longitudinal study for almost 1.5 years, analyzing in an integral manner the immunological responses triggered by the prime-boost mRNA-based vaccination schedule against COVID-19.

KEYWORDS

mRNA vaccine, COVID-19 booster, SARS-CoV-2-specific, naïve, recovered from COVID-19

Introduction

Pandemic COVID-19 has impacted worldwide to an unprecedented depth in modern times. The lockdown of entire countries, population confinement, and social distance policies were measures not even imagined before this global crisis (1). Biomedical science has responded to this challenge with the development of effective vaccines that have allowed to recover most of the regular habits known before December 2019. Among these vaccines, mRNA-based jabs have been developed and administered for the first time to human beings, with two different options such as BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna-NIAID) vaccines conferring protection against severe forms of COVID-19 (2). This is due to the triggering of combined B-cell-based humoral responses, along with cellular reactions mediated by T cells. Interestingly, although the relevance of neutralizing antibodies is clear, till which extent both branches of immunity contribute to the actual response against the infection along the time and against the COVID-19 pathology is still a matter of debate. Along these lines, it is important to highlight the importance of an adequate assessment of immunological responses ignited against both SARS-CoV-2 infection and COVID-19 vaccines (3, 4), as this might impact clinically relevant interpretations.

These brand-new mRNA-based vaccines were designed to be administered in a two-dose regimen. We and others have studied the evolution of responses ignited by this vaccination schedule along the time (5, 6). There is a quite established consensus about the generation of potent humoral and cellular responses early after vaccination that decayed after 6–8 months (7). Contemporarily to this waning, a raise of infections due to SARS-CoV-2 variants of concerns (VoC) took place, which, at first, alarmed about till which extent initial vaccines would achieve a proper coverage against them (8). These two main factors accelerated the recommendation for a third vaccination dose or boost. Indeed, studies including large cohorts such as the ZEO COVID study indicated that booster doses restore vaccine effectiveness waned after the second dose, no matter the vaccine initially administered (9). At this point, heterologous vaccination boosts as the one studied in this work, meaning the administration of a third vaccination with a different vaccine than the one administered during the prime phase, showed stronger immunological responses (10, 11). This was accompanied by a reduced incidence of SARS-CoV-2-confirmed infections in individuals receiving heterologous compared with homologous boosting (12). Notably, slight differences observed between the two mRNA vaccines after the two-dose prime phase were balanced with the booster dose (13). Interestingly, the best sequence of heterologous prime-boost schedule appears to be the combination of mRNA vaccines, even against variants of concern (14). Note that in these studies, previous infections by SARS-CoV-2 influenced the responses triggered by prime vaccination, with cellular and humoral reactions differing between naïve and subjects recovered from COVID-19 (5), even after only a single dose of the mRNA vaccine (15–17). Interestingly, strong hybrid immunity due to SARS-CoV-2 infection and the complete initial full vaccination was detected, no matter whether the infection was before or after vaccination (18). Notably, differential immunological patterns observed between the four EMA-approved vaccines against COVID-19 appeared balanced by a prior infection with SARS-CoV-2 (19).

Nevertheless, although it is interesting to address whether responses triggered after the vaccination boost are also influenced by a previous infection with SARS-CoV-2, comparative information of putative differential responses triggered by booster vaccines in naïve and COVID-19 convalescent individuals is scarce (20), most

likely due to the growing difficulty to find participants not previously infected. In this work, we hypothesize that a former infection with SARS-CoV-2 might generate differential responses triggered by the booster vaccine dose.

Considering all these factors, the rising in the incidence in the current autumn and winter, along with the beginning of the administration of a fourth booster dose, in here we have addressed the immunological responses triggered by a heterologous prime-boost regime with BNT162b2 plus mRNA-1273 boost, in a longitudinal cohort for almost 1.5 years. This work represents an integral study of such immunological features including circulating antibodies and T cell- and B cell-mediated cellular responses, against both the wild-type (WT) and Omicron BA.1 (B.1.1.529) VoC, differentiating between naïve and COVID-19-recovered individuals. We believe this is a timely study covering critical immunological features triggered after vaccination, including some not commonly analyzed such as B-cell class-switching and B-cell antigen-specific cytokine production after restimulation, to fully understand protective responses ignited by mRNA-based COVID-19 heterologous vaccination in the long time.

Material and methods

Longitudinal sampling of healthy medical personnel volunteers

Blood samples of 27 healthy medical personnel volunteers of the University Hospital La Paz Institute for Health Research in Madrid (Spain) were collected for this study before and after the vaccination against Spike protein of SARS-CoV-2 according to two doses of BNT162b2 (30 μg) followed by a booster dose of mRNA-1273 (50 μg). Samples were retrieved at seven different time points: 5 days before the first dose of BNT162b2 vaccine (sample 0, N = 27, naïve = 16, recovered = 11), 14 days after the first dose of BNT162b2 vaccine (sample 1, N = 27, naïve = 16, recovered = 11), 14 days after the second dose of BNT162b2 vaccine (sample 2, N = 27, naïve = 16, recovered = 11), 230 days after the second dose of BNT162b2 vaccine (sample 3, N = 27, naïve = 16, recovered = 11), 299 days after the second dose of BNT162b2 vaccine and 5 days before the booster dose of mRNA-1273 vaccine (sample 4, N = 21, naïve = 11, recovered = 10), 318 days after the second dose of BNT162b2 vaccine and 14 days after the booster dose of mRNA-1273 vaccine (sample 5, N = 17, naïve = 9, recovered = 8), and 493 days after the second dose of BNT162b2 vaccine and 189 days after the booster dose of mRNA-1273 vaccine (sample 6, N = 20, naïve = 8, recovered = 12) (Figure 1A). Note that all the participants were gathered exactly at the indicated time points to collect their blood samples. According to the ethical guidelines of the 1975 Declaration of Helsinki, we collected the informed consent from all healthy medical personnel volunteers in obedience with the ethical standards stablished. The study was authorized by the La Paz University Hospital Research Ethics Committee (PI-4100).

PBMC isolation procedure and culture conditions

Peripheral blood mononuclear cells (PBMCs) from BNT162b2 two-dose- and mRNA-1273 booster-vaccinated healthy medical personnel volunteers were obtained from venous blood with EDTA anticoagulant, using Ficoll-Plus (GE Healthcare Bio-Sciences, Chicago, IL) solution based on the manufacturer's instructions. PBMCs were counted using Trypan blue staining after washing them twice with phosphate-buffered saline (PBS). Before some stimulations such as activation or proliferation, the culture of fresh PBMCs was plated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, and 1% streptomycin and penicillin mix (Gibco, Billings, MT). A humidified incubator at 37°C at 5% CO_2 was used to culture fresh PBMCs.

Plasma collection

Plasma samples from healthy medical personnel vaccinated with BNT162b2 two-dose and mRNA-1273 boosters were obtained from venous blood with EDTA anticoagulant using a density gradient of Ficoll-Plus solution following the typical centrifugation method. Subsequently, they were aliquoted and stored at -80°C until use.

Anti-SARS-CoV-2 IgA and IgG antibody quantification

For the quantification of specific IgA and IgG antibodies against Spike protein of SARS-CoV-2, saved frozen plasma samples from healthy medical personnel vaccinated with BNT162b2 two-dose and mRNA-1273 boosters were thawed. Prior to use, all plasma samples were centrifuged at 1,000 relative centrifugal force (rcf) for 30 min to remove possible debris. Two bead-based immunoassays, both from BioLegend (San Diego, CA), the LEGENDplex SARS-CoV-2 Serological IgA Panel (2-plex, Spike (S1) and receptor binding domain (RBD) of Spike protein) and LEGENDplex SARS-CoV-2 Serological IgG Panel (3-plex, Spike (S1), receptor binding domain (RBD) of Spike protein and nucleocapsid (N)), were used following the manufacturer's instructions to quantify the titers of IgA and IgG antibodies, respectively, in plasma samples. A FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) were used to acquire the samples, and data were analyzed by LEGENDplex (BioLegend) v.8 software. The presence of anti-SARS-CoV-2 N immunoglobulins, indicative of a former SARS-CoV-2 viral infection, was confirmed by the CE-IVD-marked system INgezim COVID 19 DR50.CoV.KO provided by Eurofins Ingenasa.

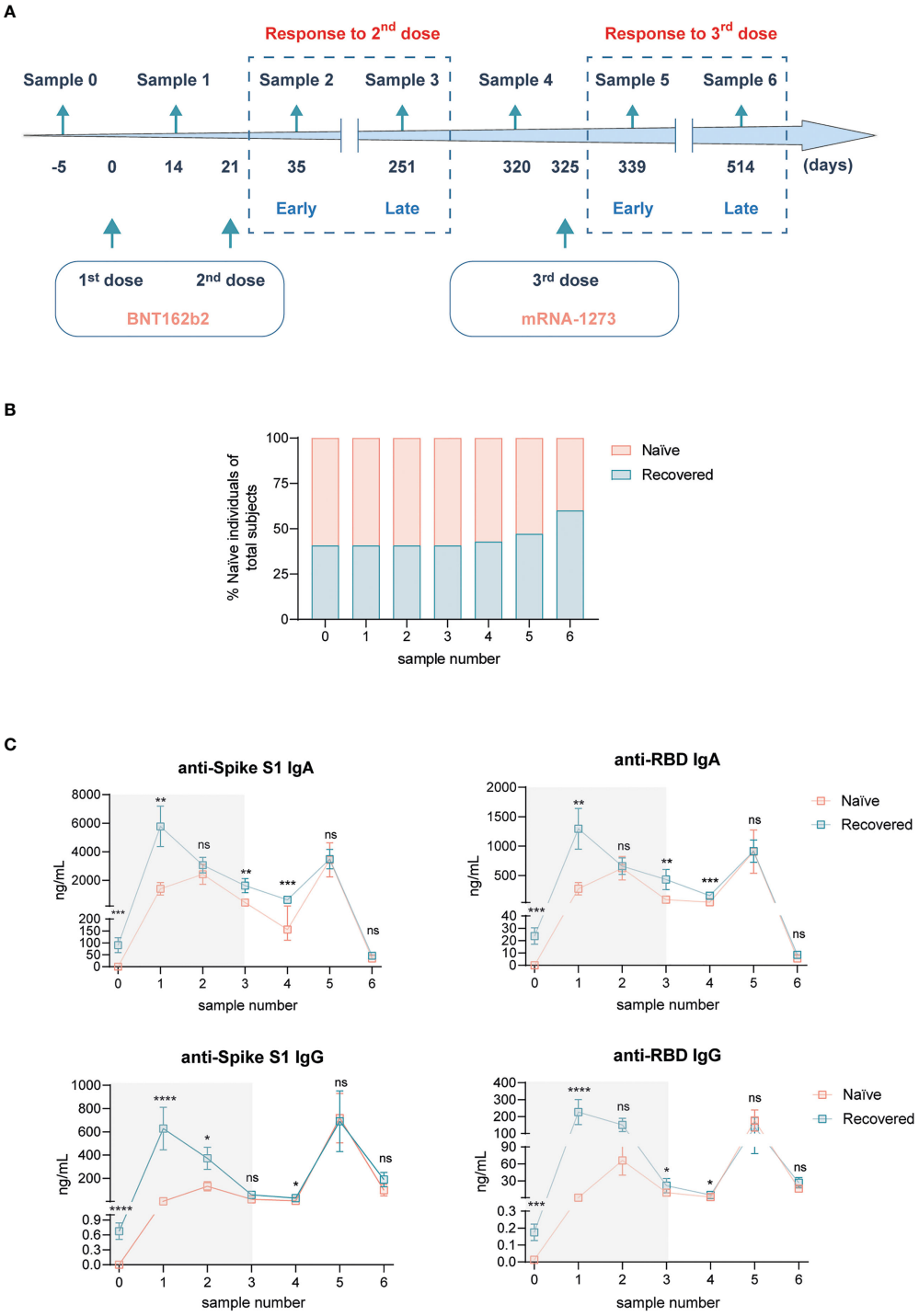

FIGURE 1
Humoral response Spike-specific of SARS-CoV-2 subsequent to BNT162b2 mRNA vaccination plus mRNA-1273 booster dose in naïve individuals and individuals recovered from COVID-19. **(A)** Experimental design. Blood samples from healthy medical personnel were collected 5 days before the first dose of BNT162b2 mRNA vaccine (sample 0), 14 days after the first dose of BNT162b2 mRNA vaccine (sample 1), 14 days (sample 2, 35 days from the beginning), 230 days (sample 3, 251 days from the beginning), and 69 days (sample 4, 320 days from the beginning) after the second dose of BNT162b2 mRNA vaccine. Subsequent blood samples were collected 14 days (sample 5, 339 days from the beginning) and 189 days (sample 6, 514 days from the beginning) following the administration of the mRNA-1273 booster dose. **(B)** Frequency of naïve participants and recovered from COVID-19 along the study. **(C)** Plasmatic levels of anti-Spike S1 IgA (upper left panel), anti-receptor binding domain (RBD) IgA (upper right panel), anti-Spike S1 IgG (lower left panel), and anti-RBD IgG (lower right panel) antibodies. Shadowed areas represent analyses performed in (5). n = 16 naïve, n = 11 recovered from COVID-19 at sample 0. Data in **(C)** are shown as mean ± SEM. Unpaired Student's *t*-test in each sample number between naïve and COVID-19-recovered subjects (ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). In every plot, all the possible experimental conditions were compared; statistically significant differences are indicated, and the lack of statistical indication means the absence of a significant difference.

Supernatant collection and SARS-CoV-2 Spike-specific T-cell proliferation assays

PBMCs from healthy medical personnel, 14 (sample 2) and 230 (sample 3) days after the second dose of the BNT162b2 vaccine, and 14 (sample 5) and 189 (sample 6) after the booster dose of the mRNA-1273 vaccine, were obtained from venous blood with EDTA anticoagulant using Ficoll-Plus based on the manufacturer's instructions. PBMCs were counted using Trypan blue staining after washing them twice with phosphate-buffered saline (PBS) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, and 1% penicillin and streptomycin mix. To evaluate the T lymphocyte proliferation, PBMCs were stained with carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. Next, CFSE-labeled PBMCs were plated in a 96-well flat-bottom plate (1.5×10^6 cells/well) in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, and 1% penicillin and streptomycin mix. CFSE-labeled PBMCs of samples 2, 3, 5, and 6 were stimulated or not with PepTivator SARS-CoV-2 Prot_S (Miltenyi Biotec, Bergisch Gladbach, Germany) for 5 days to allow T-cell proliferation at 37°C at 5% CO_2, whereas samples 5 and 6 were also stimulated or not with PepTivator SARS-CoV-2 Prot_S B.1.1.529/BA.1 wild-type reference pool or PepTivator SARS-CoV-2 Prot_S B.1.1.529/BA.1 mutation pool (both from Miltenyi Biotec) for 5 days to allow T-cell proliferation at 37°C at 5% CO_2. Note that both *PepTivator SARS-CoV-2 Prot_S* and *PepTivator SARS-CoV-2 Prot_S B.1.1.529/BA.1 wild-type reference pool* represent the sequence of the WT/Wuhan SARS-CoV-2 strain, nevertheless covering different regions of the Spike protein. Subsequently to the proliferation assay, supernatants were taken, aliquoted, and stored at -80°C until use. For the PBMC staining, PBMCs were washed with PBS and stained with fluorochrome-conjugated antibodies against surface markers listed in Supplementary Table 1. LIVE/DEAD Blue fluorescent reactive dye purchased from Invitrogen was used to exclude the debris and dead cells. To avoid the non-specific binding of certain fluorochromes on monocytes, the True-Stain Monocyte Blocker (BioLegend) reagent was added prior to the label protocol. A Cytek Aurora Spectral Cytometer (Cytek Biosciences, Fremont, CA) were used to acquire the labeled cells, and data were analyzed using FlowJo (TreeStar, Ashland, OR) v10.6.2 software.

Antibody-secreting cell generation and their functional assessment against wild-type and BA.1 Omicron variant Spike SARS-CoV-2 proteins

PBMCs from healthy medical personnel 493 days after the second dose of BNT162b2 vaccine and 189 days after the booster dose of mRNA-1273 SARS-CoV-2 mRNA vaccine (sample 6) were obtained from venous blood with EDTA anticoagulant using Ficoll-Plus following the manufacturer's instructions. PBMCs were counted using Trypan blue staining after washing them twice with phosphate-buffered saline (PBS). Following the PBMC counting, 6×10^6 PBMCs were plated in a 24-well flat-bottom plate (1.5×10^6 cells/well) in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, and 1% penicillin and streptomycin mix. They were stimulated with IL-4 (70 ng/ml, PeproTech, Cranbury, NJ) and an agonistic mouse anti-human CD40 (5 µg/ml, BioGems, Seoul, South Korea) for 6 days to perform a polyclonal stimulation of memory B cells (MBCs). High-binding 96-well plates were prehydrated with 150 µl of PBS and then coated with 50 µl of bovine serum albumin (BSA, 5 µg/ml), the recombinant wild-type SARS-CoV-2 spike full protein ECD (5 µg/ml, Sino Biological, Beijing, China), or the recombinant B.1.1.529/BA.1 Omicron variant of SARS-CoV-2 spike full protein ECD (5 µg/ml, Sino Biological) overnight at 4°C. Supernatants were removed from the coated wells and washed twice with PBS and blocked with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, and 1% penicillin and streptomycin mix for 1 h at 37°C at 5% CO_2 in a humidified incubator. After that, in a volume of 200 µl/well, 1.5×10^5 polyclonal stimulated memory B cells were seeded to evaluate the functional spike-specific memory B-cell response for 44 h at 37°C at 5% CO_2. After the functional Spike-specific memory B-cell assay, supernatants were obtained, aliquoted, and stored at -80°C until use. Cells were recovered and stained for surface markers with fluorochrome-conjugated antibodies listed in Supplementary Table 2.

Cytokine quantification in supernatants

Based on a bead-based immunoassay, LEGENDplex Human Essential Immune Response Panel (13-plex: IL-1β, IL-2, IL-4, IFNγ, TNF-α, MCP-1 (CCL2), CXCL10, IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A, and free active TGF-β1) was used to quantify the concentration of cytokines in supernatant samples from both T and B Spike-specific stimulations, following the manufacturer's instructions. A FACSCalibur flow cytometer was used to acquire the samples, and data were analyzed by the LEGENDplex v.8 software.

Statistical analysis of biological data

Continuous measurements are expressed as mean ± SEM. To assess the normality distribution to all studied variables, a D'Agostino and Pearson normality test was performed. For two-group comparisons of quantitative variables, a Student's *t*-test either unpaired (*t*-test or Mann–Whitney *U*) or paired (*t*-test or Wilcoxon) was performed, and for multiple-group comparisons of quantitative variables, an ANOVA or Kruskal–Wallis *H* was performed. For all figures, *p*-values are shown as ns: non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$, $\#\#\#\#p < 0.0001$. Note that in every plot of Figures 2 and 3, all the possible experimental conditions were compared; statistically significant differences are indicated, and

the lack of statistical indication means the absence of a significant difference.

Results

Boost mRNA vaccination (third dose) equalizes titers of SARS-CoV-2-specific antibodies between naïve and COVID-19-recovered individuals

After receiving the BNT162b2 two-dose vaccination schedule recommended by both the FDA and EMA (5), in this work we have explored the SARS-CoV-2-specific responses triggered by the boost with the COVID-19 vaccine mRNA-1273 in this well-controlled cohort. Following the two BNT162b2 doses, both early (14 days) and late (230 days ≈ 8 months) responses were addressed, labeled as samples 2 and 3, respectively (5). 69 days afterward, meaning 320 days since the starting point of the study, sample 4 was obtained to monitor the steady-state status 5 days before receiving the mRNA-1273 boost (Figure 1A). Mirroring the schedule followed for the two first doses, 14 days after the third jab, blood samples were obtained (sample 5) to study early responses. Eventually, 189 days (≈ 6 months) post-boost, a new sample was analyzed (sample 6) to monitor late responses to this third vaccination dose (Figure 1A). This scheme represents a longitudinal study for almost 1.5 years since the beginning of the vaccination, exploring SARS-CoV-2-specific responses in the same cohort of individuals (5). Hence, a total of 27 individuals received the mRNA-1273 boost. Among them, there was an almost 50% distribution between those that had not been previously exposed to SARS-CoV-2 infection (naïve) and those that had recovered from COVID-19 (Figure 1B). Nonetheless, the analysis of antibodies against SARS-CoV-2 N protein (data not shown) indicated a slight rise in the proportion of recovered participants in samples 4 and 5, which was further increased when analyzing late responses to mRNA-1273 boost in sample 6 (Figure 1B). Still, differential responses between individuals exposed or not to SARS-CoV-2 infection could be analyzed.

First, we continued the analysis of humoral responses to vaccination by means of plasma SARS-CoV-2-specific immunoglobulin quantification (Figure 1C). Shadowed results represent the already known evolution of such antibodies after the two initial doses of the BNT162b2 vaccine (5). More than 2 months after the last analysis and right before the mRNA-1273 boost, the levels of all antibodies dropped slightly, but the presence of higher titers in COVID-19-recovered individuals was still present (Figure 1C). As expected, the vaccination boost increased notoriously the concentrations of all analyzed antibodies. Interestingly, early after this boost (sample 5), the levels of antibodies were comparable with those obtained following the first vaccination jab, suggesting a maximum capacity of production. Eventually, the analysis of this humoral responses 6 months after boost showed that IgA titers were essentially negligible, whereas IgG dropped till baseline levels detected before the boost (Figure 1C). Of note, the levels of antibodies against SARS-CoV-2, both early and late after the third vaccination dose, were equivalent between naïve and subjects recovered from COVID-19 (Figure 1C).

mRNA-1273 boost maintains the SARS-CoV-2-specific T-cell responses triggered by the second vaccination dose

Next, SARS-CoV-2-specific T-cell responses were evaluated. Peripheral blood mononuclear cells (PBMCs) from both naïve and COVID-19-recovered participants were *ex vivo* stimulated with a peptide pool including the SARS-CoV-2 spike protein from the wild-type (WT) viral variant, hereafter called the S-peptide. Antigen-specific responses were evaluated in terms of cytokine production and T-cell proliferation (Figure 2A). Stimuli were performed for 5 days to allow T-cell proliferation (Supplementary Figure 1).

Among the cytokines analyzed in response to S-peptide, some of them such as TNFα, IL-6, IL-2, IFNγ, or CXCL10 were differentially produced between naïve subjects and subjects recovered from COVID-19 early after the second vaccination dose (sample 2), with higher levels in the former group. However, following this early response, no further differences were found due to the SARS-CoV-2 infection between naïve and COVID-19-recovered individuals along the study (Figure 2B).

Then, we analyzed the longitudinal behavior of those cytokines induced in response to S-peptide stimulation (Figure 2B), as some of them were not produced in an antigen-specific manner (Supplementary Figure 2). Worthy of note is that SARS-CoV-2-specific T-cell responses following the second vaccination dose dropped along the time, with the only exception of IL-1β production late after the boost jab (Figure 2B).

The parallel analysis of T-cell proliferation corroborated this pattern. S-peptide induced $CD4^+$ T-cell proliferation to a higher extent in naïve than in subjects recovered from COVID-19 early after the second vaccination dose, with a similar trend in $CD8^+$ T cells (Figure 2C). This differential response not only disappear along the time but also showed a light decreasing longitudinal pattern (Figure 2C). Importantly, the vaccination boost maintained the SARS-CoV-2-specific T-cell-mediated proliferation, with a slight decrease late after boost in recovered individuals (Figure 2C). The analysis of T-cell memory populations comparing samples 5 and 6 after boosting suggests a more dynamic behavior in COVID-19-recovered individuals, with reduced central memory $CD4^+$ T and naïve $CD8^+$ cells and a concomitant increase in effector memory $CD8^+$ T cells (Supplementary Figure 3).

Therefore, since late after the second vaccination dose onward, SARS-CoV-2-specific T-cell responses got mostly balanced between naïve individuals and subjects that had recovered from COVID-19. Furthermore, these responses triggered by the second vaccination dose were essentially maintained or slightly decreased equally in both groups along the time.

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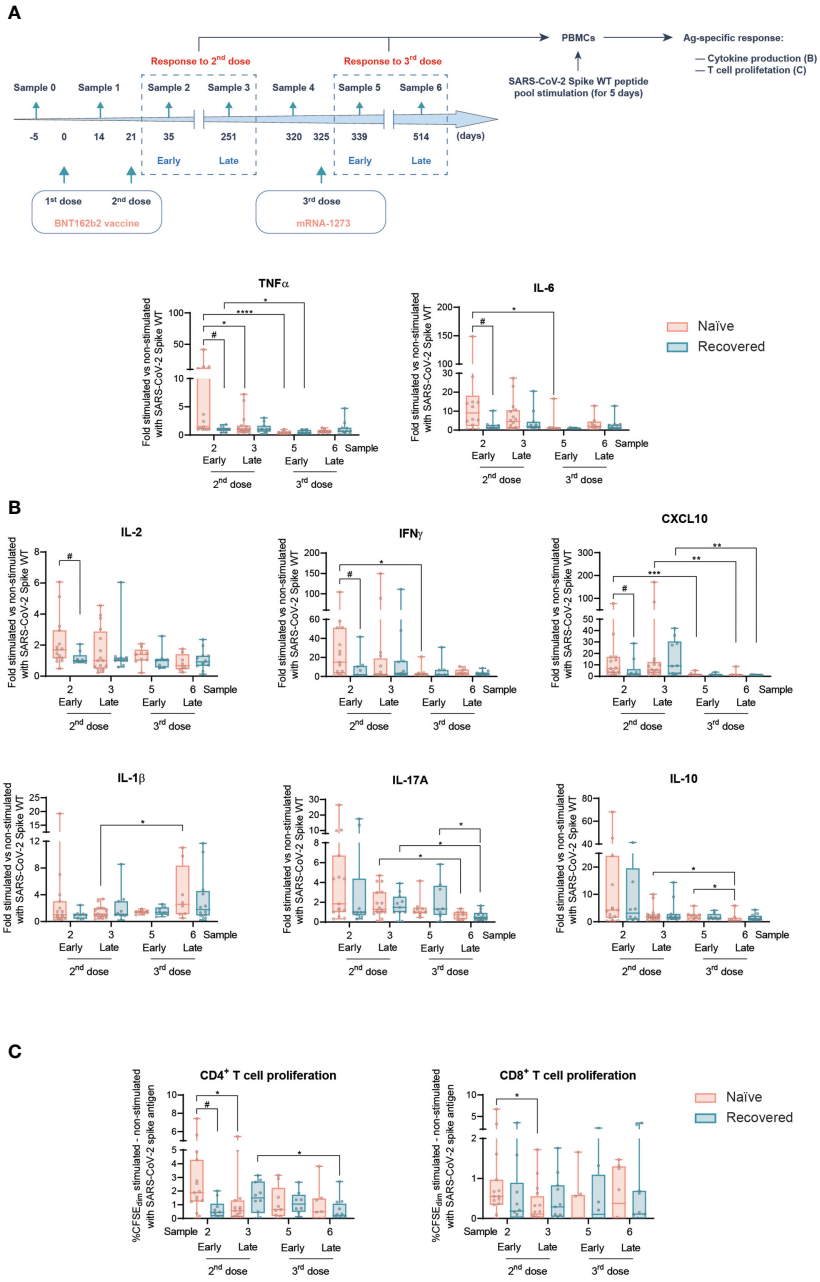

FIGURE 2
Ex vivo T cell-mediated cellular response Spike-specific of SARS-CoV-2 following prime boost vaccination in naïve and individuals recovered from COVID-19. **(A)** Experimental design of the *ex vivo* T cell-mediated cellular responses in PBMCs, to address early and late responses to the second and third (boost) vaccination, after stimulation with the SARS-CoV-2 wild-type (WT) Spike peptide pool. **(B)** TNFα, IL-6, IL-2, IFNγ, CXCL10, IL-1β, IL-17A, and IL-10 production in naïve participants and recovered from COVID-19 at the indicated sample numbers. **(C)** Increment of proliferation ($CFSE^{dim}$) comparing SARS-CoV-2 Spike peptide pool-stimulated and non-stimulated $CD4^+$ (left panel) and $CD8^+$ (right panel) T cells in naïve participants and those recovered from COVID-19 at the indicated sample numbers. Data in **(B, C)** are shown as mean ± min to max. Each dot represents a single participant. Unpaired Student's *t*-test or Mann–Whitney test according to normality, comparing naïve and subjects recovered from COVID-19 at each sample (#$p < 0.05$). Paired Student's *t*-test or Wilcoxon test according to normality, comparing samples along the time in naïve subjects and those recovered from COVID-19 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). In every plot, all the possible experimental conditions were compared; statistically significant differences are indicated, and the lack of statistical indication means the absence of a significant difference.

Dampened SARS-CoV-2-specific T-cell responses against the Omicron BA.1 variant are comparable between naïve and COVID-19-recovered subjects

Next, we wanted to evaluate SARS-CoV-2-specific T-cell responses after the booster jab against the Omicron BA.1 variant of concern, as the vaccination schedule with this third dose coincided temporally with the rise of this variant. Note that, due to the large mutation load contained in the Spike BA.1 variant (21), the WT pool of peptides used in here is different from previous experiments and represents the actual WT reference for the Spike BA.1 variant. Similarly, as observed previously at late time points (Figure 2), the WT S-peptide-specific responses showed a limited strength for most of the analyzed cytokines, with essentially no differences between naïve individuals and subjects recovered from COVID-19, along with a decreasing profile between early (sample 5) and late (sample 6) responses (Figure 3A). Importantly, for some of the analyzed cytokines such as IL-2, IL-10, IL-1β, IFNγ, and CCL-2 (Figure 3A) and even for cytokines not induced after S-peptide stimulation such as IL-6 or TGFβ (Supplementary Figure 4A), the Omicron BA.1 pool of peptides generated a poorer response in sample 6. These data suggested that this variant of concern triggered dampened T-cell-mediated responses, in particular late after the boost vaccine dose. Considering the equivalent responses between naïve subjects and those recovered from COVID-19 following the booster vaccination, we decided to analyze the readouts against Omicron BA.1 combining both groups of participants, in an attempt to have a more robust information about the responses triggered by this variant of concern. This analysis reinforced the drop in T-cell-mediated responses late (sample 6) after the third vaccination dose (Figure 3B). At the same time, it also showed that the responses to Omicron BA.1 were lower than its corresponding WT. Interestingly, this was more remarkable at the late time point (sample 6) after the vaccination boost, with five cytokines (IL-4, IL-2, IL-1b, IL-8, and TGFβ) out of seven differentially expressed between WT and BA.1 showing this temporal pattern (Figure 3B and Supplementary Figure 4B). T-cell proliferation was also analyzed in these contexts, but most likely due to the limited activation induced by S-peptides in these late samples, no statistically significant differences were detected (Supplementary Figure 5).

Omicron BA.1 variant generates reduced functional responses in B cells

Due to the relevance of humoral responses against SARS-CoV-2 infection and considering that the titers of specific antibodies late after the vaccination boost (sample 6) were dramatically reduced (Figure 1C), we decided to explore the functionality of B cells after reexposing them *ex vivo* to Spike protein, both WT and Omicron BA.1 variants of concern. In this sense, it is important to consider the dampened reactions observed in T-cell activity against the Omicron BA.1 variant of concern. Considering the apparent power of our experimental approach to detect differential behaviors by analyzing the production of cytokines (Figure 3), we decided to address SARS-CoV-2-specific functional responses in B cells against both WT and BA.1 variants using this methodology. First, we generated antibody-secreting cells (ASCs) from PBMCs by polyclonal stimulation of memory B cells (MBCs) with IL-4 and anti-CD40. Then, these cells were stimulated in an antigen-specific manner with plate-coated Spike protein, either WT or Omicron BA.1, analyzing the phenotype of the resulting ASCs and their cytokine production (Figure 4A).

Following the analysis described for B cells in a full-spectrum flow cytometry (22) (Supplementary Figure 6A), we observed that the stimulation with Spike protein either WT or Omicron BA.1 was innocuous in the IgG/IgM ratio and frequency of plasmablast or transitional cells (Figure 4B). However, WT Spike induced a class switch in ASCs that was absent in BA.1-stimulated cells (Figure 4B). Therefore, this analysis suggested a dampened response to the Omicron variant also in terms of B-cell activation.

We next analyzed the cytokine production by these cells in response to both Spike protein variants. Following the same pattern observed for class-switching, the production of nearly all the induced cytokines in response to the WT Spike protein (TNFα, IL-2, IL-4, CCL2, IL-10, and IFNγ) was reduced or even absent in response to Omicron BA.1 Spike protein (Figure 4C). This was also the case for IL-8 among the not-induced cytokines due to stimulation (Supplementary Figure 6B).

Altogether, these data indicate that functional responses of B cells against the Spike protein from the SARS-CoV-2 Omicron BA.1 variant of concern are dampened compared with those triggered in response to the Spike WT.

