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Ad5-nCoV boosted vaccine and reinfection-induced memory T/B cell responses and humoral immunity to SARS-CoV-2: based on two prospective cohorts

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ABSTRACT

Here, we regularly followed two SARS-CoV-2 infected cohorts to investigate the combined effects of neutralizing antibodies (NAbs) and B and T cell profiles during the convalescent period. Ten infected participants in December 2022 were selected to assess the effects of an inhaled adenovirus type 5 vectored COVID-19 vaccine (Ad5-nCoV) booster on B cells and humoral immunity in the first cohort. To evaluate T cell responses, eight primary and 20 reinfection participants were included in the second cohort. Blood samples from all 38 participants were collected at 1-, 2-, and 6-months post-infection. In the first cohort, eighteen monoclonal antibodies (mAbs) with neutralizing activity from memory B cells (MBC) against SARS-CoV-2 mutants were obtained by high throughput single-B-cell cloning method, which lasted from 1- month to 6- month post infection. The overall number of mAbs from MBC in the boosted immunization group was higher than that in the nonboosted immunization group at 2-, and 6-months post-infection. In the second cohort, circulating T follicular helper cells (cTfh) and AIM^+CD4^+T cells increased over time in the reinfection group ($P < 0.05$). In both cohorts, serum NAb titers showed significant immune escape, while cTfh and AIM^+CD4^+T cells in the second cohort essentially showed no immune escape to new strains (including XBB, EG.5). AIM^+CD4^+T cells against BA.5 and EG.5 were strongly negatively correlated with the time to viral clearance in the reinfected group at 6-months post-infection. We comprehensively assessed the ability of the SARS-CoV-2 boosted immunization and reinfection-induced generation of T/B cell immune memories in preventing reinfection.

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Introduction

The coronavirus disease 2019 (COVID-19) pandemic has caused an unparalleled worldwide disaster, with millions of lives lost, public health systems in shock, and economic and social devastation [1]. Inhalable vaccines based on the adenovirus type 5 vector (Ad5) were designed to mimic how SARS-CoV-2 enters the human body via the airways [2–4]. Inhaled

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Ad5 COVID-19 vaccine (Ad5-nCoV) based on a sequential vaccination strategy has been reported to be safe and capable of inducing more neutralizing antibodies (NAbs) against the prototype [5].

Several SARS-CoV-2 variants cause considerable immune escape, but a multi-dose combination vaccine produces multiple memory B cell (MBC) complexes that contain high-affinity neutralizing monoclonal antibodies against all sublineages of Omicron [6]. Neutralizing monoclonal antibodies (mAbs) against the receptor binding domain have been the most effective therapies approved by regulatory agencies (such as the US FDA) to treat SARS-CoV-2 infections [7]. One approach to generating NAbs is to clone neutralizing mAbs from single B cells in convalescent patients infected with COVID-19 [7]. Therapeutic proteins may induce anti-drug (proteins or mAbs) antibodies (ADAs) in human patients, which may alter mAb efficacy and even lead to unwanted side effects [8, 9]. Furthermore, some mAbs can neutralize multiple SARS-CoV-2 variants [10–12]. A recent study identified a mAb using a single-B cell screening platform that possessed broad-spectrum neutralization and antibody-dependent cell-mediated cytotoxic activities against SARS-CoV-2 variants, including EG.5.1 [13]. However, the B cell-mediated immune dynamics of breakthrough-infected populations with long-term follow-up in different vaccine booster statuses have not yet been studied.

Presently, increasing cases of reinfection with SARS-CoV-2 have been reported worldwide, especially with the emergence of the Omicron sublineage, which has been proven to escape the immunity of previous infections [14]. In addition, understanding the direct effects of mixed immunization is needed [15]. The SARS-CoV-2-specific T cell response is central to controlling viral infections and providing immune memory [16]. Some longitudinal studies have reported cellular immunity in SARS-CoV-2 patients, but the subjects did not have primary infections [17, 18]. Another study revealed an interaction between the temporal characteristics of SARS-CoV-2-specific T cell responses, but the T cell responses of patients with different infection statuses were not evaluated [19]. A prospective cohort study reported the titer of NAb and T cell responses after different doses of inactivated vaccine and compared the reinfection with that of non-infection; the detection index and analysis depth of the T cell response were relatively simple [20]. However, the long-term duration of T cell-mediated immunity induced by vaccines, infection superposition, and their effectiveness in preventing reinfection are largely unclear.

In this study, we conducted a six-month follow-up of patients who had received two doses of the inactivated COVID-19 vaccine and a breakthrough infection in December 2022. We used high-throughput single B cell technology to obtain multiple highly effective neutralizing mAbs and divided them into two groups according to whether they inhaled Ad5-nCoV booster immunization. Additionally, we included 28 participants who were primary infected or reinfected with SARS-CoV-2 in December 2022. We closely monitored the serum neutralization ability, circulating T follicular helper cells (cTfh), and $CD4^+$ and $CD8^+$ T cell responses. There is a continuous emergence of new vaccines, population-based vaccination and the emergence of more reinfections in the population, but the protective effect of the immune level of these objects on subsequent infection was not clear, which was worthy of our attention. The objective of this study was to compare the difference of B cell-mediated cellular immunity and humoral immunity between inhaled Ad5-nCoV booster immunized and no booster immunized SARS-CoV-2 breakthrough infection, and to compare the serum neutralization ability, Tfh, CD4 + and CD8 + T cell response between SARS-CoV-2 primary infected and reinfected participants, to reveal the effects of Ad5-nCoV boosted immunity and reinfection on the timing and persistence of immune protection.

Materials and methods

Two prospective cohorts

To assess whether heterologous booster immunization with aerosol inhalation induces robust B cell-mediated immune responses among breakthrough-infected patients, the first cohort including 10 participants in Nanjing City, Jiangsu Province, who were infected with SARS-CoV-2 for the first time in December 2022, were followed. All participants received the first two doses of the inactivated vaccine before the primary infection; six received an inhalation booster of the Ad5-nCoV; and the remaining four received no booster. In the Ad5-nCoV-booster study, the time range of breakthrough infection was from December 18, 2022, to December 25, 2022. The first batch of samples were collected on January 11, 2023, the second on February 27, 2023, and the third on June 27, 2023.

To evaluate T cell-mediated immunity with SARS-CoV-2 reinfection relative to primary infection, we included 28 participants from Changzhou City, Jiangsu Province to form the second cohort. Among them, eight cases were primary infections and 20 were reinfections with SARS-CoV-2 since December 2022. Whole blood samples were collected from all participants thrice in the first, second, and sixth months (Post 1M, 2M, and 6M) after the last infection. In the primary infection and reinfection cohorts, the subjects were enrolled from June 2023 to November 2023, and the last batch of samples were collected on

March 26, 2024. A flowchart of subject enrollment is shown in Figure 1.

