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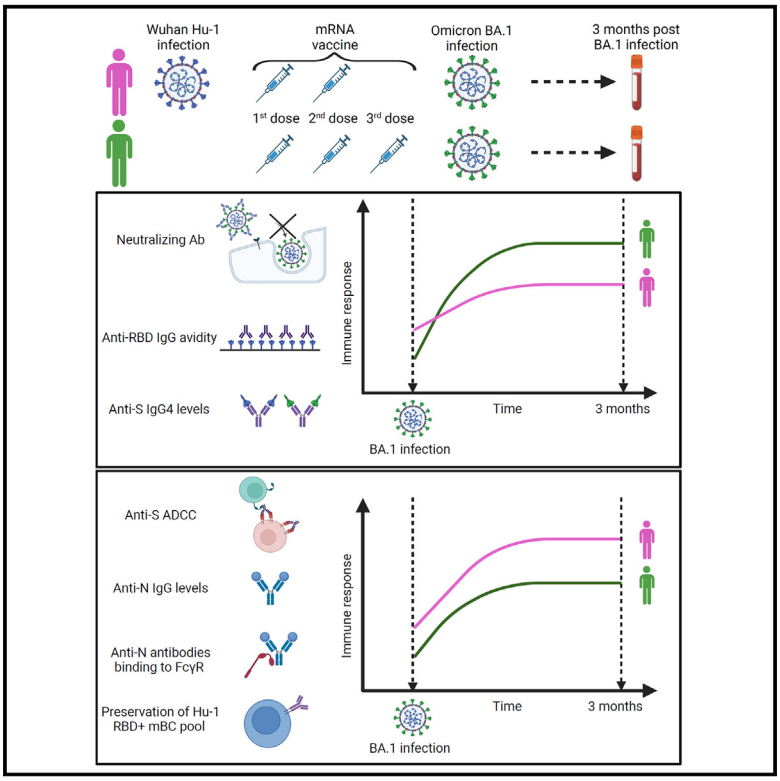
BA.1 breakthrough infection elicits distinct antibody and memory B cell responses in vaccinated-only versus hybrid immunity individuals

Graphical abstract



Highlights

- Hybrid immunity prevents decrease in the anti-S ADCC response post BA.1 infection
- Hybrid immunity limits increase in anti-S IgG4 levels post BA.1 infection
- Hybrid immunity boosts anti-N response despite anti-S imprinting post BA.1 infection

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In brief

Health sciences; Immunology; Virology

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BA.1 breakthrough infection elicits distinct antibody and memory B cell responses in vaccinated-only versus hybrid immunity individuals

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SUMMARY

Immune memory is influenced by the frequency and type of antigenic challenges. Here, we performed a cross-sectional comparison of immune parameters following a BA.1 breakthrough infection in individuals with prior hybrid immunity (conferred by infection and vaccination) versus those solely vaccinated in a cohort of health care workers in Lyon, France. The results showed higher levels of serum anti-receptor binding domain (RBD) antibodies and neutralizing antibodies against BA.1 post-infection in the vaccine-only group. Individuals in this group also showed a decrease in memory B cells against the ancestral strain but an increase in those specific and cross-reactive to BA.1, suggesting a more limited immune imprinting. Conversely, hybrid immunity prevents the decrease in antibody dependent cellular cytotoxicity (ADCC) response, possibly by limiting IgG4 class-switching and enhanced anti-N responses post-infection. This highlights that BA.1 breakthrough infection induces different immune responses depending on prior history of vaccination and infection, which should be considered for further vaccination guidelines.

INTRODUCTION

Immune memory against SARS-CoV-2 in the general population has been acquired through cycles of infections and/or vaccinations, with great heterogeneity in this pattern across individuals. Infection by SARS-CoV-2 results in broad cellular and humoral immunity against various antigens, whereas most vaccines target only the viral spike (S) protein. mRNA vaccination induces strong levels of neutralizing antibodies, especially in previously infected individuals, the latter situation defined as hybrid immunity.[1–3] Hybrid immunity is also acquired if the infection occurs after vaccination, a situation that can be defined as breakthrough immunity.[4] Breakthrough infections have started to arise when variants of concern (VOCs) have emerged, leading up to the current Omicron lineage.[5] These VOCs differ from the initial strain mainly at the level of the S protein, which accumulated immune evasive mutations, making it much less sensitive to neutralizing antibodies directed against the so-called wild-type or Wuhan Hu-1 (Hu-1) strain. The emergence of these VOCs led to new epidemic waves responsible for high infection rates, even in

vaccinated individuals, prompting health authorities to recommend vaccine boosters regardless of infection history.[6,7] For example, the World Health Organization (WHO)'s Strategic Advisory Group on immunization (SAGE) recommends a revaccination for healthcare workers (HCWs) 12 months after the most recent dose regardless of their previous infection history.[7]

However, repeated vaccination may lead to the emergence of S-specific IgG4 that have less efficient Fc-dependent immune properties than IgG1. This is especially true in individuals not previously infected by SARS-CoV-2 before their vaccination scheme.[8–10] Reciprocally, a dampening of the immune response post Omicron BA.1 breakthrough infection has been observed among individuals with hybrid immunity in comparison to those with vaccination-induced immunity.[11] In particular, most individuals increase BA.1/BA.2-specific neutralizing antibodies following infection, but the magnitude of increase is lower in individuals with established hybrid immunity compared to infection-naive vaccinated subjects.[10] This phenomenon has already been described in influenza under the term of ''original antigenic sin,'' also called ''immune imprinting,'' and can be explained by the strong response of memory B cells (mBCs) induced by infection or vaccination competing with naive B cells directed against antigenic variants.[12–16] Thus, SARS-CoV-2 infection occurring either before or after vaccination can influence antiviral immunity in distinct ways, particularly regarding antibody subclasses, antibody functions, and antigen specificity. To explore this further, we investigated a cohort of HCWs from Lyon Hospital. We conducted a comprehensive analysis of humoral and cellular immunity parameters targeting S and N antigens across five groups of individuals with diverse vaccination and infection histories. Our goal was to identify the key factors shaping immunity after a BA.1 breakthrough infection, with a particular focus on prior SARS-CoV-2 infection before vaccination and the cumulative number of antigenic exposures.

RESULTS

Clinical characteristics of study population

To understand how antiviral immunity evolves after a BA.1 breakthrough infection and how prior infection and vaccination history influence this evolution, we leveraged a cohort of 714 HCWs that we previously described,[17] and identified five groups of individuals.

First, we selected two groups of individuals who had a breakthrough infection during the Omicron BA.1 wave.

a. The first group consisted of individuals who were previously infected during the Hu-1 wave of infection and then received two doses of a monovalent mRNA vaccine before the BA.1 breakthrough infection (Hu-1/2RNA/BA.1).
b. The second group consisted of individuals with vaccination-induced immunity who were previously COVID-19-naive and received three doses of a monovalent mRNA vaccine before the BA.1 breakthrough infection (3RNA/BA.1).

Then, we selected three other groups that did not undergo breakthrough infection.

c. The third and fourth groups consisted of individuals infected during the Hu-1 wave of the pandemic then received 2 or 3 doses of a monovalent mRNA vaccine (Hu-1/2RNA and Hu-1/3RNA respectively).
d. The fifth group consisted of individuals who were COVID-19-naive and received three doses of a monovalent mRNA vaccine (3RNA).

Clinical and demographic characteristics of selected individuals are detailed in Table 1. Each group is composed of 15 individuals.

All infections, whether during the Hu-1 wave (established hybrid immunity) or the BA.1 wave (breakthrough infection), were mildly symptomatic. Importantly, blood sampling was performed 3 months (median [interquartile range, IQR] of 91 [71–104] days) post last exposure to viral antigens, i.e., 3 months post last infection (groups Hu-1/2RNA/BA.1 and 3RNA/BA.1) or vaccination (groups Hu-1/2RNA, Hu-1/3RNA, and 3RNA). Figure S1 summarizes the different groups, highlighting the timing of infections/vaccination and sampling events.

