Characterization of neutralizing versus binding antibodies and memory B cells in COVID-19 recovered individuals from India

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Humoral and B cell memory in COVID-19 recovered individuals from India

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Abstract

India is one of the countries most affected by the recent COVID-19 pandemic. Characterization of humoral responses to SARS-CoV-2 infection, including immunoglobulin isotype usage, neutralizing activity and memory B cell generation, is necessary to provide critical insights on the formation of immune memory in Indian subjects. In this study, we evaluated SARS-CoV-2 receptor-binding domain (RBD)-specific IgG, IgM, and IgA antibody responses, neutralization of live virus, and RBD-specific memory B cell responses in pre-pandemic healthy versus convalescent COVID-19 individuals from India. We observed substantial heterogeneity in the formation of humoral and B cell memory post COVID-19 recovery. While a vast majority (38/42, 90.47%) of COVID-19 recovered individuals developed SARS-CoV-2 RBD-specific IgG responses, only half of them had appreciable neutralizing antibody titers. RBD-specific IgG titers correlated with these neutralizing antibody titers as well as with RBD-specific memory B cell frequencies. In contrast, IgG titers measured against SARS-CoV-2 whole virus preparation, which includes responses to additional viral proteins besides RBD, did not show robust correlation. Our results suggest that assessing RBD-specific IgG titers can serve as a surrogate assay to determine the neutralizing antibody response. These observations have timely implications for identifying potential plasma therapy donors based on RBD-specific IgG in resource-limited settings where routine performance of neutralization assays remains a challenge.

Importance

Our study provides an understanding of SARS-CoV-2-specific neutralizing antibodies, binding antibodies and memory B cells in COVID-19 convalescent subjects from India. Our study highlights that PCR-confirmed convalescent COVID-19 individuals develop SARS-CoV-2 RBD-specific IgG antibodies, which correlate strongly with their neutralizing antibody titers. RBD-specific IgG titers, thus, can serve as a valuable surrogate measurement for neutralizing antibody responses. These finding have timely significance for selection of appropriate individuals as donors for plasma intervention strategies, as well as determining vaccine efficacy.

Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the virus responsible for the coronavirus disease 2019 (COVID-19) pandemic, emerged as a grave public health threat beginning in December 2019(1), paralyzing daily lives and causing economic downturns in many parts of the world. Currently, India is one of the countries most affected with more than 3 million COVID-19 confirmed cases and 60,000 associated deaths (2).

Intense efforts are underway to develop vaccines and antiviral therapeutics (3-11). These efforts require a detailed understanding of immune correlates of protection, formation of immune memory, and durability of these responses. Additionally, infusion of plasma derived from COVID-19 recovered individuals is also being explored as a treatment strategy (12-20). All these efforts require a detailed understanding of humoral immunity, immunoglobulin isotype usage and neutralizing activity following recovery from SARS-CoV-2 infection. Moreover, given that many of the SARS-CoV-2 neutralizing epitopes are located in the viral receptor binding domain (RBD) of the Spike (S) protein (21-29), it is important to evaluate the relationship between RBD-specific IgG titers and neutralizing antibody responses.

In this study, we evaluated IgG, IgA, IgM, neutralizing antibodies and memory B cell responses in PCR-confirmed COVID-19 convalescent subjects. Our results show that while a vast majority (38/42, 90.47%) of COVID-19 recovered individuals developed SARS-CoV-2 RBD-specific IgG responses, we were able to detect appreciable levels of neutralizing antibody responses in only half of the convalescent subjects. Neutralizing responses

104 correlated closely with RBD-specific IgG titers, but weakly with IgG titers measured against
105 crude virus concentrate using a commercial ELISA kit. Taken together, these findings suggest
106 that despite significant inter-individual variation in the RBD-specific IgG titers and
107 neutralizing antibodies, RBD-specific IgG titers can serve as a valuable and robust surrogate
108 measurement for neutralizing antibody responses. These observations not only provide a
109 glimpse of humoral immune responses in COVID-19 recovered individuals from India, but
110 also have timely implications for identifying potential plasma therapy donors using on RBD-
111 specific IgG ELISA's in India where routine performance of neutralization assays remains a
112 challenge.

Methods

Subject recruitment

COVID-19 recovered individuals were recruited at Shaheed Hasan Khan Mewati Government Medical College, Nuh, Haryana, India, Super Specialty Pediatric Hospital and Post Graduate Teaching Institute, Noida and ICMR-National Institute of Malaria Research, New Delhi. The Institutional ethical boards approved the study. Informed consent was obtained prior to inclusion in the study. All subjects (mean age 39.4 years, range 15 – 70 years) were SARS-CoV-2 PCR positive at the time of initial diagnosis, and were PCR negative when recruited for this study at 3.6 – 12 weeks post initial diagnosis **(Table 1).** Samples collected from healthy adult blood bank donors in the year 2018 are included as pre-pandemic controls.

