Identification of neutralizing human monoclonal antibodies from Italian Covid-19 convalescent patients

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One Sentence Summary: Neutralizing human monoclonal antibodies isolated from Covid-19 convalescent patients for therapeutic and prophylactic interventions.

ABSTRACT

In the absence of approved drugs or vaccines, there is a pressing need to develop tools for therapy and prevention of Covid-19. Human monoclonal antibodies have very good probability of being safe and effective tools for therapy and prevention of SARS-CoV-2 infection and disease. Here we describe the screening of PBMCs from seven people who survived Covid-19 infection to isolate human monoclonal antibodies against SARS-CoV-2. Over 1,100 memory B cells were single-cell sorted using the stabilized prefusion form of the spike protein and incubated for two weeks to allow natural production of antibodies. Supernatants from each cell were tested by ELISA for spike protein binding, and positive antibodies were further tested for neutralization of spike binding to receptor(s) on Vero E6 cells and for virus neutralization *in vitro*. From the 1,167 memory B specific for SARS-CoV-2, we recovered 318 B lymphocytes expressing human monoclonals recognizing the spike protein and 74 of these were able to inhibit the binding of the spike protein to the receptor. Finally, 17 mAbs were able to neutralize the virus when assessed for neutralization *in vitro*. Lead candidates to progress into the drug development pipeline will be selected from the panel of neutralizing antibodies identified with the procedure described in this study.

INTRODUCTION

The impact of the SARS-CoV-2 pandemic, with more than 3.5 million cases, 250,000 deaths and more than 25 million people unemployed in the United States alone, is unprecedented. This first wave of infection is likely to be followed by additional waves in the next few years, until herd immunity, acquired by vaccination or by infection, will constrain the circulation of the virus. It is therefore imperative to develop therapeutic and preventive tools to face the next waves of SARS-CoV-2 infections as soon as possible. Among the many therapeutic options available, human monoclonal antibodies (mAbs) are the ones that can be developed in the shortest period of time. In fact, the extensive clinical experience with the safety of more than 50 commercial mAbs approved to treat cancer, inflammatory and autoimmune disorders provides high confidence on their safety. These advantages, combined with the urgency of the SARS-CoV-2 pandemic, support and justify an accelerated regulatory pathway. In addition, the long industrial experience in developing and manufacturing mAbs decreases the risks usually associated with the technical development of investigational products. Finally, the incredible technical progress in the field allows to shorten the conventional timelines and go from discovery to proof of concept trials in 5-6 months (*1*). Indeed, in the case of Ebola, mAbs were the first therapeutic intervention recommended by the World Health Organization (WHO) and they were developed faster than vaccines or other drugs (*2*).

The SARS-CoV-2 spike glycoprotein (S-protein) has a pivotal role in viral pathogenesis and it is considered the main target to elicit potent neutralizing antibodies and the focus for the development of therapeutic and prophylactic tools against this virus (*3, 4*). Indeed, SARS-CoV-2 entry into host cells is mediated by the interaction between S-protein and the human angiotensin converting enzyme 2 (ACE2) (*3, 5*). The S-protein is a trimeric class I viral fusion protein which exists in a metastable prefusion conformation and in a stable postfusion state. Each S-protein monomer is composed of two distinct regions, the S1 and S2 subunits. Structural rearrangement occurs when the receptor binding domain (RBD) present in the S1 subunit binds to the host cell membrane. This

interaction destabilizes the prefusion state of the S-protein triggering the transition into the postfusion conformation which in turn results in the ingress of the virus particle into the host cell (*6*). Single-cell RNA-seq analyses to evaluate the expression levels of ACE2 in different human organs have shown that SARS-CoV-2, through the S-protein, can invade human cells in different major physiological systems including the respiratory, cardiovascular, digestive and urinary systems, thus enhancing the possibility of spreading and infection (*7*).

To identify potent mAbs against SARS-CoV-2 we isolated over a 1,100 S-protein specific-memory B cells derived from seven Covid-19 convalescent donors. As the S-protein RBD domain is mainly exposed when this glycoprotein is in its prefusion state (*6*), we screened naturally produced mAbs against either the S1/S2 subunits and the S-protein trimer stabilized in its prefusion conformation (*6*). This strategy allows us to identify mAbs able to recognize linear epitopes as well as highly neutralizing trimer specific regions on the S-protein surface. The potent neutralizing effect of trimer specific mAbs has already been shown for other pathogens including the respiratory syncytial virus (RSV) (*8, 9*).