Hybrid immunity preserves Fc receptor-dependent anti-S IgG function post breakthrough infection

We first compared serological immunity against S or receptor binding domain (RBD) antigens post BA.1 breakthrough infection, in groups Hu-1/2RNA/BA.1 and 3RNA/BA.1 (Figure 1A). These two groups had an equivalent cumulative number of exposures to the S antigen through infection or vaccination. Higher anti-RBD IgG levels were found in the 3RNA/BA.1 group (median [IQR] of 5,013 [2,783–7,030] binding antibody unit (BAU)/mL) in comparison to the Hu-1/2RNA/BA.1 group (2,740 [1,118–4,379] BAU/mL, $p = 0.1150$, Figure 1B). Anti-RBD IgG from individuals in the 3RNA/BA.1 group also had a significantly increased avidity compared to those from the Hu-1/2RNA/BA.1 group, ($p = 0.0238$, Figure 1C). We then analyzed serum IgG subtypes against Hu-1 and BA.1 S proteins in both groups. The results were similar for both proteins, with individuals in the 3RNA/BA.1 group having a higher level of anti-S IgG4 ($p < 0.0001$ for both proteins, Figure 1D). In particular, there was a 1.63-fold and a 1.48-fold increase in IgG4 levels, in these individuals compared to those from the Hu-1/2RNA/BA.1 group for the Hu-1 S and BA.1 S protein, respectively (Figure 1D).

Serum neutralizing antibody titers against SARS-CoV-2 isolates 19A (B38 lineage), BA.1, BA.4, BA.5, and XBB.1.5 were then assessed. For all tested isolates, there was no significant difference in neutralizing antibody titers between serum samples from the Hu-1/2RNA/BA.1 group and those from the 3RNA/BA.1 group ($p > 0.05$, Figure 1E). Yet, for the BA.1 isolate, the results showed a tendency toward higher neutralizing titers among the 3RNA/BA.1 subjects with a median [IQR] titer of 480 [120–960] in comparison to 240 [80–480] for the Hu-1/2RNA/BA.1 ones (Figure 1E).

ADCC was then assessed against the D614G, BA.1, and BA.4/5 S proteins. ADCC was 5.22-, 4.43-, and 4.25-fold higher among individuals belonging to the Hu-1/2RNA/BA.1 group in comparison to those belonging to the 3RNA/BA.1 group for the D614G, BA.1, and BA.4/5 S proteins, respectively ($p = 0.0039$, $p = 0.0855$, and $p = 0.0038$, respectively, Figure 1F).

Table 1. Clinical characteristics of individuals included in this study

	Hu-1/2RNA/BA.1	Hu-1/2RNA	Hu-1/3RNA	3RNA/BA.1	3RNA	Adjusted *p* value
n	15	15	15	15	15	–
Age at blood sampling (years), median [IQR]	34 [31–42]	55 [38–58]	55 [28–61]	44 [40–53]	51 [33–60]	0.303
Female, *n* (%)	12 (80)	12 (80)	12 (80)	12 (80)	10 (66.66)	1
Time elapsed between the last vaccination or infection event and blood sampling (days)	73 [65–86]	92 [86–97]	110 [94–112]	107 [65–120]	84 [70–98]	0.035
Body mass index[a], *n*	15/15	14/15	15/15	15/15	13/15	–
Body mass index, median [IQR]	23.81 [21.60–25.47]	25.01 [20.72–27.76]	23.88 [20.20–27.72]	25.10 [21.94–32.89]	22.00 [19.50–25.00]	0.868
Currently smoker, *n* (%)	1/15 (6.66)	2/15 (13.33)	1/15 (6.66)	4/15 (26.66)	2/15 (13.33)	1
Alcohol consumption[a] (daily), *n* (%)	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)	0/13 (0)	–
Comorbidities						
Presence of comorbidities, *n* (%)	2/15 (13.33)	9/15 (60)	3/15 (20)	3/15 (20)	1/15 (6.66)	0.208
Neurological disorders, *n* (%)	1 (6.66)	1 (6.66)	1 (6.66)	1 (6.66)	0 (0)	1
Cardiovascular disorders, n (%)	1 (6.66)	0 (0)	1 (6.66)	0 (0)	0 (0)	1
Hypertension, *n* (%)	0 (0)	1 (6.66)	0 (0)	0 (0)	0 (0)	1
Heart failure, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1
Diabetes, *n* (%)	0 (0)	1 (6.66)	0 (0)	0 (0)	1 (6.66)	1
Immune deficiency, *n* (%)	0 (0)	1 (6.66)	0 (0)	0 (0)	0 (0)	1
Liver disease, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1
Kidney disease, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1
History of cancer, *n* (%)	0 (0)	1 (6.66)	0 (0)	1 (6.66)	0 (0)	1
Hypothyroidy, *n* (%)	0 (0)	1 (6.66)	0 (0)	1 (6.66)	0 (0)	1
Rheumatic disease, *n* (%)	0 (0)	2 (13.33)	1 (6.66)	0 (0)	0 (0)	1
Chronic lung disease, *n* (%)	0 (0)	3 (20)	1 (6.66)	0 (0)	0 (0)	0.472
Autoimmune and inflammatory disease, *n* (%)[b]	1 (6.66)	2 (13.33)	0 (0)	0 (0)	0 (0)	1

IQR, interquartile range.
Comparison of clinical parameters between all groups were carried out using a Bonferroni's test for multiple comparisons.
[a]Missing data.
[b]Autoimmune and inflammatory disease include: Adisson's disease, Celiac disease, and Crohn's disease.

Together, these results demonstrate that a prior infection before the mRNA vaccine regimen significantly increases the ADCC activity of antibodies against S proteins carried by the tested VOCs post BA.1 reinfection, but slightly limits the emergence of neutralizing antibodies against all tested Omicron subvariants.

BA.1 breakthrough infection in individuals with prior hybrid immunity recalls anti-N serological immunity

Next, we monitored serological humoral immunity against the N protein, an antigen not present in COVID-19 vaccines available in France. Anti-N IgG levels were higher among individuals from the Hu-1/2RNA/BA.1 group in comparison to the 3RNA/BA.1 group (median [IQR] index of 7.09 [5.94–8.58] and 1.33 [0.58–2.23]; $p < 0.0001$) (Figure 2A).

Anti-N IgG subtyping was performed and higher IgG1, IgG2, and IgG4 levels were found among individuals in the Hu-1/2RNA/BA.1 group in comparison to those in the 3RNA/BA.1 group ($p = 0.0046$, $p < 0.0001$, $p < 0.0001$, respectively, Figure 2B). No significant difference was observed regarding anti-N IgG3 levels between the two groups ($p = 0.5490$, Figure 2B). Serum anti-N IgG binding to FcγRIIa and FcγRIIIa was then assessed using a Luminex assay. Sera collected from individuals in the Hu-1/2RNA/BA.1 group had antibodies with a higher capacity to bind both receptors in comparison to the 3RNA/BA.1 group ($p < 0.0001$ for both receptors, Figure 2C). The difference in the magnitude of the serological response against the N protein confirms that subjects in the Hu-1/2RNA/BA.1 group who were infected twice with the virus, exhibit a recall response against this protein.

BA.1 breakthrough infection induces different immune responses based on prior history of vaccination and infection

The preserved ADCC function, along with the robust anti-N response observed after a BA.1 breakthrough infection in individuals with prior hybrid immunity, led us to investigate whether these serological responses were driven by the BA.1 breakthrough