Estimation of sample size

Because of the high cost of B cell-mediated cellular immunity, we only conducted the study on 10 volunteers, which did not involve the estimation of the sample size. Regarding T cell immunity in individuals fully vaccinated against Omicron series strains, the specific T cell positive rate for primary infection is 95.2%, while that for reinfection is 74.3% [18]. With 1-β = 80% and α = 5%, matched according to sex and age at a ratio of 1:2, combined with the actual situation of the COVID-19 epidemic in Jiangsu Province at that time and the feasibility of the study (especially considering the difficulty in finding primary infected cases), it was calculated using Epi Info software that 8 cases of primary infection and 20 cases of reinfection were actually included.

Sample collection

Blood samples were collected from the patients in ethylenediaminetetraacetic acid-treated tubes (BD Biosciences), and plasma and PBMCs were separated using the Ficoll gradient method (Ficoll-Paque PLUS, GE Healthcare Biosciences). Plasma samples were stored at −80°C, and PBMCs were stored in liquid nitrogen before use.

Sorting of spike-specific memory B cells

B cells were magnetically purified (STEMCELL Technologies, 17954) and stained with anti-CD27-APC (BioLegend, 356410), anti-human IgM (BioLegend, 314512), anti-human IgG (BioLegend, 410708), and biotinylated spike proteins. Spike-specific memory B cells were isolated from pooled PBMCs by flow cytometry using a BD fluorescence-activated cell sorter (FACS) Aria III flow cytometer (BD Biosciences). Flow cytometry data were analyzed using FlowJo. The gating strategy for B cells is shown in Supplementary Figure S1.

Single-B cell RT–PCR and high-throughput cloning and pseudovirus neutralization

Single B cells' cDNA was prepared via RT–PCR using Sc Reverse Transcriptase (Vazyme, N721) primed with oligo (dT). The antibody expression cassettes were amplified via two rounds of PCR. Variable region genes were amplified via pre-PCR from single B cells' cDNA using gene-specific primers at both the 5' and 3' ends. Variable region genes, a human cytomegalovirus (HCMV) promoter fragment, and an antibody constant region were combined and amplified to produce linear products using overlapping PCR. Purified overlapping PCR products (two separate linear PCR products) were co-transfected into ExpiCHO cells (Life Technologies) grown in 96-deep well plates (2 mL per well). mAbs were produced

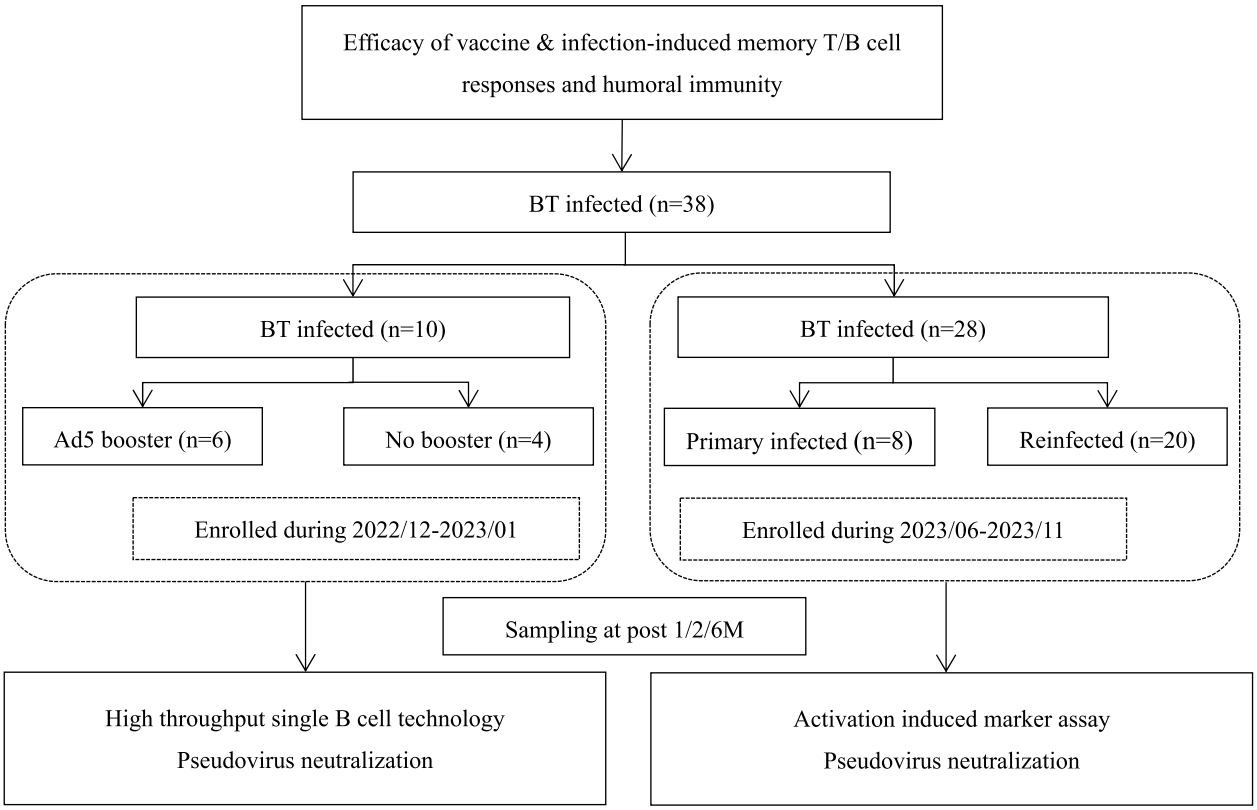

Figure 1. Flow chart of study enrollment. Note: BT denotes breakthrough.

by transient transfection of ExpiCHO cells. Antigen-specific ELISA and HTRF ACE2-RBD assays were used to detect the binding capacity and blocking activity of NAbs in culture supernatants transfected with SARS-CoV-2 RBD [21].

The lentivirus-based SARS-CoV-2 pseudoviruses were provided by Vazyme Biotech Co., Ltd. (Nanjing, China), which bear the spike protein derived from the wild-type (WT) variant and the Omicron variant (WT, BA.4/5, BF.7, XBB.1.5, BQ.1.1, CH.1.1, EG.5, JN.1). The details of pseudovirus neutralization are shown in Supplementary Material S2.

Antigen-specific T cell measurement

Activation-induced marker (AIM) assay was performed to measure antigen-specific circulating $CD4^+$ T cells, $CD8^+$ T cells, and cTfh cells. AIM assay was recently developed as a cytokine-independent method capable of detecting early antigen-specific $CD4^+$ T cells, $CD8^+$ T cells, and cTfh cells. Details of the AIM assay are provided in Supplementary Material S3 (FigureS2–S3). Staining samples were analyzed using a FACS Aria™III Cell Sorter instrument (BD Biosciences) using FlowJo software (version 10).