SARS-CoV-2 *specific PCR*

SARS-CoV-2 specific rRT-PCR was performed as per the Indian government guidelines for COVID-19 diagnosis. Nasopharyngeal and throat swabs were collected in viral transport medium (VTM) (HiMedia, #AL 167)) and transported to the testing laboratory maintaining cold chain. All the samples were subjected to the first line screening assay or the 'e' gene assay as per the guidelines (30). Samples reactive by the first line assay were subjected to the RdRp gene assay (Invitrogen SuperScript™ III Platinum® One-Step Quantitative Kit (Cat. No.11732088). Samples reactive for both the genes were labeled positive, while samples reactive to 'e' gene only were considered indeterminate and were subjected to repeat sampling. The same protocol was used to verify that the subjects were PCR negative at the time of recruitment for this study.

SARS-CoV-2 *RBD-specific direct ELISA*

Recombinant SARS-CoV-2 RDB gene was cloned, expressed, purified and standard direct ELISAs were performed as previously described (31). Briefly, purified RBD was coated on MaxiSorp plates (Thermo Fisher, #439454) at a concentration of 1 ug/mL in 100 uL phosphate-buffered saline (PBS) at 4°C overnight. The plates were washed extensively with PBS containing 0.05% Tween-20. Three-fold serially diluted plasma samples were added to the plates and incubated at room temperature for 1hr. After incubation, the plates were washed and the SARS-CoV-2 RBD specific IgG, IgM, IgA signals were detected by incubating with horseradish peroxidase (HRP) conjugated - anti-human IgG (Jackson ImmunoResearch Labs, #109-036-098), IgM (Jackson ImmunoResearch Labs, #109-036-129), or IgA (Jackson ImmunoResearch Labs, #109-036-011). Plates were then washed thoroughly and developed with o-phenylenediamine (OPD) substrate (Sigma, #P8787) in 0.05M phosphate-citrate buffer (Sigma, #P4809) pH 5.0, containing with 0.012% hydrogen peroxide (Fisher Scientific, #18755) just before use. Absorbance was measured at 490 nm.

Enumeration of SARS-CoV-2 *RBD-specific memory B cells*

Purified RBD protein (100 ug) was labeled with Alexa Fluor 488 using microscale protein labeling kit (Life Technologies, #A30006) as per manufacturer's protocol. PBMC's were stained with RBD-Alexa Fluor 488 for 1 hour at 4°C, followed by washing with PBS containing 0.25% FBS, and incubation with efluor780 Fixable Viability (Live Dead) dye (Life Technologies, #65-0865-14) and anti-human CD3, CD19, CD27, CD38 and IgD antibodies (BD Biosciences) for 30 minutes. Cells were washed twice with FACS buffer and acquired on BD LSR Fortessa X20. Data was analyzed using FlowJo software 10. SARS-CoV-2 RBD-specific

157 memory B cells were identified in cells positive for CD19, CD20, CD27 that were negative for
158 IgD and CD3.
159
160 ***IgG ELISA for SARS-CoV-2 whole virus preparation***

161 SARS-CoV-2 antigen specific IgG was detected using a commercially available assay (COVID-
162 Kavach ELISA tests kit, Zydus diagnostics), which measures responses to antigen
163 concentrated from gamma-irradiated SARS-CoV-2-infected tissue culture fluid as per the
164 manufacturer's instructions (32, 33).

165 ***SARS-CoV-2 neutralization assay***

166 Neutralization titers to SARS-CoV-2 were determined as previously described (31). Briefly
167 infectious clone of the full-length mNeonGreen SARS-CoV-2 (2019-nCoV/USA_WA1/2020)
168 was used to test heat-inactivated COVID-19 convalescent samples and healthy donor
169 samples (pre-pandemic). Heat-inactivated serum was serially diluted three-fold in duplicate
170 starting at a 1:20 dilution in a 96-well round-bottom plate and incubated between 750 FFU
171 of ic-SARS-CoV-2-mNG for 1 h at 37°C. This antibody-virus mixture was transferred into the
172 wells of a 96-well plate that had been seeded with Vero-E6 cells the previous day at a
173 concentration of 2.5×10^4 cells/well. After 1 hour, the antibody-virus inoculum was removed
174 and 0.85% methylcellulose in 2% FBS containing DMEM was overlaid onto the cell
175 monolayer. Cells were incubated at 37°C for 24 hours. Cells were washed three times with
176 1XPBS (Corning Cellgro) and fixed with 125 µl of 2% paraformaldehyde in PBS (Electron
177 Microscopy Sciences) for 30 minutes. Following fixation, plates were washed twice with 1x
178 PBS and imaged on an ELISPOT reader (CTL Analyzer). Foci were counted using Viridot (34)

(counted first under the "green light" setting followed by background subtraction under the "red light" setting). FRNT-mNG$_{50}$ titers were calculated by non-linear regression analysis using the 4PL sigmoidal dose curve equation on Prism 8 (Graphpad Software). Neutralization titers were calculated as 100% x [1- (average foci in duplicate wells incubated with the specimen) ÷ (average number of foci in the duplicate wells incubated at the highest dilution of the respective specimen).

Statistical analysis

Statistical analysis was performed using GraphPad prism 8.0 software. Non-parametric t test (Mann-Whitney) was used to calculate the differences between groups. Non-parametric Spearman's correlation coefficient (r) was used to calculate correlation between groups. A p value of ≤ 0.05 was considered as significant.

Results

SARS-CoV-2 RBD-specific humoral immunity in COVID-19 recovered individuals.

