SARS-CoV-2 Immunity following different combinations of vaccination and breakthrough infection

Jernej Pusnik
Institute of Virology, University Hospital, University of Bonn, Germany and German Center for Infection Research (DZIF), partner site Bonn-Cologne

Werner O. Monzon-Posadas
Occupational Medicine Department, University Hospital Bonn, Germany

Jasmin Zorn
Institute of Virology, University Hospital, University of Bonn, Germany and German Center for Infection Research (DZIF), partner site Bonn-Cologne

Kathrin Peters
Institute of Virology, University Hospital, University of Bonn, Germany and German Center for Infection Research (DZIF), partner site Bonn-Cologne https://orcid.org/0000-0003-0901-767X

Maximilian Baum
Institute of Virology, University Hospital, University of Bonn, Germany and German Center for Infection Research (DZIF), partner site Bonn-Cologne

Hannah Proksch
Institute of Virology, University Hospital, University of Bonn, Germany and German Center for Infection Research (DZIF), partner site Bonn-Cologne

Celina Beta Schlüter
Institute of Virology, University Hospital, University of Bonn, Germany and German Center for Infection Research (DZIF), partner site Bonn-Cologne

Galit Alter
Ragon Institute of MGH, MIT, and Harvard https://orcid.org/0000-0002-7680-9215

Tanja Menting
Occupational Medicine Department, University Hospital Bonn, Germany

Hendrik Streeck (hstreeck@uni-bonn.de)
Institute of Virology, University Hospital, University of Bonn, Germany and German Center for Infection Research (DZIF), partner site Bonn-Cologne https://orcid.org/0000-0002-0335-6390

Article

Keywords: SARS-CoV-2, immunity, COVID-19, B cells, spike, T cell, antibody, neutralization, breakthrough infection, vaccination, immunization history

Posted Date: September 27th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2053251/v1

Abstract

The elicited anti-SARS-CoV-2 immunity worldwide is becoming increasingly complex with individuals receiving a different amount of vaccine doses paired with or without recovery from breakthrough infections with different variants. To understand the variety of anti-SARS-CoV-2 immunity we analyzed the adaptive immune responses of individuals that initially received 2 doses of mRNA vaccine and either received a booster vaccination, recovered from a breakthrough infection, or both. Our data suggest that two vaccine doses and delta breakthrough infection or three vaccine doses and optionally omicron or delta infection provide better B cell immunity than the initial 2 doses of mRNA vaccine with or without alpha breakthrough infection. A particularly potent humoral response against the currently circulating omicron variant was observed for the thrice vaccinated individuals with omicron breakthrough infection; a 46-fold increase in neutralization compared to 2 vaccine doses ($P < 0.0001$). The same group also had the highest titer of spike-specific antibodies in saliva and highest frequency of spike-specific memory B cells in peripheral blood; 9.5-fold ($P < 0.001$) and 3.5-fold ($P < 0.05$) increase compared to 2 vaccine doses. The T cell response after two vaccine doses was not significantly influenced by additional immunizations. Of note, individuals with hybrid immunity showed better coordination of the adaptive immune response compared to those only vaccinated. Taken together, our data provide a detailed insight into the SARS-CoV-2 immunity following different immunization scenarios.

Introduction

The worldwide vaccination campaign against SARS-CoV-2 infections demonstrated outstanding results in cutting down the severity of infections [1–3]. The first approved vaccines were based on the novel mRNA technology and triggered a robust immune response resulting in high protection efficiency [4–7]. This was reflected in the low frequency of breakthrough infections among the vaccinated, which were mostly caused by the alpha variant, and resulted in mild disease [8, 9]. Soon it however became clear that similar to infection-induced immunity, the immune response to SARS-CoV-2 vaccines relatively rapidly declines below the level required for protection from infection [10, 11]. At the time when the first vaccines were put in use, the SARS-CoV-2 pandemics were already established in most of the populated regions on earth. With several 100,000 new infections per week globally [12], the virus had plenty of space to mutate and adapt in a way that would overcome the immune protection of the vaccinated population. The waning immunity and the emergence of new viral variants like delta fuelled the increasing frequency of breakthrough infections [13, 14]. Although severe Covid-19 cases were less frequent in previously vaccinated individuals it became clear that booster vaccinations will be needed to curb the SARS-CoV-2 transmission [15, 16]. In developed countries like Germany, the majority of the vaccinated individuals also received a third vaccine dose significantly improving the immune response [17, 18]. However, in particular against the omicron variants the induced immunity does not offer protection from infection.

While SARS-CoV-2 infection of vaccinated individuals can have in rare cases a more severe disease course, most of the infections resolve without life-threatening consequences [19]. Importantly, these breakthrough infections further strengthen the immunity established by vaccination. Studies have shown

that vaccinated individuals that also recovered from a SARS-CoV-2 infection have an immune response that is approximately equal to that after the third vaccination, and are therefore better protected from the disease than twice vaccinated individuals [20–23]. Furthermore, breakthrough infections compensate for the waning of immunity established by previous infection or vaccination [20]. However, there is a large heterogeneity among the vaccinated individuals with breakthrough infection in terms of the number of immunization and variants that cause the infection. We postulated that individuals that initially received two doses of mRNA vaccine and later got infected with different variants and/or received a third vaccine dose have distinct immunity profiles depending on the immunization. We compared the antibody levels, B cell, and T cell responses among the individuals belonging to seven different groups based on the immunization history: 2-times vaccinated (2xVacc), 2-times vaccinated followed by alpha breakthrough infection (2xVacc + α), 2-times vaccinated followed by delta breakthrough (2xVacc + δ), 3-times vaccinated (3xVacc), 3-times vaccinated followed by omicron infection (3xVacc + o), 3-times vaccinated where the third dose was preceded by an alpha infection (3xVacc + α), and 3-times vaccinated where the third dose was preceded by a delta infection (3xVacc + δ). In most cases, the immune response was augmented following the third immunization and did not further increase after the fourth immunization. Individuals with delta and omicron breakthrough infections had more robust immunity than those who recovered from alpha breakthrough infection.

Materials And Methods

Study cohort

A total of 110 individuals that were initially immunized with 2 doses of mRNA-based SARS-CoV-2 vaccine and subsequently infected and/or vaccinated were recruited for the study. All individuals were sampled 2–9 weeks following the last immunization. Breakthrough infections were confirmed by RT-PCR and the viral RNA was sequenced as a part of routine SARS-CoV-2 variant monitoring at the diagnostics department of the Institute of Virology, University Hospital Bonn. All participants were either employed or studied at the University of Bonn at the time of sampling but were not necessarily healthcare workers.

Ethics approval

All participants provided written informed consent approved by the Ethics Committee of the Medical Faculty of the University of Bonn (ethics approval numbers 125/21).

Sample collection and storage

Study participants provided peripheral blood specimens, saliva, 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.