Statistical analysis

Binding antibody or neutralization titers are expressed as geometric mean titers. The Wilcoxon matched-pairs signed-rank test was used for comparisons between the time points and SARS-CoV-2 variants. The unpaired Wilcoxon test was used for comparisons between groups. Statistical significance was set at $P < 0.05$. SPSS version 22.0 (Chicago, IL, USA) and Prism9 were used for the data analysis.

Results

A total of 38 subjects were enrolled in this study, ranging in age from 28 to 57 years. They were vaccinated with two doses of the SARS-CoV-2 inactivated vaccine before entering the group. The frequency of each symptom is shown in Table 1.

MBC-derived monoclonal antibody

As shown in Figure 2A, the number of mAbs against the WT showed a clear downward trend at the three sampling points; the number of mAbs against BA.4/5 and XBB.1.5 increased in the sixth month. The detailed numbers of mAbs generated by each individual during the three sampling periods are listed in Supplementary Table S1. The neutralization activity of cases 1 and 2 against the WT was less than the sum of the other four strains, and cases 3, 6, 7, and 8 had no neutralization activity against the XBB.1.5, EG.5.1, and CH.1.1 strains (Figure 2B). The number of mAbs neutralizing WT and BA.4/5 was the lowest in case 7. Among these, 18 showed neutralizing activity against the Omicron strain used during the same period. Only Case 5 produced mAbs against all strains in each of the three sampling assays.

Next, we analyzed whether the inhalation of Ad5-nCoV-enhanced immunization affected the number of MBC-derived mAbs. As shown in Figure 2C–E, the overall number of MBC-derived mAbs secreted by those who underwent Ad5-nCoV-boosted immunization at Post 1M was not significantly different from that of the non-boosted group ($P > 0.05$). However, at Post 2M and Post 6M, the number was higher in the boosted group ($P < 0.05$). In addition, we analyzed the changes in the two groups at each of the three time points, and the results are shown in Figure 2F–G. The overall number of MBC-derived mAbs secreted in the inhaled Ad5-nCoV-boosted immunization group decreased from Post 2M to Post 6M post-infection ($P < 0.05$), and the same trend was observed in the non-boosted immunization group ($P > 0.05$).

SARS-CoV-2-specific circulating T follicular helper cell responses

The Tfh cell response is required for the formation and maintenance of the germinal center (GC) response, which is essential for the development of a durable high-affinity antibody response [22]. Here, vaccine-induced SARS-CoV-2 RBD-specific cTfh was tracked and characterized over time in a cohort of 28 primary infections and reinfections (Figure 3A–C). Except for cTfh against BA.5 in Post 1M, which was statistically different between primary infection and reinfection, there was no statistically significant difference between all strains or between primary and reinfection. Furthermore, we compared the cTfh of the same strain at different sampling times in the groups, as shown in Figure 3D–G. In the primary infection group, cTfh against WT, BA.1, BA.2, and BA.5 did not show statistically significant differences at the three sampling time points. However, in the reinfection group, cTfh against WT, BA.1, and BA.2 showed a statistically significant difference between Post 1M and Post 3M after reinfection (Figure 3G).

To extend these findings, the phenotypic characterization of SARS-CoV-2 RBD-specific cTfh cells was investigated using CXCR 3 and CCR 6 chemokine receptor markers (Figure 4A–B). As shown in the figure, compared to Post 1M infection, the percentage of cTfh 1–17 increased significantly against each strain Post 2M infection, whereas the percentage of cTfh 2 decreased significantly. By the Post 6M infection, the percentage of cTfh 2 was significantly higher and dominant among the four subtypes, followed by a

Table 1. Demographic characteristics of the participants.

	B-cell-mediated immunological study		T-cell-mediated immunological study	
	Primary infection with Ad5 Booster n = 6	Primary infection with No Booster n = 4	Primary Infection n = 8	Reinfection n = 20
Age [Year (IQR)]	40(31.75–51.75)	40.5(34.75–41)	41.5 (30.5–45)	41.0 (32.75–45)
Gender				
Male	4	2	3	9
Female	2	2	5	11
Symptoms (n/N)				
Fever	6/6	4/4	7/8	13/20
Sore throat or cough	6/6	3/4	1/8	8/20
Stuffy or runny nose	2/6	3/4	2/8	2/20
Dizziness or headache	3/6	3/4	1/8	8/20
Muscle pain	3/6	3/4	0/8	2/20
Smell or Taste disorder	1/6	1/4	0/8	0/20
Fatigue	3/6	3/4	2/8	2/20
Diarrhea	2/6	0/4	0/8	0/20
Nausea	1/6	1/4	0/8	1/20

larger percentage of cTfh 17 and the smallest percentages of cTfh 1 and cTfh 1–17. A similar trend was observed in the reinfection group (Figure 4B). In both groups, the percentage of cTfh 17 was substantial at all three time points, especially in the reinfection group, where it was not less than 30% at any time point and for any strain.

SARS-CoV-2-specific CD4 + and CD8+ T cell responses to different SARS-CoV-2 variants

The percentages of AIM^+CD4^+ T cells against the different strains in the primary infection and reinfection groups were compared. The results showed (Figure 5A–C) that the proportion of AIM^+CD4^+ T cells against BA.5 in the first month after infection was higher in the primary infection group than in the reinfection group ($P < 0.05$), whereas the proportion of AIM^+CD4^+ T cells among the different strains in the Post 2M and 6M infection was not statistically different between the primary and reinfection groups ($P > 0.05$). In addition, we compared the proportion of AIM^+CD4^+ T cells in the primary infection and reinfection groups for the same strain at different sampling times. As shown in Figure 5D–G, the proportion of AIM^+CD4^+ T cells against WT, BA.1, BA.2, and BA.5 in the primary infection group did not change significantly over time ($P > 0.05$).

The distribution of AIM^+CD8^+ T cells (Supplementary Figure S4A–C) that there was no statistically significant difference ($P > 0.05$) between the Post 1M and Post 2M infection groups or between the strains. In contrast, after months of Post 6M infection, AIM^+CD8^+ T cells against WT, BA.2, and XBB were higher ($P < 0.05$) in the primary infection group than in the reinfection group. As shown in Supplementary Figure S4D–G, the proportion of AIM^+CD8^+ T cells showed a statistical difference between Post 2M and Post 6M infection only in the reinfection group targeting BA.1 ($P < 0.01$). However, the proportion of AIM^+CD8^+ T cells targeting other strains did not change over time between the primary infection and reinfection groups ($P > 0.05$).

Net immunosuppressive status was assessed by evaluating the CD4/CD8 T cell ratio; patients with a ratio ≤ 1 were immunosuppressed. The distribution of AIM^+ CD4/CD8 ratios is shown in Supplementary Figure S5A–G, and the results showed no statistical significance between the primary infection group and the reinfection group or for the same strain at different times ($P > 0.05$).

