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1 **Hybrid immunity improves B cell frequency, antibody potency and breadth against SARS-CoV-2 and**
2 **variants of concern**

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19 **Key words:** COVID-19; SARS-CoV-2; Monoclonal antibodies; Variants of concern; Vaccination; B
20 cell repertoire

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ABSTRACT

To understand the nature of the antibody response to SARS-CoV-2 vaccination, we analyzed at single cell level the B cell responses of five naïve and five convalescent people immunized with the BNT162b2 mRNA vaccine. Convalescents had higher frequency of spike protein specific memory B cells and by cell sorting delivered 3,532 B cells, compared with 2,352 from naïve people. Of these, 944 from naïve and 2,299 from convalescents produced monoclonal antibodies against the spike protein and 411 of them neutralized the original Wuhan SARS-CoV-2 virus. More than 75% of the monoclonal antibodies from naïve people lost their neutralization activity against the B.1.351 (beta) and B.1.1.248 (gamma) variants while this happened only for 61% of those from convalescents. The overall loss of neutralization was lower for the B.1.1.7 (alpha) and B.1.617.2 (delta) variants, however it was always significantly higher in those of naïve people. In part this was due to the IGHV2-5;IGHJ4-1 germline, which was found only in convalescents and generated potent and broadly neutralizing antibodies. Overall, vaccination of seropositive people increases the frequency of B cells encoding antibodies with high potency and that are not susceptible to escape by any of the four variants of concern. 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.

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38 **INTRODUCTION**

39 Twenty months after the beginning of the COVID-19 pandemic, with 200 million people infected, 4.2
40 million deaths, and 3.9 billion vaccines doses administered, the world is still struggling to control the
41 virus. In most developed countries vaccines have vastly reduced severe diseases, hospitalization
42 and deaths, but they have not been able to control the infections which are fueled by new and more
43 infectious variants. A large number of studies so far have shown that protection from infection is
44 linked to the production of neutralizing antibodies against the spike protein (S protein) of the virus[1-
45 4]. This is a metastable, trimeric class 1 fusion glycoprotein, composed by the S1 and S2 subunits,
46 and mediates virus entry changing from a prefusion to postfusion conformation after binding to the
47 human angiotensin-converting enzyme 2 (ACE2) receptor and heparan sulfates on the host cells[5].
48 Potent neutralizing antibodies recognize the S1 subunit of each monomer which includes the
49 receptor binding domain (RBD) and N-terminal domain (NTD) immunodominant sites[6]. The large
50 majority of neutralizing antibodies bind the receptor binding motif (RBM), within the RBD, and a
51 smaller fraction target the NTD[3,7]. Neutralizing antibodies against the S2 subunit have been
52 described, however they have very low potency[3,8]. Neutralizing antibodies generated after infection
53 derive in large part from germline IGHV3-53 and the closely related IGHV3-66 with very few somatic
54 mutations[9,10]. Starting from the summer of 2020, the virus started to generate mutations that allowed
55 the virus to evade neutralizing antibodies, to become more infectious, or both. Some of the mutant
56 viruses completely replaced the original Wuhan SARS-CoV-2. The most successful variant viruses
57 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
58 named Variants of Concern (VoCs) by the World Health Organization[11]. The delta variant is presently
59 spreading across the globe and causing big concern also in fully vaccinated populations. It is
60 therefore imperative to understand the molecular dynamics of the immune response to vaccination
61 in order to design better vaccines and vaccination policies. Several investigators have shown that
62 vaccination of convalescent people can yield neutralizing antibodies which can be up to a thousand-
63 fold higher than those induced by infection or vaccination, suggesting that one way of controlling the
64 pandemic may be the induction of a hybrid immunity-like response using a third booster dose[12-16].
65 Here we compared at single cell level the nature of the neutralizing antibody response against the
66 original Wuhan virus and the VoCs in naïve and convalescent subjects immunized with the
67 BNT162b2 mRNA vaccine. Naïve subjects were seronegative before vaccination while convalescent
68 donors were already seropositive before vaccination. They will be named seronegatives and
69 seropositives respectively in this work. Our data suggest that immunization of people already
70 seropositive to the virus, increases the frequency, potency and breadth of neutralizing antibodies
71 and may help to control emerging variants.

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RESULTS

High levels of S protein specific memory B cells and plasma activity in seropositive vaccinees

In this study we enrolled 10 donors vaccinated with the BNT162b2 mRNA vaccine, 5 of them were healthy people naïve to SARS-CoV-2 infection at vaccination (seronegative) and other 5 had recovered from SARS-CoV-2 infection before vaccination (seropositive). Subject details are summarized in **Table S1**. We initially analyzed the B cell population frequencies 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 (**Figure S1A-B**). 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 (**Figure S1A-B**). 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 (**Figure S2**). Plasma from seropositives showed a higher binding activity to the S protein and all tested domains compared to seronegatives (**Figure S2A – D**). In addition, seropositives showed a 10-fold higher neutralization activity against the original Wuhan SARS-CoV-2 virus compared to seronegatives (**Figure S2E – F**).

Frequency of neutralizing antibodies against the Wuhan virus and variants of concern

To better characterize the B cell immune response in seronegative and seropositive donors, we single cell sorted antigen-specific memory B cell (MBCs) using as bait the stabilized Wuhan SARS-CoV-2 S protein antigen which was encoded by the mRNA vaccine. The single cell sorting strategy was performed as previously described[3]. 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 (**Table S2**). 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 (**Figure 1A**; **Table S2**). 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(**Figure 1B**; **Table S2**). 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

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110 overall higher percentage of nAbs neutralizing the VoCs compared to seronegatives. The average
111 frequency of nAbs from seropositives neutralizing the alpha, beta and gamma variants was 80.6
112 (n=274), 39.4 (n=134) and 62.0% (n=211) respectively, compared to 70.4 (n=50), 22.5 (n=16) and
113 43.6% (n=31) respectively in seronegatives (**Figure 1C**; **Table S2**).
114
115 **High potency and breadth of neutralization in seropositive COVID-19 vaccinees**
116 To better characterize and understand the potency and breadth of coverage of all Wuhan SARS-
117 CoV-2 nAbs, we aimed to express as immunoglobulin G1 (IgG1) all the 411 nAbs previously
118 identified. We were able to recover and express 276 antibodies for further characterization, 224
119 (89.8%) from seropositives and 52 (10.2%) from seronegatives. Initially, antibodies were tested for
120 binding against the RBD, NTD and the S2-domain of the original Wuhan SARS-CoV-2 S protein.
121 Overall, no major differences were observed in nAbs that recognized the RBD and NTD with 71.2%
122 (n=37) and 79.5% (n=178) nAbs binding the RBD, while 17.3% (n=9) and 16.5% (n=37) nAbs binding
123 the NTD for seronegatives and seropositives respectively (**Figure S3**). None of the tested nAbs
124 targeted the S2 domain. The biggest difference between groups was seen for nAbs able to bind the
125 S protein only in its trimeric conformation i.e. not able to bind single domains. This class of nAbs was
126 almost 3-fold higher in seronegatives compared to seropositives (**Figure S3**). nAbs were then tested
127 by CPE-MN in serial dilution to evaluate their 100% inhibitory concentration (IC_{100}) against the
128 Wuhan SARS-CoV-2 virus and the VoCs. At this stage of the study, the B.1.617.2 (delta) spread
129 globally, and we were able to obtain the live virus for our experiments. Overall, nAbs isolated from
130 seropositive vaccinees had a significantly higher potency than those isolated from seronegatives.
131 The IC_{100} geometric mean (GM-IC_{100}) in seropositives was 2.87, 2.17-, 1.17-, 1.43-, and 1.92-fold
132 lower than in seronegatives for the Wuhan virus, the alpha, beta, gamma and delta VoCs respectively
133 (**Figure 2**). In addition, a bigger fraction of nAbs from seropositives retained the ability to neutralize
134 the VoCs. Indeed, when nAbs were individually tested against all VoCs, the ability to neutralize the
135 alpha, beta, gamma and delta variants was lost by 14, 61, 61 and 29% of the antibodies from
136 seropositives versus 32, 78, 75 and 46% respectively of those from seronegatives. The overall
137 number of nAbs that lost neutralizing activity against the beta and gamma VoCs was very high, (75-
138 78% in seronegatives and 61% in seropositives), while it was much lower for the alpha and delta
139 variants (32-46% for seronegatives and 14-29% for seropositives) (**Figure 2**). Finally, a major
140 difference between seronegatives and seropositives was found in the class of medium/high potency
141 nAbs (IC_{100} of 11-100 ng/mL and 101-1000 ng/mL) against all variants. Indeed, nAbs in these ranges
142 from seropositives constitute the 71.0%, 62.5%, 23.7%, 22.8%, 53,1% of the whole nAbs repertoire
143 while nAbs from seronegative donors were 48.1%, 38.5%, 17.3%, 17.3%, 34.6% against the Wuhan
144 SARS-CoV-2 virus and alpha, beta, gamma and delta VoCs respectively (**Figure 2**).
145
146 **Functional gene repertoire of neutralizing antibodies**

