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Article

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The emergence of SARS-CoV-2 variants is jeopardizing the effectiveness of current vaccines and limiting the application of monoclonal antibody-based therapy for COVID-19[1,2]. Here we analysed at single-cell level the memory B cells of five naive and five convalescent people vaccinated with the BNT162b2 mRNA vaccine to dissect the nature of the B cell and antibody response. Almost six-thousands cells were sorted, over three-thousand of them produced monoclonal antibodies against the spike protein and more than four hundred neutralized the original Wuhan SARS-CoV-2 virus. The B.1.351 (Beta) and B.1.1.248 (Gamma) variants showed to escape almost seventy per cent of these antibodies while a much smaller portion was impacted by the B.1.1.7 (Alpha) and B.1.617.2 (Delta) variants. The overall loss of neutralization was always significantly higher in the antibodies from naive people. In part this was due to the IGHV2-5;IGHJ4-1 germline, which was found only in convalescent people and generated potent and broadly neutralizing antibodies. Our data suggest that people that are seropositive following infection or primary vaccination will produce antibodies with increased potency and breadth and will be able to better control SARS-CoV-2 emerging variants.

Twenty months after the beginning of the COVID-19 pandemic, with 219 million people infected, 4.5 million deaths, and 6.3 billion vaccines doses administered, the world is still struggling to control the virus. In most developed countries vaccines have vastly reduced severe diseases, hospitalization and deaths, but they have not been able to control the infections which are fueled by new and more infectious variants. A large number of studies so far have shown that protection from infection is linked to the production of neutralizing antibodies against the spike protein (S protein) of the virus[3–6]. This is a metastable, trimeric class 1 fusion glycoprotein, composed by the S1 and S2 subunits, and mediates virus entry changing from a prefusion to postfusion conformation after binding to the human angiotensin-converting enzyme 2 (ACE2) receptor and heparan sulfates on the host cells[7]. Potent neutralizing antibodies recognize the S1 subunit of each monomer which includes the receptor binding domain (RBD) and N-terminal domain (NTD) immunodominant sites[8]. The large majority of neutralizing antibodies bind the receptor binding motif (RBM), within the RBD, and a smaller fraction target the NTD[5,9]. Neutralizing antibodies against the S2 subunit have been described, however they have very low potency[5,10]. Neutralizing antibodies generated after infection derive in large part from germline IGHV3-53 and the closely related IGHV3-66 with very few somatic mutations[11,12]. Starting from the summer of 2020, the virus started to generate mutations that allowed the virus to evade neutralizing antibodies, to become more infectious, or both. Some of the mutant viruses completely replaced the original Wuhan SARS-CoV-2. The most successful variant viruses are the B.1.1.7 (alpha), B.1.351 (beta), B.1.1.248 (gamma) and B.1.617.2 (delta) which have been named Variants of Concern (VoCs)[13]. The delta variant is presently spreading across the globe and causing big concern also in fully vaccinated populations. It is therefore imperative to understand the molecular mechanisms of the immune response to vaccination in order to design better vaccines and vaccination policies. Several investigators have shown that vaccination of convalescent people can yield neutralizing antibodies which can be up to a thousand-fold higher than those induced by infection or vaccination, suggesting that one way of controlling the pandemic may be the induction of a hybrid immunity-like response using a third booster dose[14–18]. Here we compared at single cell level the nature of the neutralizing antibody response against the original Wuhan virus and the VoCs in naïve and convalescent subjects immunized with the BNT162b2 mRNA vaccine.

Results

B cells response in COVID-19 vaccinees

We enrolled 10 donors vaccinated with the BNT162b2 mRNA vaccine, 5 of them were healthy people naïve to SARS-CoV-2 infection at

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vaccination (seronegative) and other 5 had recovered from SARS-CoV-2 infection before vaccination (seropositive). Subject details are summarized in Extended Data Table 1. Blood collection occurred at an average of 48- and 21-days post last vaccination dose for seronegative and seropositive respectively (Extended Data Table 1). This difference may affect the circulating B cell frequency and serum activity of seronegative and seropositive subjects analyzed in this study. We initially analyzed the frequency of circulating B cell populations between our groups. Seropositives showed a 2.46-fold increase in S protein specific $CD19^+CD27^+IgD^-IgM^-$ MBCs compared to seronegatives and an overall 10% higher level of $CD19^+CD27^+IgD^-IgM^-$ MBCs (Extended Data Fig. 1a–c). On the other hand, seronegatives showed a 2.3-fold higher frequency of $CD19^+CD27^+IgD^-IgM^+$ MBCs compared to seropositives. No differences were found in the levels of $CD19^+CD27^+IgD^-IgM^+$ S protein$^+$ MBCs between the two groups assessed in this study (Extended Data Fig. 1a–c). Following the MBC analyses, we characterized the polyclonal response of these donors by testing their binding response to the S protein trimer, RBD, NTD and S2-domain, and subsequently by testing their neutralization activity against the original Wuhan SARS-CoV-2 virus (Extended Data Fig. 2). Plasma from seropositives showed a higher binding activity to the S protein and all tested domains compared to seronegatives (Extended Data Fig. 2a–d). In addition, seropositives showed a 10-fold higher neutralization activity against the original Wuhan SARS-CoV-2 virus compared to seronegatives (Extended Data Fig. 2e,f).

Isolation of neutralizing antibodies

To better characterize the B cell immune response, we single cell sorted antigen-specific memory B cell (MBCs) using as bait the Wuhan SARS-CoV-2 S protein antigen which was encoded by the mRNA vaccine. The single cell sorting strategy was performed as previously described[5]. Briefly, MBCs prefusion S protein trimer-specific (S protein$^+$), class-switched MBCs ($CD19^+CD27^+IgD^-IgM^-$) were single-cell sorted and then incubated for two weeks to naturally produce and release mAbs into the supernatant. A total of 2,352 and 3,532 S protein$^+$ MBCs were sorted from seronegative and seropositive vaccinees respectively (Extended Data Table 2). Of these 944 (40.1%) and 2,299 (65.1%) respectively, released in the supernatant monoclonal antibodies (mAbs) recognizing the S protein prefusion trimer in ELISA (Fig. 1a; Extended Data Table 2). These mAbs were then tested in a cytopathic effect-based microneutralization assay (CPE-MN) with the original Wuhan live SARS-CoV-2 virus at a single point dilution (1:5) to identify SARS-CoV-2 neutralizing human monoclonal antibodies (nAbs). This first screening identified a total of 411 nAbs, of which 71 derived from seronegatives and 340 were from seropositives (Fig. 1b; Extended Data Table 2). Overall, the fraction of S protein-specific B cells producing nAbs were 7.5% for seronegatives and 14.8% for seropositives. Following this first screening, all nAbs able to neutralize the Wuhan SARS-CoV-2 virus were tested by CPE-MN against major variants of concern (VoCs) including the B.1.1.7 (alpha), B.1.351 (beta) and B.1.1.248 (gamma) to understand the breadth of neutralization of nAbs elicited by the BNT162b2 mRNA vaccine. At the time of this assessment the B.1.617.2 (delta) variant was not yet spread globally and therefore it was not available for screening. Seropositives had an overall higher percentage of nAbs neutralizing the VoCs compared to seronegatives. The average frequency of nAbs from seropositives neutralizing the alpha, beta and gamma variants was 80.6 (n=274), 39.4 (n=134) and 62.0% (n=211) respectively, compared to 70.4 (n=50), 22.5 (n=16) and 43.6% (n=31) respectively in seronegatives (Fig. 1c; Extended Data Table 2).

Potency and breadth against variants

To better characterize and understand the potency and breadth of coverage of all Wuhan SARS-CoV-2 nAbs, we aimed to express as immunoglobulin G1 (IgG1) all the 411 nAbs previously identified. We were able to recover and express 276 antibodies for further characterization, 224 (89.8%) from seropositives and 52 (10.2%) from seronegatives. Initially, antibodies were tested for binding against the RBD, NTD and the S2-domain of the original Wuhan SARS-CoV-2 S protein. Overall, no major differences were observed in nAbs that recognized the RBD and NTD, while nAbs able to bind the S protein only in its trimeric conformation, i.e. not able to bind single domains, were almost 3-fold higher in seronegatives compared to seropositives (Extended Data Fig. 3). None of the tested nAbs targeted the S2 domain. nAbs were then tested by CPE-MN in serial dilution to evaluate their 100% inhibitory concentration (IC_{100}) against the Wuhan SARS-CoV-2 virus and the VoCs. At this stage of the study, the B.1.617.2 (delta) spread globally, and we were able to obtain the live virus for our experiments. Overall, nAbs isolated from seropositive vaccinees had a significantly higher potency than those isolated from seronegatives. The IC_{100} geometric mean (GM-IC_{100}) in seropositives was 2.87, 2.17-, 1.17-, 1.43-, and 1.92-fold lower than in seronegatives for the Wuhan virus, the alpha, beta, gamma and delta VoCs respectively (Fig. 2). In addition, a bigger fraction of nAbs from seropositives retained the ability to neutralize the VoCs. Indeed, when nAbs were individually tested against all VoCs, the ability to neutralize the alpha, beta, gamma and delta variants was lost by 14, 61, 61 and 29% of the antibodies from seropositives versus 32, 78, 75 and 46% respectively of those from seronegatives (Fig. 2). Finally, a major difference between seronegatives and seropositives was found in the class of medium/high potency nAbs (IC_{100} of 11-100 ng/mL and 101–1000 ng/mL) against all variants. Indeed, nAbs in these ranges from seropositives constitute the 71.0%, 62.5%, 23.7%, 22.8%, 53,1% of the whole nAbs repertoire while nAbs from seronegative donors were 48.1%, 38.5%, 17.3%, 17.3%, 34.6% against the Wuhan SARS-CoV-2 virus and alpha, beta, gamma and delta VoCs respectively (Fig. 2).

