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RESEARCH ARTICLE

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Longer intervals between SARS-CoV-2 infection and mRNA-1273 doses improve the neutralization of different variants of concern

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Abstract

The humoral immune response against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants of concern elicited by vaccination was evaluated in COVID-19 recovered individuals (Rec) separated 1–3 months (Rec2m) or 4–12 months (Rec9m) postinfection and compared to the response in naïve participants. Antibody-mediated immune responses were assessed in 66 participants by three commercial immunoassays and a SARS-CoV-2 lentiviral-based pseudovirus neutralization assay. Immunoglobulin (Ig) levels against SARS-CoV-2 spike were lower in naïve participants after two doses than in Rec after a single dose ($p < 0.05$). After two doses in Rec, levels of total Ig to receptor-binding domain were significantly increased in Rec9m compared to Rec2m ($p < 0.001$). The neutralizing potency observed in Rec9m was consistently higher than in Rec2m against variants of concern (VOCs) Alpha, Beta, Delta, and BA.1 sublineage of Omicron with 2.2–2.8-fold increases. Increasing the

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interval between SARS-CoV-2 infection and the vaccination with messenger RNA-based vaccines to more than 3 months generates a more efficient heterologous humoral immune response against VOCs by allowing enough time to mount a strong recall memory B cell response.

KEYWORDS
hybrid immunity, mRNA-1273, neutralizing antibodies, Omicron, timing of vaccination

1 | INTRODUCTION

The magnitude of adaptive immune responses elicited by either severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection or vaccination will define the protective immunity against new infections as well as the control and clearance of an ongoing infection.[1] SARS-CoV-2-specific antibodies, $CD4^+$ T cells, and interferon-γ-producing CD8+ T cells were all identified after both SARS-CoV-2 natural infection and vaccination.[2] Neutralizing antibodies (NAbs) are associated with protective immunity against SARS-CoV-2 infection in nonhuman primates[3–5] and are highly predictive of immune protection from symptomatic infection in humans.[6,7] However, the levels of NAbs are in general not correlated with disease severity[8] and the passive transfer of NAbs to infected patients has limited efficacy on COVID-19.[9,10] On the other hand, different studies support the importance of T cells as the other arms of the adaptive immune response in the control and resolution of the infection.[11,12] Despite that, NAbs are considered a surrogate marker of protection and the immunogenicity of the most widely inoculated COVID-19 vaccines has been demonstrated by their capacity to produce robust neutralizing responses.[13–16] In the case of SARS-CoV-2 natural infection, immunoglobulin G (IgG) to the spike protein and receptor-binding domain (RBD), and NAbs decrease importantly over 8 months, although spike-specific IgG memory B cells increase at that time compared with 1 month after infection.[1] This fact suggests that the evolution and maturation of this long-lasting response generated by memory B lymphocytes require longer periods from the natural infection to the vaccination to be optimal.

The messenger RNA (mRNA)-1273 vaccine is a lipid nanoparticle–encapsulated mRNA-based vaccine that encodes the prefusion stabilized full-length spike protein of SARS-CoV-2. The phase 3 clinical trial of the mRNA-1273 vaccine showed 94.1% efficacy in preventing COVID-19 illness.[17] The immunogenicity of mRNA-1273 vaccine in terms of RBD-binding IgG and neutralizing capacity peaked at day 14 postboost.[13] Longitudinal studies revealed a 1.2-, 2.3-, and 5.3-fold reduction in the neutralization titer observed at days 28, 90, and 180 after the second dose, respectively.[18,19] The mRNA-based vaccines produce stronger antibody responses compared with adenovirus vaccines[20] and higher total anti-RBD immunoglobulin (Ig) titers have also been observed in two large cohorts of vaccinees at 2–6 and 6–10 weeks after the vaccination with mRNA-1273 compared with those vaccinated with BNT162b2.[20,21] The strong correlation between anti-RBD Ig and neutralizing titers[13] and the immunogenicity results published in their respective phase 1/2 clinical trials suggest that mRNA-1273 vaccination produce higher neutralizing titers compared with BNT162b2.[22]

The emergence of new SARS-CoV-2 variants has raised concerns about the effectiveness of vaccination strategies. Among the three compartments of the adaptive immune response elicited by vaccination, SARS-CoV-2-specific $CD4^+$ T cells and $CD8^+$ T cells are not greatly disrupted by variants of concern (VOCs).[23,24] However, a plethora of publications have shown the escape neutralization of VOCs by vaccine-induced humoral immunity.[25–34] Although there is evidence that SARS-CoV-2-specific circulating antibodies decrease rapidly after infection and vaccination, there is less information characterizing the time necessary to mount an effective and durable memory B cell response against VOCs in COVID-19-recovered patients and later vaccinated.[18,19] This knowledge is extremely valuable in Public Health to face the advent and spread of new variants and to mitigate situations like the emergence of the BA.1 wave at the beginning of 2022, promoting second and third doses of the vaccine at the optimal time for COVID-19 recovered patients.

Within this context, we evaluated the humoral response elicited in convalescent and naïve vaccinees with mRNA-1273 against VOCs Alpha, Beta, Delta, and Omicron and against the variant of interest (VOI) Mu. The population selected for this study allowed us to compare hybrid immunity versus vaccine-induced immunity. Recent studies have associated hybrid immunity with improved protection against the disease through more virus-specific memory B cells and neutralizing antibody breadth as well as a different population of spike-specific $CD4^+$ T cells compared with naïve vaccinees.[34,35] Finally, the effect of separating vaccination 1–3 months versus 4–12 months from the natural infection in COVID-19 recovered patients will be evaluated, allowing sufficient time for the latter to mount a strong recall memory B cell response.