Discussion

This work represents a longitudinal study of responses triggered by a heterologous prime-boost vaccination scheme with mRNA vaccines against COVID-19, for almost 1.5 years. The study includes a comprehensive survey of immunological features including humoral responses in terms of circulating SARS-CoV-2-specific antibodies, T-cell-mediated reactions after *ex vivo* SARS-CoV-2-specific re-stimulation, as well as the analysis of B-cell-based cellular responses after polyclonal differentiation of antibodies secreting cells from PBMCs, and subsequent *ex vivo* SARS-CoV-2-specific restimulation, analyzing the phenotype of the differentiated cells and their cytokine production profile. All these parameters have been analyzed comparing the behavior observed in naïve subjects and participants recovered from COVID-19, also including the comparative study of responses triggered by the original wild-type (WT) version of the SARS-CoV-2 Spike protein and the Omicron BA.1 variant of concern (VoC). Worthy of note is that this study has been performed based on a cohort of healthy health workers, all vaccinated at the same time, with the same vaccines and vaccination schedule, and including only COVID-19-recovered individuals that suffered from mild disease, who did not require clinical intervention. Therefore, considering the impact of different determinants on the immunological responses to SARS-CoV-2 infection and COVID-19 vaccination (23, 24), this constitutes a well-standardized cohort. To the best of our knowledge, this represents a unique integral immunological

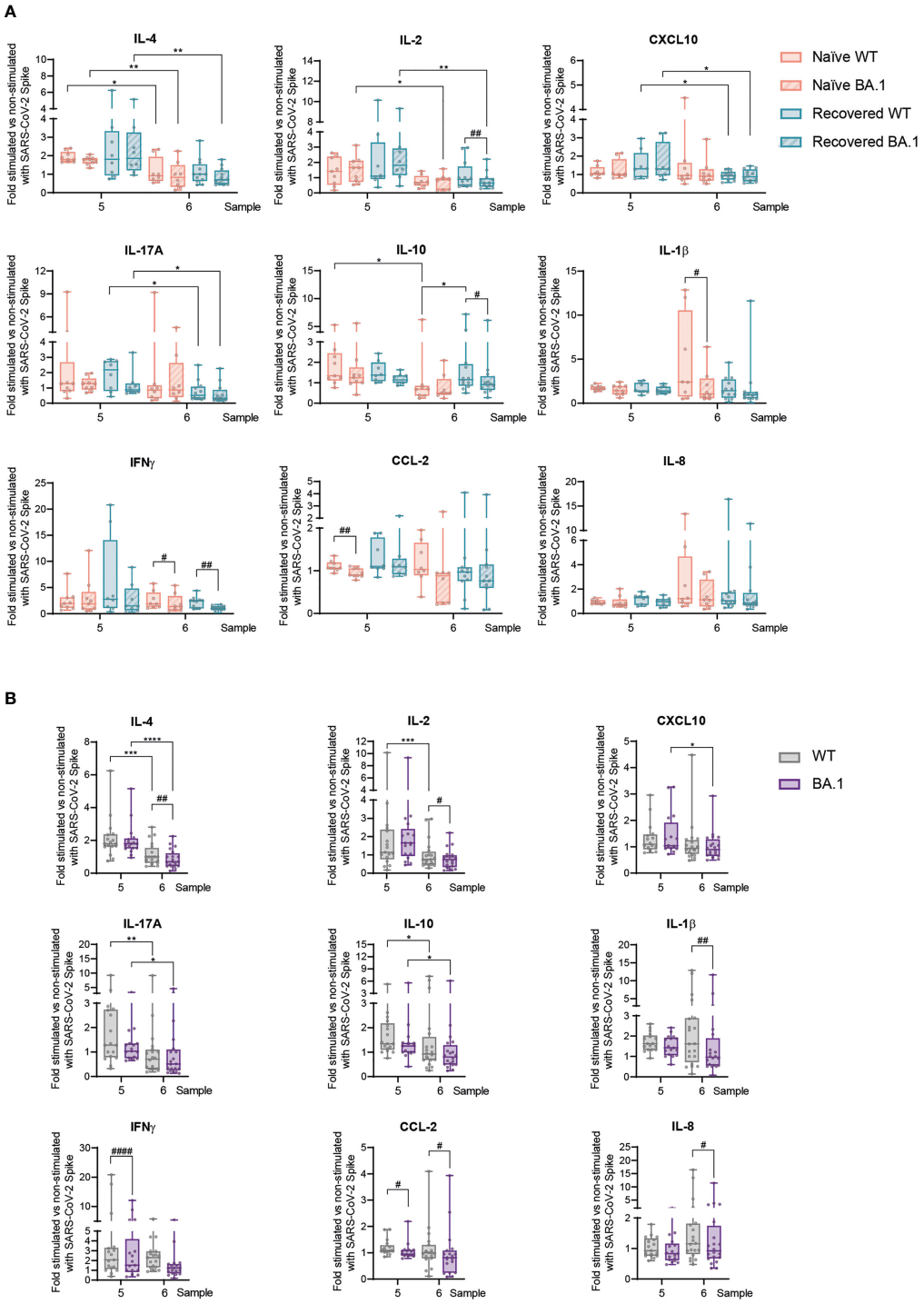

FIGURE 3
T-cell *ex vivo* responses against SARS-CoV-2 Spike wild type and Omicron BA.1 following prime-boost vaccination in naïve individuals and those recovered from COVID-19. Following the scheme shown in Figure 2A, responses to the third vaccination dose, both early and late, were studied in response to either the SARS-CoV-2 wild-type (WT) or Omicron BA.1 variant Spike peptide pool. **(A)** IL-4, IL-2, CXCL10, IL-17A, IL-10, IL-1β, IFNγ, CCL-2, and IL-8 production in naïve participants and those recovered from COVID-19 at the indicated sample numbers and Spike variant. **(B)** IL-4, IL-2, CXCL10, IL-17A, IL-10, IL-1β, IFNγ, CCL-2, and IL-8 production in pooled participants at the indicated sample number. Data are shown as mean ± min to max. Each dot represents a single participant. **(A)**, paired Student's *t*-test or Wilcoxon test according to normality, comparing samples 5 and 6 in naïve subjects and those recovered from COVID-19 (*$p < 0.05$; **$p < 0.01$), or WT *vs*. BA.1 inside each group of participants (#$p < 0.05$; ##$p < 0.01$). **(B)**, unpaired Student's *t*-test or Mann–Whitney test according to normality, comparing samples 5 and 6 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$), or WT *vs*. BA.1 inside each participant group (#$p < 0.05$; ##$p < 0.01$; ####$p < 0.0001$). In every plot, all the possible experimental conditions were compared; statistically significant differences are indicated, and the lack of statistical indication means the absence of a significant difference.

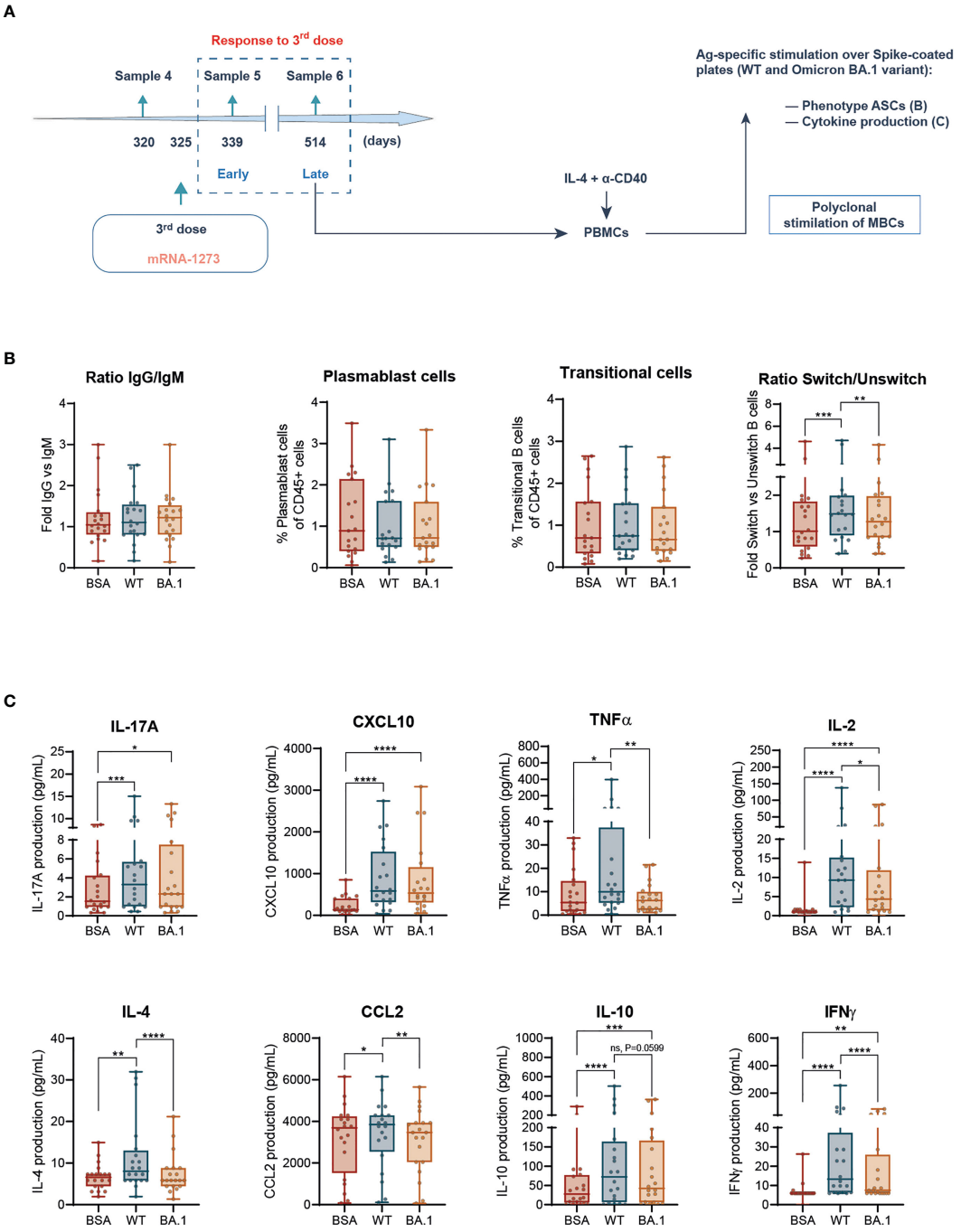

FIGURE 4
B-cell *ex vivo* responses against SARS-CoV-2 Spike wild type and Omicron BA.1 following prime-boost vaccination. **(A)** Experimental design of the B cell-mediated cellular responses *ex vivo* in PBMCs, after polyclonal stimulation of memory B cells (MBCs) with IL-4 + anti-CD40, to address late responses (sample 6) to the third (boost) vaccination dose. Following this step, generated antibody-producing cells were counted and plated on Spike-coated plates, either the WT or Omicron BA.1 variant of concern. BSA-coated plates were used as negative control. **(B)** Ratio of IgG/IgM in IgD- memory B cells, frequency of $CD27^{+}$ $CD20^{-}$ plasmablasts, and $IgD^{-}CD38^{+}$ transitional cells and ratio between class switched versus unswitched $CD19^{high}$ $CD20^{high}$ cells. **(C)** IL-17A, CXCL10, TNFα, IL-2, IL-4, CCL-2, IL-10, and IFNγ production in pooled participants following stimulation with plated BSA, or SARS-CoV-2 Spike protein, either WT or Omicron BA.1. Data are shown as mean ± min to max. Each dot represents a single participant. **(B, C)**, paired Student's *t*-test or Wilcoxon test according to normality, comparing stimuli (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). In every plot, all the possible experimental conditions were compared; statistically significant differences are indicated, and the lack of statistical indication means the absence of a significant difference.

study performed on the basis of such a long longitudinal scheme, addressing SARS-CoV-2-specific responses after prime-boost COVID-19 vaccination.

Alternative longitudinal studies support some of the information provided by our work, nevertheless segmented. Most of the studies focused their longitudinal findings in the quantification of circulating SARS-CoV-2-specific antibodies, mainly addressing the differential responses triggered against WT and Omicron Spike proteins. This is consistent with our design, as booster vaccination doses were contemporary to the rise of the Omicron BA.1 VoC (World Health Organization, 2022). Still, for most of these studies, the longitudinal duration is limited compared with ours, with analyses following boost vaccination for 1 month (25–27), 4 months (28), or 6 months (29), the latter in a comparable scheme to our study. The longest study that we have found lasted for 8 months following the booster dose, analyzing the efficacy of a rapid lateral flow assay to detect antibodies against the SARS-CoV-2 Spike receptor binding domain (RBD) (30). Importantly, none of these studies differentiate between naïve and participants recovered from COVID-19. Our data indicate that until receiving the booster vaccine dose, humoral responses were stronger in those individuals with a previous infection with SARS-CoV-2. On the other hand, early T-cell responses after the two first doses were dampened in COVID-19-recovered individuals, which might be associated with the generation of immunomodulatory T regulatory cells (5) and/or T-cell exhaustion due to SARS-CoV-2 infection, as described elsewhere (31–33). Nevertheless, T-cell-mediated responses were comparable in both naïve and recovered participants following 7 months after the second vaccine jab. Therefore, considering a previous infection by SARS-CoV-2 is relevant to fully understanding the effects triggered by prime-boost COVID-19 vaccination schedules.

The observed differential behaviors between humoral and T-cell-mediated responses are consistent with former studies. The booster effect of the third vaccination dose on the production of SARS-CoV-2-specific antibodies is key to supporting the recommendation for this recall vaccination. Furthermore, those studies analyzing in parallel humoral and T-cell responses concur with our data regarding the stable behavior of cellular reactions late after two vaccination doses (20, 26). Whether this effect has something to do with certain T-cell exhaustion requires further deeper analyses, as this effect has been mostly studied in the context of acute and severe SARS-CoV-2 infections (32, 34). As T-cell activation has been shown to correlate with reinfection (35), and infection is not rare in vaccinated populations, it is tempting to speculate that vaccine-triggered humoral responses are fundamental for the protection versus severe COVID-19, whereas cellular reactions are more decisive for reinfection, although both systems work in a concerted manner. However, this interpretation could be misleading considering that this analysis is performed during waves of different virus strains that might differentially impact both branches of immunity (36).

Along these lines, the rise of the Omicron BA.1 VoC threated the *status quo* generated by the initial two-dose vaccination schedules, due to the heavy load of mutations carried by this variant in the Spike protein and in the RBD specifically (37), which is a major target for neutralizing antibodies generated after vaccination. Very early after its characterization, it was clear that Omicron BA.1 had the capacity to escape from neutralizing antibodies (38), although vaccination was still partially efficient against this variant (39). Our data and the abovementioned longitudinal studies (27–29) corroborated this fact. However, our study provides an extra layer of complexity with the analysis of cellular functions triggered by antibody-secreting cells (ASCs). Functional interrogation *ex vivo* of these cells represents a powerful tool to identify patterns of response *in vivo* (40). Nevertheless, knowledge about functionality of B cells after specific restimulation beyond detection of SARS-CoV-2-specific BCR-expressing cells (26, 41) is scarce, in particular after such a long period following the booster vaccination, and in response to the Omicron VoC. In line with these results, no differences were found in T-cell proliferation but detected in cytokine production after T-cell activation following the booster vaccination. Furthermore, antibody-independent functions of B cells, mainly cytokine production, can play essential roles in homeostasis, activation of lymphoid organs, and development of T-cell responses (42), with relevance as therapeutic targets (43). These results highlight the relevance of extending the range of functional readouts to fully address differential responses, even in conditions of low strength activation. Thus, it appears critical to perform SARS-CoV-2-specific activation approaches to uncover functional information, beyond the characterization of antigen-specific receptor-expressing cells.

Based on these functional analyses, we have confirmed that responses triggered by the Spike protein from the Omicron BA.1 (B.1.1.529/BA.1) VoC are attenuated compared with its reference WT. These results go in line with the current knowledge (20, 26) and has prompted the recommendation of a second vaccine boost, in particular for patients endangered for severe COVID-19 such as patients receiving hemodialysis (25) or transplant recipients (44).

Summarizing, this work indicates that a previous infection with SARS-CoV-2 modulated responses to COVID-19 vaccination after the two-dose prime phase. Booster vaccination balanced these responses between naïve and subjects recovered from COVID-19. Responses triggered against the SARS-CoV-2 Omicron BA.1 VoC are dampened compared with WT virus, and after receiving a booster vaccination dose, these responses are equally diminished independently of a previous SARS-CoV-2 infection. This fact applies to both T-cell and B-cell cellular responses. Therefore, a fourth booster vaccination dose is highly recommended, where variant-specific vaccines should be considered.

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 CEIm "La Paz University Hospital" (PI-4100). The

patients/participants provided their written informed consent to participate in this study.

Author contributions

EL-C, CdF, RL-R, and JA-O designed the study. CdF wrote the manuscript. AM-Q, AB-C, MG-G, MP, IL-F, LG, GM-M, and CH-B recruited the participants and collected the samples. RL-R, JA-O, VT, KH, JC-D, MB-G and PM-M performed the experiments. LL-M and CV-O provided critical experimental data during revision of the manuscript. EL-C, CdF, RL-R, JA-O and CC-Z discussed the results. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1136029/full#supplementary-material

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Analysis and comparison of SARS-CoV-2 variant antibodies and neutralizing activity for 6 months after a booster mRNA vaccine in a healthcare worker population

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Introduction: In the context of recurrent surges of SARS-CoV-2 infections, a detailed characterization of antibody persistence over a 6-month period following vaccine booster dose is necessary to crafting effective public health policies on repeat vaccination.

Methods: To characterize the SARS-CoV-2 antibody profile of a healthcare worker population over a 6-month period following mRNA vaccination and booster dose. 323 healthcare workers at an academic medical center in Orange County, California who had completed primary vaccination and booster dose against SARS-CoV-2 were recruited for the study. A total of 690 blood specimens over a 6-month period were collected *via* finger-stick blood and analyzed for the presence of antibodies against 9 SARS-CoV-2 antigens using a coronavirus antigen microarray.

Results: The primary outcome of this study was the average SARS-CoV-2 antibody level as measured using a novel coronavirus antigen microarray. Additional outcomes measured include levels of antibodies specific to SARS-CoV-2 variants including Delta, Omicron BA.1, and BA.2. We also measured SARS-CoV-2 neutralization capacity for a subset of the population to confirm correlation with antibody levels. Although antibodies against SARS-CoV-2 wane throughout the 6-month period following a booster dose, antibody levels remain higher than pre-boost levels. However, a booster dose of vaccine based on the original Wuhan strain generates approximately 3-fold lower antibody reactivity against Omicron variants BA.1 and

BA.2 as compared to the vaccine strain. Despite waning antibody levels, neutralization activity against the vaccine strain is maintained throughout the 6-month period.

Discussion: In the context of recurrent surges of SARS-CoV-2 infections, our data indicate that breakthrough infections are likely driven by novel variants with different antibody specificity and not by time since last dose of vaccination, indicating that development of vaccinations specific to these novel variants is necessary to prevent future surges of SARS-CoV-2 infections.

KEYWORDS

serology, vaccine, mRNA, healthcare worker (HCW), SARS-CoV-2

Introduction

Since the initial 2019 outbreak of the novel beta coronavirus SARS-CoV-2, rapid international spread of the COVID-19 disease has resulted in a global pandemic. In efforts to contain the spread and severity of COVID-19, the FDA approved the emergency distribution of mRNA vaccines BNT162b2 and mRNA-1273 in December of 2020. Both vaccines provide high rates of protective efficacy of up to 95% against the targeted virus strain following two doses administered at least 3-4 weeks apart (1, 2). There has been a rapid global increase in SARS-CoV-2 cases since then, mainly due to the high infectivity and antibody escape mutations of the new Omicron variants, as well as waning immunity from the BNT162b2 and mRNA-1273 vaccines (3). The FDA has since approved the administration of additional "booster" doses of mRNA vaccines. A booster vaccine dose has previously been effective at protecting against severe COVID-19-related outcomes (4) and has been shown to substantially increase neutralizing antibodies (5, 6). The neutralizing ability of the antibodies has also differed among SARS-CoV-2 variants (7). Therefore, it is of great importance to elucidate the effectiveness of the booster vaccine in maintaining a persistent antibody response in a population consistently exposed to novel variants of SARS-CoV-2.

Here, we seek to analyze the initial rise and waning of SARS-CoV-2 antibody responses induced by the third-dose mRNA vaccine booster in a healthcare worker population over a 6-month period using a coronavirus antigen microarray, with direct comparison of antibodies against multiple variants of concern. Binding antibodies against SARS-CoV-2 antigens have been shown to correlate strongly with neutralizing antibodies, which are a critical component of clinical immunity (8–11). We confirm the correlation of measured antibody responses with SARS-CoV-2 neutralizing capacity for a subset of 30 healthcare workers utilizing an FDA-authorized surrogate neutralization assay (12, 13).

Methods

Study population

This study was approved by the institutional review board (IRB) of the University of California Irvine (UCI) prior to initiation of the study (protocol HS-20205818). Widespread mRNA vaccination of healthcare workers (HCWs) at UC Irvine Health began in December 2020, administering over 16,000 doses of mRNA-1273 (Moderna-NIAID) or the BNT162b2 (Pfizer-BioNTech) vaccines within the first 4 months. In September of 2021, the FDA approved and UCI secured and administered booster shots to all HCWs who wished to be additionally vaccinated. All HCWs working at the UCI Medical Center, located in Orange County, CA, who participated in our previous study (14) were invited to receive serological testing by providing serum blood samples *via* a fingerstick directly before vaccination, 1-2 weeks after vaccination, 2 months, 4 months, and 6 months after booster vaccination. All blood samples were brought to the Institute for Clinical and Translational Science Core Laboratory at the UCI Medical Center. Serum samples were centrifuged using the Eppendorf 5415R and spun at 3000xg for 5 minutes. Serum was quickly transferred into a clean sterile tube and frozen at -80°C until analyzed for IgGs. Reports of their serological test results were returned within 4 weeks of receiving the test. At each assessment, demographic and work-related characteristics, testing frequency, exposure risk, and symptom history were collected *via* surveys administered prior to serum sample collection. Longitudinal participation was encouraged through an aggressive email campaign as well as ensuring that participants received a report of their antibody levels, but not every subject participated at every time point. Subjects who participated provided written consent in the form of an electronic document that was physically signed using an iPad or cellular device.

Coronavirus antigen microarray

953 independent finger stick blood serum samples were collected over the 6-month period for analysis. This analysis was restricted to 690 samples to adhere to the pre-specified guidelines (Table 1). Specimens were probed and analyzed on a coronavirus antigen microarray (CoVAM) for IgG and IgM antibodies against 37 antigens from SARS-CoV-2, other coronaviruses, and other respiratory viruses using a coronavirus antigen microarray. The CoVAM contained 10 SARS-CoV-2 antigens including nucleocapsid protein (NP) and several varying fragments of the spike (S) protein, as well as 4 SARS, 3 MERS, 12 Common CoV, and 8 influenza antigens.

TABLE 1 Participant demographics.

	HCWs (n= 323)
Gender	
Female	240 (74.3%)
Male	65 (20.1%)
Non-binary	1 (0.3%)
Declined to respond	17 (5.3%)
Vaccine type	
BNT162b2	234 (72.4%)
mRNA-1273	51 (15.8%)
Declined to respond	38 (11.8%)
Age	
<55 years	256 (79.2%)
≥55 years	53 (16.4%)
Declined to respond	14 (4.3%)
Race	
Asian	84 (26.0%)
White	49 (15.2%)
Latino/Hispanic	33 (10.2%)
Black	1 (0.3%)
Pacific Islander	5 (1.5%)
Other	3 (0.9%)
Declined to respond	148 (45.8%)
Role	
Administrative	22 (6.8%)
Food/EVS	12 (3.7%)
Nurse	134 (41.5%)
Physician	35 (10.8%)
Student	9 (2.8%)
MA/technician	8 (2.5%)
Other	87 (26.9%)
Declined to respond	16 (5.0%)

A full list of antigens used in the assay can be found in Supplementary Figure 1. Samples were tested in triplicate. For more detailed information regarding the coronavirus antigen microarray, analysis, refer to our previous work (14).

In summary, the raw data were checked for quality and normalized using a composite of methods described by Duarte (2013) and Bolstad (2003). Then, using a collection of known samples (here referred as "training set", known to be positive or negative for SARS-CoV-2), a prediction model was constructed using a logistic regression, in which a combination of SARS-CoV-2 antigens were used as well as a Random Forest model using the full SARS-CoV-2 antigen collection. For single antigen reactivity prediction, a logistic regression model was built for each individual antigen, also using the training set. As a surrogate marker for exposure (in contrast to mRNA vaccination), reactivity to the nucleoprotein, as determined by the Logistic regression model, was used.

This model was found to be 93% sensitive and 98% specific in correctly classifying 91 PCR-positive cases and 88 pre-pandemic negative control (15). The model was then used to generate a weighted composite measure of IgG reactivity across antigens, with weights corresponding to each antigen's relative importance in the model. This composite IgG reactivity measure was scaled up to represent the weighted mean fluorescence intensity (MFI) of all antigens assayed in the CoVAM. Here, we utilized a model containing all SARS-CoV-2 antigens as above with the exception of NP, as this antigen was used to classify prior exposure to SARS-CoV-2 in a subgroup analysis.

Statistical analysis

In order to characterize SARS-CoV-2 antibody response over time, we fit a third-degree polynomial model of the average IgG reactivity measure using all available data from n=323 HCWs. Due to the variability in the timing of the tests across individuals, we report the model-estimated average IgG reactivity means and standard error of the mean (SEM) error bars at pre-boost, 1-2 weeks, 2 months, 4 months and 6 months post-boost dose, and compared the changes over time. We then explored differences in long-term antibody response by individual characteristics hypothesized to influence the magnitude and durability of the vaccine-induced antibody response: history of PCR positivity [yes or no], presence of systemic side effects (side effects used in this study include fever, chills, cough, difficulty breathing, congestion, loss of smell or taste, new onset fatigue, myalgia other than site of injection, and headache [none, mild, moderate, or severe] exposure in community [yes or no], HCW role [patient care vs. non-patient care role, gender [male or female, by self-report], age [≥55 vs. <55 years, by self-report]. We tested each potential moderator individually by fitting the same linear mixed effect model with the inclusion of an interaction term between that variable and time (e.g., time * age≥55 vs. <55 years).

In order to determine the correlation between reactivity of SARS-CoV-2 antigens and reactivity of antigens from the other viruses in the array, a correlation matrix using the function rcorr() from the Hmisc (version 5.0-1) package. The correlation matrix was visualized using the corrplot (version 0.92) package. PCA analysis was performed using the tidyverse (version 1.3.2) and factoextra (version 1.0.7) packages. All analyses were conducted using R programing environment v4.1.1.

Neutralization assay

In order to accurately assess neutralization capacity of SARS-CoV-2 antibodies, we utilized a FDA-authorized research use only surrogate virus neutralization test provided by GeneScript and carried out through a local commercial lab, Innovative Health Design lab located in Irvine, California. Samples and controls are

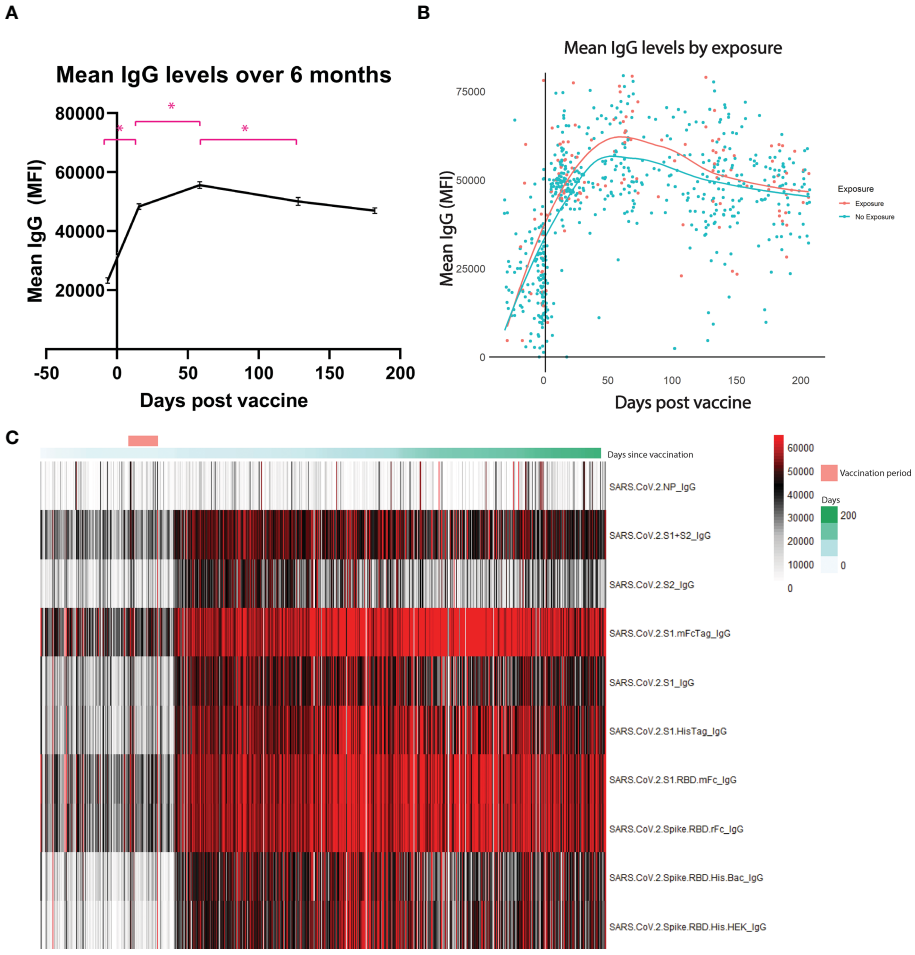

FIGURE 1
Antibody levels over 6 months. Mean IgG antibody levels measured by mean fluorescence intensity (MFI). **(A)** shows each individual sample collected over the 6-month period. **(B)** shows the difference between those who were exposed to the coronavirus at any point before the booster versus those who had no exposure. Exposure is deemed through presence of antibodies against the nucleocapsid protein, a protein only found in the virus and not in the vaccine. **(C)** is a heatmap of each antigen measured throughout the 6 months. * represents statistically significance, $p < 0.05$.

pre-incubated with HRP bound RBD to allow for interaction and binding of neutralization antibodies to RBD-HRP. The mixture is subsequently added to a pre-coated plate of hACE2 protein. Unbound RBD-HRP or RBD-HRP bound to non-neutralizing antibody will be captured by the plate. Neutralization antibodies complexed to RBD-HRP remains in the supernatant and is removed through washout steps. The reaction is quenched and samples are read at 450 nm in a microtiter plate reader. Absorbance of the sample is inversely dependent on the titer of the neutralizing antibodies, numbers are shown as a percentage of controls. Sera of 30 individuals who participated at every timepoint were tested using this surrogate neutralization assay to assess neutralizing capacity for binding of spike RBD fragments from the original Wuhan strain to human ACE2 receptors.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

SARS-CoV-2 antibody levels 6 months post-vaccination

A total of 690 tests were analyzed from 323 HCWs throughout a 6-month period following booster vaccine dose (Table 1). Composite IgG antibody levels as measured by mean fluorescence intensity significantly increased in the 2 weeks following the booster vaccination (mean 23,303 vs 48,384, p <0.01) (Figure 1). Antibody levels continued to increase from 2 weeks post-boost until 2 months post-boost (mean 48,384 vs. 55,613, $p < 0.01$) but decreased significantly between months 2 and 4 (mean 55,613 vs. 50,105, $p < 0.01$) with a non-significant trend towards decreasing antibodies between months 4 and 6 (mean 50,105 vs 46,946, $p = 0.14$) Despite waning, antibody levels at 6 months post booster dose remain significantly higher than pre-boost levels.

To infer natural exposure, we assessed reactivity to the SARS-CoV-2 nucleocapsid protein, as this antigen is not present in currently approved SARS-CoV-2 vaccines. Exposure threshold

was determined by logistic regression using the training set as reference. As seen on Figure 1B, no significant difference was observed between the groups at any of the timepoints tested.

Antibody levels by demographic

The cohort of HCWs were divided into subgroups based on their gender, age, presence of exposure in their community, worker role, presence of comorbidities, and history of PCR-confirmed SARS-CoV-2 infection. Individuals with a history of PCR-confirmed infection after their booster were found to have higher antibody levels at the 4 month timepoint (57,262 vs 40,846, $p < 0.01$) and at the 6 month timepoint (47,222 vs 42,451, $p = 0.04$) (Figure 2A). Vaccination systemic side effects were assessed through a 45-point subjective scale of severity encompassing 9 systemic side effects with subjective, self-reported severity from 0 to 5 points. Baseline antibody levels were equivalent between those with no systemic side effects and those with severe systemic side effects (21,639 vs 20,872, $p = 0.85$), but those with severe systemic side effects had significantly higher antibody levels at 2 months after booster vaccination (51,313 vs 69,849, $p < 0.01$), with a continued non-significant trend towards higher antibody levels compared to those without systemic side effects at later time points (Figure 2B). Stratification by community exposure, healthcare worker role, gender, and age did not yield any significant differences between groups (Figures 2C–F).

Antibody levels against variants of concern

Blood specimens from 30 healthcare workers were retrospectively chosen, based on specimen volume and availability at multiple time points and representativeness compared to the study population, to undergo further analysis using a more advanced microarray that included antigens against the original Wuhan strain and 3 variants of concern: Delta, Omicron BA.1, and BA.2. This analysis was restricted to analyze only the receptor-binding domain (RBD) for unbiased comparison between variants and included a total of 213 samples over the 6-month period, with a minimum of 27 samples per time point (Figure 3). Prior to booster vaccine dose, antibody levels against the Wuhan strain were not significantly different from the Delta strain (mean 3473 vs. 2449, $p = 0.24$) but were significantly lower against Omicron strains BA.1 and BA.2 (mean 3473 vs. 300, $p < 0.01$ and 3473 vs. 542, $p < 0.01$). At 2 weeks post booster vaccination, all variants had significantly lower antibody levels directed against them as compared to the Wuhan strain (Delta: 1.3 fold-reduction, $p < 0.01$ Omicron BA.1 6.0 fold-reduction, $p < 0.01$; BA.2 3.4 fold reduction, $p = <0.01$). This trend persisted over the 6-month period.

Antibody levels against other respiratory viruses

Reactivity to antigens from all viruses represented on the array was observed for multiple samples. However, no significant correlation between the reactivity to SARS-CoV-2 antigens and either endemic coronavirus (229E, NL63, OC43, HKU1) or influenza virus antigens was observed. In accordance with our previous observations and the literature, this can be explained by a low cross reactivity between SARS-CoV-2 S1 and RBD antigens and the same antigens on other coronaviruses. Nevertheless, as shown on Figure 4, the observed correlation between different fragments of SARS-CoV-2 spike antigen was high (averaging $r > 0.8$) except for the nucleoprotein (averaging a $r < 0.3$). This observation confirms that the microarray can distinguish between antibody responses induced by vaccines that include spike protein antigens and natural infection which includes exposure to both spike and nucleocapsid protein antigens.

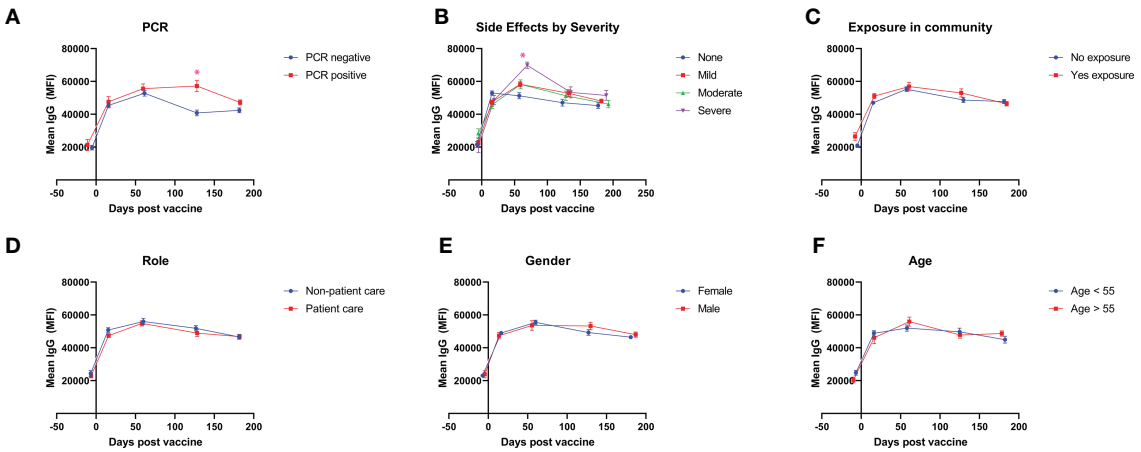

FIGURE 2
Antibody levels after vaccination for demographic subgroups. Mean IgG antibody levels measured throughout 6 months are compared for self-identified subgroups divided by **(A)** history of PCR-confirmed SARS-CoV-2 infection after booster vaccination, **(B)** severity of vaccine side-effects **(C)** exposure in community after booster vaccination, **(D)** healthcare worker role, **(E)** gender, and **(F)** age. * represents statistically significance, $p < 0.05$.