Determination of SARS-CoV-2 S- and N-specific antibodies in plasma

N-specific antibody levels were assessed using the Roche Cobas® SARS-CoV-2 assay following the manufacturer's protocol. For the determination of S1-specific IgG, an in-house quantitative ELISA was used. For that 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). Coated plates were covered and incubated overnight at 4°C. After washing with wash buffer (PBS with 0.05% (v/v) Tween®-20) plates were blocked (PBS containing 1% (w/v) BSA) to prevent unspecific binding. Cryopreserved EDTA plasma samples were thawed and diluted 1:3200 in blocking buffer. Blocked plates were washed, incubated with plasma and standard samples, washed again, and incubated with 100 µl HRP-conjugated anti-IgG antibody (Goat anti-Human IgG (Heavy chain) Secondary Antibody, HRP, Invitrogen) diluted 1:8000 in wash buffer. Incubation steps were performed for 1 hour at 37°C. Afterward, plates were washed and 100 µl of the substrate solution was added (TMB Chromogen Solution, Life technologies). The reaction took place at room temperature for 5 min until the addition of 50 µl of 0.2 M H_2SO_4. Finally, optical density at 450 nm was measured. The background-subtracted OD_{450} readings were interpolated to the standard dilution curve calibrated to the international WHO standard (NIBSC reference number: 20/136). The positivity cutoff was determined based on measurements of plasma samples from healthy individuals collected before the Covid-19 outbreak.

Determination of SARS-CoV-2 S-specific IgG and IgA in saliva

The relative amounts of S1-specific IgA and IgG in saliva were measured by in-house quantitative ELISA. High-binding microtiter plates 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). After overnight incubation at 4°C the plates were washed (PBS with 0.05% (v/v) Tween®-20), blocked (PBS containing 3% (w/v) BSA), and washed again. Frozen saliva samples were thawed, diluted in sample buffer (PBS containing 1% (w/v) BSA), and pipetted onto the plate. Following incubation with saliva samples and standard dilutions plates were washed and incubated with 100 µl HRP-conjugated anti-IgG antibody (Goat anti-Human IgG (Heavy chain) Secondary Antibody, HRP, Invitrogen) diluted 1:8000 in wash buffer or 100 µl HRP-conjugated anti-IgA antibody (Goat anti-Human IgA (Heavy chain) Secondary Antibody, HRP, Invitrogen) diluted 1:1000 in wash buffer. Plates were then washed and 100 µl of substrate were added (TMB ELISA Substrate, High Sensitivity, Abcam). The reaction took place at room temperature for 5 min, followed by the addition of 100 µl of 1 M H_2SO_4. Finally, optical density at 450 nm was measured. The background-subtracted OD_{450} readings were interpolated to the standard dilution curve. The same concentrations of S1-specific monoclonal antibody (anti-SARS-CoV-2-RBD antibody, clone CR3022, Abcam) with IgA or IgG constant region was measured to make the OD_{450} readings comparable between the assays.

Plaque reduction neutralization assay

The neutralization capacity of plasma samples was determined by a plaque reduction neutralization assay as previously described [24]. In brief, plasma was heat-inactivated and serially two-fold diluted

starting with 2-fold up to 32768-fold dilution. Each dilution was combined with 80 plaque-forming units of SARS-CoV-2 (either wild-type, delta, or omicron variant) and added to Vero E6 cells. After the removal of the inoculum cells were overlaid with carboxymethylcellulose-containing media. Following three days of incubation, plates were fixed and stained with crystal violet solution 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.

B cell isolation

B cells were enriched from cryopreserved PBMC samples by immunomagnetic isolation (Human CD19 MultiSort Kit, Miltenyi Biotec). Isolation was performed following the manufacturer's instructions. Briefly, PBMCs, which had been thawed and rested overnight, were resuspended in recommended isolation buffer and labeled with anti-CD19 antibody coupled to magnetic beads. Labeled cells were then immobilized onto a magnetic column. B cell-depleted flow-through was used for the assessment of CD4 + T cell responses. Immobilized B cells were washed out of the column and enzymatically released from magnetic beads.

Detection of S1-specific memory B cells by flow cytometry

SARS-CoV-2 S1-specific B cells were identified by immunofluorescent tagging with recombinant wild-type SARS-CoV-2 S1 protein as previously described [24]. Briefly, the cells were incubated with the S1 protein conjugated to two different fluorophores. Afterward, the cells were washed, stained for viability, washed, and incubated with an antibody mixture blocking the Fc receptors and a mixture of fluorescently labeled antibodies binding to surface antigens. Following incubation, the cells were washed again and acquired on a flow cytometer (BD FACS Celesta). 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.

Ex vivo stimulation of T cells

Overnight-rested B-cell-depleted 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 or N proteins, in presence of anti-CD107a-APC (clone H4A3; Biolegend) antibody. One million cells were stimulated per condition and the final concentration of each peptide was 1 µg/ml for both peptide pools. Co-stimulatory antibodies (BD FastImmune™ D28/CD49d) were added to a final concentration of 1 µg/ml. Stimulation was performed at 37°C for 6 hours. For each sample, an equally treated DMSO-stimulated negative control was included. As positive control cells were stimulated with PMA (20 ng/ml) and ionomycin (1 µg/ml). One hour into stimulation Golgi Stop and Golgi Plug (BD Bioscience) were added (final concentration 1 µg/ml) to inhibit vesicular transport and prevent the secretion of the cytokines.

Detection of SARS-CoV-2-specific T cells by flow cytometry

Following stimulation, cells were 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, fixed, and permeabilized in CytoFix/CytoPerm Solution (BD Bioscience) for 15 min at 4°C. Fixed cells were then washed with 1x Perm/Wash Buffer (BD Bioscience), and stained for intracellular markers for 15 min at 4°C using the following antibody conjugates; anti-CD3-APC-Cy7 (clone UCHT1; Biolegend), anti-CD4-BV786 (clone SK3; BD Bioscience), anti-IFNγ-PE (clone B27; Biolegend), anti-TNFα-BV421 (clone Mab11; Biolegend), and anti-IL2-AF488 (clone MQ1-17H12; Biolegend). Finally, cells were washed 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 was performed using R software [25]. Differences between the groups were assessed using the Mann-Whitney test or Wilcoxon test for matched data with Holm's correction for multiple testing. 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.

Results

Not only the number but also the type of immunization is important for potent humoral immunity against the SARS-CoV-2

Antibodies are the best-defined correlate of protection against the SARS-CoV-2 infection, therefore, we first investigated levels of neutralizing antibodies among individuals with different immunization histories. Based on the immunization status we defined the following groups: 2-times vaccinated (2xVacc, n = 54), 2-times vaccinated followed by alpha breakthrough infection (2xVacc + α, n = 7), 2-times vaccinated followed by delta breakthrough (2xVacc + δ, n = 13), 3-times vaccinated (3xVacc, n = 23), 3-times vaccinated followed by omicron infection (3xVacc + o, n = 10), 3-times vaccinated where the third dose was preceded by an alpha infection (3xVacc + α, n = 7), and 3-times vaccinated where the third dose was preceded by a delta infection (3xVacc + δ, n = 7) (Fig. 1a).

