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PATHOGENESIS AND IMMUNITY

Persistent Maintenance of Intermediate Memory B Cells Following SARS-CoV-2 Infection and Vaccination Recall Response

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ABSTRACT Robust population-wide immunity will help to curb the SARS-CoV-2 pandemics. To maintain the immunity at protective levels, the quality and persistence of the immune response elicited by infection or vaccination must be determined. We analyzed the dynamics of B cell response during 12 months following SARS-CoV-2 infection on an individual level. In contrast to antibodies, memory B cells specific for the spike (S) protein persisted at high levels throughout the period. These cells efficiently secreted neutralizing antibodies and correlated with IFN-γ-secreting $CD4^+$ T cells. Interestingly, the $CD27^-CD21^+$ intermediate memory B cell phenotype was associated with high B cell receptor avidity and the production of neutralizing antibodies. Vaccination of previously infected individuals triggered a recall response enhancing neutralizing antibody and memory B cell levels. Collectively, our findings provide a detailed insight into the longevity of SARS-CoV-2-infection-induced B cell immunity and highlight the importance of vaccination among previously infected.

IMPORTANCE To efficiently maintain immunity against SARS-CoV-2 infection, we must first determine the durability of the immune response following infection or vaccination. Here, we demonstrated that, unlike antibodies, virus-specific memory B cells persist at high levels for at least 12 months postinfection and successfully respond to a secondary antigen challenge. Furthermore, we demonstrated that vaccination of previously infected individuals significantly boosters B cell immunity.

KEYWORDS SARS-CoV-2, immunity, COVID-19, memory B cells, spike, $CD4^+$ T cell, antibody, neutralization, recovered, vaccination, longevity, longitudinal, kinetics, immunization

Both immune responses after severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) recovery and the global vaccination campaigns will help to curb the SARS-CoV-2 pandemic. However, with the continuous emergence of new SARS-CoV-2 variants partially evading immunity, and immune response waning over time, the overall reduction of worldwide SARS-CoV-2 transmissions, seems unlikely in the near future. The key to coexistence with SARS-CoV-2 in the presence of infections and the absence of high mortality is to establish and maintain robust immunity. To achieve this, we need to first determine the longevity and quality of protection offered by the previous infection and/or vaccination and investigate the immune mechanisms associated with protection from severe disease.

Upon the establishment of viral infection, the human body responds with the production of virus-specific antibodies, B cells, and T cells that limit viral replication and help to clear the virus. Recent reports suggest that SARS-CoV-2 infection elicits a robust

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immune response with neutralizing antibodies being the best-defined correlate of protection (1, 2). However, the persistence of SARS-CoV-2-specific antibodies was found to be relatively short-lived (3, 4). Virus-specific antibodies can be detected in the blood of infected individuals 7 to 15 days after the first positive PCR test or onset of the symptoms and reach their peak 3 to 7 weeks following infection (5, 6). The peak levels of these antibodies are highly variable among individuals and correlate with the severity of the disease (4, 5). After the buildup-phase antibodies begin to decay in a biphasic manner with an initial half-life of 2 to 3 months followed by a slower decline (3, 4). Nevertheless, it has been shown, that a previous infection decreases the risk of subsequent infection by 80 to 93% for a minimum of 6 to 9 months (7, 8). In addition, studies have suggested that protection from severe disease may be long-lived (9, 10).

There is increasing evidence, that virus-specific memory B and T cells play an important role in SARS-CoV-2 immunity (11, 12). However, their protective value is less clearly defined at this time. It has been demonstrated that T cell frequencies peak during the first month postinfection and then gradually decline with the average half-life of 5 months for $CD4^+$ T cells and 13 months for the $CD8^+$ T cells (3, 4, 13). The most persistent component of the adaptive immune response during the 8 months postinfection was found to be memory B cells whose frequencies keep increasing over the first months and afterward remain relatively stable (3, 4, 13).

Similar dynamics of the adaptive immune response were observed after vaccination. Neutralizing antibodies induced by vaccination decay with an average half-life of 4 months while memory T cells remain relatively stable for up to 7 months (14–16). Memory B cell frequencies increase between months 3 and 6 postvaccination (16). Furthermore, vaccination of previously infected individuals with one dose of mRNA vaccine substantially boosts immunity against SARS-CoV-2 infection, and according to some studies, even provides higher protection from infection than full vaccination of uninfected individuals (17, 18). Interestingly, the durability of both vaccine- and infection-induced immunity was shown to be dependent on age with lower rates of seroconversion and accelerated waning of immune components among the elderly (19, 20).

Taken together, these findings demonstrated superior durability of cellular immunological memory compared to antibodies and point out memory B cells as particularly interesting in terms of long-term protection from SARS-CoV-2 infection. To shed light on the generation and persistence of B cell immunity after SARS-CoV-2 infection, we longitudinally assessed neutralizing antibody levels, frequency of memory B cells, and associated $CD4^+$ T cells responses in a cohort of 43 individuals who recovered from asymptomatic to moderate disease. The immune responses were monitored during a period of 12 months starting 0 to 5 weeks postinfection on an individual level. Furthermore, we evaluated the effect of vaccination on B cell immunity in 13 previously infected individuals.

RESULTS

Magnitude but not quality of humoral response to SARS-CoV-2-infection declines over time. We first assessed the long-term kinetics and functional properties of SARS-CoV-2-specific B cell memory in 43 SARS-CoV-2-recovered individuals from a cross-sectional cohort study (21). All had either tested positive by RT-PCR or had IgG against SARS-CoV-2 spike (S) protein indicating the previous infection. Most individuals likely became infected during the outbreak of coronavirus disease 2019 (COVID-19) in Germany end of February 2020. The initial sampling time point was at the beginning of April 2020 (21). Follow-up samples were taken at 6, 9, and 12 months after the first time point. All individuals had asymptomatic to moderate disease. For the time points at 0, 6, and 9 months, we were able to collect samples from all 43 participants. At month 12, we followed up with 38 participants, 13 of which were vaccinated against COVID-19 between months 9 and 12.

To assess the persistence of humoral response after SARS-CoV-2-infection we first measured plasma levels of S-specific antibodies by ELISA. We found that IgG antibody levels were waning rapidly during the first 6 months (2.8-fold decrease, $P < 0.0001$) and continued to decline until month 9 of monitoring (1.3-fold decrease, $P < 0.0001$).