2 | MATERIALS AND METHODS

2.1 | Study design and population

Authors investigated the impact of SARS-CoV-2 variants on recognition by sera from individuals who received two 100 µg doses

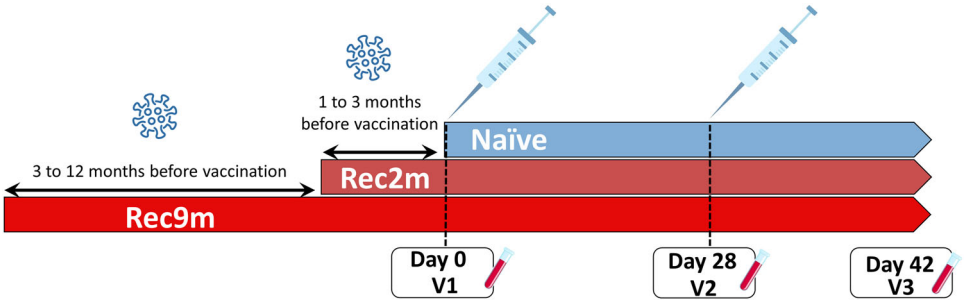


FIGURE 1 Study design, indicating the time of previous infection, vaccine administration, and blood collection for the three groups included in the study (Naïve, Rec2m, and Rec9m).

of the SARS-CoV-2 vaccine messenger RNA (mRNA)-1273 (Moderna), including COVID-naïve participants ($n = 34$) and individuals with a prior SARS-CoV-2 infection with polymerase chain reaction test positivity ($n = 32$). The mRNA-1273 vaccine encodes the full-length stabilized spike protein of a D614 variant (WA1), administered in two doses, 28 days apart. Sera from 66 healthcare workers from the University Hospital Puerta de Hierro (Majadahonda, Spain) were collected in three time points: visit 1 (V1) at prevaccination (on the day of the first dose of mRNA-1273 administration), visit 2 (V2) at preboost (on the day of the second dose administration), and visit 3 (V3) at postboost (14 days after the second dose) on days 0, 28, and 42 after the first dose, respectively (Figure 1). To evaluate the effect of the time elapsed from SARS-CoV-2 infection on the humoral immune response against the virus, vaccine recipients with a documented prior history of COVID-19 were included. Among these convalescent recipients of mRNA-1273, 17 (53.1%) were late-infected during the first 3 months before vaccination (Rec2m) and 14 (43.7%) with an early infection documented from 3 to 12 months before vaccination (Rec9m). None had a known history of re-exposure to SARS-CoV-2 after the initial infection.

2.2 | SARS-CoV-2-specific humoral immune response

We used three different commercial immunoassays to assess antibody-mediated immune responses. The three commercial assays quantitatively reported the concentration of IgG/IgA Ig in united antibody units (BAU/mL). First, the Elecsys anti-SARS-CoV-2 electrochemiluminescent immunoassay (Roche Diagnostic) to test for total Ig to SARS-CoV-2 RBD spike on the Cobas e411 module (Roche Diagnostic). Measuring range from 0.4 to 250.0 U/mL (up to 2500 U/mL with onboard 1:10 dilution, and up to 12 500 U/mL with onboard 1:50 dilution). Values higher than 0.8 BAU/mL were considered positive. The conversion factor between U/mL and BAU/mL was 1 U/mL is equal to 0.972 BAU/mL.

Second, the LIAISON® SARS-CoV-2 TrimericS chemiluminescent immunoassay (DiaSorin) to test for IgG to trimeric spike glycoprotein on the Liason XL (DiaSorin). This technique has a measuring range from 4.81 to 2080.0 BAU/mL. As per the manufacturer's instructions, values more than 2080.0 BAU/mL were diluted 1:20, and values higher than 33.8 BAU/mL were considered positive.

Third, the enzyme-linked immunosorbent assay for the detection of IgA antibodies to the stabilized trimer of the SARS-CoV-2 spike glycoprotein (Immunostep). IgA quantification was expressed in BAU/mL after the calibration with serial dilutions of positive control with a known concentration included in the kit.

2.3 | SARS-CoV-2 pseudovirus neutralization assay

The codon-optimized sequence from Ou et al.[36] was modified by synthesis (GeneArt Gene Synthesis; ThermoFisher Scientific) to generate the different spikes of SARS-CoV-2 virus: D614G (as the reference variant), Alpha (Δ69-70, Δ144, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H), Beta (L18F, D80A, D215G, Δ242–244, K417N, E484K, N501Y, D614G, A701V), Delta (T19R, Δ156-157, R158G, L452R, T478K, D614G, P681R, D950N), Mu (T95I, Y144S, Y145N, R346K, E484K, N501Y, D614G, P681H, D950N), and Omicron (A67V, Δ69–70, T95I, G142D, Δ143-145, N211I, Δ212, G339D, S371L, S373P, S375F, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, D796Y, N856K, Q954H, N969K, L981F). All these sequences were synthesized removing the last 19 amino acids and were inserted in pcDNA3.1D/V5-His-TOPO between BamHI and XbaI sites. The pcDNA-VSV-G plasmid contains the complementary DNA (cDNA) encoding the vesicular stomatitis virus G protein and was obtained from Dr. Arenzana-Seisdedos (Institute Pasteur). VSV-G pseudoviruses were used as a control of specificity in neutralization testing.

NL4.3 pseudotypes were generated with the previously described plasmid pNL4-3ΔenvRen.[37] Briefly, Renilla luciferase reporter pseudovirus was prepared by co-transfecting HEK-293T cells with pNL4-3ΔenvRen backbone and viral envelope protein expression plasmid pcDNA3.1-S-CoV2Δ19 or pcDNA-VSV-G using the calcium phosphate method. The medium was changed 18 h after transfection, and 48 h posttransfection cell culture supernatants were harvested, clarified by centrifugation at 500*g* for 5 min, and frozen at −80°C. The amount of HIV p24 antigen in the supernatants

was quantified by electrochemiluminescence Immunoassay (Roche Diagnostic) on the Cobas e411 module (Roche Diagnostic).