Determination of plasma IgG titer against the S1 subunit of the SARS-CoV-2 spike protein was carried out by the in-house ELISA calibrated to the international WHO standard (NIBSC reference number: 20/136). The data revealed that 2xVacc + δ, 3xVacc, 3xVacc + o, and 3xVacc + δ groups had significantly higher S1-specific IgG titers compared to the baseline immunization 2xVacc group and the groups with an alpha breakthrough infection; 2xVacc + α, 3xVacc + α (Fig. 1b). The largest difference (5.2-fold, P < 0.0001) was observed between the 2xVacc + δ and 2xVacc groups. To investigate whether the same pattern can be observed for only neutralizing antibodies we performed plaque reduction assays using live

unmanipulated SARS-CoV-2 isolates (wild-type, delta, and omicron variants). We observed similar differences between the groups as for the S1-binding IgG measured by ELISA for all variants. Of note, the 2xVacc + δ group showed particularly high neutralization potency against the wild-type (6.5-fold compared to 2xVacc, $P < 0.0001$) and delta variants (7.8-fold compared to 2xVacc, $P < 0.0001$), while the 3xVacc + o group was the most efficient at neutralizing the omicron variant (46-fold compared to 2xVacc, $P < 0.0001$) (Fig. 1c). Comparing the three SARS-CoV-2 variants and their susceptibility to neutralization we observed that the omicron variant was significantly more resistant to neutralization than delta and wild-type. The most profound reduction in the neutralization capacity against the omicron variant was observed for the 2xVacc and 2xVacc + α groups (16-fold and 20-fold respectively when compared to the wild-type). The only exception was the 3xVacc + o group that equally neutralized all three variants. Importantly, delta showed significantly higher resistance to neutralization than the wild-type exclusively in the case of the groups without breakthrough infections; 2xVacc, 3xVacc (Fig. 1d). To assess the relationship between the S1-specific IgG levels and neutralization capacity against the wild-type virus we correlated the two parameters for each of the immunization groups. The level of S1-binding antibodies correlated with neutralization regardless of the immunization history. The strongest correlations were observed in case of the 2xVacc + α ($r = 0.89$, $p = 0.012$), 3xVacc + α ($r = 0.93$, $p = 0.0067$), 2xVacc + δ ($r = 0.93$, $p < 0.0001$), and 3xVacc + δ ($r = 0.82$, $p = 0.034$) groups (Fig. 1e). Moreover, we correlated the S1-specific IgG titers with neutralization of the delta variant. The groups with the strongest correlations were in this case 2xVacc + δ ($r = 0.93$, $p < 0.0001$), 3xVacc + δ ($r = 0.86$, $p = 0.024$), and 2xVacc + α ($r = 0.89$, $p = 0.012$) (Fig. 1f). Correlations with neutralization of the omicron variant were notably weaker compared to the other two variants and significant only for the 3 out of 7 groups; 2xVacc, 2xVacc + δ, and 3xVacc + o. Relatively strong associations were observed only for the 2xVacc + δ ($r = 0.81$, $p = 0.0014$) and 3xVacc + o ($r = 0.81$, $p = 0.0082$) groups (Fig. 1f). Furthermore, we assessed the presence of antibodies specific for the nucleocapsid (N) protein of the SARS-CoV-2 using a commercial Roche Cobass assay. Complying with findings for the S1-specific IgG levels we observed that 77% of the delta, and 100% of omicron, but only 33% of the alpha breakthrough infections led to seroconversion.

Taken together our findings demonstrate that two vaccine doses and delta breakthrough infection or three vaccine doses and optionally omicron or delta infection provide significantly better humoral immunity to SARS-CoV-2 infection compared to the baseline immunization with 2 doses of mRNA vaccine. A particularly strong humoral response against different SARS-CoV-2 variants was observed among twice-vaccinated individuals that got infected with the delta variant and thrice-vaccinated individuals that recovered from omicron breakthrough infection.

Salivary S1-specific antibody levels are highly dependent on the immunization scenario

SARS-CoV-2 initially replicates in the upper respiratory tract where it is exposed to antibodies present in mucosa and saliva [26]. Therefore, the SARS-CoV-2-specific antibody titer in saliva might be a better correlate of protection from infection than the level of antibodies in plasma [27, 28]. To determine the titer of IgG and IgA specific for the S1 subunit of the SARS-CoV-2 spike protein we developed an ELISA detecting comparatively low amounts of antibodies present in saliva. We demonstrated that similar to S1-

specific plasma IgG levels, 2xVacc + δ, 3xVacc, 3xVacc + o, and 3xVacc + δ groups had significantly higher IgG levels compared to the baseline 2xVacc group, while the groups with an alpha breakthrough infection; 2xVacc + α, 3xVacc + α had relatively few S-specific IgG in saliva. Particularly high S1-specific IgG levels were observed in the 3xVacc + o group which had a 13-fold higher titer than the baseline 2xVacc group ($P < 0.0001$) (Fig. 2a). In contrast to IgG, salivary S1-specific IgA levels were only significantly increased in the case of 2xVacc + δ and 3xVacc + o groups (Fig. 2b). Since we used the same monoclonal antibody with variable Fc region as a calibrator for the IgG and IgA ELISAs we were able to compare the relative binding strengths of the two antibody isotypes. The highest cumulative binding strength of S1-specific IgG and IgA was observed for the 3xVacc + o group (a 9.5-fold increase compared to 2xVacc; $P < 0.001$) followed by 2xVacc + δ, 3xVacc, and 3xVacc + δ. 2xVacc and 2xVacc + α groups were significantly lower than most of the other groups (Fig. 2c). Regardless of the immunization group, IgG showed a stronger binding capacity than IgA. The highest proportion of IgA binding was observed for the 2xVacc group and the lowest for the 3xVacc group (Fig. 2d). We next investigated whether S1-specific IgG levels in saliva reflect those measured in plasma. This was partially true since we only observed significant correlations for the 2xVacc ($r = 0.28$, $p = 0.044$), 2xVacc + α ($r = 0.93$, $p = 0.0067$) and 2xVacc + δ ($r = 0.75$, $p = 0.012$) groups (Fig. 2e).

Collectively, we have shown that the individuals that received 3 vaccine doses and afterward acquired an omicron infection show the highest S1-specific antibody titer in saliva. Interestingly, IgG represented the majority of S1-binding antibodies in saliva and its levels correlated with S1-specific plasma IgG only in case of particular immunization combinations.

The frequency of S1-specific memory B cells is influenced by the immunization history

When assessing the quality of SARS-CoV-2 immunity it is important to not only consider the humoral but also cellular components that are particularly important for the long-term protection from the disease [29].

We, therefore, measured the frequency of S1-specific memory B cells in the peripheral blood of individuals with different immunization histories utilizing multiparameter flow-cytometry (Fig. 3a). Our data demonstrate increased frequencies of IgG + S1-specific memory B cells for 2xVacc + δ, 3xVacc, and 3xVacc + o groups when compared to the 2xVacc and 2xVacc + α groups. In the case of the 3xVacc + δ and 3xVacc + α groups, the frequencies of these cells were relatively low and comparable to the baseline 2xVacc group (Fig. 3b). Similar differences between the immunization groups were also observed for the IgA + and IgM + S1-specific memory B cells but were not statistically significant due to the high variability among individuals (Fig. 3c and 3d respectively). When considering the frequency of the total S1-specific memory B cells, regardless of the B cell receptor (BCR) isotype, the 3xVacc + o group had the highest frequency of these cells (a 3.5-fold increase compared to the 2xVacc group; $P < 0.05$) followed by 2xVacc + δ and 3xVacc groups. The frequencies observed within 3xVacc + δ, 3xVacc + α, and 2xVacc + α groups were comparable to the 2xVacc group (Fig. 3e). We next investigated the relative proportions of S1-specific memory B cells with different BCR isotypes. We observed that IgG + S1-specific memory B cells

were the most frequent for all groups compared. 2xVacc and 2xVacc + α groups had moderately increased levels of IgA + and IgM + S1-specific memory B cells (Fig. 3f).

Taken together our findings demonstrate increased frequencies of S1-specific memory B cells among individuals that either received 3 doses of mRNA vaccine, 3 doses of mRNA vaccine plus omicron infection, or 2 doses of vaccine and delta infection. IgG was the most prevalent BCR isotype of S1-specific memory B cells regardless of the immunization history.