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147 The analysis of the immunoglobulin G heavy chain variable (IGHV) and joining (IGHJ) gene
148 rearrangements of 58 and 278 sequences recovered from seronegative and seropositive subjects
149 respectively, showed that they use a broad range of germlines and share the most abundant. In
150 particular, both groups predominantly used the IGHV1-69;IGHJ4-1 and IGHV3-53;IGHJ6-1, which
151 were shared by three out five subjects per each group (**Figure 3A**). In addition, the IGHV3-30;IGHJ6-
152 1 and IGHV3-33;IGHJ4-1 germlines, more abundant in seronegative donors, and IGHV1-2;IGHJ6-
153 1, mainly expanded in seropositive vaccinees, were also used with high frequency in both groups.
154 Only the IGHV2-5;IGHJ4-1 germline was seen to be predominantly expanded only in seropositive
155 donors (**Figure 3A**). To better characterize these predominant gene families, we evaluated their
156 neutralization potency and breadth against SARS-CoV-2 and VoCs. In this analyses we could not
157 evaluate IGHV3-33;IGHJ4-1 nAbs, as only three of these antibodies were expressed, but we
158 included the IGHV3-53 closely related family IGHV3-66;IGHJ4-1, as this family was previously
159 described to be mainly involved in SARS-CoV-2 neutralization[9,17]. A large part of nAbs deriving from
160 these predominant germlines had a very broad range of neutralization potency against the original
161 Wuhan SARS-CoV-2 virus with IC_{100} spanning from less than 10 to over 10,000 ng/mL (**Figure 3B -**
162 **G**). However, many of them lost the ability to neutralize SARS-CoV-2 VoCs. The loss of neutralizing
163 activity occurred for most germlines and it was moderate against the alpha and delta variants, while
164 it was dramatic against the beta and gamma variants (**Figure 3 B - G**). A notable exception was the
165 IGHV2-5;IGHJ4-1 germline, present only in nAbs of seropositive patients, that showed potent
166 antibodies able to equally neutralize all SARS-CoV-2 VoCs (**Figure 3D**). Finally, we evaluated the
167 CDRH3-length and V-gene somatic hyper mutation (SHM) levels for all nAbs retrieved from
168 seronegatives and seropositives and for predominant germlines. Overall, the two groups show a
169 similar average CDRH3-length (15.0 aa and 15.1 aa for seronegatives and seropositives
170 respectively), however seropositives showed almost 2-fold higher V-gene mutation levels compared
171 to seronegatives (**Figure S4**). As for predominant gene derived nAbs, we observed heterogenous
172 CDRH3-length, with the only exception of IGHV3-53;IGHJ6-1 nAbs, and higher V-gene mutation
173 levels in seropositives predominant germlines compared to seronegatives (**Figure S5**).
174

175 **Epitope mapping of neutralizing antibodies**

176 To map the regions of the S protein recognized by the identified nAbs we used a competition assay
177 with four known antibodies: J08, which targets the top loop of the receptor binding motif (RBM)[3],
178 S309, which binds the RBD but outside of the RMB region[18], 4A8, that recognized the NTD[19], and
179 L19, that binds the S2-domain[3](**Figure S6**). The nAbs identified in this study were pre-incubated with
180 the original Wuhan SARS-CoV-2 S protein and subsequently the four nAbs labeled with different
181 fluorophores were added as single mix. 50% signal reduction for one of the four fluorescently labelled
182 nAbs, was used as threshold for positive competition. The vast majority of nAbs from both
183 seronegative (50.0%; n=26) and seropositive (51.3%; n=115) vaccinees competed with J08 (**Figure**
184 **4A; Table S3**). For seronegatives, the second most abundant population was composed by nAbs

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185 that did not compete with any of the four fluorescently labelled nAbs (25.0%; n=13) followed by nAbs
186 targeting the NTD (17.3%; n=9). As for seropositives, the second most abundant population was
187 composed by nAbs that competed with S309 (21.4%; n=48) followed by nAbs competing with 4A8
188 (15.6%; n=35) and not-competing nAbs (11.6%; n=26). None of our nAbs did compete with the S2
189 targeting antibody L19 (**Figure 4A; Table S3**). nAbs competing with J08, which are likely to bind the
190 RBM, derived from several germlines, including the predominant IGHV3-53;IGHJ6-1 (10.6%; n=14),
191 IGHV1-69;IGHJ4-1 (8.3%; n=11) and IGHV1-2;IGHJ6-1 (6.8%; n=9) (**Figure 4B**). In contrast, those
192 competing with S309 derived mostly from germline IGHV2-5;IGHJ4-1 (13.7%; n=7) which were
193 isolated exclusively from seropositive vaccinees (**Figure 4C**). As for NTD-directed nAbs the non-
194 predominant gene family IGHV1-24;IGHJ6-1 was the most abundant confirming what was reported
195 in previous studies (**Figure 4D**)[20]. Finally, for nAbs that did not compete with any of the known
196 antibodies used in our competition assay, the non-predominant gene families IGHV1-69;IGHJ3-1
197 (9.7%; n=3) and IGHV1-69;IGHJ6-1 (9.7%; n=3) were the most abundant (**Figure 4E**).

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198 **DISCUSSION**

199 Our study analyzed for the first time at single cell level the repertoire of B cells producing neutralizing
200 antibodies following vaccination of naïve and previously infected people. The most important
201 conclusion from this work is that vaccinating people that are already seropositive to the virus,
202 because of previous SARS-CoV-2 infection, induces neutralizing antibodies which are more potent
203 and less susceptible to escape from SARS-CoV-2 variants. This conclusion is not surprising since
204 several papers have already reported that vaccination of convalescent people induces a hybrid
205 immunity with titers of neutralizing antibodies up to 50-fold higher than those induced by vaccination
206 of naïve people[15,16]. The novelty of our work lies in the molecular analysis of this response which
207 shows that seropositive subjects respond to vaccination with more B cells producing antibodies
208 which show higher neutralization potency and are not susceptible to escape variants. In part this is
209 due to potent antibodies derived from the IGHV2-5;IGHJ4-1 germline which was only found in
210 seropositive people. The absence of this germline in naïve vaccinees is also confirmed by previous
211 studies[16]. One limitation of our study is that we did not include people that received a third booster
212 dose of vaccine, as at the time that this work was being performed no policies for a third booster
213 dose were implemented. In spite of this limitation, we believe that our conclusions are likely to be
214 extendable to people that are seropositives following primary vaccination. Indeed, the S protein
215 produced following vaccination with mRNA, viral vectors or following infection is produced in all
216 cases by the cell of the host and it is likely to be presented to the immune system in a similar manner
217 and generate a similar antibody response. This is confirmed by the fact that neutralizing antibodies
218 following infection and vaccination derive mostly from the same immunodominant germlines, i.e.
219 IGHV3-53, 3-30 and 3-66[9,10,16,17]. Our analysis suggests that a booster dose of vaccine will increase
220 the frequency of memory B cells producing potent neutralizing antibodies not susceptible to escape
221 variants and allow better control of this pandemic. Our work also shows that more than three quarters
222 of antibodies neutralizing the Wuhan virus do not neutralize at all the beta and gamma variants, while
223 the fraction of antibodies not neutralizing the alpha and delta variants is much smaller and in all
224 cases below 50%. This suggest that the beta and gamma variants were originally selected to escape
225 natural immunity, while the alpha and gamma were selected mostly for their increased infectivity and
226 ability to spread in the population[21,22]. The massive escape from predominant germlines, such as
227 IGHV3-53, 3-66, 3-30 and 1-69, and the presence of antibodies deriving from germline IGHV2-5 that
228 are completely insensitive to the existing variants, suggest that the design of vaccines that
229 preferentially promote or avoid the expansion of selected germlines can generate broad protection
230 against SARS-CoV-2 variants. Germline-targeting vaccination, which has been pioneered in the HIV
231 field[23,24], may be a promising strategy to fight the COVID-19 pandemic.

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232 **MATERIALS & METHODS**

233 **Enrollment of COVID-19 vaccinees and human sample collection**

234 This work results from a collaboration with the Azienda Ospedaliera Universitaria Senese, Siena (IT)
235 that provided samples from COVID-19 vaccinated donors, of both sexes, who gave their written
236 consent. The study was approved by local ethics committees (Parere 17065 in Siena) and conducted
237 according to good clinical practice in accordance with the declaration of Helsinki (European Council
238 2001, US Code of Federal Regulations, ICH 1997). This study was unblinded and not randomized.
239 No statistical methods were used to predetermine sample size.