To measure the neutralizing activity of plasma from donors, fourfold serial dilutions of heat-inactivated sera (1:32–1:131 072) were preincubated with titrated pseudoviruses (10 ng of p24 Gag/well) for 1 h at 37°C. Thereafter, 100 µL of the mixture was added to Vero E6 cells plated the previous day at 5×10^3 cells/well in 100 µL medium in 96-well plates. The culture medium was refreshed after 16 h. At 48 h postinfection, cells were lysed, and viral infectivity was assessed by measuring luciferase activity (Renilla Luciferase Assay; Promega) using a 96-well plate luminometer LB 960 Centro XS³ (Berthold Technologies). The titers of NAbs were calculated as 50% inhibitory dose (neutralizing titer 50, NT50), expressed as the highest dilution of plasma which resulted in a 50% reduction of luciferase activity compared to control without plasma. Sigmoid curves were generated and NT50s were calculated by nonlinear regression using GraphPad Prism version 9.3.1 (GraphPad Software Inc.).

2.4 | Statistical analysis

All analyses were carried out using the statistical software GraphPad Prism version 9.0.1 (GraphPad Software Inc.). All continuous variables were summarized using the following descriptive statistics: *n* (nonmissing sample size), geometric mean, first quartile, median, third quartile, maximum, and minimum. Absolute and relative frequencies (based on the nonmissing sample size) of observed levels were reported for all categorical variables. All continuous variables were tested for normality hypothesis assumption with the Kolmogorov–Smirnov test, using the nonparametric rank-based Kruskal–Wallis or the equivalent one-way analysis of variance tests to determine whether the means of two or more groups are different. *p* values lower than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Overall clinical characteristics of study participants

Sixty-six vaccinated participants were included, with a median age of 31.8 years (interquartile range [IQR], 24.7–46.1); 69.7% were women and 18.2% were aged 50 years or older. Baseline characteristics including age, sex, comorbidities, concomitant medications, and the severity of the disease were evenly distributed between naïve and convalescent participants with serologic evidence of prior COVID-19. Among convalescent participants, the median time from infection was 45 days (IQR: 35–54 days) for the lately-infected individuals (Rec2m) and 306 days (IQR: 171–347 days) for the early-infected vaccine recipients (Rec9m) (Table 1).

TABLE 1 Characteristics of the study population vaccinated with mRNA-1273.

	Naïve (*n* = 34)	Recovered (*n* = 32)
Age	32.6 (24.4–44.3)	32.1 (26.5–47.2)
Sex		
Female	23 (67.6%)	23 (71.9%)
Comorbidities		
Obesity	2 (5.9%)	1 (3.1%)
Arterial hypertension	2 (5.9%)	1 (3.1%)
Asthma	1 (2.9%)	0
Diabetes	1 (2.9%)	0
Immunosuppression	1 (2.9%)	1 (3.1%)
Severity of disease[a]		
No clinical or virological evidence of infection	34 (100%)	1 (3.1%)
Ambulatory, no limitation of activities	–	6 (18.7%)
Ambulatory, limitation of activities	–	20 (62.5%)
Hospitalized, mild disease, no oxygen therapy	–	4 (12.5%)
Hospitalized, mild disease, oxygen therapy	–	0
Hospitalized, noninvasive ventilation	–	0
Hospitalized, intubation, and mechanical ventilation	–	1 (3.1%)
Hospitalized, ventilation + additional organ support	–	0
Death	–	0
Previous COVID-19		
<3 months from vaccination (mean: 48 days)	–	17 (53.1%)
>3 months from vaccination (mean: 266 days)	–	14 (43.7%)

Abbreviations: mRNA, messenger RNA; WHO, World Health Organization.

[a]WHO Ordinal Scale for Clinical Improvement, COVID-19 trial design synopsis (0–8 scale).

3.2 | Humoral immune response against SARS-CoV-2

Levels of total Ig to SARS-CoV-2 spike RBD were lower in naïve participants than Rec at both preboost (154 [95% confidence interval, CI, 98.3–241.0 BAU/mL] vs. 8234 BAU/mL [95% CI, 5325–12 732]) ($p < 0.0001$) and postboost immunization

(4921 [95% CI, 3701–6544 BAU/mL] vs. 14 754 BAU/mL [95% CI, 11 880–18 323]) ($p < 0.0001$). Lower levels were even found after comparing naïve participants after two doses with Rec after a single dose ($p < 0.01$) (Figure 2A). The same pattern was observed for anti-trimeric spike IgG levels, comparing naïve recipients and Rec. Specifically, a total of 834 (95% CI, 618–1126 BAU/mL) and 5459 BAU/mL (95% CI, 4036–7383) were found after one dose ($p < 0.0001$), whereas 3587 (95% CI, 2760–4662) and 6526 BAU/mL (95% CI, 4779–8913) were found after two doses ($p < 0.01$) for naïve recipients and convalescents, respectively. Lower levels were also found comparing anti-trimeric IgG for naïve recipients at postboost and the levels of convalescents at preboost ($p < 0.05$) (Figure 2B). The levels of anti-SARS-CoV-2 spike IgA in naïve recipients were 680 BAU/mL (95% CI, 374–1236 BAU/mL) compared with 3161 BAU/mL (95% CI, 2213–4514 BAU/mL) observed in Rec at V2. After two doses, no differences were found between naïve and Rec vaccine recipients with titers of 5755 (95% CI, 4600–7198 BAU/mL) versus 4477 BAU/mL (95% CI, 3378–5934 BAU/mL), respectively (Figure 2C).