Different immunization combinations do not significantly affect SARS-CoV-2-specific T cells

T cells contribute to the defense against viral infections by coordinating the production of antibodies and killing the infected cells. It has been previously shown that T cells specific for SARS-CoV-2 successfully limit the infection and positively correlate with protection from the severe disease [30, 31].

Given their importance we next measured the frequencies of CD4 + and CD8 + T cells specific for the spike (S) protein of the SARS-CoV-2 in peripheral blood of individuals with different immunization histories. Antigen-specific cells were detected by peptide stimulation and subsequent detection of cytokine expression by multiparameter flow cytometry. Four major functions of the T cells were monitored; cytotoxicity (CD107a and IFNγ expression), IFNγ-expression, IL-2 expression, and TNFα expression (Fig. 4a). Interestingly, we did not observe any significant differences between the immunization groups for the S-specific CD4 + T cells regardless of their function. Nevertheless, a similar pattern was observed in the cases of cytotoxic, IFNγ-expressing and IL-2 expressing CD4 + T cells as for the S1-specific plasma IgG; 2xVacc + δ, 3xVacc, 3xVacc + o, and 3xVacc + δ were higher than the baseline 2xVacc group and the groups with an alpha breakthrough infection; 2xVacc + α, 3xVacc + α (Fig. 4b). Also, the frequencies of S-specific CD8 + T cells were not significantly different between the immunization groups. However, the 3xVacc + o group had the highest median frequency of those cells for all assessed functions (Fig. 4c). We have previously demonstrated that unvaccinated individuals infected with SARS-CoV-2 develop high frequencies of the nucleocapsid-specific T cells [24, 32]. To check whether this is also true for breakthrough infections we stimulated the peripheral blood T cells with peptides spanning the entire sequence of the SARS-CoV-2 nucleocapsid (N) protein. Antigen-specific cells were detected by monitoring the same functions as for the S protein. Strikingly, we observed no significant differences between the immunization groups with and without breakthrough infections indicating that previously vaccinated individuals do not efficiently develop N-specific T cell responses. This was true for both CD4+ (Fig. 4d) and CD8+ (Fig. 4e) T cells. Regarding the overall SARS-CoV-2-specific T cell response (not discriminating the CD4 + and CD8 + T cell or their specificity for S or N proteins) 3xVacc group had significantly increased frequencies of cytotoxic (1.7-fold; $P < 0.01$) and IFNγ-expressing (2.1-fold; $P < 0.01$) cells compared to the 2xVacc group (Fig. 4f). The largest proportion of SARS-CoV-2-specific cytotoxic T cells represented CD4 + S-specific T cells followed by CD8 + S-specific T cells, CD8 + N-specific T cells, and CD4 + N-specific T cells respectively for all groups. Similar was true for the IFNγ-expressing cells. In the case of IL-2- and TNFα-expressing T cells CD4 + cells specific for the S-protein represented the large majority of the response. Comparing the immunization groups 2xVacc group had a relatively high

proportion of the CD8 + T cells specific for the S-protein, while the 3xVacc + o group had increased levels of N-specific CD8 + and CD4 + T cells (Fig. 4g). Most of the study participants had detectable S-specific CD4 + T cells for all of the measured functions. Particularly high proportions were observed within the 3xVacc, 3xVacc + o, and 3xVacc + δ groups. The second most frequent response was CD8 + T cells specific for the S-protein. Here, the 3xVacc + o, 3xVacc + α, and 3xVacc + δ groups harbored the highest percentage of responders. More rare were individuals with detectable N-specific CD4 + and CD8 + T cells, especially within the 2xVacc and 2xVacc + α groups (Fig. 4h).

To sum up, additional immunizations of twice vaccinated individuals do not significantly boost the frequencies of SARS-CoV-2-specific T cells except in the case of three vaccine doses. CD4 + T cell responses were more frequent than CD8 + T cells and more T cells were specific for S than N protein. Individuals with omicron breakthroughs had an increased proportion of N-specific T cells.

Individuals with breakthrough infections have a better coordinated adaptive immune response

The quality of immunization is not only dependent on the magnitudes of individual immune components but also on their coordination. Apart from antibodies, many studies have demonstrated the importance of SARS-CoV-2-specific memory B and T cells [29–31]. Considering their distinct mechanism of action a multilayer immune response might be more effective at preventing SARS-CoV-2 infection.

We, therefore, correlated the measured immune parameters including antibody, memory B cell, and memory T cell responses for each of the seven groups with different immunization histories. For the 2xVacc and 3xVacc groups, we observed a moderate to low degree of correlation among the parameters defining antibody response and among the parameters defining the T cell response. The rest of the immune response was poorly correlated (Fig. 5). For the rest of the immunization groups, all with breakthrough infections, we observed a strong to moderate degree of correlation within the antibody compartment, and also the T cell compartment. For the 3xVacc + α and 3xVacc + δ groups, a considerable proportion of correlations among the T cell parameters was inverse (Fig. 5). Strong correlations between the antibodies and T cells and antibodies and memory B cells were mostly observed in the cases of the 2xVacc + δ, 3xVacc + δ, 2xVacc + α, and 3xVacc + α groups. Moreover, groups with breakthrough infections showed a higher degree of correlation between the memory B cell and T cell parameters compared to only vaccinated groups. Of note, 3xVacc + o, 3xVacc + δ, and 3xVacc + α groups had a high proportion of inverse correlations (Fig. 5). The parameters defining memory B cell response were weakly correlated regardless of immunization group (Fig. 5).

Taken together, our data suggest improved coordination of immune response among the individuals with breakthrough infection when compared to those only vaccinated. Moreover, individuals with four immunizations had a higher proportion of inverse correlations.

Discussion

There is increasing evidence that multiple immunization events might be needed for robust immunity against SARS-CoV-2 [16, 18, 20, 23]. Furthermore, studies have suggested that hybrid immunity in terms of vaccination and infection offers improved protection from the disease [33, 34]. It is, however, not clear how different combinations of vaccination and breakthrough infection with SARS-CoV-2 variants shape the immune response. Here we systematically compared the antibody, memory B cell, and memory T cell responses of individuals that initially received 2 doses of mRNA vaccine and were later boosted by either breakthrough infection, vaccination, or both. We discriminated the infections with wild-type, delta, and omicron variants. Our findings suggest augmented immune response among twice vaccinated individuals that recovered from the delta variant infection and thrice vaccinated individuals that recovered from omicron breakthrough infection.

The assessment of the SARS-CoV-2 immunity most often relies on the measurement of spike-specific antibodies in plasma. We have shown that, following the initial two doses of mRNA vaccine, a breakthrough infection with delta, or a third vaccine dose with or without additional omicron or delta breakthrough significantly boosts the production of spike-specific and neutralizing antibodies. Conversely, the alpha breakthrough infection did not significantly enhance antibody production. Furthermore, we have shown that the group with delta variant breakthrough infection most efficiently neutralized delta SARS-CoV-2 and the group with omicron breakthrough the omicron virus suggesting that the pre-existing immunity is shaped by the variant causing breakthrough infection. Further supporting this hypothesis, individuals who recovered from the infection with a particular variant showed a stronger correlation between the spike-specific antibody levels and neutralization against that variant. In line with previous studies, the currently circulating omicron variant was significantly more resistant to neutralization than wild-type or delta and delta was more resistant to neutralization than wild-type for vaccinated individuals only [35, 36]. The only exception was the group with the omicron breakthrough that equally neutralized all three variants. Overall, these findings suggest superior humoral immunity against SARS-CoV-2 for vaccinated individuals with delta or omicron breakthrough infections.