240

241 **Single cell sorting of SARS-CoV-2 S protein^{+} memory B cells from COVID-19 vaccinees**

242 Peripheral blood mononuclear cells (PBMCs) and single cell sorting strategy were performed as
243 previously described[3]. Briefly, PBMC were isolated from heparin-treated whole blood by density
244 gradient centrifugation (Ficoll-Paque™ PREMIUM, Sigma-Aldrich). After separation, PBMC were
245 stained with Live/Dead Fixable Aqua (Invitrogen; Thermo Scientific) diluted 1:500 at room
246 temperature RT. After 20 min incubation cells were washed with PBS and unspecific bindings were
247 saturated with 20% normal rabbit serum (Life technologies). Following 20 min incubation at 4°C cells
248 were washed with PBS and stained with SARS-CoV-2 S-protein labeled with Strep-Tactin®XT DY-
249 488 (iba-lifesciences cat# 2-1562-050) for 30 min at 4°C. After incubation the following staining mix
250 was used CD19 V421 (BD cat# 562440), IgM PerCP-Cy5.5 (BD cat# 561285), CD27 PE (BD cat#
251 340425), IgD-A700 (BD cat# 561302), CD3 PE-Cy7 (BioLegend cat# 300420), CD14 PE-Cy7
252 (BioLegend cat# 301814), CD56 PE-Cy7 (BioLegend cat# 318318) and cells were incubated at 4°C
253 for additional 30 min. Stained MBCs were single cell-sorted with a BD FACS Aria III (BD Biosciences)
254 into 384-well plates containing 3T3-CD40L feeder cells and were incubated with IL-2 and IL-21 for
255 14 days as previously described[25].

256

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

258 mAbs and plasma binding specificity against the S-protein trimer was detected by ELISA as
259 previously described[3]. Briefly, 384-well plates (microplate clear, Greiner Bio-one) were coated with
260 3 µg/mL of streptavidin (Thermo Fisher) diluted in carbonate-bicarbonate buffer (E107, Bethyl
261 laboratories) and incubated at RT overnight. The next day, plates were incubated 1 h at RT with
262 3µg/mL of SARS-CoV-2 S protein diluted in PBS. Plates were then saturated with 50 µL/well of
263 blocking buffer (phosphate-buffered saline, 1% BSA) for 1 h at 37°C. After blocking, 25 µL/well of
264 mAbs diluted 1:5 in sample buffer (phosphate-buffered saline, 1% BSA, 0.05% Tween-20) were
265 added to the plates and were incubated at 37°C. Plasma samples derived from vaccinees were
266 tested (starting dilution 1:10; step dilution 1:2 in sample buffer) in a final volume of 25 µL/well and
267 were incubated at 37°C. After 1 h of incubation, 25 µl/well of alkaline phosphatase-conjugated goat
268 antihuman IgG and IgA (Southern Biotech) diluted 1:2000 in sample buffer were added. Finally, S
269 protein binding was detected using 25 µL/well of PNPP (p-nitrophenyl phosphate;Thermo Fisher)

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270 and the reaction was measured at a wavelength of 405 nm by the Varioskan Lux Reader (Thermo
271 Fisher Scientific). After each incubation step, plates were washed three times with 100µl/well of
272 washing buffer (phosphate-buffered saline, 0.05% Tween-20). Sample buffer was used as a blank
273 and the threshold for sample positivity was set at 2-fold the optical density (OD) of the blank.
274

275 **ELISA assay with RBD, NTD and S2 subunits**

276 mAbs identification and plasma screening of vaccinees against RBD, NTD or S2 SARS-CoV-2
277 protein were performed by ELISA. Briefly, 3 µg/mL of RBD, NTD or S2 SARS-CoV-2 protein diluted
278 in carbonate-bicarbonate buffer (E107, Bethyl laboratories) were coated in 384-well plates
279 (microplate clear, Greiner Bio-one). After overnight incubation at 4°C, plates were washed 3 times
280 with washing buffer (phosphate-buffered saline, 0.05% Tween-20) and blocked with 50 µL/well of
281 blocking buffer (phosphate-buffered saline, 1% BSA) for 1h at 37°C. After washing, plates were
282 incubated 1 h at 37 °C with mAbs diluted 1:5 in samples buffer (phosphate-buffered saline, 1% BSA,
283 0.05% Tween-20) or with plasma at a starting dilution 1:10 and step diluted 1:2 in sample buffer.
284 Wells with no sample added were consider blank controls. Anti-Human IgG −Peroxidase antibody
285 (Fab specific) produced in goat (Sigma) diluted 1:45000 in sample buffer was then added and
286 samples were incubated for 1 h at 37°C. Plates were then washed, incubated with TMB substrate
287 (Sigma) for 15 min before adding the stop solution (H_2SO_4 0.2M). The OD values were identified
288 using the Varioskan Lux Reader (Thermo Fisher Scientific) at 450 nm. Each condition was tested in
289 triplicate and samples tested were considered positive if OD value was 2-fold the blank.
290

291 **Flow cytometry-based competition assay**

292 To classify mAbs candidates on the basis of their interaction with Spike epitopes, we performed a
293 flow cytometry-based competition assay. In detail, magnetic beads (Dynabeads His-Tag, Invitrogen)
294 were coated with histidine tagged S protein according to the manufacturers' instructions. Then, 20
295 µg/ml of coated S-protein-beads were pre-incubated with unlabeled nAbs candidates diluted 1:2 in
296 PBS for 40 minutes at RT. After incubation, the mix Beads-antibodies was washed with 100 µL of
297 PBS-BSA 1%. Then, to analyze epitope competition, mAbs able to bind RBD (J08, S309), NTD (4A8)
298 or S2 (L19) domain of the S-protein were labeled with 4 different fluorophores (Alexa Fluor 647, 488,
299 594 and 405) using Alexa Fluor NHS Ester kit (Thermo Scientific), were mixed and incubated with
300 S-protein-beads. Following 40 minutes of incubation at RT, the mix Beads-antibodies was washed
301 with PBS, resuspended in 150 µL of PBS-BSA 1% and analyzed using BD LSR II flow cytometer
302 (Becton Dickinson). Beads with or without S-protein incubated with labeled antibodies mix were used
303 as positive and negative control respectively. Analysis was performed using FlowJo (version 10).
304

305 **SARS-CoV-2 authentic viruses neutralization assay**

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306 All SARS-CoV-2 authentic virus neutralization assays were performed in the biosafety level 3 (BSL3)
307 laboratories at Toscana Life Sciences in Siena (Italy) and Vismederi Srl, Siena (Italy). BSL3
308 laboratories are approved by a Certified Biosafety Professional and are inspected every year by local
309 authorities. To evaluate the neutralization activity of identified nAbs against SARS-CoV-2 and all
310 VoCs and evaluate the breadth of neutralization of this antibody is a cytopathic effect-based
311 microneutralization assay (CPE-MN) was performed[3]. Briefly, the CPE-based neutralization assay
312 sees the co-incubation of J08 with a SARS-CoV-2 viral solution containing 100 $TCID_{50}$ of virus and
313 after 1 hour incubation at 37°C, 5% CO_2. The mixture was then added to the wells of a 96-well plate
314 containing a sub-confluent Vero E6 cell monolayer. Plates were incubated for 3-4 days at 37°C in a
315 humidified environment with 5% CO_2, then examined for CPE by means of an inverted optical
316 microscope by two independent operators. All nAbs were tested a starting dilution of 1:5 and the
317 IC_{100} evaluated based on their initial concentration while plasma samples were tested starting from
318 a 1:10 dilution. Both nAbs and plasma samples were then diluted step 1:2. Technical duplicates were
319 performed for both nAbs and plasma samples. In each plate positive and negative control were used
320 as previously described[3].
321
322 **SARS-CoV-2 virus variants CPE-MN neutralization assay**
323 The SARS-CoV-2 viruses used to perform the CPE-MN neutralization assay were the original Wuhan
324 SARS-CoV-2 virus (SARS-CoV-2/INMI1-Isolate/2020/Italy: MT066156), SARS-CoV-2 B.1.1.7 (INMI
325 GISAID accession number: EPI_ISL_736997), SARS-CoV-2 B.1.351 (EVAg Cod: 014V-04058),
326 B.1.1.248 (EVAg CoD: 014V-04089) and B.1.617.2 (GISAID ID: EPI_ISL_2029113)[26].
327
328 **Single cell RT-PCR and Ig gene amplification and transcriptionally active PCR expression**
329 The whole process for nAbs heavy and light chain recovery, amplification and transcriptionally active
330 PCR (TAP) expression was performed as previously described[3]. Briefly, 5 µL of cell lysate were
331 mixed with 1 µL of random hexamer primers (50 ng/µL), 1 µL of dNTP-Mix (10 mM), 2 µL 0.1 M DTT,
332 40 U/µL RNAse OUT, $MgCl_2$ (25 mM), 5x FS buffer and Superscript IV reverse transcriptase
333 (Invitrogen) to perform RT-PCR. Reverse transcription (RT) reaction was performed at 42°C/10',
334 25°C/10', 50°C/60' and 94°/5'. Two rounds of PCR were performed to obtain the heavy (VH) and
335 light (VL) chain amplicons. All PCR reactions were performed in a nuclease-free water (DEPC) in a
336 total volume of 25 µL/well. For PCR I, 4 µL of cDNA were mixed with 10 µM of VH and 10 µM VL
337 primer-mix ,10mM dNTP mix, 0.125 µL of Kapa Long Range Polymerase (Sigma), 1.5 µL MgCl2 and
338 5 µL of 5x Kapa Long Range Buffer. PCRI reaction was performed at 95°/3', 5 cycles at 95°C/30'',
339 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'.
340 Nested PCR II was performed as above starting from 3.5 µL of unpurified PCR I product. PCR II
341 products were purified by Millipore MultiScreen® PCRµ96 plate according to manufacture
342 instructions and eluted in 30 µL of nuclease-free water (DEPC). As for TAP expression, vectors were
343 initially digested using restriction enzymes AgeI, SalI and Xho as previously described and PCR II