Correlation of $CD4^+$ and $CD8^+$ T cells with the timing of viral clearance

Prior vaccination is associated with accelerated viral clearance from days 4 and 6, despite similar peak viral loads between vaccinated and unvaccinated individuals [23, 24]. To further assess the potential role of T cells in viral clearance, we performed exploratory analyses to see if T cell kinetics correlated with the timing of viral clearance in individuals in the primary infection versus reinfection groups, and the results are presented in Table 2, which shows only statistically significant results. In the Post 1M infection, the proportions of AIM^+CD4^+ and AIM^+CD8^+ T cells targeting WT and BA.1 showed a strong positive correlation with the time to viral clearance in the primary infection group. AIM^+CD4^+ T cells targeting BA.2 in the reinfection group after Post 2M infection showed a strong negative correlation with the time to viral clearance. AIM^+CD4^+ T cells against BA.5 and EG.5 were strongly negatively correlated with the time to viral clearance in the reinfected group after months of 6M infection.

Serum pseudovirus neutralization

Serum samples from the first two months were tested for pseudovirus neutralization using WT, BA.4/5, BF.7, BQ.1.1, and XBB.1.5. In contrast, WT, BA.4/5, XBB.1.5, CH.1.1, and EG.5.1, which were prevalent

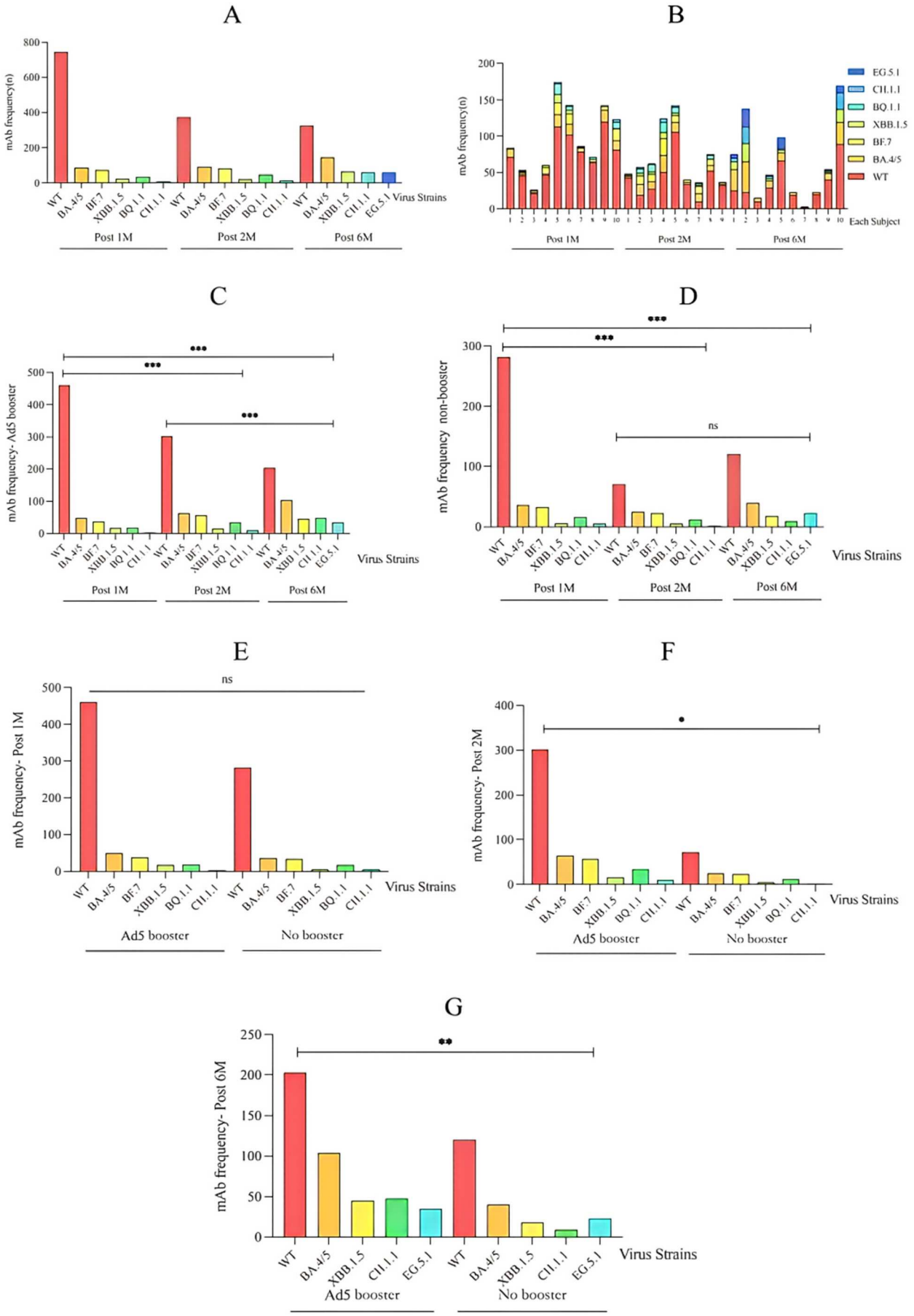

Figure 2. Analysis of monoclonal neutralizing antibodies from memory B cells in the study subjects with 6 months post-infection. A Analysis of the ability of monoclonal antibodies from MBC to neutralize each variant was investigated three times overall at 1, 2, and 6 months post-infection during the high-intensity epidemic. B Analysis of the ability of monoclonal antibodies from MBC to neutralize each variant was investigated three times in each individual at 1, 2, and 6 months post-infection in infected individuals during the high-intensity epidemic. C-D Analysis of changes over time in the Ad5-nCoV-booster group versus the non-booster group. E-G Ad5-nCoV-booster immunization neutralized titers of MBC-derived mAbs against each variant at 1, 2, and 6 months post-infection, respectively.