When evaluating protection from SARS-CoV-2 infection, the measurement of antibodies in saliva might be more informative since they can neutralize the virus immediately after it enters the upper respiratory tract [27, 28]. We demonstrated increased levels of salivary anti-spike antibodies for the same immunization groups as in the case of plasma IgG. Exceptionally high levels of spike-specific IgG and IgA in saliva were observed for the three times vaccinated individuals that recovered from omicron infection. This may be due to the increased replication of omicron in the upper respiratory tract compared to the previous variants and consequently stronger stimulation of the local mucosal immunity [37, 38]. Moreover, given the abundance of IgG in comparison to IgA in the saliva our data demonstrate that the majority of the spike binding activity in saliva was by IgG and not IgA antibodies regardless of the immunization history. The highest proportion of IgA spike binding was among the twice vaccinated individuals, while the third vaccination did not lead to a further increase in IgA immunity. Interestingly, we observed that salivary spike-specific IgG does not correlate well with the plasma IgG levels for most of the immunization groups, suggesting that a significant part of the salivary IgG is locally produced.

Following the SARS-CoV-2 infection or vaccination, the antibody levels rapidly decline increasing the chance of breakthrough infections. This is, however, not true for the memory B cells that are more persistent and therefore particularly important for long-term protection against severe SARS-CoV-2 infection [24, 29]. We found that most of the spike-specific memory B cells found in the peripheral blood of vaccinated individuals with or without breakthrough infections bear IgG BCR. The highest frequencies of those cells were observed for the groups with strong antibody responses except for the thrice vaccinated group that recovered from delta infection. Together with the antibody measurements, this implies that the fourth immunization does not further boost the B cell immunity except in the case of the breakthrough with a genetically distinct omicron variant.

Besides antibodies, T cells represent an important mechanism for limiting viral infections and were previously associated with protection from SARS-CoV-2 infection [30, 31]. Importantly, the frequency of these cells remains at elevated levels for a longer time after infection or vaccination than the antibody level [24, 39]. Our data show that further immunizations, either by infection or vaccination, of the twice vaccinated individuals, mostly do not improve the SARS-CoV-2-specific T cell response. The only exception was the thrice vaccinated group that had significantly elevated cytotoxic and IFNγ-secreting SARS-CoV-2-specific T cells. Of note, SARS-CoV-2-specific CD4 + T cells were considerably more frequent than the CD8 + T cells and T cells specific for the spike protein prevailed over those recognizing the nucleocapsid protein. This is likely because most immunizations for either of the groups were vaccinations with the spike encoding mRNA vaccine. Interestingly, some of the vaccinated individuals without breakthrough infection had detectable nucleocapsid-specific T cells which could be explained by the high levels of pre-existing cross-reactive T cells previously documented among SARS-CoV-2 naïve individuals [31, 40]. To sum up, individuals boosted by infection or vaccination do not have significantly augmented T cell immunity compared to the subjects that received only two vaccine doses.

The quality of immunity against viral infections does not only depend on a single but rather on the synchronization of multiple immune mechanisms. We demonstrated that individuals with breakthrough infections have better correlated parameters defining the adaptive immune response than those only vaccinated. Increased coordination of the immune response after breakthrough infection complies with the findings demonstrating better protection among individuals with hybrid immunity [33, 34].

Taken together we compared the adaptive immune response of individuals with different immunization histories in terms of vaccination and breakthrough infection. Our findings suggest that delta but not an alpha breakthrough infection or third vaccination of doubly vaccinated individuals considerably improves the SARS-CoV-2 immunity. Strikingly, the fourth immunization did not further boost the immune response except for the omicron breakthrough infection.

Declarations

Funding

The study was supported financially by the State of North Rhine-Westphalia, Germany (grant number: CPS-1-1C). No other financial support by any third parties was received.

Acknowledgments

We thank the participants of this study that generously provided their samples.

Author contributions

Conceptualization, J.P., W.M.P., H.S; Methodology, J.P., G.A. H.S.; Investigation, J.P., W.M.P., K.P., J.Z., M.B., C.B.S. and H.P.; Resources, J.P., and W.M.P; Writing-Original Draft, J.P.; Writing-Review & Editing, H.S., and G.A.; Funding acquisition, J.P., H.S.; Supervision, H.S.

Declaration of interests

The authors declare no competing interests. The idea, the plan, the concept, the protocol, the conduct, the data analysis, and the writing of the manuscript of this study were independent of any third parties, including the funding agency.

References

1. Eyre, D.W., et al., *Effect of Covid-19 Vaccination on Transmission of Alpha and Delta Variants*. New England Journal of Medicine, 2022. **386**(8): p. 744–756.
2. Prunas, O., et al., *Vaccination with BNT162b2 reduces transmission of SARS-CoV-2 to household contacts in Israel*. Science, 2022. **375**(6585): p. 1151–1154.
3. Macchia, A., et al., *Evaluation of a COVID-19 Vaccine Campaign and SARS-CoV-2 Infection and Mortality Among Adults Aged 60 Years And Older in a Middle-Income Country*. JAMA Network Open, 2021. **4**(10): p. e2130800-e2130800.
4. Turner, J.S., et al., *SARS-CoV-2 mRNA vaccines induce persistent human germinal centre responses*. Nature, 2021. **596**(7870): p. 109–113.
5. Walsh, E.E., et al., *Safety and Immunogenicity of Two RNA-Based Covid-19 Vaccine Candidates*. New England Journal of Medicine, 2020. **383**(25): p. 2439–2450.
6. Baden, L.R., et al., *Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine*. New England Journal of Medicine, 2020. **384**(5): p. 403–416.
7. Polack, F.P., et al., *Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine*. New England Journal of Medicine, 2020. **383**(27): p. 2603–2615.
8. Peter, J.K., et al., *SARS-CoV-2 Vaccine Alpha and Delta Variant Breakthrough Infections Are Rare and Mild but Can Happen Relatively Early after Vaccination*. Microorganisms, 2022. **10**(5).
9. Rovida, F., et al., *SARS-CoV-2 vaccine breakthrough infections with the alpha variant are asymptomatic or mildly symptomatic among health care workers*. Nature Communications, 2021. **12**(1): p. 6032.