The demographic profile of COVID-19 recovered individuals recruited for this study is shown in **Table 1.** All subjects were at least 3.6 weeks past their initial SARS-CoV-2 positive diagnosis. RBD-specific ELISA curves for IgG, IgA and IgM at different dilutions of plasma in pre-pandemic healthy versus COVID-19 recovered individuals are shown in **Figure 1.** RBD-specific responses were highly elevated in COVID-19 recovered individuals as compared to pre-pandemic healthy controls (**Figure 1A,B,C, left versus middle panels)**. Titers of IgG, IgA and IgM in the COVID-19 recovered individuals showed substantial inter-individual variation **(Figure 1 A, B, C, right panel)** - with IgG endpoint titers ranging from below detection to 24484 (2000±619); IgA titers from below detection to 5686 (386±136) and IgM titers from below detection to 2958 (515±90). Four individuals had undetectable RBD-specific IgG and IgA titers. One of these individuals was also below detection for IgM **(Table 2)**. Inter-individual heterogeneity was not related to the age of the individuals **(Figure 2A)** or the number of days that elapsed between PCR confirmation of infection and sample collection **(Figure 2B)**.

SARS-CoV-2 specific neutralizing titers in COVID-19 recovered individuals.

To assess plasma neutralizing titers from COVID-19 convalescent individuals, we performed a live virus neutralization assay using a focus-reduction neutralization mNeonGreen (FRNT-mNG) assay (31). The neutralizing activity at different dilutions of plasma for pre-pandemic

213 healthy individuals **(Figure 3A)** and COVID-19 recovered individuals is shown in (**Figure**
214 **3B). Figure 3C** shows FRNT-mNG$_{50}$ titers calculated based on the plasma dilution that
215 neutralized 50% of the virus. While all pre-pandemic healthy individuals had undetectable
216 FRNT-mNG$_{50}$ titers, only half of the COVID-19 recovered individuals showed 50% or more
217 neutralization even at a 1:20 dilution of plasma. Similar to RBD-specific IgG titers, the FRNT-
218 mNG$_{50}$ titers were heterogeneous with the latter reaching titers as high as 682 **(Figure 3C)**.
219
220 Previous studies in other viral infections have shown that all three antibody isotypes (IgG,
221 IgA and IgM) can potentially neutralize (35-39)**.** We next determined if any correlation exists
222 between SARS-CoV-2 neutralizing titers and RBD-specific IgG, IgA, IgM binding antibody
223 titers. We observed a positive correlation ($r=0.83$; $p<0.001$) between SARS-CoV-2
224 neutralizing titers and RBD-specific IgG titers **(Figure 4, left graph)** but not with IgA
225 **(Figure 4, middle graph)** or IgM titers **(Figure 4, right graph)**.
226
227 Plasma infusion therapy has recently been started in India as an intervention therapy for
228 COVID-19. For this, plasma donors are being typically identified by the presence of IgG to
229 SARS-CoV-2 by commercial ELISA tests (40). One of these tests detects IgG towards viral
230 antigens concentrated from gamma-irradiated SARS-CoV-2-infected tissue culture fluid (32,
231 33)**.** It was therefore of interest to examine the correlation between neutralization titers and
232 IgG responses measured using this test. We observed that, of the 42 COVID-19 recovered
233 individuals tested, 33 were IgG positive whereas 9 were below the assay cut off **(Figure 5A)**.
234 Of the 9 individuals that were below cut off, 4 also tested negative by the RBD-specific IgG
235 ELISA **(Table 2)**. All of the samples from the pre-pandemic healthy individuals were below

the limit of detection using both the ELISA methods. Most importantly, the IgG values obtained by whole virus-based ELISA did not show as robust a correlation (r=0.56) with neutralizing antibody titers **(Figure 5B)** as compared to those observed with RBD-specific IgG titers (r=0.83) **(Figure 4, left graph)**.

Characterization of RBD-specific memory B cells in COVID-19 recovered individuals.

While circulating neutralizing antibodies help prevent re-infection by viruses, memory B cells allow for rapid production of new antibodies in case of re-infection. To address whether the COVID-19 recovered individuals generated memory B cells, we enumerated RBD-specific memory B cells using fluorescently-conjugated RBD antigen. An example of the flow cytometric gating strategy and RBD staining among the gated memory B cells is shown in **Figure 6A and 6B**. **Figure 6C** shows the frequency of RBD-specific memory B cells in a subset of the individuals where sufficient PBMCs were available. Though we found that there was substantial inter-individual variation in the frequency of SARS-CoV-2 RBD-specific memory B cells, their frequencies modestly correlated with RBD-specific IgG titers.

Discussion

Our study provides a detailed understanding of humoral immunity and memory B cells in COVID-19 recovered individuals from India. We examined SARS-CoV-2 neutralizing antibodies, IgG, IgM, IgA and memory B cells in pre-pandemic healthy versus COVID-19 recovered individuals and further evaluated inter-individual variation and relation among these.

Our correlative analysis of RBD-specific IgG binding titers with neutralizing antibody titers and memory B cells has important implications for not only identifying potential donors for plasma therapy but also for understanding humoral and cellular memory post COVID-19. Though current plasma therapy guidelines in India do not consider neutralizing antibody titers, United States Food and Drug Administration (FDA) guidelines recommend, when available, a neutralizing titer of 1:160 or 1:80 to be used for identifying potential plasma donors (41). Our correlation analysis shows that RBD-specific titers of more than 3668 can provide a suitable surrogate for identifying the individuals with neutralizing titers of above 1:160 and RBD-specific IgG titers 1926 for neutralizing titers of 1:80. Though larger scale studies are needed to establish robustness, these observations have timely implications to identify potential plasma therapy donors.