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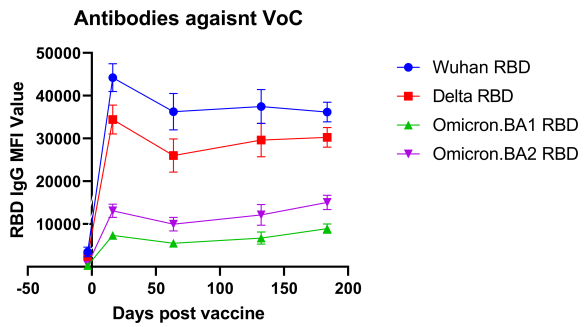

FIGURE 3
Antibody reactivity against variant RBD antigens. The antibody reactivity as measured by mean fluorescence intensity (MFI) against variant-specific receptor-binding domain (RBD) antigens is shown for different variants of concern (VoC) at different time points after vaccination.

Persistence of neutralizing antibodies against Wuhan strain despite waning antibody levels

The same 30 healthcare workers who were selected to undergo variant testing were also selected for analysis of neutralization capacity. These 30 individuals expressed a similar trend to the overall cohort, with their average antibody levels initially increasing from pre-boost to 2 months post-boost, but significant waning between months 2 to 6. Signal inhibition of neutralizing titers increased from 77% before booster vaccination to 96% immediately after booster vaccination ($p < 0.01$). Despite waning antibody levels from months 2 to 6, signal inhibition did not wane significantly over this time (Supplementary Figure 2).

Discussion

In summary, we report data on the boosting of both composite IgG antibody levels and antibody neutralizing activity by a third dose of mRNA vaccine against SARS-CoV-2. The strengths of our study include a 6-month follow-up period, the comparison of antibody responses against SARS-CoV-2 variants of concern, and inclusion of subgroup analyses with respect to demographics, vaccine side-effects, and breakthrough SARS-CoV-2 infection. The weaknesses of our study include limited power to detect differences in subgroups and limitations of the HCW population, which is a relatively young and healthy population with few individuals who are immunocompromised or over the age of 65.

Both IgG antibody levels and neutralization activity have been shown to wane over the course of months following vaccination (14, 16). The booster vaccine dose has been shown to significantly increase both IgG antibody levels and neutralization activity (5, 17). Similarly, the booster vaccine dose has been shown to reduce risk of SARS-CoV-2 breakthrough infections (6). However, previous studies have focused on short-term effect of booster vaccination on SARS-CoV-2 antibody responses (18), while we

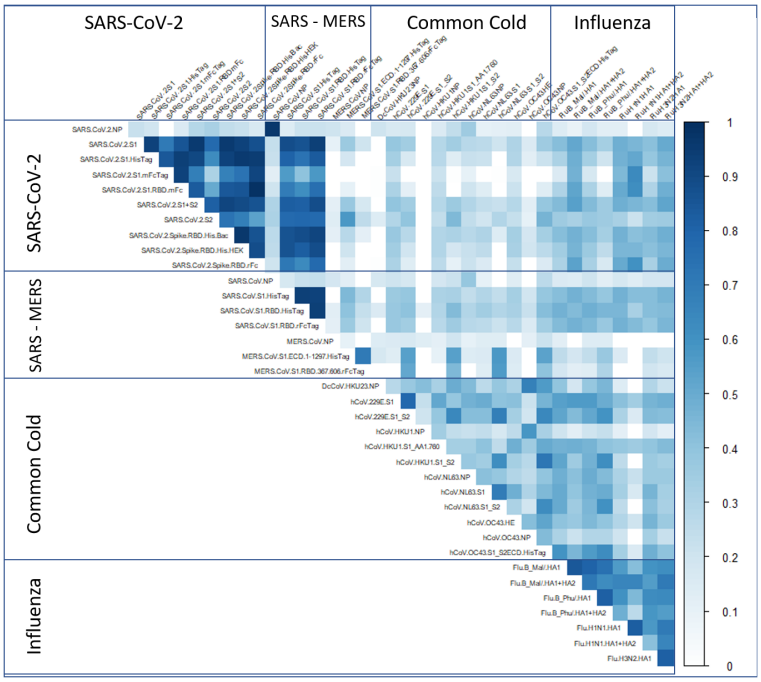

FIGURE 4
Correlation of antibodies against other respiratory viruses. Using COVAM 5, we are able to measure antibodies against SARS, MERS, CoV, and influenza in addition to SARS-CoV-2. This figure represents the correlation coefficient for each antibody against antibodies from other respiratory viruses.

chose to observe the study population over a longer 6-month follow-up period.

Using our CoVAM assay, we observed a significant increase in antibody levels following the booster dose of the vaccine. Despite significant waning between months 2-6, the antibody levels at 6 months remain higher than pre-boost levels. We also show no significant difference between individuals who were exposed to SARS-CoV-2 (as determined through reactivity to the nucleocapsid protein) compared to those who had no previous exposure. This data is limited in the ability to detect a small effect of previous exposure by our small sample size of individuals with previous exposure. Others have shown longer-lasting immunity as exhibited by higher antibody levels and neutralization activity in those with previous infection 9 months after primary 2-dose COVID-19 mRNA vaccination (19). In the context of the high rate of breakthrough infections prior to 6 months observed in recent surges of SARS-CoV-2 infections, these data indicate that waning of antibody levels over time is not the primary driver of SARS-CoV-2 breakthrough infections.

We stratified study participants based on gender, age, community exposure, worker role, and breakthrough COVID-19 infection based on a history of PCR positivity. We did not find significant differences in antibody levels for subgroups stratified by gender, age, self-reported community exposure to COVID-19, or clinical role, although the study may have been underpowered to detect small differences within these subgroups. We had a small cohort of individuals who had breakthrough infections confirmed through PCR positivity, and these individuals showed less waning in antibody responses with significantly higher antibody levels at 4 and 6 months.

We, among others, hypothesized that the quantity and severity of systemic side effects may be correlated with a more robust immune response, resulting in higher antibody titers. We show that the presence of systemic side effects is associated with higher antibody titers up to 4 months following booster vaccination. Severe systemic side effects are associated with higher antibody levels in a longitudinal fashion throughout the 6-months period. These data can reassure patients that systemic side-effects associated with vaccination may represent a more robust immune response to the vaccine dose.

The neutralizing capacity of SARS-CoV-2 antibodies has been shown to increase quickly following the vaccine booster dose (20). While we focused on binding antibody levels in this study, we did confirm correlation between binding antibodies and neutralizing antibodies in a subset of patients using an FDA-authorized surrogate neutralization assay (12). We retroactively selected 30 individuals who participated at every time point available, including before booster vaccination, in order to get a complete timeline of neutralization throughout the 6 month period. We acknowledge that this may lead to some selection bias, as those who come to every collection offered may be more likely to be more compliant with masking and exposure guidelines. We report significant increase in neutralizing antibodies against the original Wuhan strain following booster vaccine dose with maintenance throughout the 6-month period in this cohort, despite waning binding antibody levels measured on the CoVAM. It is important to note that our neutralization data are limited in that our assay was likely saturated at post-boost time points, with our values being at 96% inhibition, which may not fully allow us to detect small differences between post-boost timepoints. However, based on these data, we hypothesize that the decrease in antibody levels over the course of the 6-month period is not sufficient to reduce neutralization capacity and therefore unlikely clinically significant given correlation between neutralization capacity and clinical immunity.

We acknowledge that others have directly compared the neutralization capacity of vaccinee sera against SARS-CoV-2 variants of concern (20), however, estimates for the magnitude of antibody escape by the Omicron variant of concern vary widely from as low as 1.5-fold to as high as 32-fold (21) (22) (23, 24). Using an updated version of the CoVAM, we were able to directly compare antibodies elicited by booster vaccination against the spike protein receptor-binding domain antigens of multiple variants of concern. Our data show that antibodies elicited by booster vaccination directed against the original Wuhan strain show only a small non-significant decrease in binding to RBD antigen from the Delta variant but show significant decreases in binding to Omicron variants BA.1 (4-fold decrease) and BA.2 (3-fold decrease). Although the booster dose is effective in maintaining antibodies against Wuhan and Delta strain antigens, these data suggest that vaccines directed against Omicron strain antigens are needed to effectively prevent breakthrough COVID-19 infections.

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 UCI IRB. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SH, GK, ML, AP, SS, PF, and SK conceived and designed research. SH, GK, ML, AH, ES, and NG collected samples. PH, JA, and FZ prepared and stored samples. PF and SK designed the microarray. RA, AJ, RN, and AJ constructed the microarray and probed samples. RA analyzed data. SH, GK, SS, PF, and SK interpreted results of data. SH and RA prepared figures. SH, GK, ML, NG, and SK drafted the manuscript. SH and SK edited and revised the manuscript. SH, SS, PF, and SK obtained funding for the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The coronavirus antigen microarray is intellectual property of the Regents of the University of California that is licensed for commercialization to Nanommune Inc. Irvine, CA, a private company for which PF is the largest shareholder and several co-authors RA, AaJ, RN, and SK also own shares. Nanommune Inc. has a business partnership with Sino Biological Inc. Beijing, China which expressed and purified the antigens used in this study.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1166261/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Antibodies tested through COVAM 5.

SUPPLEMENTARY FIGURE 2
Neutralizing capacity of vaccinee sera. Neutralizing capacity of sera, measured as percent signal inhibition of binding of RBD antigen from the Wuhan strain to human ACE2 receptors compared to negative control sera, is shown for different time points after vaccination.

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The effectiveness of the first dose COVID-19 booster vs. full vaccination to prevent SARS-CoV-2 infection and severe COVID-19 clinical event: a meta-analysis and systematic review of longitudinal studies

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Background: The effectiveness of full Coronavirus Disease 2019 (COVID-19) vaccination against COVID-19 wanes over time. This study aimed to synthesize the clinical effectiveness of the first dose of COVID-19 booster by comparing it to the full vaccination.

Methods: Studies in PubMed, Web of Science, Embase, and clinical trials databases were searched from 1 January 2021 to 10 September 2022. Studies were eligible if they comprised general adult participants who were not ever or currently infected with SARS-CoV-2, did not have impaired immunity or immunosuppression, and did not have severe diseases. The seroconversion rate of antibodies to S and S subunits and antibody titers of SARS-CoV-2, frequency, phenotype of specific T and B cells, and clinical events involving confirmed infection, admission to the intensive care unit (ICU), and death were compared between the first booster dose of COVID-19 vaccination group and full vaccination group. The DerSimonian and Laird random effects models were used to estimate the pooled risk ratios (RRs) and corresponding 95% confidence intervals (CIs) for the outcomes of clinical interest. While a qualitative description was mainly used to compare the immunogenicity between the first booster dose of COVID-19 vaccination group and full vaccination group. Sensitivity analysis was used to deal with heterogenicity.

Results: Of the 10,173 records identified, 10 studies were included for analysis. The first dose COVID-19 booster vaccine could induce higher seroconversion rates of antibodies against various SAS-CoV-2 fragments, higher neutralization antibody titers against various SARS-CoV-2 variants, and robust cellular immune response compared to the full vaccination. The risk of SARS-CoV-2 infection, the risk of admission to the ICU, and the risk of death were all higher in the non-booster group than those in the booster group, with RRs of 9.45 (95% CI 3.22–27.79; total evaluated population 12,422,454 vs. 8,441,368; I^2 = 100%), 14.75 (95% CI 4.07–53.46; total evaluated population 12,048,224 vs. 7,291,644; I^2 = 91%), and 13.63 (95% CI 4.72–39.36; total evaluated population 12,385,960 vs. 8,297,037; I^2 = 85%), respectively.

Conclusion: A homogenous or heterogeneous booster COVID-19 vaccination could elicit strong humoral and cellular immune responses to SARS-CoV-2. Furthermore, it could significantly reduce the risk of SARS-CoV-2 infection and severe COVID-19 clinical events on top of two doses. Future studies are needed to investigate the long-term clinical effectiveness of the first booster dose of the COVID-19 vaccine and compare the effectiveness between homogenous and heterogeneous booster COVID-19 vaccination.

Systematic review registration: https://inplasy.com/inplasy-2022-11-0114/, identifier: INPLASY2022110114.

KEYWORDS

COVID-19, SARS-CoV-2, vaccine, booster, effectiveness, meta-analysis

1. Introduction

As of 23 December 2022, Coronavirus Disease 2019 (COVID-19) caused 651.9 million cases and 6.7 million deaths globally (1). As a major prevention strategy, COVID-19 vaccination plays a vital role in reducing rates of mortality and severe events during this pandemic. The booster COVID-19 vaccine promoted humoral and cellular immunity through the recall of memory B cells, the *de novo* activation of B cells, and B cell maturation through the activation and development of follicular helper T (Tfh) cells. These were the physiopathology basis for minimizing the risk of COVID-19 infection and progression to severe diseases (2). The first dose of COVID-19 booster vaccination has been promoted worldwide to strengthen the effect of the full COVID-19 vaccination. As of 28 December 2022, the global booster administration rate was 33.5% (1). Previous research has investigated the effects of the COVID-19 vaccine booster on disease prevention. Various studies, including randomized controlled clinical trials (RCTs), used cell immunity and humoral immunity parameters to indicate the potential preventive effects of COVID-19, but no direct prevention effects have been discovered to date (3, 4). More clinical indicators have been recommended in the evaluation of the effects of the COVID-19 vaccine booster, such as the SARS-CoV-2 incidence rate, the effect on the prevention of severe COVID-19 disease, hospitalization, intensive care unit (ICU) admission, and mortality. The inclusion of these factors could provide a more objective and complete picture of the effects of the COVID-19 vaccine booster. Except for a few developed countries, most countries in the world are still vaccinated for wild SARS-CoV-2 strains. With the frequent emergence of mutant strains, it is urgent and crucial to determine whether the COVID-19 vaccines for wild strains can also prevent mutant SARS-CoV-2 strains in the real world. The evidence could provide vital guidance on the administration of COVID-19 vaccine boosters and inform associated COVID-19 prevention strategies locally and nationally. At present, there are two major types of COVID-19 vaccine booster approaches, namely heterologous booster vaccination and homologous booster vaccination (based on two shots of COVID-19 vaccine, injecting the first shot of booster vaccination with the same technical route vaccine is defined as homologous booster vaccination, or then injecting a first shot of booster vaccination with different technical routes vaccine is defined heterogeneous booster vaccination). Heterologous COVID-19 vaccine booster strategy has been promoted in many developing countries, including China (5, 6). However, the literature lacks systematic reports on the clinical effects of the heterologous COVID-19 vaccination on the prevention of SARS-CoV-2 infections (3, 6).

A recent meta-analysis study compared the preventive efficacy of the first dose of the COVID-19 booster with less than three doses of COVID-19 vaccination (7). Nevertheless, the preventive efficacy between the first dose of the COVID-19 booster and only two doses of the COVID-19 vaccination remained unclear. In addition, the studies that this meta-analysis synthesized included various research designs, including, RCTs, cohort studies, cross-sectional studies, and case–control studies (7). The findings must be more convincing when only studies with study designs of interest were chosen for the review. With full doses becoming the majority of the vaccinated population, it was necessary to investigate the effects of the COVID-19 vaccine booster by comparing it to the full doses of the COVID-19 vaccination. This pooled meta-analysis aimed to synthesize the literature of cohort studies and RCTs on COVID-19 booster efficacy compared to full doses (two doses) of COVID-19 vaccination by comparing the differences in the incidence of COVID-19, hospitalization rate, ICU rate, and mortality rate. With approximately two-thirds of the world's population not received the first dose of the COVID-19 vaccine booster, the findings of the study have implications on eliminating the hesitation of COVID-19 booster administration, increasing the awareness of COVID-19 booster vaccination, and providing robust evidence on COVID-19 booster promotion.

2. Methods

This systematic review was reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (8). We register this review to the INPLASY register. The registration number is INPLASY2022110114.

Abbreviations: COVID-19, Coronavirus disease 2019; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; WHO, World Health Organization; ICU, intensive care unit; RCT, randomized controlled clinical trial; CI, confidence interval; RR, relative risk.

2.1. Literature search strategy

Between 1 January 2021 and 10 September 2022, we searched relevant studies that were published in PubMed, Embase, Web of Science, and clinical trials databases using a combination of comprehensive keywords, including "COVID-19," "SARS-CoV-2," "vaccination," "vaccine," "third," "boost," and "four" with Boolean operators and MeSH terms (Supplementary Table 1 for search strategy). We also searched relevant systematic reviews to add additional eligible studies. The searching, reviewing, and selecting literature were independently and blindly performed by two authors (Lan X and Zhang L). Discrepancies were resolved through consultation with a third author (Song M).

2.2. Study selection

Published articles were eligible for this study if they meet the following inclusion criteria: (1) observational studies (prospective or retrospective cohort) or RCTs with a minimum of 10 general adult participants in any study group; (2) at least involved one type of the booster COVID-19 vaccination after full vaccination (e.g., one dose of mRNA vaccine booster after two doses of mRNA vaccines); (3) a control group comprising participants who completed full COVID-19 vaccination but did not receive a booster; and (4) reported at least one of the outcomes of interest in both the booster group and full vaccination group with comparable time periods: serum antibodies against different SAS-CoV-2 fragments regardless of continuous or binary outcomes, cell-mediated immune, laboratory-confirmed infection, COVID-19-related hospitalization, COVID-19-related ICU admission, or death.

We did not include the following studies: (1) comprised participants who were ever or currently infected with SARS-CoV-2; (2) comprised participants who had impaired immunity or immunosuppression; (3) comprised participants who had severe diseases, such as patients who needed hemodialysis; (4) the studies did not have baseline data; (5) review studies; and (6) non-English publications.

2.3. Data extraction

We extracted the data according to a standardized form in Microsoft Excel 2016 (Microsoft Office, CA, USA). This process was also conducted by two authors independently and checked by a third author. The following study characteristics were collected: first author, study setting, year of publication, study design, and sample size. Other information was also summarized, such as participant characteristics comprising age and sex and immunization-related data including vaccine type and brand, the interval between prime full vaccination and booster vaccination, dosing schedule, and the number of participants who received each type of vaccine. Outcome-related data comprised the interval between booster vaccination and the assessed outcomes, antibody measured and the methods, frequency, and phenotype of specific T and B cells, mean or median of cytokine levels, and the number of events involving infection, hospitalization, admission to the ICU, and death.

2.4. Risk of bias assessment

We used the Risk of Bias in Non-randomized Studies of Interventions (*ROBINS-I*) for the quality assessment of all cohort studies, which consisted of seven domains: risk of bias from confounding, selection of participants, classification of interventions, deviations from intended interventions, missing data, measurement of outcomes, and selection of the reported results (9). In addition to cohort studies, we also included RCTs in this review. The risk of bias in RCTs was assessed using version 2 of the Cochrane risk-of-bias tool for randomized trials (10).

2.5. Statistical analysis

We performed all meta-analytical evaluations on R 4.0.3 using the *meta-packages*. We used the DerSimonian and Laird random effects model to estimate the pooled risk ratios (*RRs*) and corresponding 95% confidence intervals (*CI*s) for the outcomes of interest. *RR* was estimated as the event rate in the control group divided by the same rate in the booster group. We also estimated the summary vaccine effectiveness (VE) against various clinical outcomes. VE was obtained from the effect size (RR) defined as $(1-1/RR) \times 100\%$. Statistical heterogeneity was assessed using the Cochrane Q test and I^2 statistics. We considered heterogeneity to be significant when the p-value was <0.10 or the I^2 statistic was $\geq 50\%$. Unless specified otherwise, we considered a two-sided p-value of <0.05 to be statistically significant.

The techniques used to measure SARS-CoV-2 specific antibodies and criteria for positivity varied in different studies. Thus, meta-analysis was inappropriate to compare the antibody titers and seroconversion of different studies. Instead, a qualitative description was mainly used to compare and pool the immunogenicity.

Sensitivity analysis was used to deal with the heterogenicity by removing the studies with the highest effect value. The funnel plots, Egger's test, and Begg rank correlation test were used to assess the potential publication bias (11).

3. Results

3.1. Summary of included studies

Figure 1 shows a flow chart of the study selection. Finally, 10 studies that met the eligibility criteria were included in the analysis (12–21). The characteristics of the included studies are summarized in Table 1. They were conducted one in each of the following countries: India (21), Singapore (20), Malaysia (16), Israel (13), UK (19), Qatar (12), Brazil (18), Abu Dhabi (17), Turkey (15), and China (14). The proportion of the female ranged from 7.1 to 78.5% and the median age ranged from 33 to 56 years. Five studies reported the proportion of comorbidities ranging from 7 to 75.4%. Of the 10 studies, one was a randomized trial (21) and nine

were observational studies (12–20). Five studies included patients who received an mRNA (BNT162b2 or mRNA-1273) booster after the standard two-dose mRNA full vaccination (12, 13, 16, 19, 20); five studies included patients who received an inactivated booster vaccine after two-dose inactivated full vaccination (15–17, 20, 21); three studies included patients who received an mRNA booster vaccine after two-dose inactivated full vaccination (14–16); and one study included patients who received an mRNA-1273 booster vaccine after two-dose MVC-COV1901 vaccination (14). The qualitative analysis included the following studies: two studies reported the seroconversion rates of antibodies against different SAS-CoV-2 fragments (15, 21), three studies reported antibody titers (14, 17, 21), and two studies reported cell-mediated immune response after booster vaccination (14, 21). The meta-analysis included the following studies: six studies reported laboratory-confirmed SARS-CoV-2 infection (12, 13, 16, 18–20), three studies reported COVID-19-related ICU admission (16, 18, 20), and four studies reported mortality after that booster vaccination (12, 13, 16, 20).

The quality assessment scores for included studies are shown in Supplementary Table 2. The randomized trial was considered of having some concerns of bias. Of the nine observational studies, seven studies (12, 13, 15, 16, 18–20), were considered to have a moderate risk of bias, one study (14) was at low risk of bias, and one study (17) was at serious risk of bias.

3.2. Seroconversion rates following booster shot

Two studies (15, 21) with three comparisons reported seroconversion rates after a booster dose. Vadrevu et al. (21) reported that one-dose booster of BBV152 induced higher seroconversion rates of antibody against spike protein (93.8 vs. 81.6%) and receptor binding domain (89.8 vs. 74.7%) and neutralization antibody by PRNT (98.7 vs. 79.8%) and MNT (100 vs. 92.9%) 1 month after the booster administration compared to full doses of the BBV152 vaccination. Moreover, after 6 months of the first booster dose administration, seroconversion of neutralization antibody against the D614G (96.8 vs. 59.5%), Delta (96.8 vs. 59.5%), and Omicron variant (93.5 vs. 56.8%) in the booster group was higher than those in the control group (21). Among individuals who received two doses of CoronaVac (Sinovac), both homologous vaccination (CoronaVac; 100 vs. 83.3%) and heterologous vaccination (BNT162b2; 100 vs. 83.3%) induced a higher seroconversion rate of antibody against receptor binding domain (15). In summary, these studies implied that booster vaccination could induce robust humoral response regardless of the booster vaccine type and anti-SARS-CoV-2 specific antibodies (Supplementary Table 3).

3.3. Antibody titers post-booster dose

Three studies (14, 17, 21) reported neutralization antibody titers against various SARS-CoV-2 variants. Vadrevu et al. (21) reported that the neutralization antibody titers of PRNT [746.2 (514.9–1,081) vs. 100.7 (43.6–232.6)] and MNT [641 (536.8–765.3) vs. 359.3 (267.4–482.7)] were higher in BBV152 booster group than those in the control group 1 month after the first booster dose administration. Moreover, after 6 months of the first booster administration, neutralization antibody titers against the D614G [178.9 (82.6–387.5) vs. 10.7 (2.6–44.5)], Delta [115.9 (55.8–240.8) vs. 7.3 (2.0–27.0)], and Omicron variants [25.7 (13.0–50.6) vs. 2.9 (0.99–8.3)] in the booster group maintained higher levels than those in the control group. Mahmoud et al. (17) reported that three doses of BBIBP-CorV induced higher neutralization antibody titers against SARS-CoV-2 Alpha (289.23 ± 186.30 vs. 138.46 ± 94.68), Beta (103.53 ± 62.94 vs. 34.12 ± 21.52), and Delta (156.89 ± 104.44 vs. 45.78 ± 29.96) variants compared with those received two doses of BBIBP-CorV. Similarly, Chiu et al. (14) reported that one-dose booster of mRNA-1273 after two doses of MVC-COV1901 induced 16.3-, 17.7-, and 32.2-fold higher neutralization antibody titers for the Alpha, Delta, and Omicron variants compared to those who received two doses of ChAdOx1-S and were 8.8-, 8.4-, and 26.0-fold higher than those received two doses of MVC-COV1901. In general, these studies suggested that the booster vaccination could induce higher neutralization antibody titers against various SARS-CoV-2 variants (Supplementary Table 4).

3.4. Cell-mediated immune post-booster dose

Two studies (14, 21) reported cell-mediated immunity. Vadrevu et al. (21) reported that the median Th1:Th2 index increased from 10.0 (IQR, 1.0–32.0) to 16.0 (IQR, 4.0–32.0) after the booster dose administration. The IFN-γ level was similar with a median of 48 (15.0–85.0) and 48 (29.0–95.0) in the booster and non-booster group, respectively, after 6 months of third-dose administration (21). Similarly, the median IgG secreting memory B cells (MBC) per 10^6 PBMCs were increased in the booster group (50, IQR 12.0–60.0) compared to the non-booster group (21.3, IQR 14.2–43.5) (21). Chiu et al. (14) reported that the administration of an additional dose of mRNA vaccine after two doses of the subunit vaccine could significantly enhance the cellular immune response for both the wild type and the Delta variant (Supplementary Table 5).

3.5. Laboratory-confirmed infection, admission to the ICU, and death

This meta-analysis found that the risk of SARS-CoV-2 infection was higher in the non-booster group than that in the booster group with a relative risk (RR) of 9.45 (95% CI 3.22–27.79) and with a summary of VE of 89.42% (95% CI 68.94%—96.40%), which was significantly heterogenous (I^2 = 100%; Figure 2 and Table 2). We undertook further subgroup analyses to compare the results in the booster vaccination group and full doses vaccination group. A significant difference was found among the three groups ($p < 0.05$). The pooled RR of comparison between the two-dose inactivated vaccines vs. two-dose inactivated vaccines plus one-dose BNT162b2 was 27.53 (95% CI 20.46–37.05) with a summary

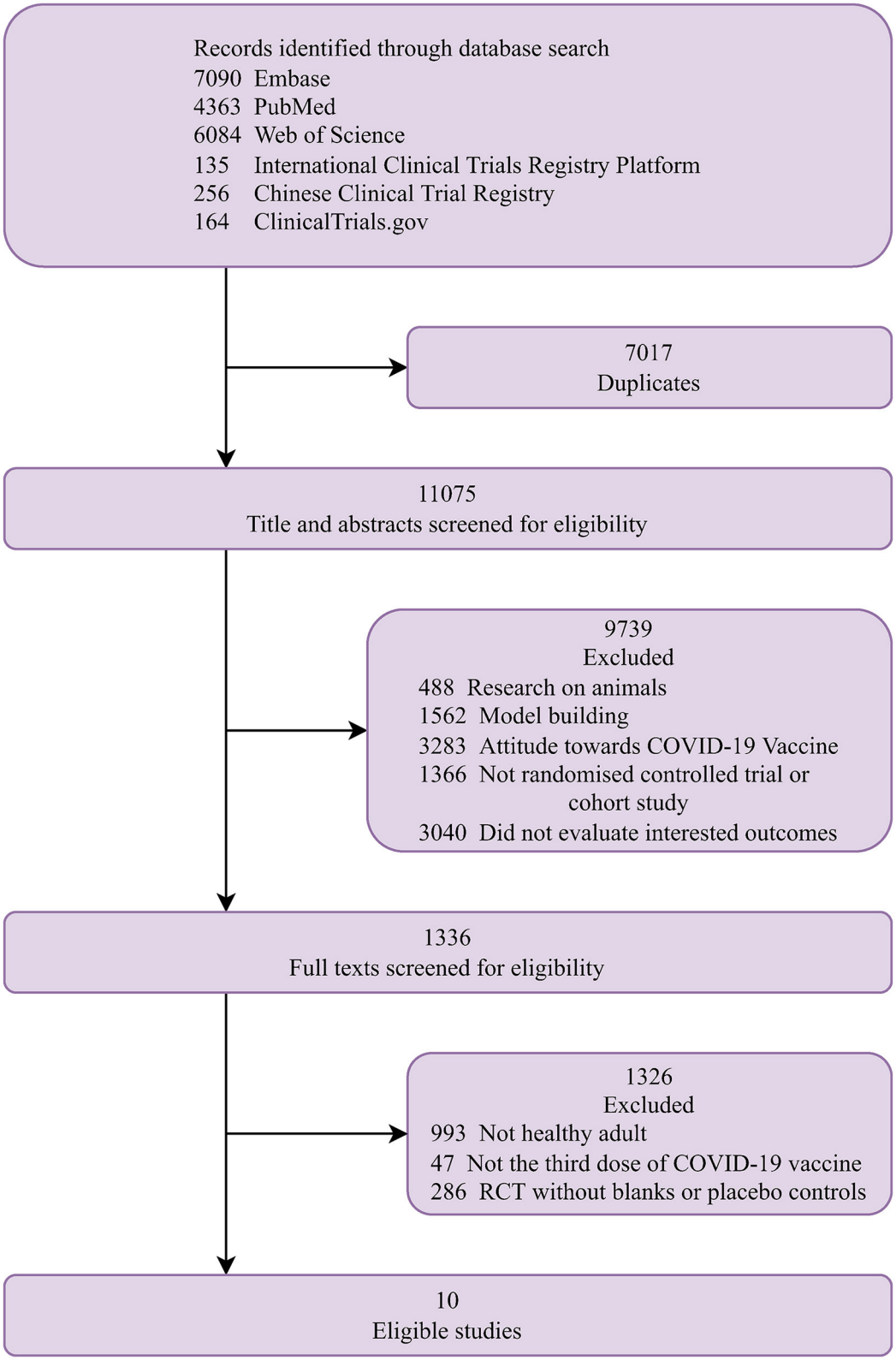

FIGURE 1
Study selection.

TABLE 1 The characteristics of the included studies.