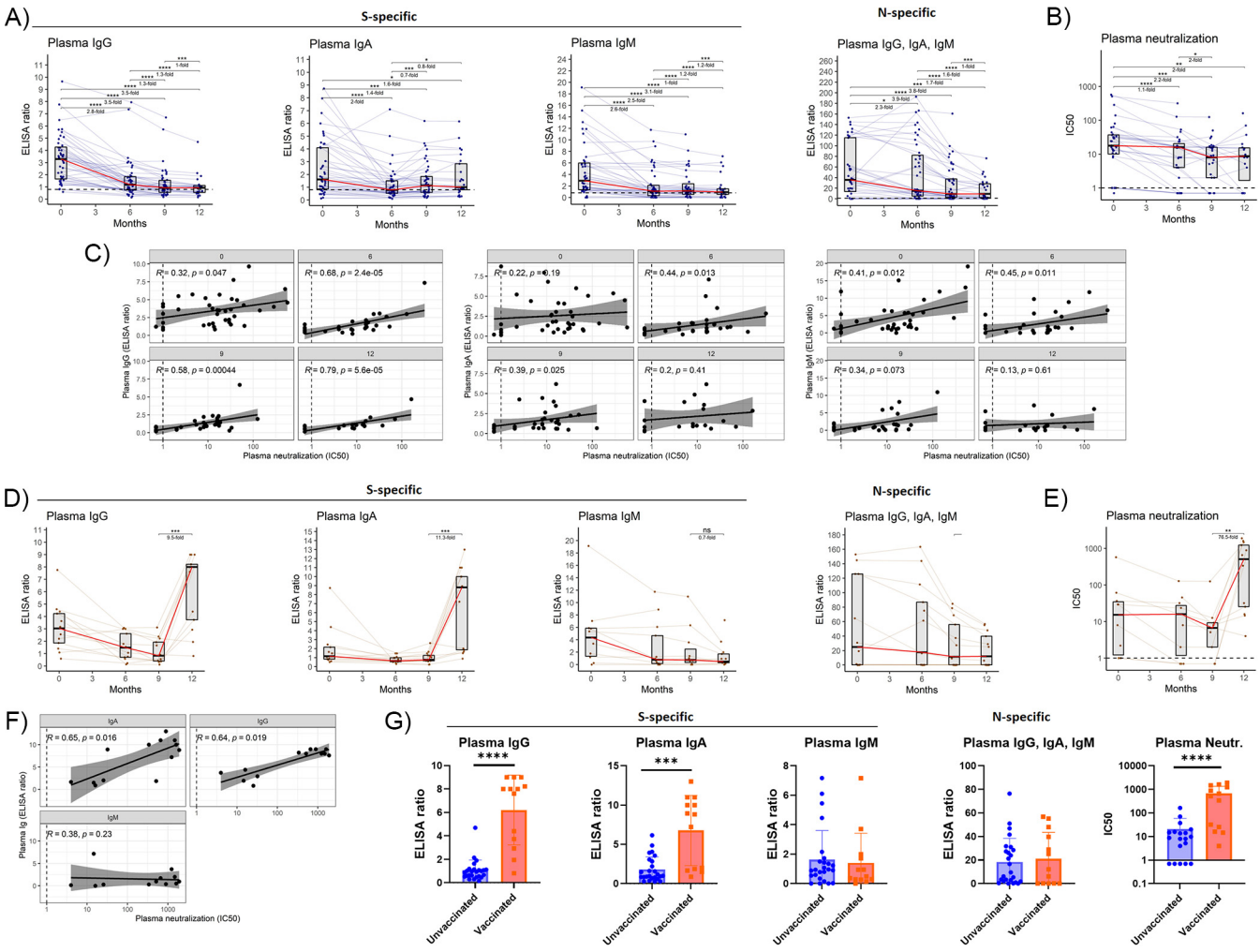

FIG 1 Dynamics of SARS-CoV-2 S-specific antibodies and plasma neutralization. Relative plasma levels of antibodies specific for the S- or N-protein at different time points after the infection for (A) unvaccinated and (D) boosted individuals. Immunoglobulin isotypes were not distinguished in the case of N-specific antibodies. Plasma neutralization capacity given as IC_{50} is plotted against the time for (B) unvaccinated and (E) vaccinated individuals recovered from SARS-CoV-2 infection. Data are represented as individual points and box plots. The red line connects the median values of each time point and the dashed line represents the positivity cutoff. Differences between the time points were assessed by the Wilcoxon test and the fold change was calculated as a ratio of the medians of compared time points. Correlations between the plasma levels of S-specific antibodies and neutralization are depicted in (C) vaccinated and (F) unvaccinated recovered individuals. Separate graphs are shown for different time points and isotypes. The strength of correlations was assessed by spearman's correlation test. (G) Comparison of antibody levels and plasma neutralization between unvaccinated and vaccinated recovered individuals. Differences between the groups were assessed by the Mann-Whitney test.

9 months after infection S-specific IgG antibody levels reached a semistable level (Fig. 1A) as previously described (22). The nucleocapsid (N)-specific IgG responses showed similar kinetics (Fig. 1A). In contrast, S-specific serum IgA antibody levels decayed rapidly during the first 6 months (2-fold decrease, $P < 0.0001$), but then increased between months 6 and 9 (1.4-fold increase, $P < 0.001$) and remained stable until the end of the acquisition period (Fig. 1A). The kinetics of S-specific IgM antibodies closely resembled those of IgG antibodies. There was a rapid decrease between months 0 and 6 (2.6-fold, $P < 0.0001$), after which the S-specific IgM levels remained stable (Fig. 1A).

We next investigated whether the antibody kinetics were also reflected in the serum neutralization capacity. We, therefore, performed a plaque reduction neutralization assay with live wild-type SARS-CoV-2 as previously described (21). Our data showed a gradual decline in plasma neutralization capacity during the first 6 months of the study (1.1-fold decrease, $P < 0.0001$) and a more rapid decrease between the months 6 and 9 (2-fold decrease, $P < 0.05$). Neutralization capacity did not change significantly between months

9 and 12 (Fig. 1B). Our findings indicate that serum neutralization capacity as well as IgG antibody levels gradually decline to a semistable level in the first 9 months after infection.

To investigate the associations between the antibodies of different isotypes and plasma neutralization we next performed a correlation analysis. We found that S-specific IgG levels weakly correlated with plasma neutralization at the first time point ($r = 0.32$, $P = 0.047$). The association became stronger at months 6, 9 and 12 ($r = 0.68$, $P < 0.0001$; $r = 0.58$, $P = 0.0004$; $r = 0.79$, $P < 0.0001$, respectively) indicating increased neutralization potency of S-specific IgG with time, most likely due to affinity maturation (Fig. 1C). In the case of S-specific IgA, we only observed significant correlations for months 6 and 9 ($r = 0.44$, $P = 0.013$; $r = 0.39$, $P = 0.025$, respectively), while S-specific IgM significantly correlated with neutralization at months 0 and 6 ($r = 0.41$, $P = 0.012$; $r = 0.45$, $P = 0.011$, respectively) (Fig. 1C). These correlations suggest that IgG is the main isotype mediating viral neutralization of SARS-CoV-2, especially 6 or more months after the infection. IgM mostly contributes to neutralization early after the recovery from infection, while the contribution of IgA becomes significant only 6 or more months after the infection for a limited period.

Collectively, our data demonstrated that the S- and N-specific plasma antibodies of individuals who recovered from SARS-CoV-2 infection rapidly decay during the first 6 months followed by a slower decay. The neutralization capacity decreases more gradually, presumably due to the progressive affinity maturation of S-specific IgG.

Vaccination of previously SARS-CoV-2-infected individuals induces a recall humoral response. Given the pivotal role of booster vaccinations in the control of the pandemic, we next assessed how vaccination of previously infected individuals changes the humoral anti-SARS-CoV-2 immune response. We, therefore, measured plasma antibody levels of 13 study participants that had been infected and were afterward vaccinated between months 9 and 12. The measurements of plasma antibody levels in these individuals revealed profound increases in S-specific IgG, and IgA antibody levels (9.5-fold increase, $P < 0.001$, 11.3-fold increase, $P < 0.001$, respectively). The levels of S-specific IgM antibodies did not change significantly after vaccination indicating that immunization of individuals with preexisting immunological memory triggers a recall response rather than priming of naive cells (Fig. 1D). Moreover, the N-specific antibodies remained at the same level in vaccinated individuals confirming that the immunization succeeded by vaccination and not reinfection (Fig. 1D).

Of note, plasma neutralization capacity against the wild-type virus greatly increased after vaccination (76.5-fold increase, $P < 0.01$) (Fig. 1E) and significantly correlated with the S-specific IgG and IgA ($r = 0.64$, $P = 0.019$; $r = 0.65$, $P = 0.016$, respectively), but not IgM levels (Fig. 1F).

Comparing the S-specific antibody levels of unvaccinated and vaccinated individuals with a history of SARS-CoV-2 infection, we observed significantly higher levels of IgG, IgA, and plasma neutralization ($P < 0.0001$, $P < 0.001$, and $P < 0.0001$, respectively) in vaccinees at month 12 of the study. Levels of S-specific IgM and N-specific antibodies were comparable between the groups (Fig. 1G).

Taken together, vaccination of previously SARS-CoV-2 infected individuals resulted in a surge of S-specific plasma IgG, IgA, and neutralization capacity, but not IgM levels, indicating a recall response.

SARS-CoV-2-specific memory B cells persisted at high levels for at least 12 months after the infection and expand following vaccination. Previous reports showed that memory B cells generated in response to SARS-CoV-2 infection do not wane as rapidly as antibodies (3, 4, 13), suggesting their key role in long-term immunity against SARS-CoV-2. We, therefore, investigated memory B cell kinetics in individuals who recovered from the infection and individuals who also received a vaccination booster following infection. We first measured the frequency of S-specific memory B cells in the peripheral blood of the 43 study participants by flow cytometry (Fig. S1). Our data revealed the presence of S-specific IgG^+ memory B cells already at the earliest time point after the infection (maximum 5 weeks) in most of the recovered individuals (82%). The frequency of these cells was substantially increased 6 months later (3.8-times, $P < 0.0001$), indicating a prolonged formation of the B cell memory pool.