3.3 | Neutralizing activity against SARS-CoV-2 variants of concern

The pseudovirus-based assay was used to measure D614G SARS-CoV-2 neutralization, by estimating the NT50. Sera from

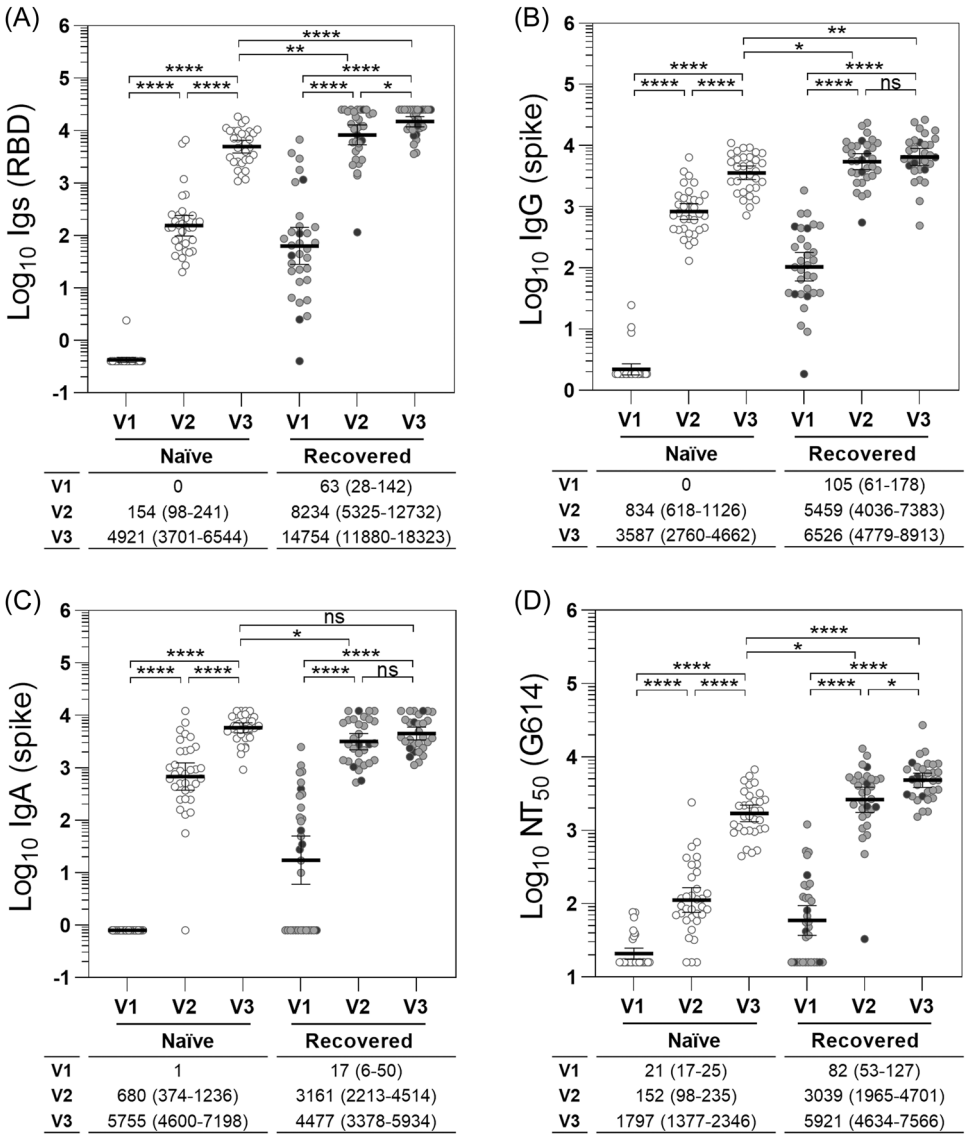


FIGURE 2 Humoral immune response against SARS-CoV-2 spike. Total anti-RBD immunoglobulins (A), IgG to trimeric spike glycoprotein (B), IgA to trimeric spike glycoprotein (C), and pseudotyped neutralization assay (D). Levels of immunoglobulins are reported in BAU/mL and neutralizing activity in neutralizing titer 50 (NT50). All measures were summarized as geometric mean and 95% confidence interval. Black circles represent the values of the recovered participants who required hospitalization. BAU/mL, binding antibody units per mL; IgG, immunoglobulin G; RBD, receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ns, $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ****$p \leq 0.0001$.