In this paper we report the identification of a panel of 318 mAbs from which we plan to select lead candidates for clinical development.

RESULTS

SARS-CoV2 induces a strong antibody response in patients contracting infection

Patients recovered from SARS-CoV-2 infection were enrolled in two ongoing clinical studies based in Rome, Italy (National Institute for Infectious Diseases, IRCCS, Lazzaro Spallanzani) and Siena, Italy (Azienda Ospedaliera Universitaria Senese). We firstly examined whether these patients had anti-SARS-CoV-2 S-protein antibodies. Plasma samples were evaluated by enzyme linked immunosorbent assay (ELISA) to assess the polyclonal response to the S-protein trimer, for their ability to neutralize the binding of the spike protein to Vero E6 cells (neutralization of binding or NOB assay) and for their potency in neutralizing the cytopathic effect caused by SARS-CoV-2 infection *in vitro*. Results shown in Table 1 and Figure 1 show that, among the seven donors included in this study, six were able to produce high titers of SARS-CoV-2 S-protein specific antibodies and in particular donors R-042, R-122 and R-188 showed the highest virus neutralizing titers. Only one patient (R-276) mounted low anti-spike polyclonal response (Fig. 1A-B). Interestingly, despite no statistically significant correlation was observed when Pearson correlation analysis was performed, it is possible to observe a trend of correlation between S-protein binding, NOB titer and neutralization titer, suggesting that an abundant response against the S-protein trimer in its prefusion conformation may be indicative of immunity against SARS-CoV-2 (Fig. 1C-D). A bigger dataset may be needed to support this observation.

Isolation of naturally induced S-protein specific antibodies from SARS-CoV-2 convalescent patients

To retrieve mAbs specific for SARS-CoV-2 S-protein, peripheral blood mononuclear cells (PBMCs) from the seven convalescent patients enrolled in this study were collected and stained with fluorescent labeled S-protein trimer to identify antigen specific memory B cells (MBCs). The gating strategy described in Fig. 2 was used to single cell sort into 384-well plates IgG^+ and IgA^+

MBCs binding to the SARS-CoV-2 S-protein trimer in its prefusion conformation. A total of 1,167 S-protein-binding MBCs were successfully retrieved with frequencies ranging from 0,17% to 1,41% (Table 2). Following the sorting procedure, S-protein^{+} MBCs were incubated over a layer of 3T3-CD40L feeder cells in the presence of IL-2 and IL-21 stimuli for two weeks to allow natural production of immunoglobulins (*10*). Subsequently, MBC supernatants containing IgG or IgA were tested for their ability to bind either the SARS-CoV-2 S-protein S1 + S2 subunits (Fig. 3A) or trimer in its prefusion conformation (Fig. 3B) by enzyme linked immunosorbent assay (ELISA). A panel of 318 mAbs specific for the SARS-CoV-2 S-protein were identified showing a broad range of signal intensities (Table 2 and Fig. 3).

Functional characterization of S-protein specific mAbs against SARS-CoV-2

Of the 318 supernatants containing S-protein specific mAbs, 265 were screened *in vitro* for their ability to block the binding of the S-protein to Vero E6 cell receptors (Table 2 and Fig. 4A) and for their neutralization activity against the SARS-CoV-2 virus (Fig. 5A). In the NOB assay, 74 of the 265 tested (28%) S-protein specific mAbs were able to neutralize the antigen/receptor binding showing a broad array of neutralization potency ranging from 52% to over 90% (Fig. 4B). In the viral neutralization assay, supernatants containing naturally produced IgG or IgA were tested for their ability to protect the layer of Vero E6 cells from the cytopathic effect triggered by SARS-CoV-2 infection (Fig. 5A). When mAbs were able to protect Vero E6 cells from infection, i.e. showing neutralization capacity against SARS-CoV-2, no cytopathic effect was observed (Fig. 5B, bottom-left box). On the contrary, when mAbs were not able to prevent infection, cytopathic effect on Vero E6 was clearly visible (Fig. 5B, bottom-right box). Out of the 265 mAbs tested in this study, a panel of 17 mAbs neutralized the virus and prevented infection of Vero E6 cells (Table 2).