Functional gene repertoire

The analysis of the immunoglobulin G heavy chain variable (IGHV) and joining (IGHJ) gene rearrangements of 58 and 278 sequences recovered from seronegative and seropositive subjects respectively, showed that they use a broad range of germlines and share the most abundant. In particular, both groups predominantly used the IGHV1-69;IGHJ4-1 and IGHV3-53;IGHJ6-1, which were shared by three out five subjects per each group (Fig. 3a). In addition, the IGHV3-30;IGHJ6-1 and IGHV3-33;IGHJ4-1 germlines, more abundant in seronegative donors, and IGHV1-2;IGHJ6-1, mainly expanded in seropositive vaccinees, were also used with high frequency in both groups. Only the IGHV2-5;IGHJ4-1 germline was seen to be predominantly expanded only in seropositive donors (Fig. 3a). Despite selected germlines were boosted following vaccination, no major clonal families were identified, and the biggest family observed contained only four antibodies. To better characterize these predominant gene families, we evaluated their neutralization potency and breadth against SARS-CoV-2 and VoCs. In this analysis we could not evaluate IGHV3-33;IGHJ4-1 nAbs, as only three of these antibodies were expressed, but we included the IGHV3-53 closely related family IGHV3-66;IGHJ4-1, as this family was previously described to be mainly involved in SARS-CoV-2 neutralization[11,19]. A large part of nAbs deriving from these predominant germlines had a very broad range of neutralization potency against the original Wuhan SARS-CoV-2 virus with IC_{100} spanning from less than 10 to over 10,000 ng/mL (Fig. 3b–g). However, many of them lost the ability to neutralize SARS-CoV-2 VoCs. The loss of neutralizing activity occurred for most germlines and it was moderate against the alpha and delta variants, while it was dramatic against the beta and gamma variants (Fig. 3b–g). A notable exception was the IGHV2-5;IGHJ4-1 germline, present only in nAbs of seropositive patients, that showed potent antibodies able to equally neutralize all SARS-CoV-2 VoCs (Fig. 3d). Finally, we evaluated the CDRH3-length and V-gene somatic hyper mutation (SHM) levels for all nAbs retrieved from seronegatives and seropositives and for predominant germlines.

Overall, the two groups show a similar average CDRH3-length (15.0 aa and 15.1 aa for seronegatives and seropositives respectively), however seropositives showed almost 2-fold higher V-gene mutation levels compared to seronegatives (Extended Data Fig. 4). As for predominant gene derived nAbs, we observed heterogenous CDRH3-length, with the only exception of IGHV3-53;IGHJ6-1 nAbs, and higher V-gene mutation levels in seropositives predominant germlines compared to seronegatives (Extended Data Fig. 5).

S protein epitope mapping

To map the regions of the S protein recognized by the identified nAbs we used a competition assay with four known antibodies: J08, which targets the top loop of the receptor binding motif (RBM)[5], S309, which binds the RBD but outside of the RMB region[20], 4A8, that recognized the NTD[21], and L19, that binds the S2-domain[5](Extended Data Fig. 6). The nAbs identified in this study were pre-incubated with the original Wuhan SARS-CoV-2 S protein and subsequently the four nAbs labeled with different fluorophores were added as single mix. 50% signal reduction for one of the four fluorescently labelled nAbs, was used as threshold for positive competition. The vast majority of nAbs from both seronegative (50.0%; n=26) and seropositive (51.3%; n=115) vaccinees competed with J08 (Extended Data Fig. 7a; Extended Data Table 3). For seronegatives, the second most abundant population was composed by nAbs that did not compete with any of the four fluorescently labelled nAbs (25.0%; n=13) followed by nAbs targeting the NTD (17.3%; n=9). As for seropositives, the second most abundant population was composed by nAbs that competed with S309 (21.4%; n=48) followed by nAbs competing with 4A8 (15.6%; n=35) and not-competing nAbs (11.6%; n=26). None of our nAbs did compete with the S2 targeting antibody L19 (Extended Data Fig. 7a; Extended Data Table 3). nAbs competing with J08, which are likely to bind the RBM, derived from several germlines, including the predominant IGHV3-53;IGHJ6-1 (10.6%; n=14), IGHV1-69;IGHJ4-1 (8.3%; n=11) and IGHV1-2;IGHJ6-1 (6.8%; n=9) (Extended Data Fig. 7b). In contrast, those competing with S309 derived mostly from germline IGHV2-5;IGHJ4-1 (13.7%; n=7) which were isolated exclusively from seropositive vaccinees (Extended Data Fig. 7c). As for NTD-directed nAbs the non-predominant gene family IGHV1-24;IGHJ6-1 was the most abundant confirming what was reported in previous studies (Extended Data Fig. 7d)[22]. Finally, for nAbs that did not compete with any of the known antibodies used in our competition assay, the non-predominant gene families IGHV1-69;IGHJ3-1 (9.7%; n=3) and IGHV1-69;IGHJ6-1 (9.7%; n=3) were the most abundant (Extended Data Fig. 7e).

Discussion

Our study analyzed at single cell level the repertoire of B cells producing neutralizing antibodies following vaccination of naïve and previously infected people. The most important conclusion from this work is that people previously exposed to SARS-CoV-2 infection, respond to vaccination with more B cells producing antibodies that are not susceptible to escape variants and that have higher neutralization potency. This can be explained in part by the increased number of somatic mutations and by the fact that seropositives expand potent antibodies derived from the IGHV2-5;IGHJ4-1 germline which were not described in naïve vaccinees[18]. One limitation of our study is that we did not include people that received a third booster dose of vaccine. In spite of this limitation, we believe that our conclusions are likely to be extendable to seronegative people as a third vaccine dose could lead to a hybrid immunity-like response as neutralizing antibodies following infection and vaccination derive mostly from the same immunodominant germlines[11,12,17–19]. Our analysis suggests that a booster dose of vaccine will increase the frequency of memory B cells producing potent neutralizing antibodies not susceptible to escape variants and allow better control of this pandemic. The massive variant escape from predominant germlines, such as IGHV3-53, 3-66, 3-30 and 1-69, and the presence of antibodies deriving from germline IGHV2-5 that are resistant to variants, suggest that the design of vaccines that preferentially promote or avoid the expansion of selected germlines can generate broad protection against SARS-CoV-2 variants. Germline-targeting vaccination, which has been pioneered in the HIV field[23,24], may be a promising strategy to fight the COVID-19 pandemic.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-021-04117-7.

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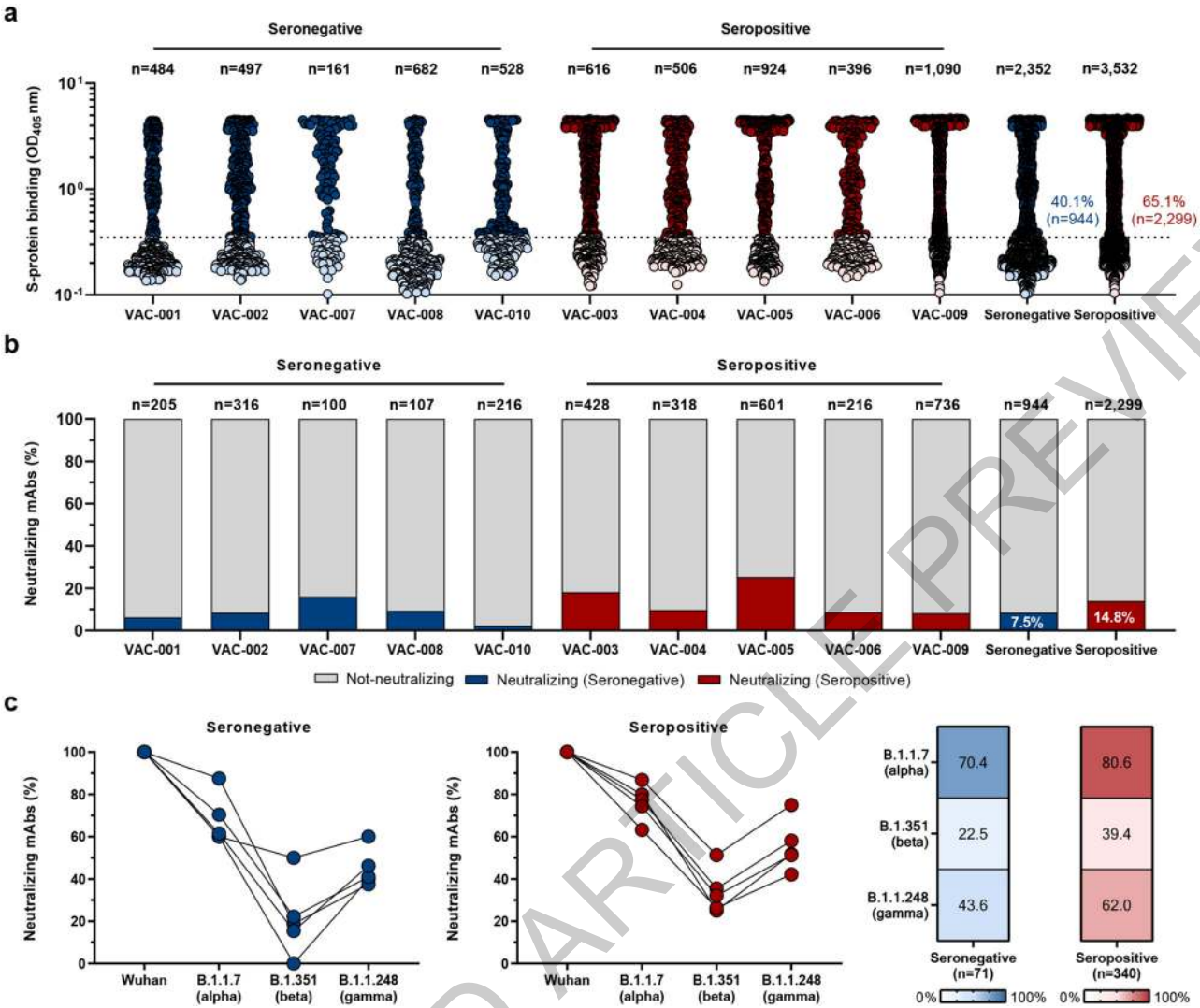
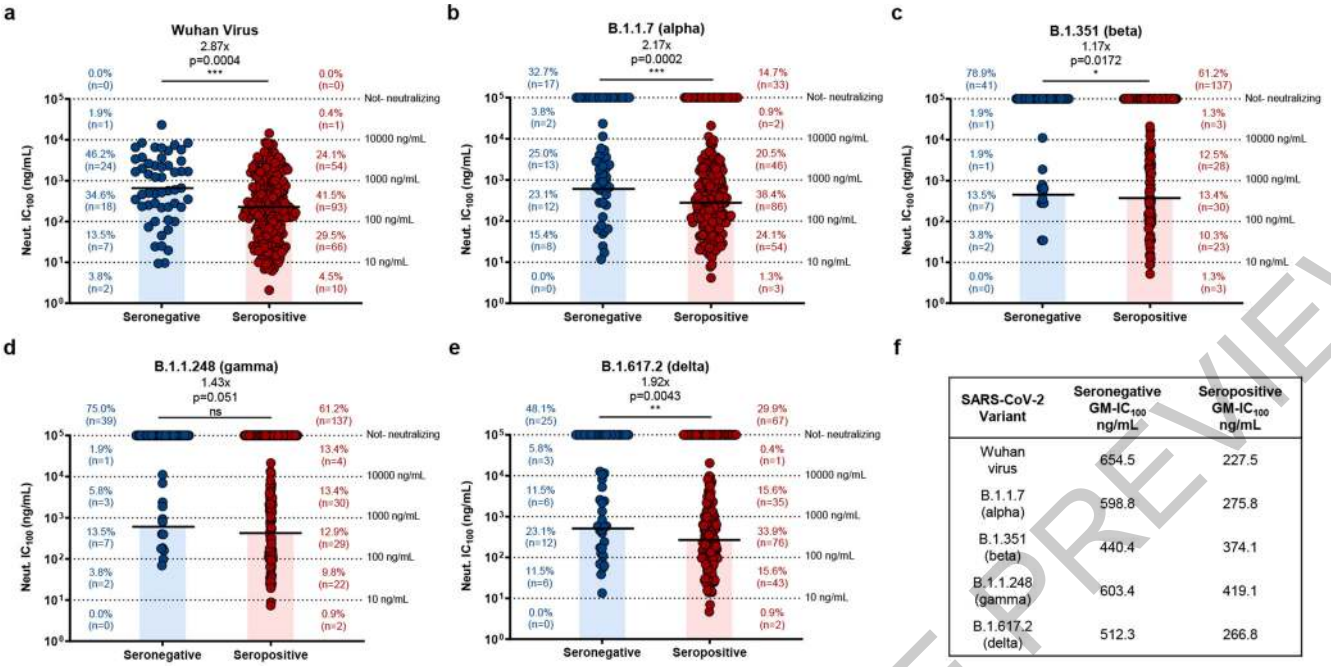