Our study raises important questions on formation of protective immune memory after recovering from COVID-19. We found that nearly half of the COVID-19 recovered individuals did not induce 50% neutralizing titers even at 1:20 dilution of plasma. This raises the

question of whether these individuals with low neutralizing antibodies also differ in formation of cellular immune memory. Our data show that individuals with low neutralizing antibodies indeed had lower memory B cells. Given that T cells may also contribute to COVID-19 protection, studies are needed to understand whether these individuals may also differ in the generation of memory CD8 and CD4 T cells (42-44).

The reason why only half of the COVID-19 recovered individuals developed appreciable levels of neutralizing antibody titers requires further investigation. This may be related to inter-individual differences in human immune responses associated with the expected heterogeneity in initial viral inoculum(45), initial viral loads (46-48), incubation period (49), host genetic factors (50-52) and disease severity (53, 54). This is consistent with previous studies that show relatively higher neutralizing antibodies in COVID-19 hospitalized patients during the acute febrile phase, or in recovered individuals that were previously hospitalized with severe COVID-19 disease (53, 54). It is noteworthy that the COVID-19 recovered individuals from our study had mild to moderate symptoms during the initial diagnosis. In light of these studies, our findings warrant future studies to seek an understanding of whether the individuals that have generated low or no neutralizing antibodies, IgG titers or memory B cells past recovery will be protected if they were re-exposed to SARS-CoV-2 or a related virus.

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Author Contributions

Experimental work, data acquisition and analysis of data by K.N, K.G, S.K, E.S.R, V.V.E., K.F, R.K, S.L. C.D, J.W, M.S.S, and D.S. Clinical site coordination by D.S, P.K.G, S.A, A.S and M.R. Conceptualization and implementation by A.S, R.A, K.M, A.C. Manuscript writing by A.C and K.M. All authors contributed reviewing and editing the manuscript.

Figure legends

Figure 1: Evaluation of SARS-CoV-2 RBD specific IgG, IgA and IgM antibody responses.

(A) RBD-specific IgG, **(B),** RBD-specific IgA; **(C),** RBD-specific IgM. Left, pre-pandemic healthy (n-22), middle COVID-19 recovered (n=42); right, endpoint titers. ELISA cutoff values are calculated using the average plus 3 standard deviations of the 22 healthy controls at 1:100 dilution (shown as a dotted line). The unpaired analysis was done using non-

parametric Mann-Whitney-U test. $p \leq 0.05$ was considered significant. Assay cutoff value is marked with dotted line.

Figure 2. Correlation of age and day post initial diagnosis of COVID-19 recovered individuals with SARS-CoV-2 IgG, IgM and IgA titers.

(A). Age versus IgG (left, n=42), IgA (middle, n=42) or IgM (right, n=42) titers. (**B).** Time post initial diagnosis versus IgG (left, n=42), IgA (middle, n=42) or IgM (right, n=42) titers. Correlations were calculated by Spearman's correlation coefficient r. $p \leq 0.05$ is considered significant. Note that none of the data sets above reached significant values of correlation.

Figure 3. Evaluation of SARS-CoV-2 neutralizing antibodies in COVID-19 recovered individuals.

SARS-CoV-2 neutralizing activity at indicated dilutions of plasma is shown in pre-pandemic healthy (n=22, in grey) **(A)** and in COVID-19 recovered individuals (n=42, in blue) **(B)**. Dotted line represents the plasma dilution that leads to 50% neutralization. **(C)** Scatter plot shows neutralization titers (FRNT-mNG$_{50}$) in pre-pandemic healthy (n=22) and COVID-19 recovered (n=42) individuals. The unpaired analysis was done using non-parametric Mann-Whitney-U test. $p \leq 0.05$ was considered significant. Limit of detection is marked with a dotted line.

335 ***Figure 4. Correlation analysis of SARS-CoV-2-specific antibody responses versus***
336 ***neutralization titers.***

337 Correlation analysis shows FRNT-mNG$_{50}$ titers (x-axis) versus RBD-specific IgG (Left), IgA
338 (middle) and IgM (right) titers on y-axis in COVID-19 recovered individuals (n=42, blue
339 dots). Correlation analysis was performed by log transformation of the endpoint ELISA titers
340 followed by linear regression analysis. Correlations were calculated by Spearman's
341 correlation coefficient r. $p \leq 0.05$ was considered significant. Dotted line on x-axis and y-axis
342 indicate limit of detection.

343

344 ***Figure 5. Correlation analysis of SARS-CoV-2 whole virus specific IgG versus neutralizing***
345 ***titers.***

346 **(A).** Scatter plots shows SARS-CoV-2 whole virus specific IgG measured using measured
347 using commercial kit (Zydus diagnosis, Covid Kavach) in pre-pandemic healthy (n=5) and
348 COVID-19 recovered (n=42). The unpaired analysis was done using non-parametric Mann-
349 Whitney-U test. $p \leq 0.05$ was considered significant. **(B).** Correlation analysis of SARS-CoV-2
350 whole virus antigen specific IgG ELISA kit values (y-axis) versus neutralizing titers (x-axis)
351 in COVID-19 recovered individuals (n=42). Correlations were calculated by Spearman's
352 correlation coefficient r. $p \leq 0.05$ was considered significant. Dotted line on x-axis indicate
353 limit of detection and on y-axis assay cut off.