References	Country/ region	Male (*n*, %)	Age (Years)	Comorbidities (*n*, %)	Study design	Boost Vaccine	Prime-boost interval (months)	Boost-outcome interval (days)	Comparison
Vadrevuet al. (21)	India	3 Dose: 67/91 (73.6 %) 2 Dose with placebo: 73/93 (78.5%)	Median (IQR) 3 Dose: 35 (25–44) 2 Dose with placebo: 36 (26–44)	NR	RCT	BBV152 after 2 Dose BBV152	7.2 months	28	3 vs. 2 Dose BBV152 with placebo
Ng et al. (20)	Singapore	3 Dose: 1,058,786/2,201,604 (48.1%) 2 Dose: 103,748/239,977 (43.2%)	Median (IQR) 3 Dose: 53 (42–64) 2 Dose: 44 (36–60)	NR	Retrospective cohort study	mRNA after 2 Dose mRNA Inactivated vaccine after 2 Dose Inactivated vaccine	5 months (no later than 9 months)	15–60	3 Dose vs. 2 Dose mRNA 3 vs. 2 Dose inactivated vaccine
Low et al. (16)	Malaysia	3 Dose: 2,522,806/5,081,641 (49.6%) 2 Dose: 11,557,790/22,598,839 (51.1%)	Mean (SD) 39.9 (15.5)	4 451 180 (32.2)	Retrospective cohort study	BNT after 2 Dose BNT BNT after 2 Dose CoronaVac CoronaVac after 2 Dose CoronaVac	6 months after 2 dose BNT 3 months after 2 dose CoronaVac:	14	3 vs. 2 Dose BNT 3 vs. 2 Dose CoronaVac 2 Dose CoronaVac + BNT vs. 2 Dose BNT 2 Dose CoronaVac + BNT vs. 2 Dose CoronaVac
Arbel et al. (13)	Israel	3 Dose: 357,818/758,118 (47%) 2 Dose: 37118/85090 (44%)	Proportion of ≥65yr 3 Dose:470,808/758,118 (62) 2 Dose:35,208/85,090 (41) Proportion of 50–64 years 3Dose: 287,310/758,118 (38) 2 Dose: 49,882/85,090 (59)	NR	Prospetive cohort study	BNT after 2 Dose BNT	At least 5 months	7	3 vs. 2 Dose BNT
Menni et al. (19)	UK	3 Dose BNT: 70,699/20,4731 (34.5%) 2 Dose ChAd + BNT: 162,410/405,239 (40.1%) 2 Dose BNT + Mod: 70,699/20,4731 (34.5%) 2 Dose ChAd + Mod: 162,410/405,239 (40.1%) 2 Dose BNT: 70,699/204,731 (34.5%) 2 Dose ChAd: 162,410/405,239 (40.1%) 2 Dose Mod: 4,588/10,823 (42.4%)	Median (IQR) 3 Dose BNT: 52 (38–62) 2 Dose ChAd + BNT: 56 (46–63) 2 Dose BNT + Mod: 52 (38–62) 2 Dose ChAd + Mod: 56 (46–63) 2 Dose BNT: 52 (38–62) 2 Dose ChAd: 56 (46–63) 2 Dose mRNA-1273: 39 (33–46)	3 Dose BNT: 41,136/20,4731 (20.1%) 2 Dose ChAd + BNT: 66,471/40,5239 (16.4%) 2 Dose Mod: 755/10,823 (7%)	Prospetive cohort study	BNT after 2 Dose BNT BNT after 2 Dose ChAd Mod after 2 Dose BNT Mod after 2 Dose ChAd	NA	150–240	3 Dose mRNA vs. 2 Dose BNT 3 Dose mRNA vs. 2 Dose ChAd 2 Dose ChAd + mRNA vs. 2 Dose BNT 2 Dose ChAd + mRNA vs. 2 Dose ChAd

(Continued)

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TABLE 1 (Continued)

References	Country/ region	Male (*n*, %)	Age (Years)	Comorbidities (*n*, %)	Study design	Boost Vaccine	Prime-boost interval (months)	Boost-outcome interval (days)	Comparison
Abu-Raddad et al. (12)	Qatar	3 Dose BNT: 122,435/189,483 (64.6%) 3 Dose mRNA-1273: 45,443/66,191 (68.7%) 2 Dose BNT: 122,435/189,483 (64.6%) 2 Dose mRNA-1273: 45,443/66,191 (68.7%)	Median (IQR) 3 Dose BNT: 41 (34–50) 3 Dose mRNA-1273: 39 (33–46) 2 Dose BNT: 41 (34–50) 2 Dose mRNA-1273: 39 (33–46)	NR	Retrospective cohort study	BNT after 2 Dose BNT Mod after 2 Dose Mod	Median (IQR) BNT: 8.3 (7.7–9) Mod: 7.7 (7.4–8.3)	Median (IQR) BNT: 22 (12–28) Mod: 18 (8–32)	3 vs. 2 Dose BNT 3 vs. 2 Dose Mod
Marra et al. (18)	Brazil	2 Dose CoronaVac + BNT: 1,183/4,472 (26.5%) 2 Dose ChAd + BNT: 1,161/3,927 (29.6%) 2 Dose CoronaVac: 327/1,157 (28.3%) 2 Dose ChAd: 601/1,871 (32.1%)	Median (IQR) 2 Dose CoronaVac + BNT: 37 (31–43) 2 Dose ChAd + BNT: 37 (30–43) 2 Dose CoronaVac: 34 (28–40) 2 Dose ChAd: 33 (26–40)	2 Dose CoronaVac + BNT: 879/4,472 (25.7%) 2 Dose ChAd + BNT: 1,016/3,927 (29.4%) 2 Dose CoronaVac: 177/1,157 (21.2%) 2 Dose ChAd: 344/1,871 (24.7%)	Retrospective cohort study	BNT after 2 Dose CoronaVac or 2 Dose ChAd	NA	14	2 Dose Coronavac + BNT vs. 2 Dose Coronavac 2 Dose ChAd + BNT vs. 2 Dose ChAd
Mahmoud et al. (17)	Abu Dhabi	3 Dose BBIBP-CORV: 16/20 (66.1%) 2 Dose BBIBP-CORV: 20/35 (57.1%)	Mean (SD) 3 Dose BBIBP-CORV: 41.71 (9.86) 2 Dose BBIBP-CORV:41.84 (10.45)	NR	Retrospective cohort study	BBIBP-CORV after2 Dose BBIBP-CORV	NA	82	3 Dose vs. 2 Dose BBIBP-CORV
Demirhindi et al. (15)	Adana Turkey	3 Dose CoronaVac: 7.1% 2 Dose CoronaVac + BNT booster: 68.4% 2 Dose CoronaVac: 24%	Median (IQR) 3 Dose CoronaVac: 39.29 (8.18) 2 Dose CoronaVac + BNT booster: 40.67 (10.94) 2 Dose CoronaVac: 35.66 (8.13)	3 Dose CoronaVac: 6.6% 2 Dose CoronaVac + BNT booster: 75.4% 2 Dose CoronaVac: 18%	Prospetive cohort study	CoronaVac after 2 Dose CoronaVac BNT after 2 Dose CoronaVac	CoronaVac: 4.3–6 months BNT: 4.5–6.2 months	14	3 Dose vs. 2 Dose CoronaVac 2 Dose CoronaVac + BNT vs. 2 Dose CoronaVac
Chiu et al. (14)	Taiwan China	2 Dose MVC + Mod: 6/14 (42.9%) 2 Dose ChAd: 8/15 (53.3%) 2 Dose MVC: 6/14 (42.9%)	Median 2 Dose MVC + Mod: 44.5 2 Dose ChAd: 40 2 Dose MVC: 44.5	2 Dose MVC + Mod: 3/14 (21.4%) 2 Dose ChAd: 2/15 (13.3%) 2 Dose MVC: 3/14 (21.4%)	Prospetive cohort study	Mod after 2 Dose MVC	Median (IQR) 1.0 (0.97–1.07) months	14	2 Dose MVC + Mod vs. 2 Dose ChAd 2 Dose MVC + Mod vs. 2 Dose MVC

BNT, BNT162b2 (Pfizer-BioNTech); ChAd, ChAdx1-S (Oxford-AstraZeneca); Mod, mRNA-1273 (Moderna); MVC, MVC-COV1901; NA, not applicable; NR, not reported.

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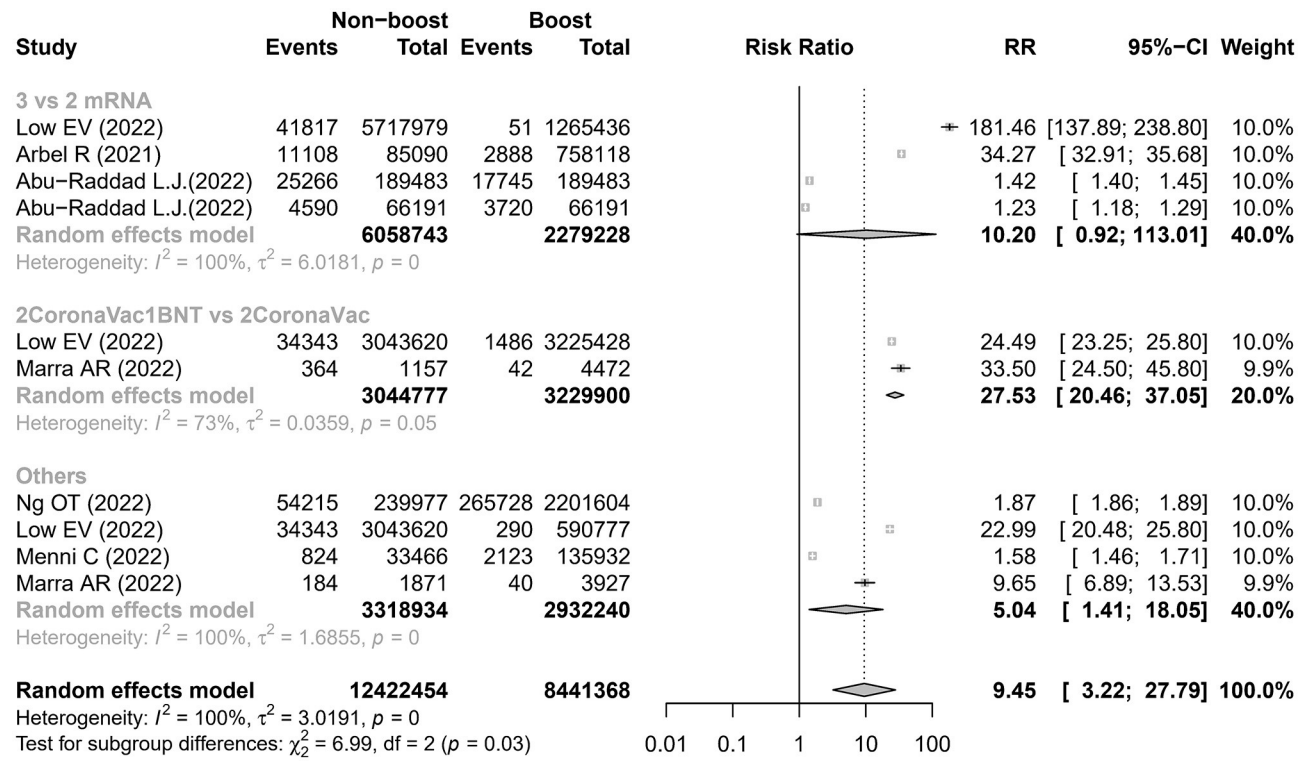

FIGURE 2
Estimates of the risk ratio of SARS-CoV-2 infection in the non-booster group vs. the booster group. BNT, BNT162b2(Pfizer-BioNTech); CI, confidence intervals; RR, risk ratio.

VE of 96.37% (95% CI 95.11%−97.30%), which was higher than that in the comparison of two-dose mRNA vaccines vs. three-dose mRNA vaccines [10.20 (95% CI 0.92–113.01)]. Four studies (16, 18–20) used various prime and booster combinations, such as two doses of mRNA plus an mRNA booster, two doses of inactivated vaccine plus an inactivated booster in Ng et al.'s study (20), two doses of mRNA plus an mRNA booster and two doses of inactivated vaccine plus an inactivated booster or BNT162b2 in Low et al.'s study (16), two doses of mRNA or ChAdOx1-S plus an mRNA booster in Menni C's study (19), and two doses of ChAdOx1-S plus a BNT162b2 booster in Marra et al.'s study (18). However, three of these studies (18–20) did not report the number of SARS-CoV-2 infections associated with the specific booster vaccination. The pooled estimation also showed that individuals who received two doses of vaccination had a higher risk of SARS-CoV-2 infections than individuals who also received a booster vaccination (RR 5.04, 95% CI 1.41–18.05) with a summary VE of 80.16% (95% CI 29.08%−94.46%). After removing the studies with the highest effect value, sensitivity analysis gave similar results (Supplementary Figure 1).

Three studies (16, 18, 20) reported six comparisons on the admission rate to ICU. The meta-analysis showed that the risk of admission to the ICU was higher in the non-booster group than that in the booster group with an RR value of 14.75 (95% CI 4.07–53.46), which was significantly heterogeneous (I^2 = 91%; Figure 3 and Table 2). After removing the studies with the highest effect value, sensitivity analysis gave similar results (Supplementary Figure 2). Four studies (12, 13, 16, 20) reported seven comparisons on the rate of death. The meta-analysis found that the risk of death was higher in the non-booster group than that in the booster group with an RR value of 13.63 (95% CI 4.72–39.36), which was significantly heterogenous (I^2 = 85%; Figure 4 and Table 2). After removing the studies with the highest effect value, sensitivity analysis gave similar results (Supplementary Figure 3). Due to the limited number of included studies, we did not take further subgroup analyses on admission to the ICU and death by prime and booster vaccination group. No significant statistical publication bias was detected by Egger's test (p = 0.075, 0.852, and 0.993, respectively), Begg rank correlation test (p = 0.380, 0.0.719, and 1.000, respectively), and funnel plot (Supplementary Figures 4–6).

3.6. Heterogeneity

The p-value for Cochrane's Q-test suggested high heterogeneity across studies in the assessment of all events.

4. Discussion

COVID-19 remains a public health concern worldwide, and hence clarifying and optimizing the vaccination effects is urgently needed to guide disease prevention and control. This

TABLE 2 The prevention effect comparison of a third COVID-19 boost vaccines vs. the full COVID-19 vaccine.

References	Country/ region	Diagnosis of COVID-19 infection	Boost vaccine comparison	SARS-CoV-2 infection (95% CI)	Admission to ICU (95% CI)	Death (95% CI)
Vadrevuet al. (21)	India	NA	NA	NA	NA	NA
Ng et al. (20)	Singapore	Official COVID-19 database	Others*	RR: 1.87 (1.86, 1.89) VE: 0.47 (0.46, 0.47)	RR: 5.47 (3.42, 8.73) VE: 0.82 (0.71, 0.89)	RR: 13.99 (10.55, 18.55) VE: 0.93 (0.91, 0.95)
Low et al. (16)	Malaysia	Reverse transcription-polymerase chain reaction (RT-PCR) or rapid antigen test result	3 vs. 2 mRNA	RR: 181.46 (137.89, 238.80) VE: 0.99 (0.99, 1.00)	RR: 121.06 (7.55, 1,940.28) VE: 0.99 (0.87, 1.00)	RR: 80.78 (5.03, 1,296.34) VE: 0.99 (0.80, 1.00)
Low et al. (16)	Malaysia	Reverse transcription-polymerase chain reaction (RT-PCR) or rapid antigen test result	2 CoronaVac1BNT vs. 2 CoronaVac	RR: 24.49 (23.25, 25.80) VE: 0.959 (0.956, 0.961)	RR: 62.87 (35.59, 111.40) VE: 0.98 (0.97, 0.99)	RR: 82.92 (30.93, 222.33) VE: 0.99 (0.97, 1.00)
Low et al. (16)	Malaysia	Reverse transcription-polymerase chain reaction (RT-PCR) or rapid antigen test result	Others	RR: 22.99 (20.48, 25.80) VE: 0.96 (0.95, 0.96)	RR: 23.07 (10.33, 51.51) VE: 0.96 (0.90, 0.98)	RR: 20.25 (6.50, 63.13) VE: 0.95 (0.85, 0.98)
Arbel et al. (13)	Israel	Reverse-transcriptase–quantitative polymerase-chain-reaction (RT-qPCR)	3 vs. 2 mRNA	RR: 34.27 (32.91, 35.68) VE: 0.970 (0.969, 0.972)	NA	RR: 18.78 (13.98, 25.22) VE: 0.95 (0.93, 0.96)
Menni et al. (19)	UK	Self-reported lateral flow or PCR test	Others	RR: 1.58 (1.46, 1.71) VE: 0.37 (0.32, 0.42)	NA	NA
Abu-Raddad et al. (12)	Qatar	Polymerase chain-reaction (PCR) tests	3 vs. 2 BNT	RR: 1.42 (1.40, 1.45) VE: 0.30 (0.29, 0.31)	NA	RR: 2.88 (1.29, 6.43) VE: 0.19 (0.15, 0.22)
Abu-Raddad et al. (12)	Qatar	Polymerase chain-reaction (PCR) tests	3 vs. 2 mRNA-1273	RR: 1.23 (1.18, 1.29) VE: 0.65 (0.22, 0.84)	NA	RR: 1.00 (0.14, 7.10) VE: 0.00 (−6.14, 0.86)
Marra et al. (18)	Brazil	Reverse transcription–polymerase chain-reaction (RT-PCR) testing	2 CoronaVac1BNT vs. 2 CoronaVac	RR: 33.50 (24.50, 45.80) VE: 0.97 (0.96, 0.98)	RR: 15.46 (1.73, 138.19) VE: 0.94 (0.42, 0.99)	NA
Marra et al. (18)	Brazil	Reverse transcription–polymerase chain-reaction (RT-PCR) testing	Others	RR: 9.65 (6.89, 13.53) VE: 0.90 (0.85, 0.93)	RR: 1.05 (0.19, 5.72) VE: 0.05 (-4.26, 0.83)	NA
Mahmoud et al. (17)	Abu Dhabi	NA	NA	NA	NA	NA
Demirhindi et al. (15)	Adana Turkey	NA	NA	NA	NA	NA
Chiu et al. (14)	Taiwan China	NA	NA	NA	NA	NA

*The used booster vaccine includes the inactivated vaccine, or other types of COVID-19 vaccine (the homologous or heterologous vaccines).
NA, not applicable; Mod, mRNA-1273 (Moderna); BNT, BNT162b2 (Pfizer-BioNTech); VE, 1–1/RR.

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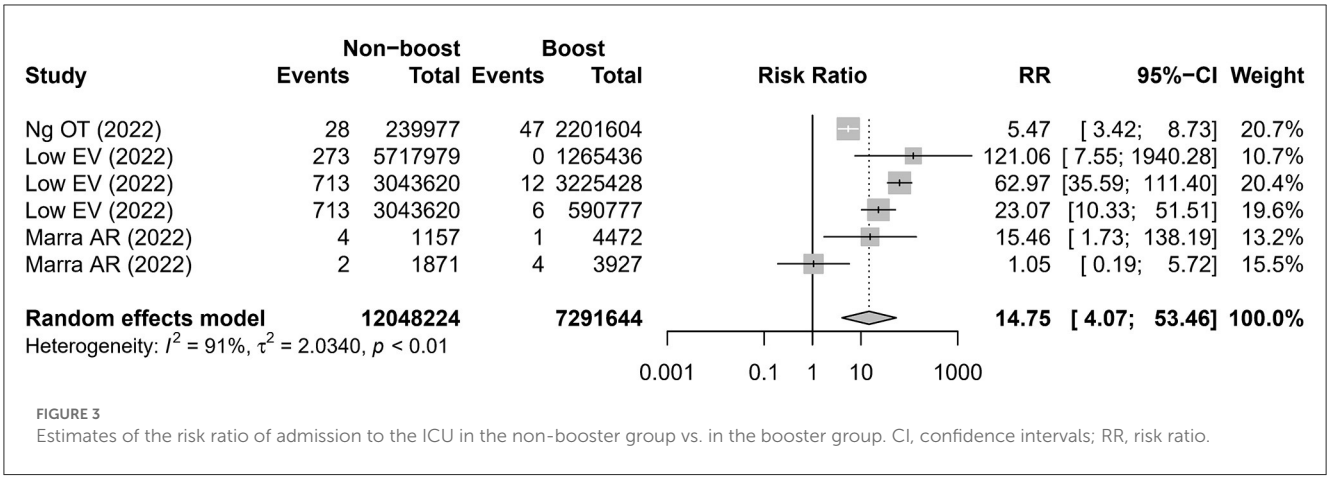

FIGURE 3
Estimates of the risk ratio of admission to the ICU in the non-booster group vs. in the booster group. CI, confidence intervals; RR, risk ratio.

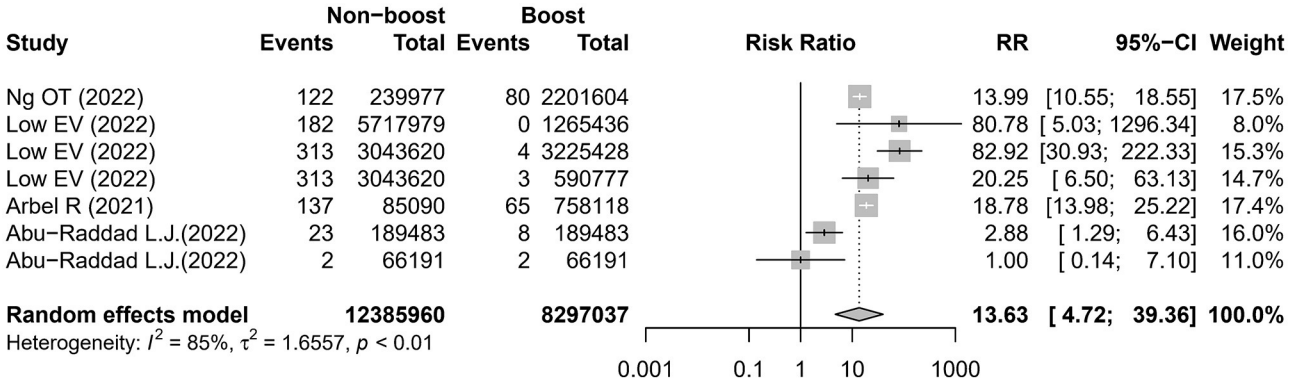

FIGURE 4
Estimates of the risk ratio of death in the non-booster group vs. in the booster group. CI, confidence intervals; RR, risk ratio.

systematic review and meta-analysis found that the provision of a booster COVID-19 vaccination induced a higher seroconversion rate and antibody levels compared to the primary vaccination alone. Furthermore, this study showed that a booster COVID-19 vaccination resulted in the improvement of some indicators, including SARS-CoV-2 infection, admission to the ICU, and death, which reflected a substantial clinical protective efficacy of a booster vaccination. This systematic review provided comprehensive and solid evidence supporting the promotion of one booster dose COVID-19 vaccine in the general adult population on top of the full vaccination.

SARS-CoV-2 infection begins when the RBD of the S protein of the virus binds to the angiotensin-converting enzyme 2 (ACE-2) receptor in human cells. Thus, positive serologic tests for the vaccine antigenic target (S and S subunits, including RBD) or antibody titers have been regarded as the most useful surrogate endpoint for COVID-19 vaccine effectiveness. However, it has been proven that the effectiveness of COVID-19 vaccines wanes around 4–6 months after the primary series of vaccination has been completed (22, 23). Moreover, newly emerging variants of SARS-CoV-2 resulted in break-through viral infections in vaccinated individuals and recovered patients (24, 25). A booster dose of COVID-19 vaccine could be a promising strategy by inducing a higher seroconversion rate of vaccine antigenic target and a higher level of antibody titers. Four studies included in this systematic review found that a booster dose of the COVID-19 vaccine could induce a higher seroconversion rate of antibodies or higher level of antibody titers among individuals compared to those who only received full COVID-19 vaccination (14, 15, 17, 21). Specifically, two of the four studies showed that a booster dose increased the neutralization efficiency against the Alpha, Delta, and Omicron variants (14, 21). Among the two, one study investigated an inactivated vaccine booster vaccination after two-dose inactivated full vaccination (21) and the other was involved in an mRNA-1273 booster vaccine after two-dose MVC-COV1901 vaccination (14). It suggested that a booster vaccine regardless of whether it was heterologous or homologous with the prime vaccination had a good humoral response against various variants including Omicron, which was the dominant strain globally to date.

T cells can recognize viral protein segments of deep hid and are less susceptible to immune evasion via mutation, even for variants that were considered able to escape neutralizing antibodies (26, 27). Chiu et al. (14) found that the administration of an additional dose of mRNA vaccine after two doses of the subunit vaccine can significantly enhance the cellular immune response for both the wild type and the Delta variant. In addition, Vadrevu et al. (21) showed that IgG-secreting memory B cells were higher in the booster arm compared to the non-booster arm. These results

implied that the provision of a booster COVID-19 vaccination could elicit both strong humoral and cellular immune responses to SARS-CoV-2.

In this study, the seroconversion rate or antibody titers were used as indicators to reflect the immune responses to a vaccine, and they worked as proxies on the effects of the vaccine regarding infection rates and severity of COVID-19. At the same time, the infection rate, admission rate to ICU, and death were direct indicators reflecting the effectiveness of COVID-19 vaccination. The protection effect against SARS-CoV-2 infection and severe disease had been widely confirmed, but this effect decreased in months after the prime vaccination. Furthermore, the frequent emergence of various variants of SARS-CoV-2 could also result in reduced protection effects in vaccinated individuals. Our meta-analysis showed that a third dose of the COVID-19 vaccine could significantly reduce the risk of SARS-CoV-2 infection, admission to the ICU, and death, regardless of whether it was heterologous or homologous with the prime vaccination. In addition, three of 10 comparisons in SARS-CoV-2 infection (12, 20), one of five comparisons in admission to the ICU (20), and three of seven comparisons (12, 20) were assessed during the Omicron wave. Thus, these results provided the most updated evidence on the clinical effects of the COVID-19 booster vaccination. The global average proportion of COVID-19 vaccine booster administration rate was 33.5% as of 28 December 2022 (1). With a huge gap remaining between the actual booster vaccination coverage and the ideal coverage, the findings of this study could reduce the booster vaccination hesitancy and add solid evidence to the current World Health Organization (WHO) recommendations on adult booster vaccination (28).

This study has limitations. First, we only included studies with either longitudinal or RCT study design in this review, the effects of some potential factors might not be fully ruled out due to the inclusion of observational studies, such as age and comorbidities. The uneven status of baseline information between the exposed group and the non-exposed group may affect the analysis results between the exposure factors and the outcome time. Therefore, relevant limitations need to be considered when interpreting the research results. Second, only 10 studies were eligible and were included in the analysis. Some subgroup analyses comparing the effectiveness of various booster vaccination strategies were unable to be performed (e.g., heterologous and homologous with the booster vaccination and the time interval between the prime and booster vaccinations). Third, most of the included studies assessed the clinical outcomes during the Delta wave and only two studies during the Omicron wave. The findings for the Omicron variant could be strengthened when more studies are available reporting the effectiveness of the booster vaccination in the Omicron wave in the future. Four, the heterogeneity was high in the pooled estimation of clinical effectiveness, which may be due to the small number of included studies with different study designs, including the different types of used SARS-CoV-2 vaccines and different vaccination duration. Nonetheless, this meta-analysis synthesized the study results on COVID-19 booster effectiveness by comparing the first dose of COVID-19 booster and full vaccination. Most of the included studies had large samples, which ensured a large sample of participants as a whole for the meta-analysis. Five, most of the included studies assessed clinical outcomes during the Delta wave. While the Omicron wave is the current predominant variant, so further studies need to focus on the effectiveness of booster vaccinations during the Omicron wave and any subsequent variants that may emerge in the future. Finally, the number of included studies is small (10), which may limit the robustness of our study findings, so the generalizability of your results should be cautious.

5. Conclusion

This systematic review and meta-analysis indicated that both types of homogenous or heterogeneous booster COVID-19 vaccination could elicit both strong humoral and cellular immune responses to SARS-CoV-2. Furthermore, a third dose of the COVID-19 vaccine could significantly reduce the risk of SARS-CoV-2 infection, admission to the ICU, and death. These results were also applicable to the Omicron variant. Future studies were needed to investigate the long-term clinical effectiveness of the first booster dose of the COVID-19 vaccine and compare the effectiveness between homogenous and heterogeneous booster COVID-19 vaccination.

Data availability statement

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

Author contributions

JX and JL drafted the manuscript and analyzed the data. XL, LZ, and MS performed the searching, reviewing, and selecting of literature. JL, XL, LZ, JZ, MS, and XZ collected the epidemiological data. JL and XZ designed and revised the manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

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

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Supplementary material

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Low vaccination and infection rate of Omicron in patients with inflammatory bowel disease: a comparative study of three unique cohorts

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Background: The SARS-CoV-2 Omicron variant caused a large-scale outbreak of COVID-19 in Shanghai, China. Patients with inflammatory bowel disease (IBD) are at high risk of infection due to immunosuppressive interventions. We aimed to investigate the vaccination information of patients with IBD and update a vaccination guide based on a comparison of vaccination in asymptomatic carriers and healthy individuals.

Methods: This retrospective study was conducted during an Omicron variant wave. We assessed the vaccination status in patients with IBD, asymptomatic carriers and healthy individuals. Factors with unvaccinated status and adverse events following vaccination were also determined in patients with IBD.

Results: The vaccination rate was 51.2% in patients with IBD, 73.2% in asymptomatic carriers, and 96.1% in healthy individuals. Female sex ($p = 0.012$), Crohn's disease ($p = 0.026$), and disease behavior of B3 ($p = 0.029$) were factors that indicated a lower vaccination rate. A significantly higher proportion of healthy individuals had received one booster dose (76.8%) than asymptomatic carriers (43.4%) and patients with IBD (26.2%). Patients with IBD received vaccination without an increased risk of adverse events ($p = 0.768$).

Conclusion: The vaccination rate of patients with IBD remains much lower than that of asymptomatic carriers and healthy individuals. The COVID-19 vaccine has been found to be safe among all three groups and patients with IBD are not more susceptible to adverse events.

KEYWORDS

Omicron, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), coronavirus disease-19 (COVID-19), inflammatory bowel disease (IBD), asymptomatic

1. Introduction

The Omicron lineage of SARS-CoV-2 became globally dominant within a few days of its first detection in Africa in November 2021 because of its powerful infectivity and poor detectability by the immune system (1). It split into multiple sub-lineages, among which the BA.2 variant is spreading rapidly throughout the world and has sparked a new wave of fulminant infection in Shanghai, China. As of June 1, 2022, a total of 626,737 individuals tested positive for the Omicron BA.2 and BA.2.2 variants during the Shanghai 2022 SARS-CoV-2 Omicron variant wave, most of which were asymptomatic carriers (2, 3).

Compared with the original variant of SARS-CoV-2, Omicron variants seem to be less severe, particularly in vaccinated individuals, but still lead to a number of severe situations in individuals with low immunity. Therefore, it is necessary to implement measures to prevent highly transmissible infections. Vaccination is currently the best available tool to protect people from SARS-CoV-2 infection and appears to be particularly necessary in those with pre-existing health conditions, although SARS-CoV-2 Omicron variants are believed to be partially resistant to infection- and vaccine-induced immunity.

Inflammatory bowel disease (IBD), mainly comprising ulcerative colitis (UC) and Crohn's disease (CD), is an immune-mediated inflammatory gastrointestinal disease with steadily increasing prevalence worldwide. Patients with IBD are frequently treated with immunomodulators, which can increase the risk of infection. Facing the omicron variant pandemic, particular attention should be paid to the prevention of infection. Major IBD societies recommend the vaccination of patients with IBD at the earliest possible stage for better protection (4, 5). In practice, not all IBD clinicians suggest vaccination during the active disease stage or immunosuppressive situation, and a number of patients with IBD still hesitate to be vaccinated for various reasons, leading to suboptimal vaccination coverage (6, 7). To date, there existed a limited number of documented cases of SARS-CoV-2 infection in patients with IBD on a global scale, with only one case of infection reported locally during this local Omicron wave. Nonetheless, the risk of Omicron variants in patients with IBD should not be underestimated and SARS-CoV-2 may be mutated to other variants in the future. Breakthrough infections in vaccinated people are noticed in asymptomatic patients with COVID-19, and vaccination strategies for patients with IBD should be dynamically adjusted according to real-world settings.

In this study, we assessed the COVID-19 vaccination rate, factors related to unvaccination, and the post-vaccination adverse events (AEs) in patients with IBD. In particular, we included a detailed comparative analysis of the vaccination status of asymptomatic carriers and uninfected healthy people during the 2022 Omicron wave. The analysis of vaccination information for three specific cohorts conveys more practical vaccination information for patients with IBD, beyond the highly transmissible Omicron BA.2 and BA.2.2 variants.

2. Methods

2.1. Study design and participants

This was a retrospective, cross-sectional study. The study was conducted during the new wave of SARS-CoV-2 Omicron variant infections in Shanghai, making it possible for us to evaluate the impact of vaccination and restrictions on public gatherings. Patients with IBD who were regularly followed up at Ren Ji Hospital, Shanghai, China between January 1, 2020, and June 1, 2022, were enrolled in this study. Patients were followed up closely during the Shanghai COVID-19 outbreak using the telephone and WeChat app. Asymptomatic carriers with Omicron variants were double-confirmed by PCR testing, and vaccination data were included via the hospital information system of Meipu Temporary Hospital, which is a designated COVID-19 treatment campus affiliated to Baoshan Branch, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine. These asymptomatic carriers were non-IBD patients and had no history of immune-related disorders. Healthy individuals were enrolled from the physical examination center of Ren Ji Hospital.

The inclusion criteria were: (i) patients with IBD diagnosed based on the European Crohn's and Colitis Organization guidelines (8), (ii) actively followed-up *via* the hospital information system, and (iii) no history of SARS-CoV-2 variant infection. Asymptomatic carriers were double-checked by PCR testing and diagnosed according to the Corona Virus Disease-19 Prevention and Control Consensus Diagnosis and Treatment of Corona Virus Disease-19 (9th edition, China) (9), which is quite similar to the Centers for Disease Control and Prevention of the US. Healthy individuals without a history of SARS-CoV-2 variant infection were confirmed by PCR testing and none of them had any other immune-related disorders. Participants with missing vaccination data or those living outside Shanghai during the regional outbreak were excluded from the study (Figure 1). The need for informed consent was waived in view of the retrospective observational nature of the study. The study protocol was approved by the Institutional Review Board of Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine (KY2022-162-B). This study was conducted in accordance with the Declaration of Helsinki to protect the patients' confidentiality of information.

2.2. Data collection

We conducted a survey and analysis of vaccination data for three cohorts: patients with IBD, asymptomatic carriers, and healthy individuals. Participants vaccinated with at least one dose were recognized as vaccinated, and the unvaccinated rate was documented until June 1, 2022.

For patients with IBD, demographic characteristics and data related to IBD diagnosis during their recent hospitalization were retrospectively extracted from medical records. They were actively followed up every 3 months via a hospital information system phone call and/or WeChat app. Extra follow-up was performed between May 20 and June 1, 2022, by telephone regarding the overall disease situation, the SARS-CoV-2 Omicron variant infection condition, and vaccination information, including vaccination status, number of doses, AEs within 7 days of vaccination, or factors related to unvaccination. The characteristics and vaccination information of asymptomatic carriers were summarized from medical records with concealed identification information. For healthy individuals, relevant information was obtained from medical records at the time of medical examination at our hospital.

2.3. Statistical analysis

Statistical analysis was performed using R version 4.0.3 and STATA version 15.0 for Windows (Stata Corp LLC, College Station,

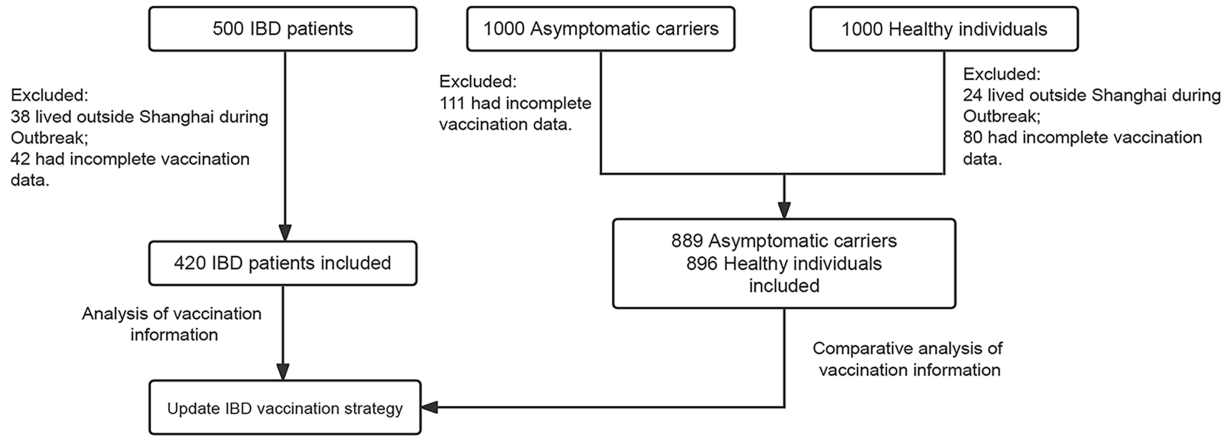

FIGURE 1
Flowchart of patients' recruitment.

TX, United States). Normally distributed continuous variables are presented as means and standard deviations, and non-normally distributed variables are presented as medians and interquartile ranges. Categorical variables are presented as proportions. Comparisons between the two groups were made using Student's *t*-test, Mann–Whitney U test, chi-squared test, or Fisher's exact test, as appropriate. The proportion of unvaccinated participants was calculated in the full analysis set and compared between cohorts. An additional safety analysis was conducted in patients with IBD and healthy individuals who received at least one dose of COVID-19 vaccination. Univariate analysis was used to identify variables that may be associated with the unvaccinated status.

Sample size estimation was performed using an online sample size calculator.[1] Based on previously reported vaccination data for patients with IBD and asymptomatic carriers (50% and 70%, respectively), we estimated the sample size with a significance level of $\alpha = 0.05$, and the matching ratio of the two groups was set to 1:2. A ratio 1:1 was used to evaluate the sample size of asymptomatic carriers and healthy individuals. All statistical tests were two-sided, and statistical significance was set at $p < 0.05$.