Fig. 1 | Identification of cross-neutralizing SARS-CoV-2 S protein-specific nAbs. a, The graph shows supernatants tested for binding to the Wuhan SARS-CoV-2 S protein antigen. Threshold of positivity has been set as two times the value of the blank (dotted line). Dark blue and red dots represent mAbs that bind to the S protein for seronegative and seropositive vaccinees respectively. Light blue and red dots represent mAbs that do not bind the S protein for seronegative and seropositive vaccinees. **b**, The bar graph shows the percentage of not-neutralizing (gray), neutralizing mAbs from seronegatives (dark blue), and neutralizing mAbs for seropositives (dark red). The total number (n) of antibodies tested per individual is shown on top of each bar. **c**, Graphs show the fold change percentage of nAbs in seronegatives and seropositives against the alpha, beta and gamma VoCs compared to the original Wuhan SARS-CoV-2 virus. The heatmaps show the overall percentage of Wuhan SARS-CoV-2 nAbs able to neutralize tested VoCs.



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SARS-CoV-2 Variant	Seronegative GM-IC_{100} ng/mL	Seropositive GM-IC_{100} ng/mL
Wuhan virus	654.5	227.5
B.1.1.7 (alpha)	598.8	275.8
B.1.351 (beta)	440.4	374.1
B.1.1.248 (gamma)	603.4	419.1
B.1.617.2 (delta)	512.3	266.8

Fig. 2 | Potency and breadth of neutralization of nAbs against SARS-CoV-2 and VoCs. a-e, Scatter dot charts show the neutralization potency, reported as IC_{100} (ng/mL), of nAbs tested against the original Wuhan SARS-CoV-2 virus and the B.1.1.7, B.1.351, B.1.1.248 and B.1.617.2 VoCs. The number and percentage of nAbs from seronegatives vs seropositives, fold-change and statistical significance are denoted on each graph. A nonparametric Mann–Whitney t test was used to evaluate statistical significances between groups. Two-tailed p-value significances are shown as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. (f) The table shows the IC_{100} geometric mean (GM) of all nAbs pulled together from each group against all SARS-CoV-2 viruses tested. Technical duplicates were performed for each experiment.

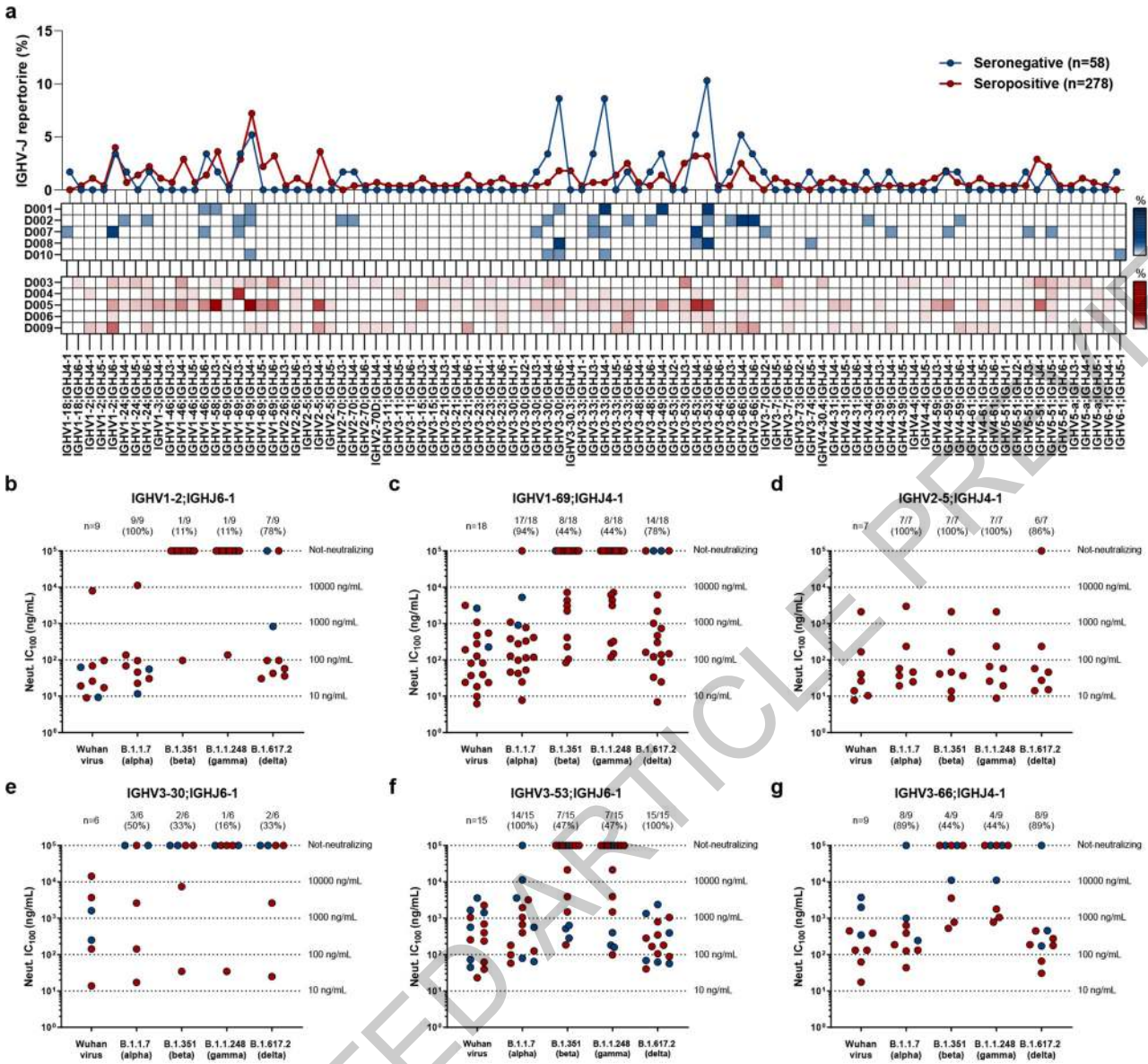

Fig. 3 | Repertoire analyses and functional characterization of predominant gene derived nAbs. a, The graph shows the IGHV-J rearrangement frequencies between seronegative and seropositive vaccinees (top panel), the frequency within subjects (middle panels) and the Log2 fold change (FC) between groups (bottom panel). **b**–**g**, Graphs show the neutralization potency (IC_{100}) of predominant gene derived nAbs from the IGHV1-2;IGHJ6-1, IGHV1-69;IGHJ4-1, IGHV2-5;IGHJ4-1, IGHV3-30;IGHJ6-1, IGHV3-53;IGHJ6-1 and IGHV3-66;IGHJ4-1 families, against the he original Wuhan SARS-CoV-2 virus and the B.1.1.7, B.1.351, B.1.1.248 and B.1.617.2 VoCs.

Methods

Enrollment of COVID-19 vaccinees and human sample collection

This work results from a collaboration with the Azienda Ospedaliera Universitaria Senese, Siena (IT) that provided samples from COVID-19 vaccinated donors, of both sexes, who gave their written consent. The study was approved by the Comitato Etico di Area Vasta Sud Est (CEAVSE) ethics committees (Parere 17065 in Siena) and conducted according to good clinical practice in accordance with the declaration of Helsinki (European Council 2001, US Code of Federal Regulations, ICH 1997). This study was unblinded and not randomized. No statistical methods were used to predetermine sample size.

Single cell sorting of SARS-CoV-2 S protein$^+$ memory B cells from COVID-19 vaccinees

Peripheral blood mononuclear cells (PBMCs) and single cell sorting strategy were performed as previously described[5]. Briefly, PBMC were isolated from heparin-treated whole blood by density gradient centrifugation (Ficoll-Paque™ PREMIUM, Sigma-Aldrich). After separation, PBMC were stained with Live/Dead Fixable Aqua (Invitrogen; Thermo Scientific) diluted 1:500 at room temperature RT. After 20 min incubation cells were washed with PBS and unspecific bindings were saturated with 20% normal rabbit serum (Life technologies). Following 20 min incubation at 4 °C cells were washed with PBS and stained with SARS-CoV-2 S-protein labeled with Strep-Tactin®XT DY-488 (iba-lifesciences cat# 2-1562-050) for 30 min at 4 °C. After incubation the following staining mix was used CD19 V421 (BD cat# 562440, 1:320), IgM PerCP-Cy5.5 (BD cat# 561285, 1:50), CD27 PE (BD cat# 340425, 1:30), IgD-A700 (BD cat# 561302, 1:15), CD3 PE-Cy7 (BioLegend cat# 300420, 1:100), CD14 PE-Cy7 (BioLegend cat# 301814, 1:320), CD56 PE-Cy7 (BioLegend cat# 318318, 1:80) and cells were incubated at 4 °C for additional 30 min. Stained MBCs were single cell-sorted with a BD FACS Aria III (BD Biosciences) into 384-well plates containing 3T3-CD40L feeder cells and were incubated with IL-2 and IL-21 for 14 days as previously described[25].