10. Pérez-Alós, L., et al., *Modeling of waning immunity after SARS-CoV-2 vaccination and influencing factors*. Nature Communications, 2022. **13**(1): p. 1614.
11. Levin, E.G., et al., *Waning Immune Humoral Response to BNT162b2 Covid-19 Vaccine over 6 Months*. New England Journal of Medicine, 2021. **385**(24): p. e84.
12. Dong, E., H. Du, and L. Gardner, *An interactive web-based dashboard to track COVID-19 in real time*. The Lancet Infectious Diseases, 2020. **20**(5): p. 533–534.
13. Wang, S.Y., et al., *Severe breakthrough COVID-19 cases in the SARS-CoV-2 delta (B.1.617.2) variant era*. The Lancet Microbe, 2022. **3**(1): p. e4-e5.
14. Bergwerk, M., et al., *Covid-19 Breakthrough Infections in Vaccinated Health Care Workers*. N Engl J Med, 2021. **385**(16): p. 1474–1484.
15. Altmann, D.M. and R.J. Boyton, *Waning immunity to SARS-CoV-2: implications for vaccine booster strategies*. The Lancet Respiratory Medicine, 2021. **9**(12): p. 1356–1358.
16. Menni, C., et al., *COVID-19 vaccine waning and effectiveness and side-effects of boosters: a prospective community study from the ZOE COVID Study*. The Lancet Infectious Diseases, 2022. **22**(7): p. 1002–1010.
17. Impfdashboard.*de*. Available from: https://impfdashboard.de/en/.
18. Chu, L., et al., *Immune response to SARS-CoV-2 after a booster of mRNA-1273: an open-label phase 2 trial*. Nature Medicine, 2022. **28**(5): p. 1042–1049.
19. Bergwerk, M., et al., *Covid-19 Breakthrough Infections in Vaccinated Health Care Workers*. New England Journal of Medicine, 2021. **385**(16): p. 1474–1484.
20. Evans, J.P., et al., *Neutralizing antibody responses elicited by SARS-CoV-2 mRNA vaccination wane over time and are boosted by breakthrough infection*. Science Translational Medicine, 2022. **14**(637): p. eabn8057.
21. Bates, T.A., et al., *Vaccination before or after SARS-CoV-2 infection leads to robust humoral response and antibodies that effectively neutralize variants*. Science Immunology, 2022. **7**(68): p. eabn8014.
22. Walls, A.C., et al., *SARS-CoV-2 breakthrough infections elicit potent, broad, and durable neutralizing antibody responses*. Cell, 2022. **185**(5): p. 872–880.e3.
23. Wratil, P.R., et al., *Three exposures to the spike protein of SARS-CoV-2 by either infection or vaccination elicit superior neutralizing immunity to all variants of concern*. Nature Medicine, 2022. **28**(3): p. 496–503.
24. Pušnik, J., et al., *Persistent Maintenance of Intermediate Memory B Cells Following SARS-CoV-2 Infection and Vaccination Recall Response*. Journal of Virology. **0**(0): p. e00760-22.
25. *R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL* https://www.R-project.org/.
26. V'kovski, P., et al., *Coronavirus biology and replication: implications for SARS-CoV-2*. Nature Reviews Microbiology, 2021. **19**(3): p. 155–170.

27. Russell, M.W., et al., *Mucosal Immunity in COVID-19: A Neglected but Critical Aspect of SARS-CoV-2 Infection*. Frontiers in Immunology, 2020. **11**(3221).
28. Isho, B., et al., *Persistence of serum and saliva antibody responses to SARS-CoV-2 spike antigens in COVID-19 patients*. Science Immunology, 2020. **5**(52): p. eabe5511.
29. Terreri, S., et al., *Persistent B cell memory after SARS-CoV-2 vaccination is functional during breakthrough infections*. Cell Host Microbe, 2022. **30**(3): p. 400–408.e4.
30. Moss, P., *The T cell immune response against SARS-CoV-2*. Nature Immunology, 2022. **23**(2): p. 186–193.
31. Kundu, R., et al., *Cross-reactive memory T cells associate with protection against SARS-CoV-2 infection in COVID-19 contacts*. Nature Communications, 2022. **13**(1): p. 80.
32. Pušnik, J., et al., *Memory B cells targeting SARS-CoV-2 spike protein and their dependence on CD4(+) T cell help*. Cell Rep, 2021. **35**(13): p. 109320.
33. Andreano, E., et al., *Hybrid immunity improves B cells and antibodies against SARS-CoV-2 variants*. Nature, 2021. **600**(7889): p. 530–535.
34. Crotty, S., *Hybrid immunity*. Science, 2021. **372**(6549): p. 1392–1393.
35. Rössler, A., et al., *SARS-CoV-2 Omicron Variant Neutralization in Serum from Vaccinated and Convalescent Persons*. New England Journal of Medicine, 2022. **386**(7): p. 698–700.
36. Sheward, D.J., et al., *Neutralisation sensitivity of the SARS-CoV-2 omicron (B.1.1.529) variant: a cross-sectional study*. The Lancet Infectious Diseases, 2022. **22**(6): p. 813–820.
37. Hui, K.P.Y., et al., *SARS-CoV-2 Omicron variant replication in human bronchus and lung ex vivo*. Nature, 2022. **603**(7902): p. 715–720.
38. Menni, C., et al., *Symptom prevalence, duration, and risk of hospital admission in individuals infected with SARS-CoV-2 during periods of omicron and delta variant dominance: a prospective observational study from the ZOE COVID Study*. The Lancet, 2022. **399**(10335): p. 1618–1624.
39. Dan, J.M., et al., *Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection*. Science, 2021: p. eabf4063.
40. Loyal, L., et al., *Cross-reactive CD4 < sup>+</sup > T cells enhance SARS-CoV-2 immune responses upon infection and vaccination*. Science, 2021. **374**(6564): p. eabh1823.

Figures

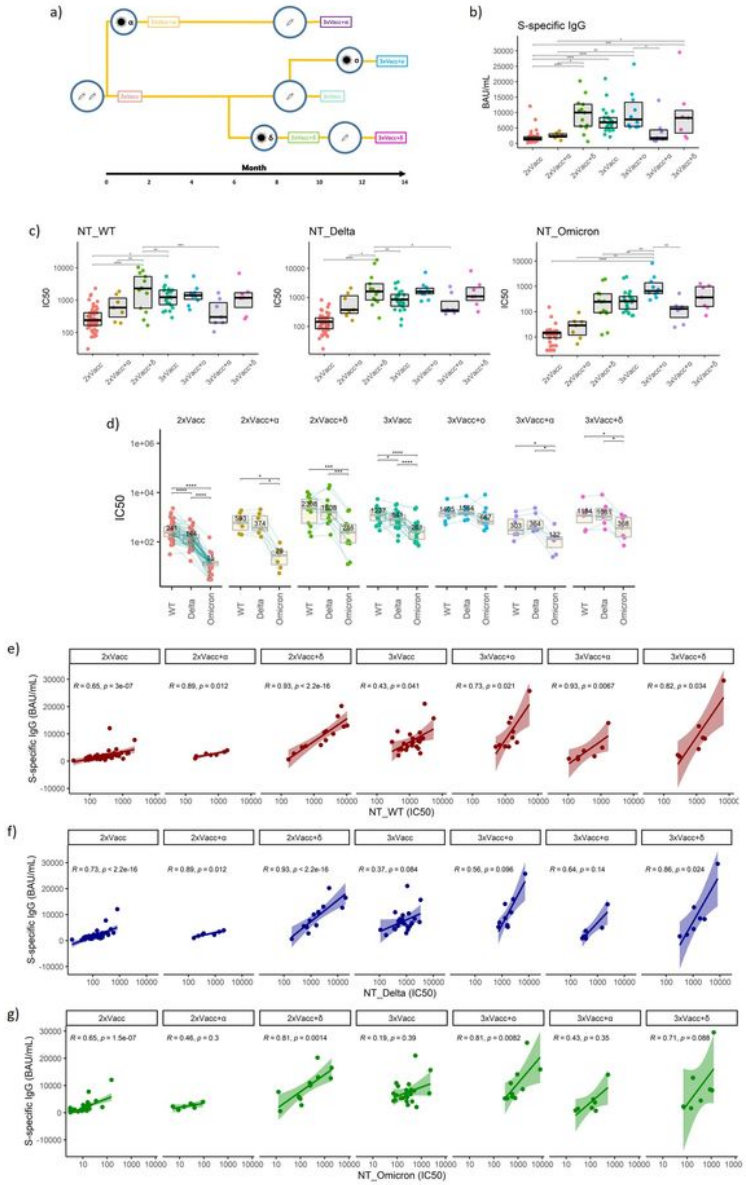

Figure 1

Plasma antibody response after different combinations of vaccination and breakthrough infection. a) Chronological representation of immunization events defining the seven groups compared in this study. b) S1-specific IgG levels in international units measured for the seven groups with different immunization histories. c) Plasma neutralization capacity against wild-type, delta, and omicron variants. d) Comparison of neutralization susceptibility of SARS-CoV-2 variants for each of the analyzed groups. Correlation

between plasma levels of S1-specific IgG and plasma neutralization capacity for e) wild-type, f) delta, g) omicron variants. Differences between the groups were assessed using the Mann-Whitney test or Wilcoxon test for matched data. Correction for multiple testing was performed using Holm's method. The strength of correlations was assessed by Spearman's correlation test.