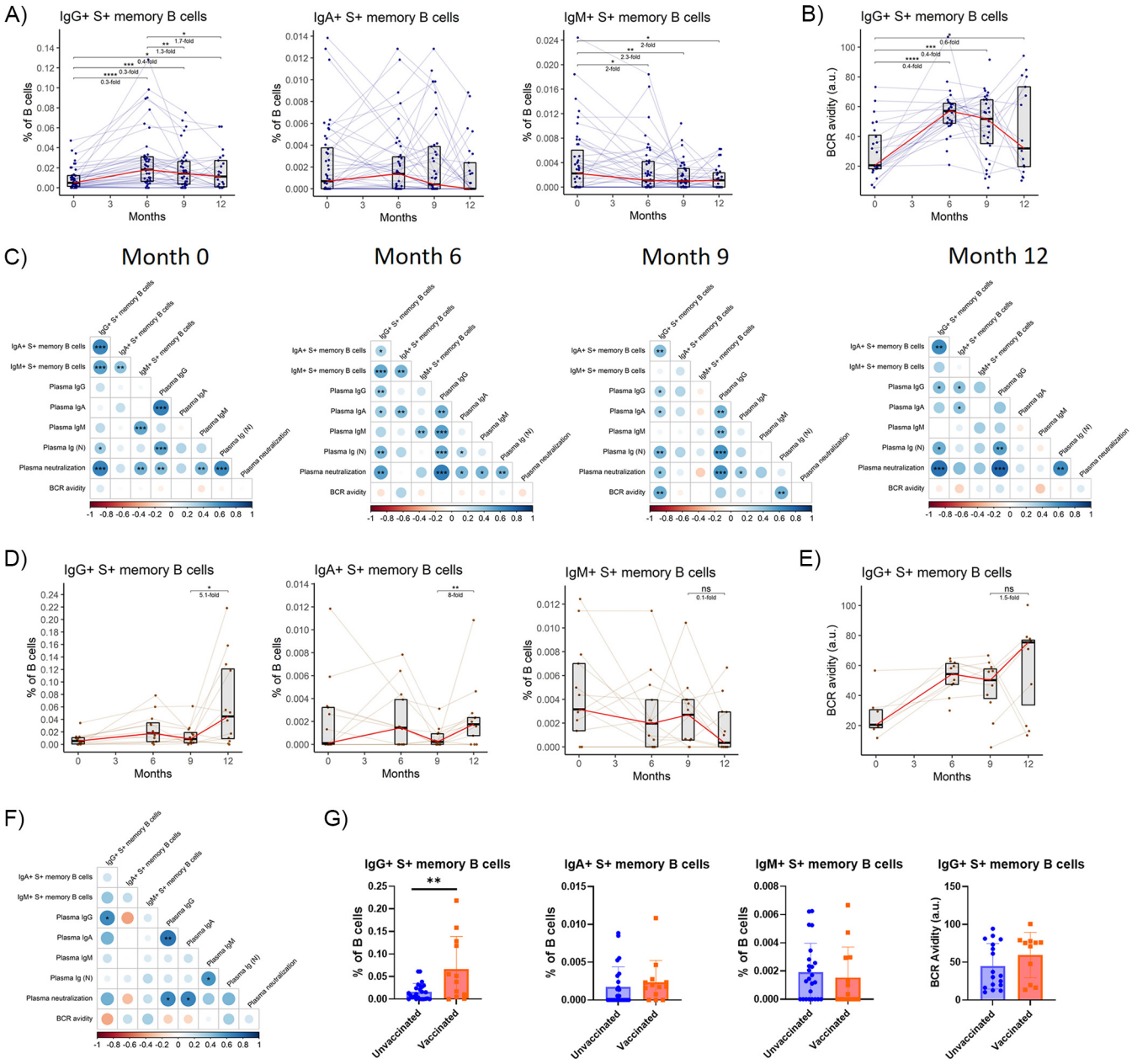

FIG 2 Dynamics of SARS-CoV-2 S-specific memory B cells. Frequency of S-specific memory B cells as a percentage of the bulk B cell population at four different time points for (A) unvaccinated and (D) vaccinated recovered individuals. Memory B cells were discriminated based on the isotype of their B cell receptor. Data are represented as individual points and box plots. The red line connects the median values of each time point and the dashed line represents the positivity cutoff. Differences between the time points were assessed by the Wilcoxon test and the fold change was calculated as a ratio of the medians of compared time points. Longitudinal changes in avidity of IgG BCR on the surface of S-specific memory B cells for (B) unvaccinated and (E) vaccinated recovered individuals. Correlograms demonstrated the associations between the memory B cells plasma antibodies and neutralization for (C) unvaccinated and (F) vaccinated recovered individuals. The strength of correlations was assessed by spearman's test and is coded by the size and color of the circles. (G) Comparison of memory B cells frequency and BCR avidity between unvaccinated and vaccinated recovered individuals. Differences between the groups were assessed by the Mann-Whitney test.

Following this time point, the frequency of IgG^+ memory B cells gradually declined (1.7-fold decrease, $P < 0.05$) but remained well above initial levels (2.3-fold increase, $P < 0.05$) (Fig. 2A). These findings indicate that IgG^+ memory B cells persist at high frequencies for at least 12 months after the SARS-CoV-2 infection and might therefore be crucial for the durability of immunological memory. S-specific memory B cells bearing IgA B cell receptor (BCR) were about 10-times less frequent than those with IgG BCR but showed a similar longitudinal behavior (Fig. 2A). Similarly, S-specific IgM^+ memory

B cells were observed at low levels. However, their frequency peaked already at month 0 and rapidly declined until month 6 (2-fold, $P < 0.05$) (Fig. 2A) in line with previously described antibody class switching (23–25). After that, they remained stable until the end of the study.

Not only the frequency of memory B cells but also their ability to bind the cognate antigen determines the quality of memory B cell response. We, therefore, measured the avidity of B cell receptors (BCR) present on the surface of S1-specific IgG^+ memory B cells. These experiments were analyzed by normalizing the mean fluorescence intensity of bound S1-probe with the level of BCR expression as previously described (26). Our data demonstrated a 2.8-fold ($P < 0.0001$) increase in the avidity of IgG present on the surface of S-specific memory B cells during the first 6 months. While no significant changes were observed between months 6 and 12, the avidity remained significantly elevated for the time of this study with increasingly variable levels among individuals (Fig. 2B).

To evaluate the coordination of overall B cell response, we next correlated the parameters describing humoral and memory B cell responses. We observed that the frequencies of S-specific IgG^+ and IgA^+ memory B cells correlated throughout monitoring, indicating synchronized generation and decay of these cells, while the frequency of S-specific IgM^+ memory B cells correlated with the other isotypes only at the first two time points (Fig. 2C). The level of S-specific IgG antibodies correlated with the frequency of S-specific IgG^+ memory B cells at the last three time points but not at month 0, demonstrating that IgG levels only predict the frequency of S-specific IgG^+ memory B cells after the initial drop of IgG in plasma. Interestingly, N-specific antibodies correlated with the frequency of IgG^+ memory B cells at all time points (Fig. 2C). Furthermore, the frequency of S-specific IgG^+ memory B cells correlated with plasma neutralization capacity at all time points, while the frequency of S-specific IgM^+ memory B cells only correlated with neutralization at month 0 reflecting the associations between IgG/IgM antibodies and neutralization (Fig. 2C). These findings indicate partially coordinated formation of memory B cells and antibodies after SARS-CoV-2 infection.

We have shown that vaccination of previously infected individuals efficiently boosts the levels of neutralizing antibodies. To determine whether this is also true for memory B cells, we measured the levels of SARS-CoV-2-specific memory B cells in individuals who received a vaccine booster between months 9 and 12 of the study. The data showed increased frequencies of IgG^+ and IgA^+ S-specific memory B cells at the last time point of monitoring. The frequency of IgG^+ cells increased 5.1-fold ($P < 0.05$) and that of IgA^+ cells increased 8-fold ($P < 0.01$). The frequency of IgM^+ cells remained unchanged further confirming that vaccination primarily triggered reactivation of memory and not naive B cells (Fig. 2D). While vaccination efficiently boosted the levels of class-switched S-specific memory B cells, the BCR avidity of S-specific IgG^+ memory B cells did not change significantly following vaccination (Fig. 2E).

To assess the coordination of the B cell response triggered by the vaccine booster we correlated the parameters describing humoral and memory B cell responses. We observed a significant correlation between the frequencies of S-specific IgG^+ memory B cells and IgG antibodies ($P < 0.05$). In contrast to unvaccinated individuals, the frequencies of S-specific memory B cells with different BCRs did not correlate. Moreover, the frequency of S-specific IgG^+ memory B cells did not correlate with plasma neutralization (Fig. 2F) suggesting limited synchronization of B cell response after the vaccination of previously infected individuals.

We next directly compared the frequencies of S-specific memory B cells at month 12 of the study between vaccinated and unvaccinated individuals who previously recovered from SARS-CoV-2 infection. The frequency of IgG^+ cells was significantly higher in the vaccinated group ($P < 0.01$), while the frequencies of IgA^+ and IgM^+ cells and avidity of IgG BCR did not change significantly (Fig. 2G).

Taken together our data demonstrated the persistence of S-specific IgG^+ memory B cells during the period of a minimum of 12 months after the SARS-CoV-2 infection. The

frequency of these cells was associated with plasma neutralization, and their BCR avidity increased over time, further demonstrating their pivotal role in the maintenance of long-term immunological memory. Importantly, vaccination of previously infected individuals resulted in increased frequencies of S-specific IgG^+ memory B cells.

IgG^+ memory B cells with high avidity for the S protein predominantly exhibited $CD27^-CD21^+$ phenotype and were activated by vaccination. To determine the dynamics of S-specific IgG^+ memory B cells more closely, we investigated the changes in the phenotypic composition of these cells over time. We distinguished four commonly described memory B cell subsets found in peripheral blood: activated memory (AM), resting memory (RM), tissue-like memory (TLM), and intermediate memory (IM). The distinctions were made based on the expression of CD21 and CD27 (AM: $CD27^+CD21^-$; RM: $CD27^+CD21^+$; TLM: $CD27^-CD21^-$; IM: $CD27^-CD21^+$) as previously described (27–29). Our data demonstrated that the frequency of activated subsets, including AM and TLM, decreased over time with a fast decay during the initial months (14.4-fold decrease, $P < 0.01$ for AM and 3.2-fold decrease, $P < 0.001$ for TLM) (Fig. 3A). Surprisingly, the frequency of resting memory B cells also decreased during the first 9 months (1.7-fold decrease, $P < 0.01$) but then rebounded at the 12-month time point (1.4-fold increase, $P < 0.01$) (Fig. 3A). The only subset that stayed significantly expanded during the time frame of this study was IM whose kinetics resemble those of the total S-specific IgG^+ memory B cells. The frequency of IM most rapidly increased between months 0 and 9 (1.6-times increase, $P < 0.0001$) and remained stable until month 12 (Fig. 3A).