DISCUSSION

Human monoclonal antibodies are an industrially mature technology with more than 50 products already approved in the field of cancer, inflammation and autoimmunity. The well-established safety profile and the large experience for their development, make mAbs ideal candidates for rapid development especially in epidemic and pandemic settings. So far mAbs have rarely been used in the field of infectious diseases, mostly because the large quantities needed for therapy made them not cost effective. However, in recent years, the incredible technological progress to isolate and screen memory B cells made possible to identify highly potent neutralizing mAbs and to further improve their potency of several orders of magnitude through established engineering procedures. This possibility resulted into a decreased amount of antibodies necessary for therapy thus making non-intravenous delivery of potent neutralizing mAbs possible. Several candidates are presently under development in the field of HIV, pandemic influenza, RSV and many other infectious diseases (*11*). Perhaps the most striking demonstration of the power of mAbs for emerging infections came from the Ebola experience. In this case rapidly developed potent mAbs were among the first drugs to be tested in the Ebola outbreak and showed remarkable efficacy in preventing mortality (*2*). Given the remarkable efficacy of this intervention, potent mAbs became the first, and so far the only, drug to be recommended for Ebola by the WHO.

In the case of SARS-CoV-2, where so far we do not have any effective therapeutic nor prophylactic interventions, mAbs have the possibility to become one of the first drugs that can be used for immediate therapy of any patient testing positive for the virus, and even to provide immediate protection from infection in high risk populations. Preliminary evidences showed that plasma from infected people improves the outcome of patients with severe disease, therefore it is highly possible that a therapeutic and/or prophylactic mAb-based intervention can be highly effective (*12*). Furthermore, vaccination strategies inducing neutralizing antibodies have shown to protect non-

161 human primates from infection (*13*). These results further stress the importance of mAbs as a
162 measure to counterattack SARS-CoV-2 infection and to constrain its circulation.
163 In this work we addressed the question of whether mAbs recognizing SARS-CoV-2 can be
164 recovered from memory B cells of people who survived Covid-19 and whether some of them are
165 able to neutralize the virus. Our data show that SARS-CoV-2 specific mAbs can be successfully
166 isolated from most convalescent donors even if the frequency of S-protein specific memory B cells
167 is highly variable among them. In addition, approximately 28% of isolated mAbs were able to
168 inhibit the binding of the S-protein to the receptor(s) on Vero E6 cells. Finally a fraction of isolated
169 mAbs (N=17) were able to effectively neutralize SARS-CoV-2 with high potency when tested *in*
170 *vitro*. These data suggest that the method we implemented allows us to successfully retrieve mAbs
171 with potent neutralizing activity against SARS-CoV-2 and we plan to select the most promising
172 candidate(s) for drug development. Lead candidates will be further tested for the ability to generate
173 resistant viruses and for the ability to induce antibody-dependent disease enhancement in
174 appropriate models.
175

MATERIALS & METHODS

Enrollment of SARS-COV-2 convalescent donors and human sample collection

This work results from a collaboration with the National Institute for Infectious Diseases, IRCCS – Lazzaro Spallanzani Rome (IT) and Azienda Ospedaliera Universitaria Senese, Siena (IT) that provided samples from SARS-CoV-2 convalescent donors who gave their written consent. The study was approved by local ethics committees (Parere 18_2020 in Rome and 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.

Human peripheral blood mononuclear cells (PBMCs) isolation from SARS-CoV-2 convalescent donors

Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-treated whole blood by density gradient centrifugation (Lympholyte-H; Cedarlane). After separation, PBMC were: i) frozen in liquid nitrogen at concentration of 10 x 10^6 PBMC/vial using 10% DMSO in heat-inactivated fetal bovine serum (FBS) or ii) resuspended in RPMI 1640 (EuroClone) supplemented with 10% FBS (EuroClone), 2 mmol/L L-glutamine, 2 mmol/L penicillin, and 50 µg/mL streptomycin (EuroClone). Cells were cultured for 18 hour at 37°C with 5% CO_2. Blood samples were screened for SARS-CoV-2 RNA and for antibodies against HIV, HBV and HCV.