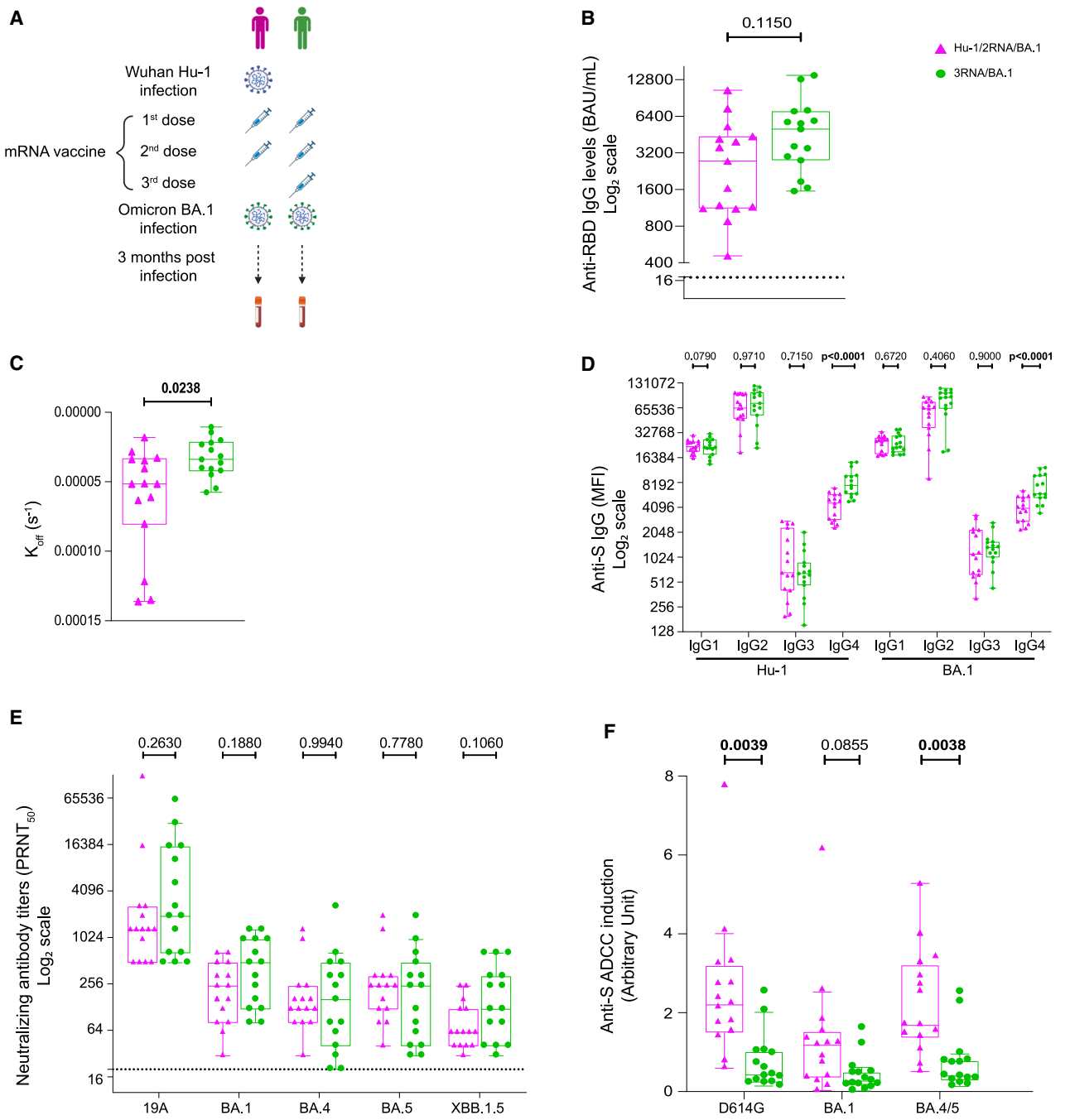

Figure 1. Prior hybrid immunity status prevents the decline of Fc receptor-dependent anti-Spike IgG functions post breakthrough infection

(A) Serum samples were collected from individuals with established hybrid (Hu-1/2RNA/BA.1, purple, $n = 15$) or vaccination-induced (3RNA/BA.1, green, $n = 15$) immunity 3 months post BA.1 breakthrough infection.

(B) IgG levels specific to the Wuhan Hu-1 (Hu-1) RBD were assessed and concentrations were expressed in binding antibody unit (BAU)/mL. Each serum sample was evaluated as a single measurement. The data are represented using a $\log_2$ scale. The dotted line represents the positivity threshold ($\geq$20.33 BAU/mL).

(C) Purified total IgGs from each individual were assayed for Hu-1 RBD-specific IgG off-rate measurement using biolayer interferometry. K_{off} values are expressed as s^{-1} and each sample was assayed as a single measurement.

(D) Subtyping of Hu-1 and BA.1 S-specific IgG was carried out on serum samples. The data are expressed using a $\log_2$ scale. Each sample was assayed as a single measurement.

(E) Antibody neutralization capacity was evaluated against the 19A, BA.1, BA.4, BA.5 and XBB.1.5 live isolates. The data are represented using a $\log_2$ scale. Serum samples were tested as technical duplicates. The dotted line represents the positivity threshold ($PRNT_{50} \geq 20$).

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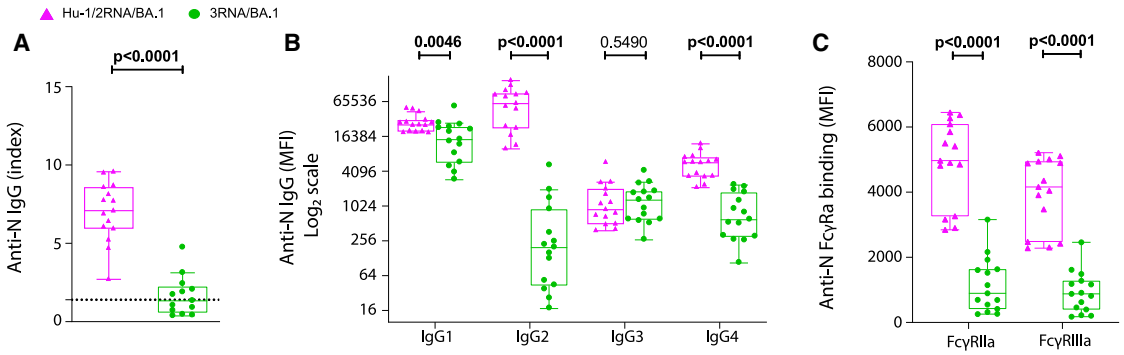


Figure 2. BA.1 breakthrough infection in individuals with prior hybrid immunity recalls anti-nucleocapsid serological immunity

Serum samples were collected from individuals with established hybrid- (Hu-1/2RNA/BA.1, purple, $n = 15$) or vaccination-induced (3RNA/BA.1, green, $n = 15$) immunity 3 months post BA.1 breakthrough infection.

(A) Anti-N total IgG levels were quantified and expressed as an index. Each sample was assayed as a single measurement. The dotted line represents the positivity threshold (index $\geq$ 1.4) according to manufacturer's instruction.

(B) Subtyping of N-specific IgG was carried out on serum samples. The data are expressed using a $\log_2$ scale. Each sample was assayed as a single measurement.

(C) Antibodies specific to the N protein were assessed for their ability to bind the FcγRIIa and FcγRIIIa receptors. Each sample was assayed as a single measurement. Data for all parameters are expressed as box-and-whiskers plots according to the Tukey method with the line inside the box indicating the median, the upper and lower line of the box indicating the interquartile range [IQR]. Multiple regressions (ANCOVA) were realized for each parameter, adjusted on covariate "age," to compare the results obtained between the two groups. Exact p-values are indicated for each parameter. MFI: mean fluorescence intensity.