Previous studies suggested limited affinity maturation of the BCR found on memory B cells with IM phenotype (29, 30). Because IM was the predominant subset of S-specific IgG^+ memory B cells we compared the avidity of its BCR with other memory subsets. Strikingly, IM had significantly increased avidity compared to AM ($P < 0.0001$, $P < 0.01$, $P < 0.05$ for months 0, 6, and 12, respectively) and RM ($P < 0.05$, $P < 0.05$, and $P < 0.05$ for months 0, 6, and 9, respectively) while no significant differences were observed between IM and TLM (Fig. 3B). These findings suggested that, despite the association of $CD27^-$ memory B cells with reduced affinity maturation, SARS-CoV-2-specific memory B cells with high-avidity BCR mostly exhibit a $CD27^-CD21^+$ intermediate memory phenotype.

We next investigated possible changes in the phenotype of S-specific IgG^+ memory B cells in vaccinated individuals with a history of SARS-CoV-2 infection. The proportions of RM and IM phenotypes significantly decreased between months 9 and 12 (1.7-fold decrease, $P < 0.05$ and 3.3-fold decrease, $P < 0.05$, respectively), while those of AM (0% to 25%, $P < 0.05$) and TLM expanded (2.9-fold increase, $P < 0.05$) (Fig. 3C). Unlike in the unvaccinated, no differences in BCR avidity were observed among the memory subsets at month 12 of the study for boosted individuals (Fig. 3D).

Directly comparing the proportions of memory subsets between vaccinated and unvaccinated individuals who recovered from SARS-CoV-2 infection, the former had a significantly higher proportion of AM and TLM subsets ($P < 0.001$ and $P < 0.001$, respectively) subsets but a lower proportion of IM subset ($P < 0.01$). The fractions of cells with an RM phenotype were not significantly different among the two groups of individuals (Fig. 3E). This indicated that the activation of S-specific IgG^+ memory B cells by vaccination was causing a transition from resting to activated memory phenotypes. Furthermore, the BCR avidity of the activated subsets like AM and TLM was significantly increased following vaccination ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 3F). This was likely due to the preferable activation of IgG^+ memory B cells with high avidity BCRs.

To sum up, we showed that most S-specific IgG^+ memory B cells exhibit an unusual $CD27^-CD21^+$ memory phenotype, especially at the later time points after SARS-CoV-2 infection. In contrast to previous reports associating $CD27^-$ memory B cells with reduced affinity maturation, S-specific IgG^+ memory B cells with IM phenotype bear high-avidity BCRs. Vaccination of previously infected individuals caused activation of high-avidity S-specific IgG^+ memory B cells that was reflected in an increased proportion of activated memory subsets.

Vaccination in previously infected individuals boosted the *in vitro* production of neutralizing antibodies by S-specific memory B cells. We next assessed the functionality of S-specific memory B cells present in peripheral blood of recovered

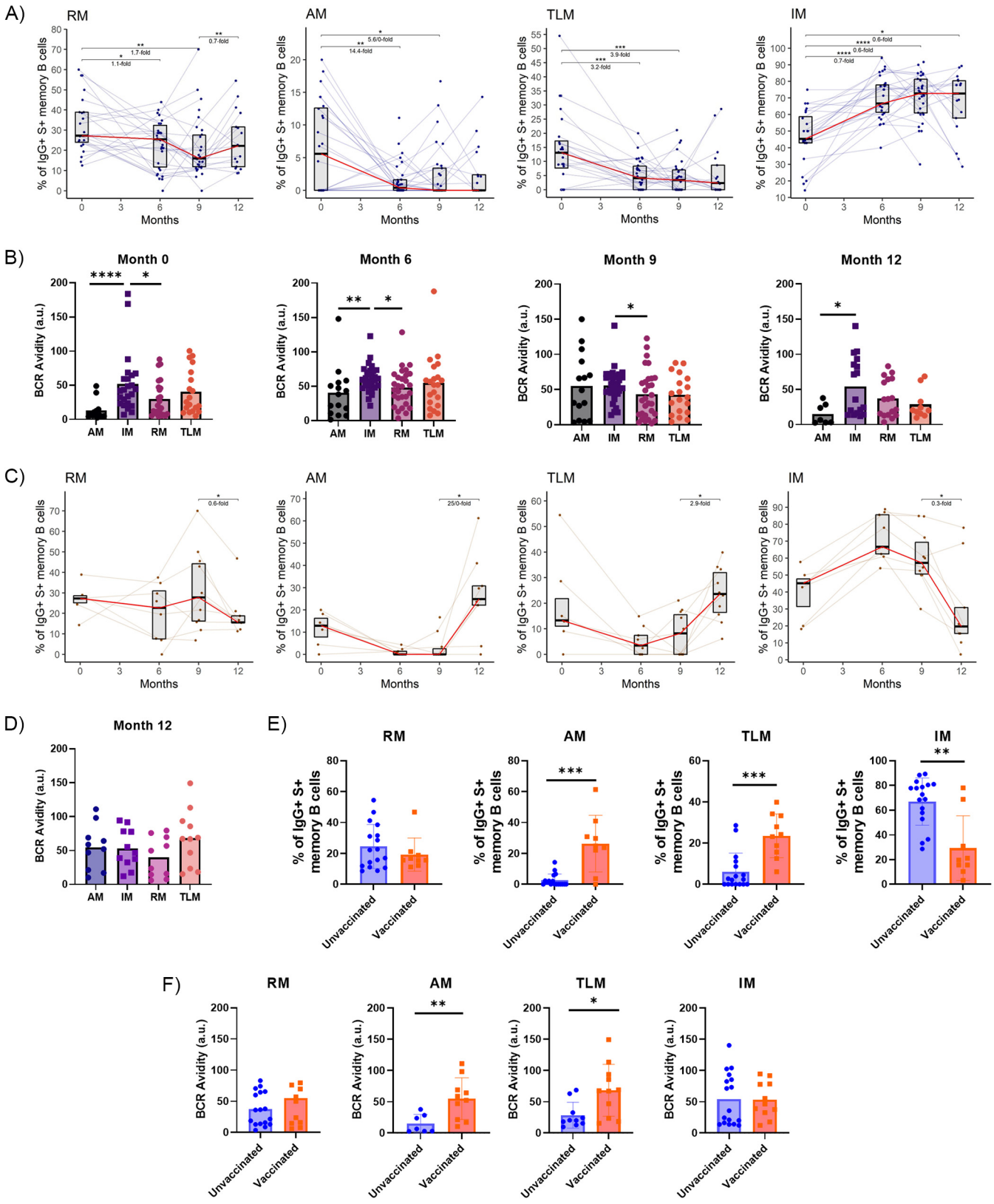

FIG 3 Phenotype of SARS-CoV-2 S-specific IgG$^+$ memory B cells. Longitudinal changes in proportions of different memory phenotypes within the S-specific IgG$^+$ memory B cell population. Graph (A) represents unvaccinated and graph (C) vaccinated individuals who recovered from SARS-CoV-2 infection. Data are represented as individual points and box plots. The red line connects the median values of each time point and the dashed line represents the positivity cutoff. Differences between the time points were assessed by the Wilcoxon test and the fold change was calculated as a ratio of the medians of compared time points. Comparison of BCR avidity among the different memory subsets at different time points for (B) unvaccinated and (D) vaccinated recovered individuals. Comparisons of (E) prevalence and (F) BCR avidity of memory subsets between unvaccinated and vaccinated recovered individuals. Differences between the groups were assessed by the Mann-Whitney test.