Expression and purification of SARS-CoV-2 S-protein prefusion trimer

The expression vector coding for prefusion S ectodomain (kind gift of Dr. Jason Mc Lellan) was used to transiently transfect Expi293F cells (Thermo Fisher #A14527) using Expifectamine (Thermo Fisher # A14525). The protein was purified from filtered cell supernatants using NiNTA resin (GE Healtcare #11-0004-58), eluted with 250 mM Imidazole (Sigma Aldrich #56750), dialyzed against PBS, and then stored at 4°C prior to use.

Single cell sorting of SARS-CoV-2 S-protein^{+} memory B cells

Human peripheral blood mononuclear cells (PBMCs) from SARS-CoV-2 convalescent donors were stained with Live/Dead Fixable Aqua (Invitrogen; Thermo Scientific) in 100 µL final volume diluted 1:500 at room temperature (RT). After 20 min incubation cells were washed with phosphate buffered saline (PBS) and unspecific bindings were saturated with 50 µL of 20% rabbit serum in PBS. 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), IgM PerCP-Cy5.5 (BD cat# 561285), CD27 PE (BD cat# 340425), IgD-A700 (BD cat# 561302), CD3 PE-Cy7 (BioLegend cat# 300420), CD14 PE-Cy7 (BioLegend cat# 301814), CD56 PE-Cy7 (BioLegend cat# 318318) and cells were incubated at 4°C for additional 30 min. Stained MBCs were single cell-sorted with a BD FACSAria 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 (*10*).

ELISA assay with S1 and S2 subunits of SARS-CoV-2 S-protein

The presence of S1- and S2-binding antibodies in culture supernatants of monoclonal S-protein-specific memory B cells was assessed by means of an ELISA assay implemented with the use of a commercial kit (ELISA Starter Accessory Kit, Catalogue No. E101; Bethyl Laboratories, Montgomery, TX, USA). Briefly, 384-well flat-bottom microtiter plates (Nunc MaxiSorp 384-well plates; Sigma-Aldrich) were coated with 25 µl/well of antigen (1:1 mix of S1 and S2 subunits, 1 µg/ml each; The Native Antigen Company, Oxford, United Kingdom) diluted in coating buffer (0.05 M carbonate-bicarbonate solution, pH 9.6), and incubated overnight at 4°C. The plates were then washed three times with 100 µl/well washing buffer (50 mM Tris Buffered Saline (TBS) pH 8.0, 0.05% Tween-20) and saturated with 50 µl/well blocking buffer containing Bovine Serum Albumin (BSA) (50 mM TBS pH 8.0, 1% BSA, 0.05% Tween-20) for 1 hour (h) at 37°C. After

228 further washing, samples diluted 1:5 in blocking buffer were added to the plate. Blocking buffer
229 was used as a blank. After an incubation of 1 h at 37°C, plates were washed and incubated with 25
230 µl/well secondary antibody (horseradish peroxidase (HRP)-conjugated goat anti-human IgG-Fc
231 Fragment polyclonal antibody, diluted 1:10,000 in blocking buffer, Catalogue No. A80-104P;
232 (Bethyl Laboratories, Montgomery, TX, USA) for 1 h at 37°C. After three washes, 25 µl/well TMB
233 One Component HRP Microwell Substrate (Bethyl Laboratories, Montgomery, TX, USA) was
234 added and incubated 10–15 minutes at RT in the dark. Color development was terminated by
235 addition of 25 µl/well 0.2 M H_2SO_4. Absorbance was measured at 450 nm in a Varioskan Lux
236 microplate reader (Thermo Fisher Scientific). The threshold for sample positivity was set at twice
237 the OD of the blank.
238
239 **ELISA assay with SARS-CoV-2 S-protein prefusion trimer**
240 ELISA assay was used to detect SARS-CoV-2 S-protein specific mAbs and to screen plasma from
241 SARS-CoV-2 convalescent donors. 384-well plates (Nunc MaxiSorp 384 well plates; Sigma
242 Aldrich) were coated with 3µg/mL of streptavidin diluted in PBS and incubated at RT overnight.
243 Plates were then coated with SARS-CoV-2 S-protein at 3µg/mL and incubated for 1h at room
244 temperature. 50 µL/well of saturation buffer (PBS/BSA 1%) was used to saturate unspecific binding
245 and plates were incubated at 37°C for 1h without CO_2. Supernatants were diluted 1:5 in PBS/BSA
246 1%/Tween20 0,05% in 25 µL/well final volume and incubated for 1h at 37°C without CO_2. 25
247 µL/well of alkaline phosphatase-conjugated goat anti-human IgG (Sigma-Aldrich) and IgA
248 (Jackson Immuno Research) were used as secondary antibodies. In addition, twelve two-fold serial
249 dilutions of plasma from SARS-CoV-2 infected patients were analyzed in duplicate. Plasma
250 samples were diluted in PBS/BSA 1%/Tween20 0,05% (25 µL/well final volume; Starting Dilution
251 1:80) and incubated for 1h at 37°C without CO_2. Next, 25 µL/well of alkaline phosphatase-
252 conjugated goat anti-human IgG (Sigma-Aldrich) was added for 1h at 37°C without CO_2. Wells
253 were washed three times between each step with PBS/BSA 1%/Tween20 0.05%. PNPP (p-