ELISA assay with SARS-CoV-2 S protein prefusion trimer

mAbs and plasma binding specificity against the S-protein trimer was detected by ELISA as previously described[5]. Briefly, 384-well plates (microplate clear, Greiner Bio-one) were coated with 3 µg/mL of streptavidin (Thermo Fisher) diluted in carbonate-bicarbonate buffer (E107, Bethyl laboratories) and incubated at RT overnight. The next day, plates were incubated 1 h at RT with 3 µg/mL of SARS-CoV-2 S protein diluted in PBS. Plates were then saturated with 50 µL/well of blocking buffer (phosphate-buffered saline, 1% BSA) for 1 h at 37 °C. After blocking, 25 µL/well of mAbs diluted 1:5 in sample buffer (phosphate-buffered saline, 1% BSA, 0.05% Tween-20) were added to the plates and were incubated at 37 °C. Plasma samples derived from vaccinees were tested (starting dilution 1:10; step dilution 1:2 in sample buffer) in a final volume of 25 µL/well and were incubated at 37 °C. After 1 h of incubation, 25 µL/well of alkaline phosphatase-conjugated goat antihuman IgG and IgA (Southern Biotech) diluted 1:2000 in sample buffer were added. Finally, S protein binding was detected using 25 µL/well of PNPP (p-nitrophenyl phosphate; Thermo Fisher) and the reaction was measured at a wavelength of 405 nm by the Varioskan Lux Reader (Thermo Fisher Scientific). After each incubation step, plates were washed three times with 100 µL/well of washing buffer (phosphate-buffered saline, 0.05% Tween-20). Sample buffer was used as a blank and the threshold for sample positivity was set at 2-fold the optical density (OD) of the blank. Technical duplicates were performed for mAbs and technical triplicates were performed for sera samples.

ELISA assay with RBD, NTD and S2 subunits

mAbs identification and plasma screening of vaccinees against RBD, NTD or S2 SARS-CoV-2 protein were performed by ELISA. Briefly, 3 µg/mL of RBD, NTD or S2 SARS-CoV-2 protein diluted in carbonate-bicarbonate buffer (E107, Bethyl laboratories) were coated in 384-well plates (microplate clear, Greiner Bio-one). After overnight incubation at 4 °C, plates were washed 3 times with washing buffer (phosphate-buffered saline, 0.05% Tween-20) and blocked with 50 µL/well of blocking buffer (phosphate-buffered saline, 1% BSA) for 1h at 37 °C. After washing, plates were incubated 1 h at 37 °C with mAbs diluted 1:5 in samples buffer (phosphate-buffered saline, 1% BSA, 0.05% Tween-20) or with plasma at a starting dilution 1:10 and step diluted 1:2 in sample buffer. Wells with no sample added were consider blank controls. Anti-Human IgG –Peroxidase antibody (Fab specific) produced in goat (Sigma) diluted 1:45000 in sample buffer was then added and samples were incubated for 1 h at 37 °C. Plates were then washed, incubated with TMB substrate (Sigma) for 15 min before adding the stop solution (H_2SO_4 0.2M). The OD values were identified using the Varioskan Lux Reader (Thermo Fisher Scientific) at 450 nm. Each condition was tested in triplicate and samples tested were considered positive if OD value was 2-fold the blank.

Flow cytometry-based competition assay

To classify mAbs candidates on the basis of their interaction with Spike epitopes, we performed a flow cytometry-based competition assay. In detail, magnetic beads (Dynabeads His-Tag, Invitrogen) were coated with histidine tagged S protein according to the manufacturers' instructions. Then, 20 µg/mL of coated S protein-beads were pre-incubated with unlabeled nAbs candidates diluted 1:2 in PBS for 40 minutes at RT. After incubation, the mix Beads-antibodies was washed with 100 µL of PBS-BSA 1%. Then, to analyze epitope competition, mAbs able to bind RBD (J08, S309), NTD (4A8) or S2 (L19) domain of the S protein were labeled with 4 different fluorophores (Alexa Fluor 647, 488, 594 and 405) using Alexa Fluor NHS Ester kit (Thermo Scientific), were mixed and incubated with S-protein-beads. Following 40 minutes of incubation at RT, the mix Beads-antibodies was washed with PBS, resuspended in 150 µL of PBS-BSA 1% and analyzed using BD LSR II flow cytometer (Becton Dickinson). Beads with or without S-protein incubated with labeled antibodies mix were used as positive and negative control respectively. FACSDiva Software (version 9) was used for data acquisition and analysis was performed using FlowJo (version 10).

SARS-CoV-2 authentic viruses neutralization assay

All SARS-CoV-2 authentic virus neutralization assays were performed in the biosafety level 3 (BSL3) laboratories at Toscana Life Sciences in Siena (Italy) and Vismederi Srl, Siena (Italy). BSL3 laboratories are approved by a Certified Biosafety Professional and are inspected every year by local authorities. To evaluate the neutralization activity of identified nAbs against SARS-CoV-2 and all VoCs and evaluate the breadth of neutralization of this antibody is a cytopathic effect-based microneutralization assay (CPE-MN) was performed[5]. Briefly, the CPE-based neutralization assay sees the co-incubation of J08 with a SARS-CoV-2 viral solution containing 100 median Tissue Culture Infectious Dose (100 $TCID_{50}$) of virus and after 1 hour incubation at 37 °C, 5% CO_2. The mixture was then added to the wells of a 96-well plate containing a sub-confluent Vero E6 cell monolayer. Plates were incubated for 3-4 days at 37 °C in a humidified environment with 5% CO_2, then examined for CPE by means of an inverted optical microscope by two independent operators. All nAbs were tested a starting dilution of 1:5 and the IC_{100} evaluated based on their initial concentration while plasma samples were tested starting from a 1:10 dilution. Both nAbs and plasma samples were then diluted step 1:2. Technical duplicates were performed for both nAbs and plasma samples. In each plate positive and negative control were used as previously described[5].

SARS-CoV-2 virus variants CPE-MN neutralization assay

The SARS-CoV-2 viruses used to perform the CPE-MN neutralization assay were the original Wuhan SARS-CoV-2 virus (SARS-CoV-2/

INMI1-Isolate/2020/Italy: MT066156), SARS-CoV-2 B.1.1.7 (INMI GISAID accession number: EPI_ISL_736997), SARS-CoV-2 B.1.351 (EVAg Cod: 014V-04058), B.1.1.248 (EVAg CoD: 014V-04089) and B.1.617.2 (GISAID ID: EPI_ISL_2029113)[26].

Single cell RT-PCR and Ig gene amplification and transcriptionally active PCR expression

The whole process for nAbs heavy and light chain recovery, amplification and transcriptionally active PCR (TAP) expression was performed as previously described[5]. Briefly, 5 µL of cell lysate were mixed with 1 µL of random hexamer primers (50 ng/µL), 1 µL of dNTP-Mix (10 mM), 2 µL 0.1 M DTT, 40 U/µL RNAse OUT, $MgCl_2$ (25 mM), 5x FS buffer and Superscript IV reverse transcriptase (Invitrogen) to perform RT-PCR. Reverse transcription (RT) reaction was performed at 42 °C/10', 25 °C/10', 50 °C/60' and 94°/5'. Two rounds of PCR were performed to obtain the heavy (VH) and light (VL) chain amplicons. All PCR reactions were performed in a nuclease-free water (DEPC) in a total volume of 25 µL/well. For PCR I, 4 µL of cDNA were mixed with 10 µM of VH and 10 µM VL primer-mix, 10mM dNTP mix, 0.125 µL of Kapa Long Range Polymerase (Sigma), 1.5 µL MgCl2 and 5 µL of 5x Kapa Long Range Buffer. PCRI reaction was performed at 95°/3', 5 cycles at 95 °C/30'', 57 °C/30'', 72 °C/30'' and 30 cycles at 95 °C/30'', 60 °C/30'', 72 °C/30'' and a final extension of 72°/2'. Nested PCR II was performed as above starting from 3.5 µL of unpurified PCR I product. PCR II products were purified by Millipore MultiScreen® PCRµ96 plate according to manufacture instructions and eluted in 30 µL of nuclease-free water (DEPC). As for TAP expression, vectors were initially digested using restriction enzymes AgeI, SalI and Xho as previously described and PCR II products ligated by using the Gibson Assembly NEB into 25 ng of respective human Igγ1, Igκ and Igλ expression vectors[27,28]. TAP reaction was performed using 5 µL of Q5 polymerase (NEB), 5 µL of GC Enhancer (NEB), 5 µL of 5X buffer,10 mM dNTPs, 0.125 µL of forward/reverse primers and 3 µL of ligation product, using the following cycles: 98°/2', 35 cycles 98°/10'', 61°/20'', 72°/1' and 72°/5'. TAP products were purified under the same PCRII conditions, quantified by Qubit Fluorometric Quantitation assay (Invitrogen) and used for transient transfection in Expi293F cell line following manufacturing instructions.

Functional repertoire analyses

nAbs VH and VL sequence reads were manually curated and retrieved using CLC sequence viewer (Qiagen). Aberrant sequences were removed from the data set. Analyzed reads were saved in FASTA format and the repertoire analyses was performed using Cloanalyst (http://www.bu.edu/computationalimmunology/research/software/)[29,30].

Statistical analysis

Statistical analysis was assessed with GraphPad Prism Version 8.0.2 (GraphPad Software, Inc., San Diego, CA). Nonparametric Mann-Whitney t test was used to evaluate statistical significance between the two groups analyzed in this study. Statistical significance was shown as * for values ≤ 0.05, ** for values ≤ 0.01, *** for values ≤ 0.001, and **** for values ≤ 0.0001.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

Source data are provided with this paper. All data supporting the findings in this study are available within the article or can be obtained from the corresponding author upon request. Source data are provided with this paper.

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Author contributions Conceived the study: E.A. and R.R.; Enrolled COVID-19 vaccinees: F.M., M.F., I.R. and M.T.; Performed PBMC isolation and single cell sorting: E.A and I.P.; Performed ELISAs and competition assays: I.P.; Recovered nAbs VH and VL and expressed antibodies: I.P. and N.M.; Recovered VH and VL sequences and performed the repertoire analyses: P.P. and E.A.; Produced and purified SARS-CoV-2 S protein constructs: E.P. and V.A.; Performed neutralization assays in BSL3 facilities: E.A., G.P., I.H., M.L., L.B. and G.G.; Supported day-by-day laboratory activities and management: C.D.S.; Manuscript writing: E.A. and R.R.; Final revision of the manuscript: E.A., I.P., G.P., N.M., P.P., I.H., M.L., E.P., V.A., L.B., G.G., C.D.S., M.F., I.R., M.T., F.M., C.S., E.M. and R.R.; Coordinated the project: E.A., C.S., E.M. and R.R.