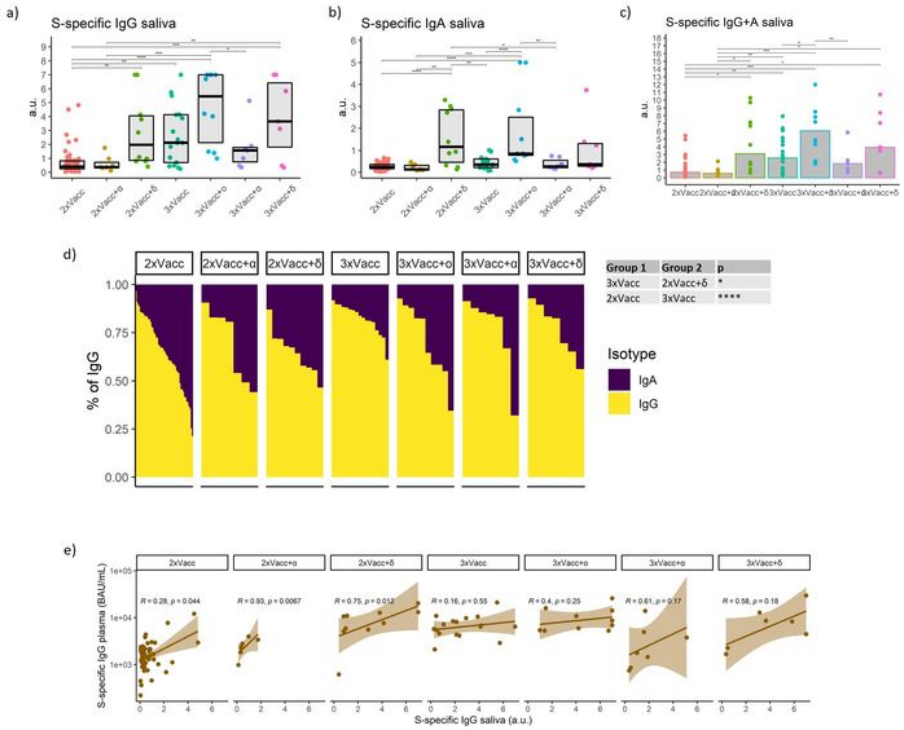

Figure 2

SARS-CoV-2-specific antibodies in saliva. S1-specific a) IgG, b) IgA, and c) IgG+IgA levels in saliva for the seven groups with different immunization histories. d) Relative proportions of IgA and IgG isotypes among the S1-specific antibodies in the saliva of individuals with different immunization histories. e) Correlations between the plasma and salivary S1-specific IgG for the seven immunization groups. Differences between the groups were assessed using the Mann-Whitney test with Holm's correction for multiple testing. The strength of correlations was assessed by Spearman's correlation test.

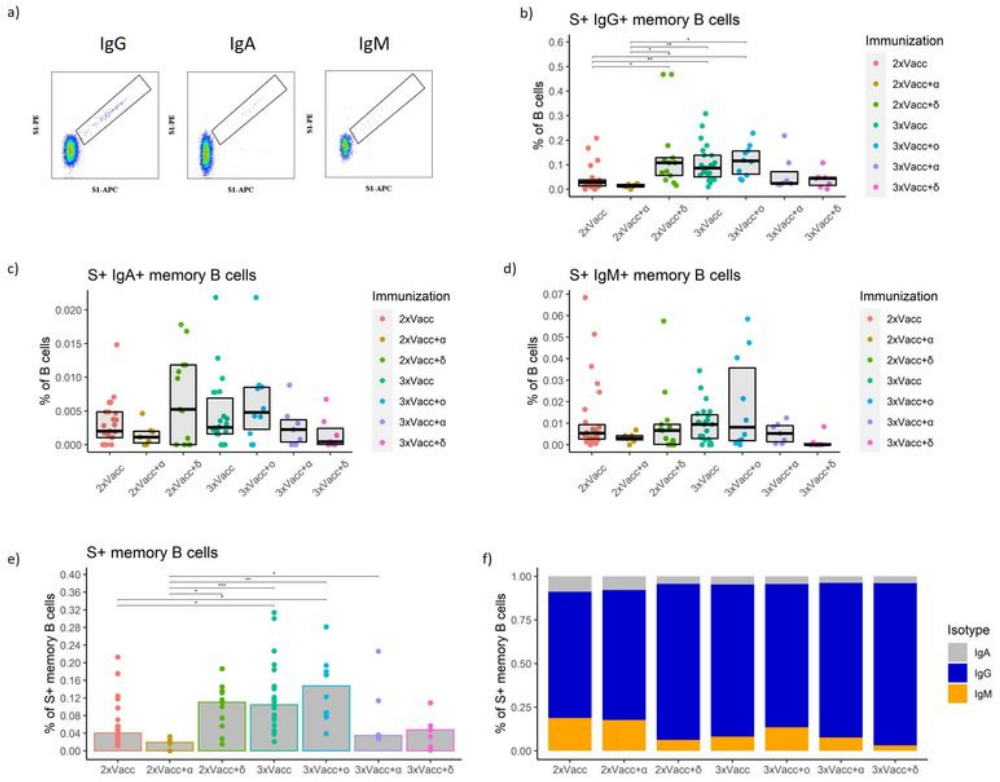

Figure 3

SARS-CoV-2-specific memory B cell response after different combinations of vaccination and breakthrough infection. a) Representative flow cytometry plots for detection of S1-specific memory B cells with different BCRs. Frequencies of b) IgG+, c) IgA+, d) IgM+ and e) total S1-specific memory B cells in peripheral blood of individuals belonging to the seven immunization groups expressed as a percentage of B cells. f) Relative proportions of S1-specific memory B cells bearing BCRs of a different isotype. Differences between the groups were assessed using the Mann-Whitney test with Holm's correction for multiple testing.