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344 products ligated by using the Gibson Assembly NEB into 25 ng of respective human Igγ1, Igκ and
345 Igλ expression vectors[27,28]. TAP reaction was performed using 5 µL of Q5 polymerase (NEB), 5 µL
346 of GC Enhancer (NEB), 5 µL of 5X buffer,10 mM dNTPs, 0.125 µL of forward/reverse primers and 3
347 µL of ligation product, using the following cycles: 98°/2', 35 cycles 98°/10'', 61°/20'', 72°/1' and 72°/5'.
348

349 **Functional repertoire analyses**

350 nAbs VH and VL sequence reads were manually curated and retrieved using CLC sequence viewer
351 (Qiagen). Aberrant sequences were removed from the data set. Analyzed reads were saved in
352 FASTA format and the repertoire analyses was performed using Cloanalyst
353 (http://www.bu.edu/computationalimmunology/research/software/)[29,30].
354

355 **Statistical analysis**

356 Statistical analysis was assessed with GraphPad Prism Version 8.0.2 (GraphPad Software, Inc., San
357 Diego, CA). Nonparametric Mann-Whitney t test was used to evaluate statistical significance
358 between the two groups analyzed in this study. Statistical significance was shown as * for values ≤
359 0.05, ** for values ≤ 0.01, *** for values ≤ 0.001, and **** for values ≤ 0.0001.
360

361 **Data availability**

362 All data supporting the findings in this study are available within the article or can be obtained from
363 the corresponding author upon request.

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364 ## ACKNOWLEDGMENTS

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379

380 ## AUTHOR CONTRIBUTIONS

381 EA and RR conceived the project. FM, MF, IR and MT enrolled COVID-19 vaccinees to the study.
382 EA and IP performed PBMC isolation and single cell sorting. IP performed ELISAs and competition
383 assays. IP and NM recovered nAbs VH and VL and expressed antibodies. PP and EA recovered VH
384 and VL sequences and performed the repertoire analyses. EP and VA produced and purified SARS-
385 CoV-2 S protein constructs. EA, GP, IH, ML, LB and GG performed neutralization assays in BSL3
386 facilities. CDS supported day-by-day laboratory activities and management. EA and RR wrote the
387 manuscript. All authors contributed to the final revision of the manuscript. EA, CS, EM and RR
388 coordinated the project.

389

390 ## DECLARATION OF INTERESTS

391 Rino Rappuoli is an employee of GSK group of companies. EA, IP, NM, PP, EP, CDS, CS and RR
392 are listed as inventors of full-length human monoclonal antibodies described in Italian patent
393 applications n. 102020000015754 filed on June 30th 2020, 102020000018955 filed on August 3rd
394 2020 and 102020000029969 filed on 4th of December 2020, and the international patent system
395 number PCT/IB2021/055755 filed on the 28th of June 2021.

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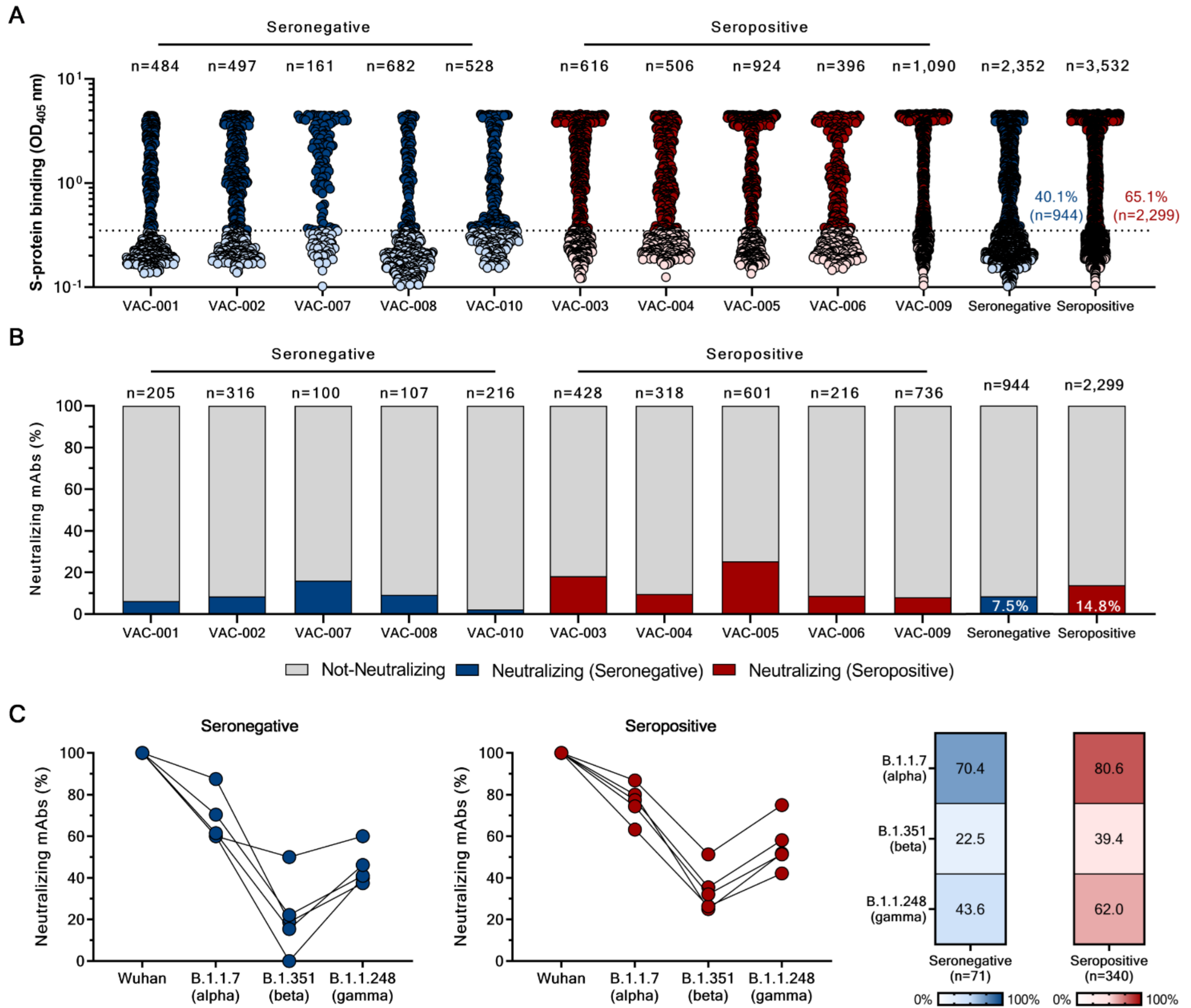
396 FIGURES



397

398 **Figure 1. Identification of cross-neutralizing SARS-CoV-2 S protein-specific nAbs.** (A) The graph
399 shows supernatants tested for binding to the Wuhan SARS-CoV-2 S protein antigen stabilized in its
400 prefusion conformation. Threshold of positivity has been set as two times the value of the blank
401 (dotted line). Dark blue and red dots represent mAbs that bind to the S protein for seronegative and
402 seropositive vaccinees respectively. Light blue and red dots represent mAbs that do not bind the S
403 protein for seronegative and seropositive vaccinees. (B) The bar graph shows the percentage of not-
404 neutralizing (gray), neutralizing mAbs from seronegatives (dark blue), and neutralizing mAbs for
405 seropositives (dark red). The total number (n) of antibodies tested per individual is shown on top of
406 each bar (C) Graphs show the fold change percentage of nAbs in seronegatives and seropositives
407 against the alpha, beta and gamma VoCs compared to the original Wuhan SARS-CoV-2 virus. The
408 heatmaps show the overall percentage of Wuhan SARS-CoV-2 nAbs able to neutralize tested VoCs.