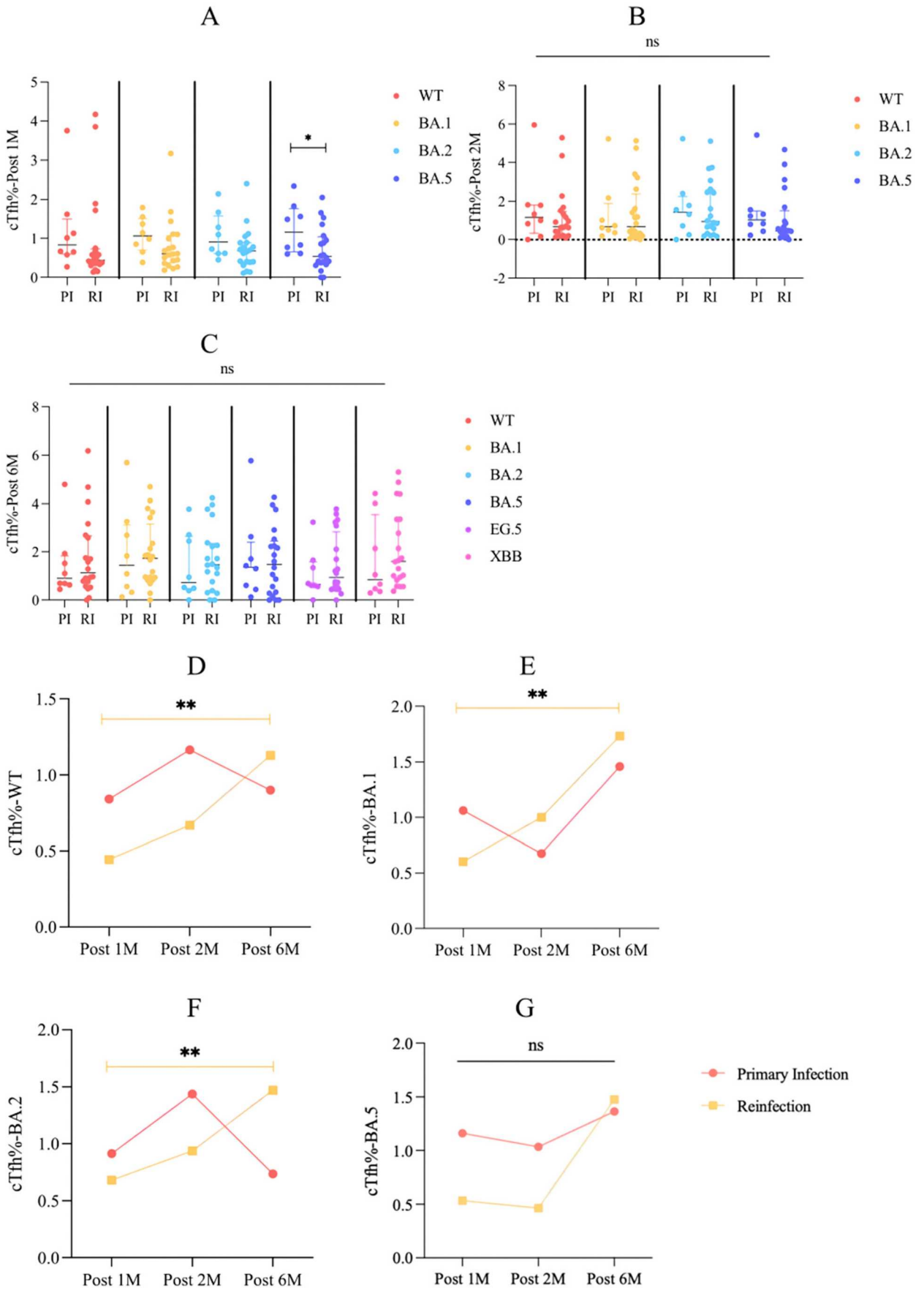

Figure 3. Specific cTfh response in the cohort of primary infection and reinfection. A-C: The cTfh frequencies of WT, BA.1, BA.2, BA.5 EG.5 and XBB in the first, second and sixth month after primary infection and reinfection. D-G: Trends over time in cTfh frequency against WT, BA.1, BA.2, and BA.5 in primary infected versus reinfected individuals. Note: PI denotes primary infection, RI denotes reinfection. * denotes $P < 0.05$, ** denotes $P < 0.01$, and *** indicates $P < 0.001$, and ns indicates no statistical difference.

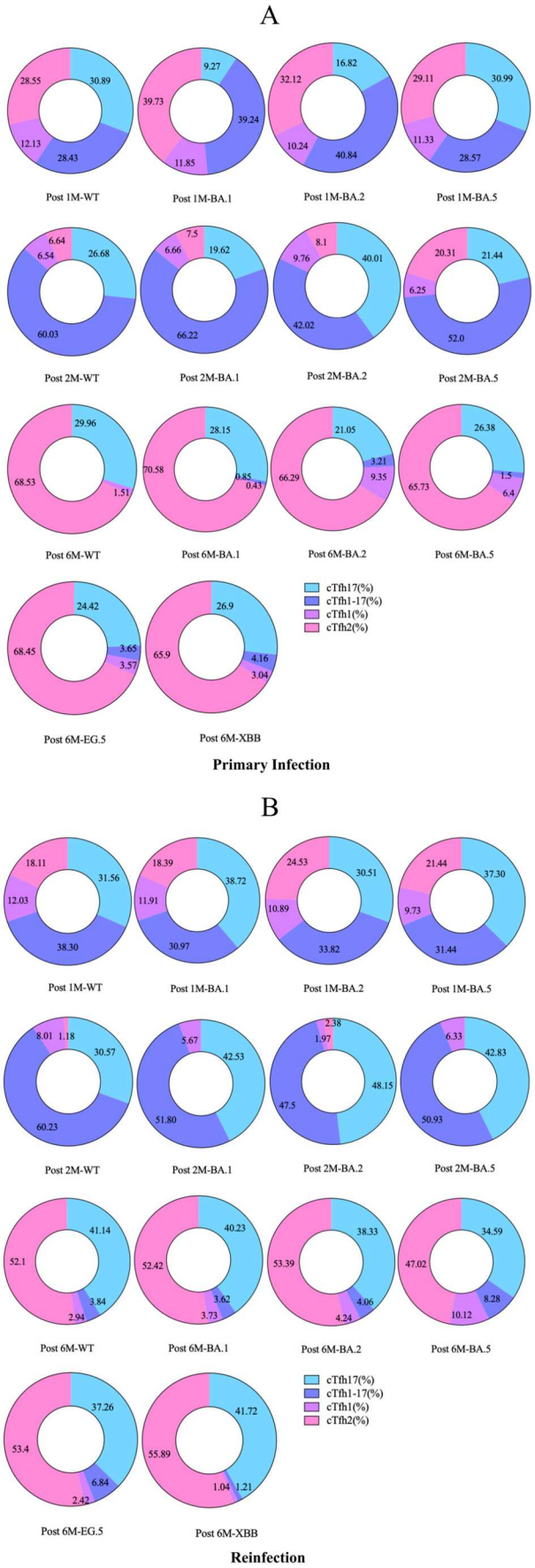

Figure 4. Dynamics of cTfh subpopulations. A dynamics of cTfh 1, cTfh 2, cTfh 17, and cTfh 1 −17, at months 1, 2, and 6 post infection in primary infection group. B dynamics of cTfh 1, cTfh 2, cTfh 17, and cTfh 1 −17, at months 1, 2, and 6 post infection in reinfection group.

during the sampling period, were used for neutralization in the Post 6M infection. In the Ad5-nCoV booster group, NAb titers against WT at Post 1M were statistically different from the remaining four strains (Figure 6A) ($P < 0.05$). The titers of NAb against WT were significantly higher than those against BQ.1.1 and XBB.1.5 in the Post 2M, and the titers of NAb against WT and BA.4/5 were significantly higher than those against the other strains at post 6M ($P < 0.001$). The results in the non-booster group showed that the NAb titers of WT mice were significantly different from those of BQ.1.1 and XBB.1.5 at Post 1M and 2M, and the NAb titers of WT mice were significantly different from those of XBB.1.5, CH.1.1, and EG.1.5 at Post 6M (Figure 6B, $P < 0.05$). In addition, the comparison of NAb titers of the same strain at different times is also demonstrated in Supplementary Figure S6A–E. No significant difference in the results of NAbs at Post 1M, 2M, and 6M against all the strains between the Ad5-nCoV booster group and the non-booster group was observed (Supplementary Figure S6).