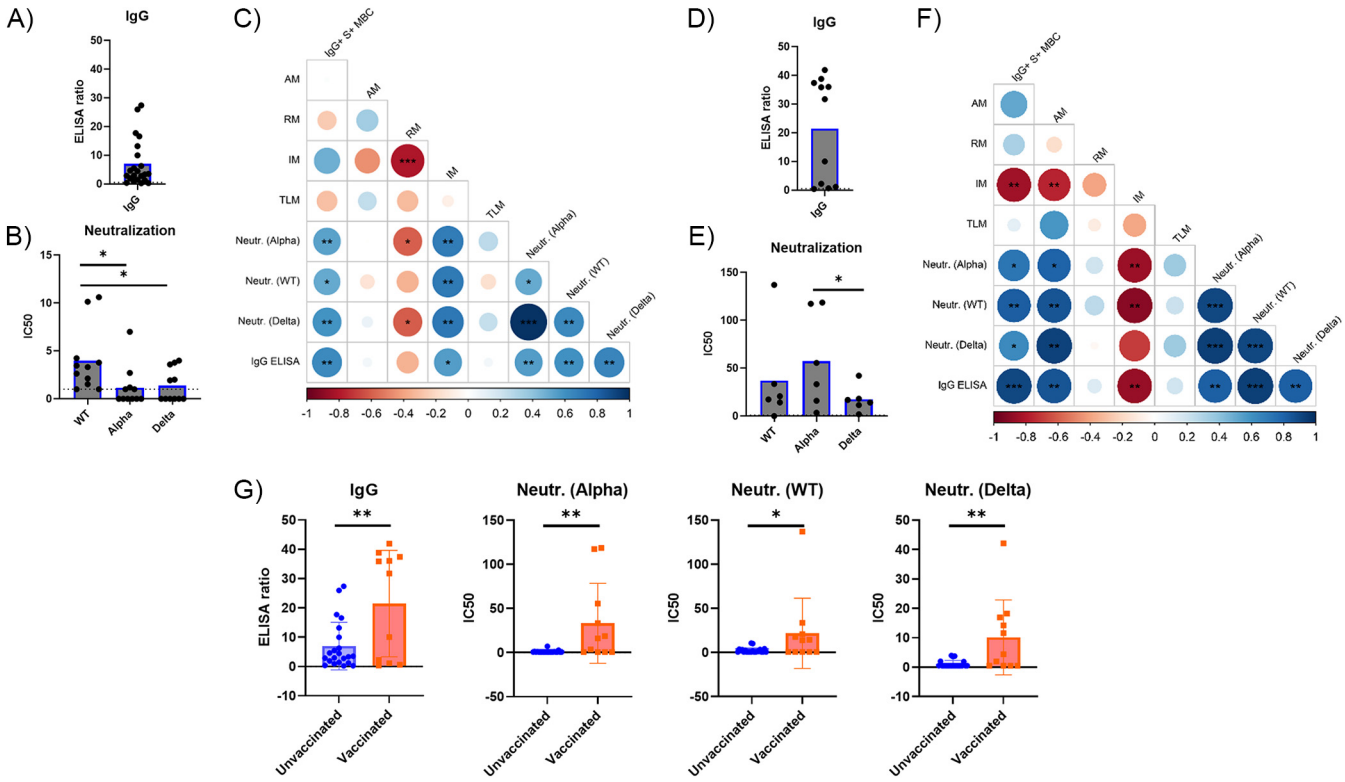

FIG 4 Functionality of S-specific memory B cells. Relative levels of (A) S-specific IgG and (B) neutralization potency of supernatants from stimulated memory B cell cultures. The data represent unvaccinated recovered individuals on month 12 of the study. For the comparison of neutralization efficiency against different SARS-CoV-2 variants only samples neutralizing at least one of the variants are included. (D) and (E) are corresponding graphs for vaccinated recovered individuals. Frequency and phenotype of S-specific IgG$^+$ memory B cells were correlated with levels of S-specific IgG and neutralizing antibodies in stimulated memory B cell cultures. Graph (C) represents unvaccinated and graph (F) vaccinated individuals who recovered from SARS-CoV-2 infection. The strength of correlations was assessed by Spearman's test and is coded by the size and color of the circles. (G) S-specific IgG levels and neutralization potency of stimulated memory B cell culture supernatants. Compared are vaccinated and unvaccinated individuals recovered from SARS-CoV-2 infection. Differences between the groups were assessed using the Mann-Whitney test or Wilcoxon test for matched data.

individuals at about 12 months after infection. We stimulated PBMC with a memory B cell stimulation cocktail and investigated the characteristics of secreted antibodies. S-specific IgG antibodies measured by ELISA were detectable in 82% of the stimulated cell cultures at various levels (Fig. 4A). The neutralization capacity, as measured by plaque reduction neutralization assay, was tested against the three most common SARS-CoV-2 variants at the time of this study: wild-type, alpha (B.1.1.7), and delta (B.1.617.2). Among the cell cultures where we were able to measure a neutralizing effect for at least one of the variants, all samples efficiently neutralized the wild-type virus while alpha and delta were more resistant with only 45% of the samples showing neutralization against each variant (Fig. 4B). The neutralization capacities against the variants were nevertheless positively associated and correlated with the level of S-specific IgG in cell cultures (Fig. 4C). Furthermore, the frequency of S-specific memory B cells at month 12 correlated with S-specific IgG levels and neutralization capacities of the cell culture supernatants. The memory phenotype of these cells played an important role since the proportion of the IM subset positively correlated with the neutralization and secreted IgG levels (Fig. 3B). This is most likely due to the high BCR avidity that we observed in the IM subset.

We also stimulated memory B cells of recovered and then vaccinated participants, collected at month 12 of the study. S-specific IgG antibodies were detectable in 91% of the cases at highly variable levels (Fig. 4D). Among the cell cultures that showed neutralization against at least one of the tested SARS-CoV-2 variants delta showed to be significantly more resistant to neutralization compared to the alpha variant (70% reduced sensitivity to neutralization) but not wild-type (Fig. 4E). The neutralization

capacities against all three variants highly correlated between each other and correlated well with the level of S-specific IgG in cell culture (Fig. 4F). Similar to unvaccinated individuals the frequency of S-specific IgG$^+$ memory B cells correlated with the neutralization and IgG levels in cell culture suggesting that vaccination expanded S-specific memory B cells' ability to secrete neutralizing antibodies effective against all viral variants. The memory B cell phenotype associated with secretion of neutralizing antibodies *in vitro* was AM while IM correlated inversely (Fig. 4F). This contrasted with unvaccinated individuals where IM was the phenotype, which positively correlated with the production of neutralizing antibodies after *in vitro* stimulation. We postulated that this was due to the relatively recent (maximum of 3 months) activation of S-specific IgG$^+$ memory B cells by vaccination compared to unvaccinated individuals where immunization occurred at least 12 months ago and most of the highly S-specific memory B cells already acquired resting phenotype.

To further investigate the effect of vaccination on previously infected individuals, we directly compared the secretion of neutralizing antibodies from *in vitro* activated memory B cells among the vaccinated and unvaccinated groups. The secretion of S-specific IgG was significantly augmented in vaccinated individuals (3-fold increase, $P < 0.01$), as were the neutralization capacities against the wild type (10-fold increase, $P < 0.05$), alpha (33-fold increase, $P < 0.01$), and delta (9-fold increase, $P < 0.01$) variants (Fig. 4G). This is likely due to the increased frequencies of S-specific memory B cells in vaccinated individuals and demonstrates the beneficial effect of a booster vaccination.

Taken together, we demonstrated that S-specific memory B cells of individuals recovered from SARS-CoV-2 infection produced neutralizing antibodies after *in vitro* stimulation. Production of these antibodies and their neutralizing potency was augmented following vaccination. Together, these findings demonstrated the functionality of S-specific memory B cells that emerge in response to SARS-CoV-2 infection and vaccination, underpinning their role in long-term immunity.

SARS-CoV-2-specific IFN-γ^+ CD4$^+$ memory T cells were associated with S-specific IgG$^+$ memory B cells in recovered individuals and after vaccination booster. To better understand the observed dynamics of S-specific IgG$^+$ memory B cells we next measured the frequency of SARS-CoV-2-specific CD4$^+$ T cells. We stimulated CD4$^+$ T cells with peptides derived from the M, N, and S proteins of the wild-type SARS-CoV-2 and monitored the expression of molecules crucial for modulation of the B cell response (CD40L, IFN-γ, IL-4/13, and IL-21) by flow cytometry (Fig. S2). Our data demonstrated that the frequency of CD40L$^+$ CD4$^+$ memory T cells remained relatively stable over the 12 months of monitoring but showed a declining trend. Only in the case of N-protein did we observe a significant decline in the frequency of those cells between months 6 and 12 (2-fold decrease, $P < 0.05$) (Fig. 5A). In comparison, the frequencies of IFN-γ^+ CD4$^+$ memory T cells declined more rapidly throughout the 12 months. IFN-γ^+ CD4$^+$ memory T cells specific for the M protein declined 5.9-fold ($P < 0.05$), those specific for the N protein 3.7-fold ($P < 0.0001$), and those specific for the S protein 2.3-fold ($P < 0.01$) between months 0 and 12 (Fig. 5A). Even more rapid was the decline in the frequency of rare IL-4/13-secreting CD4$^+$ memory T cells whose median frequency fell from 0.003% for M, 0.003% for N, and 0.01% for the S protein to 0% on month 12 regardless of specificity ($P < 0.01$, $P < 0.05$, $P < 0.05$, respectively) (Fig. 5A). The kinetics of IL-21-secreting CD4$^+$ memory T cells proved to be complex, in that their median frequency decreased to 0% on month 6, significantly expanded on month 9, and decreased again on month 12 for all three proteins. In the case of the M protein, the decrease between months 0 and 12 was from 0.03% to 0% ($P < 0.05$), for the N protein 1.7-fold ($P < 0.05$), and for the S protein 6.6-fold ($P < 0.05$) (Fig. 5A). Collectively, these data suggest that the SARS-CoV-2-specific CD4$^+$ T cells with functions important for the formation and maintenance of the B cell response gradually decrease during at least 12 months after SARS-CoV-2 infection.