nitrophenyl phosphate) (Thermo Fisher) was used as soluble substrate to detect SARS-CoV-2 S-protein specific monoclonal antibodies and the final reaction was measured by using the Varioskan Lux Reader (Thermo Fisher Scientific) at a wavelength of 405 nm. Samples were considered as positive if optical density at 405 nm (OD_{405}) was two times the blank.

SARS-CoV-2 virus and cell infection

African green monkey kidney cell line Vero E6 cells (American Type Culture Collection [ATCC] #CRL-1586) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) - High Glucose (Euroclone, Pero, Italy) supplemented with 2 mM L- Glutamine (Lonza, Milano, Italy), penicillin (100 U/mL) - streptomycin (100 µg/mL) mixture (Lonza, Milano, Italy) and 10% Foetal Bovine Serum (FBS) (Euroclone, Pero, Italy). Cells were maintained at 37°C, in a 5% CO_2 humidified environment and passaged every 3-4 days.

Wild type SARS CoV-2 2019 (2019-nCoV strain 2019-nCov/Italy-INMI1) virus was purchased from the European Virus Archive goes Global (EVAg, Spallanzani Institute, Rome). For virus propagation, sub-confluent Vero E6 cell monolayers were prepared in T175 flasks (Sarstedt) containing supplemented D-MEM high glucose medium. For titration and neutralization tests of SARS-CoV-2, Vero E6 were seeded in 96-well plates (Sarstedt) at a density of $1{,}5x10^4$ cells/well the day before the assay.

Neutralization of Binding (NOB) Assay

To study the binding of the Covid-19 Spike protein to cell-surface receptor(s) we developed an assay to assess recombinant Spike protein specific binding to target cells and neutralization thereof. To this aim the stabilized Spike protein was coupled to Streptavidin-PE (eBioscience # 12-4317-87, Thermo Fisher) for 30 min at 4°C and then incubated with VERO E6 cells. Binding was assessed by flow cytometry. The stabilized Spike protein bound VERO E6 cells with high affinity (data not shown).

To assess the content of neutralizing antibodies in sera or in B-cell culture supernatants, two microliters of SARS-CoV-2 Spike-Streptavidin-PE at 15-30 µg/ml in PBS-1%FCS were mixed with two microliters of various dilutions of sera or B-cell culture supernatants in U bottom 96-well plates. After incubation at 37°C for 1 hr, $25x10^3$ Vero E6 cells suspended in two microliters of PBS 1% FCS were added and incubated for additional 1 hr at 4°C. Non-bound protein and antibodies were removed and cell-bound PE-fluorescence was analyzed with a FACScantoII flow cytometer (Becton Dickinson). Data were analyzed using the FlowJo data analysis software package (TreeStar, USA). The specific neutralization was calculated as follows: NOB (%) = 1 – (Sample MFI value – background MFI value) / (Negative Control MFI value – background MFI value).