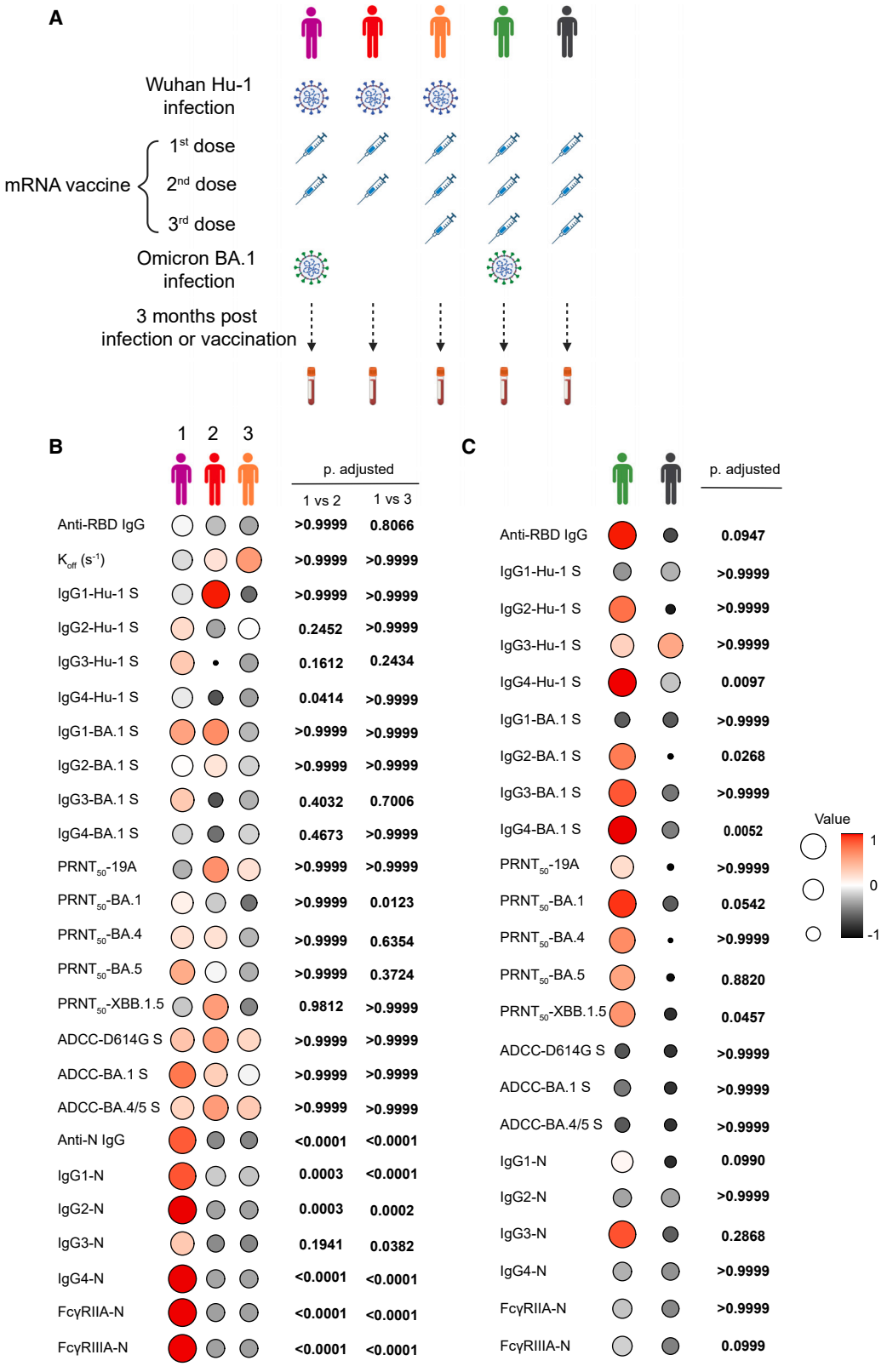
infection itself or were a consequence of the pre-existing hybrid immunity. To address this question, we leveraged the groups without breakthrough infection that consisted of subjects infected during the Hu-1 wave of the pandemic and that received 2 (Hu-1/2RNA) or 3 (Hu-1/3RNA) doses of a monovalent mRNA vaccine (Figures 3A, S2, and S3). Serum IgG were analyzed in individuals from these groups 3 months post vaccination, in comparison to those from the Hu-1/2RNA/BA.1 group. No significant difference was observed between the 3 groups in terms of ADCC, as well as other anti-S serological parameters ($p > 0.05$, Figure 3B). Nevertheless, a tendency toward higher neutralizing antibody titers against BA.1 was observed among individuals from the Hu-1/2RNA/BA.1 group (median [IQR] of 240 [80–480]) in comparison to those from the Hu-1/2RNA (160 [30–320]) and Hu-1/3RNA (60 [30–120]) groups (Figures 3B and S2F). As for the anti-N immune response, anti-N IgG levels, subtypes, and FcγR binding were significantly higher in subjects from the Hu-1/2RNA/BA.1 group compared to the other 2 groups ($p < 0.001$, Figures 3B and S3). Thus, although a BA.1 breakthrough infection does not significantly impact the anti-S serological response among individuals with established hybrid immunity, it leads to an increase in neutralization capacity against BA.1. Additionally, a breakthrough infection triggers a recall of pre-existing immunity targeting the N protein among individuals with established hybrid immunity.

In parallel, we examined whether the dampening of ADCC associated with IgG4 class-switching in individuals with a BA.1 breakthrough infection after vaccination was a result of the breakthrough infection itself or a consequence of repeated mRNA vaccinations. For this, we compared the serological immune response induced by 3 mRNA vaccine doses between subjects with (3RNA/BA.1) or without (3RNA) BA.1 breakthrough infection (Figures 3A, S2, and S3). No significant difference was observed between the groups in terms of anti-RBD IgG levels, anti-S ADCC and anti-N IgG subtypes ($p > 0.05$, Figures 3C, S2, and S3). Interestingly, significantly higher anti-S IgG4 levels were observed among individuals in the 3RNA/BA.1 group, for both the Hu-1 and BA.1 S proteins ($p = 0.0097$ and $p = 0.0052$ respectively, Figures 3C, S2D, and S2E). In addition, there was a tendency toward higher BA.1 and XBB.1.5 neutralizing antibody titers in the 3RNA/BA.1 group ($p = 0.0542$ and $p = 0.0457$ respectively, Figures 3C and S2F). Thus, among individuals with vaccination-induced immunity, a BA.1 breakthrough infection resulted in an increase in neutralization capacity against BA.1 despite an increase in anti-S IgG4 levels.

Furthermore, we observed an inverse trend between anti-S IgG4 levels and anti-S ADCC activity and sought to further investigate this relationship. Correlation analyses were performed between anti-Hu-1 S IgG4 levels and D614G S ADCC activity as well as anti-BA.1 S IgG4 levels and BA.1 S ADCC activity across all individuals in the cohort's five groups. As illustrated in Figure S2J, both IgG4 levels showed a significant negative association with ADCC activity, supporting a potential causal link

(F) The induction of the CD16 pathway was used as a surrogate of ADCC using the ADCC reporter assay. 293T cells stably expressing D614G, BA.1 and BA.4/5 S proteins, or a control plasmid, were used as target cells. The fold change of CD16 activation was calculated in each condition, and the control condition (cells transduced with an empty vector) was subtracted to account for interindividual variations of the background. The y axis indicates the calculated ADCC induction, with a value of zero indicating no induction. Each sample was assayed as a single measurement.

Data for all parameters are expressed as box-and-whiskers plots according to the Tukey method with the line inside the box indicating the median, the upper and lower line of the box indicating the interquartile range [IQR]. Multiple regressions (ANCOVA) were realized for each parameter, adjusted on covariate "age," to compare the results obtained between the two groups. Exact p values are indicated for each parameter. MFI, mean fluorescence intensity.



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between these parameters. It has been reported that IgG4 levels tend to increase following a third dose of vaccination in COVID-19-naive patients.[8,9] In our study, we aimed to compare anti-S IgG4 levels between triple vaccinated individuals infected before or after the vaccination scheme; i.e., Hu-1/3RNA and 3RNA/BA.1 groups. Our results showed that despite both groups having the same number of vaccinations and infections, individuals in the 3RNA/BA.1 group had significantly higher anti-S IgG4 levels ($p = 0.003$ for Hu-1 S, Figure S2D, and $p = 0.0380$ for BA.1 S, Figure S2E). This would suggest that hybrid immunity limits the increase in IgG4 levels following repeated exposure to viral antigens in comparison to vaccination alone.

In summary, a BA.1 breakthrough infection enhanced neutralization capacity against BA.1 regardless of prior history of vaccination and infection. For individuals with established hybrid immunity, a BA.1 breakthrough infection recalled the anti-N serological response. As for individuals with vaccination-induced immunity, a BA.1 breakthrough infection led to an increase in anti-S IgG4 levels. Hence, a BA.1 breakthrough infection does not induce the same immune response depending on prior history of vaccination and infection.

Prior hybrid immunity limits the emergence of mBCs specific to BA.1 RBD upon breakthrough infection

Next, we examined the pool of mBCs specific to the Hu-1 and BA.1 RBD for subjects belonging to the Hu-1/2RNA/BA.1, Hu-1/2RNA, Hu-1/3RNA, and 3RNA/BA.1 groups (Figure 4A). RBD-specific mBCs were analyzed by multiparameter flow cytometry approach using fluorescently labeled tetrameric Hu-1 and BA.1 RBDs as previously described.[2] The gating strategy to identify mBCs is depicted in Figure S4A. Two distinct couples of fluorophores were used to detect Hu-1 and BA.1 RBD specific mBCs allowing the identification of mBCs only specific to either the Hu-1 or BA.1 RBD and those cross-reactive to both RBDs (Figures S4B and S4C).

Figures 4B and 4C illustrate the percentage of mBCs specific to the Hu-1 RBD and mBCs specific and cross-reactive to the BA.1 RBD (BA.1 RBD+), respectively, as a proportion of the total mBC population. The results showed a significantly lower percentage of Hu-1 RBD-specific mBCs and a significantly higher percentage of BA.1 RBD+ mBCs following a breakthrough infection in individuals previously only vaccinated, compared to those with prior hybrid immunity ($p = 0.095$, Figures 4B and 4C). This would suggest that established hybrid immunity restricts the diversification of the RBD+ mBCs pool by preserving the Hu-1 RBD-specific mBCs and limiting the expansion of BA.1 RBD+ mBCs.