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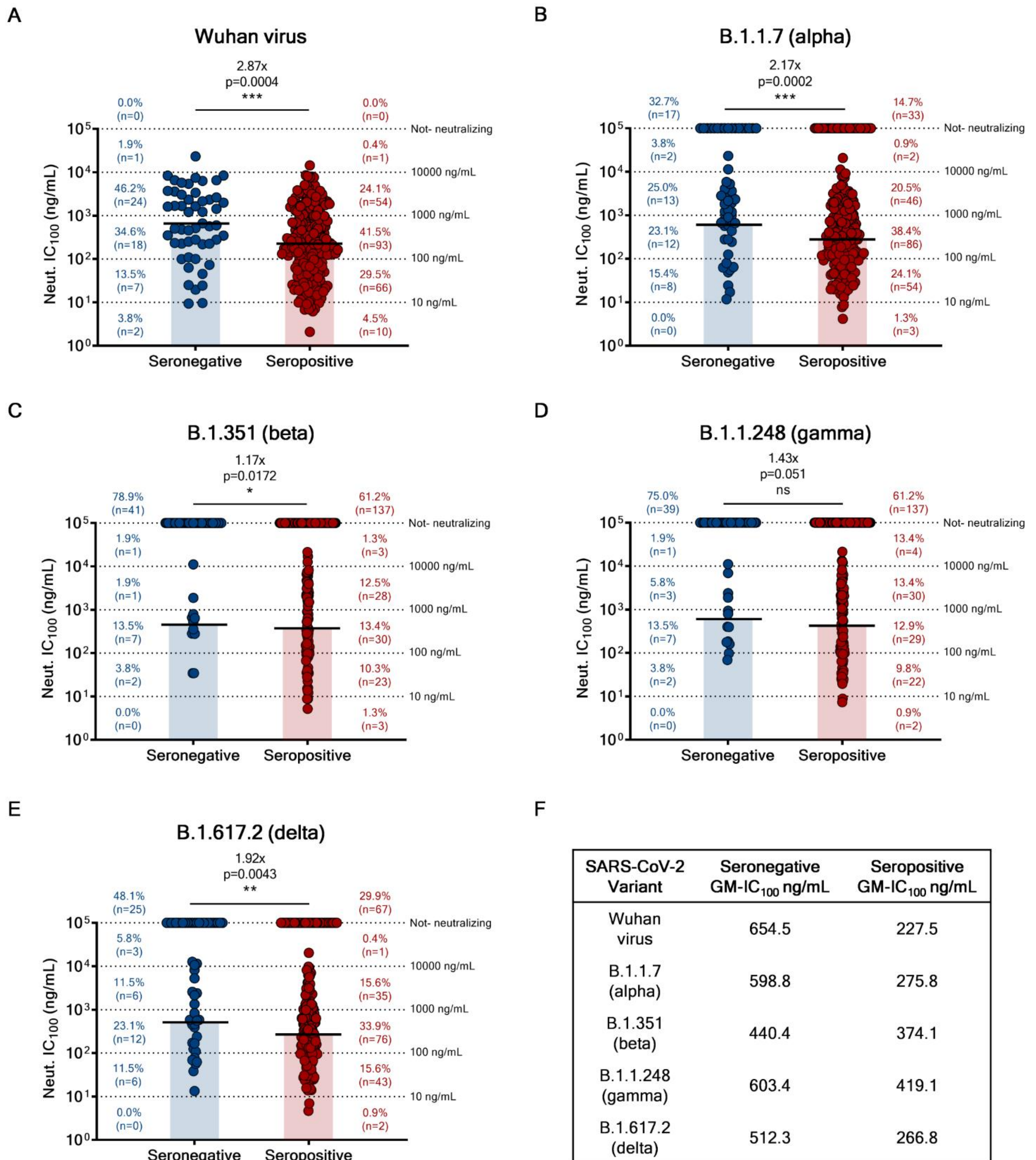

SARS-CoV-2 Variant	Seronegative GM-IC_{100} ng/mL	Seropositive GM-IC_{100} ng/mL
Wuhan virus	654.5	227.5
B.1.1.7 (alpha)	598.8	275.8
B.1.351 (beta)	440.4	374.1
B.1.1.248 (gamma)	603.4	419.1
B.1.617.2 (delta)	512.3	266.8

409

410 **Figure 2. Potency and breadth of neutralization of nAbs against SARS-CoV-2 and VoCs.** (A - E)
411 Scatter dot charts show the neutralization potency, reported as IC_{100} (ng/mL), of nAbs tested against
412 the original Wuhan SARS-CoV-2 virus (A) and the B.1.1.7 (B), B.1.351 (C), B.1.1.248 (D) and
413 B.1.617.2 (E) VoCs. The number and percentage of nAbs from seronegatives vs seropositives, fold-
414 change and statistical significance are denoted on each graph. A nonparametric Mann-Whitney t test
415 was used to evaluate statistical significances between groups. Significances are shown as *$p < 0.05$,

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416 **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. (F) The table shows the IC_{100} geometric mean (GM) of
417 all nAbs pulled together from each group against all SARS-CoV-2 viruses tested.

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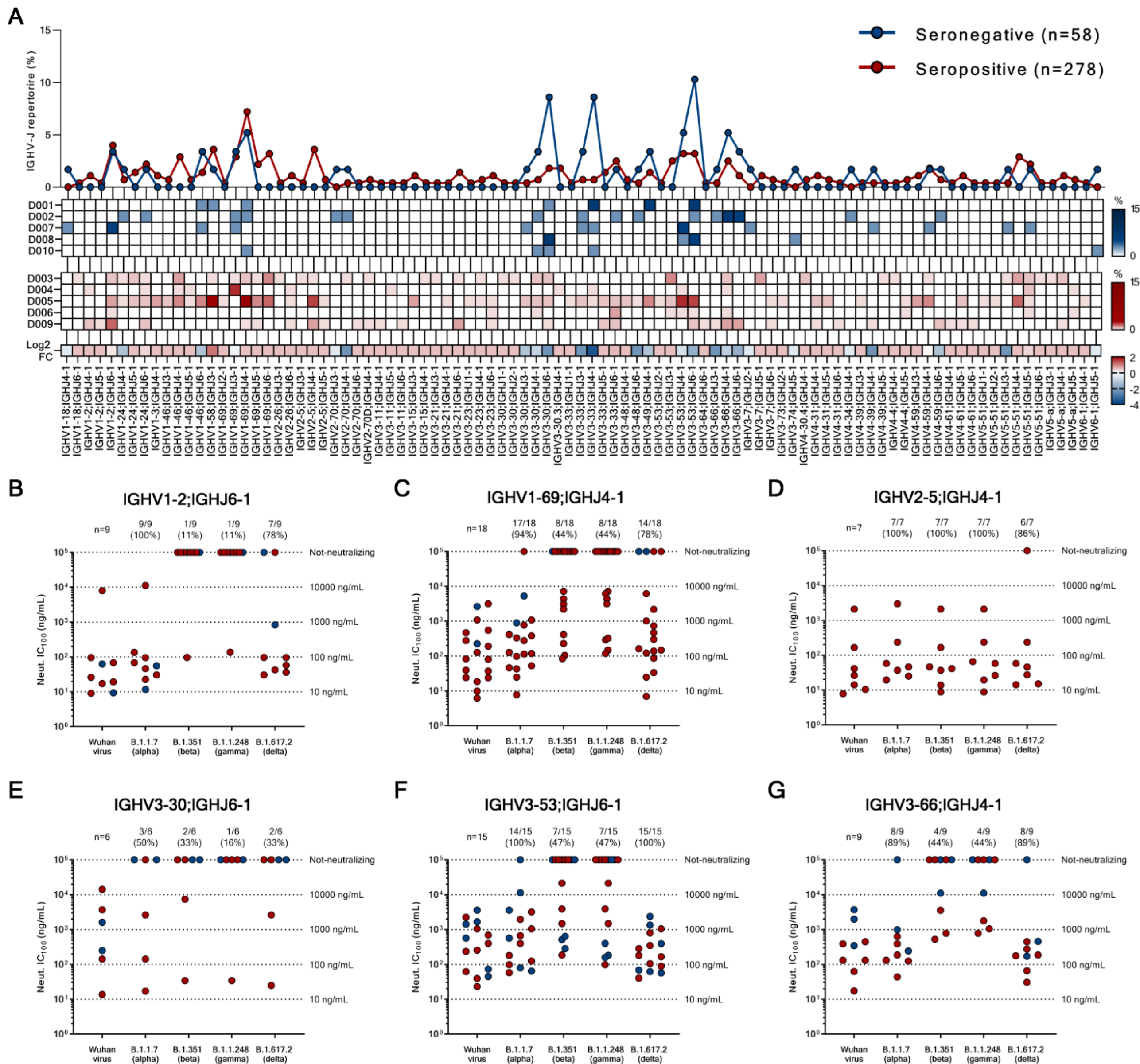

Figure 3. Repertoire analyses and functional characterization of predominant gene derived nAbs. (A) The graph shows the IGHV-J rearrangement frequencies between seronegative and seropositive vaccinees (top panel), the frequency within subjects (middle panels) and the Log2 fold change (FC) between groups (bottom panel). (B - G) Graphs show the neutralization potency (IC_{100}) of predominant gene derived nAbs from the IGHV1-2;IGHJ6-1 (B), IGHV1-69;IGHJ4-1 (C), IGHV2-5;IGHJ4-1 (D), IGHV3-30;IGHJ6-1 (E), IGHV3-53;IGHJ6-1 (F) and IGHV3-66;IGHJ4-1 (G) 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.