Competing interests R.R. is an employee of GSK group of companies. E.A., I.P., N.M., P.P., E.P., C.D.S., C.S. and R.R. are listed as inventors of full-length human monoclonal antibodies described in Italian patent applications n. 102020000015754 filed on June 30th 2020, 102020000018955 filed on August 3rd 2020 and 102020000029969 filed on 4th of December 2020, and the international patent system number PCT/IB2021/055755 filed on the 28th of June 2021. All patents were submitted by Fondazione Toscana Life Sciences, Siena, Italy. Remaining authors have no competing interests to declare.

Additional information

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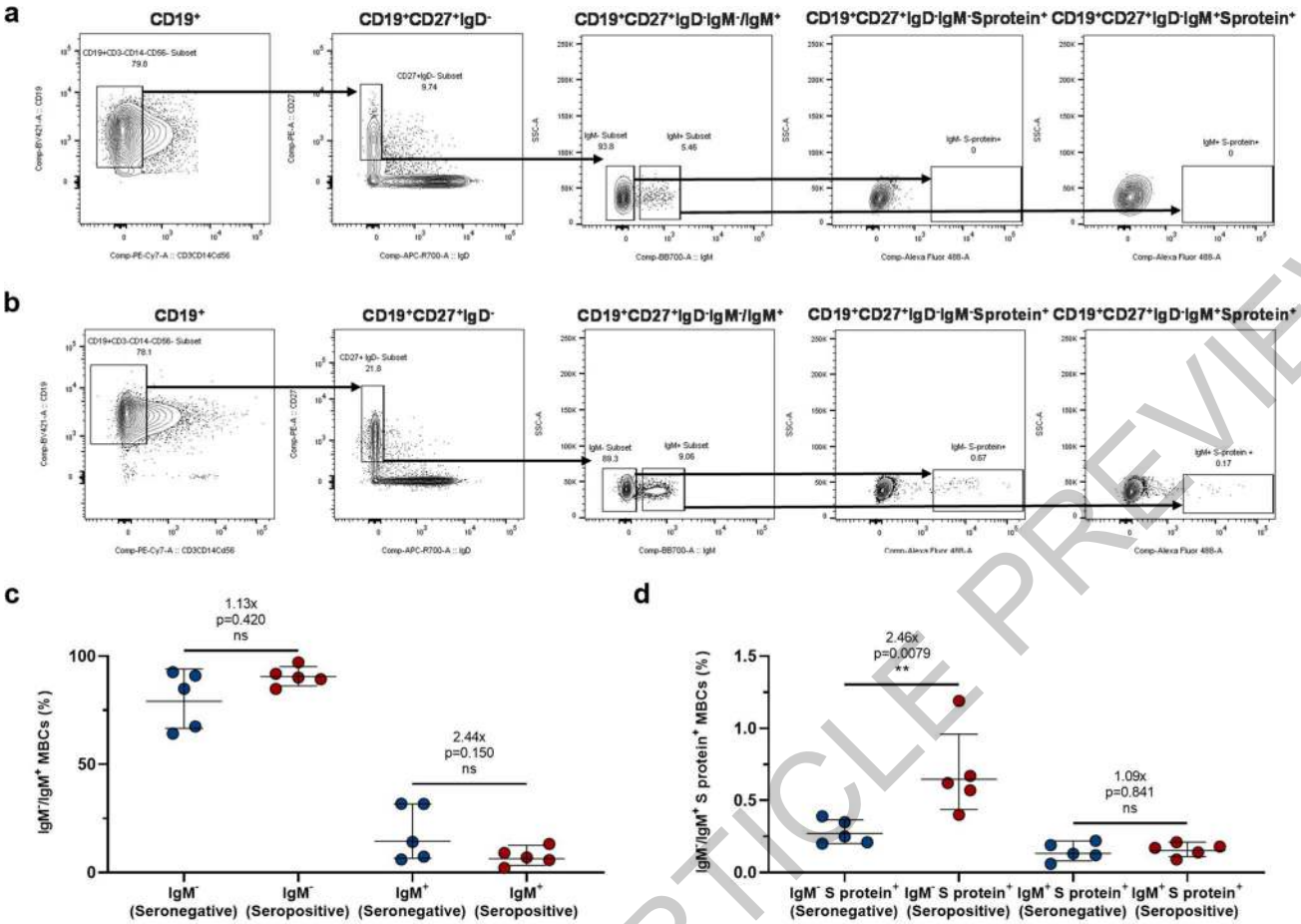
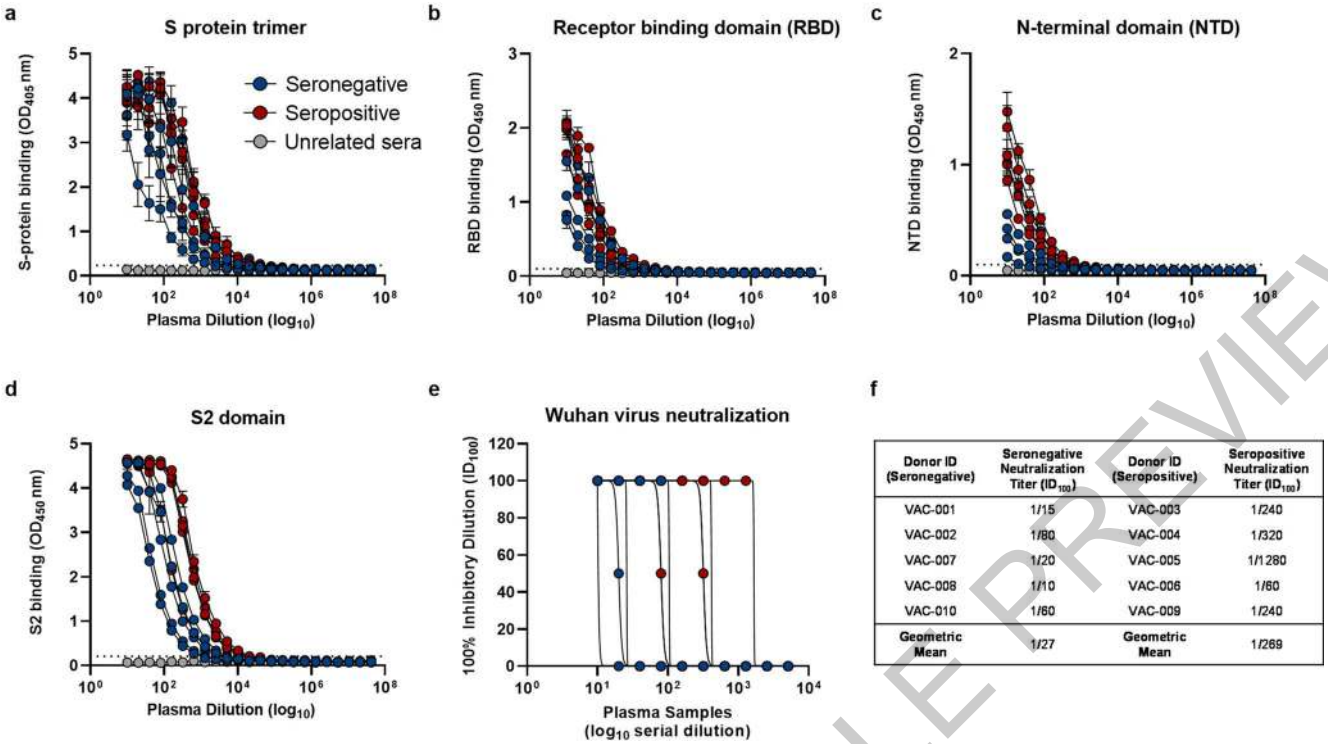


e

Subject	SARS-CoV-2 serology	IgM⁻ Subset (%)	IgM⁻ S protein⁺ Subset (%)	IgM⁺ Subset (%)	IgM⁺ S protein⁺ Subset (%)
VAC-001	Seronegative	67.50	0.21	31.70	0.13
VAC-002	Seronegative	64.20	0.25	31.80	0.06
VAC-007	Seronegative	92.60	0.35	6.02	0.22
VAC-008	Seronegative	84.90	0.39	14.10	0.12
VAC-010	Seronegative	91.00	0.20	7.27	0.19
VAC-003	Seropositive	91.80	0.57	5.77	0.21
VAC-004	Seropositive	97.10	0.62	2.14	0.14
VAC-005	Seropositive	89.30	0.67	9.06	0.17
VAC-006	Seropositive	90.10	0.40	6.94	0.18
VAC-009	Seropositive	84.80	1.19	13.20	0.09

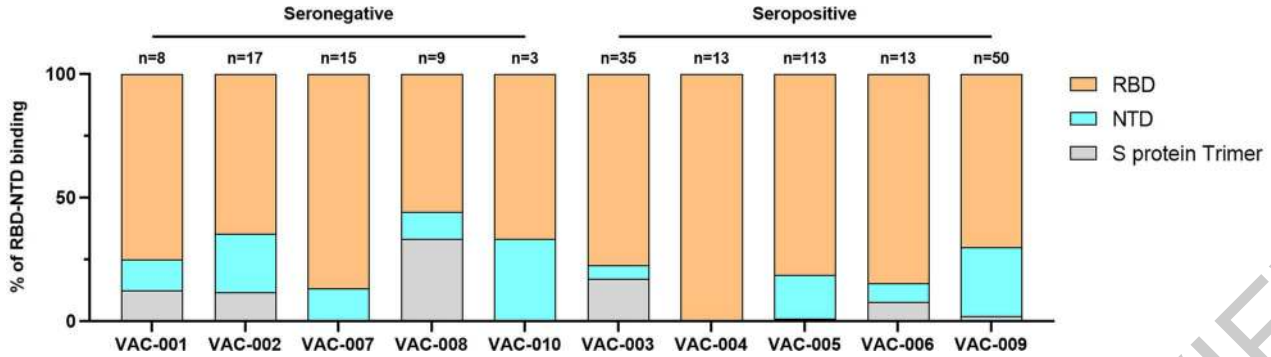
Extended Data Fig. 1 | Single cell sorting and memory B cell frequencies. **a-b**, The gating strategy shows from left to right: CD19⁺ B cells; CD19⁺CD27⁺IgD⁻; CD19⁺CD27⁺IgD⁻IgM⁻/IgM⁺; CD19⁺CD27⁺IgD⁻IgM⁻Sprotein⁺; CD19⁺CD27⁺IgD⁻IgM⁺Sprotein⁺ for a healthy donor (used as negative control for S protein staining) and a vaccinated subject. **c**, The graph shows the frequency of CD19⁺CD27⁺IgD⁻IgM⁻ and IgM⁺ in seronegative (n=5) and seropositive donors (n=5). **d**, The graph shows the frequency of CD19⁺CD27⁺IgD⁻IgM⁻ and IgM⁺ able to bind the SARS-CoV-2 S protein trimer (S protein⁺) in seronegative (n=5) and seropositive (n=5) donors. Geometric mean and standard deviation are denoted on the graphs. A nonparametric Mann–Whitney t test was used to evaluate statistical significances between groups. Two-tailed p-value significances are shown as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. **e**, The table summarizes the frequencies of the cell population above described for all subjects enrolled in our study.