354

Figure 6. SARS-CoV-2 RBD-specific memory B cell analysis in COVID-19 recovered individuals.

(A) Gating strategy used to identify memory B cells. **(B)** SARS-CoV-2 RBD-specific memory B cells on gated total memory B cells that were CD19 positive, CD20 high, IgD negative and CD27 high is shown. **(C)** Frequency of RBD-specific memory B cells of the total memory B cells in the COVID-19 recovered individuals (n= 13). **(D)** Correlation analysis shows frequency of RBD-specific memory B cells (x-axis) and the RBD-specific IgG titers (y-axis) in COVID-19 recovered individuals.

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Table 1. COVID-19 recovered individuals characteristics (n=42)*

Age in years Mean (Range)	39.4 (15-70)
Males/Females	38/4
Days post PCR diagnosis Mean (Range)	47.3 (25-84)

*COVID-19 recovered individuals were recruited at Shaheed Hasan Khan Mewati Government Medical College, Nuh, Haryana, India. Super Speciality Paediatric Hospital and Post Graduate Teaching Institute, Noida and ICMR-National Institute of Malaria Research, New Delhi. All subjects were SARS-CoV-2 PCR positive at the time of initial diagnosis and were PCR negative when recruited for this study at 4.8 – 11 weeks post initial diagnosis.

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Table 2. Individual characteristics of the COVID-19 recovered subjects

Subject number	Age	Gender (Male,M Female,F)	Days Post PCR Diagnosis	SARS CoV-2 RBD specific Immunoglobulin titers*			SARS Cov-2 whole Virus specific IgG ELISA values**	Neutralization titer ($FRNT\text{-}mNG_{50}$)***
				IgG	IgM	IgA		
1	23	M	84	2220	565	220	26	39
2	22	F	84	354	283	<100	3	26
3	68	M	40	464	<100	<100	19	<20
4	35	M	51	4547	393	545	6	113
5	50	M	37	1354	301	275	7	81
6	29	M	34	<100	866	<100	<1.5	<20
7	27	M	34	422	104	450	<1.5	<20
8	25	M	34	222	1031	<100	26	<20
9	21	M	40	650	588	153	9	25
10	39	M	38	612	539	5686	12	23
11	46	M	38	2011	325	224	24	55
12	31	M	38	494	828	183	10	<20
13	20	M	41	944	274	<100	14	49
14	36	M	41	228	279	1614	<1.5	<20
15	34	M	44	282	302	<100	4	<20
16	70	M	44	1250	220	518	14	43
17	40	M	45	464	112	101	16	<20
18	32	M	41	867	381	399	<1.5	<20
19	57	M	45	1069	354	231	<1.5	<20
20	27	F	49	1935	528	<100	23	80
21	36	M	49	3156	355	593	28	166
22	24	M	45	<100	387	<100	<1.5	<20
23	55	F	45	<100	778	<100	<1.5	<20
24	15	M	45	212	496	<100	<1.5	<20
25	49	M	45	4183	2958	397	17	657
26	26	M	48	2352	<100	<100	16	48
27	54	F	54	1202	<100	182	15	49
28	53	M	52	799	197	417	12	<20
29	52	M	48	2611	249	157	23	46
30	45	M	62	1490	401	<100	15	50
31	52	M	56	10127	421	437	21	434
32	26	M	47	<100	<100	<100	<1.5	<20
33	32	M	57	701	177	<100	14	<20
34	44	M	49	815	428	<100	20	<20
35	32	M	40	829	140	<100	6	29
36	44	M	42	4685	494	295	26	167
37	22	M	77	3954	764	690	24	209
38	49	M	25	24484	2828	459	22	682
39	55	M	51	371	753	<100	17	<20
40	36	M	51	621	350	104	17	<20
41	60	M	51	156	459	<100	17	34
42	62	M	47	467	354	<100	6	<20

*ELISA end point titre limit of detection is 100.

**ELISA was performed with a commercial kit (Covid Kavach, Zydus) using 1:100 dilution of plasma as per by the manufacturer's recommendation. Assay cut off is 1.5.

***Neutralization titres: Neutralization assay were performed using 3 fold dilution of plasma, starting at 1:20 up to 1:43740. Limit of detection for $FRNT\text{-}mNG_{50}$ is 20.