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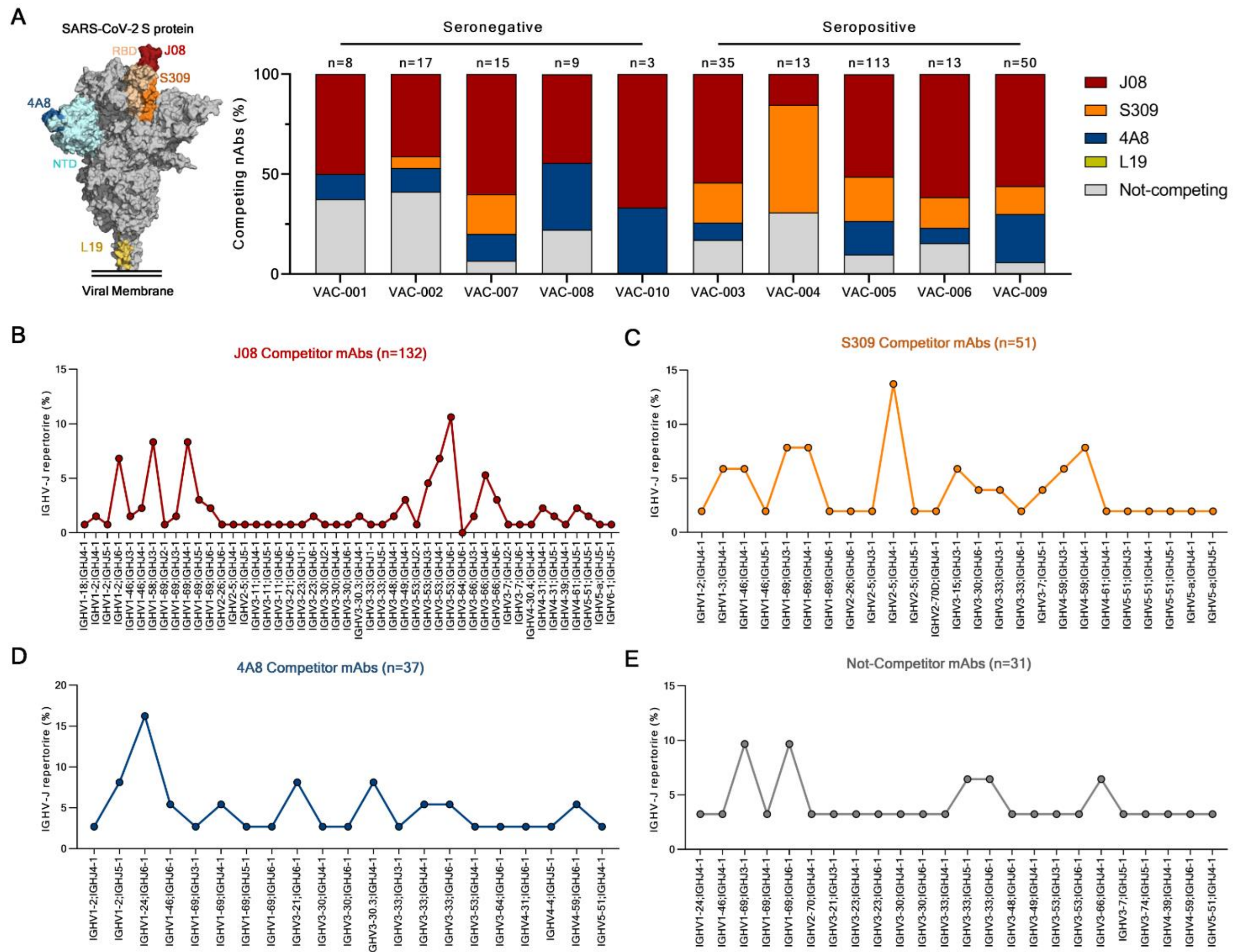

427

428 **Figure 4. Epitope binning and genetic characterization of competing nAbs.** (A) The bar graph shows
429 the percentage (%) of nAbs competing with J08 (dark red), S309 (orange), 4A8 (dark blue) and L19
430 (gold), or antibodies that did not compete with any of the previous mAbs (gray). A schematic
431 representation of J08, S309, 4A8 and L19 epitopes on the S protein surface is shown on the left side
432 of the panel. (B - D) Graphs show the IGHV-J rearrangement percentage for nAbs that competed
433 against J08 (B), S309 (C), 4A8 (D), or that did not compete with any of these mAbs (E). The total
434 number (n) of competing nAbs per group is shown on top of each graph.

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435 ## SUPPLEMENTARY TABLES

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	Second Dose	Blood Collection
VAC-001	M	38	16/12/2020	Not-applicable	Swab	Not-applicable	Seronegative	27/12/2020	18/01/2021	02/03/2021
VAC-002	F	38	31/12/2020	Not-applicable	Swab	Not-applicable	Seronegative	01/01/2021	22/01/2021	02/03/2021
VAC-007	M	38	29/12/2020	Not-applicable	Swab	Not-applicable	Seronegative	04/01/2021	25/01/2021	31/03/2021
VAC-008	M	43	28/12/2020	Not-applicable	Swab	Not-applicable	Seronegative	03/01/2021	24/01/2021	31/03/2021
VAC-010	F	51	15/02/2021	Not-applicable	Swab	Not-applicable	Seronegative	18/02/2021	11/03/2021	07/04/2021
VAC-003	M	38	15/10/2020	26/10/2020	Swab	Asymptomatic	Seropositive	08/01/2021	15/02/2021	09/03/2021
VAC-004	F	25	Not-applicable	22/10/2020	Swab	Mild	Seropositive	08/02/2021	01/03/2021	09/03/2021
VAC-005	M	25	01/08/2020	02/11/2020	Serological	Mild	Seropositive	11/01/2021	16/02/2021	16/03/2021
VAC-006	F	57	27/04/2020	24/10/2020	Swab	Asymptomatic	Seropositive	16/01/2021	11/02/2021	16/03/2021
VAC-009	M	46	29/09/2020	06/11/2020	Serological	Moderate	Seropositive	20/03/2021	Not-applicable	07/04/2021

436

437 **Table S1. Clinical details of COVID-19 vaccinees.** This table summarizes all the clinically relevant
438 information for the 5 seropositive and 5 seronegative vaccinated donors enrolled in this study.

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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

439

440 **Table S2. Summary of COVID-19 vaccinees.** This table summarizes the number and percentages
441 of single cell sorted S protein-specific MBCs, number and percentages of S protein binding mAbs,
442 and neutralizing mAbs against the original Wuhan SARS-CoV-2 virus and VoCs. This table includes
443 only data from the initial screening where nAbs were tested at a single point dilution against the
444 original Wuhan virus and the B.1.1.7, B.1.351 and B.1.1.248 VoCs. At this stage the B.1.617.2 (delta)
445 variant was not available.

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446

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

447 **Table S3. Competition assay.** This table summarizes the number and percentages of competing
448 nAbs from seronegative and seropositive vaccinees against J08, S309, 4A8 and L19. Not-competing
449 nAbs are also reported in this table.