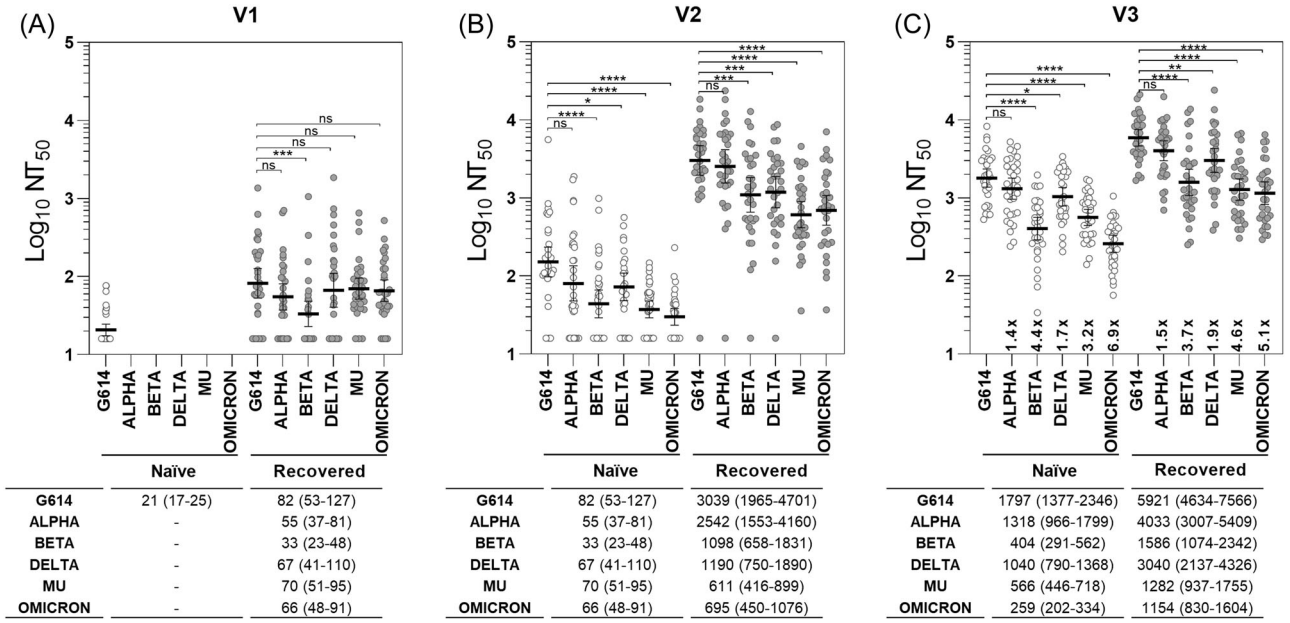
naïve recipients reached lower levels of neutralization compared with Rec's sera after a single dose (152 NT50 [95% CI, 98–235 NT50] vs. 3039 NT50 [95% CI, 1965–4701]; $p < 0.0001$) and after two doses (1797 NT50 [95% CI, 1377–2346 NT50] vs. 5921 NT50 [95% CI, 4634–7566]; $p < 0.0001$). The neutralization observed in Rec participants after a single dose was higher than that observed in naïve participants after two doses ($p < 0.05$) (Figure 2D). The neutralization of Alpha (B.1.1.7 lineage), Beta (B.1.351 lineage), Delta (B.1.617.2 lineage), Mu (B.1.621 lineage), and Omicron (BA.1 lineage) variants were also measured. The same pattern was consistently found in naïve and Rec recipients at every time point, with the D614G variant showing the higher neutralizing activity, Beta, Omicron, and Mu the lowest NT50 levels, and Alpha and Delta between them (Figure 3).

Scarce neutralization levels were observed before vaccination in Rec (NT50 levels under 100). However, we consistently found statistically significant higher values of NT50 in Rec recipients compared to naïve participants and for each tested variant in the three time points. Thus, naïve recipients showed a 2.3–3.3-fold reduction against Alpha, Beta, Delta, and Mu variants after two doses and compared to convalescent participants, whereas the reduction against Omicron was even higher (4.5-fold reduction) (Figure 3C). This higher fold reduction observed for Omicron was the result of the low NT50 value of naïve participants (259 NT50) compared with convalescents (1154 NT50), being both the lowest values observed against a variant. NT50 values observed in convalescents after a single dose (V2) were consistently higher than in naïve individuals after the completion of the vaccination regime (V3) (Figure 3). The second dose in Rec assured NT50 levels above 200 for all the participants (Figure 3C).

After completion of the vaccination regime, the naïve recipients experienced 1.4-, 4.4-, 1.7-, 3.2-, and 6.9-fold reductions in the neutralizing activity of Alpha, Beta, Delta, Mu, and Omicron variants, respectively, as compared with D614G. Similarly, in the Rec vaccine recipients, a 1.5-, 3.7-, 1.9-, 4.6-, and 5.1-fold reduction was observed in the neutralization of Alpha, Beta, Delta, Mu, and Omicron variants, respectively (Figure 3).

3.4 | Rec9m show higher levels of Ig to SARS-CoV-2 spike compared with Rec2m

To quantify the effect of the time from SARS-CoV-2 infection in Rec, we split the group into those late-infected within the 3 months before receiving the first dose of mRNA-1273 (Rec2m) and those early-infected, at least 3 months before the first shot (Rec9m). Overall, the levels of anti-RBD Ig, IgG to trimeric spike, and anti-spike IgA were consistently higher in the Rec9m group compared to Rec2m at both pre- and postboost time points. After the second dose, levels of total Ig to RBD were 10 219 (95% CI, 7572–13 792 BAU/mL) and 21 618 BAU/mL (95% CI, 18 092–25 831) for Rec2m and Rec9m groups, respectively ($p < 0.001$); levels of IgG to trimeric spike were 5020 (95% CI, 3478–7246 BAU/mL) and 8470 BAU/mL (95% CI, 4843–14 816 BAU/mL) for Rec2m and Rec9m groups, respectively ($p > 0.05$); and levels of anti-spike IgA were 2530 (95% CI, 1922–3332 BAU/mL) and 7687 BAU/mL (95% CI, 5803–10 184 BAU/mL) for Rec2m and Rec9m groups, respectively ($p < 0.0001$) (Figure 4).



	V1 Naïve	V1 Recovered	V2 Naïve	V2 Recovered	V3 Naïve	V3 Recovered
G614	21 (17-25)	82 (53-127)	82 (53-127)	3039 (1965-4701)	1797 (1377-2346)	5921 (4634-7566)
ALPHA	-	55 (37-81)	55 (37-81)	2542 (1553-4160)	1318 (966-1799)	4033 (3007-5409)
BETA	-	33 (23-48)	33 (23-48)	1098 (658-1831)	404 (291-562)	1586 (1074-2342)
DELTA	-	67 (41-110)	67 (41-110)	1190 (750-1890)	1040 (790-1368)	3040 (2137-4326)
MU	-	70 (51-95)	70 (51-95)	611 (416-899)	566 (446-718)	1282 (937-1755)
OMICRON	-	66 (48-91)	66 (48-91)	695 (450-1076)	259 (202-334)	1154 (830-1604)

FIGURE 3 NT50 in pseudovirus neutralization assay against SARS-CoV-2 variants Alpha, Beta, Delta, Mu, and Omicron. NT50 levels were obtained in V1 at prevaccination (A), V2 at preboost (B), and V3 at postboost (C). NT50 was summarized as a geometric mean and 95% confidence interval. NT50, neutralizing titer 50; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; V1, visit 1; ns, $p > 0.05$; *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; ****$p \le 0.0001$.