To investigate this hypothesis further, we compared the pool of RBD+ mBCs between the three groups with established hybrid immunity, with or without a breakthrough infection. The results indicate no significant differences among the three groups for either Hu-1 RBD-specific mBCs or BA.1 RBD+ mBCs ($p > 0.05$, Figure 4D). This suggests a similar prevalence of Hu-1 RBD specific and BA.1 RBD+ mBCs across the three groups with established hybrid immunity.

Taken together, these results suggest that a BA.1 breakthrough infection increases the frequency of BA.1 RBD+ mBCs among individuals previously only vaccinated in comparison to those with established hybrid immunity. These results suggest that hybrid immunity results in stronger immune imprinting than sole vaccination, limiting the emergence of mBCs specific or cross-reactive to the BA.1 RBD upon breakthrough infection.

DISCUSSION

Previous studies have reported contradictory results regarding the impact of prior hybrid immunity on the immune response post breakthrough infection.[8,11,18–20] We show here that prior hybrid immunity is generally not a situation that prevents the development of the response against Omicron post breakthrough infection. It is even rather beneficial as it maintains a strong ADCC capacity of anti-S IgG possibly by preventing the class-switch to IgG4, and it significantly increases the humoral immunity against N, without altering the level of neutralizing antibodies against different variants contrary to findings reported in earlier studies.[11] Conversely, we report that prior hybrid immunity slightly limits the emergence of BA.1 RBD-specific mBCs linked to the preservation of Hu-1 RBD-specific mBCs compartment.

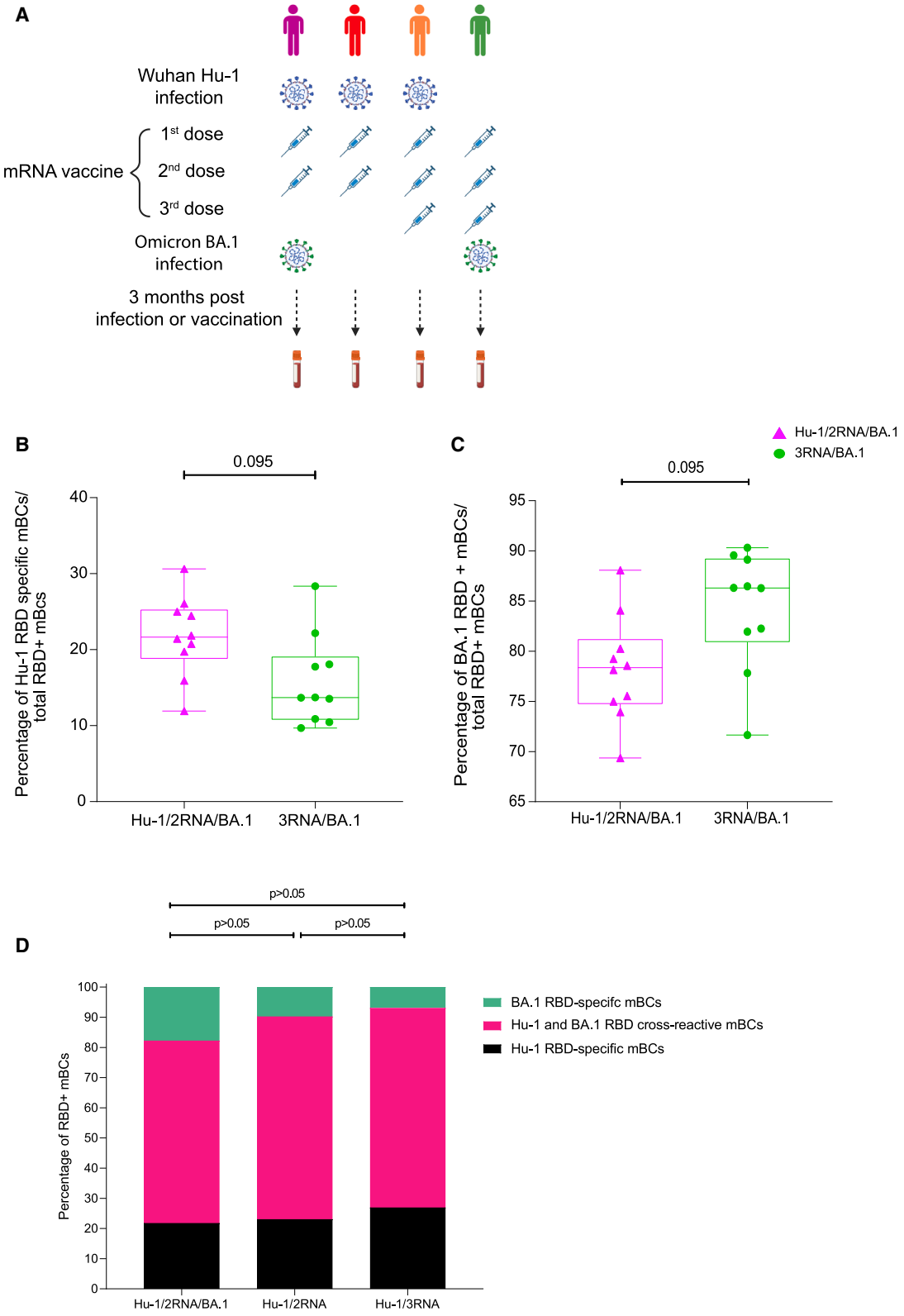
Our results reveal significantly higher anti-S IgG4 levels following a BA.1 breakthrough infection in individuals with vaccination-only immunity compared to those with prior hybrid immunity, whereas IgG1 levels were similar between the two groups. These findings align with previous findings indicating high anti-S IgG4 levels among COVID-19-naive individuals upon repeated vaccination or breakthrough infection[8,21] and low anti-S IgG4 levels in individuals with hybrid immunity.[21,22] Interestingly, we also show that low levels of anti-S IgG4 are maintained post BA.1 breakthrough infection among those individuals, suggesting a sustained protective effect of the initial infection. IgG4 have been described as anti-inflammatory antibodies secreted in response to chronic antigen exposure with a reduced capacity to induce Fc-mediated antibody effector functions such as ADCC.[23,24] Here, we demonstrate that alongside a

Figure 3. BA.1 breakthrough infection changes immune responses based on previous history of vaccination/infection

(A) Serum samples were collected from individuals with established hybrid immunity with (Hu-1/2RNA/BA.1, purple, $n = 15$) or without (Hu-1/2RNA, red, $n = 15$; Hu-1/3RNA, orange; $n = 15$) BA.1 breakthrough infection. Additionally, serum samples were collected from individuals with vaccination-induced immunity with (3RNA/BA.1, green, $n = 15$) or without (3RNA, black, $n = 15$) BA.1 breakthrough infection. All blood sampling was performed 3 months post last infection or vaccination.

(B) Bubble plot representing a comparison of the measured humoral parameters between individuals with established hybrid immunity. The bubble plot was generated by scaling and centering the values.

(C) Bubble plot representing a comparison of the measured humoral parameters between individuals with vaccination-induced immunity. The bubble plot was generated by scaling and centering the values. The intensity of the color as well as the size of the circles illustrated at the right of the bubble plots is proportional to the level of different immune parameters measured. To compare immune parameters between the groups, a statistical analysis was carried out using a two-way ANOVA mixed model followed by a Bonferroni's test to correct for multiple comparisons.



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significant increase in anti-S IgG4 levels among individuals experiencing a breakthrough infection post-vaccination alone, there is a significantly reduced anti-S ADCC function, despite a slight improvement in BA.1 neutralization capacity. This indicates that hybrid immunity maintains ADCC function, possibly by preventing the IgG4 class-switch post breakthrough infection and maintaining IgG1 that have the highest ADCC potential.[25]

In the context of COVID-19, most studies have focused on neutralizing antibodies, with limited attention given to ADCC.[26–30] Nevertheless, a study demonstrated that in an animal model, the ADCC function of antibodies was essential for their protective effect when used as a treatment for SARS-CoV-2 infection.[31] Moreover, in the context of respiratory syncytial virus (RSV) infections, ADCC function has been described as essential for the control of respiratory infections.[32,33] Bartsch et al. showed that individuals with higher IgG4 levels and lower Fc-mediated antibody effector functions were more susceptible to infection after RSV challenge.[32] Therefore, the influence of additional vaccinations on ADCC function should also be considered when establishing recommendations for COVID-19 vaccination.