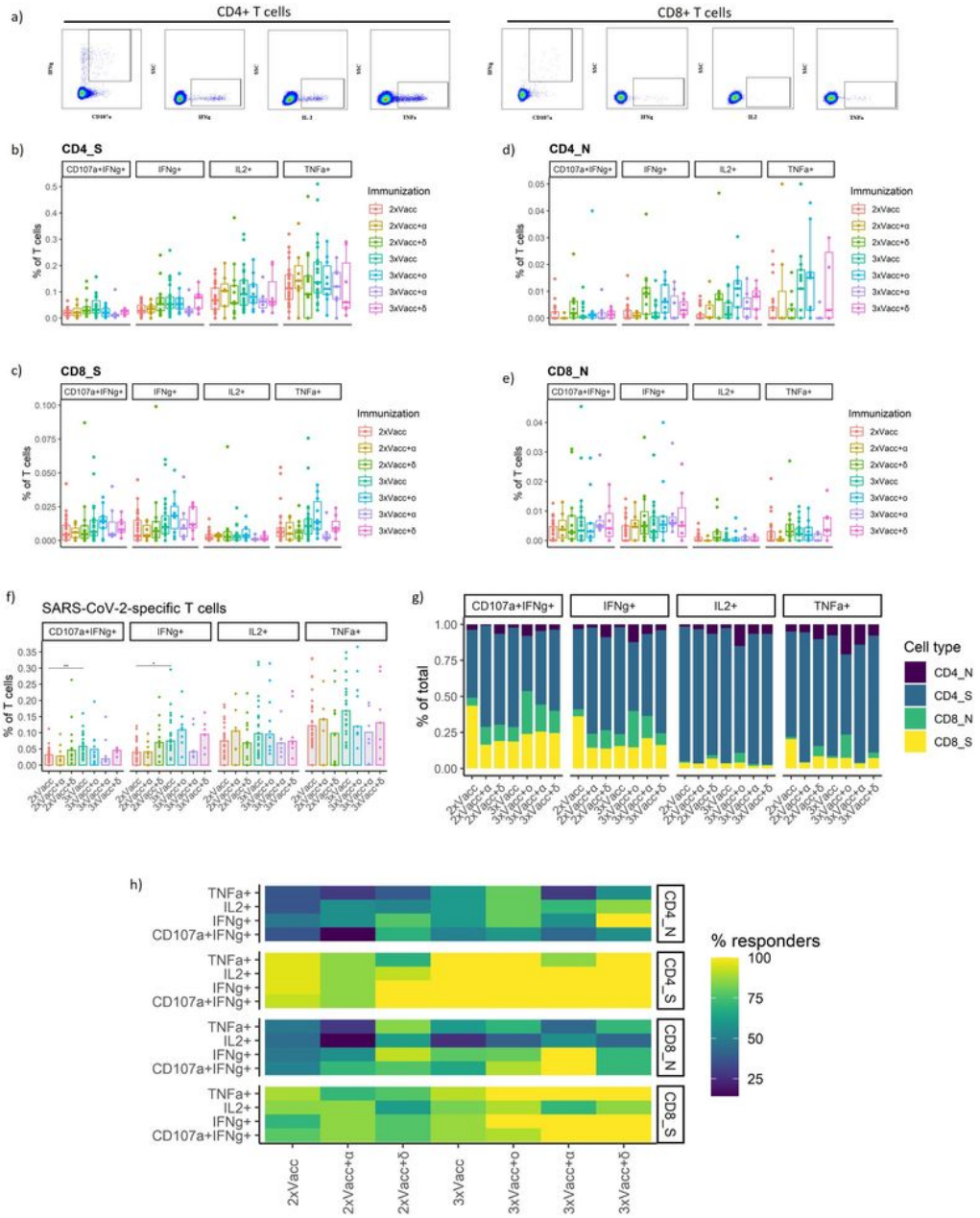

Figure 4

SARS-CoV-2-specific T cell response to different immunization scenarios. Frequencies of S-specific a) Representative flow cytometry plots demonstrating the detection of SARS-CoV-2-specific CD4+ and CD8+ T cells with different effector functions; cytotoxicity (CD107a and IFNγ expression), IFNγ-expression, IL-2 expression, and TNFα expression. b) CD8+ T cells and c) CD4+ T cells with different effector functions as a percentage of bulk T cells. Frequencies of N-specific d) CD4+ T cells and e) CD8+ T cells with different

effector functions as a percentage of bulk T cells. Different immunization groups are color-coded. f) Frequencies of S or N-specific T cells with different functions, not discriminating the CD4+ and CD8+ T cells. g) Relative proportions of S-specific CD4+, N-specific CD4+, S-specific CD8+, and N-specific CD8+ T cells performing different functions for each immunization group. h) Percentage of individuals with detectable SARS-CoV-2-specific CD4+ and CD8+ T cells within each group. Differences between the groups were assessed using the Mann-Whitney test with Holm's correction for multiple testing.

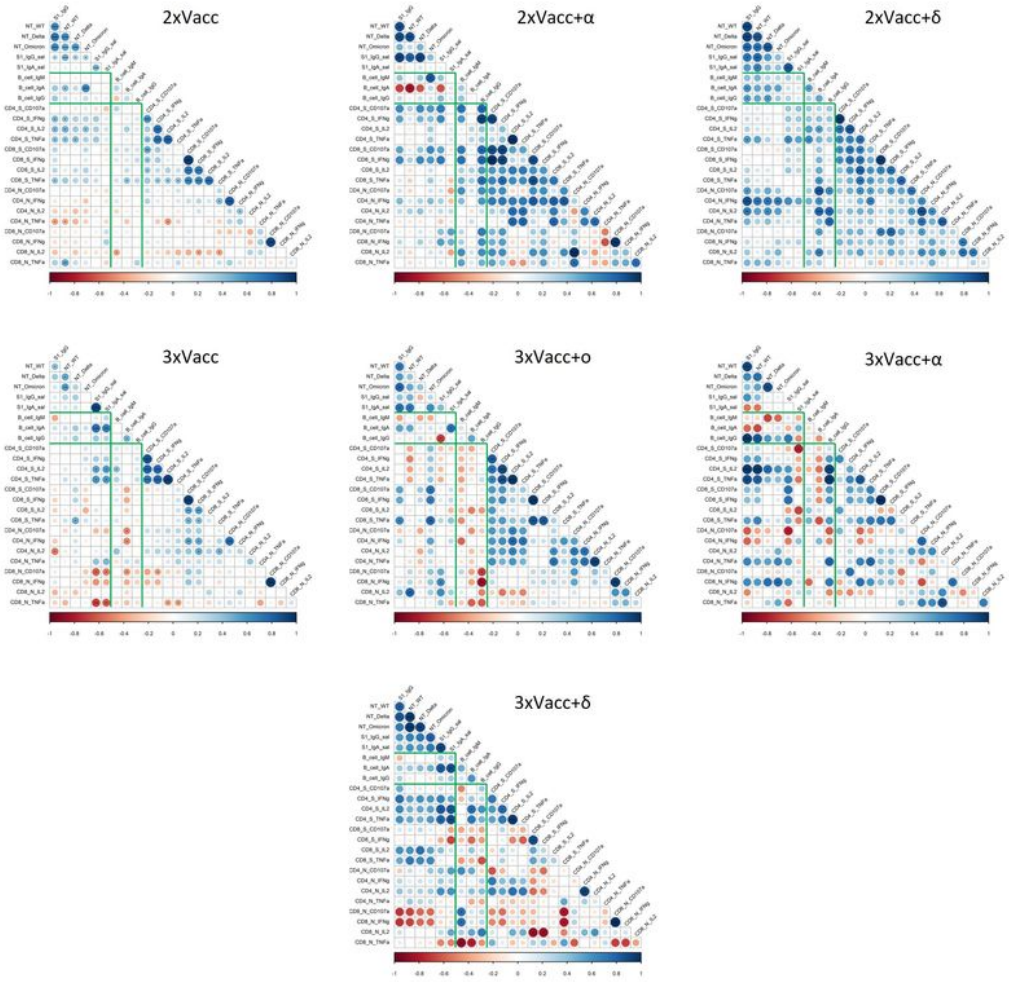

Figure 5

Coordination of the adaptive immune response to different immunization scenarios. Correlation matrices demonstrate the strength of correlations between the measured immune parameters for each of the immunization groups. Pearson's r is depicted by the size and color of the circle, significance is indicated by asterisks. Green lines separate correlations between different branches of the measured immune parameters; antibodies, memory B cells, and memory T cells. The strength of correlations was assessed by Spearman's correlation test.

Supplementary Files

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