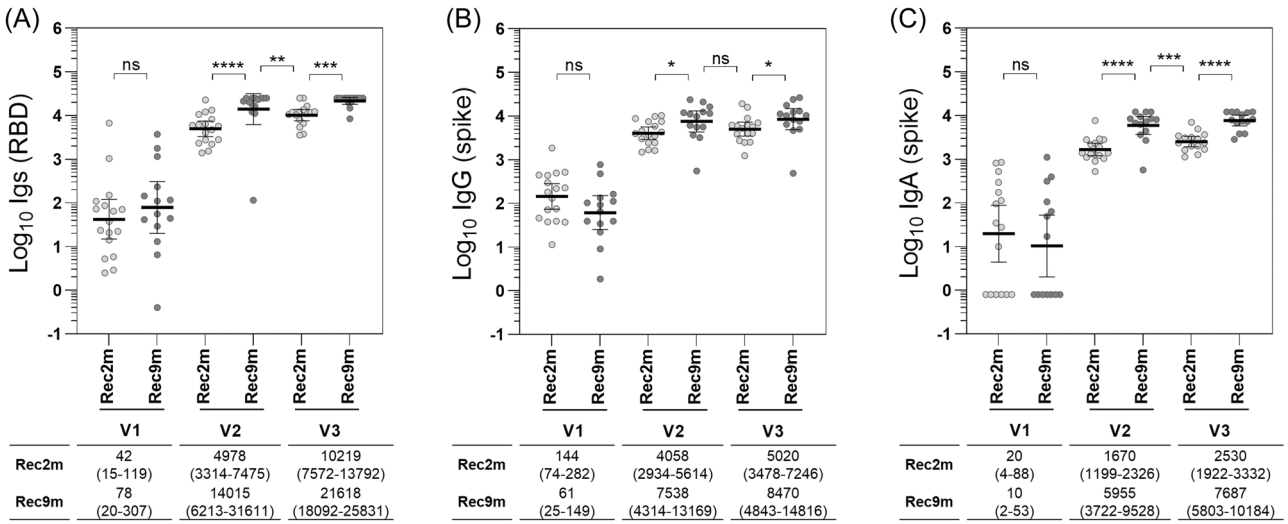


FIGURE 4 Levels of immunoglobulins to SARS-CoV-2 spike in Rec2m and Rec9m vaccine recipients. Total anti-RBD immunoglobulins (A), IgG to trimeric spike glycoprotein (B), and IgA to trimeric spike glycoprotein (C) were expressed as BAU/mL in geometric mean and 95% confidence interval. BAU/mL, binding antibody units per mL; IgG, immunoglobulin G; RBD, receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ns, $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

3.5 | Neutralizing activity of the variants of concern in Rec2m and Rec9m

The neutralizing activity of sera from Rec9m vaccine recipients was consistently higher than the NT50 found in Rec2m group against all the variants analyzed in the study at V2 and V3. Specifically, after the second dose of mRNA-1273, the neutralization levels of Alpha were 2575 NT50 (95% CI, 1737–3817 NT50) for Rec2m and 6306 NT50 (95% CI, 4548–8743 NT50) for Rec9m; 922 NT50 (95% CI, 553–1536 NT50) versus 2607 NT50 (95% CI, 1614–4211 NT50) for Beta in Rec2m and Rec9m, respectively; 1795 NT50 (95% CI, 1135–2838 NT50) versus 4991 NT50 (95% CI, 3319–7506 NT50) for Delta in Rec2m and Rec9m, respectively; 815 NT50 (95% CI, 540–1232 NT50) versus 1911 NT50 (95% CI, 1350–2705 NT50) for Mu in Rec2m and Rec9m, respectively; and 765 (95% CI, 485–1206) versus 1656 NT50 (95% CI, 1106–2480 NT50) for Omicron in Rec2m and Rec9m, respectively (Figure 5). These levels entail reductions in Rec2m compared with the Rec9m group of 2.4-fold against Alpha, 2.8-fold against Beta, 2.8-fold against Delta, 2.3-fold against Mu, and 2.2-fold against Omicron. The second dose in Rec9m guarantees neutralizing activity of at least 400 NT50 for all participants and against all the SARS-CoV-2 variants. This premise is only achieved in Rec2m against D614G and Alpha variants and is not reached in naïve participants for any VOC tested.

4 | DISCUSSION

SARS-CoV-2 infection induces a robust humoral and cellular immune response and seroprevalence rates range from 90% to more than 99% in patients who self-reported or had laboratory-documented SARS-CoV-2 infection.[8,38] Circulating SARS-CoV-2-specific IgA, IgG, and IgM isotypes can be detected in blood a few days after infection, with IgM and IgA decreasing rapidly and IgG being more durable.[39] In our study, no differences were found comparing the levels of anti-spike IgG and IgA, anti-RBD Ig, and NAbs between Rec2m and Rec9m patients before vaccination, suggesting that a single natural infection elicits low levels of long-lasting SARS-CoV-2-specific responses which are robustly boosted after a single dose of vaccination. A recent study involving 188 COVID-19 cases determined the SARS-CoV-2 antibody dynamics in the first 6–8 months after infection.[1] Dan et al.[1] also estimated the half-life of IgG and IgA to the RBD, as this region is the target of the antibodies with neutralizing capacity against the virus.[1,39,40] In the case of IgG to RBD, a half-life of 83 days was estimated, and a proportion of 88% of subjects was seropositive at 6–8 months after symptom onset. For RBD IgA, the half-life was estimated at 27 days, and no IgA responses were detected after 90 days. On his part, a half-life of 27 days was determined for the NAbs with 90% of individuals maintaining titers of NAbs after 6–8 months.[1]

Multiple factors could distort these antibodies' kinetics, including but not limited to: the severity of the disease (with higher antibody levels in persons with more severe COVID-19),[41] age (with lower neutralizing potency in the elderly population),[42] and the presence of immunocompromising conditions (with a suboptimal immune response in affected individuals).[43] These factors were controlled in the present study with no differences between groups and suggesting that the observed differences between naïve, Rec2m and Rec9m individuals are not attributable to confounding factors.