3. Results

3.1. Study population

To evaluate the impact of vaccination and restrictions on public gatherings during the local outbreak of SARS-CoV-2 Omicron variant infection, only participants who lived in Shanghai during the local outbreak were included. Forty-two patients with IBD, 111 asymptomatic carriers, and 80 healthy individuals were excluded from our study owing to loss to follow-up or incomplete information records. Finally, 420 patients with IBD, 889 asymptomatic carriers, and 896 healthy individuals were included in the current analyses. Participants who received at least one dose of the vaccine were administered inactivated vaccines. The recruitment process is shown in Figure 1.

3.2. COVID-19 vaccination and factors related to unvaccination in patients with IBD

The characteristics of the patients with IBD included in this study are summarized in Table 1, and comparative analyses are presented between the unvaccinated and vaccinated cohorts. There were predominantly male patients (66.2%) with CD (69.8%), undergoing biological treatment during or within the last 12 months of vaccination (85.2%) with a mean age of 34.4 ± 11.1 years. None of the patients were infected with the SARS-CoV-2 Omicron variant during the Shanghai 2022 outbreak.

Among 420 patients, 215 (51.2%) received at least one dose of the vaccine. Compared with vaccinated patients, those who remained unvaccinated were more likely to be female (odds ratio [OR] 0.58, 95% confidence interval [CI] 0.39–0.87, $p = 0.012$). As a subtype of inflammatory bowel disease, patients with CD had a lower vaccination rate than patients with UC (47.4% vs. 59.8%, $p = 0.026$). In addition, patients with CD with the disease behavior of B3, a penetrating type of Montreal classification which indicates a more severe condition, appeared to be more reluctant to be vaccinated ($p = 0.029$) (Table 2). In our study, most of the patients ($n = 358$, 85.2%) underwent biological therapy. Although the concern regarding immunosuppression of biologics seems to affect the vaccination compliance of patients, patients receiving biological agents showed no significant difference in vaccination rates ($p = 0.357$).

Among the 205 patients who remained unvaccinated, the majority (n = 72, 35.1%) refused or delayed vaccination due to fear of IBD aggravation, 57 (27.8%) were concerned about vaccine allergies, 43 were temporarily unable to get vaccinated due to other events such as pregnancy, 24 (11.7%) were worried about the interaction between vaccine and biologics, and 9 (4.4%) claimed lack of positive medical advice (Figure 2).

1 http://www.powerandsamplesize.com/

TABLE 1 Characteristics and factors associated with vaccination status in patients with IBD.

	All (*n* =420)	Unvaccinated (*n* =205)	Vaccinated (*n* =215)	*p*
Sex				0.012
Female	142 (33.8)	82 (40.0)	60 (27.9)	
Male	278 (66.2)	123 (60.0)	155 (72.1)	
Age	34.4 (11.1)	35.2 (11.0)	33.5 (11.2)	0.123
IBD type				0.026
CD	293 (69.8)	154 (75.1)	139 (64.7)	
UC	127 (30.2)	51 (24.9)	76 (35.3)	
Extension CD[a] (L1; L2; L3)		48 (31.2); 19 (12.3); 87 (56.5)	48 (34.5); 22 (15.8); 69 (49.6)	0.465
Behavior CD[b]				
(B1; B2; B3)		83 (53.9); 40 (26.0); 31 (20.1)	90 (64.7); 36 (25.9); 13 (9.35)	0.029
P[b]		89 (57.8)	69 (49.6)	0.200
Surgical history	180 (42.9)	90 (43.9)	90 (41.9)	0.746
Biological therapy				0.357
IFX	231 (55.0)	117 (57.1)	114 (53.0)	
ADA	8 (1.90)	4 (1.95)	4 (1.86)	
VDZ	86 (20.5)	39 (19.0)	47 (21.9)	
UST	33 (7.86)	20 (9.76)	13 (6.05)	
Not in use[c]	62 (14.8)	25 (12.2)	37 (17.2)	

Values represent the mean (standard deviation) or *n* (%). [a,b]Subtypes of montreal classifications; [c]no use of biologics in the 12 months prior to vaccination.
CD, Crohn's disease; UC, ulcerative colitis; P, perianal lesion; IFX, infliximab; ADA, adalimumab; VDZ, vedolizumab; UST, ustekinumab.

3.3. Comparison of COVID-19 vaccination among patients with IBD, asymptomatic carriers and healthy individuals

To comprehensively evaluate the protective impact of vaccination on COVID-19 disease control across multiple populations, vaccination data were also collected and analyzed from asymptomatic carriers and healthy individuals during a local outbreak of SARS-CoV-2 Omicron variant infection (Supplementary Table S1). Of 889 asymptomatic carriers, 651 (73.2%), and 861/896 healthy individuals (96.1%), were vaccinated with at least one dose within 12 months before the outbreak, which indicated that the vaccination rate of asymptomatic carriers was significantly lower than that of healthy individuals ($p < 0.001$). There was no significant difference in age, but a significant difference in sex (OR 1.34, 95% CI 1.11–1.62, $p = 0.003$) between the two groups, indicating that men were more likely to be infected with Omicron. Moreover, patients with IBD had the lowest vaccination rate among the three groups (Figure 3). In the subgroup analysis, healthy individuals had a higher proportion of one booster dose (76.8%) than asymptomatic carriers (43.4%) and patients with IBD (26.2%) ($p < 0.001$), while the fully vaccinated individuals were similar in proportion among healthy individuals (18.1%), asymptomatic carriers (26.5%), and patients with IBD (21.4%).

3.4. Safety analysis of COVID-19 vaccine In patients with IBD and healthy individuals

A total of 215 patients with IBD and 861 healthy individuals who had received at least one dose of the COVID-19 vaccine were included in the safety analysis after elimination of missing data or reluctance to disclose health information. Thirty-one patients with IBD and 115 healthy individuals reported at least one adverse event after vaccination, accounting for 14.4% and 13.4%, respectively. No statistically significant difference was detected in the incidence of AEs ($p = 0.768$) (Supplementary Table S2).

The most common AEs of the two groups were "Fatigue" and "Injection-site pain" (Table 3). Most injection-site or systematic AEs were transient and could be resolved without medication. However, six patients with IBD suffered from aggravation of diarrhea, and one developed gastrointestinal bleeding 1 week after vaccination, all of whom recovered smoothly without hospitalization.

4. Discussion

The SARS-CoV-2 Omicron variant is highly transmissible and may escape vaccine-induced immunity, thereby leading to the rapid spread of COVID-19 worldwide. Thus, vaccination and isolation are necessary, especially for immunosuppressed patients. Our study was performed during the outbreak of SARS-CoV-2 Omicron variant in Shanghai, China to investigate the vaccination status of patients with IBD and compare the vaccination data between asymptomatic carriers and healthy individuals. To the best of our knowledge, no previous study has reported the differences in vaccination between patients with IBD and other non-IBD populations, including infected and non-infected individuals.

Although surprisingly few patients actually got SARS-CoV-2 Omicron variant infection during the local outbreak, the vaccination coverage of Chinese patients with IBD is of concern. Only 51.2% of

TABLE 2 Characteristics and factors associated with vaccination status in Crohn's disease and ulcerative colitis.

	CD			UC		
Characteristics	Unvaccinated (*n* =154)	Vaccinated (*n* =139)	*p*	Unvaccinated (*n* =51)	Vaccinated (*n* =76)	*p*
Age	32.9(8.62)	30.6 (9.40)	0.026	42.3 (14.1)	38.9 (12.3)	0.172
Sex			0.003			0.707
Female	60 (39.0)	31 (22.3)		22 (43.1)	29 (38.2)	
Male	94 (61.0)	108 (77.7)		29 (56.9)	47 (61.8)	
Surgical history	82 (53.2)	76 (54.7)	0.898	8 (15.7)	14 (18.4)	0.873
Times vaccinated (1; 2; 3)	11 (7.91); 65 (46.8); 63 (45.3)			4 (5.26); 25 (32.9); 47 (61.8)		
Extension CD[a]			0.465			
L1	48 (31.2)	48 (34.5)				
L2	19 (12.3)	22 (15.8)				
L3	87 (56.5)	69 (49.6)				
Behavior CD[b]			0.029			
B1	83 (53.9)	90 (64.7)				
B2	40 (26.0)	36 (25.9)				
B3	31 (20.1)	13 (9.35)				
P	89 (57.8)	69 (49.6)	0.200			
Biologic therapy			0.894			0.313
IFX	102 (66.2)	98 (70.5)		15 (48.39)	16 (51.61)	
ADA	4 (2.60)	4 (2.88)		0	0	
VDZ	14 (9.09)	12 (8.63)		25 (41.67)	35 (58.33)	
UST	20 (13.0)	13 (9.35)		0	0	
Not in use[c]	14 (9.09)	12 (8.63)		11 (30.56)	25 (69.44)	

Values represent the mean (standard deviation) or *n* (%). [a,b]Subtypes of Montreal classifications; [c]no use of biologics in the 12 months prior to vaccination.
CD, Crohn's disease; UC, ulcerative colitis; P, perianal lesion; IFX, infliximab; ADA, adalimumab; VDZ, vedolizumab; UST, ustekinumab.

patients with IBD enrolled in our study had been vaccinated, whereas vaccination rates in other countries were above 60% and even up to 95% (7, 10, 11). Our investigation indicated that women and patients with CD and relatively severe disease status were more reluctant to be vaccinated—possibly because they worried that this might aggravate their condition, which mirrors the findings of similar studies in other countries (5, 10, 12). As revealed in our study, "Fear of IBD aggravation" is the primary concern of patients with IBD. Indeed, there appears to be a widespread concern among patients with autoimmune diseases that their illnesses may be exacerbated as a result of post-vaccination AEs (13–15). Suspicion of vaccines in these patients usually stems from the misconception that the vaccines have not been studied sufficiently, despite their safety and effect have been demonstrated widely (16–18). To address these concerns, healthcare providers and IBD specialists should cooperate to proactively inform patients about the benefits of vaccination in their health, such as creating health knowledge brochures and holding vaccination lectures to dispel any misconceptions or myths that patients may have about vaccines and their potential effects on IBD.

Although the vaccination rate of patients with IBD was significantly lower than that of asymptomatic carriers and healthy individuals, no SARS-CoV-2 Omicron variant infection of IBD was reported in this local outbreak. It has also been demonstrated that patients with IBD have no increased risk of SARS-CoV-2 infection compared with the general population (19). This is probably the result of these patients' education to avoid gatherings, alongside their tendency to self-protect more than the general population and avoid contact with high-risk populations. Nevertheless, the vaccine does prevent infections in patients with IBD. Since asymptomatic carriers have significantly lower vaccination rates than healthy individuals, the vaccine could reduce the risk of SARS-CoV-2 Omicron variant infection, despite the pronounced ability of the Omicron variant to escape being detected from the immune system. Several large cohort studies have pointed out and provided evidence that a booster vaccine could more effectively prevent the infection of Omicron variant (20). Besides, we observed an infection sex bias, with men being more susceptible than women, which confirmed the findings of other studies (21, 22). Hence, improved vaccine coverage, especially among males, is warranted.

However, quite a few patients with IBD still show a high level of vaccine reluctance and safety concerns. Previous studies on other vaccine types have considered inactivated vaccines to be safe for patients with IBD regardless of their immunosuppressive therapies (23, 24), which was further confirmed in this real-world COVID-19 vaccine study. We found that the incidence of COVID-19 vaccine AEs was similar in patients with IBD and healthy individuals, both of whom did not exceed 15%. The predominant reported AEs of healthy individuals and patients with

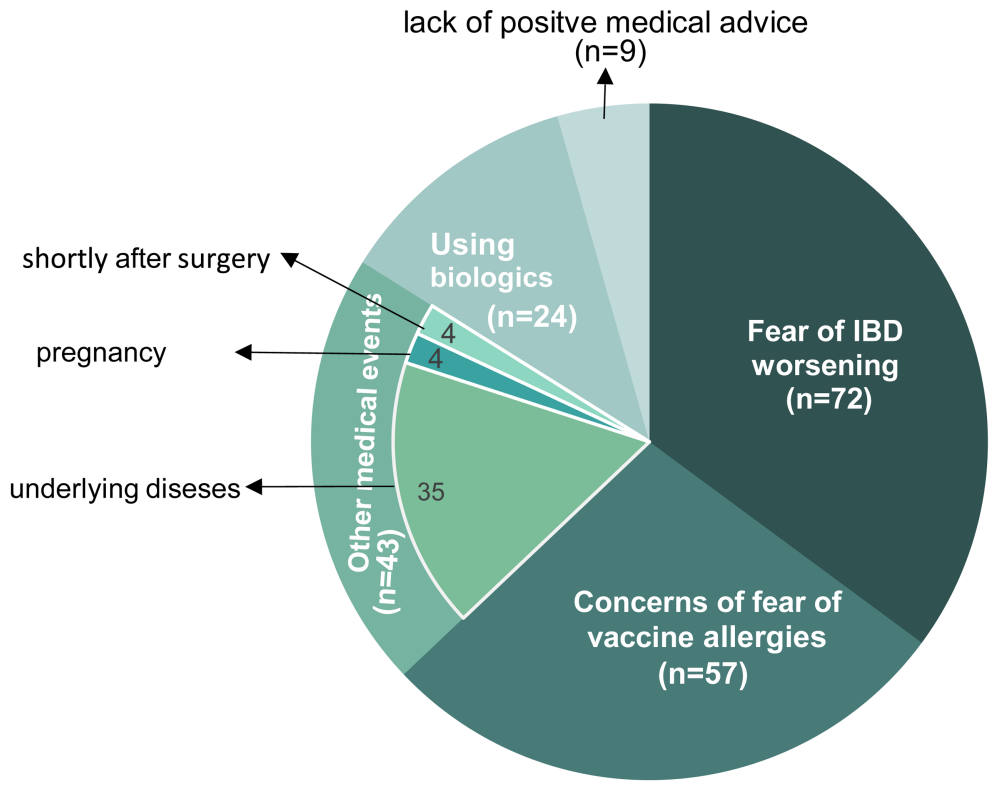

FIGURE 2
Reasons for remaining unvaccinated in patients with IBD.

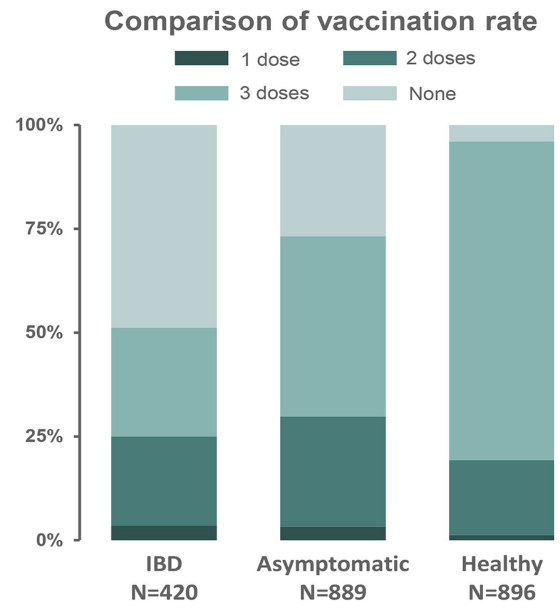

FIGURE 3
Comparison of vaccination rate among three populations.

TABLE 3 Adverse events in patients with IBD and healthy individuals.

Adverse events	IBD (*n* =31)	Healthy (*n* =115)
Local		
Injection-site pain	5	61
Systemic		
Fatigue	11	26
Fever	1	14
Headache	5	9
Somnolence	4	24
Muscle aches	1	2
Nausea	1	1
Cough	2	2
Allergic reaction	3	3
Other*	**10**	**6**

*Six patients with IBD experienced exacerbation of diseases, such as diarrhea. Four patients with IBD presented with elevated blood pressure, gastrointestinal bleeding, swollen feet, and a 2-cm increase in breast nodules. Among the healthy individuals, one had elevated blood pressure, blood glucose, and lipids, two had irregular menstruation, one had diarrhea, and one had swollen thighs.

IBD were mostly common, such as fatigue, injection-site pain, and headache, which can be relieved spontaneously within a week. Patients with IBD were not at an increased risk of developing AEs or more serious AEs after vaccination. To date, the Shanghai 2022 Omicron BA.2 infection has not yet ended, while Omicron BA.5 has already raised a new wave of infections worldwide. To better combat the new wave of the epidemic, on the one hand, patients with IBD should further strengthen their personal protection and

receive vaccination whenever possible, especially men who are at a higher risk of infection. On the other hand, the government and healthcare providers should educate patients with IBD more about the efficacy of vaccines and offering a special AEs monitoring service may be a good way to allay their concerns about vaccines. Continuously developing new COVID-19 vaccines and improving the existing vaccines is also essential.

In conclusion, the COVID-19 vaccine is generally safe and effective in providing resistance against SARS-CoV-2 Omicron variant infection; however, the vaccination rate was rather low in patients with IBD. Females and patients with severe CD were less likely to accept vaccination, while males were at a higher risk of infection. It is essential for healthcare and IBD specialists to proactively convince patients with IBD of the importance and safety of vaccines and encourage them to intensify their personal protection to minimize their infection risk in face of the successive waves of COVID-19.

This study has several strengths, including a large sample size and multidimensional analysis. To our knowledge, this is currently the first largest real-world study to perform a comparative analysis of vaccination data from three unique cohorts in the same Omicron variant exposure environment: patients with IBD, asymptomatic carriers, and healthy individuals. The study provides important insights into the vaccination coverage and attitudes towards vaccination among patients with IBD in China. However, there were also some limitations to our study. First, all the participants were from a single center in Shanghai, which may lead to a regional bias. Second, there has been a lack of vaccination data for patients with IBD infected with Omicron variant. Third, the data on AEs are insufficient owing to mission information or reluctance to disclose health information. The information collected in our study was not comprehensive enough because of the tight timelines of the outbreak and the limited resources allocated to the creation of the data collection and reporting system.

Data availability statement

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

Author contributions

JF contributed significantly to perform the research and manuscript preparation. TY and RY participated in data collection. JF and BF analyzed and interpreted the clinical data. RH helped analyze part of the data. YQ helped create tables and figures. JT helped revise the manuscript. JS conceived the study and critically reviewed the content of the paper and supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1115127/full#supplementary-material

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Comparison of vaccination and booster rates and their impact on excess mortality during the COVID-19 pandemic in European countries

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Aim: To evaluate the effect of vaccination/booster administration dynamics on the reduction of excess mortality during COVID-19 infection waves in European countries.

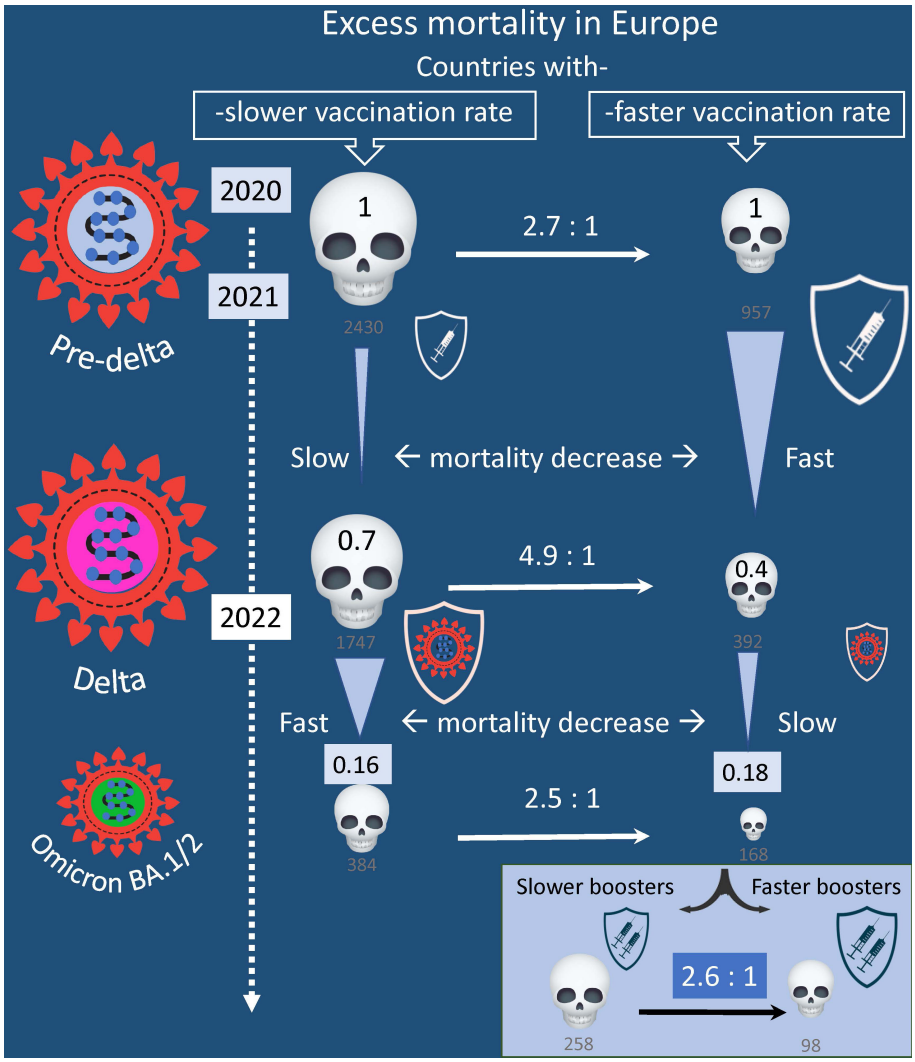
Methods: We selected twenty-nine countries from the OurWorldInData project database according to their population size of more than one million and the availability of information on dominant SARS-CoV-2 variants during COVID-19 infection waves. After selection, we categorized countries according to their "faster" or "slower" vaccination rates. The first category included countries that reached 60% of vaccinated residents by October 2021 and 70% by January 2022. The second or "slower" category included all other countries. In the first or "faster" category, two groups, "boosters faster'' and "boosters slower" were created. Pearson correlation analysis, linear regression, and chi-square test for categorical data were used to identify the association between vaccination rate and excess mortality. We chose time intervals corresponding to the dominance of viral variants: Wuhan, Alpha, Delta, and Omicron BA.1/2.

Results and discussion: The "faster" countries, as opposed to the "slower" ones, did better in protecting their residents from mortality during all periods of the SARS-CoV-2 pandemic and even before vaccination. Perhaps higher GDP per capita contributed to their better performance throughout the pandemic. During mass vaccination, when the Delta variant prevailed, the contrast in mortality rates between the "faster" and "slower" categories was strongest. The average excess mortality in the "slower" countries was nearly 5 times higher than in the "faster" countries, and the odds ratio (OR) was 4.9 (95% CI 4.4 to 5.4). Slower booster rates were associated with significantly higher mortality during periods dominated by Omicron BA.1 and BA.2, with an OR of 2.6 (CI 95%. 2.1 to 3.3). Among the European countries we analyzed, Denmark, Norway, and Ireland did best, with a pandemic mortality rate of 0.1% of the population or less. By comparison, Bulgaria, Serbia, and Russia had a much higher mortality rate of up to 1% of the population.

Conclusion: Thus, slow vaccination and booster administration was a major factor contributing to an order of magnitude higher excess mortality in "slower" European countries compared to more rapidly immunized countries.

KEYWORDS

SARS-CoV-2, COVID-19, vaccination rate, excess mortality, booster administration, GDP, European countries



GRAPHICAL ABSTRACT

Introduction

To deal with infectious waves caused by different variants of SARS-CoV-2, governments around the world have had to make many difficult decisions, including unpopular business closures, quarantines, and so on. An important tool in the fight against the pandemic was vaccination, which was also far from being always popular among the people of different countries and even among governments. Almost three years after the beginning of the pandemic, it is time to analyze how effective vaccination and booster administration have been during different waves of infections in various countries. One way to address this question is to compare excess mortality in different countries in which some infectious waves were synchronized, but vaccination rates varied widely. There are various vaccines that have been used to immunize populations in Europe to prevent hospitalizations and deaths from COVID-19. As shown in Supplementary Figure 1, approximately 70% of the doses administered in the EU in 2021 and 2022 are RNA vaccines produced by Pfizer or Moderna; the other 15% are non-replicating adenovirus vector vaccines produced by AstraZeneca and Johnson & Johnson. The origin of the remaining doses of vaccines administered in the EU and in other European countries according to the European vaccine tracker is unknown (1). Russia and China partially provided these vaccine portions for the EU and other European countries. For example, Russia supplied Hungary and Slovakia and predominantly vaccinated its own citizens with the adenovirus two-dose vector vaccine Sputnik V, which was not approved for use by WHO or EMA due to a lack of proper documentation (2, 3). China supplied several European countries with inactivated viral vaccines, which were approved by WHO (4). There are no studies comparing the efficacy of these vaccines back-to-back. However, many published studies have compared other vaccines used in Europe (5).

A meta-analysis of many studies shows similar effectiveness of the mRNA-based vaccines among themselves (6) and with the two-dose adenovirus-based vaccine produced by AstraZeneca (5, 7). Some studies show that the Sputnik V vaccine was just as effective (8). However, there is evidence that mRNA-based vaccines are more effective than the single-dose adenovirus vector-based vaccine produced by Johnson & Johnson (9). In addition, Lau ret al. (10) showed that virus-inactivated vaccines made in China had a shorter protection time compared with mRNA-based vaccines. It is also worth noting that several studies observed a significant reduction in the efficacy of all vaccines regarding protection against hospitalization or death caused by the Omicron virus variant compared with earlier virus variants (11–13).

In our analysis, we considered vaccination without analyzing which vaccine was predominantly used to vaccinate the country's population. We believe that this is not a serious flaw in our study, since the vaccines used in Europe had similar efficacy. We have chosen to estimate excess all-cause mortality as a measure of the negative impact of the pandemic. This estimate can be made for countries that regularly publish all-cause mortality data. There are several models for estimating excess mortality that have been suggested for use during the COVID-19 pandemic (14–17). We worked with the first of these (14) for several reasons (see below), including because its estimates are available in a very user-friendly format from the OWID database, which was the source of most of the data we analyzed. At present, however, all important findings can be substantiated by employing different models.

By comparing excess mortality estimates and other characteristics of different countries, it is possible to determine which countries did better in reducing excess mortality during each infectious wave and to try to understand why. Our study is not the first one to address such questions. It is consistent with others that have analyzed international heterogeneous mortality socio-economic, regulatory, and biological consequences of the COVID-19 pandemic (15–19). In our work, however, we focused on European countries and tried to determine exactly how much vaccination contributed to reduction of mortality.

During the coronavirus pandemic, countries around the world consistently adopted various measures to reduce mortality from COVID-19. In our work, we attempted to separate the effect that these measures had before and during mass vaccination and the effect of vaccination itself on reducing excess mortality.

Both political and biological factors influence the magnitude of all-cause mortality during pandemic infection waves (15, 16, 18). All these factors can be divided into those that change slightly and those that change or may change more radically in population during a pandemic. The first category of these factors includes the age structure of the population, GDP per capita, health care structure, and so on. The second category includes factors such as 1) the rate (ratio) of lethality in SARS-CoV-2 infections, 2) COVID-19 prevention strategies that do not include vaccination, 3) vaccination rates, 4) vaccine type, 5) population accumulation of immunity from natural COVID-19 infections, 6) the length of immune protection a person receives from a vaccine or natural infection, and 7) immune escape from the virus. Each of these factors contributes to the excess number of deaths in each infectious wave. Estimating the weight of each factor in each infectious wave is not a straightforward task. It is even more difficult to assess the causal relationship between each factor and its effect on excess mortality.

Several studies have found a negative correlation between vaccination rates and excess mortality associated with COVID-19 (20–24). In theory, the mere fact that such a correlation exists cannot be proof of a causal relationship. Both high vaccination rates and low pandemic-associated mortality occur in higher-income communities or countries (25–27). This inverse relationship between income and mortality was demonstrated for the 1918-1920 influenza pandemic in Europe when individual countries were analyzed (26). A similar observation was made for the pandemic COVID-19 (2020–2021). The analysis was done at the level of individual counties and zip codes (25), and globally at the individual county level (27, 28) in the US.

High GDP per capita and high vaccination rates are factors in reducing excess pandemic mortality. This is not surprising, since countries with higher income levels have more resources to deal with pandemics. Thus, they likely had more effective protective measures before mass vaccination and had higher rates of vaccination as well. There is also a collinearity between GDP and vaccination rates; a strong correlation has been demonstrated in previously published studies as well (29). Therefore, it is important to analyze how each of the factors, namely slow rate of vaccination or low GDP per capita in the population, influenced the excess mortality rate.

In our study, we tried to assess exactly how national vaccination rates were related to the mortality peaks in Europe during the Delta wave and during the first Omicron wave. European countries are leveling off in terms of excess mortality associated with waves of infections in the second half of 2022. An increase in population immunity due to natural infections probably plays a major role in this process, especially in slower vaccinated countries. Along with this process, viral immune can escape, and antigenic drift of the virus occurs relatively quickly. Even the first Omicron variants that emerged, for example, such as B.1.1.529, were characterized by an unusually large number of mutations in their spike proteins, compared with the original strain of SARS-CoV-2 (30). As additional infections and booster immunizations occur, the level of protection of the population against the virus may increase, while at the same time it may decrease due to viral immune escape.

Materials and methods

Datasets of countries

We chose European countries for our work because waves of infection caused by different variants of SARS-CoV-2 were better synchronized in Europe compared to many other regions in the world. For our analysis, we selected countries that are located entirely in Europe, except for Russia, and those with a population of more than one million people. Another selection criterion was regular information updates about the coronavirus variants that were circulating in the country at any given time interval. As a

result, we selected 29 countries and assigned each country a number, which we then used to refer to the country in the graphical representation of the data analysis. The countries and their assigned numbers for presentation/display in the Tables and Figures are listed in Figure 1 and Figure 2 legend. The names of the countries that fall into each category are shown in Supplementary Figure 1. Collected country characteristics for each analyzed time interval (explanation is below) were recorded in an Excel file, available as Table 1 in the Supplementary Material.

Data availability

Actual GDP per capita data were retrieved from the World Bank dataset (31). The time intervals when different variants of the coronavirus dominated were calculated from the information provided in the Our World in Data (OWID) project database.

Vaccination values were taken from the OWID database (32, 33) for each selected European country. We retrieved the percentage of fully vaccinated people for three dates: July 2, 2021, October 2, 2021, and January 2, 2022. For the countries included in our analysis, full vaccination usually means receiving a primary series: two doses for vaccines with a two-dose course and one dose for vaccines with a one-dose course. We also collected information on how many people in each country got booster doses of the vaccine by January 2, 2022. A booster dose refers to a third or fourth injection of the vaccine. The OWID database does not tell you whether the booster dose was given once or twice.

Mortality estimates were extracted from the OWID database, as numbers of confirmed COVID-19 deaths and excess deaths over various pandemic time intervals. The latter estimates were obtained using the Karlinsky-Kobak model. The model algorithm was created first by fitting a regression model for each country using historical mortality data for 2015-2019, second by using the resulting model to predict the number of deaths that can be expected in 2020-2022, and third by subtracting expected deaths from those reported for each country (14).

Classification and analysis of data

Categorization of countries was based on their vaccination rate and countries were identified as “faster”, or “slower”. The “faster” category included countries that had reached 60% of vaccinated residents by Oct. 2, 2021, and 70% by Jan.2, 2022. The “slower” category included remaining countries that had not reached these levels of vaccination during the relevant time intervals. In the “faster” category, a subcategory with a vaccination rate of 35% achieved as of January 2, 2022, or more, was created and named “boosters fast”. We show the names of the countries that fall into each category in Supplementary Figure 1.

Three time periods, labeled I, II, and III, were used to estimate mortality in our analysis and in the graphical presentation of the data. The first of the periods (I) is the time interval before the start of mass vaccination from February 2020 to June 2021. The second (II) period is July 2021 to January 2022, and the third (III) is February 2022 to May 2022, both of which correspond to the time when vaccination was in full swing.

Two virus variants, namely Wuhan and Alpha, dominated the first period in sequential order, Delta dominated the second time interval, and Omicron BA.1 and BA.2 dominated the third. The precise data collection time points of the Delta/Omicron dominance

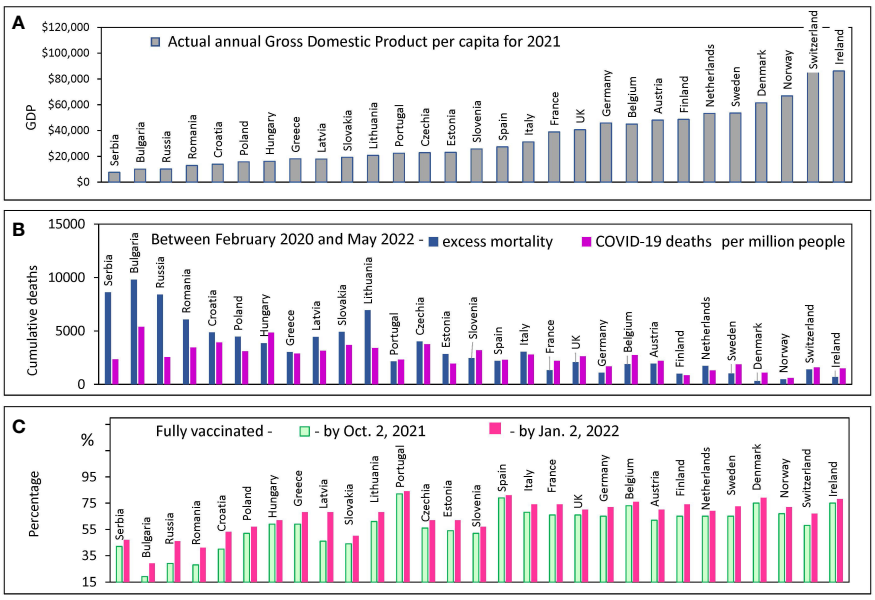


FIGURE 1
Country characteristics: GDP, Mortality and Vaccination Rates. Countries are ranked in order of increasing GDP per capita in 2021 in all panels. Standard deviations and error bars are not shown. **(A)** Actual annual GDP values from the World bank database for 29 European countries. **(B)** Excess mortality estimates, and COVID-19 confirmed deaths from the OWID database **(C)** The percentage of people who received two doses of vaccine (primary series) at certain dates.

switch in January or February were defined individually for each country. For some countries, this switch occurred in January and for some in February 2022. Technically, using OWID available information, we determined the date when the Delta/Omicron balance in a country approached 50% and estimated excess mortality or mortality from COVID-19 in that country after three weeks from this date. We assumed that one week after the 50% point of presence in the country, Omicron dominates. Thus, we chose the time point at which Omicron dominates, and we assumed that the mortality is mainly due to the infection associated with this strain two weeks after the corresponding date. In other words, three weeks after the date when Delta and Omicron accounted for half of the infections, we assumed that deaths occurred mainly from Omicron infection.