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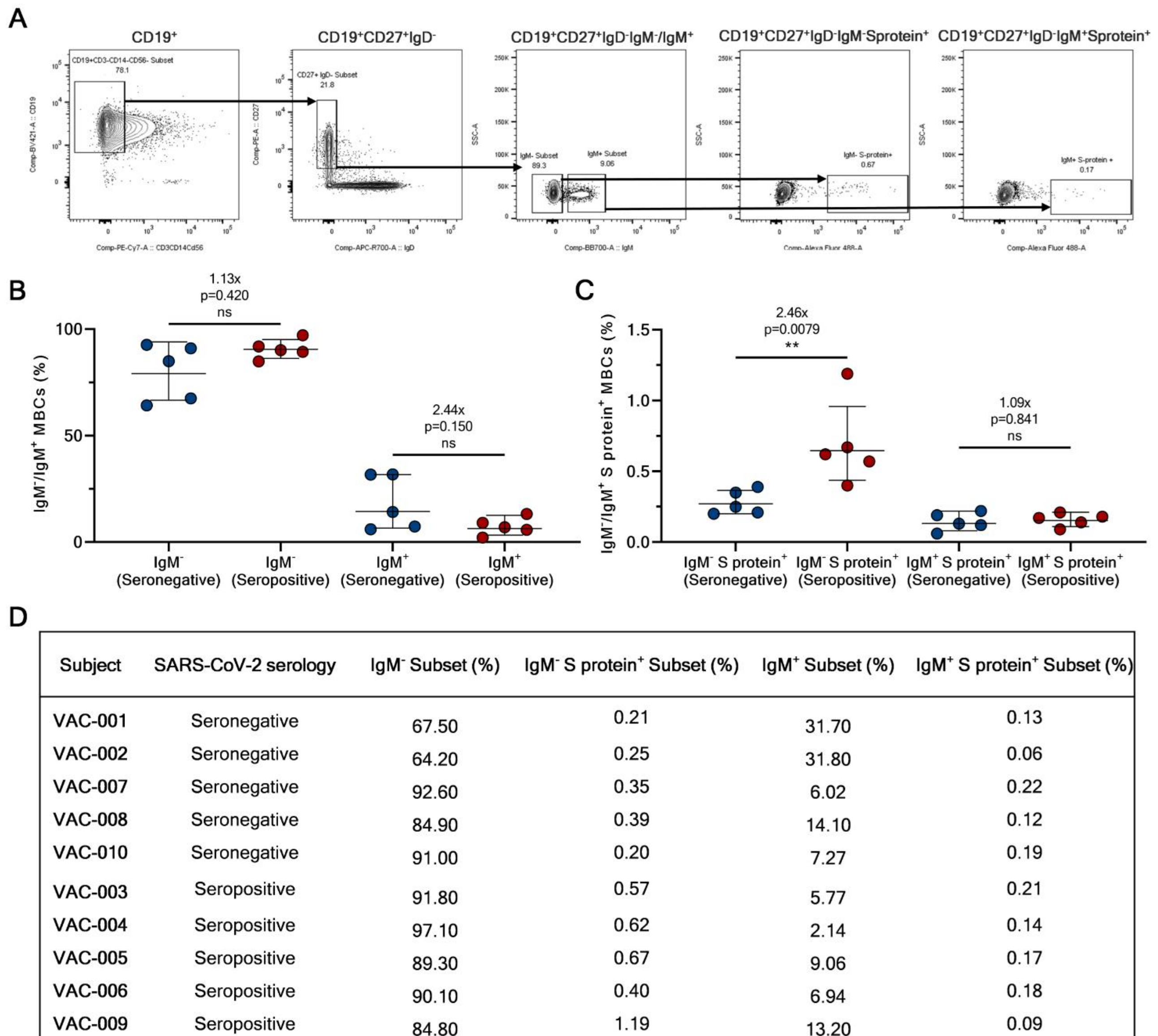
450 ## SUPPLEMENTARY FIGURES



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

451

452 **Figure S1. Single cell sorting and memory B cell frequencies.** (A) The gating strategy shows from
453 left to right: $CD19^+$ B cells; $CD19^+CD27^+IgD^-$; $CD19^+CD27^+IgD^-IgM^-/IgM^+$; $CD19^+CD27^+IgD^-IgM^-$
454 $Sprotein^+$; $CD19^+CD27^+IgD^-IgM^+Sprotein^+$. (B) The graph shows the frequency of $CD19^+CD27^+IgD^-$
455 IgM^- and IgM^+. (C) The graph shows the frequency of $CD19^+CD27^+IgD^-IgM^-$ and IgM^+ able to bind
456 the SARS-CoV-2 S protein trimer (S $protein^+$). (D) The table summarizes the frequencies of the cell
457 population above described for all subjects enrolled in our study.

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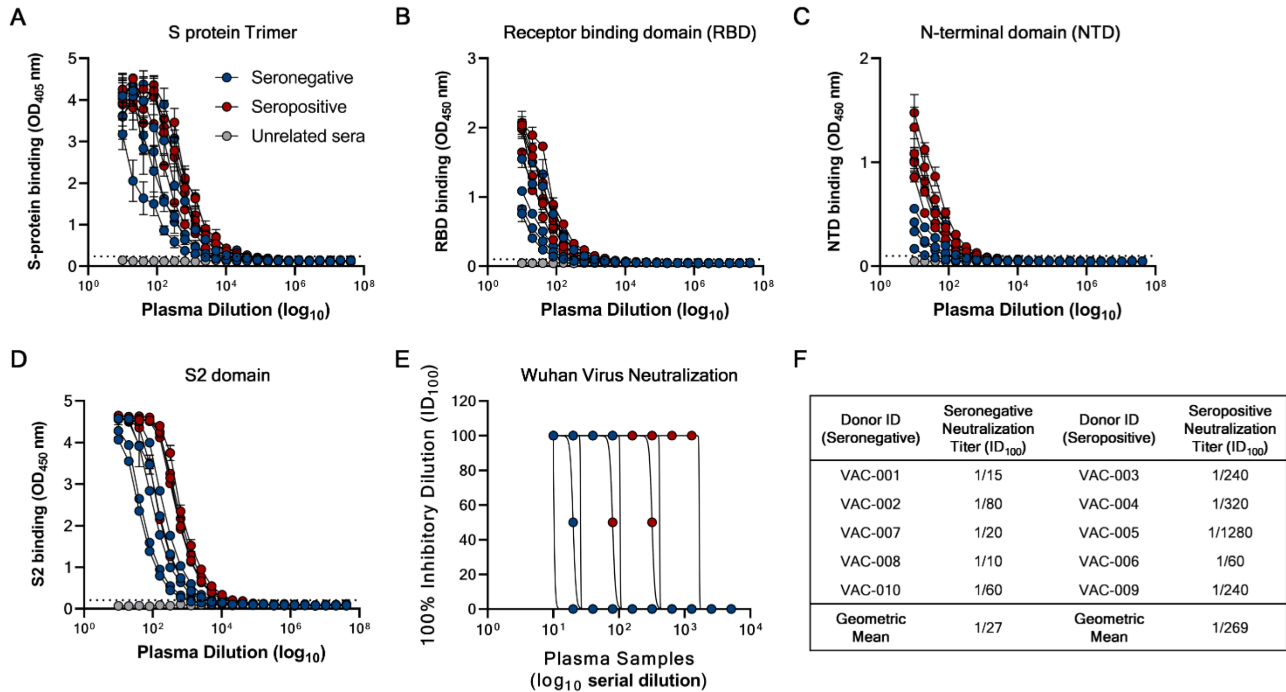

Donor ID (Seronegative)	Seronegative Neutralization Titer (ID_{100})	Donor ID (Seropositive)	Seropositive Neutralization Titer (ID_{100})
VAC-001	1/15	VAC-003	1/240
VAC-002	1/80	VAC-004	1/320
VAC-007	1/20	VAC-005	1/1280
VAC-008	1/10	VAC-006	1/60
VAC-010	1/60	VAC-009	1/240
Geometric Mean	1/27	Geometric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

Figure S2. 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 (A), RBD (B), NTD (C) and S2 domain (D). (E) The graph shows the neutralizing activity of plasma samples against the original Wuhan SARS-CoV-2 virus. (F) The table summarizes the 100% inhibitory dilution (ID_{100}) of each COVID-19 vaccinee and the geometric mean for seronegative and seropositive donors.

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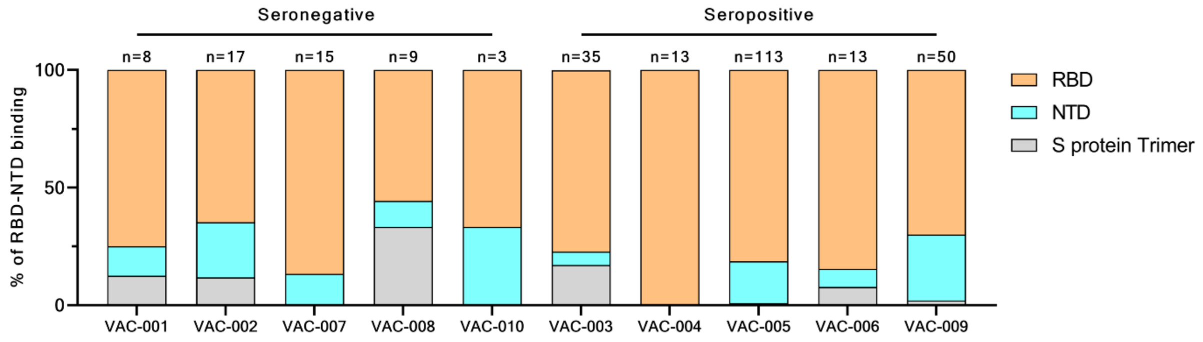

464

465 **Figure S3. RBD and NTD binding distribution of nAbs.** The graph shows the percentage of antibodies
466 that bind specifically the RBD (light orange) or the NTD (cyan) or that did not bind single domains
467 but recognized exclusively the S protein in its trimetric conformation (gray).