To reveal whether reinfection affected the serum neutralization titers of primary-infected versus reinfected individuals, we performed a pseudovirus neutralization assay using WT, XBB.1.5, XBB.1.22, EG.5.1, and JN.1. As shown in Figure 6C, the NAb titers of the reinfection group targeting XBB.1.22 and EG.5.1 were significantly higher than those of the primary infection group in the Post 1M infection ($P < 0.05$); the NAb levels of the primary infection group targeting WT were significantly higher than those of the primary infection group targeting XBB.1.5, XBB.1.22, EG.5.1, and JN.1 ($P < 0.001$), and those of the reinfection group targeting WT were significantly higher than those of EG.5.1 and JN.1 ($P < 0.001$). As shown in Figure 6D, NAb levels against WT in the primary infection group after Post 2M infection were consistent with those in Figure 6C. The Post 6M infection showed that the NAb of both the primary infection and reinfection groups against WT was significantly higher than that against XBB.1.22, EG.5.1, and JN.1 (Figure 6E, $P < 0.05$).

The changes in the same strain at different times were visualized, as shown in Supplementary FigureS7A-E, and a significant decreasing trend of NAb against WT, XBB.1.5, XBB.1.22, EG.5.1, and JN.1 was observed in the reinfection group from Post 2M to 6M infection ($P < 0.05$). However, there were statistical differences in NAb levels in the primary infection group against XBB.1.5, XBB.1.22, and EG.5.1 from the Post 1M to 2M infection ($P < 0.05$).

Discussion

Basic demographic information and blood samples were collected from the 38 infected participants after

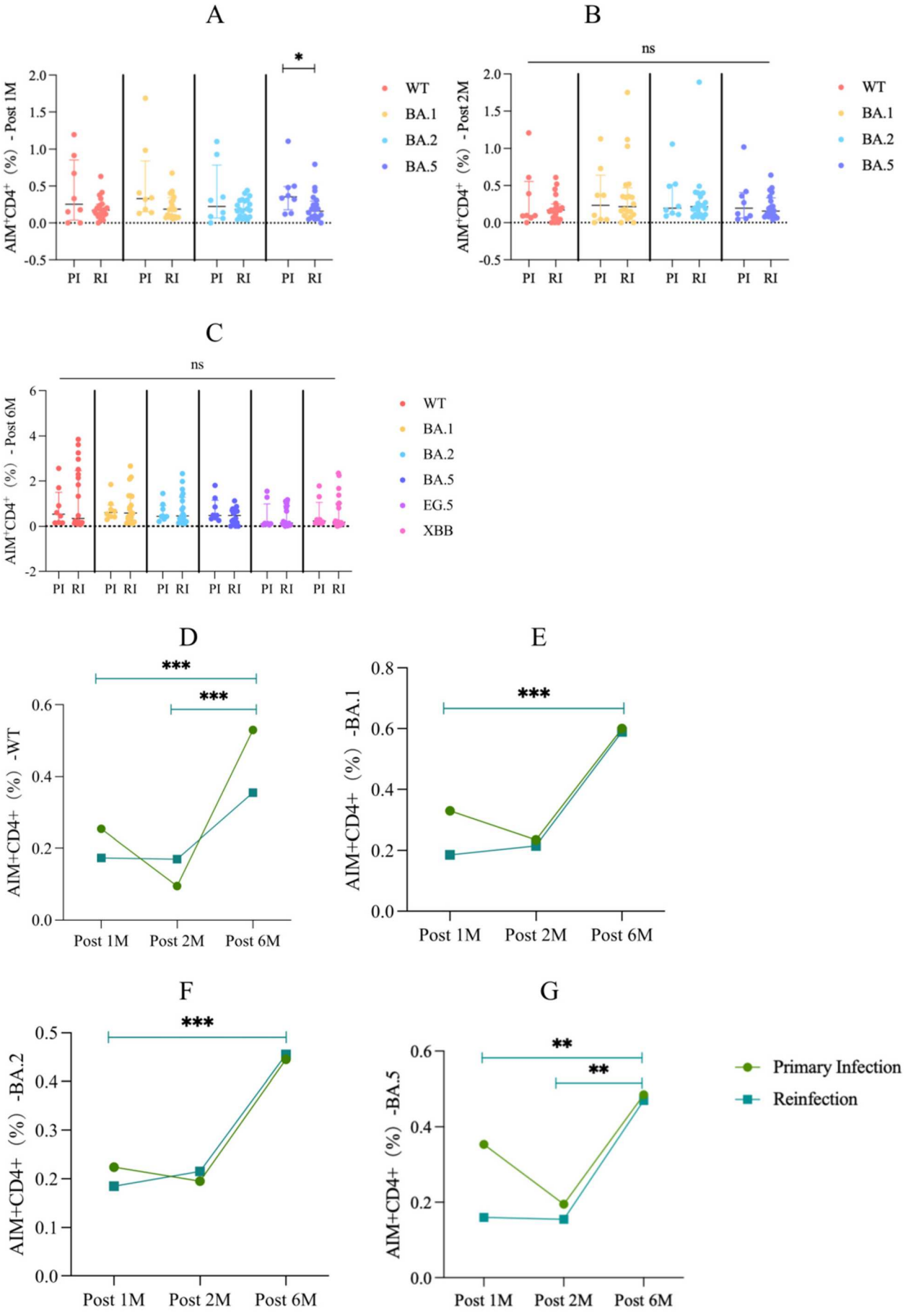

Figure 5. AIM$^+$CD4$^+$, AIM$^+$CD8$^+$T cell response in the primary infection versus reinfection cohort. A-C, H-J, O-Q Proportion of AIM$^+$CD4$^+$, AIM$^+$CD8$^+$T cells and AIM$^+$CD4/CD8 in primary-infected versus reinfected individuals at months 1, 2, and 6 post-infection against WT, BA.1, BA.2, BA.5, EG.5, and XBB. D-G, K-N, R-U Trends in the proportion of AIM$^+$CD4$^+$, AIM$^+$CD8$^+$T cells and AIM$^+$CD4/CD8 in primary-infected versus reinfected individuals against WT, BA.1, BA.2, BA.5 over time. Note: PI denotes primary infection, RI denotes reinfection. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, and ns indicates no statistical difference.