Data analysis, including Pearson correlation analysis, linear regression fitting, and Odds Ratio calculations were performed using Excel and in-house software. We also verified our results of categorical Chi-Square test calculation by using online tools (34, 35).

Results

Comparative analysis of GDP, mortality, and vaccination rates in European countries

In this study, we were most interested in the relationship between COVID-19 mortality and vaccination rates. However, when examining this aspect, we need to consider that countries differ not only in vaccination rates but also in other characteristics that may be related to population survival in a pandemic. For example, one of the most important characteristics of a country is the actual GDP per capita. This parameter can correlate strongly with the level of health care and the effectiveness of pandemic mitigation strategies, which are directly related to the reduction of excess deaths.

European countries are extremely diverse by this criterion: their GDP per capita varies by orders of magnitude: from $9,200 in Serbia to more than $90,000 in Switzerland and Ireland (Figure 1A). We examined how the diversity of annual income is related to the diversity of other country characteristics of interest. COVID-19 mortality rates, the excess mortality rate during a pandemic, and the vaccination rate achieved in a country at different intervals are shown in Figures 1B, C. All countries are displayed (X-axis) in the order of national GDP per capita growth according to the order of the countries in Figure 1A.

Countries with relatively low GDP per capita (less than $15K) tend to have COVID-19 mortality rates that are significantly lower than the excess all-cause mortality estimates (Figure 1B). In addition, these same countries with lower GDPs have relatively low vaccination rates (Figure 1C). The discrepancy between COVID-19 deaths and excess deaths may indicate a problem of deficiencies in disease diagnosis. A similar discrepancy was observed in several other studies published previously (17, 36). Given the problem of possible underreporting of COVID-19 deaths in some countries, we decided to analyze the data solely using excess mortality estimates.

We found a significant negative correlation between excess mortality and a country's actual GDP per capita. The correlation is much more pronounced for poorer countries - those with GDP per capita of less than 40K (R=-0.79, p<0.0001). For richer countries, the correlation is much weaker and does not reach the 95% level of significance (Figure 2A).

For countries with a GDP below 40K, we found a positive correlation of GDP per capita with vaccination rates (Figure 2B), as well as with boosters' administration rates (Figure 2C). No such correlation was observed in countries with higher GDP (Figure 2B). In addition, a strong positive correlation between GDP and life expectancy was found (R=0.82, p<10^{-4}) for countries with GDP below 40K (Figure 2D). The trend was less significant for higher-income countries (R=0.69, p<0.05). Greece and France compared favorably with countries of similar income levels on average with higher life expectancy. While Russia and Lithuania stand out negatively, people in these countries lived less on average than in countries with similar income levels. During the COVID-19 pandemic, Bulgaria lost the most lives per million population and had the lowest vaccination rate among close income countries. While Denmark had the highest vaccination rate and lost the fewest lives among the European countries we analyzed.

Estimates of excess mortality during waves of COVID-19 infection caused by different dominant virus variants

Coronavirus infection has spread in Europe in several waves caused by different variants of SARS-CoV-2. As reported smoothened weekly COVID-19 deaths, these waves are shown in Figure 3A. The first wave shows deaths caused by the ancestral Wuhan variant of the virus. The second large wave with several peaks represents deaths caused mainly by SARS-CoV-2 Alpha variant, which was dominant in Europe before the Delta variant emerged. The next large wave with two peaks shows deaths during the time interval when Delta and Omicron BA.1/2 variants dominated. Finally, the last visible comparatively small waves represent COVID-19 deaths that occurred during the time intervals when Omicron BA.5, BQ.1, and XBB 1.5 dominated.

In Europe, as noted above, the Delta-driven wave and the Omicron BA.1 wave were very close to each other and appeared as one big wave with two peaks (Figure 3A). However, by analyzing individual data for each country, these waves caused by different dominant virus variants could often be distinguished from each other.

The overall estimates of excess mortality, which range from a few hundred to a few thousand per million people in each country, show a contrast in the impact of the pandemic on European countries (Figure 3B). At the extremes of the spectrum,

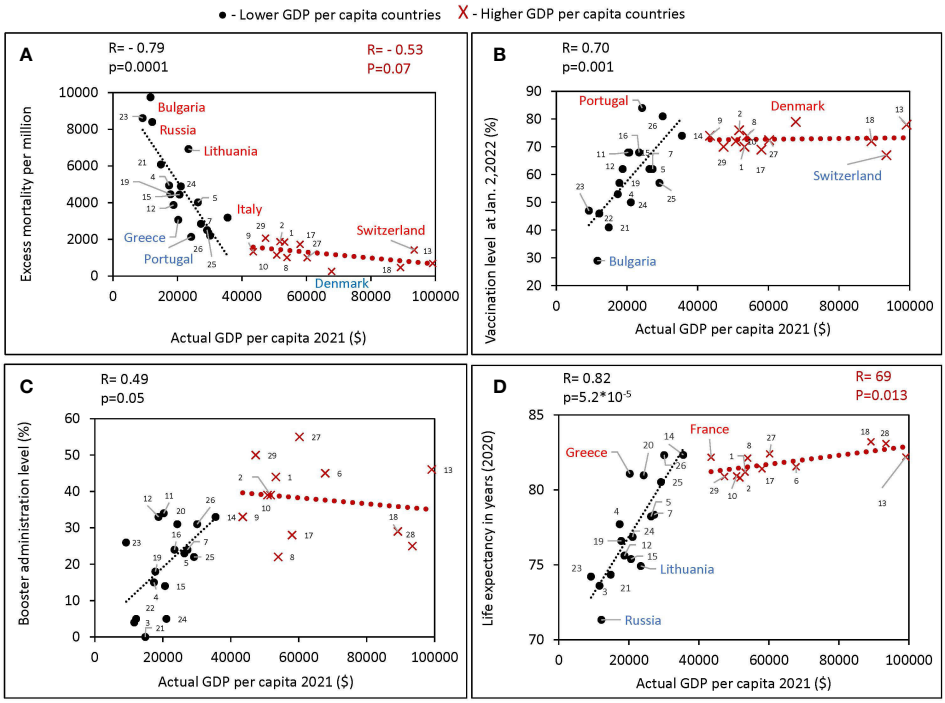


FIGURE 2
Relationship between GDPs per capita and various characteristics of European countries obtained during different periods of the pandemic. All estimates of excess mortality in the scatter plots are per million population. Higher GDP per capita countries were defined in this study as those with a GDPs equal to or greater than the threshold of $40,000, lower GDPs per capita countries defined as those with a GDPs per capita below the threshold. Countries were numbered according to the alphabetical order of their names as follows: Austria 1, Belgium 2, Bulgaria 3, Croatia 4, Czechia 5, Denmark 6, Estonia 7, Finland 8, France 9, Germany 10, Greece 11, Hungary 12, Ireland 13, Italy 14, Latvia 15, Lithuania 16, Netherlands 17, Norway 18, Poland 19, Portugal 20, Romania 21, Russia 22, Serbia 23, Slovakia 24, Slovenia 25, Spain 26, Sweden 27, Switzerland 28, United Kingdom 29. **(A)** Excess mortality during the COVID-19 pandemic from February 1, 2020, to May 30, 2022, in relation to actual GDP per capita as of 2021. **(B)** Vaccination rates, as of October 2, versus actual GDP per capita. **(C)** Boosters administration rates, as of January 2, versus actual GDP per capita. **(D)** Life expectancy versus actual GDP per capita.

illustrating this contrast, are countries that have lost less than one-tenth of their residents and countries that have lost nearly one percent of their citizens' lives. Figure 3B shows that countries differ in excess mortality not only throughout the entire pandemic period, but also in each time interval corresponding to each infectious wave. Thus, we showed that some countries handled the pandemic better than others, and often a country's success in reducing excess mortality was consistent across different waves of COVID-19 infections. For example, Denmark, Norway, and Ireland handled the pandemic best in all waves, while Bulgaria, Serbia, and Russia suffered the greatest losses of life.

The excess mortality estimates presented in Figure 3B were calculated using the algorithms of the Karlinsky-Kobak model (14). To avoid presenting too much detail in Figure 3B, we have provided estimates of the standard deviation (SD) of the model calculation separately in Figure 3C. This analysis was done solely to reflect the confidence intervals of the calculations at the time the model description was published. The negative excess mortality values for some countries can be explained by lockdowns and quarantine, which reduced the number of car accidents. Also, social distancing and mask-wearing, reduced the prevalence of influenza and other viral infections affecting national mortality.

Comparison of excess deaths that occurred early and late in the pandemic

COVID-19 vaccination began in late 2020, but by February 2021, only two percent of the population in Europe had been vaccinated. We compared the number of COVID-19-related casualties in European countries before and after mass vaccination began, which had a protective effect on the population and affected excess deaths. The beginning of July 2021 was chosen as the date to separate the two periods. By this date, most European countries had vaccinated over 8% but less than 40% of their fellow citizens. We present this information in more detail in Supplementary Figure 2.

Until July 2021, the Wuhan and Alpha virus variants prevailed in Europe, and after that month, the Delta variant, followed by Omicron. Thus, most of the excess deaths before July 2021 happened before vaccination protected people in masse. The periods we compared differ both in the level of vaccination and in the type of dominant viral variant. Wuhan and Alpha dominated for almost a year and a half and claimed a certain number of lives. Delta dominated for six months and claimed many more lives in some countries than previous variants of the virus over the same period. In other countries, though, Delta claimed far fewer lives. To

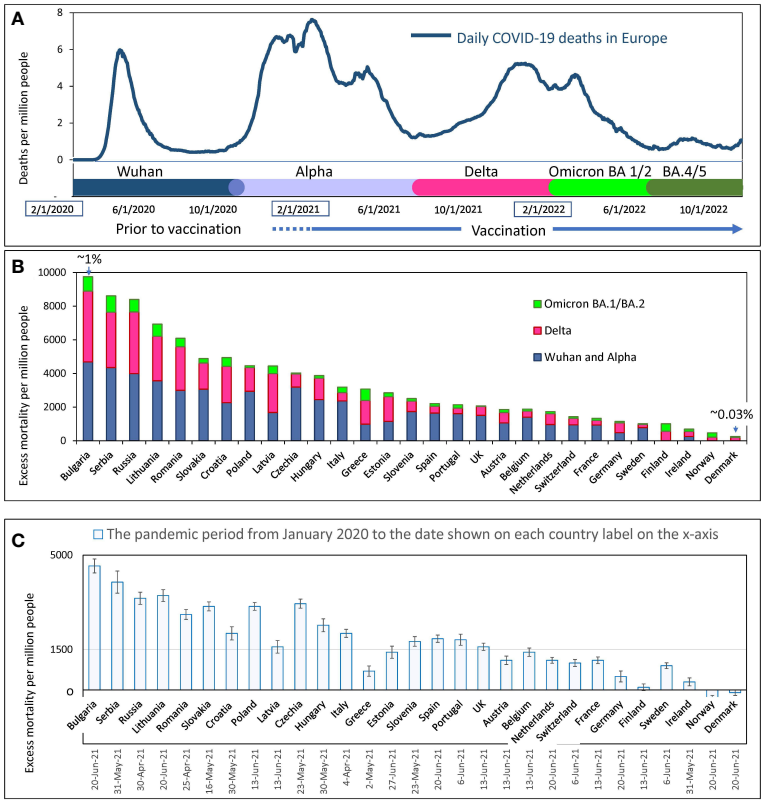


FIGURE 3
Mortality during COVID-19 pandemics in Europe **(A)** Visualization of infection waves as weekly averaged confirmed COVID-19 deaths in Europe during the pandemic through October 15, 2022. Different time intervals are highlighted in distinct colors. Information on excess mortality and dominant virus variants in each time interval was taken from the OWID database. The information is shown in more detail in the Supplementary Figure 1. **(B)** Excess mortality estimates for each European country for the COVID-19 pandemic period from February 2020 to May 2022. Estimates are from the OWID database and are derived from the Karlinsky-Kobak model. Each column represents three different time intervals, which are highlighted with the same colors as in "A". **(C)** Karlinsky - Kobak model estimates of excess mortality values over the time from the beginning of the pandemic up to the summer of 2021 with the standard deviation intervals.

address the question related to the differences in Delta associated mortality, we compared the number of deaths in each country before and after mass vaccination, when the Delta appeared. This analysis allowed us to understand how well countries protected their populations in the periods separated by July 2021 (Figure 4). Figure 4A shows that there is a significant correlation between the rates of excess mortality that occurred during the two periods of interest (R=0.82, p<<0.001).

Figure 4B shows the ratio of deaths between the two periods for each country relative to deaths during the first period. It illustrates how effectively countries have improved the protection of the lives of their citizens in the second period compared to the first. Portugal, Sweden, Italy, the Czech Republic, and Belgium reduced mortality by a factor of four or more, and all these countries with rapid vaccination rates. At the same time, countries with very low vaccination rates, such as Serbia. Bulgaria, Russia, and Lithuania had virtually no reduction in losses. Thus, some countries, after experiencing high mortality in the first period, radically changed their trajectory and protected their populations much better in the second period, when faced with a Delta variant of the virus. The results indicated that those with higher vaccination rates reduced mortality in the Delta wave more than those with lower vaccination rates.

Correlation analysis of excess mortality estimates versus vaccination rates

In the context of factors that may affect COVID-19-related mortality, we analyzed the effect of vaccination/boosters rates on estimated excess mortality over time. For each country, an excess mortality value was presented versus vaccination or booster administration rates achieved at specific time points (Figure 5).

Analysis of the data showed that a higher vaccination rate in a country corresponded to a lower excess mortality rate. By sampling the data and linear interpolation between points, we found that the best linear trend of decreasing mortality as the vaccinated population in a country increased was in countries with vaccination rates below a certain threshold. This threshold is somewhere between 60 and 70% of the vaccinated population in the country. Thus, we found that for countries with vaccination rates below the threshold; the correlation is very strong and significant, while for countries with vaccination rates above the threshold, the correlation is weak, marginal (Figure 5A) or virtually not-detectable (Figure 5B). Low excess mortality, which was not significantly different between countries, was observed in all countries with vaccination rates above the threshold. This

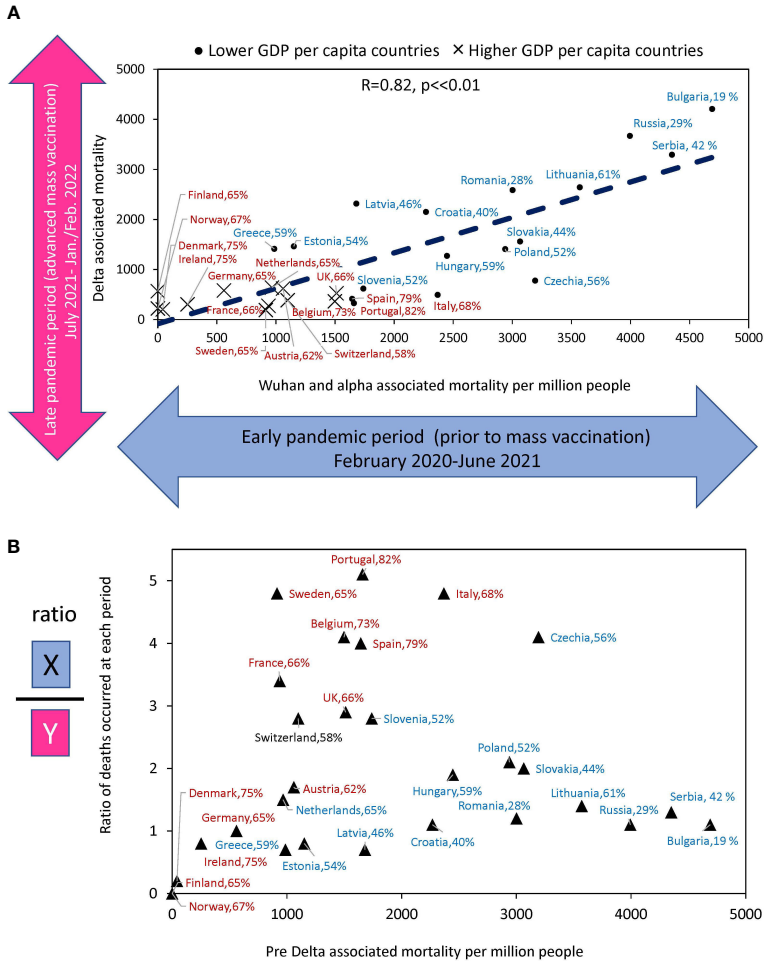


FIGURE 4
Relationship between estimates of excess deaths occurring earlier and later during the COVID-10 pandemic. **(A)** The relationship between deaths that occurred in the country during Delta domination relative to deaths whose culprits were pre-Delta virus variants (Alpha and Wuhan). **(B)** The ratio of excess death numbers that occurred in the two periods for the same countries versus to excess death numbers in the first period. The names of countries that fall into the "faster" vaccination category are shown in brown font. The names of countries tht fall into "slower" vaccination category are in blue. The percentage of the country population that got primary vaccines series by the time Delta variant arrived is shown after the name of the country.

observation was accurate for the Delta-dominated period (Figure 5A) and for the Omicron BA.1/2 dominated period as well (Figure 5B). Such an analysis of the data allows us to divide more accurately the countries into two categories: those that vaccinated faster and those that vaccinated slower. The countries with higher vaccination rates reached some threshold level and equaled them in terms of excess mortality rates.

At the same time, for the Omicron BA.1 and BA.2 period, we found that in countries in the "faster" vaccination category, while mortality was practically independent of vaccination rate, it depended on booster rate (Figure 5C).

In summary, a significant negative linear relationship between excess mortality and vaccination rates was found only for "slower" countries. This was true both for the Delta dominant period (R=-0.8, p<0.01) and for the Omicron BA.1 and BA.2 dominant period (R=-0.5, p=0.045). We did not find significant relationships for countries in the "faster" category, likely because all countries in the "faster" category have relatively low mortality rates. However, an inverse linear relationship between mortality and the rate of booster administration was found for the "faster" countries during the Omicron-dominated period. The greater the percentage of the population that received booster doses, the lower the mortality rate (R=-0.8, p<<0.01).

Far more people died from COVID-19 in countries that were slow to vaccinate their populations compared to countries that did it faster. The same can be said for the speed at which countries provided additional doses of booster vaccine - the higher the speed, the fewer deaths.

Age characteristics and excess mortality

To examine how a country's age characteristics were related to excess mortality we chose three features: the average age of the

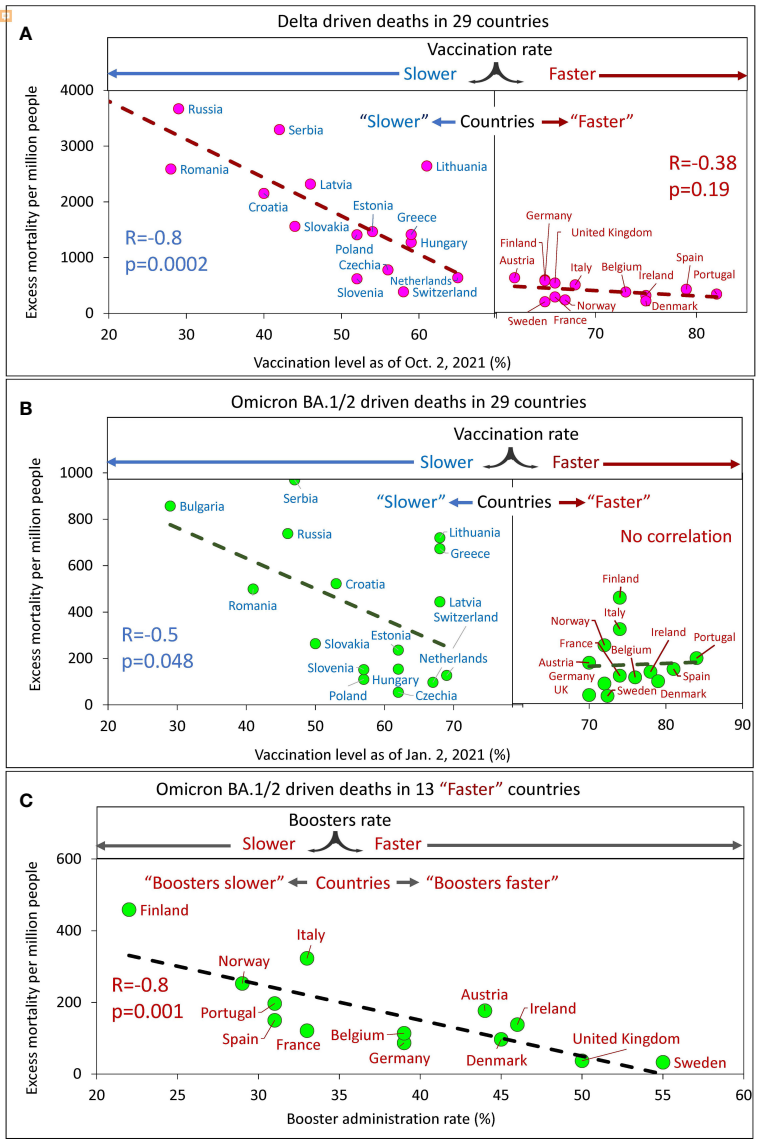


FIGURE 5
Excess mortality and vaccination rates in European countries estimated for different time intervals. **(A)** Delta associated excess mortality by country *vs.* vaccination rates. **(B)** Omicron BA.1/2 associated excess mortality by country *vs.* vaccination rates. **(C)** Omicron BA.1/2 associated excess mortality by country *vs.* boosters' administration rate in the "faster" vaccination rate country category.

country's population, the percentage of the elderly population (65+), and life expectancy. From the three characteristics listed, only life expectancy of the population is the most related to the quality of life and health care. The average age and the percentage of the elderly population (65+) are less dependent as they are strongly influenced by the percentage of young people in the country. The results of a correlation analysis of these three characteristics with excess mortality, which correspond to infection waves caused by Delta and Omicron BA.1/BA.2 virus variants, are presented in Figure 6.

There is no significant correlation of excess mortality in a country with the average age of a population (Figure 6A) or with the percentage of the elderly population (Figure 6B). However, we do see a strong significant negative correlation between life expectancy and excess mortality (Figure 6C). The longer a life expectancy in a country, the lower the pandemic excess mortality for both the Delta wave and the Omicron wave.

We performed a similar correlation analysis using the same parameters separately for the categories of countries that vaccinated their populations faster and slower (Supplementary Figure 3). In this analysis, we found a trend with marginal significance for set of countries that vaccinated more rapidly, showing that the higher the average national age, the higher the excess mortality. However, we found no correlation between excess mortality in a country and the percentage of the elderly population, either when analyzing all countries together or when analyzing the categories of countries in which the populations were vaccinated at different rates (Supplementary Figure 3).

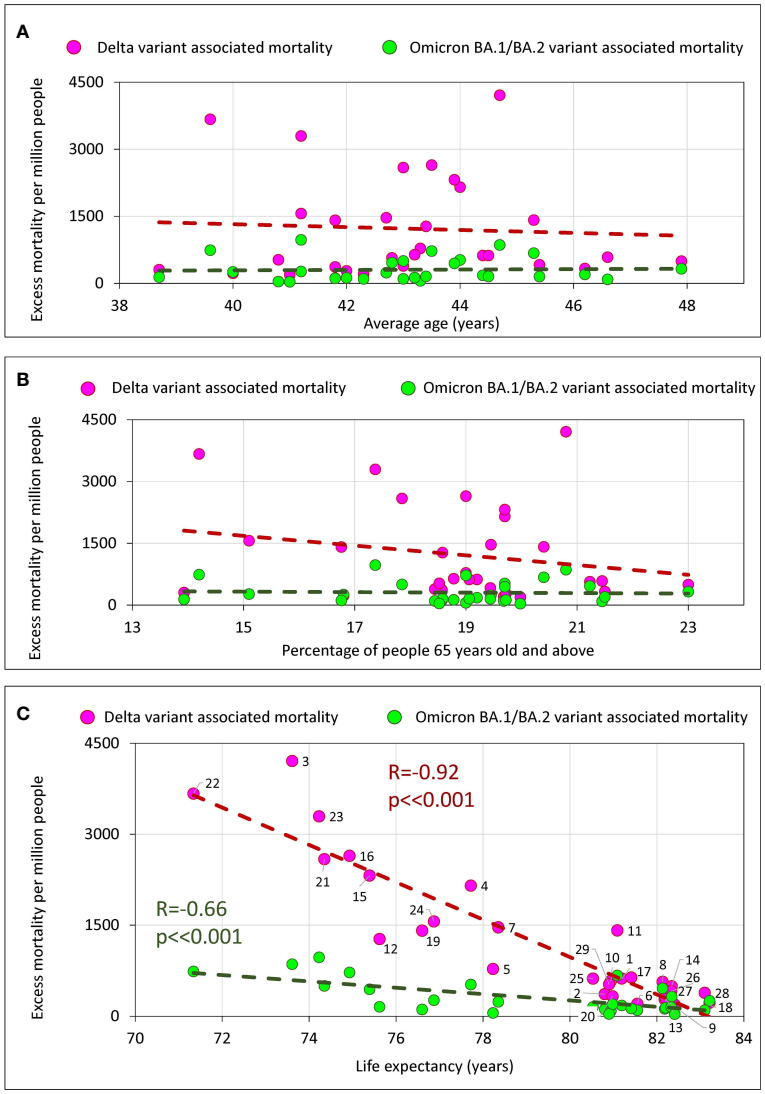


FIGURE 6
Excess mortality and the average age of the population in the country, the percentage of the elderly, and life expectancy in the country. **(A)** Excess mortality and the average age of the population in the country. **(B)** Excess mortality and the percentage of the elderly population (65+) in the country. **(C)** Relationship between excess mortality and life expectancy.

Analysis of GDP per capita and vaccination rate using regression models

In trying to establish a causal relationship between vaccination rates and reductions in excess mortality, we tried to understand what factors other than vaccination contribute to saving lives of the nation's citizens. Considering that some of these factors may be related to GDP, this is not a straightforward task. We showed that the level of a country's GDP itself is significantly correlated with the level of vaccination (Figure 2B), and this result is consistent with previously published data (19, 29).

To address this issue, a set of linear regression models with variable inputs was created. In all proposed models, mortality was used as the dependent variable, while GDP per capita and country vaccination or booster rates were treated as independent variables. The results of the linear regression analysis are described in detail in Table 2 in Supplementary Material and are presented in Figure 7 as columns showing the level of significance assigned to each parameter in that each model.

We found that to predict excess mortality, depending on the time interval when the deaths occur, either the levels of GDP or the vaccination rate or both may be significant as input parameters (Figure 7A). Model 1, which considers mainly COVID-19 mortality in the period before mass vaccination and before the dominance of the Delta variant, assigned a level of significance only to the GDP parameter. In contrast, Model 2, which considers mortality that occurred when the vaccination process was advanced and the Delta variant dominated, assigned significance levels to both input parameters: namely, GDP and vaccination. Finally, model 3, which analyzes mortality during the period of advanced vaccination, when the Omicron variant dominates, assigns significance levels only to the vaccination rate. It should be noted

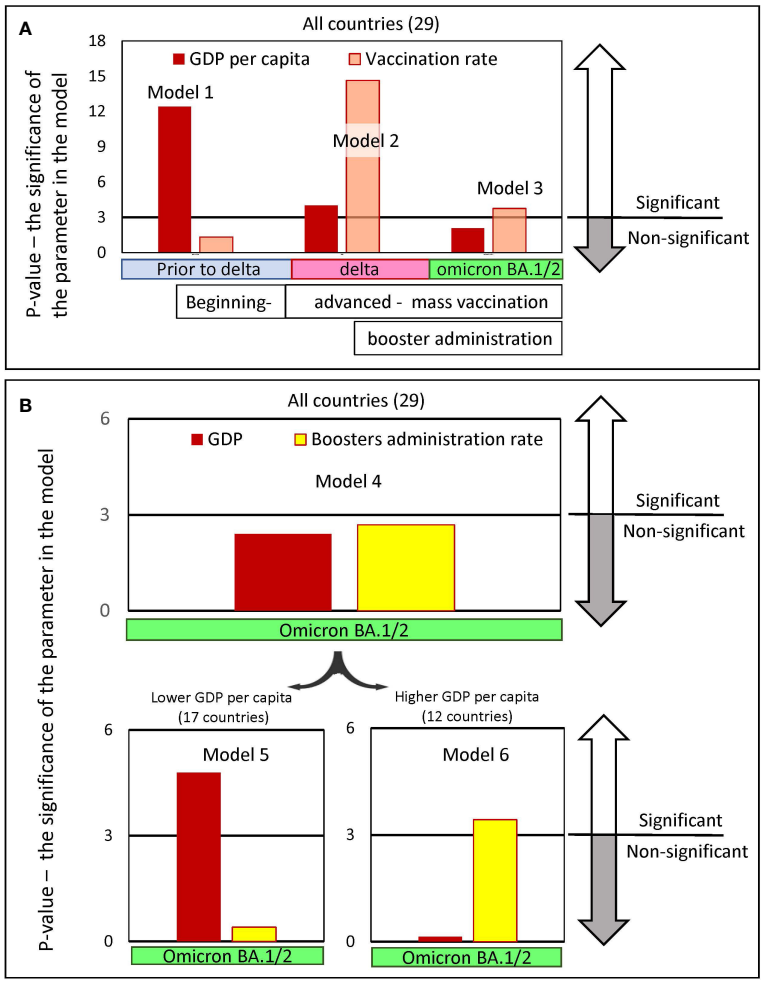


FIGURE 7
Significance levels of input parameters in linear regression models predicting pandemic excess mortality. The regression models were created to predict excess mortality. Mortality was chosen as the dependent variable, while GDP per capita and the vaccine immunization rate of the country's population were chosen as the independent variables. The models' outputs are presented in Table 2 in Supplementary Material. Each figure column represents a negative natural logarithmic value of the significance level of the corresponding model input parameter. The pandemic periods, for which we built models, differed in the dominance of the viral variant. Wuhan and Alpha variants dominated Europe, before July 2021, afterwards the Delta variant dominated until January/February 2022 and later until May 2022 Omicron BA.½. **(A)** Input parameters are GDP and vaccination rates in the country, achieved in the respective time periods. Models 1-3 were created for the set of all 29 countries. **(B)** Input parameters are GDP and the level of boosters in the country reached by a certain time. Model 4 was created for the set of all 29 countries, and models 5 and 6 were created only for countries subsets with GDP per capita below or above 40K, respectively. There are 17 countries in the first subset and 12 in the second.

that the vaccination parameter appears to be much more significant in Model 2 compared to Model 3.

Thus, summarizing the results, we can conclude that the factors affecting mortality reduction related to the country's income played the greatest role before mass vaccination began, but the least afterward. We can also conclude that the level of vaccination played a greater role in preventing Delta-induced deaths compared to Omicron-induced deaths.

We examined models in which GDP and the level of boosters achieved in a country by the time the Omicron virus variant emerged were considered as input parameters (Model 4-6, Figure 7B). The analysis of all countries in Model 4 showed that neither GDP nor booster administration are significant input parameters that can dramatically affect mortality during Omicron dominance. However, we found that GDP and vaccination rate can affect excess mortality in the Omicron wave when categories of countries with lower and higher GDP per capita are analyzed separately.

During the Omicron wave, the GDP parameter is most significant in determining high mortality for relatively low-income countries (Figure 7B, Model 5). In contrast, for countries with higher income, GDP is not significant, but the rate of booster administration is highly significant in the model (Figure 7B, Model 6). Because the level of booster administration in low-income countries is not quite high, it is difficult to expect an important role for this parameter in Model 5. This analysis demonstrated that the rate of booster administration was important for preventing deaths in high-income countries during the period when Omicron BA.1/BA.2 dominated. Thus, we showed that when mixing countries with different levels of GDP, models could not produce reliable results.

Comparison of COVID-19-related mortality in "faster" and "slower" countries during the dominance of different SARS-CoV-2 variants

Four epidemic waves caused by Wuhan, Alpha, Delta, and Omicron viruses BA.1, as well as BA.2, resulted in different excess mortality rates in different countries, as shown in Figure 3B. We were interested in knowing how the magnitude of excess mortality in different periods of the pandemic depended on which category a country was in, namely whether it was "faster" or "slower" in its vaccination.

To answer this question, the distributions of excess mortality values were visualized as box plots for both categories of countries for the three intervals of the pandemic (Figure 8A). The first interval (I) included the time prior to mass vaccination where viral variants Wuhan and Alpha (pre-Delta strains) dominated. The second (II) and third (III) periods correspond to the time when vaccination was in full swing, where Delta dominated in the second interval and Omicron BA 1/BA.2. in the third. We then assessed the mean values in each data category and their ratios. A comparison of these averages and their ratios showed how mortality during each pandemic period was related to the category of country in which the individual lived (Figure 8B). A low probability of dying during a pandemic was associated with better protection against all-cause mortality provided by the countries in the corresponding category.

The "faster" countries, compared to the "slower" ones, were much better at protecting their residents throughout the pandemic. However, the difference in protection effectiveness depended on the time interval. For example, the odds of dying in the first pre-Delta time interval were nearly three times higher for residents of countries that failed to provide prompt vaccination in the future compared with those that did (OR 2.7 (95% CI 2.5-3)). The contrast in the odds of dying between these same categories of countries became much more pronounced, reaching almost fivefold in the second time interval, when vaccination was advanced, and the Delta variant dominated (OR 4.9 (95% CI 4.4-5.4)). However, the contrast halved in the third time interval, when people died mostly from the Omicron variant to an OR of 2.5 (95% CI 2.1- 2.9). For the same wave of Omicron infections, there was more than a twofold difference in mortality for the "boosters slower" versus "boosters faster" subcategory with an OR of 2.6 (95% CI2.1-3.3).

Analysis of excess mortality during successive waves of COVID-19 infections

To evaluate the dynamics of mortality-change in transitions between different COVID-19 pandemic waves, we measured odds ratios between mortality in the late and early infectious waves for "faster" and "slower" vaccinated countries separately. We visualized the excess mortality distributions as box plots and estimated the odds ratios of mortality between Delta and pre-Delta, or Omicron and Delta waves and (Figures 8A, B).

The comparative analysis of the odds ratio in Figure 8C demonstrated that the first transition was much more pronounced for the "faster" countries compared to the "slower". Mortality during the Delta wave was less than half of pre-Delta mortality in the "faster" category (OR 0.4 (95% CI 0.36-0.46)) and more than half in the "slower" category (OR 0.7 (95% CI 0.68-0.76). In other words, during the transition to the Delta wave, mortality rates declined strongly in the "faster" countries and weakly in the "slower" ones. In contrast, the transition between Delta and Omicron waves was more pronounced for the "slower" countries. In this category, Omicron-associated mortality was only one fifth of that of the Delta wave (OR 0.22 (95% CI 0.2-0.25)). In contrast, in the "faster" category, Omicron accounted for just under half of the Delta deaths (0.43 (95% CI 0.36-0.53)). Nevertheless, the odds ratios of pre-Delta versus Omicron deaths represent similar values in both categories of countries (Figure 8C).