Vaccination of previously infected individuals led to significant enhancement of the B cell response. To find out whether the same was true for the CD4$^+$ T cell response, we measured the frequencies of S-specific SARS-CoV-2-specific CD4$^+$ T cells in

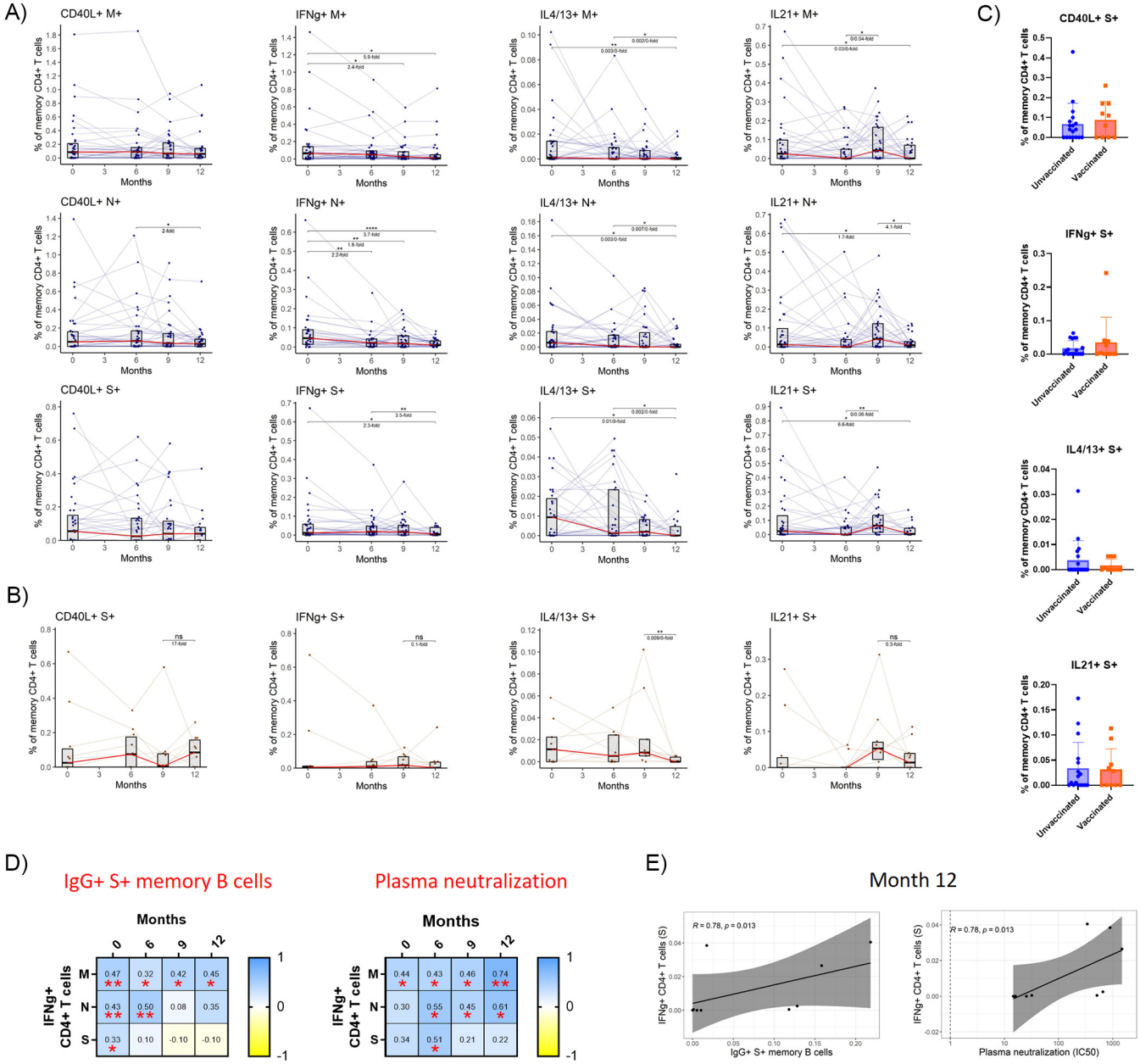

FIG 5 Dynamics of SARS-CoV-2-specific memory CD4$^+$T cells important for the formation and maintenance of B cell response. (A) Frequencies of SARS-CoV-2-specific CD4$^+$ memory T cells with different effector functions; CD40L, IFN-γ, IL-4/13, and IL-21 as a percentage of bulk CD4$^+$ T cells for the four points of monitoring. Cells specific for M, N, and S proteins were differentiated. (B) Temporal changes in the frequency of S-specific CD4$^+$ memory T cells with different effector functions for vaccinated individuals recovered from SARS-CoV-2-infection. (C) Comparison of S-specific CD4$^+$ memory T cells with different effector functions for unvaccinated and vaccinated recovered individuals. Differences between the groups were assessed using the Mann-Whitney test. (D) Heat-map demonstrating the strength of correlations between M-, N-, and S-specific CD4$^+$ T cells secreting IFN-γ and frequency of S-specific IgG$^+$ memory B cells or plasma neutralization. Shown are the associations at different time points of monitoring. Cells are color-coded concerning Spearman's correlation coefficient. Significant correlations are marked by asterisks. (E) Correlations between the frequency of S-specific CD4$^+$ T cells secreting IFN-γ and frequency of S-specific IgG$^+$ memory B cells or plasma neutralization on month 12 for vaccinated individuals. The strength of correlations was assessed by Spearman's correlation test.

previously infected individuals who were vaccinated between months 9 and 12. While there was a trend toward the expansion of CD40L-expressing CD4$^+$ memory T cells, the rest of the measured helper functions remained unchanged or significantly declined in the case of IL-4/13-expressing cells (from 0.009% to 0%, $P < 0.01$) between months 9 and 12 (Fig. 5B). Direct comparison of S-specific CD4$^+$ T cells frequencies between unvaccinated and vaccinated individuals recovered from SARS-CoV-2 infection showed no significant differences for any of the effector functions indicating that

vaccination failed to boost CD4$^+$ T cell functions important for the B cells in previously infected individuals (Fig. 5C).

We next investigated the association between the B cell and helper T cell responses. We, therefore, correlated the frequencies of SARS-CoV-2-specific CD4$^+$ T cells with those of S-specific IgG$^+$ memory B cells and plasma neutralization capacity. Only IFN-γ-secreting CD4$^+$ T cells significantly correlated with memory B cell frequency and plasma neutralization throughout the 12 months of monitoring. Similar to our previous findings (26), cells specific to the M protein showed the strongest associations and those specific to the S protein the weakest (Fig. 4D). Interestingly, while the S-specific IFN-γ^+ memory CD4$^+$ T cells correlated with memory B cells and plasma neutralization only at the first two time points, these associations were restored in vaccinated individuals on month 12 (Fig. 4E).

Collectively, our data showed that the frequencies of SARS-CoV-2-specific CD4$^+$ T cells, important for the regulation of B cell response, decline during the 12 months postinfection and do not expand following vaccination. The frequency of SARS-CoV-2-specific IFN-γ-secreting CD4$^+$ T cells correlates with the frequency of S-specific IgG$^+$ memory B cells and plasma neutralization proposing their important role in the maintenance of protective B cell responses.

DISCUSSION

Studies have demonstrated that SARS-CoV-2 infection successfully induces both a humoral and cellular immune response (1, 2). However, the initial antibody levels rapidly deteriorate opening a window of opportunity for reinfections (3, 4). In addition, the frequencies of memory B and T cells decline over time but at a lower rate and might, therefore, play a major role in protection from the severe disease at later time points (3, 4, 13). To control the pandemics and compensate for the waning immunity, booster vaccinations have been recommended for previously SARS-CoV-2-infected individuals. In our present study, we investigated the kinetics of the B cell response in individuals who recovered from asymptomatic to moderate SARS-CoV-2 infection who were either unvaccinated or received a vaccination booster. Our findings demonstrated the waning of humoral and cellular immune responses after SARS-CoV-2 infection and suggest memory B cells as the most persistent component of immunological memory. Importantly, vaccination of recovered individuals triggered a recall response notably boosting B cell immunity.