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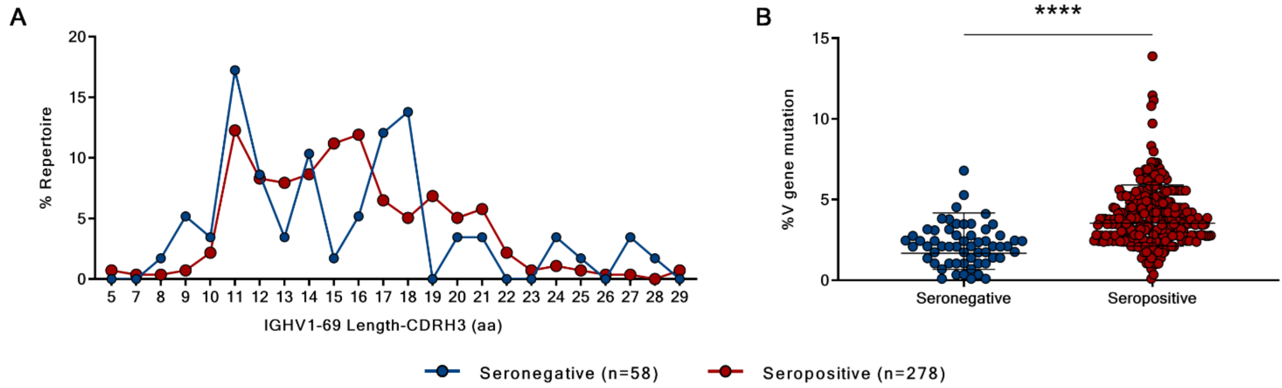

468

469 **Figure S4. Heavy chain CDR3 length and somatic hypermutation levels in seronegative and**
470 **seropositive vaccinees.** (A) The graph shows the heavy chain CDR3 length represented in amino
471 acids (aa). (B) The graph shows the overall somatic hypermutation level of nAbs isolated from
472 seronegative and seropositive vaccinees.

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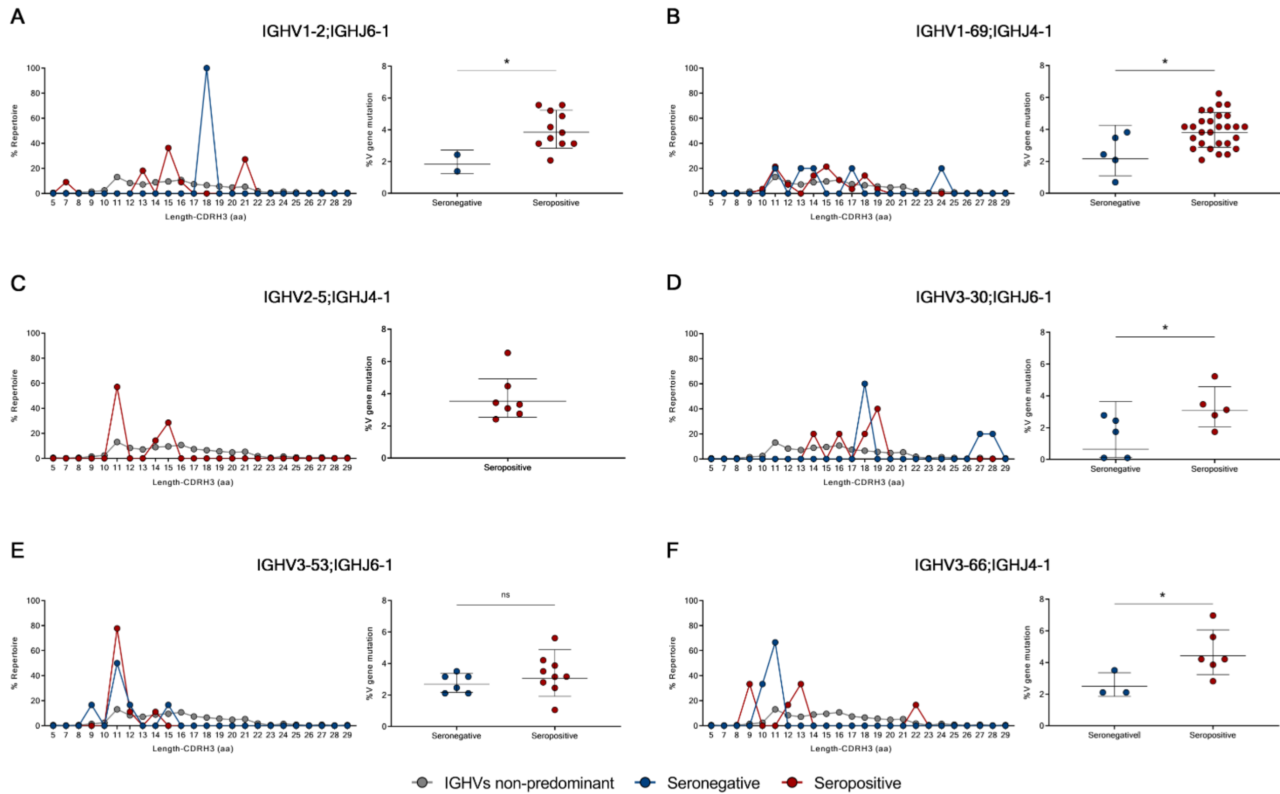

473

474 **Figure S5. Heavy chain CDR3 length and somatic hypermutation levels of predominant gene derived**
475 **nAbs.** (A - F) Graphs show the amino acidic heavy chain CDR3 length (left panel) and the somatic
476 hypermutation level (right panel) of nAbs derived from the IGHV1-2;IGHJ6-1 (A), IGHV1-69;IGHJ4-
477 1 (B), IGHV2-5;IGHJ4-1 (C), IGHV3-30;IGHJ6-1 (D), IGHV3-53;IGHJ6-1 (E) and IGHV3-66;IGHJ4-
478 1 (F) gene families.

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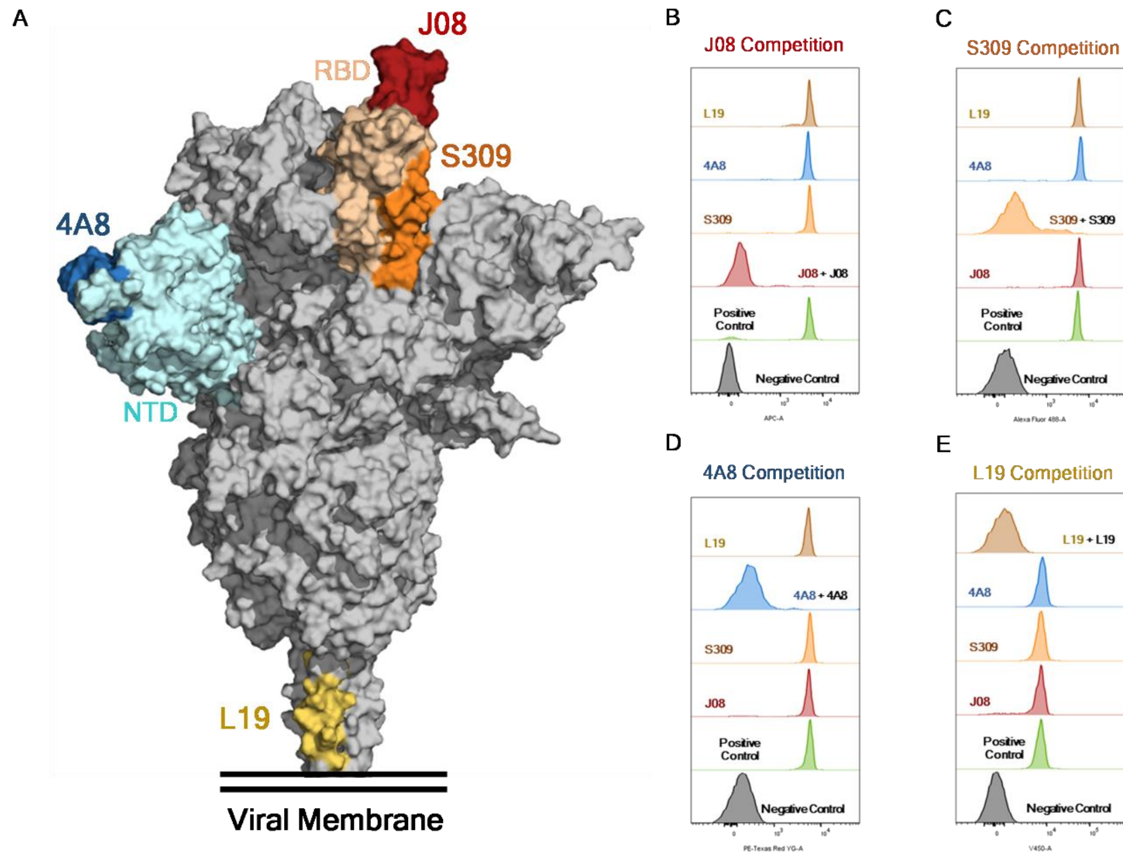

479

480 **Figure S6. Heavy chain CDR3 length and somatic hypermutation levels of predominant gene derived**
481 **nAbs.** (A) Schematic representation of the epitopes recognized by J08 (dark red), S309 (orange),
482 4A8 (dark blue) and L19 (gold), mAbs on the S protein surface. (B - E) Representative cytometer
483 peaks per each of the four mAbs used for the competition assay. Positive (beads conjugated with
484 only primary labeled antibody) and negative (un-conjugated beads) controls are shown as green and
485 gray peaks, respectively.

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