Table 2. Correlation between each T cell and the time to viral clearance in the primary infection and reinfection groups.

	Post 1M			
	Primary infection		Reinfection	
Indicators that correlate with viral clearance time	Correlation coefficient r	*P* value	Correlation coefficient r	*P* value
WT-AIM $^+$CD8	0.875	0.023	–	–
WT-AIM $^+$CD4	0.905	0.013	–	–
BA.1-AIM $^+$CD8	0.935	0.006	–	–
BA.1-AIM $^+$CD4	0.970	0.001	–	–
BA.2-AIM $^+$CD4	0.843	0.035	–	–
BA.5-AIM $^+$CD4	0.833	0.040	–	–
	Post 2M			
BA.2-AIM $^+$CD4	–	–	−0.860	0.006
	Post 6M			
BA.5-AIM $^+$CD4	–	–	−0.790	0.020
EG.5-AIM $^+$CD4	–	–	−0.756	0.030

December 2022. Ten of them underwent isolation of PBMC, followed by isolation of MBC by high-throughput single B cell technology to obtain highly specific mAbs, and 944, 678, and 645 mAbs with neutralizing activity were obtained after Post 1M, 2M, and 6M infection, respectively. Eighteen mAbs showed neutralizing activity against all Omicron strains used during the same period and were the best means of antibody therapy. This simple and efficient method may be useful in developing human therapeutic antibodies for other diseases and next pandemic.

Immune memory develops in B cells after infection. It has two main components: (1) long-lived plasma cells that produce antibodies to protect against homologous challenges and (2) MBCs that are activated after re-exposure and rapidly generate an antibody response against homologous or heterologous challenges [25]. The results of the MBC-derived neutralizing mAb showed that the number of neutralizing mAbs against the WT was overwhelmingly dominant at all three sampling times and decreased over time, most likely because the first two doses of the vaccine for all subjects were against the WT, and six of them underwent Ad5-nCoV booster immunization. In the Post 6M infection, the number of neutralizing mAbs against VOC (BA.4/5, XBB.1.5, CH.1.1, EG.5.1) accounted for more than in the first two months, with a significant increase in the number of mAbs against XBB.1.5 vs. CH.1.1. Our study showed that the total number of neutralized mAb was higher in Ad5-nCoV booster immunizers than in none boosted immunizers at Post 2, and 6M infection, and that the number of neutralized mAb declined over time in both Ad5-nCoV-boosted and none boosted immunizers. The follow-up period of this study was six months, and due to the uncontrollable disseminated infections of the COVID-19 variant, two subjects (one in each group) had reinfections on June 3, 2023, and May 21, 2023 (before the third sampling). In order to avoid bias, we excluded two reinfected subjects and compared the number of mAbs between the two groups, and the results remained the same (the total number of neutralized mAb was higher in Ad5-nCoV booster immunizers than in none boosted immunizers at Post 6M infection, $P < 0.05$). Overall, inhaled Ad5-nCoV booster immunization enhanced the neutralizing activity of MBC-derived mAbs.

Previous studies have described how the cTfh response correlates with humoral immunity during viral infection and vaccination [26] and that S-specific cTfh cells in the breakthrough infection cohort remain at a low frequency [27]. In the present study, cTfh levels were largely preserved in the first infection group and did not change significantly over time. However, cTfh in the reinfection group increased over time when targeting WT, BA.1, and BA.2, and cTfh targeting BA.5 did not show this trend in the reinfection group. cTfh3-subtypes (cTfh2 vs. cTfh17) accounted for an absolute predominance of cTfh cells in the Post 1M and Post 6M infection in both the primary infection and reinfection groups, and these subpopulations contributed to the spiking specificity of cTfh maintenance and the recall response of high-affinity antibodies [28].

In addition to antibodies and memory B cells, T cells play a protective role upon re-exposure to the virus. $CD4^+$ and $CD8^+$ T cell memory may be important mediators of vaccine immunity [29, 30]. Although the unvaccinated and vaccinated groups showed similar peak viral loads after infection, vaccinated individuals demonstrated accelerated viral clearance in the upper respiratory tract beginning 4–6 days after symptom onset [31, 32]. T cell protection is reflected by the fact that higher levels of $CD8^+$ T cell activation in peripheral blood correlate with lower peak viral loads and faster rates of viral clearance in the upper respiratory tract [33]. Our results showed that Spike-specific CD4+ T cell and CD8+ T cell activation positively correlates with the rate of viral decay in primary infection group, but CD4 T cell inversely correlates with the rate of viral decay in reinfection group. It might suggest that as the number of infections increases, specific T cells play a progressively greater role in viral clearance. However, it was not fully explored and would benefit from further validation in larger cohorts. Importantly, associations based on viral load and T cell immunity do not simply reflect the temporal relationship between activated immune responses and declining viruses. Because T cell responses are less affected by VOC than humoral immune responses [34], the cross-reactivity of $CD4^+$ T cell responses from different SARS-CoV-2 variants in our cohort was examined. Our results suggest that SARS-CoV-2-specific $CD4^+$ T cells are largely preserved in the reinfected individuals. As for SARS-CoV-2-specific $CD8^+$ T cells, they were higher in the primary infected group from the WT, BA.2, and XBB variants than in the reinfected group at the Post 6M infection. Furthermore, the effect of