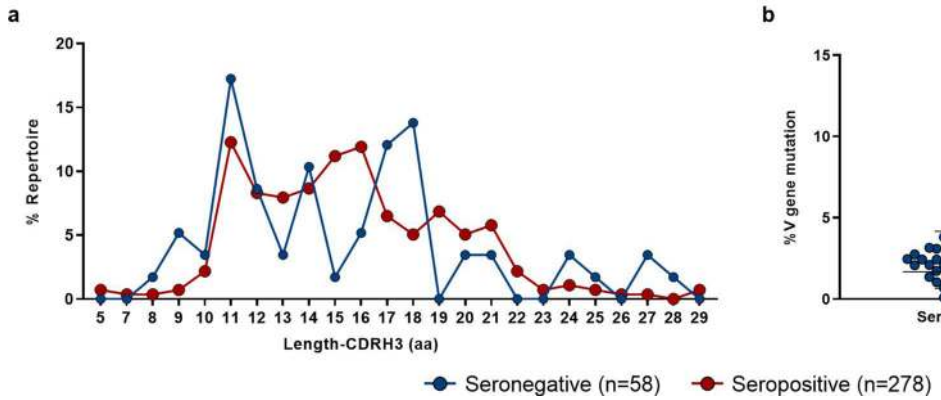
f

Donor ID (Seronegative)	Seronegative Neutralization Titer (ID_{100})	Donor ID (Seropositive)	Seropositive Neutralization Titer (ID_{100})
VAC-001	1/15	VAC-003	1/240
VAC-002	1/80	VAC-004	1/320
VAC-007	1/20	VAC-005	1/1280
VAC-008	1/10	VAC-006	1/60
VAC-010	1/60	VAC-009	1/240
Geometric Mean	1/27	**Geometric Mean**	1/269

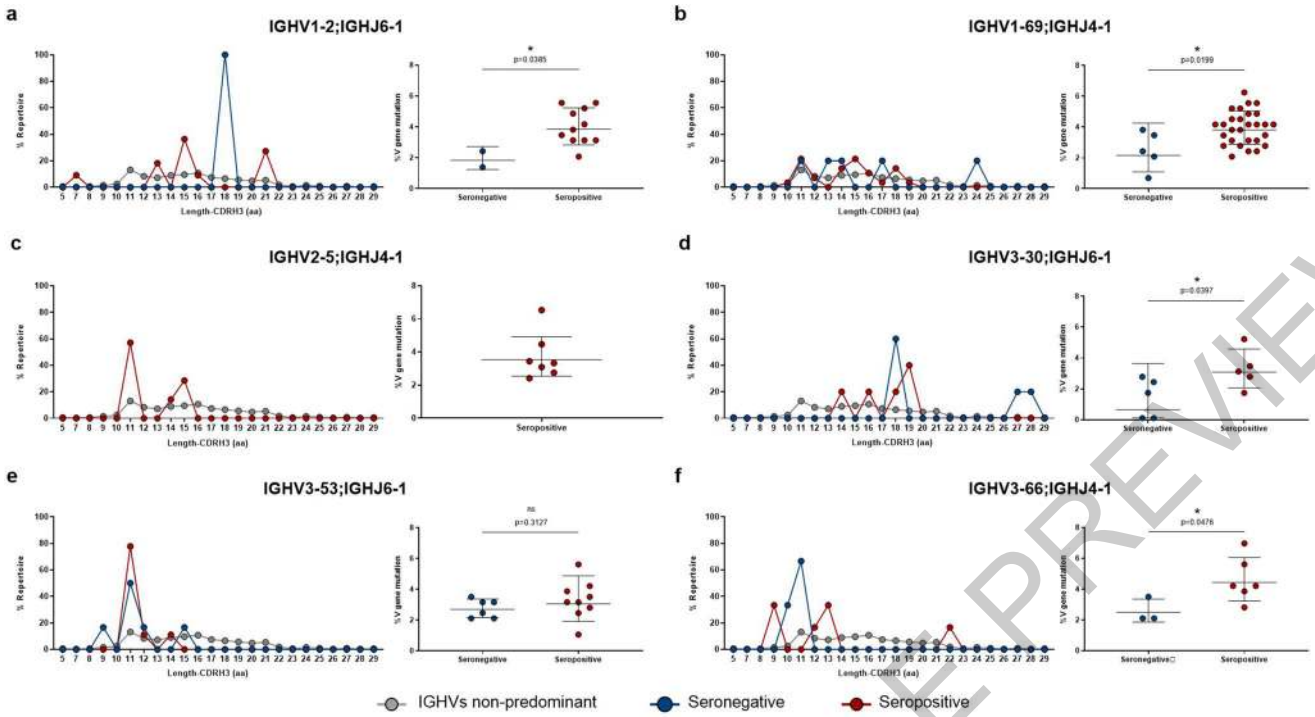
Extended Data Fig. 2 | Plasma response of COVID-19 vaccinees. a-d, Graphs show the ability of plasma samples from seronegative and seropositive vaccinees to bind the S protein trimer, RBD, NTD and S2 domain. Mean and standard deviation are denoted on each graph. Technical triplicates were performed for each experiment. **e**, The graph shows the neutralizing activity of plasma samples against the original Wuhan SARS-CoV-2 virus. Technical duplicates were performed for each experiment. **f**, The table summarizes the 100% inhibitory dilution (ID_{100}) of each COVID-19 vaccinee and the geometric mean for seronegative and seropositive donors.



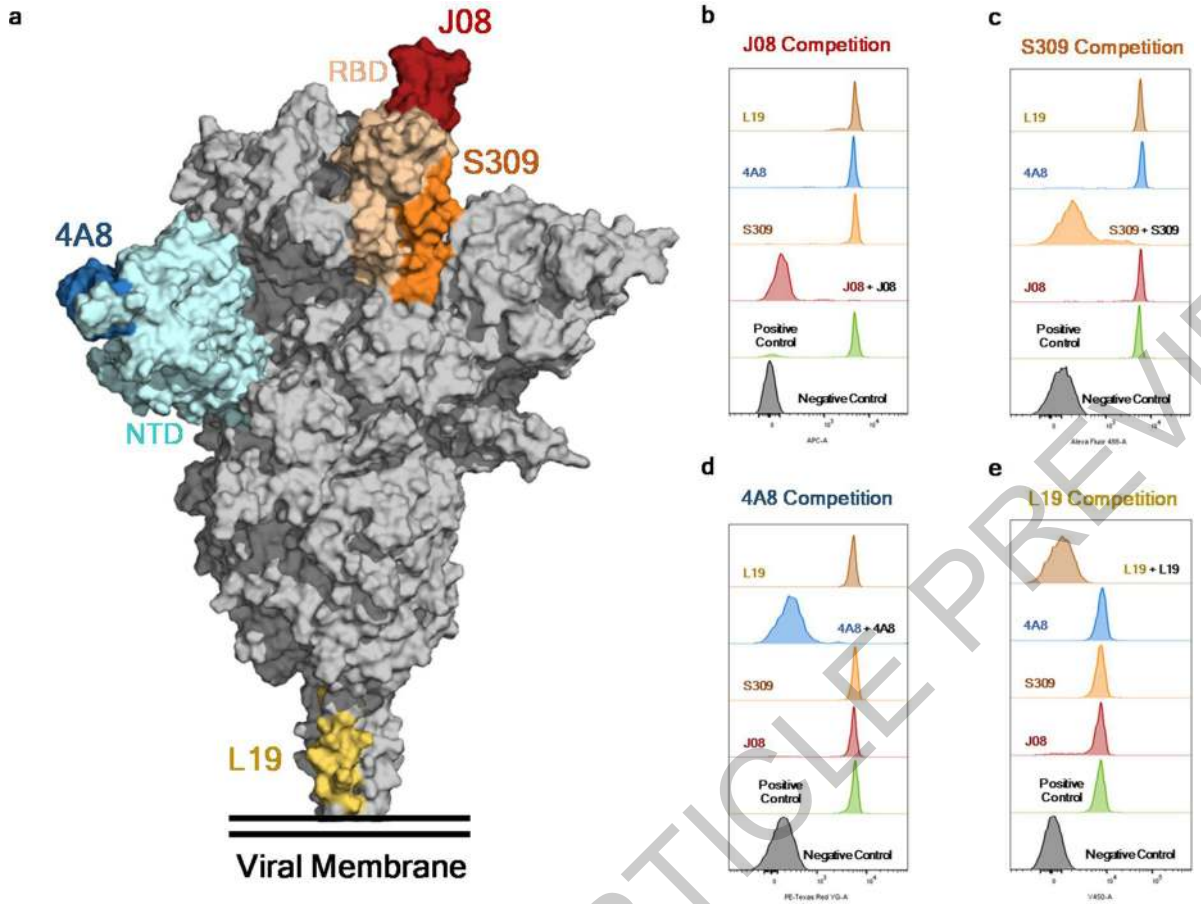
Extended Data Fig. 3 | RBD and NTD binding distribution of nAbs. The graph shows the percentage of antibodies that bind specifically the RBD (light orange) or the NTD (cyan) or that did not bind single domains but recognized exclusively the S protein in its trimetric conformation (gray). The number (n) of tested nAbs per donor is reported on top of each bar. Technical duplicates were performed for each experiment.