Neutralizing antibodies induced by either infection or vaccination are the best correlate of protection against the symptomatic SARS-CoV-2 infection (11, 31). Their plasma titers peak 3 to 5 weeks following infection and then decay in a biphasic manner with an initially higher rate of decline (5, 6). In line with these findings, we demonstrated a rapid decline in plasma levels of S-specific IgG, IgA, and IgM during the first 6 months of monitoring. The antibody levels then continued decreasing at a slower pace until month 12 of the study. Similar kinetics were also observed for the N-specific antibodies. The plasma neutralization capacity waned more gradually than S-specific antibody levels. This was most likely due to the affinity maturation and increasing neutralization potency of IgG because we showed that the correlation between the IgG levels and plasma neutralization becomes stronger over time. In line with this hypothesis, previous studies have demonstrated the maturation of the SARS-CoV-2-specific B cell response over time (24, 32). IgM and IgA also correlated with neutralization but only at earlier time points indicating that these antibodies significantly contribute to protection only up to about 9 months after the infection. Similar to earlier studies (18, 33–36), vaccination of individuals with a history of SARS-CoV-2 infection increased S-specific IgG and IgA levels which were reflected in substantially augmented plasma neutralization capacity. The levels of S-specific IgM did not increase following vaccination indicating a recall response. Overall, these findings indicated that the antibody levels induced by SARS-CoV-2 infection wane with time while their neutralizing

potency increases. Moreover, vaccination of recovered individuals triggers a recall response boosting humoral immunity.

Based on the currently available reports rapidly waning antibodies might not provide long-term immunity against SARS-CoV-2 infection (2, 5, 37). Memory B cells, on the other hand, were found more persistent at least during the first months following infection (3, 24, 32). We showed that S-specific IgG^+ memory B cells expand during the first 6 months after asymptomatic to moderate infection and only afterward begin to slowly decline. Their frequency at month 12 was 2.3-times higher than at the beginning of the monitoring suggesting robust B cell memory 1-year postinfection. Moreover, the avidity of IgG present on the surface of S-specific memory B cells increased after the initial time point indicating improved antigen-binding. In comparison, IgA^+ memory B cells were about 10 times less frequent than IgG^+ memory B cells but showed the same longitudinal trend for the time of monitoring. Conversely, IgM^+ memory B cells, which were also rare, followed similar biphasic decay kinetics as the antibodies. The frequencies of S-specific memory B cells of the three isotypes were correlated only during the first two time points of monitoring indicating coordinated generation of these cells followed by a transition from IgM to IgG and IgA B cell receptor isotypes. Similar to its influence on antibody levels, vaccination of previously infected individuals caused activation of preexisting memory B cells because we observed expansion of S-specific IgG^+ and IgA^+ but not IgM^+ memory B cells. Together, these findings suggest memory B cells as the key component of long-term immunity and demonstrated the beneficial effect of booster vaccinations.

To better understand the kinetics of S-specific IgG^+ memory B cells, we also investigated the four common maturational subsets of these cells (27–29). The proportions of activated subsets like AM and TLM, that become expanded during SARS-CoV-2 infection (26, 38), rapidly decreased after the initial time point, mirroring the general downregulation of the immune response. The only memory subset whose fraction increased during the 12 months was IM suggesting that this was the predominating phenotype of the expanding memory B cells. IM is an unconventional memory phenotype characterized by the lack of CD27 expression. It has been shown that $CD27^-$ class-switched memory B cells are increased in some autoimmune diseases, cancer, and the elderly (39, 40), and combine the typical morphology of memory B cells with traits of naive cells like limited affinity maturation (29, 30). Surprisingly, we observed that S-specific IgG^+ memory B cells bind the S-protein with high avidity, which is in line with the findings demonstrating that SARS-CoV-2-specific antibodies do not need extensive somatic hypermutation to efficiently bind the S protein (41–43). The phenotype of S-specific IgG^+ memory B cells was shifted from the resting IM subset toward the activated subsets like AM and TLM following booster vaccination. Furthermore, the BCR avidity was increased in activated subsets compared to unvaccinated individuals indicating that vaccination of previously infected individuals predominantly activated high-avidity memory B cells.

We demonstrated increased frequencies of S-specific memory B cells at about 1 year following SARS-CoV-2 infection. It is, however, not clear whether these cells clonally expand and produce neutralizing antibodies in case of re-exposure to the antigen. Our findings implied that S-specific memory B cells present in peripheral blood of recovered individuals at month 12 of our study indeed secreted S-binding antibodies after *in vitro* activation that efficiently neutralized the wild-type virus but showed limited neutralization capacity against the alpha and delta variants. This conforms with previous reports of the decreased sensitivity of alpha and delta variants to antibody neutralization (44, 45). The production of neutralizing antibodies was associated with an expanded IM phenotype suggesting that these cells might be the primary source of protective antibodies upon re-exposure to the virus. This agreed with the increased BCR avidity that we observed within this subset and the activation of the IM subset following booster vaccination.

The *in vitro* reactivation of memory B cells from recovered and afterward vaccinated individuals showed increased production of S-specific IgG and higher neutralization

potency against all three variants compared to unvaccinated individuals. These antibodies equally neutralized the wild-type and alpha variants, but not the delta variant likely due to its superior resistance to neutralization (45). After vaccination of recovered individuals, the proportion of IM phenotype inversely correlated with the production of neutralizing IgG in cell culture, while the activated (AM) phenotype showed a positive association. This further supports our hypothesis that S-specific IgG^+ memory B cells with IM phenotype preferentially get activated, expanded, and differentiated into antibody-secreting cells in case of a reencounter with the cognate antigen, most likely due to their high-avidity BCRs.

To better understand the dynamics of memory B cells following SARS-CoV-2 infection, we also measured the frequencies of $CD4^+$ T cells with relevant helper functions. The latter included CD40L, IFN-γ, IL-4/13, and IL-21 expression. In consent with previous studies (3, 4, 13), we showed that the frequencies of memory $CD4^+$ T cells specific for M, N, and S proteins decrease with time. However, the CD40L-expressing cells seemed to be more persistent than those secreting IFN-γ, IL-4/13, or IL-21. Surprisingly, vaccination did not significantly affect the frequency of SARS-CoV-2-specific $CD4^+$ T cells with the assessed effector functions, although other studies showed expansion of SARS-CoV-2-specific $CD4^+$ T cells after vaccination (46, 47). Regarding the crosstalk between the virus-specific B and T cells, we found that IFN-γ-secreting memory $CD4^+$ T cells correlated with the frequency of S-specific IgG^+ memory B cells and plasma neutralization. This is in line with the data demonstrating Th1-driven response following SARS-CoV-2 infection (48–50). The M-specific IFN-γ-secreting$+CD4^+$ T cells correlated with memory B cells and plasma neutralization throughout the whole study, while those specific for the N and S proteins did so only at some time points. The higher degree of correlation in the case of M-specific $CD4^+$ T cells could be due to their higher frequency. Moreover, in vaccinated, but not unvaccinated individuals, the frequency of S-specific IFN-γ-secreting $CD4^+$ T cells significantly correlated with S-specific memory B cells and plasma neutralization, indicating a restoration of B cell-T cell crosstalk after vaccination.

Taken together our data provide a detailed insight into the dynamics of B cell immunity during the 12 months starting 0 to 5 weeks after infection and following booster vaccination. We demonstrated waning B cell response with exception of S-specific IgG^+ memory B cells, which stay at elevated levels for at least 12 months postinfection. These cells mostly exhibit a $CD27^-CD21^+$ memory phenotype, that was associated with increased BCR avidity, and are highly functional in terms of secondary antigen challenge. Furthermore, we observed augmented humoral and cellular B cell responses after vaccination of previously infected individuals demonstrating the beneficial effect of booster vaccinations.

MATERIALS AND METHODS

Ethics approval. Forty-three individuals with previous SARS-CoV-2 infection were randomly selected from a cross-sectional study cohort (21). All participants provided written informed consent approved by the Ethics Committee of the Medical Faculty of the University of Bonn (ethics approval numbers 085/20 and 372/20).

Sample collection and storage. Study participants provided peripheral blood specimens and pharyngeal swabs. Blood was centrifuged and EDTA-plasma was stored until analysis (–80°C). PBMC were isolated by density gradient centrifugation and cryopreserved in liquid nitrogen.

SARS-CoV-2 S- and N-specific IgA, IgG, IgM ELISA. N-specific antibody levels were assessed using the Roche Cobas SARS-CoV-2 test following the manufacturer's protocol. For the determination of S-specific IgG and IgA commercially available assays (Anti-SARS-CoV-2 ELISA IgG/IgA, Euroimmun) was used following the manufacturer's instructions. For the determination of S-specific IgM an in-house assay was used. In brief, microtiter plates with high binding capacity were coated with 100 μL of coating buffer (carbonate-bicarbonate buffer, pH = 9.6) containing 1 μg/mL of recombinant SARS-CoV-2 S1 protein (Biotinylated SARS-CoV-2 [COVID-19] S1 protein, Acrobiolabs). Plates were then covered and incubated overnight at 4°C. After washing with wash buffer (PBS with 0.05% [vol/vol] Tween-20) plates were blocked (PBS containing 1% [wt/vol] BSA) to minimize unspecific binding. Cryopreserved EDTA plasma samples were thawed and diluted at 1:25 in blocking buffer. Blocked plates were washed, incubated with plasma samples, washed again, and incubated with 100 μL HRP-conjugated anti-IgM antibody (Goat anti-Human IgM [Heavy chain] Secondary Antibody, HRP, Invitrogen) diluted 1:8000 in wash buffer. All incubation steps were performed for 1 h at 37°C. Finally, plates were washed and 100 μL of the substrate solution was added to each well (TMB Chromogen Solution, Life technologies). The

reaction was carried out at room temperature for 5 min until the addition of 50 μL of stop solution (0.2 M H_2SO_4). Optical density at 450 nm was measured directly afterward. The background-subtracted OD_{450} readings were normalized to a calibrator (62.5 ng/mL anti-SARS-CoV-2-RBD antibody, IgM isotype, clone: CR3022, Abcam). The positivity cutoff was determined based on measurements of plasma samples from healthy individuals collected before the COVID-19 outbreak.