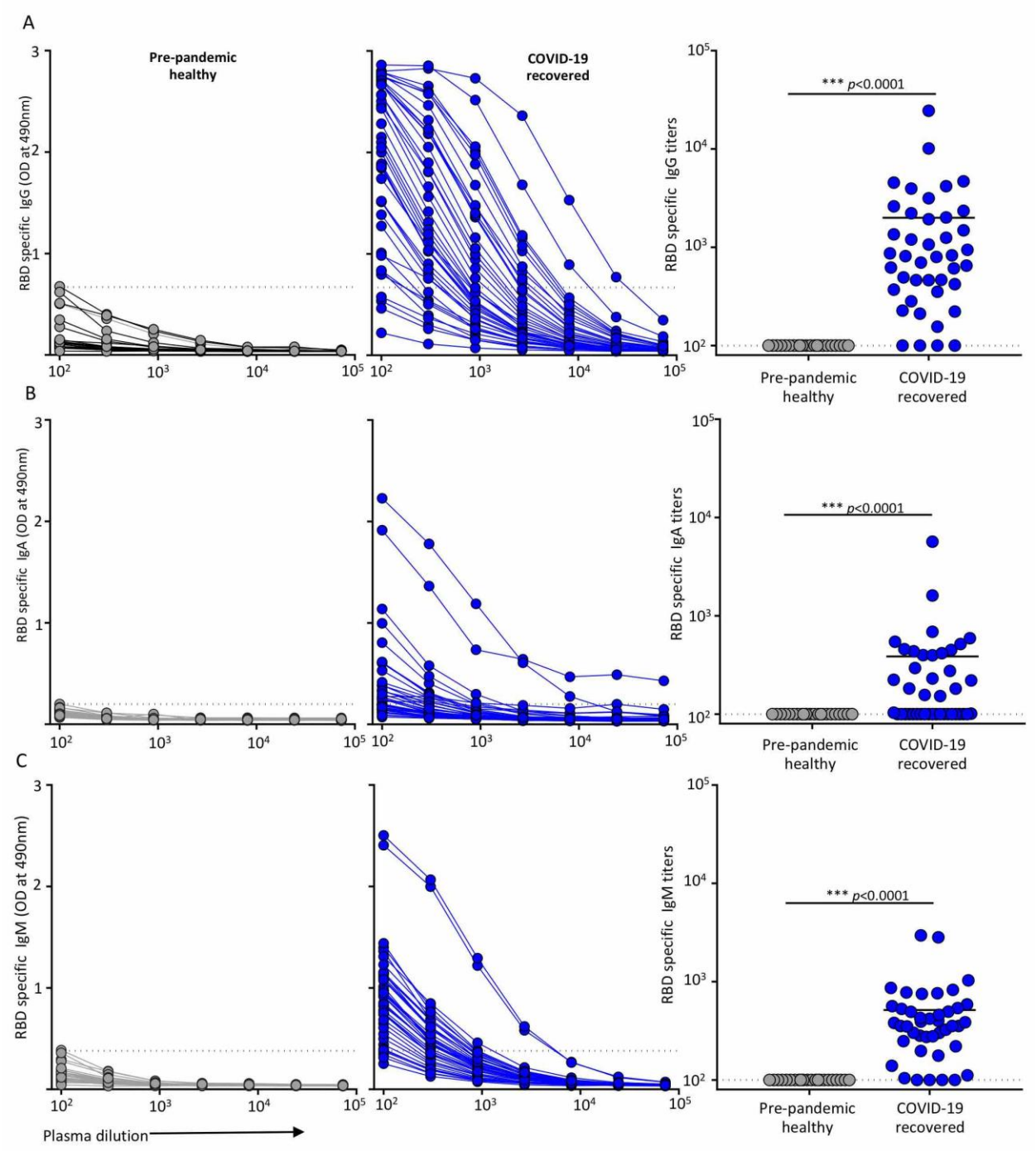

Figure 1

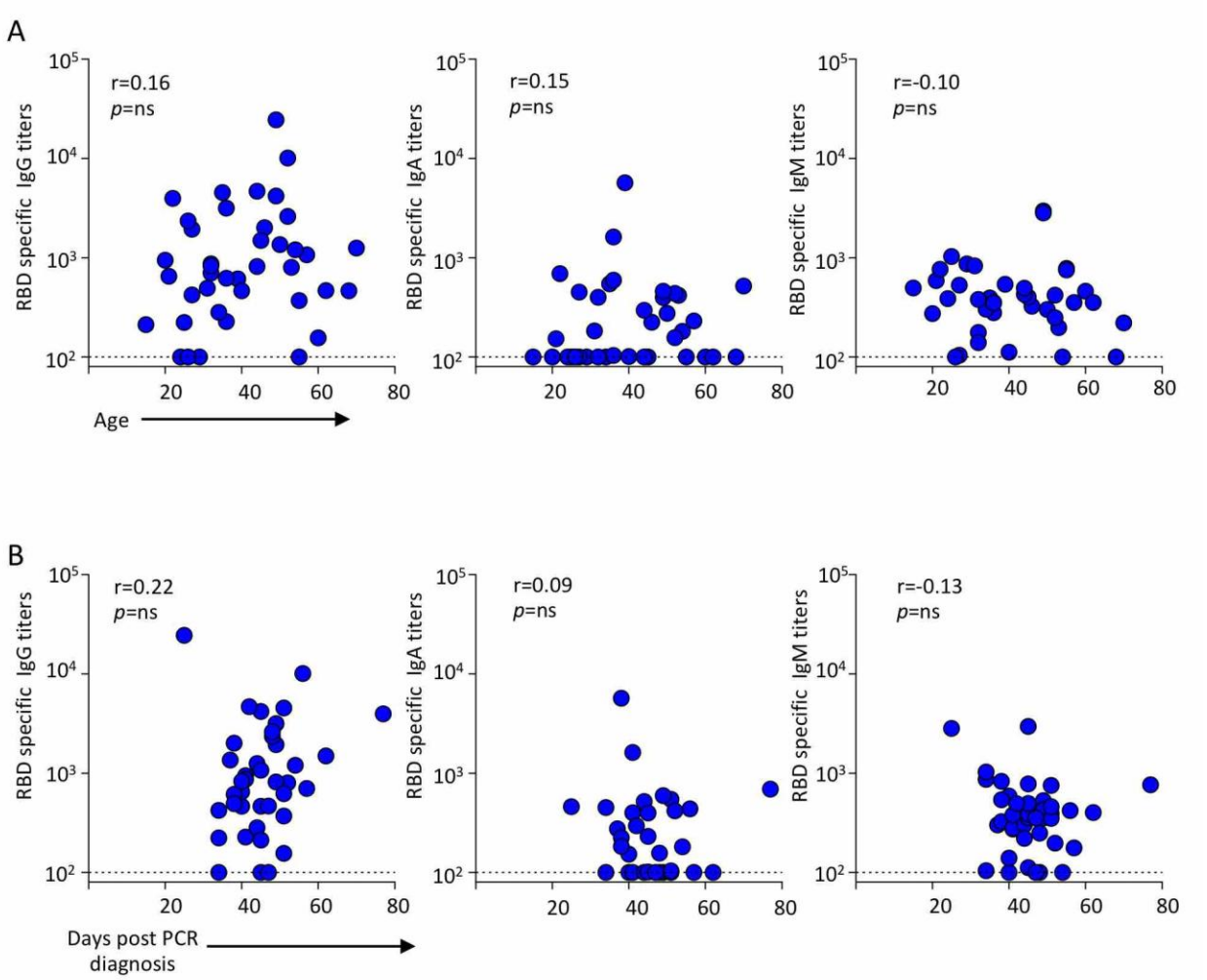

Figure 2

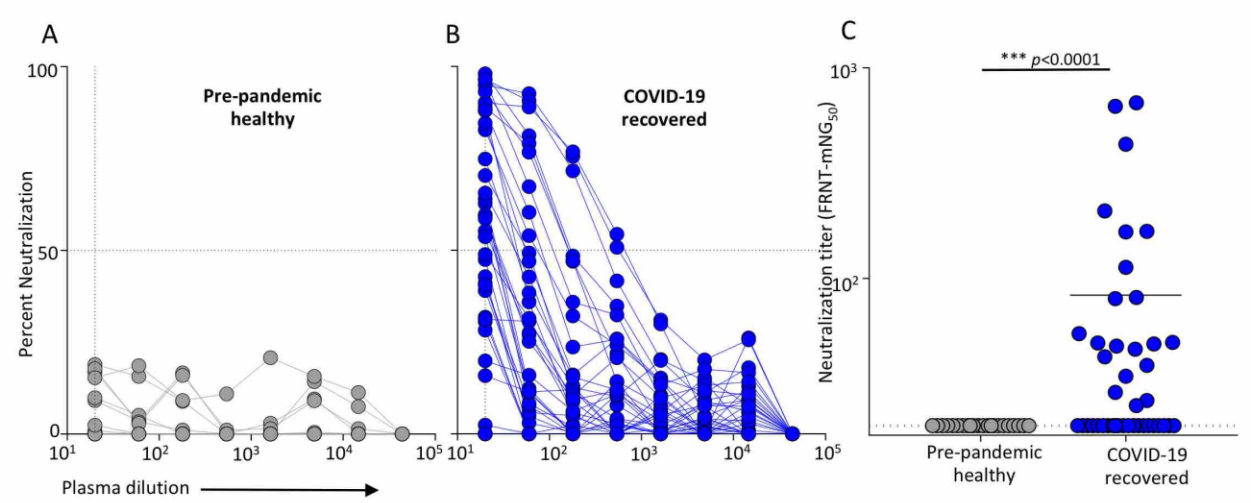