In the present study, we also show that vaccination-induced immunity leads to a decrease in the proportion of Hu-1 RBD specific mBCs paralleled by an increase in the proportion of BA.1 RBD+ mBCs, indicating a diversification of the RBD+ mBCs pool post BA.1 breakthrough infection. Conversely, hybrid immunity preserves the pool of Hu-1 RBD specific mBCs. These results are in line with previous studies reporting the limited *de novo* B cell response in individuals with prior hybrid immunity post Omicron breakthrough infection, described as "hybrid immune damping."[11] This would indicate that prior hybrid immunity limits the diversification of the RBD+ mBCs pool due to immune imprinting.

Regarding the humoral response against the N protein, an antigen not present in most vaccines, the effect of a breakthrough infection was significant in individuals with established hybrid immunity, compared to those with vaccination-induced immunity. It has been previously reported that a breakthrough infection in previously infection-naive vaccinated individuals does not lead to a robust anti-N humoral response with only 40% of individuals with a PCR-confirmed SARS-CoV-2 infection seroconverting to anti-N antibodies.[34] In addition, Allen et al. compared the anti-N humoral response between individuals who were first infected and then vaccinated, and those who were infected after vaccination. Their findings revealed that 26% of previously vaccinated individuals seroconverted to anti-N antibodies, compared to 82% of individuals who were first infected.[35] These results would suggest that a single exposure to antigens other than the S protein, such as N, might not be sufficient to induce a robust immune response in previously vaccinated individuals. Nevertheless, the possibility of vaccinating with antigens other than the S to avoid immune fatigue against the S protein should still be considered. Indeed, previous studies have described that a two-dose vaccination regimen targeting the N protein in animal models as well as clinical trials results in long-lasting N specific humoral and T cell immune responses in humans and decreased infection severity in animals.[36–39] The N protein exhibits high conservation across SARS-CoV-2 variants. The XBB.1.5 variant, for example, carries 43 mutations in the S protein and only 7 mutations in the N protein in comparison to Hu-1 strain.[40] Together these data would suggest that combining S antigens with more conserved antigens such as N in vaccines may enhance and diversify the immune response against SARS-CoV-2 in the face of a plateau in the anti-S immune response. Such recommendation would particularly apply to individuals with high SARS-CoV-2 exposure levels, such as HCWs.

In conclusion, our results highlight that a breakthrough infection induces different immune responses depending on prior history of vaccination and infection, which should be considered for further vaccination guidelines.

Limitations of the study

One of the limitations of this study is the low number of individuals included in each group, and the inclusion of individuals with comorbidities. The latter point could not be avoided due to the constraints imposed by the selection criteria for the five groups. Moreover, it remains to be assessed whether the limitation of IgG4 class-switch is beneficial to immune protection against SARS-CoV-2 and its viral escape mutants. Our study did not include COVID-19-naive individuals vaccinated with only two doses in order to evaluate the impact of IgG4 levels with repeated vaccination. Due to the unavailability of PBMC samples from the 3RNA group, we were unable to perform a comparison with the 3RNA/BA.1 group to assess the impact of a breakthrough infection on the RBD+ mBC pool. Another limitation of our study is the absence of an analysis of the T cell response, particularly against the N antigen, as well as the mucosal response, both of which play crucial roles in protection.[41,42] These parameters were

Figure 4. Hybrid immunity preserves anti-Hu-1 RBD memory B cell pool post BA.1 breakthrough infection and limits the emergence of BA.1-specific memory

(A) Peripheral blood mononuclear cells (PBMC) samples were collected from individuals with established hybrid immunity with (Hu-1/2RNA/BA.1, purple, $n = 10$) or without (Hu-1/2RNA, red, $n = 9$; Hu-1/3RNA, orange; $n = 10$) a BA.1 breakthrough infection. Additionally, PBMC samples were collected from individuals with vaccination-induced immunity with a BA.1 breakthrough infection (3RNA/BA.1, green, $n = 10$). All blood sampling was performed 3 months post last infection or vaccination.
(B and C) We then used flow cytometry to analyze the percentage of mBCs specific to the Hu-1 RBD (B) and mBCs specific and cross-reactive to the BA.1 RBD+ (C) relative to total memory B cells recognizing the RBD in individuals with vaccination-induced immunity and those with prior hybrid immunity, post breakthrough infection.
(D) Stacked bar plot representing the mean proportion of Hu-1 RBD-specific, Hu-1 and BA.1 RBD cross-reactive and BA.1 RBD-specific mBCs relative to all RBD+ mBCs among individuals with established hybrid immunity. Data are expressed as box-and-whiskers plots according to the Tukey method with the line inside the box indicating the median, the upper and lower line of the box indicating the interquartile range. Multiple regressions (ANCOVA) were realized for each parameter, adjusted on covariate "age," to compare the results obtained between the Hu-1/2RNA/BA.1 and 3RNA/BA.1 groups. A Kruskal-Wallis test followed by Dunn's multiple comparison test were carried out to compare the percentages of RBD+ mBCs between the three groups with established hybrid immunity. Exact *p* values are indicated for each parameter.

addressed in a recent report, which specifically demonstrated that T cell responses to non-S antigens increase significantly in vaccinated individuals following a BA.1 breakthrough infection, regardless of their prior infection status.[10] Studying the T cell response could also have allowed us to confirm that all individuals classified as naive for COVID-19 were truly unexposed prior to vaccination. Several studies have shown that in some convalescent individuals, serology may be negative after infection, whereas the T cell response is detectable[43,44] and does not wane[45] over time.

RESOURCE AVAILABILITY

Lead contact

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Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data associated with this study are a result of quantification and functional tests carried out as described. All data described herein are present in the paper or the supplemental information, in addition to the key resources table. Raw data have been deposited on Mendeley Data.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

All authors read and approved the final version of the manuscript. Data collection and data analysis, C S., T.B., M.K., B.P., A.D., L.K., N.M., C. Mignon, L.B., L.-L.V., and W.H.B. Data interpretation, C.S. performed all statistical analyses. Resources, J.-B.F., M.V., and B.L. The COVID-Ser study members collected the samples and performed experiments. Supervision, V.B., M.E., O.T., O.S., S.P., and T.W. Validation, B.P., T.B., M.K., T.W., and S.T.-A. Funding acquisition, B.L., S.T.-A., T.W., and T.B. Study design and writing of original draft, C.S., S.P., T.B., T.W., and S.T.-A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-Human IgG1 Fc-PE	Southern Biotech	Cat#9054-09; RRID: AB_2796628
Mouse anti-Human IgG2 Fc-PE	Southern Biotech	Cat#9070-09; RRID: AB_2796639
Mouse anti-Human IgG3 Hinge-PE	Southern Biotech	Cat#9210-09; RRID: AB_2796701
Mouse anti-Human IgG4 Fc-PE	Southern Biotech	Cat#9200-09; RRID: AB_2796693
anti-IgG AlexaFluor647	Jackson ImmunoResearch	Cat#A-21445; RRID: AB_2535862
Anti-CD3 APC Fire 810	Biolegend	Ref# 344858; RRID: AB_2860895
Anti-CD19 PE-Vio770	Miltenyi	Ref# 130-113-170; RRID: AB_2733209
Anti-CD20 BV421	BD	Ref# 562873; RRID: AB_2737857
Anti-CD27 PerCP-Vio® 700	Miltenyi	Ref# 130-113-632; RRID: AB_2784096
Anti-CD45 PerCP	Biolegend	Ref# 368506; RRID: AB_2566358
Anti-IgM PE-CF594	BD	Ref# 562539; RRID: AB_2737641
Anti-IgD BV605	Biolegend	Ref# 348232; RRID: AB_2563336
Fixable Viability Dye eFluor™ 780	ebiosciences	65-0865-18
Chemicals, peptides, and recombinant proteins		
SARS-CoV-2 (2019-nCoV) Spike Protein (RBD, His Tag)	Sino Biological	Cat #40592-V08B
Tetanus toxoid	Sigma Aldrich	Cat#582231
Influenza hemagglutinin H1Cal2009	Sino Biologicals	Cat#11085-V08H
Wuhan Hu-1 Spike	Sino Biologicals	Cat#40591-V08H
Wuhan Hu-1 Nucleocapsid	Sino Biologicals	Cat#40588-V08B
BA.1 Spike	Sino Biologicals	Cat#40589-V08H30
FcγRIIa-H131 dimer	Wines et al.[43]	N/A
FcγRIIIa-V158 dimer	Wines et al.[43]	N/A
Hoechst 33342	Invitrogen	Cat#H3570
Paraformaldehyde 4%	Alfa Aesar	Cat#J19943.K2
Recombinant SARS-CoV-2 RBD (HEK)-Biotin 50ug	Miltenyi	130-127-457
Recombinant SARS-CoV-2 RBD B.1.1.529/BA.1 omicron (HEK)-Biotin 50ug	Miltenyi	130-130-419
Critical commercial assays		
Magne Protein G Beads	Promega	Cat #G7471
Octet® amine-reactive biosensors, AR2G	Sartorius	Cat #18-5092
Octet® amine-coupling reagents	Sartorius	Cat #18-5095
ADCC Reporter Bioassay	Promega	Cat#G7010
Bright-Glo Luciferase Assay System	Promega	Cat#E2620
Deposited data		
Raw and analyzed data	Mendeley data	Mendeley data: https://data.mendeley.com/datasets/8tpxx5cxdg/1
Experimental models: Cell lines		
Vero E6	ATCC	Cat#CRL-1586; RRID: CVCL_0574
293T	ATCC	Cat#CRL-3216 RRID: CVCL_0063
Recombinant DNA		
pLV-Empty-PuroR	This paper	N/A
pLV- Spike(D614G)-PuroR	This paper	N/A
pLV- Spike(BA.1)-PuroR	This paper	N/A
pLV- Spike(BA.4/5)-PuroR	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Octet Analysis studio 12.2 software	Sartorius	Octet® Software Download | Sartorius
Harmony High-Content Imaging and Analysis Software	PerkinElmer	Cat#HH17000012
Excel 365	Microsoft	https://www.microsoft.com/en-ca/microsoft-365/excel
Prism 8	Graphpad	https://www.graphpad.com/
FlowJo v10	Tree Star	https://www.flowjo.com/
R software v4.4.1	R Foundation for Statistical Computing	https://www.r-project.org/
Other		
Octet R8 instrument	Sarorius	OCTET-R8
Bio-Plex Pro Magnetic COOH beads	Bio-Rad	Cat#MC100XX-01
Streptavidin-PE conjugate	Invitrogen	Cat#S866
PE Streptavidin	BD	554061; RRID: AB_10053328
APC Streptavidin	BD	554067; RRID: AB_10050396
BUV737 Streptavidin 100ug	BD	612775; RRID: AB_2869560
BB515 Streptavidin 0,1mg	BD	564453; RRID: AB_2869580
Brilliant Stain Buffer	BD	566349; RRID: AB_2869750
FcR Blocking Reagent human	Miltenyi	130-059-901; RRID: AB_2892112