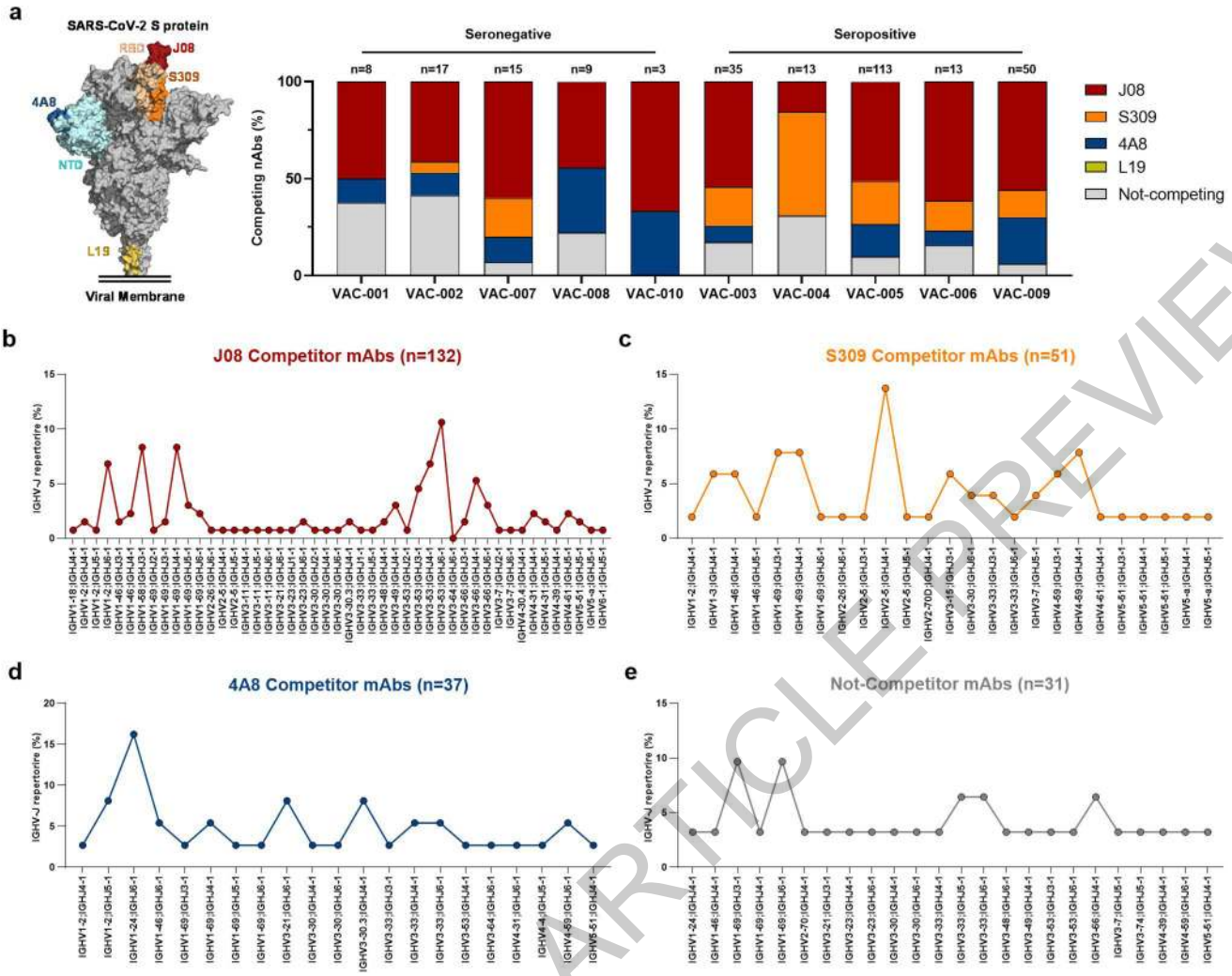
Extended Data Fig. 4 | Heavy chain CDR3 length and somatic hypermutation levels in seronegative and seropositive vaccinees. a, The graph shows the heavy chain CDR3 length represented in amino acids (aa). **b**, The graph shows the overall somatic hypermutation level of nAbs isolated from seronegative and seropositive vaccinees. Geometric mean and standard deviation are denoted on the graphs. A nonparametric Mann–Whitney t test was used to evaluate statistical significances between groups. Two-tailed p-value significances are shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.



Extended Data Fig. 5 | Heavy chain CDR3 length and somatic hypermutation levels of predominant gene derived nAbs. a-f, Graphs show the amino acidic heavy chain CDR3 length (left panel) and the somatic hypermutation level (right panel) of nAbs derived from the IGHV1-2;IGHJ6-1 (n=13), IGHV1-69;IGHJ4-1 (n=33), IGHV2-5;IGHJ4-1 (n=7), IGHV3-30;IGHJ6-1 (n=10), IGHV3-53;IGHJ6-1 (n=15) and IGHV3-66;IGHJ4-1 (n=9) gene families. Geometric mean and standard deviation are denoted on the graphs. A nonparametric Mann–Whitney t test was used to evaluate statistical significances between groups. Two-tailed p-value significances are shown as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.



Extended Data Fig. 6 | Epitope binning assay. a, Schematic representation of the epitopes recognized by J08 (dark red), S309 (orange), 4A8 (dark blue) and L19 (gold), mAbs on the S protein surface. **b-e**, Representative cytometer peaks per each of the four mAbs used for the competition assay. Positive (beads conjugated with only primary labeled antibody) and negative (un-conjugated beads) controls are shown as green and gray peaks, respectively.



Extended Data Fig. 7 | Epitope binning and genetic characterization of competing nAbs. a, The bar graph shows the percentage (%) of nAbs competing with J08 (dark red), S309 (orange), 4A8 (dark blue) and L19 (gold), or antibodies that did not compete with any of the previous mAbs (gray). A schematic representation of J08, S309, 4A8 and L19 epitopes on the S protein surface is shown on the left side of the panel. **b**–**e**, Graphs show the IGHV-J rearrangement percentage for nAbs that competed against J08, S309, 4A8, or that did not compete with any of these mAbs. The total number (n) of competing nAbs per group is shown on top of each graph.

Extended Data Table 1 | Clinical details of COVID-19 vaccinees

Subject ID	Gender	Age	Previous COVID-19 negative test	COVID-19 positive test	Type of test	Severity of Infection	SARS-CoV-2 Serology	First Dose (dd/mm/yy)	Second Dose (dd/mm/yy)	Blood Collection (dd/mm/yy)	Days from Infection to First Dose	Days from Last Dose to Blood Collection
VAC-001	M	38	16/12/2020	Not-applicable	Swab	Not-applicable	Seronegative	27/12/2020	18/01/2021	02/03/2021	Not-applicable	43
VAC-002	F	38	31/12/2020	Not-applicable	Swab	Not-applicable	Seronegative	01/01/2021	22/01/2021	02/03/2021	Not-applicable	39
VAC-007	M	38	29/12/2020	Not-applicable	Swab	Not-applicable	Seronegative	04/01/2021	25/01/2021	31/03/2021	Not-applicable	65
VAC-008	M	43	28/12/2020	Not-applicable	Swab	Not-applicable	Seronegative	03/01/2021	24/01/2021	31/03/2021	Not-applicable	66
VAC-010	F	51	15/02/2021	Not-applicable	Swab	Not-applicable	Seronegative	18/02/2021	11/03/2021	07/04/2021	Not-applicable	27
VAC-003	M	38	15/10/2020	26/10/2020	Swab	Asymptomatic	Seropositive	08/01/2021	15/02/2021	09/03/2021	74	22
VAC-004	F	25	Not-applicable	22/10/2020	Swab	Mild	Seropositive	08/02/2021	01/03/2021	09/03/2021	78	8
VAC-005	M	25	01/08/2020	02/11/2020	Serological	Mild	Seropositive	11/01/2021	16/02/2021	16/03/2021	71	28
VAC-006	F	57	27/04/2020	24/10/2020	Swab	Asymptomatic	Seropositive	16/01/2021	11/02/2021	16/03/2021	79	33
VAC-009	M	46	29/09/2020	06/11/2020	Serological	Moderate	Seropositive	20/03/2021	Not-applicable	07/04/2021	134	18

Extended Data Table 2 | Summary of B cell frequencies and antibodies of COVID-19 vaccinees

Subject	SARS-CoV-2 serology	S protein$^+$ MBCs Sorted	S protein$^+$ mAbs (n)	S protein$^+$ mAbs (%)	Wuhan - Neutralizing antibodies (n)	Wuhan - Neutralizing antibodies (%)	B.1.1.7 - Neutralizing antibodies (n)	B.1.1.7 - Neutralizing antibodies (%)	B.1.351 - Neutralizing antibodies (n)	B.1.351 - Neutralizing antibodies (%)	B.1.1.248 - Neutralizing antibodies (n)	B.1.1.248 - Neutralizing antibodies (%)
VAC-001	Seronegative	484	205	42.3	13	6.3	8	61.5	2	15.4	6	46.2
VAC-002	Seronegative	497	316	63.6	27	8.5	19	70.4	6	22.2	11	40.7
VAC-007	Seronegative	161	100	62.1	16	16.0	14	87.5	3	18.8	6	37.5
VAC-008	Seronegative	682	107	15.7	10	9.3	6	60.0	5	50.0	6	60.0
VAC-010	Seronegative	528	216	40.9	5	2.3	3	60.0	0	0.0	2	40.0
Total (Seronegative)		**2,352**	**944**	**40.1**	**71**	**7.5**	**50**	**70.4**	**16**	**22.5**	**31**	**43.6**
VAC-003	Seropositive	616	428	69.5	78	18.2	58	74.4	25	32.1	40	51.3
VAC-004	Seropositive	506	318	62.8	31	9.7	24	77.4	11	35.5	18	58.1
VAC-005	Seropositive	924	601	65.0	152	25.3	132	86.8	78	51.3	114	75.0
VAC-006	Seropositive	396	216	55.8	19	8.8	12	63.2	5	26.3	8	42.1
VAC-009	Seropositive	1,090	736	67.5	60	8.1	48	80.0	15	25.0	31	51.7
Total (Seropositive)		**3,532**	**2,299**	**65.1**	**340**	**14.8**	**274**	**80.6**	**134**	**39.4**	**211**	**62.0**

Extended Data Table 3 | Competition assay summary

Subject	SARS-CoV-2 serology	Distribution Competition J08 (n)	Distribution Competition J08 (%)	Distribution Competition S309 (n)	Distribution Competition S309 (%)	Distribution Competition 4A8 (n)	Distribution Competition 4A8 (%)	Distribution Competition L19 (n)	Distribution Competition L19 (%)	Distribution Competition Not-competing (n)	Distribution Competition Not-competing (%)
VAC-001	Seronegative	4	50.0	0	0.0	1	12.5	0	0.0	3	37.5
VAC-002	Seronegative	7	41.2	1	5.9	2	11.8	0	0.0	7	41.2
VAC-007	Seronegative	9	60.0	3	20.0	2	13.3	0	0.0	1	6.7
VAC-008	Seronegative	4	44.4	0	0.0	3	33.3	0	0.0	2	22.2
VAC-010	Seronegative	2	66.7	0	0.0	1	33.3	0	0.0	0	0.0
Total (Seronegative)		26	50.0	4	7.7	9	17.3	0	0.0	13	25.0
VAC-003	Seropositive	19	54.3	7	20.0	3	8.6	0	0.0	6	17.1
VAC-004	Seropositive	2	15.4	7	53.8	0	0.0	0	0.0	4	30.8
VAC-005	Seropositive	58	51.3	25	22.1	19	16.8	0	0.0	11	9.7
VAC-006	Seropositive	8	61.5	2	15.4	1	7.7	0	0.0	2	15.4
VAC-009	Seropositive	28	56.0	7	14.0	12	24.0	0	0.0	3	6.0
Total (Seropositive)		115	51.3	48	21.4	35	15.6	0	0.0	26	11.6