Thus, we can summarize that the degree of mortality reduction during the transition from infection waves caused by pre-Delta virus variants to the Omicron variant was independent of the rate of vaccination in the countries. However, the trajectory of this decrease depended on this rate. We have seen a sharp decline between pre-Delta and Delta mortality for the "faster" countries. At the same time, we observed a strong mortality reduction between Delta and Omicron waves for the "slower" countries. The difference in trajectories led to the major difference in mortality between the two categories of countries during the Delta infection wave. The ability of rapid vaccination to save lives was best exemplified by the Delta wave, and the ability of rapid booster administration to save lives was best exemplified by the first Omicron wave.

Discussion

Estimates of excess mortality versus COVID-19 confirmed deaths

The underreporting of COVID-19 deaths in some countries is a well-known phenomenon, which was thoroughly discussed in previously published studies (14). The global worldwide estimate is 18 million excess deaths between early 2020 and the end of 2021, while reported COVID-19 deaths over the same period are about 6 million, three times less (37).There are several reasons for the discrepancy between reported and excess COVID-19 deaths. For example, medical reporting systems may not list COVID-19 as a cause of death if a person has not been tested for SARS-CoV-2, and thus deaths caused by the virus may be missed in official counts in countries with low testing rates. Early in the pandemic, before widespread testing, many COVID-19 deaths among the elderly were not related to the disease, causing a significant underreporting in some countries (16).

Thus, our study found that in some countries there is a disparity between excess mortality and deaths directly related to COVID-19 which is not unexpected. The existence of this discrepancy, and the fact that it occurs primarily in countries with relatively low GDP per capita, is consistent with what has already been found and published (14, 17). In summary, all these findings underscore the fact that excess mortality is a more reliable indicator of pandemic deaths than COVID-19 direct mortality, which has been diagnosed as a direct result of COVID-19.

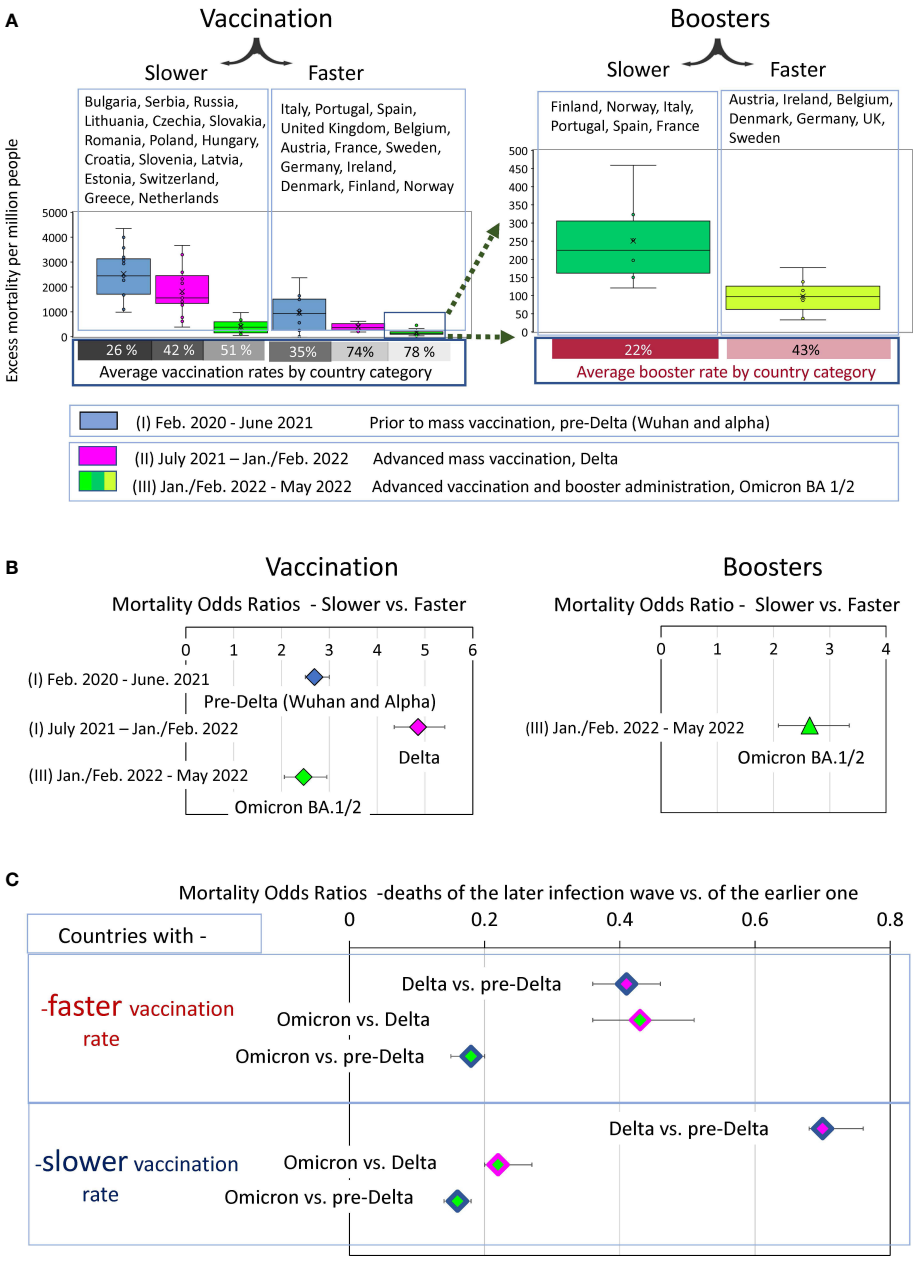


FIGURE 8
Analysis of COVID-19 associated excess deaths averaged for country categories. **(A)** Distribution of excess mortality values in two categories of countries at three different time intervals of the pandemic, visualized as boxplots. **(B)** Odds ratio of dying in each time interval depending on the country category. **(C)** Odds ratio of dying in later infection waves versus the previous infection waves depending on the country category.

Low GDP, low vaccination rates and high pandemic-associated mortality

Our study showed that countries with low GDP per capita have higher mortality rates. A negative correlation between excess mortality and GDP per capita has been observed before. It has been seen for Spanish flu (26) and for COVID-19 pandemic (27, 28). Even at the single-country level, the excess mortality associated with COVID-19 is inversely correlated with the average family income that existed in the area of residence (25). Thus, our observations are consistent with those found earlier in published studies. Not surprisingly, richer countries have more resources to deal with the pandemic-induced problems, so they do a better job of reducing excess mortality overall.

Also not surprisingly, our analysis demonstrated a positive correlation between GDPs per capita and vaccination rates. The data showed that countries with relatively low incomes were slower to vaccinate their citizens and ended up with lower vaccinated populations. Similar observations have been described in detail in the research publications (19, 29). It has also previously been observed that low vaccination rates in countries coincide with underreporting of COVID-19 mortality (18). In this context, the results of our analysis of European countries, which show a discrepancy between COVID-19 and excess mortality as well as

low rates of vaccination in countries with low GDP, are consistent with previous findings.

Age, life expectancy and excess mortality

COVID-19 is more dangerous for elderly people, and deaths occur primarily in the older population (38). In our analysis, however, we found only a weak relationship between the average age of people in a country and excess mortality (Figure 6A). The correlation did not reach statistical significance and was detected only in those countries where vaccination was faster (Supplementary Figure 3, lower left panel). We also didn't find any correlation between percentage 65+ people in a country and excess mortality (Figure 6B; Supplementary Figure 3, middle panels.) This may indicate that the level of medical care and vaccination rate play a greater role in saving lives than the average age of the population or percentage of elderly population in a country. This conclusion is also supported by the strong negative correlation between life expectancy and excess mortality (Figure 6C). The higher the life expectancy, the fewer lives the country lost during the pandemic. This is true for both the Delta wave and the Omicron wave.

Finding a causal relationship between COVID-19 pandemic mortality and rates of vaccination and booster

Analysis of pandemic mortality across countries allows us to examine the effectiveness of vaccines and boosters during different periods of the pandemic when different variants of the coronavirus were prevalent. In addition, such analyses allow an assessment of how well vaccination worked against the background of immunity triggered by natural infections. The significant and strong negative correlations we observed between national vaccination rates and excess mortality seemed to answer the question of vaccination effectiveness in reducing COVID-19 mortality in an obvious way. However, a closer analysis of the data showed that other factors, namely public health effectiveness, quality of healthcare, and the efficacy of pandemic mitigation strategies, must also be considered to assess the impact of vaccination on saving lives. Isolation of the impact of these factors is not a straightforward task. They are all linked and act synergistically.

In our work, we analyzed the data to distinguish the contribution of these listed factors from the vaccination rate factor. In doing so, we assumed that annual actual GDP per capita largely determines the amount of funding available to national governments to implement all life-saving strategies, including those not related to vaccination rates.

Our analysis shows that "faster" countries that achieved higher vaccination rates had lower pre-vaccination excess mortality compared to countries with low vaccination rates. However, the difference between "faster" and "slower" countries became much more pronounced when mass vaccination was in full swing. From this we conclude that although countries differed in the effectiveness of COVID-19 mortality control measures before vaccination, vaccination made these differences much more pronounced. Thus, vaccines greatly improved the effectiveness of pandemic control measures.

In this study, we found the existence of a certain threshold level of vaccination, namely 60-70% of the country's population. Countries that reach this threshold quickly differ little in their mortality rates in comparison to the slower vaccinating countries, where the difference was significant. However, during Omicron dominance, despite the threshold reached, the countries that reached it, still differed in terms of excess mortality, and the magnitude of this excess mortality correlated inversely with the level of booster vaccinations. The immuno-compromising characteristics of Omicron likely contributed to diminished protective effect of the vaccination.

Our work pointed to the great importance of rapid administration of boosters before January 2022. Mortality in countries with rapid booster administration was significantly lower than in countries with the same per capita GDP, the same vaccination rate, but lower booster rates. The results of our data analysis are consistent with the observation that additional booster doses of both mRNA- and adenovirus-vector-based vaccines significantly increase the protective efficacy of vaccine against severe disease (13).

The transition from one infectious wave to another and the associated change in mortality in "faster" and "slower" countries

In our work, we have shown that the overall rate of COVID-19 related deaths varies across countries and depends on many factors, including the level of vaccination. However, even before vaccination, countries in the category where the population would subsequently be vaccinated more quickly had lower excess mortality rates. Apparently, this is due to the fact that these countries have on average, higher GDP per capita and, accordingly, more capacity to mitigate the epidemic consequences. At the same time, during the period of mass vaccination, and especially during the period of dominance of the Delta variant, the ability to reduce mortality increased sharply in the category of countries that were rapidly vaccinating their population. The contrast in terms of excess mortality between rapidly or slowly vaccinating countries became particularly strong. Accordingly, the inverse correlation between the number of vaccinated people in the country and excess mortality became particularly pronounced during the Delta infection wave. This correlation weakened in the next infectious wave, namely, the Omicron-dominated wave. In fact, the difference between fast and slow vaccinating countries became the same as it was before mass vaccination began. There are several explanations for this phenomenon. First, the time has passed since the vaccination and the immune vaccine defense has weakened. Second, Omicron has a more pronounced ability to resist immune defenses, and finally, immune defenses increased after natural infections in countries with low vaccination rates.

It is worth noting that booster vaccinations played a hugely positive role in reducing mortality during the Omicron BA.1/BA.2 dominated period. In countries with comparable levels of GDP per capita and similar rates of primary series vaccination, excess mortality was largely determined by the level of booster vaccination administration during the Omicron-dominated period. This observation tells us that even though Omicron antigenically is very different from the ancestral SARS-CoV-2 strain, for which the vaccines were produced, booster vaccination effectively prevented excess mortality in the first waves of Omicron, caused by BA.1 and BA.2 virus variants.

Our work revealed an interesting pattern, namely that the degree of reduction in excess mortality when comparing the pre-Delta to Omicron waves was independent of the rate of vaccination. In Figure 7C, we showed that Omicron mortality is about one-sixth that of pre-Delta mortality in both the "faster" and "slower" categories. However, mortality decreases equally only in the transition from the pre-Delta wave to the Omicron one. As for the intermediate transitions, they are very different in the two categories of countries. The most pronounced reduction in excess mortality occurs in the transition to Delta in the category of countries with rapid vaccination and in the transition to the Omicron wave in the category of countries with slower vaccination rates.

A possible explanation is that rapidly vaccinated countries developed immunity faster, mostly in the pre-Delta pandemic phase, and slow-vaccinated countries developed hybrid (vaccine plus disease) immunity in later phases, likely during the Delta wave of infection. Thus, immunity, which saves from death, developed more rapidly in some countries, mainly due to vaccination, and more slowly in others, because of the hybrid influence of vaccine administration and natural infections. Despite all this, however, the minimum number of deaths in the first wave of Omicron was in countries with the highest rates of booster vaccination. Our results demonstrated that booster protection can still have a significant impact and reduce excess mortality despite high levels of immunity in the populations of countries in both categories.

Hybrid immunity

Excess mortality during the Omicron BA.1/BA.2 waves is approximately one-sixth that of pre-Delta mortality in both "fast" and "slow" countries (Figure 7C). We believe that this finding indicates that by the time the Omicron variant appeared, the general immunity of the population had already developed and could be comparable between countries from both categories. The protective shield of established immunity significantly reduced the number of deaths during the Omicron wave compared to previous periods. It has been shown that prior SARS-CoV-2 infection and booster vaccinations provide strong protection from Omicron caused ICU admission or deaths (39–41). Therefore, we can assume that the immunity protective shield was formed as a result of vaccination or COVID-19 disease and, importantly, sometimes as a combination of both, in the same person, namely as a results of hybrid immunity. The latter is particularly important and has recently been shown to be able to protect more effectively than natural immunity acquired by people from vaccine alone or disease alone (42–44). It is likely that the percentage of people with hybrid immunity increased significantly in subsequent waves of Omicron variants and thereby strengthened population immunity. The existence and maintenance of population immunity can probably explain, at least in part, the absence of large infectious waves in the second half of 2022 in most countries.

Many studies have compared immunity from vaccines, natural and breakthrough infections and have shown that hybrid immunity protects better against SARS-CoV-2 variants compared to immunity from vaccine alone or COVID-19 alone (42–44). When vaccinating previously infected individuals, just one dose of vaccine enhances both B- and T-cell response to various variants of the virus (45). In individuals previously infected with COVID-19, vaccination induces the production of cross-variant neutralizing antibodies (46).

In some countries, especially in China, the formation of hybrid immunity, in particular its component derived from natural immunity against the disease, has been severely delayed. This is primarily due to a three-year policy of rigorous testing, contact tracing combined with strict quarantines and even lockdowns, commonly referred to as the "zero covid" policy. This policy was introduced in China in January 2020 and continued until December 2022 (10, 47).

Vaccine immunity is gradually lost, and the rate of loss of protective effectiveness depends on the type of vaccine. China used types of vaccines based on an inactivated virus. A comparison of these vaccines and mRNA-based vaccines used in many European countries and Hong Kong during the Omicron BA.2 outbreak in March 2022 showed that vaccines based on an inactivated virus protected people for a shorter period of time than mRNA based vaccines (10).

In addition, the percentage of the booster-vaccinated population among the elderly, who are particularly at risk for fatal or severe COVID-19, was relatively small in China. Some scientists familiar with the epidemiological situation in China have advised urgently increasing this percentage (47). Thus, the abrupt cancellation of China's zero-covid policy in late 2022 led to a dramatic outbreak of disease, leading to a significant increase in hospitalizations in December and January. Already in the first week of January, the number of hospitalizations, according to the WHO, doubled and mortality rates also rose sharply (48).

Natural immunity to COVID-19 in Hong Kong in January 2023 was probably better than in mainland China because the island survived a severe outbreak of Omicron BA.2 in March 2022. Some

experts estimated that about 40% of Hong Kong's population was infected during the outbreak (OurworldInData (32),). It is worth noting that the vaccination situation in Hong Kong is better than in mainland China because almost half of the population received the mRNA vaccine, which has a longer protection period than the inactivated virus-based vaccines used in China (10). Considering these data, it can be assumed that the situation in mainland China can only be worse than in Hong Kong. The daily mortality rate on the mainland seems to be reaching a higher level than on the island, where there were nine deaths per million people in January 2023. However, using Hong Kong data, even conservative estimates for China's population of 1.4 billion predict a daily death rate of 12600.

Hybrid immunity has probably developed faster in countries that do not have such radical policies as China. It has been shown that regardless of the vaccine used, hybrid immunity induces a stronger humoral response than vaccination (44). Hybrid immunity may also provide greater protection than immunity induced by vaccination alone against the Omicron variant (49). We assume that some infectious background of continuing circulating SARS-CoV-2 variants along with booster vaccinations will maintain hybrid immunity in most countries going forward. As a result, we can expect that in 2023 most of the world, and especially the European countries considered in this study, will avoid major infectious outbreaks such as the one that occurred in China in January of this year.

Conclusions

A. Slow vaccination and slow booster administration have been associated with high excess mortality from COVID-19 in European countries. In contrast, high vaccination rates provided robust protection against virus-associated mortality. Vaccine protection peaked in the Delta wave but became weaker in the Omicron wave.
B. However, additional booster vaccination was very effective in preventing excess mortality caused by the Omicron BA.1/BA.2 infectious wave.
C. The main trend found in this study was that the European countries that vaccinated their populations faster were mostly the same countries that had higher GDP per capita. They also provided better protection against COVID-19-related deaths even before vaccination campaigns began.
D. Although a small number of countries protected their populations from COVID-19 deaths poorly before vaccination campaigns began, they did much better afterwards by ensuring fast vaccination of their citizens.
E. The excess mortality during the COVID-19 pandemic correlates not only with a county's vaccination rate, but also with its per capita GDP. The latter parameter likely reflects and is related to the quality of healthcare in the country, the availability of mass COVID-19 testing, and funding for other pandemic mitigation strategies.

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.

Author contributions

OM - Data curation, Formal analysis, Investigation, Figures design, Writing. SS - Conceptualization, Methodology, Writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

OM was employed by the company Sendai Viralytics.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1151311/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Share of people that completed the primary series vaccination at a given date and distribution of different vaccine types in EU.

SUPPLEMENTARY FIGURE 2
SARS-CoV-2 sequences share by variant.

SUPPLEMENTARY FIGURE 3
Relationships between national excess mortality in countries and their age characteristics. The correlation coefficient values corresponding to statistical significance ($p<0.05$) are shown in white on a light brown background for the Delta period and in dark green for the Omicron period.

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Upregulated CD8$^+$ MAIT cell differentiation and *KLRD1* gene expression after inactivated SARS-CoV-2 vaccination identified by single-cell sequencing

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Background: The primary strategy for reducing the incidence of COVID-19 is SARS-CoV-2 vaccination. Few studies have explored T cell subset differentiation and gene expressions induced by SARS-CoV-2 vaccines. Our study aimed to analyze T cell dynamics and transcriptome gene expression after inoculation with an inactivated SARS-CoV-2 vaccine by using single-cell sequencing.

Methods: Single-cell sequencing was performed after peripheral blood mononuclear cells were extracted from three participants at four time points during the inactivated SARS-CoV-2 vaccination process. After library preparation, raw read data analysis, quality control, dimension reduction and clustering, single-cell T cell receptor (TCR) sequencing, TCR V(D)J sequencing, cell differentiation trajectory inference, differentially expressed genes, and pathway enrichment were analyzed to explore the characteristics and mechanisms of postvaccination immunodynamics.

Results: Inactivated SARS-CoV-2 vaccination promoted T cell proliferation, TCR clone amplification, and TCR diversity. The proliferation and differentiation of CD8$^+$ mucosal-associated invariant T (MAIT) cells were significantly upregulated, as were *KLRD1* gene expression and the two pathways of nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, and translational initiation.

Conclusion: Upregulation of CD8$^+$ MAIT cell differentiation and *KLRD1* expression after inactivated SARS-CoV-2 vaccination was demonstrated by single-cell sequencing. We conclude that the inactivated SARS-CoV-2 vaccine elicits adaptive T cell immunity to enhance early immunity and rapid response to the targeted virus.

KEYWORDS

CD8$^+$ MAIT cell differentiation, *KLRD1* gene, single-cell sequencing, inactivated SARS-CoV-2 vaccine, COVID-19

Background

Coronavirus disease 2019 (COVID-19) has attracted global attention since its emergence in December 2019 in Wuhan, China (1–3) due to its high transmissibility and rapidly surging case numbers (4, 5). On 30 January 2020, by which time severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) had infected nearly 10,000 people and had caused over 200 deaths, the World Health Organization (WHO) declared the COVID-19 outbreak a public health emergency of international concern (6, 7). Soon afterward, on 11 March 2020, the WHO upgraded its classification of the COVID-19 outbreak to a global pandemic (8).

The primary strategy for reducing the incidence of COVID-19 is SARS-CoV-2 vaccination. Ongoing research on SARS-CoV-2 vaccine mechanisms of action is comprised primarily of qualitative and quantitative studies of antibody and cytokine responses (9–13). Only a few studies have explored T cell subset differentiation and gene expression. Single-cell sequencing is a sensitive method for in-depth analysis of the cellular and genetic mechanisms of vaccine response. Single-cell sequencing has identified an antigen-specific cellular basis of BNT 162b2 mRNA vaccine-induced immunity (14) and cell type-specific interferon responses to an Ad5-nCoV adenovirus vaccine that enhanced cellular immunity (15). However, single-cell sequencing has rarely been used to explore the mechanism of inactivated SARS-CoV-2 vaccines. In this study, single-cell sequencing was used to analyze the dynamics of T cell-mediated immunity and transcriptome gene expression for 3 months in three healthy Chinese adults who received three doses of inactivated SARS-CoV-2 vaccine.

Methods

Participants

Healthy adult volunteers were recruited on 8-14 January 2021. Inclusion criteria were an age of 18–59 years and good health without underlying diseases. Exclusion criteria were age younger than 18 years or older than 60 years, underlying diseases, serious adverse reactions during vaccination, illnesses requiring hospitalization, pregnancy, a history of miscarriage, or withdrawal from the study for any reason during follow-up. This study was approved by the Research Ethics Committee of the Third Affiliated Hospital of Shenzhen University and written informed consent was obtained before enrollment (The EC approval number: 2021-LHQRMYY-KYLL-033).

Sample collection and preparation

A total of 1 mL blood samples were collected from each participant at four time points: immediately before the first SARS-CoV-2 vaccination dose, 14 and 90 days after the second dose, and 90 days after the third dose (V0, V1, V2, and V3, respectively). Peripheral blood mononuclear cells (PBMCs) were extracted by density gradient centrifugation. The whole blood sample was diluted with phosphate-buffered saline (PBS, Solarbio, Beijing, China) at a ratio of 1:1. The diluted sample was added to a tube with 2/3 volume of Ficoll-Pague PLUS lymphocyte separation medium (GE Healthcare, Sweden). Cells were divided into three layers because of different sizes and densities after centrifugation at 400g for 35 min. The supernatant was removed, and the intermediate cell suspension layer was transferred into a 15 ml centrifuge tube, supplemented with PBS, and centrifuged at 300g for 7 min. The supernatant was again discarded, and the pellet was washed twice and resuspended in PBS to obtain PBMCs. PBMCs were extracted and frozen at -80°C. PMBC concentrations and activities were measured before the study began. PBMCs were taken from the -80°C freezer and thawed. The cell mixture sample was stained with 0.4% Trypan blue solution (Sigma, UK), and viable cells were counted under a microscope (ECLIPSE Ts2, Nikon, Japan). When the final concentration was 2×10^5 cells/mL and cell viability exceeded 85%, subsequent processing was performed.

Library preparation and single-cell T cell receptor sequencing

Cell suspensions (2×10^5 cells/ml, 100μl) were loaded into microfluidic devices (Matrix1.0.1) and the separation of single cells was completed according to the principle of Poisson distribution. scTCR-seq libraries were constructed following the protocol of GEXSCOPE Single Cell Immuno-TCR Kit (Biotechnologies). Specifically, poly(A) tails and TCR regions of mRNA were captured by magnetic beads with molecular markers. Cells and mRNA were labeled after the cells were lysed. The magnetic beads in the chip were collected, and mRNAs were reverse-transcribed into complementary DNA (cDNA) and amplified. After local cDNAs were fragmented and spliced, transcriptome sequencing libraries suitable for the Illumina sequencing platform were constructed. The remaining cDNA was enriched to the immune receptor (TCR), and TCR sequencing libraries suitable for the Illumina sequencing platform were constructed by PCR amplification of the enriched products. Finally, sequencing of the libraries was performed on Illumina Nova 6000, with a pair-end length of 150 bp.

TCR V(D)J sequencing and analysis

The Cell Ranger (v4.0.0) vdj (variable, diversity, joining region) pipeline was used to analyze TCR clonotype, with Genome Reference Consortium Human Genome Build 38(GRCh38) as reference. After the analysis, a TCR diversity metric of clonotype frequency and barcode information was acquired. For TCR, only cells with one productive TCR α-chain (TRA) and one productive TCR β-chain (TRB) were retained for subsequent analysis. Each unique TRA(s)-TRB(s) pair was defined as a clonotype. If one clonotype was present in at least two cells, cells harboring this clonotype were considered as clonal and the number of cells with

such pairs indicated the degree of clonality of the clonotype (16). Clonotype diversity was calculated with Chao1.

Primary analysis of raw read data

Using an internal pipeline (a data conversion process), raw reads from scRNA-seq were converted into gene expression matrices. Cell barcodes and unique molecular identifiers (UMI) were extracted by first removing low-quality data of raw reads with FastQC v0.11.4 and fastp (17), and then trimming poly-A tails and adapter sequences with Cutadapt (18). UMI and gene counts of each cell were then acquired with featureCounts v1.6.2 (19) after the reads were mapped to the reference genome GRCh38 by using STAR v2.5.3a (20). Expression matrix files were thus generated.

Quality control, dimension-reduction, and clustering

During the quality control process, the gene expression matrix was filtered after excluding the following cells: cells with gene count top 2% or < 200, cells with top 2% UMI count, cells with 50% mitochondrial content, and cells in which genes were expressed in < 5 cells. After quality control, dimension reduction and clustering were performed by using Seurat v3.1.2 (21). All gene expressions were then normalized and scaled with NormalizeData and ScaleData functions, and the top 2,000 variable genes were selected for principal component analysis with FindVariableFeatures. Cells were separated into multiple clusters according to the top 20 principle components by using FindClusters. Harmony (22) was used to remove the batch effect between samples. Finally, two-dimensional visualization of cells was achieved by using uniform manifold approximation and projection (UMAP).

Inference of cell differentiation trajectories

Cell differentiation trajectories were reconstructed by Monocle2 v2.22.0. Cell spatiotemporal differentiation sequencing was performed by evaluating highly variable genes. FindVariableFeatures and dimensional reduction were performed by DDRTree. Finally, the trajectories were visualized by using the plot_cell_trajectory function.

Differentially expressed gene analysis

The genes expressed in > 10% of the cells in a cluster and with an average log(fold change) value > 0.25 were selected and identified as DEGs by using the Seurat FindMarkers function based on the Wilcox likelihood-ratio test with default parameters. The cell type annotation of each cluster was displayed with dot plots/violin plots by using Seurat DotPlot/Vlnplot, according to the expression of canonical markers found in the DEGs and knowledge from the literature. Cells expressing markers of different cell types were identified as doublets and removed during subsequent quality control.

Pathway enrichment analysis

Pathway enrichment analysis was performed by using Gene Ontology (GO) analysis together with the clusterProfiler R package (23), aiming to investigate the functions of DEGs. Molecular function, biological process, and cellular component categories in GO gene sets were used as references. Protein-protein interactions of DEGs in each cluster were predicted according to the interactions between the known genes and the relevant GO terms in StringDB v1.22.0. Pathways with $p\_adj < 0.05$ were considered significantly enriched.

Statistical analysis

Statistical analyses and visualization were performed with the R package (R Foundation for Statistical Computing, Vienna, Austria). Data were presented as mean ± standard deviation. Comparisons between the two groups were analyzed using a two-sample Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Participants

Three subjects were recruited, including one man and two women, with an average age of 33.00 ± 13.08 years and an average body mass index of 20.13 ± 2.40 kg/m^2. All subjects received three doses of inactivated SARS-CoV-2 vaccine, with a 4-week interval between the first and second dose and a 32-week interval between the second and third dose. None of the subjects experienced serious adverse reactions.

PBMC concentration and activity

As shown in Table 1, the final PBMC concentrations were > 2×10^5 cells/mL at all time points, and cell viability exceeded 85%. All PBMCs were qualified; thus subsequent procedures were processed.

Cell type identification

A total of 130,082 PBMCs were obtained from the 12 blood samples taken from the three subjects at the four time points. After clustering, the cells were classified into six cell types, including B cells (corresponding B cell genes were *MS4A1*, *CD79A*, *CD79B*, *JCHAIN*, *MZB1*, *IGHG1*, and *IGHA1*), T cells (*CD2*, *CD3D*, *TRAC*, *TRBC2*, *KLRD1*, and *NKG7*), NK cells (*KLRD1*, *KLRF1*, *NKG7*,

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TABLE 1 PMBC concentrations and activities of each subject at each time point.

Each study time point of each subject	Cell concentration (cells/mL)	Cell activity (%)
S1_V0	1.55×10^6	90.41
S1_V1	1.75×10^6	94.60
S1_V2	5.51×10^5	95.50
S1_V3	1.03×10^6	90.40
S2_V0	1.11×10^6	90.56
S2_V1	9.48×10^5	85.87
S2_V2	1.20×10^6	93.91
S2_V3	1.47×10^6	96.40
S3_V0	1.62×10^6	91.11
S3_V1	5.87×10^5	94.79
S3_V2	1.63×10^6	93.94
S3_V3	1.14×10^6	96.10

S1, S2, and S3 refer to the three study subjects.

NCR1, *XCL1*, and *CD3D*), monocytes (*CD14*, *FCN1*, *VCAN*, *FCGR3A*, and *IFITM3*), conventional dendritic cells (cDCs) (*CD1C*, *FCER1A*, *XCR1*, and *CLEC10A*), and plasmacytoid dendritic cells (pDCs) (*IL3RA*, *CLEC4C*, and *LILRB4*), as shown in Table 2 and Figure 1. The numbers of NK and especially T cells increased significantly after vaccination. However, no significant expansions of B cells, monocytes, cDCs, or pDCs were observed. Furthermore, the numbers of these cells at the V3 time point had decreased from V0 baseline values.

Subdivision of T cell subsets

A total of 61,599 T cells were classified into 8 T cell subsets: naïve T cells, $CD4^+$ effector T cells, $CD8^+$ effector T cells, $CD8^+$ mucosal-associated invariant T ($CD8^+$ MAIT) cells, helper T cells, regulatory T cells (Treg), Gamma Delta T cells (GDT cells), and proliferating T cells, as shown in Table 3 and Figure 2.

Dimension reduction analysis of TCR-amplified clonotypes

After dimension reduction analysis, TCR clonotypes were classified into large, medium, and single groups according to the frequency of amplified clonotypes. Large, medium, and single clonotype frequencies were defined as >10, >1 and ≤10, and 1, respectively. The results are shown in Figure 3. In Figure 3A, red and gray dots indicate T cells with or without clonotype expansion, respectively. Figure 3B shows the overall dynamic proportion of TCR clonotype amplification at different time points. Figure 3C shows the proportion of TCR clonotype large, medium, and single frequencies at different time points. Figure 3D shows the TCR clonotype amplification of different T cell subsets at different time points. Figure 3E illustrates the TCR diversity analysis results. T cell subsets with significant TCR clonotype amplification were $CD4^+$ effector T cells, $CD8^+$ effector T cells, and $CD8^+$ MAIT cells. The proportion of large clone amplification in these three T cell subsets was higher after inoculation. As shown in Figure 3E, TCR diversity was higher at V2 and V3 than before inoculation (although P >0.05), with the highest value at V3, suggesting that TCR diversity increased after inactivated SARS-CoV-2 vaccination.

TABLE 2 Quantities of six cell types obtained by clustering from 130,082 cells of three individuals at different time points after SARS-CoV-2 vaccine inoculation.

	B cells	T cells	NK cells	Monocytes	cDCs	pDCs
S1_V0	791	4837	296	2061	47	44
S2_V0	1196	1183	1272	5298	443	97
S3_V0	642	1034	606	2975	143	69
S1_V1	575	5483	1498	2775	115	76
S2_V1	650	1254	1316	5382	596	231
S3_V1	864	2875	1578	4962	203	230
S1_V2	1111	11036	2033	2167	156	99
S2_V2	725	5711	925	1787	195	65
S3_V2	1396	11292	1846	2069	136	73
S1_V3	576	4976	915	2522	161	71
S2_V3	785	6472	2045	3182	199	61
S3_V3	715	6838	723	3093	173	56

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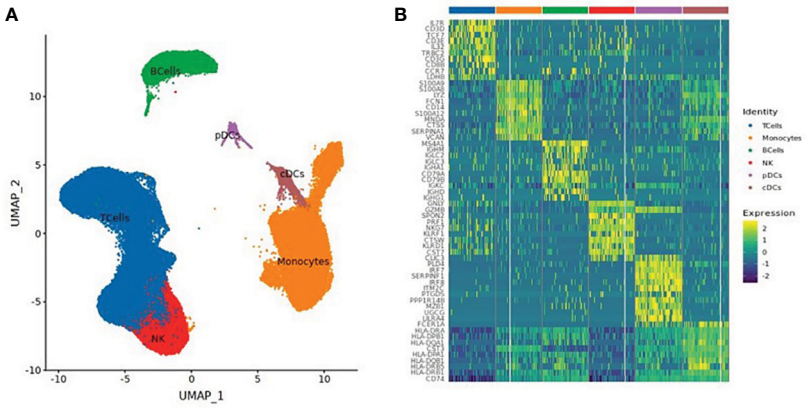

FIGURE 1
(A) UMAP Clustered by cell type coloring for all cells. **(B)** Heatmap of the top 10 differential genes of each cell type.

CD8$^+$ MAIT cell differentiation trajectories

The results of the pseudo-time sequence analysis of CD8$^+$ MAIT cells are shown in Figure 4. Figure 4A shows the overall trajectory of CD8$^+$ MAIT cell differentiation. Figure 4B shows the three stages of CD8$^+$ MAIT cell differentiation. Figure 4C shows the trajectory analysis of CD8$^+$ MAIT cells from different time points V0, V1, V2, and V3. Figure 4D (spindle diagram) shows the CD8$^+$ MAIT cell differentiation process at each time point. CD8$^+$ MAIT cells were typically within a relatively late stage of differentiation at V0. However, these cells differentiated significantly after vaccination. A large proportion of CD8$^+$ MAIT cells with relatively advanced differentiation appeared at V1. At V2, CD8$^+$ MAIT cells continued to differentiate. At the final V3 time point, after the third dose, many CD8$^+$ MAIT cells had reached the differentiation endpoint.