Vaccination of naïve individuals with mRNA-1273 vaccine also generates a durable response based on the production of anti-spike, anti-RBD, and NAbs.[13,17–19] The results obtained in this study and in other published work reveal that if this vaccination occurs in individuals previously infected with SARS-CoV-2, the immune

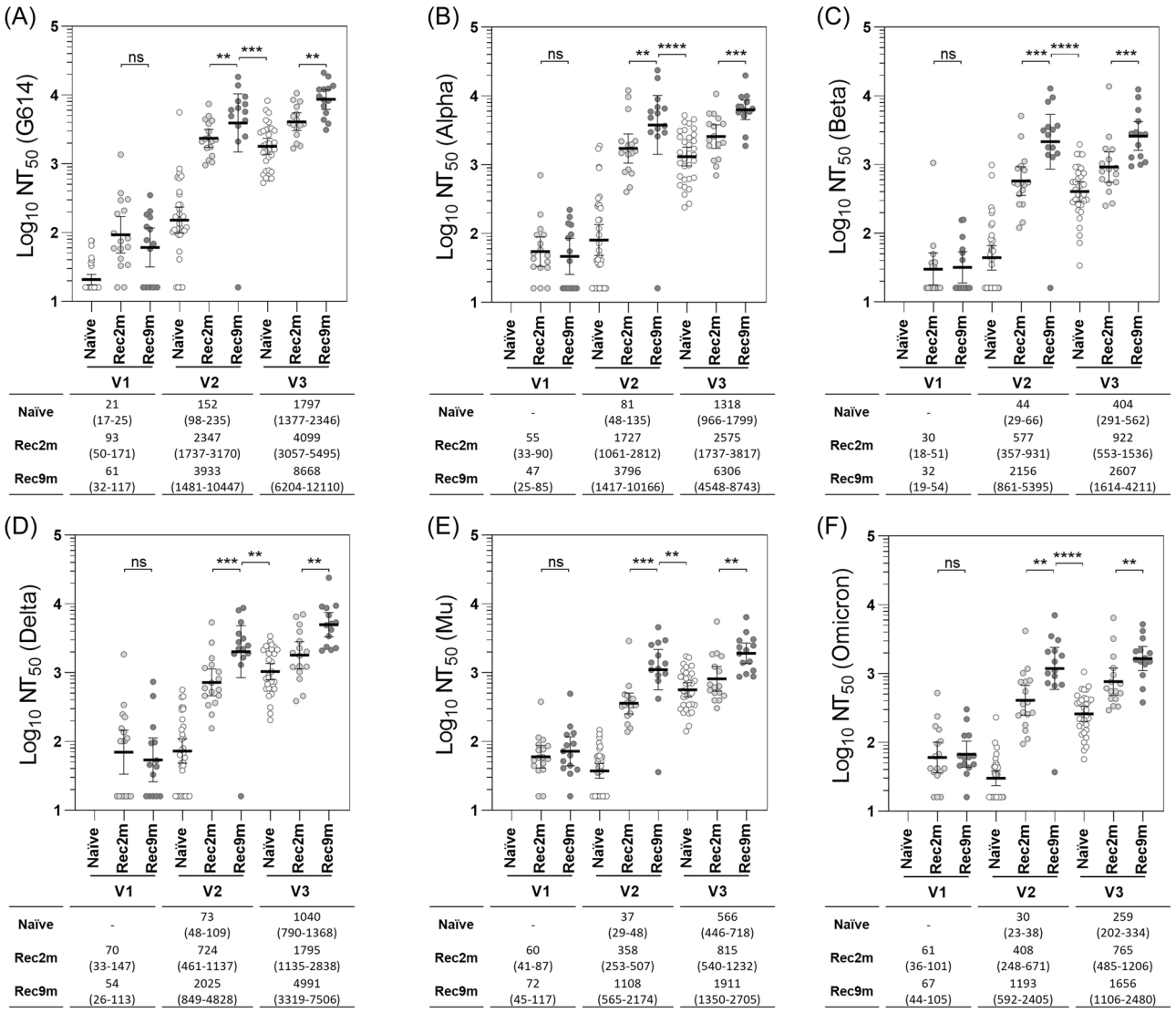


FIGURE 5 NT50 levels in pseudovirus neutralization assay in naïve, Rec2m, and Rec9m vaccine recipients against SARS-CoV-2 variants D614G (A), Alpha (B), Beta (C), Delta (D), Mu (E), and Omicron (F). NT50 was summarized as a geometric mean and 95% confidence interval. NT50, neutralizing titer 50; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ns, $p > 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

response after a single dose of mRNA-1273 is not only equivalent to two doses but elicits a greater response in terms of anti-spike, anti-RBD, and NAbs responses.[5,27] Moreover, this improved immune response after a single dose is observed for the original D614G variant, and also for the VOCs Alpha, Beta, Delta, Omicron and VOI Mu. In this regard, the present study includes individuals infected with SARS-CoV-2 between 2020 and January 2021. During this stage, the B.1.177 lineage (a D614G variant) dominated the SARS-CoV-2 pandemic in Spain previously to the Alpha, Delta, and Omicron waves,[44] suggesting that the neutralization titers observed in the present study are not distorted by homologous infections, since the probability that some recovered patients included was infected with any VOC is very low. A recent study in 43 COVID-19-recovered individuals has compared the effect to receive a single dose of an mRNA vaccine after around 6 months versus around 18 months from infection. Similarly to our study, increased neutralizing activities of wild-type and VOC Delta were elicited in individuals in whom natural infection was separated around 18 months from vaccination.[45] However, these differences disappeared after the second dose of the mRNA vaccine contrary to our observation. This discrepancy could be explained by the difference in the time spanning from natural infection and vaccination, being at least 3 months sufficient to mount a stronger response to a second encounter with the antigen.