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	- Thermo Fisher SkanIt Software Microplate Readers 6.0.1 - BD Biosciences BD FACSDiva Software v9.0
Data analysis	- GraphPad Prism 8.0.2 was used to perform statistical analyses - BD FlowJo 10.5.3 - Qiagen CLC sequence viewer 350 8.0.0 - Boston University, Cloanalyst (http://www.bu.edu/computationalimmunology/research/software/)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

Source data are provided with this paper. All data supporting the findings in this study are available within the article or can be obtained from the corresponding author upon request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	10 subjects in total, 5 seronegative and 5 seropositive, were analyzed in this study. A total of 2,352 and 3,532 spike protein specific memory B cells from seronegative and seropositive subjects were tested in this study. Given the exploratory nature of the study, we did not use statistical methods to predetermine sample size. Sample size was based on previous studies that applied a similar technology. The authors believed that 5 subjects/group were a good balance between feasibility of analyzing at single cell level several thousands of memory B cells and the ability to represent the antibody response of seronegative and seropositve people.
Data exclusions	No data was excluded.
Replication	All experiments were performed in technical duplicates or triplicates as indicated in the figure legends and methods section.
Randomization	The experiments were not randomized and all available samples were tested. The authors aimed to specifically assess the antibody response of seronegative and seropositve subjects. Donors were specifically recruited based on their previous infection and vaccination history. Randomization would have not allowed to enroll 5 subjects/group which was our technical limit for single cell analysis of the antibody response. Based on what stated above, the authors believed that randomization was not appropriate.
Blinding	The investigators were not blinded during group allocation, data collection and analyses. The clinical protocol established to enroll subjects in this study reports information regarding previous infection and vaccination in order to allocate 5 subjects/group. Pseudonymized information received in the lab reports the same information and therefore blinding for group allocation was not possible.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	BD Biosciences CD19 BV421, Cat#562440, Clone ID HIB19, Lot#8270584 BD Biosciences IgM PerCP-Cy5.5, Cat#561285, Clone ID G20-127, Lot#9269055 BD Biosciences CD27 PE, Cat#340425, Clone ID L128, Lot#9288842 BD Biosciences IgD-A700, Cat#561302, Clone ID IA6-2, Lot#9199226 BioLegend CD3 PE-Cy7, Cat#300420, Clone ID UCHT1, Lot#B303315 BioLegend CD14 PE-Cy7, Cat#301814, Clone ID M5E2, Lot#B272337 BioLegend CD56 PE-Cy7, Cat#318318, Clone ID HCD56, Lot#B297987 Southern Biotech Goat Anti-Human IgG-Alkaline Phosphatase, Cat#2040-04, polyclonal, Lot#K2119-XG00B Southern Biotech Goat Anti-Human IgA-Alkaline Phosphatase, Cat#2050-04, polyclonal, Lot#G0919-W620C Sigma-Aldrich Anti-Human IgG (Fab specific)–Peroxidase antibody produced in goat, Cat#A0293, polyclonal, Lot#019M4876V
Validation	BD Biosciences CD19 BV421, Cat#562440, Clone ID HIB19, QC testing, reactivity human, application flow cytometry (https://www.bdbiosciences.com/content/bdb/paths/generate-tds-document.us.562440.pdf). BD Biosciences IgM PerCP-Cy5.5, Cat#561285, Clone ID G20-127, QC testing, reactivity human, application flow cytometry (https://www.bdbiosciences.com/content/bdb/paths/generate-tds-document.us.561285.pdf). BD Biosciences CD27 PE, Cat#340425, Clone ID L128, QC testing, reactivity human, application flow cytometry (https://

www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/clinical-discovery-research/single-color-antibodies-ruo-gmp/pe-mouse-anti-human-cd27.340425)
BD Biosciences IgD-A700, Cat#561302, Clone ID IA6-2, QC testing, reactivity human, application flow cytometry (https://www.bdbiosciences.com/content/bdb/paths/generate-tds-document.us.561302.pdf)
BioLegend CD3 PE-Cy7, Cat#300420, Clone ID UCHT1, reactivity human and cross-reactivity with chimpanzee, application flow cytometry (https://www.biolegend.com/en-us/global-elements/pdf-popup/pe-cyanine7-anti-human-cd3-antibody-3070?filename=PECyanine7%20anti-human%20CD3%20Antibody.pdf&pdfgen=true)
BioLegend CD14 PE-Cy7, Cat#301814, Clone ID M5E2, Reactivity Human, African Green, Capuchin Monkey, Cattle (Bovine, Cow), Chimpanzee, Common Marmoset, Cotton-topped Tamarin, Cynomolgus, Dog (Canine), Rhesus, Pigtailed Macaque, Squirrel Monkey, application flow cytometry (https://www.biolegend.com/en-us/global-elements/pdf-popup/pe-cyanine7-anti-human-cd14-antibody-2729?filename=PECyanine7%20anti-human%20CD14%20Antibody.pdf&pdfgen=true)
BioLegend CD56 PE-Cy7, Cat#318318, Clone ID HCD56, Reactivity Human, African Green, Baboon, Cynomolgus, Rhesus, application flow cytometry (https://www.biolegend.com/en-us/global-elements/pdf-popup/pe-cyanine7-anti-human-cd56-ncam-antibody-3802?filename=PECyanine7%20anti-human%20CD56%20NCAM%20Antibody.pdf&pdfgen=true)
Southern Biotech Goat Anti-Human IgG-Alkaline Phosphatase, Cat#2040-04, polyclonal, reactivity heavy chain of human IgG, application ELISA (https://www.southernbiotech.com/techbul/2040.pdf)
Southern Biotech Goat Anti-Human IgA-Alkaline Phosphatase, Cat#2050-04, polyclonal, reactivity heavy chain of human IgA, application ELISA (https://www.southernbiotech.com/techbul/2050.pdf)
Sigma-Aldrich Anti-Human IgG (Fab specific)–Peroxidase antibody produced in goat, Cat#A0293, polyclonal, reactivity human, application ELISA (https://www.sigmaaldrich.com/IT/en/product/sigma/a0293#)

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	VERO E6 cell line ATCC Cat#CRL-1586; Expi293F cells Thermo Fisher Cat#A14527; 3T3-msCD40L Cells NIH AIDS Reagent Program Cat#12535.
Authentication	These cell lines were obtained from vendors that sell authenticated cell lines, they grew, performed and showed morphology as expected. No additional specific authentication was performed.
Mycoplasma contamination	Vero E6 cell lines are routinely tested on a monthly basis and tested negative for mycoplasma. 3T3-msCD40L cell line was tested negative to mycoplasma by the provider and Expi293F cells were not tested for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used in this study.

Human research participants

Policy information about studies involving human research participants

Population characteristics	This work results from a collaboration with the Azienda Ospedaliera Universitaria Senese, Siena (IT) that provided samples from COVID-19 vaccinated donors, of both sexes (4 females and 6 males), who gave their written consent. All data relevant to enrolled subjects are reported in this study. Subjects elegible for this study were of all sexes (aged 18-85) naïve or previously infected by SARS-CoV-2 and then vaccinated with the COVID-19 BNT162b2 mRNA vaccine.
Recruitment	Individuals with or without previous SARS-CoV-2 infection vaccinated with the COVID-19 BNT162b2 mRNA vaccine were enrolled by the clinicians involved in the study entitled "Isolamento di anticorpi monoclonali umani contro SARS-CoV-2 per lo sviluppo di nuove terapie e vaccini", Prot. n. TLS_SARS-CoV-2, at the Azienda Ospedaliera Universitaria Senese, Siena (IT). The authors do not see any potential bias in the generation or interpretation of the data reported in this study.
Ethics oversight	The study was approved by the Comitato Etico di Area Vasta Sud Est (CEAVSE) ethics committees (Parere 17065 in Siena) and conducted according to good clinical practice in accordance with the declaration of Helsinki (European Council 2001, US Code of Federal Regulations, ICH 1997). This study was unblinded and not randomized. No statistical methods were used to predetermine sample size.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Human PBMC were isolated from heparin-treated whole blood by density gradient centrifugation (Ficoll-Paque™ PREMIUM,

Sample preparation	Sigma-Aldrich). After separation, PBMC were stained with Live/Dead Fixable Aqua (Invitrogen; Thermo Scientific) diluted 1:500 at room temperature RT. After 20 min incubation cells were washed with PBS and unspecific bindings were saturated with 20% normal rabbit serum (Life technologies). Following 20 min incubation at 4°C cells were washed with PBS and stained with SARS-CoV-2 S-protein labeled with Strep-Tactin®XT DY-488 (iba-lifesciences cat# 2-1562-050) for 30 min at 4°C. After incubation the following staining mix was used CD19 V421 (BD cat# 562440, 1:320), IgM PerCP-Cy5.5 (BD cat# 561285, 1:50), CD27 PE (BD cat# 340425, 1:30), IgD-A700 (BD cat# 561302, 1:15), CD3 PE-Cy7 (BioLegend cat# 300420, 1:100), CD14 PE-Cy7 (BioLegend cat# 301814, 1:320), CD56 PE-Cy7 (BioLegend cat# 318318, 1:80) and cells were incubated at 4°C for additional 30 min. Stained MBCs were single cell-sorted with a BD FACS Aria III (BD Biosciences).
Instrument	BD FACS Aria III Cell Sorter BD Biosciences
Software	BD Biosciences BD FACSDiva Software v9.0
Cell population abundance	Single cell sorted S protein trimer-specific (S protein+), class-switched memory B cells (CD19+CD27+IgD-IgM-) were 0.21, 0.25, 0.35, 0.39, 0.20, 0.57, 0.62, 0.67, 0.40 and 1.19% for subject VAC-001, VAC-002, VAC-007, VAC-008, VAC-010, VAC-003, VAC-004, VAC-005, VAC-006 and VAC-009 respectively. Sorted cells were gated on the CD19+CD27+IgD-IgM-S protein+ population based on the negative control as reported in the Extended Data (Extended Data Figure 1a,b).
Gating strategy	The gating strategy used for the single cell sorting of spike protein specific memory B cells is shown in the Extended Data (Extended Data Figure 1). Boundaries between “positive” and “negative” cells are defined and denoted on each graph.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.