DEG analysis

The results of the DEG analysis are shown in Figure 5. DEGs included *TSC22D3*, *GZMB*, *PRF1*, *KLRD1*, *DUSP2*, *TNFAIP3*, and *PER1* (Figure 5A). Average expressions of *KLRD1* in NK cells, CD8$^+$ effector T cells, and CD8$^+$ MAIT cells were significantly increased after vaccination when compared with V0 baselines. The average expressions of *GZMB* and *PRF1* in NK cells, CD8$^+$ effector T cells, and CD8$^+$ MAIT cells were also significantly increased after vaccination (*$P<0.05$).

Enrichment pathway analysis

Pathways that were significantly enriched at V1, V2, and V3 were selected (Figure 6). The Y-axis shows pathways that were significantly enriched at the postvaccination time points when compared with V0 baselines, and the X-axis shows the *P*_adjust value compared between pathways. The larger the *P*_adjust value, the more significant the statistical difference. In the T cell biological process pathways (Figure 6A), we found that meaningful enrichment pathways were significantly upregulated. These included the nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, and translational initiation pathways, which play important roles in limiting viral replication and regulating post-vaccination immunity. Furthermore, in the B-cell biological process pathways (Figure 6B), several B-cell and B-cell receptor enrichment pathways, which included B cell receptor

TABLE 3 Quantities of eight T cell subsets obtained by clustering from 61,599 T cells of three individuals at different time points after SARS-CoV-2 vaccine inoculation.

T cell subsets	V0	V1	V2	V3
Naïve T cells	3655	3665	14236	8548
CD4$^+$ effector T cells	218	500	1010	837
CD8$^+$ effector T cells	1440	3232	5935	3769
D8$^+$ MAIT cell	331	443	1429	911
Helper T cell	820	846	3002	2246
Treg	179	151	563	461
GDT cells	286	445	1074	657
Proliferating T cells	64	138	250	258

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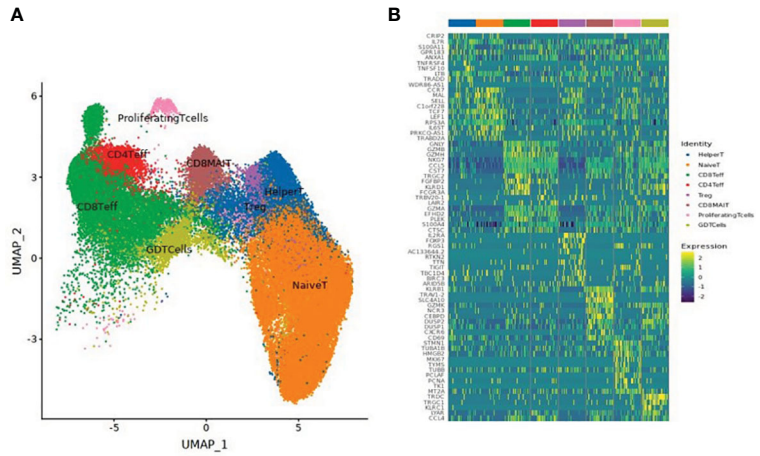

FIGURE 2
(A) UMAP clustering of T cell subsets. **(B)** Heatmap of the top 10 differential genes of each T cell subset.

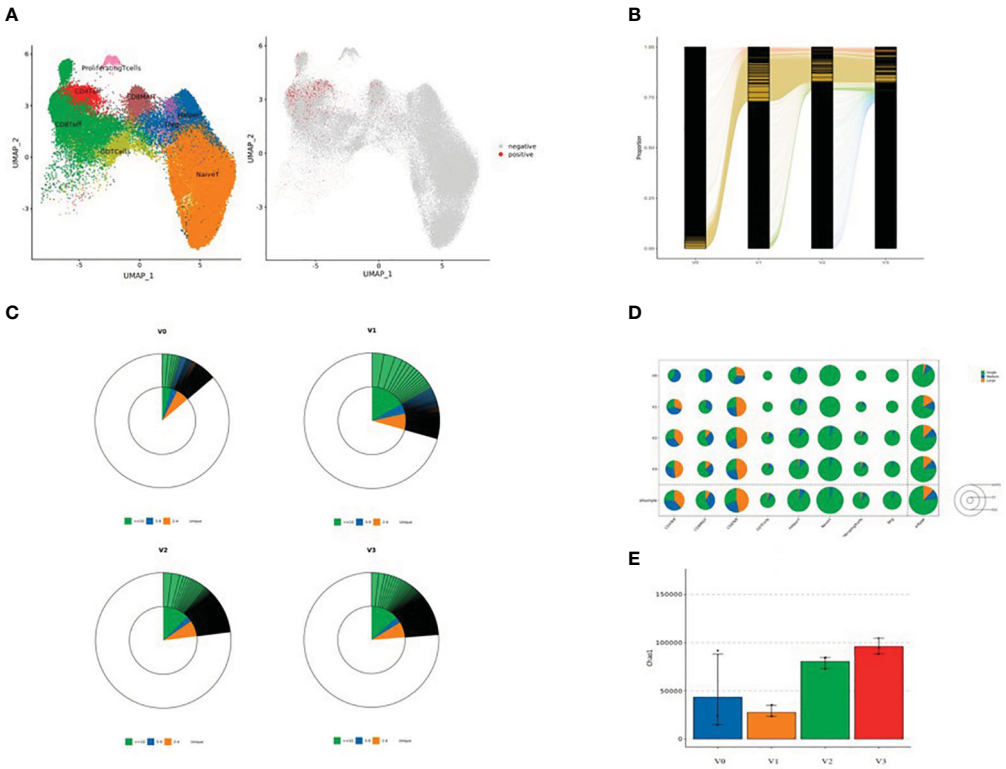

FIGURE 3
(A) Dimensional reduction analysis of TCR-amplified clonotypes (red dots indicate T cells with clonotype expansion and gray dots indicate T cells without clonotype expansion); **(B)** Overall dynamic proportion of TCR clonotype amplification at different time points; **(C)** Proportion of TCR clonotype frequencies (large, medium and single) at time points V0, V1, V2, and V3 (green: clonotype frequency ≥10; blue: clonotype frequency 5-9; yellow: clonotype frequency 2-4; and white: clonotype frequency single); **(D)** TCR clonotype amplification of different T cell subsets at four time points: V0, V1, V2, and V3 (green: single, blue: medium, and yellow: large). The area of each circle represents the number of cells; the larger the circle, the greater the number of T cell subsets with TCR-amplified clonotypes; and **(E)** TCR diversity analysis (V0: blue, V1: yellow, V2: green, and V3: red).

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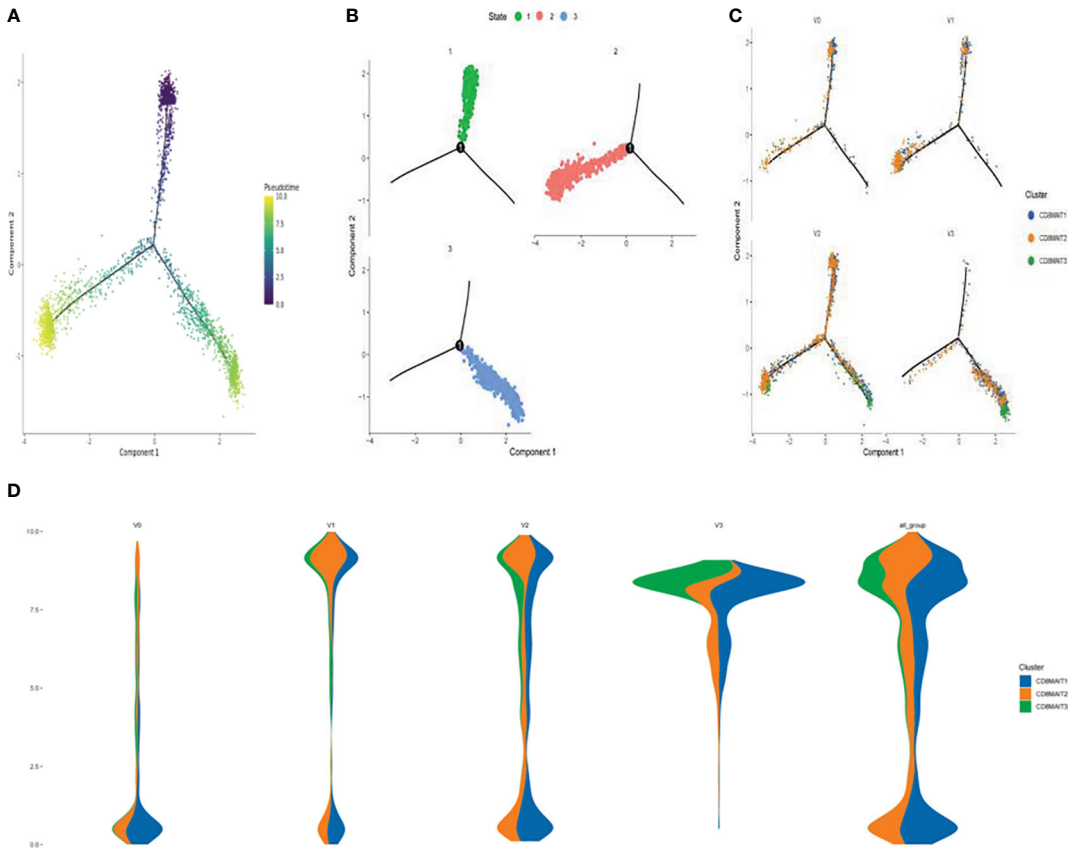

FIGURE 4
Differentiation trajectory of CD8$^+$ MAIT cells **(A)** Pseudotime trajectory of CD8$^+$ MAIT cells; **(B)** Three stages of CD8$^+$ MAIT cells differentiation; **(C)** Trajectory analysis of CD8$^+$ MAIT cells from different time points V0, V1, V2, and V3; and **(D)** Differentiation process of CD8$^+$ MAIT cells at different inoculation time points.

signaling pathway, positive regulation of B cell activation, B cell activation, and humoral immune response pathways, were all significantly enhanced.

Discussion

The deployment of SARS-CoV-2 vaccines marked an important milestone in the COVID-19 pandemic (24–26). To date, the WHO reported that more than 100 vaccine candidates have been in development globally and 26 have been evaluated in phase III clinical trials. Although SARS-CoV-2 vaccines prevent COVID-19 (27–33), vaccine-breakthrough cases still occur in fully vaccinated individuals (34–38). Several variants of concern have emerged, which include Alpha (501Y.V1 with GISAID nomenclature or B.1.1.7 with PANGO nomenclature), Beta (501Y.V2 or B.1.351), Gamma (501Y.V3 or P1),and Delta (G/478K.V1 or B.1.617.2). Variants of concern are generally associated with higher transmission, mortality, and breakthrough infections than the original strain or D614G variant.

Different SARS-CoV-2 vaccines have demonstrated varying protective efficacies. A meta-analysis (39) showed that after full vaccination, mRNA vaccine efficacy against symptomatic SARS-CoV-2 infection was 89-100% against unsequenced strains, 88-100% against Alpha, 76-100% against Beta/Gamma, and only 47.3-88% against Delta; while the adenovirus-vectored vaccine AZD1222 was 74.5% protective against Alpha and 67% against Delta. These results suggest that mRNA vaccines and AZD1222 are effective in preventing symptomatic SARS-CoV-2 infection against the original strain and Alpha and Beta variants, but less effective against the Delta strain. Meanwhile, vaccine efficacy decays after completion of the immunization series. Antibody levels after BNT162b2, mRNA-1273, and Ad26.COV2.S vaccinations were sustained for at least 6 months but then decreased over time. At 6 months, neutralizing antibody activities against Alpha, Gamma, Delta, and Epsilon were maintained, but declined against Beta in half of the participants of mRNA-1273 vaccination. Observa- tional studies stratified by time since vaccination showed that the efficacy of mRNA vaccine and AZD1222 vaccine in preventing Delta infection decreased significantly at 4-6 months after inoculation (42-57% and 47.3%, respectively). In the USA, mRNA vaccine efficacy against symptomatic SARS-CoV-2 infection decreased from 94.3% in June to 65.5% in July 2021.

Because the efficacies and durations of protection of different COVID-19 vaccines against particular SARS-CoV-2 strains have varied, vaccine targets, mechanisms, and duration of protection must be explored further. In this study, we used single-cell sequencing to track the immune status after three doses of

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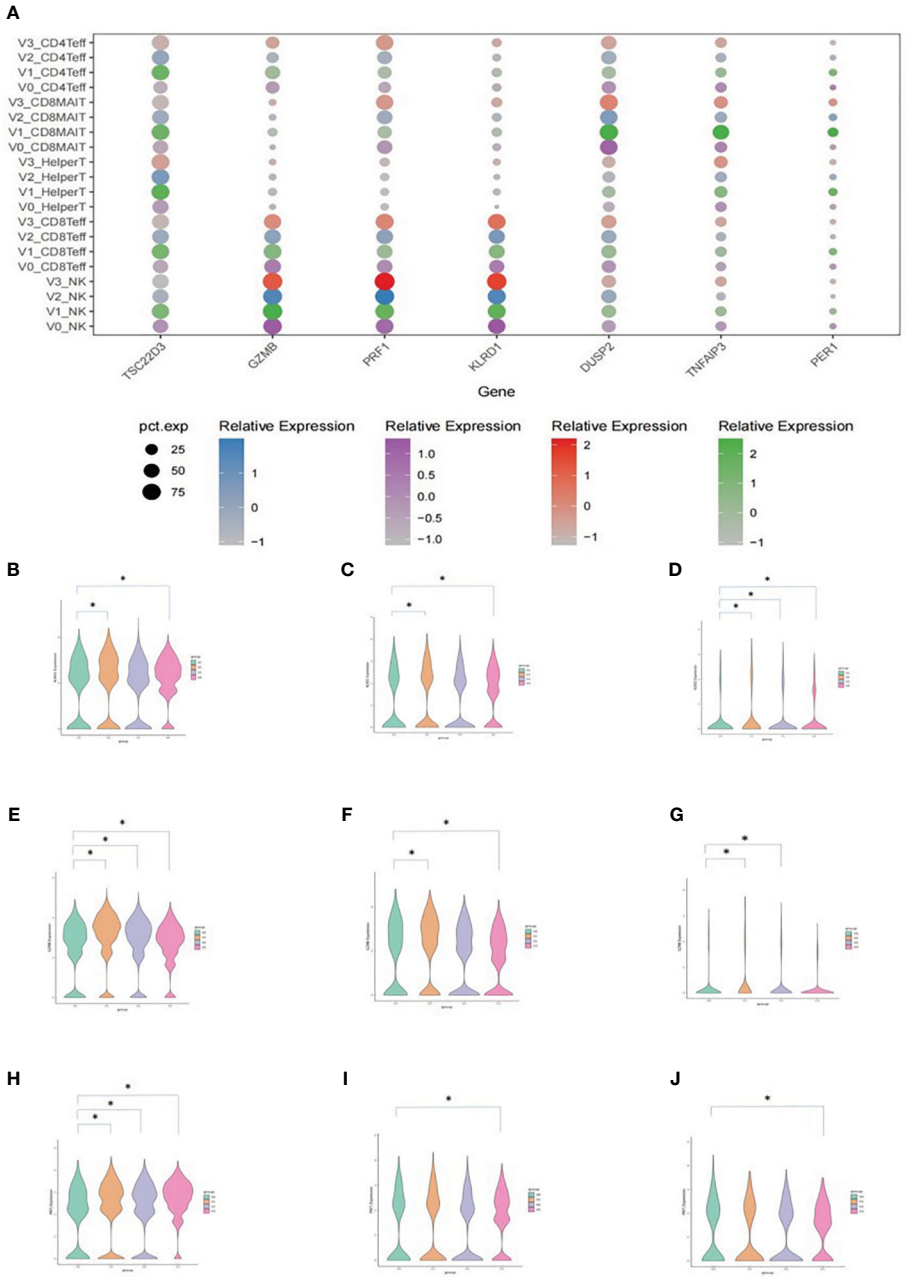

FIGURE 5
Average expression levels of DEGs. **(A)** Dotplot expressing the average expression of *TSC22D3*, *GZMB*, *PRF1*, *KLRD1*, *DUSP2*, *TNFAIP3*, and *PER1* in T cell subsets. The X-axis indicates the DEGs and the Y-axis shows different T cell subsets at each time point. V0: purple, V1: green, V2: blue, and V3: red. The size of the dot indicates the proportion of cells that expressed the genes in its cell subset, and the shade of the color of the dot indicates the average level of gene expression in all cells. **(B)** Vlnplot of the average *KLRD1* expression in NK cells. **(C)** Vlnplot of the average *KLRD1* expression in $CD8^+$ effector cells. **(D)** Vlnplot of the average *KLRD1* expression in $CD8^+$ MAIT cells. **(E)** Vlnplot of the average *GZMB* expression in NK cells. **(F)** Vlnplot of the average *GZMB* expression in $CD8^+$ effector cells. **(G)** Vlnplot of the average *GZMB* expression in $CD8^+$ MAIT cells. **(H)** Vlnplot of the average *PRF1* expression in NK cells. **(I)** Vlnplot of the average *PRF1* expression in $CD8^+$ effector cells. **(J)** Vlnplot of the average *PRF1* expression in $CD8^+$ MAIT cells. In panels **(B-J)**, the X-axis shows the four time points and the Y-axis shows the average expression levels of the corresponding gene. V0: green, V1: yellow, V2: purple, and V3: red. *P<0.05.

inactivated SARS-CoV-2 vaccine. Results revealed the T cell dynamics, characteristics, and mechanisms of human immune dynamics.

Our previous research work (40) showed that serum IgM, IgG, and neutralizing antibody titers peaked on the 14th day after the second dose of inactivated SARS-CoV-2 vaccine and decreased gradually thereafter. Therefore, in the present study, we chose four time points: V0 (pre-vaccination), V1(14 days after 2nd inoculation), V2 (90 days after the 2nd inoculation), and V3 (90 days after the 3rd inoculation) to explore the changes of immune statuses of healthy adults during the course of three doses of SARS-CoV-2 inactivated vaccine.

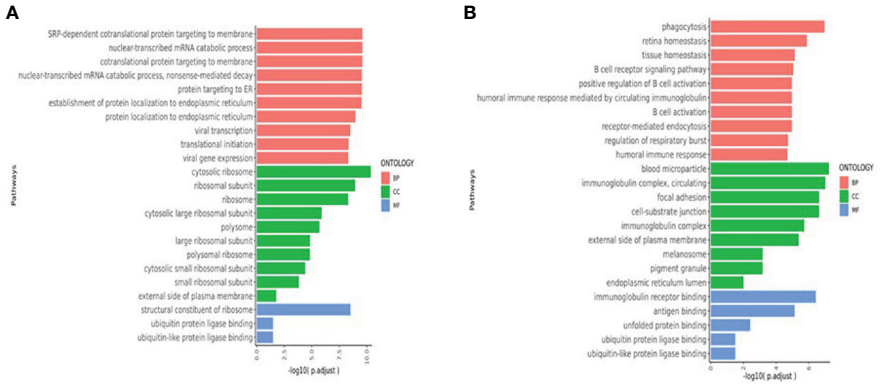

FIGURE 6
(**A**)T cell enriched upregulated pathways (V1V2V3 *vs.* V0). (**B**) B-cell enriched upregulated pathways (V1V2V3 *vs.* V0). The Y-axis shows pathways that were significantly enriched at V1, V2, and V3 time points compared with V0 baselines and the X-axis shows the *P*_adjust value compared between pathways. The larger the *P*_adjust value, the more significant the statistical difference. BP, biological processes; CC, cellular component; MF:,molecular function.

Single-cell RNA sequencing is a powerful tool for elucidating transcriptome gene expression and dynamics of cell subsets (41). Changes in the immune landscape at different time points after inactivated SARS-CoV-2 vaccination were analyzed by first determining the different proportions of cell subsets, then tracking cell differentiation trajectories through pseudo-time sequence analyses, and, finally, by analyzing DEGs and pathway enrichment between cells of amplified and unamplified clonotypes, thereby exploring the dynamics of transcriptome gene expression.

The serum-neutralizing antibody titer is usually taken as an important marker of the immunogenicity of an anti-viral vaccine. Zhang et al. (42) found that transcription levels of PBMC were changed 14 days after the first dose of inactivated SARS-CoV-2 vaccine in 13 healthy participants while serum-neutralizing antibody concentrations remained very low. On day 28, upon the second vaccine dose, the subjects' PBMC transcriptomics achieved an immune status similar to natural immunity, suggesting that PBMC single-cell sequencing is more sensitive than classical neutralizing antibody assays. Horns et al. (43) studied influenza vaccine response and found that single-cell transcriptional profiling reveals a program of memory B cell activation characterized by *CD11c* and *T-bet* expression associated with clonal expansion and differentiation toward effector function. Kong et al. (44) used single-cell transcriptomic measurements to demonstrate that Bacillus Calmette-Guérin vaccination (BCG) reduces systemic inflammation and to identify a group of genes that are putatively responsible for the non-specific protection conferred by the Bacillus Calmette-Guérin (BCG) vaccination. Because single-cell sequencing is not only more sensitive in evaluating immunogenicity and can further explore the molecular and cellular characteristics and mechanisms of the immune response, we chose single-cell sequencing as our research method.

Our study showed that the numbers of NK and especially T cells increased significantly after vaccination. However, the numbers of B cells, monocytes, cDCs, and pDCs were not increased. Moreover, the numbers of these cells at the V3 time point were reduced when compared with pre-inoculation baselines.

Our study showed that the number of T cell subsets increased significantly on the 14th day after the 2nd dose, 90 days after the 2nd dose, and 90 days after the 3rd dose of inactivated SARS-CoV-2 vaccination. Subsets included naïve T cells, $CD4^+$ effector T cells, $CD8^+$ effector T cells, $CD8^+$MAIT cells, helper T cells, Tregs, GDT cells, and proliferating T cells (Table 3 and Figure 2). Similar to our study, Mateus et al. (45) evaluated subjects in multiple age groups who received low-dose (25ug) mRNA-1273 COVID-19 vaccine and found that vaccine-generated spike-specific $CD4^+$ and $CD8^+$ T cell immune memory 6 months after the second dose of the vaccine was comparable in quantity and quality to natural immunity.

We further performed dimension reduction analysis of TCR-amplified clonotypes and found significant amplification of TCR clonotypes after inactivated SARS-CoV-2 vaccination, especially in $CD4^+$ effector T cells, $CD8^+$ effector T cells, and $CD8^+$ MAIT cells. We observed a trend toward increased TCR diversity at 90 days after the second inoculation and 90 days after the third inoculation when compared to the pre-vaccination baseline (Figure 3). TCR is the characteristic surface marker of T cells, whose function is antigen recognition. TCR is a heterodimer composed of α and β peptide chains. Each peptide chain contains variable (V) and constant (C) regions. The antigen specificity of TCR is conferred by the V region, in which there are three highly variable regions, namely, complementarity determining regions (CDR) 1, CDR2, and CDR3. When TCR recognizes a peptide-MHC complex, CDR1 and CDR2 bind to the lateral wall of the MHC molecular antigen-binding slot, while CDR3 binds directly to the antigen-binding peptide, determining the antigen-binding specificity of TCR. Crucial to immune function is the ability to recognize the millions of antigens that may be presented *via* MHC complexes on the surfaces of antigen-presenting cells. This is achieved by the enormous clonal diversity of TCRs generated by combining different CDRs within α and β TCR chains and by the pairing of

differently combined α and β TCR chains (46). Carreno et al. showed that a dendritic cell vaccine increased naturally occurring neoantigen-specific immunity and promoted a diverse neoantigen-specific TCR repertoire in terms of both TCR-β usage and clonal composition, thus broadening the antigenic breadth and clonal diversity of antitumor immunity (47). To elucidate the molecular basis of the 5~10% failure rate of the hepatitis B vaccine, Yang et al. (48) conducted high-throughput sequencing and bioinformatics analysis of TRB CDR3 repertoires and found that the diversity of TRB CDR3 was significantly increased in responders compared to non-responders, which suggested that individuals with increased TCR diversity had better vaccine responses. Our results showed an increased TCR diversity after vaccination, suggesting that the SARS-CoV-2 inactivated vaccine elicits adaptive T cell immunity that can facilitate the recognition of multiple antigens.

Our study showed that the number of $CD8^+$ MAIT cells was significantly increased in inactivated SARS-CoV-2 vaccine recipients. Furthermore, TCRs also underwent significant clone amplification. MAIT cells are unconventional innate-like T cells. They recognize antigens derived from the riboflavin biosynthetic pathway produced by a wide range of microbes and presented by the MHC class-I related (MR1) protein (49–51). Following activation, MAIT cells rapidly produce cytokines that include IFN-γ, TNF, IL-17, and IL-22 and mediate the cytolysis of infected cells, leading to the control of various infections. Innate cytokines, such as IL-12 and IL-18, can activate some MAIT cellular functions in an MR1-independent fashion, and enhance MAIT cell TCR-dependent activation. MR1-independent responses are likely important in MAIT cell responses to viral infections and in diseases driven by cytokine storms provoked by bacterial exotoxins. Human MAIT cells predominantly express the CD8α coreceptor ($CD8^+$), with a smaller subset lacking both CD4 and CD8 (double-negative, DN). $CD8^+$ MAIT cells have higher levels of IL-12 and IL-18 receptors. $CD8^+$ MAIT cells display a higher diversity of T cell receptor repertoires than DN MAIT cells. Furthermore, $CD8^+$ MAIT cells had significantly higher *GZMB*, *PRF1*, and *granulysin* levels than DN MAIT cells. These data indicate that peripheral blood $CD8^+$ MAIT cells display higher baseline expression of coactivating receptors and cytotoxic effector molecules than DN MAIT cells (52). Provine et al. (53) showed that ChAdOx1 (chimpanzee adenovirus Ox1) immunization activated MAIT cells robustly. Activation of MAIT cells correlated with vaccine-induced T cell responses in human volunteers. MAIT cell-deficient mice displayed impaired $CD8^+$ T cell responses to multiple vaccine-encoded antigens, suggesting that MAIT cells contribute to the immunogenicity of adenovirus-vectored vaccines. Boulouis et al. (54) found that pre-and postvaccination levels of MAIT cells correlated positively with the magnitude of SARS-CoV-2 spike protein-specific $CD4^+$T cell and antibody responses in healthy vaccinees and that the MAIT cell compartment is involved in the early stages of priming of adaptive immune responses, which may be important for vaccine-induced immunity. Khaitan et al. (55) showed that HIV-infected children between the ages of 3 to 18 years have significantly decreased $CD8^+$ MAIT cell populations compared to uninfected healthy children. $CD8^+$ MAIT levels gradually increased with antiretroviral therapy, with greater recovery with the initiation of treatment at a younger age. Diminished $CD8^+$ MAIT cell frequencies are associated with low CD4:CD8 ratios and elevated sCD14, suggesting a link with HIV disease progression. Moreover, $CD8^+$ MAIT cell levels correlate tightly with other antibacterial and mucosa-protective immune subsets, namely, neutrophils, innate-like T cells, and Th17 and Th22 cells. These findings suggested that decreased MAIT cell populations in HIV-infected children are part of a concerted disruption of the innate and adaptive immune compartments specialized in sensing and responding to pathogenic or commensal bacteria.

Our study also showed that the differentiation as well as the number of $CD8^+$ MAIT cells was promoted after inactivated SARS-CoV-2 vaccination. As shown in Figure 4, during the vaccination timeline, $CD8^+$ MAIT cells advanced toward full differentiation. After the third injection, a large number of $CD8^+$ MAIT cells were located at the endpoint of differentiation, indicating that three doses of inactivated SARS-CoV-2 vaccine significantly enhanced $CD8^+$ MAIT cell differentiation, with the most enhancement after the third inoculation. Walker et al. (56) indicated that $CD8^+$ MAIT cells are important tissue-homing cell populations, characterized by high expression of CD161 (++) and type-17 differentiation. By transcriptional and functional analyses, a pool of polyclonal, pre-committed type-17 CD161(++)CD8αβ(+) T cells circulate in cord blood, from which a prominent MAIT cell (TCR+) population emerges after postnatal antigen exposures and readily transitions to a CD8αα status in peripheral blood or at tissue sites. The potent cytokine secretion and homing pattern of expanded $CD8^+$ MAIT-cell populations studied here suggest a central role in host defense and tissue inflammation in health and disease. To examine circulating MAIT cell levels and function in a healthy population, Lee et al. (57) enrolled 133 healthy subjects and measured MAIT cells and their subsets by flow cytometry. Circulating MAIT cell levels varied widely (0.19% to 21.7%) and were significantly lower in older individuals (age 61-92 years) than in young subjects (age 21-40 years). Although circulating MAIT cell levels were similar between male and female subjects, linear regression revealed that levels declined annually by 3.2% among men and 1.8% among women. Notably, the proportion of $CD4^+$ MAIT cells increased, whereas that of $CD8^+$ MAIT cells decreased with age. These studies showed that the proliferation and differentiation of $CD8^+$ MAIT cells may be important markers of immune response. In our study, SARS-CoV-2 vaccination induced both $CD8^+$MAIT cell proliferation and differentiation, indicating enhanced early immunity and rapid response to the target virus.

Our study found that NK cellular expressions of DEGs *KLRD1*, *GZMB*, and *PRF1* increased significantly after SARS-CoV-2 inactivated vaccination. At the same time, the expressions of these three DEGs by $CD8^+$ effector T cells and $CD8^+$ MAIT cells were also increased (Figure 5). Adaptive NK cells are currently grouped into three major categories, including cytokine-induced, memory-like, and true antigen-specific NK cells (58, 59). Cytokine-induced memory NK cells respond to specific cytokine profiles and seem to retain "memory" of a previous activation (60), memory-like NK cells are potent effector cells *via* antibody-dependent cellular cytotoxicity (61), and true antigen-specific NK cells respond to

cytomegalovirus and adenoviral vaccine vectors in a peptide-specific manner and may utilize the NKG2C-CD94 heterodimer to identify specific target cells (62, 63). Bongen et al. (64) found that a gene associated with NK cells, *KLRD1*, which encodes CD94, was expressed at lower levels in symptomatic influenza virus shedders at baseline in discovery and validation cohorts. *KLRD1* expression in circulating NK cells at baseline negatively correlated with influenza susceptibility and symptom severity. In addition, *KLRD1* expression was positively correlated with several cytotoxic granule-associated genes, which included *PRF1* and those encoding granzymes (*GZMA*, *GZMB*, and *GZMH*), suggesting that higher *KLRD1* expression may correlate with increased proportions of cytotoxic cells. These results imply that *KLRD1*-expressing NK cells may serve as a novel biomarker for influenza susceptibility and that their early response may reduce and potentially prevent symptoms entirely. Our study also showed that *KLRD1* expression in NK cells increased significantly after inactivated SARS-CoV-2 vaccination. Furthermore, *GZMB* and *PRF1* expressions in T cells were also promoted. Therefore, whether the postvaccination upregulation of *KLRD1* expression can confer protection by promoting the expressions of *GZMB* and *PRF1*, and whether *KLRD1* expression is related to COVID-19 susceptibility needs to be clarified.

Our T cell enrichment pathway analysis found that nuclear-transcribed mRNA catabolic process, nonsense-mediated decay (NMD), and translational initiation were significantly upregulated after inactivated SARS-CoV-2 vaccination (Figure 6). NMD, which degrades flawed cellular mRNA by translating codons, not only contributes to a cellular quality control system to prevent abnormal protein synthesis but dynamically adapts the transcriptome and proteome to varying physiological conditions. The upregulation of NMD is closely related to the stress response. NMD confers positive-sense single-stranded RNA virus-restricting capacities, suggesting that the cellular RNA decay process may act as a primitive mechanism of intracellular antiviral immunity (65). In addition to its quality control function, which usually involves mRNA degradation, NMD also controls the abundance of ~10% of the cellular transcriptome (66). NMD has the capacity to co-regulate the abundance of entire groups of genes. Furthermore, as a post-transcriptional mechanism, NMD can facilitate rapid cellular responses to various stimuli. These processes are utilized during both cellular development and stress but may be circumvented by infecting viruses. The postvaccination upregulation of this pathway demonstrated in our study suggests that mechanisms of the SARS-CoV-2 vaccine may be related to quality control, limitation of SARS-CoV-2 replication, and rapid response.

Our study showed significant upregulation of the translation initiation T cell enrichment pathway after inactivated SARS-CoV-2 vaccination. Translation can initiate at alternate, non-canonical start codons in response to stressful stimuli. Viral infections and antiviral responses alter sites of translation initiation, and in some cases, lead to the production of novel immune epitopes. Machkovech et al. (67) showed that the subset of host transcripts induced by the antiviral response is enriched for alternate initiation sites upon influenza infection or during the antiviral response. Their results systematically mapped the landscape of translation initiation during influenza virus infection, and shed light on the evolutionary forces shaping this landscape. Thus, whether translation initiation promotes the production of protective antigen epitopes after inactivated SARS-CoV-2 vaccination deserves further study.

Our study showed that B cells did not proliferate after inactivated SARS-CoV-2 vaccination, while B-cell and B-cell receptor signaling enriched pathways were significantly upregulated, which is consistent with the study by Yin et al. (68). However, Yin et al. indicated that a SARS-CoV-2 inactivated vaccine induced activation of regulatory $CD4^+$ T cells and $CD8^+$ cytotoxic T cells, which may contribute to vaccine-induced T cell memory. The present study, however, focused on the vaccination-enhanced $CD8^+$ MAIT cells, which enhanced early and quick immune response.

Several limitations of this study should be noted. Only three subjects were recruited. Follow-up tracked only to day 90 after the third inoculation. A larger population of subjects and longer follow-ups are required in subsequent studies to further characterize the overall immunological landscape after vaccination. Related molecular mechanisms need to be explored. In addition, the effects of innate immunity on adaptive immunity in the context of vaccination remain to be elucidated.

Conclusion

Inactivated SARS-CoV-2 vaccination promoted T cell proliferation, TCR clone amplification, and TCR diversity, conferring adaptive T cell immunity to recognize a variety of antigens. The proliferation and differentiation of $CD8^+$ MAIT cells and the *KLRD1* gene expression were significantly enhanced. Furthermore, the two pathways of the nuclear-transcribed mRNA catabolic process, NMD, and translational initiation were upregulated, which enhanced early immunity and rapid anti-viral responses.

Data availability statement

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA005241) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa-human.

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committee of the Third Affiliated Hospital of Shenzhen University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XD and MP were major contributors in designing the study and writing the manuscript. RJ collected and analysed the data. WL and XZ designed the study and revised the article. 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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