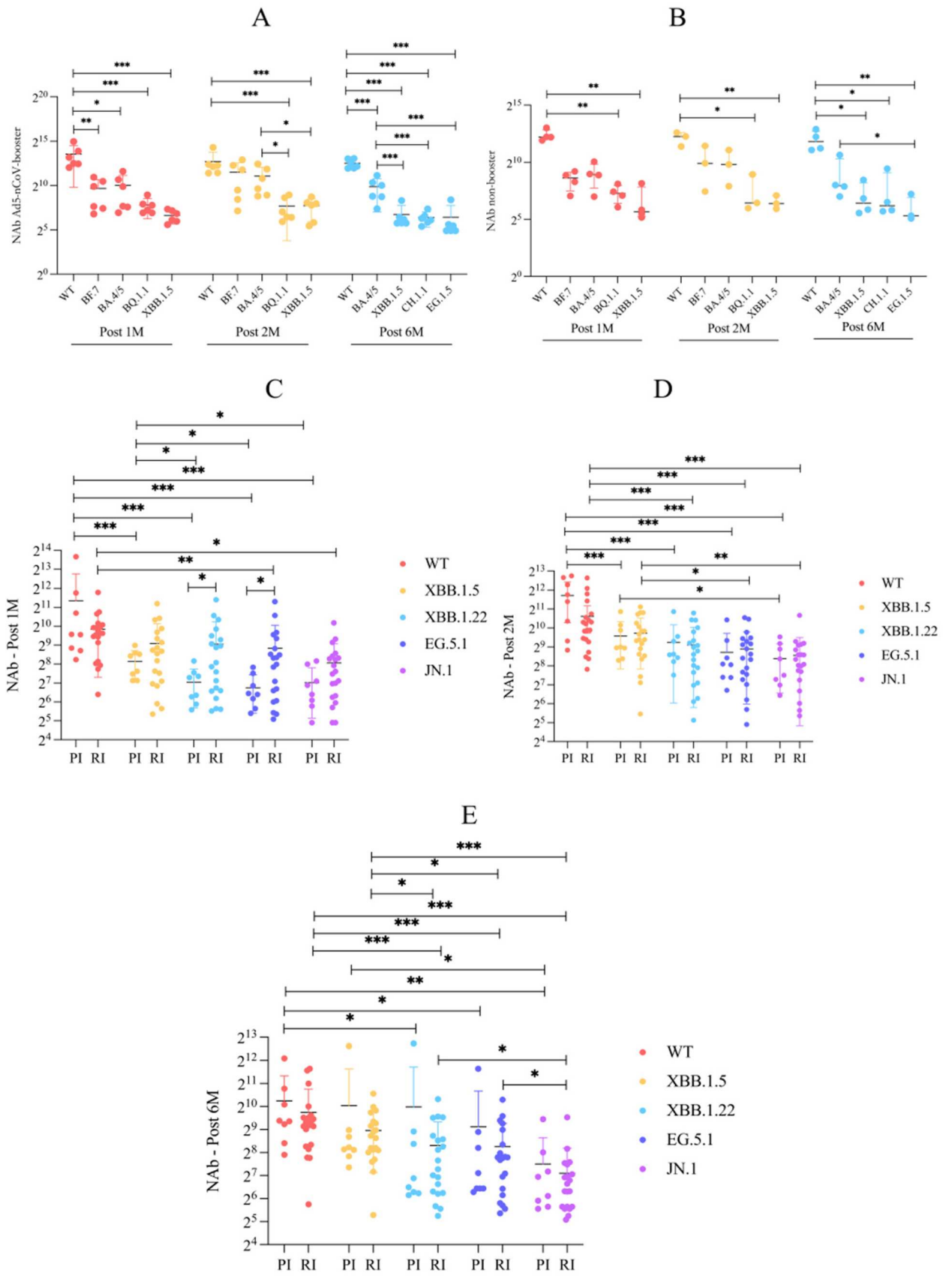

Figure 6. Neutralization assays for pseudoviruses in serum samples from the 1, 2, and 6 months after infection. A Inhaled Ad5-nCoV-booster group tested for pseudovirus neutralization in serum samples at months 1, 2, and 6 post infection; B Non-booster group tested for pseudovirus neutralization in serum samples at months 1, 2, and 6 postinfection; C Pseudovirus neutralization testing of serum samples at 1month post-infection in the primary versus reinfection groups; D Pseudovirus neutralization testing of serum samples at month 2 post-infection in the primary versus reinfection groups; E Pseudovirus neutralization testing of serum samples at month 6 post-infection in the primary versus reinfection groups; * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

the mutation of variant strains on the overall $CD4^+$ and $CD8^+$ T cell responses was negligible because of the highly conserved $CD4^+$ and $CD8^+$ T cell epitopes in the viral mutant strains [35], a finding that is generally consistent with our study. The study of the CD4/CD8 ratio as a quantitative trait is important for patient care because it can be used as a prognostic risk factor [36, 37]. Abnormal CD4/CD8 ratios are usually regarded as clinically relevant [38], and it is rare to see measurements of less than 1.0 or more than 2.5%. Imbalances that are either too high or too low always provide information about immune malfunction.

Several studies have demonstrated the safety and immunogenicity of Ad5 booster immunization; that is, inhalation of nebulized Ad5-nCoV induces greater NAbs and robust mucosal immunity [39–42]. However, in our study, there was no statistically significant difference in NAb titers against all strains between the heterologous booster Ad5-nCoV vaccine and the non-booster group. However, due to the small sample size of our study, this conclusion should be generalized with caution. In addition, Jiangsu Province, China was mainly based on inactivated vaccine basic immunization, so there was a lack of control groups that received other types of vaccines, which limited to provide a more comprehensive comparison of immune responses across different vaccination strategies. NAb titers were higher in the reinfected group than in the primary infected group only in the first month after infection against the XBB.1.22 and EG.5.1 groups, which shows that reinfection may affect humoral immunity only in the early stages of recovery.

Our study had some limitations. First, the sample size of our two cohorts were relatively small, particularly for the B cell immunity cohort, and expansion was hampered by the high cost of high-throughput single B cell technology and difficulty in enrolling primary infected individuals, which may limit the generalizability of the findings. Second, the analysis of SARS-CoV-2-specific T cells was limited to a few markers (CD3, CD4, CD8, OX40, CD45, CD69) and selected SARS-CoV-2 proteins. Third, the study focuses primarily on immune responses to specific SARS-CoV-2 variants, potentially limiting the applicability of the results to other circulating or future variants. In the future, more comprehensive results can be obtained by carrying out JN.1, XDV for suitable populations, as well as the response ability of memory T/B cells of the upcoming SARS-CoV-2 epidemic strains.

Overall, the convalescent patients showed weak cross-NAb responses to emerging Omicron strains (BA. 4/5, BF.7, BQ.1.1, CH.1.1, XBB.1.5, and EG.5.1) at 6 months post-infection. The cTfh and AIM^+CD4^+ T cells in the primary infection group were essentially immune escape-free and did not change significantly during the follow-up period. Multiple infections may impair the specific immunity of infected individuals. Serum NAb titers in both primary infection and reinfection groups showed significant immune escape during the follow-up period. These findings not only provide important clues to better understand the immunological characteristics of COVID-19, but also guide the development and application of SARS-CoV-2 vaccines. Booster immunization with inhaled Ad5-nCoV can increase the number of MBC derived mAbs, so Ad5-nCoV-booster immunization is worth popularizing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Competing interests

The authors declare that they have no competing interests.

Ethics statement

The Institutional Review Board of Jiangsu Provincial Center for Disease Control and Prevention reviewed and approved the studies involving human participants. Written informed consent to participate in this study was provided by the participant's legal guardian/next of kin.

Author contribution

Aidibai Simayi: Research design, formal analysis, data curation, laboratory detection, statistical analysis, plotting, writing original draft preparation, writing review, and editing. Yuanfang Qin, Qian Zhen, Yong Liu, Jinjin Chu, Huiyan Yu: Field survey and Laboratory detection. Shihan Zhang: Data Curation. Fengcai Zhu, Changjun Bao, Yuxin Chen: Supervision. Hui Jin: Investigation and supervision. Liguo Zhu: Research design, supervision, original draft preparation, writing review, and editing.

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