Figure 3

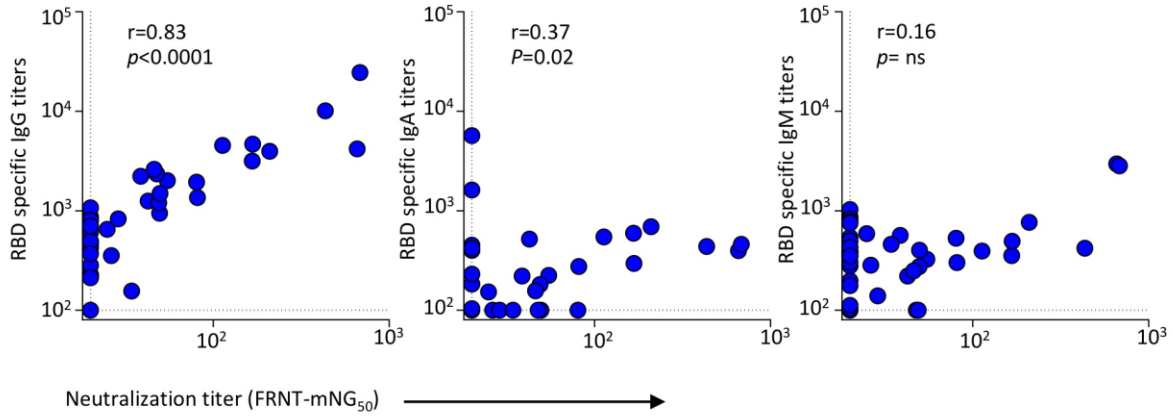

Figure 4

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