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

For the Covid-Ser cohort, clinical data were recorded by a trained clinical research associate using the Clinsight software (version _ Csonline 7.5.720.1). Seven hundred and fourteen with different histories of vaccination and infection were included in a prospective longitudinal cohort study conducted in Hospices Civils de Lyon (HCL; Lyon, France). Blood sampling was performed 3 months after the end of the vaccination schedule or the last infection event, and PBMC and serum samples were collected and stored. Written informed consent was obtained from all participants; ethics approval was obtained from the national review board for biomedical research in April 2020 (Comité de Protection des Personnes Sud Méditerranée I, Marseille, France; ID RCB 2020-A00932-37), and the study was registered on ClinicalTrials.gov (NCT04341142).[17] The inclusion criteria for this study were as follows: (i) seropositivity for anti-SARS-CoV-2 Abs or record of COVID-19 vaccination; (ii) age > 18; (iii) written consent ; and (iv) affiliation to social security. The exclusion criteria of this study were pregnancy or lactation for women.

COVID-19-naive vaccinated individuals were from two different cohorts. The first cohort, COVIMMUNITY, aimed to characterize the immune response in HCWs. Ethics approval was obtained from the national review board (ClinicalTrials.gov: NCT04648709).[46] The second cohort, ABCOVID, aimed to study the kinetics of COVID-19 antibodies in patients with confirmed SARS-CoV-2 infection as well as the kinetics of neutralizing antibodies post vaccination.[47] This study was approved by the ILE DE FRANCE IV ethical committee (ClinicalTrials.gov: NCT04750720). Written informed consent was collected at enrolment for all subjects.

The occurrence of an infection, since the start of the COVID-19 pandemic and up until the Omicron BA.1 wave, was monitored in our cohorts by assessing anti-RBD IgG titers through longitudinal blood sampling. Additionally, HCWs underwent frequent COVID-19 testing due to hospital regulations. A breakthrough infection was identified by a rebound of anti-RBD Ab titers thus excluding the subject from the present study.

All individuals in the three cohorts received either the BNT162b2 or the mRNA-1273 vaccine. SARS-CoV-2 infection before vaccination occurred during the Wuhan Hu-1 wave of the pandemic, before the emergence of variants of concern (VOCs). SARS-CoV-2 infection after vaccination occurred during the Omicron BA.1 wave. All selected individuals were divided into 5 groups, according to their infection and vaccination history. Each group is composed of 15 individuals.

Ethics statement

For the Covid-Ser cohort, ethics approval was obtained from the national review board for biomedical research in April 2020 (Comité de Protection des Personnes Sud Méditerranée I, Marseille, France; ID RCB 2020-A00932-37), and the study was registered on ClinicalTrials.gov (NCT04341142). For the COVIMMUNITY cohort, ethics approval was obtained from the national review board (ClinicalTrials.gov: NCT04648709). For the ABCOVID cohort, ethics approval was obtained from ILE DE FRANCE IV ethical committee (ClinicalTrials.gov: NCT04750720). Written informed consent was obtained from all participants.

METHOD DETAILS

Quantification of IgG titers

Serum samples were immediately stored at -80°C after blood sampling. Anti-RBD IgG were measured using the bioMerieux Vidas SARS-CoV-2 IgG II (9COG) diagnosis kit (BioMérieux, #424114), according to the manufacturer's recommendation. For standardization of these assays to the first WHO international standard, the concentrations were transformed in Binding Antibody Unit (BAU)/mL using the conversion factors provided by the manufacturer. Anti-N IgG were quantified using the Abbott Architect instrument using the Abbott SARS-CoV-2 IgG assay (ref. 6R86-20) following the manufacturer's instructions. The results are expressed as an index.

Anti-RBG IgG avidity

Avidity of RBD-specific IgG from patient sera is based on off-rate constant (K_{off}, s^{-1}) measurement because of its inverse relationship with the stability of the Ag/Ab complex, independent of antibody concentration. Total IgG were purified from 100 μL of each individual serum with Magne Protein G Beads (Promega, #G7471) according to manufacturer's instructions. BLI studies were performed on purified IgG with an Octet R8 instrument (Sartorius) using Octet® amine-reactive biosensors (AR2G; Sartorius, #18-5092). Kinetics assays were carried out at 30°C using Standard Kinetics Acquisition rate settings (5.0 Hz, averaging by 20, 1000 rpm). Briefly, after hydration in water, AR2G sensors were activated 10 min in 20 mM EDC and 10 mM s-NHS before antigen loading. RBD-His prefusion S (Sino Biological, cat 40592-V08B), 10 μg/ml in 10 mM sodium acetate pH6 was amine coupled to AR2G sensors during 10 min using Octet® amine-coupling reagents (Sartorius, #18-5095). Then, the sensor surface was inactivated with a solution of 1 M ethanolamine pH 8.5. RBD loaded AR2G sensors were dipped in 1X PBS (pH7.4) for 2 min to establish a baseline time course before 10 min association in purified serum IgG. Purified IgG were assayed from undiluted to diluted 1:50 in PBS depending on RBD-specific IgG titers of each sample. The dissociation step was monitored for 10 min by dipping sensors in 1X PBS into the wells used to collect the baseline. Dissociation rates were determined using Octet Analysis studio 12.2 software by Local full fitting of association and dissociation steps according to a 1:1 binding model.

Anti-S and anti-N IgG subtyping and anti-N FcγR binding

A custom SARS-CoV-2 multiplex assay was designed with the Wuhan Hu-1 S (Sino Biological, #40591-V08H) and N (Sino Biological, #40588-V08B) and the BA.1 S (Sino Biological, #40589-V08H30) antigens. Tetanus toxoid (Sigma Aldrich, #582231) and influenza hemagglutinin H1Cal2009 (Sino Biological, #11085-V08H) were also added to the assay as positive controls, while BSA-blocked beads were included as negative controls. The antigens were covalently coupled to Bio-Plex Pro Magnetic COOH beads (Bio-Rad) using a two-step carbodiimide reaction as per manufacturer's instructions, in a ratio of 10 million beads-to-100 μg of antigen. The antigen-coupled beads were resuspended in a storage buffer (PBS, 0.05% sodium azide) as one million beads per 100 μL, and stored in the dark at 4°C before use.