Plaque reduction neutralization assay. The neutralization capacity of plasma samples and cell culture supernatants was determined by a plaque reduction neutralization assay. Thawed plasma was heat-inactivated for 30 min at 56°C and subsequently centrifuged for 5 min at 5000 g. Similarly, cell culture supernatants were spun down at 1200 g for 5 min to remove cell debris. Samples were then serially 2-fold diluted starting with 2-fold up to 16384-fold dilution. Each plasma/supernatant dilution was combined 1:1 with 80 plaque-forming units of SARS-CoV-2 (either wild type, alpha, or delta variant) in a cell culture medium (OptiPROSFM, Gibco). After 1 h of incubation at 37°C, 200 μL of each neutralization mixture were added to 1.5×10^5 Vero E6 cells seeded in 24-well plates the day before. The inoculum was removed following 1 h incubation at 37°C, and cells were overlaid with a 1:1 mixture of 1.5% (wt/vol) carboxymethylcellulose (Sigma-Aldrich) and 2xMEM (Biochrom) supplemented with 4% (vol/vol) FBS (Pan Biotech), 0.44% (wt/vol) $NaHCO_3$ (Sigma), and 200 U/mL penicillin/streptomycin (Thermo Fisher). Inoculated cell cultures were then incubated at 37°C and 5% CO_2 for 3 days, after which the overlay was aspirated. Plates were fixed using a 6% (vol/vol) formaldehyde solution and stained with 1% (wt/vol) crystal violet in 20% (vol/vol) ethanol revealing the formation of plaques. The number of plaques was plotted against the serum/supernatant dilutions and IC_{50} was determined using the GraphPad Prism software.

***In vitro* memory B cell stimulation.** Cryopreserved PBMC were rested overnight, counted, and seeded in 12-well plates in R10 media (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES) at a density of 2×10^6 cells/well. The R10 medium was exchanged for the stimulation media (R10 supplemented with 20 ng/mL of IL-2 (Immunotools), 50 ng/mL of IL-10 (Immunotools), 1 μg/mL of R484 (Sigma-Aldrich), and 50 μM BME (Sigma-Aldrich)) and cultures were incubated at 37°C and 5% CO_2 for 14 days. Afterward, the medium was collected and centrifuged at 1200 g for 5 min to remove cell debris. Supernatant aliquots were stored at −20°C.

B cell isolation. Due to their relatively low frequency, B cells were first immunomagnetically isolated from cryopreserved PBMC samples using a positive selection approach. A commercially available kit (Human CD19 MultiSort kit, Miltenyi Biotec) was used for this purpose. Isolation was performed following the manufacturer's instructions. Briefly, PBMCs, which had been thawed and rested overnight, were resuspended in isolation buffer (PBS supplemented with 0.5% [wt/vol] BSA and 2 mM EDTA) and labeled with anti-CD19 antibody coupled to magnetic beads. Labeled cells were then immobilized by passing through a magnetic column. B cell-depleted flowthrough was preserved for assessment of $CD4^+$ T cell responses. Immobilized B cells were washed out of the column and enzymatically released from magnetic beads.

Detection of S-specific memory B cells by flow cytometry. SARS-CoV-2 specific B cells were identified by immunofluorescent tagging with recombinant wild-type SARS-CoV-2 S1 protein (Biotinylated SARS-CoV-2 [COVID-19] S1 protein, Acrobiolabs) for 30 min at 4°C in FACS buffer (PBS supplemented with 2% (vol/vol) FCS, 0.05% (wt/vol) NaN_3 and 2 mM EDTA). To distinguish the unspecific binding of the probe, two different conjugates were used: S1-streptavidin-PE and S1-streptavidin-APC in an equimolar ratio. After 15 min of labeling with S1 protein, the anti-IgG-BV421 antibody (clone G18-145) was added to the cell suspension, and incubation was continued for another 15 min. Cells were then washed with PBS and stained for viability (ZombieAqua, Biolegend) for 15 min at 4°C. To further minimize the nonspecific binding, fluorescently labeled antibody cells were incubated with a cocktail of antibodies blocking human Fc receptors (FcR block, Miltenyi Biotec) for 10 min at 4°C. After blocking the Fc receptors, a mixture of fluorescently labeled antibodies binding to surface antigens of B cells was added; anti-CD3-BV510 (clone UCHT1), anti-CD19-APC-Cy7 (clone HIB19), anti-CD21-PE-Cy7 (clone Bu32), anti-CD27-BV605 (clone O323), anti-IgM-BV785 (clone MHM-88), anti-IgA-VioBright 515 (clone REA1014). Staining was performed at 4°C for 15 min after which cells were washed with PBS and acquired on a flow cytometer (BD FACS Celesta). Possible longitudinal fluctuations in laser intensity were monitored daily before each experiment using fluorescent beads (Rainbow beads, Biolegend). If needed, PMT voltages were adjusted to ensure constant signal intensity over time. The frequency of S-specific memory B cells was calculated by subtracting the average frequency of S-binding memory B cells in eight healthy donor samples collected before the outbreak of SARS-CoV-2 pandemics. The avidity of the BCR for S1 protein was assessed based on the MFI of fluorescent S1-probe normalized to the MFI of the corresponding BCR isotype.

***Ex vivo* stimulation of $CD4^+$ T cells.** B-cell-depleted PBMC were allowed to rest overnight in R10 media at 37°C and 5% CO_2. Rested PBMC were seeded in 96-well U bottom plates and stimulated with wild-type SARS-CoV-2 PepTivator (Miltenyi Biotec) overlapping peptide pools, spanning the entire sequences of SARS-CoV-2 S, N, or M proteins. One million cells were stimulated per condition and the final concentration of each peptide was 1 μg/mL for all three pools. As a costimulatory signal, BD FastImmune Co-Stimulatory Antibodies (CD28/CD49d) were added to a final concentration of 1 μg/mL. Stimulation was performed at 37°C for a total of 6 h. For each sample, an equally treated DMSO-stimulated negative control was included. Positive controls were stimulated with PMA (20 ng/mL) and ionomycin (1 μg/mL). To inhibit vesicular transport and prevent the secretion of the cytokines, Golgi Stop and Golgi Plug (BD Bioscience) were added to a final concentration of 1 μg/mL 1 h into the stimulation.

Detection of SARS-CoV-2-specific $CD4^+$ T cells by flow cytometry. Following stimulation, cells were transferred to a U-bottom plate, washed with PBS, and stained with Zombie Aqua (Biolegend) dye to discriminate viable cells. The staining was performed for 15 min at 4°C. Subsequently, samples were washed with FACS buffer and stained for extracellular markers for 15 min at 4°C using the following fluorescently

conjugated antibodies: anti-CD8-BV510 (clone RPA-T8; Biolegend) and anti-CD45RO-BV605 (clone UCHL1; Biolegend). After washing with staining buffer, samples were fixed and permeabilized in CytoFix/CytoPerm Solution (BD Bioscience) for 20 min at 4°C, washed with 1× Perm/Wash Buffer (BD Bioscience), and stained for intracellular markers for 15 min at 4°C. Following antibody conjugates were used; anti-CD3-APC-Cy7 (clone UCHT1; Biolegend), anti-CD4-BV786 (clone SK3; BD Bioscience), anti-IFN-γ-PE (clone B27; Biolegend), anti-CD40L-CF594 (clone TRAP1; BD Bioscience), anti-IL-4-BV421 (clone MP4-25D2; Biolegend), anti-IL-13-BV421 (clone JES10-5A2; Biolegend) and anti-IL-21-AF647 (clone 3A3-N2; Biolegend). Finally, cells were washed 3 times with PBS and acquired on FACS Celesta (BD Bioscience). Frequencies of antigen-specific $CD4^+$ T cells were calculated as negative-control-subtracted data. Possible longitudinal fluctuations in laser intensity were monitored daily before the experiment using fluorescent beads (Rainbow beads, Biolegend). If needed PMT voltages were adjusted to ensure constant signal intensity over time. The data were analyzed with the FlowJo Software version 10.0.7 (TreeStar).

Statistical analysis. Statistical analysis and graphing of the data were performed using GraphPad Prism and R software (51). Differences between the groups were assessed using the Mann-Whitney test or Wilcoxon test for matched data. The strength of correlations was evaluated by Spearman's test. Statistical significance is indicated by the following annotations: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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