Beyond circulating antibodies, after SARS-CoV-2 infection, memory B cell responses are the basis to mount a long-lasting protective immunity against virus infection and severe disease. The differences observed in the adaptive humoral responses in early- and late-infected individuals may be a consequence of this specific response. In this sense, during the first 60 days after symptom onset, SARS-CoV-2 spike-specific IgG^+ and IgM^+ memory B cells seem to be the equally dominating isotypes and over IgA^+ memory B cells, which barely represent a 5% stable fraction over 8 months after infection.[1]

After these 60 days, IgM^+ memory B cells decrease, and IgG^+ memory B cells dominated by 6 months. Around 10%–30% of the spike-specific memory B cells were specific for the RBD domain.[1] SARS-CoV-2 infection elicits an RBD-specific memory B cell response that is not detected in unexposed subjects, appeared as early as 16 days, constantly increased in the following 5 months, and is maintained at lower titers after 12 months from infection.[1,46] Low levels of somatic mutations were observed in recovered individuals during the first month after infection, suggesting that longer periods are necessary to mount stronger memory B cell responses.[47] In this sense, clonal evolution of memory B cells has been evidenced by the increase in the somatic hypermutation of antibody genes observed in the dark zone of germinal centers during the first 12 months after infection and especially during the 2–6 months period.[46,47] This antibody evolution is driven by a persistent recognition of the antigen trapped in follicular dendritic cells in the light zone of the germinal centers by B cells.[46,47] Regarding our study, it is possible that the Rec2m group did not have enough time for its memory B cell response to evolve through somatic mutation, something that may have occurred in the Rec9m group.

On the other hand, vaccination after COVID-19 recovery significantly increases the number of circulating RBD-specific memory B cells between 6 and 12 months after infection, which could also explain the differences in the neutralizing potency observed in the present study between Rec2m and Rec9m individuals.[27,46,48] Vaccination in COVID-19-recovered patients triggers an increase in the affinity and neutralizing capacity of the antibodies obtained from clonally expanded B cells 12 months after infection by only retaining RBD-specific NAbs with the capacity to effectively interact with angiotensin-converting enzyme-2.[46] In this respect, a recent study showed that memory B cells circulating after a third dose of mRNA vaccine were similar to those observed in COVID-19 recovered and vaccinated individuals, suggesting that clonal evolution of memory B cells after three doses of mRNA vaccine also leads to increased potency and breadth of NAbs against the virus, including Omicron.[49]

In conclusion, our study confirms the advantage of hybrid immunity over vaccine-induced humoral response and reinforces the idea that vaccines based on the ancestral SARS-CoV-2 spike protein effectively confer protection against more recent variants in COVID-19 recovered individuals. This protection in terms of neutralizing potency and breadth was achieved despite the infection with heterologous variants circulating before the definition of VOCs at the end of 2020. Finally, this study also remarks on the importance of proper timing for boosting COVID-19-recovered individuals as a significant factor to face current VOCs, including the highly diversified Omicron variants.

AUTHOR CONTRIBUTIONS

Cristina Avendaño-Solá and **José Alcamí**: Conceptualization. **Javier García-Pérez**, **Mercedes Bermejo**, **Almudena Ramírez-García**, and **Mayte Pérez-Olmeda**: Methodology. **Javier García-Pérez**, **Almudena Ramírez-García**, and **Mayte Pérez-Olmeda**: Validation. **Javier García-Pérez**, **Mercedes Bermejo**, **Almudena Ramírez-García**, **Mayte Pérez-Olmeda**, and **Francisco Díez-Fuertes**: Formal analysis. **Javier García-Pérez**, **Mercedes Bermejo**, **Almudena Ramírez-García**, **Humberto Erick De LaTorre-Tarazona**, **Almudena Cascajero**, **María Castillo de la Osa**, **Paloma Jiménez**, **Marta Aparicio Gómez**, **Esther Calonge**, **Aránzazu Sancho-López**, **Concepción Payares-Herrera**, **Rocio Layunta Acero**, **Laura Vicente-Izquierdo**, **Mayte Pérez-Olmeda**, and **Francisco Díez-Fuertes**: Investigation. **Javier García-Pérez**, **Almudena Ramírez-García**, and **Mayte Pérez-Olmeda**: Data curation. **Javier García-Pérez** and **Francisco Díez-Fuertes**: Writing—original draft. **Cristina Avendaño-Solá**, **José Alcamí**, **Mayte Pérez-Olmeda**: Writing—review and editing. **Javier García-Pérez**, **Mayte Pérez-Olmeda**, and **Francisco Díez-Fuertes**: Visualization. **Javier García-Pérez**, **Mercedes Bermejo**, **Cristina Avendaño-Solá**, **José Alcamí**, **Mayte Pérez-Olmeda**, and **Francisco Díez-Fuertes**: Supervision. **Javier García-Pérez**, **Cristina Avendaño-Solá**, **José Alcamí**, and **Mayte Pérez-Olmeda**: Project administration. **Javier García-Pérez**, **Cristina Avendaño-Solá**, **José Alcamí**, and **Mayte Pérez-Olmeda**: Funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

All the subjects included in the study gave their informed consent for the use of clinical data, including blood donation. The study was approved by the Research Ethics Committee of the Hospital Universitario Puerta de Hierro Majadahonda.

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