The IgG1-4 subclasses of antigen-specific antibodies present in the collected serum were assessed using a multiplex assay as previously described.[48] Briefly, the antigen-coupled beads were mixed, resuspended in 0.1% PBS-BSA and added to wells of a 96-well Bio-Plex Pro flat bottom plate (Bio-Rad, #171025001) containing serum in a 1:100 final dilution in 0.1% PBS-BSA. The sealed plates were incubated on a plate shaker overnight at 4°C. After incubation, the plates were centrifuged and washed with PBS-0.05% Tween 20 using a magnetic Bio-Plex Pro wash station (Bio-Rad, #30034376).

For IgG subtyping, mouse anti-human IgG1, IgG2, IgG3, IgG4 antibodies conjugated with phycoerythrin (PE, Southern Biotech) were added at a final concentration of 1.3 μg/mL and the mixture was incubated for 2h at room temperature on a plate shaker. For the detection of antigen-specific FcγR binding, soluble recombinant FcγRIIa (higher affinity polymorphisms FcγRIIa-H131) and FcγRIIIa (higher affinity polymorphisms FcγRIIIa-V158) dimers were biotinylated and added at a final concentration of 1.3 μg/mL.[49] After incubation at room temperature for 2h on a plate shaker, the plate was washed with PBS-0.05% Tween 20, and streptavidin-PE conjugate (SAPE, Invitrogen) was added at 1 μg/ml for another 2h incubation time on a plate shaker.

After the last washing with PBS-0.05% Tween 20, the beads were resuspended in MAGPIX® Drive Fluid PLUS (Luminex, #40-50030), the plates were read on a Bio-Plex MAGPIX® multiplex reader (Bio-Rad), and analyzed using Bio-Plex Manager software (Bio-Rad). The binding of the PE-detectors was measured to calculate the median fluorescence intensity (MFI). Double background subtraction was conducted, first removing background of blank (buffer only) wells followed by removal of BSA-blocked control bead background signal for each well.

IgG subtype quantifications are relative and thus the method does not allow for comparisons between IgG subtype levels.

Live virus neutralization experiments

A 50% Plaque Reduction Neutralization Test ($PRNT_{50}$) was used for the detection and titration of neutralizing Abs as previously described.[2,50] A 10-fold dilution of each serum specimen in Dulbecco's Modified Eagle's (DMEM) high glucose (Sigma-Aldrich, #D6429) culture medium was first heated for 30 min at 56°C to avoid complement-linked reduction of the viral activity. Two percent of heat inactivated Fetal Bovine Serum (FBS, Eurobio scientific, #CVFSF06-01) and 1% of penicillin (10,000 UI/mL) and streptomycin (10,000 UI/mL) (Eurobio scientific, #CABPES01-0U) were added to the culture medium. Serial 2-fold dilutions (tested in duplicate) of the serum specimens in culture medium were mixed in equal volume with the live SARS-CoV-2 virus. After gentle shaking and a

contact of 30 min at room temperature in plastic microplates, 150 μL of the mix was transferred into 96-well microplates covered with Vero E6 cells (ATCC CRL-1586). The plates were incubated at 37°C in a 5% CO_2 atmosphere. Infection efficiency was evaluated by microscopy 5 days later when the cytopathic effect of the virus control reached 100 to 500 Tissue Culture Infectious Dose ($TCID_{50}$/150 μL). Neutralization was recorded if more than 50% of the cells present in the well were preserved. The neutralizing titer was expressed as the inverse of the higher serum dilution that exhibited neutralizing activity; a threshold of 20 was used ($PRNT_{50}$ titer $\geq$20). All experiments were performed in a biosafety level 3 laboratory. The different viral strains that were used were sequenced and deposited on GISAID [GISAID accession numbers: EPI_ISL_1707038, 19A (B.38); EPI_ISL_7608613, Omicron BA.1 (B.1.1.529), EPI_ISL_12396843, BA.4; EPI_ISL_12852091, BA.5 and EPI_ISL_16335276, XBB.1.5.

Antibody dependent cellular cytotoxicity (ADCC)

ADCC was quantified using the ADCC Reporter Bioassay (Promega) as previously described.[51] Briefly, 293T cells stably expressing the indicated S proteins ($3x10^4$ per well) were co-cultured with Jurkat-CD16-NFAT-rLuc cells ($3x10^4$ per well) in presence or absence of mAbs at the indicated concentration. Luciferase was measured after 18 h of incubation using an EnSpire plate reader (PerkinElmer). ADCC was measured as the fold induction of Luciferase activity compared to the ''no serum'' condition. Sera were tested at a 1:30 dilution. For each serum, the control condition (cells transduced with an empty vector) was subtracted to account for interindividual variations of the background. We previously reported correlations between the ADCC Reporter Bioassay titers and an ADCC assay based on primary NK cells and cells infected with an authentic virus.[52]

Flow cytometry analysis of SARS-CoV-2 RBD-specific B cells

Flow cytometry was carried out as previously described.[2] Cryopreserved PBMCs were thawed, then centrifuged and suspended in PBS + 0.5% FBS and 2 mM EDTA (PEB buffer) and incubated with Fc receptor block (Miltenyi Biotec 130-059-901, 1:10 dilution) for 15 minutes at 4°C. Cells were then washed in PEB and stained for 30 minutes in brilliant stain buffer at 4°C in the dark using the following Abs: anti-CD45-PerCP (BD Biosciences, ref. 368506, 1:10 dilution), anti-CD3-APC Fire 810 (BioLegend, ref. 344858, 1:10 dilution), anti-CD19-PE Vio770 (Miltenyi Biotec, ref. 130-113-170, 1:25 dilution), anti-CD20-BV421 (BD Biosciences, ref. 562873, 1:10 dilution), anti-CD27-PerCPVio700 (Miltenyi Biotec, ref. 130-113-632, 1:25 dilution), anti-IgM-PE- CF594 (BD Biosciences, ref. 562539, 1:20 dilution), anti-IgD-BV605 (BioLegend 348232, 1:25 dilution) together with both the PE- and APC-conjugated recombinant Wuhan Hu-1 RBD tetramers as well as BUV737- and BB515-conjugated recombinant BA.1 RBD tetramers. Fluorescent SARS-CoV-2 RBD tetramers were prepared as follows. Biotinylated recombinant RBD domains of SARS-CoV-2 (Wuhan Hu-1 and BA.1 strains) were purchased from Miltenyi Biotec (Wuhan Hu-1 strain ref. 130-127-457, BA.1 strain ref. 130-130-419) and tetramerized either with streptavidin-PE (BD Biosciences ref. 554061), streptavidin-allophycocyanin (APC) (BioLegend, ref. 405243), streptavidin-BUV737 (BD Biosciences ref. 612775) or with streptavidin-BB515 (BD Biosciences ref. 564453). PE and APC were used to label Hu-1 RBD while BUV737 and BB515 were used to label BA.1 RBD. The identification of mBCs was based on the exclusion of IgD+ CD27- naive B cells (Figure S3A). Gating of PE and APC double positive mBCs enabled the identification of Hu-1 RBD-specific mBCs while gating of BUV737 and BB515 double positive mBCs enabled the identification of BA.1 RBD + mBCs (Figures S3B and S3C respectively).

QUANTIFICATION AND STATISTICAL ANALYSIS

Comparison of clinical parameters between all groups was carried out using a Kruskal-Wallis or a Fisher's exact test, both followed by Bonferroni's test for multiple comparisons. For the comparisons of immune parameters between the Hu-1/2RNA/BA.1 and the 3RNA/BA.1 groups, multiple regressions (ANCOVA) were realized for each variable, incorporating age as a covariate, given the age difference between the two groups, using R software. When comparing immune parameters between the 3RNA/BA.1 and 3RNA groups and between the Hu-1/2RNA/BA.1, Hu-1/2RNA and Hu-1/3RNA groups, a two-way ANOVA mixed model was conducted followed by a Bonferroni's test for multiple comparisons. Comparisons of the percentage of RBD+ mBCs between the 3 groups with established hybrid immunity were conducted using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Comparisons for immune parameters between all 5 groups were carried out using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. Analyses were conducted using GraphPad Prism® software (version 8; GraphPad software) and R software (https://cran.r-project.org/). Exact p-values are represented on all graphs. All statistical details can be found in the figure legends.