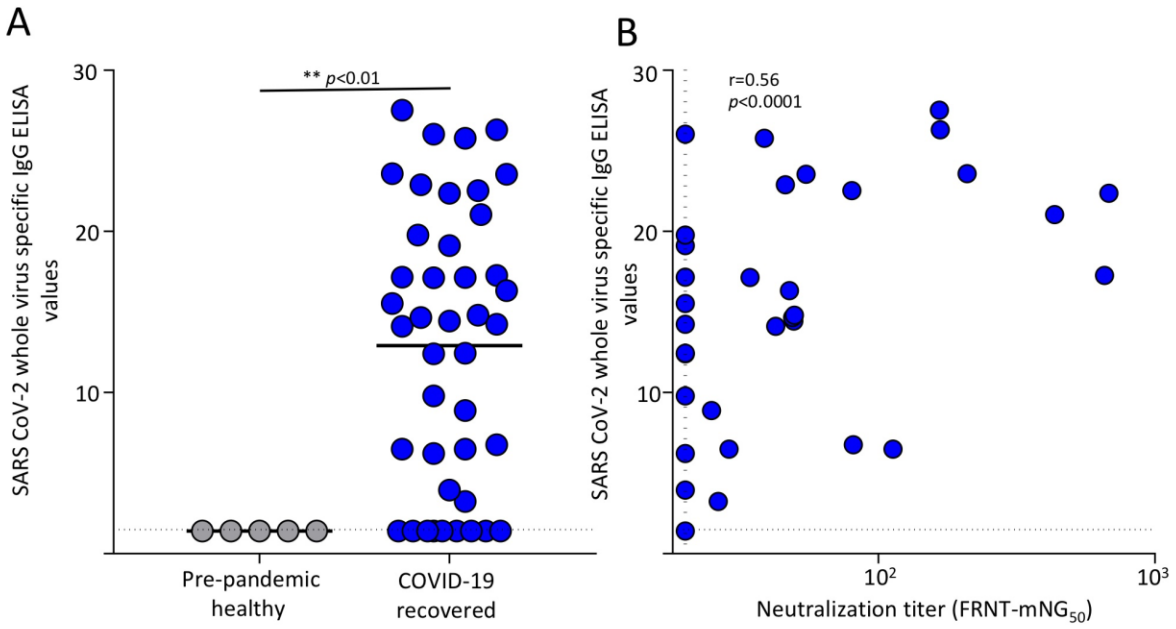

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Figure 5

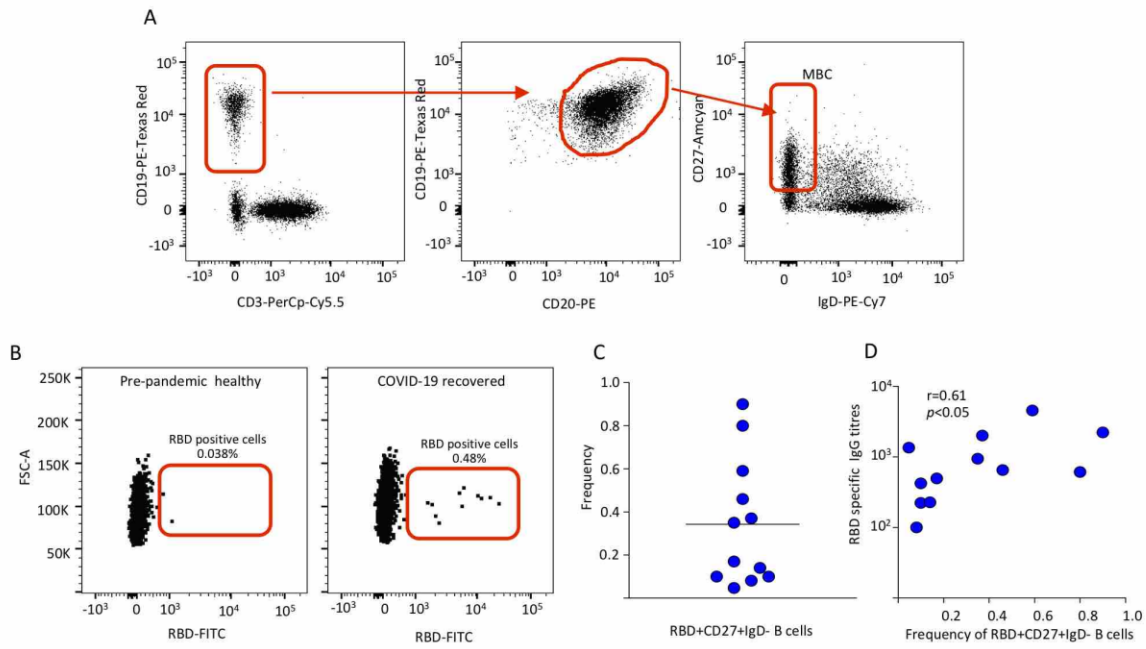

Figure 6