Viral propagation and titration

The SARS-CoV-2 virus was propagated in Vero E6 cells cultured in DMEM high Glucose supplemented with 2% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin. Cells were seeded at a density of $1x10^6$ cells/mL in T175 flasks and incubated at 37°C, 5% CO_2 for 18-20 hours. The sub-confluent cell monolayer was then washed twice with sterile Dulbecco's phosphate buffered saline (DPBS). Cells were inoculated with 3.5 ml of the virus properly diluted in DMEM 2% FBS at a multiplicity of infection (MOI) of 0.001, and incubated for 1h at 37°C in a humidified environment with 5% CO_2. At the end of the incubation, 50 mL of DMEM 2% FBS were added to the flasks. The infected cultures were incubated at 37°C, 5% CO_2 and monitored daily until approximately 80-90% of the cells exhibited cytopathic effect (CPE). Culture supernatants were then collected, centrifuged at 4°C at 1,600 rpm for 8 minutes to allow removal of cell debris, aliquoted and stored at -80°C as the harvested viral stock. Viral titers were determined in confluent monolayers of Vero E6 cells seeded in 96-well plates using a 50% tissue culture infectious dose assay (TCID50). Cells were infected with serial 1:10 dilutions (from 10-1 to 10-11) of the virus and incubated at 37°C, in a humidified atmosphere with 5% CO_2. Plates were monitored daily for the presence of SARS-CoV-2 induced CPE for 4 days using an inverted optical microscope. The virus titer was estimated

306 according to Spearman-Karber formula (*14*) and defined as the reciprocal of the highest viral
307 dilution leading to at least 50% CPE in inoculated wells.
308
309 **Semi-quantitative live SARS-CoV-2-based neutralization assay**
310 To assess the neutralization titer of anti-SARS-CoV-2 plasma samples from Covid-19 convalescent
311 donors, a semi-quantitative neutralization method was used. Plasma samples were heat-inactivated
312 for 30 minutes at 56°C and 2-fold serially diluted starting from 1:10 to 1:2,560 dilution, then mixed
313 with an equal volume of viral solution containing 100 TCID50 of SARS-CoV-2 diluted in D-MEM
314 high Glucose 2% FBS. After 1 hour incubation at 37°C, 5% CO2, 100 µl of the virus-plasma
315 mixture at each dilution was passed to a cell plate containing a sub-confluent Vero E6 cell
316 monolayer. Plates were incubated for 3 days at 37°C in a humidified environment with 5% CO_2,
317 then checked for development of CPE by means of an inverted optical microscope. The reciprocal
318 of the highest plasma dilution that resulted in more than 50% inhibition of CPE was defined as the
319 neutralization titer.
320
321 **Qualitative live SARS-CoV-2-based neutralization assay**
322 The neutralization activity of culture supernatants from monoclonal S-protein-specific memory B
323 cells was evaluated by means of a qualitative live-virus based neutralization assay against a one-
324 point dilution of the samples. Supernatants were mixed in a 1:3 ratio with a SARS-CoV-2 viral
325 solution containing 25 TCID50 of virus (final volume: 30 µl). After 1 hour incubation at 37°C, 5%
326 CO_2, 25 µl of each virus-supernatant mixture was added to the wells of a 96-well plate containing a
327 sub-confluent Vero E6 cell monolayer. Following a 2-hour incubation at 37°C, the virus-serum
328 mixture was removed and 100 µl of DMEM 2% FBS were added to each well. Plates were
329 incubated for 3 days at 37°C in a humidified environment with 5% CO_2, then examined for CPE by
330 means of an inverted optical microscope. Absence or presence of CPE was defined by comparison
331 of each well with the positive control (plasma sample showing high neutralizing activity of SARS-

CoV-2 in infected Vero E6 cells) and negative control (human serum sample negative for SARS-CoV-2 in ELISA and neutralization assays).

Author contribution

Emanuele Andreano, Ida Paciello isolated single memory B cells and identified S-protein specific mAbs (cell sorting and ELISA).

Piero Pileri, Noemi Manganaro, Elisa Pantano performed NOB assay and produced recombinant spike protein.

Giulia Piccini, Alessandro Manenti and Emanuele Montomoli performed ELISA and viral neutralization assay.

Marco Troisi, Fabiola Vacca, Concetta De Santi, Dario Cardamone, Anna Kabanova contributed to the characterization of positive memory B cells.

Emanuele Nicastri, Chiara Agrati, Concetta Castilletti, Francesca Montagnani, Arianna Emiliozzi, Massimiliano Fabbiani, Maria Rosaria Capobianchi enrolled patients and isolated PBMCs.

Claudia Sala, Giuseppe Ippolito, Rino Rappuoli coordinated the project.

Conflict of interest statement

RR is an employee of GSK group of companies.

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TABLES

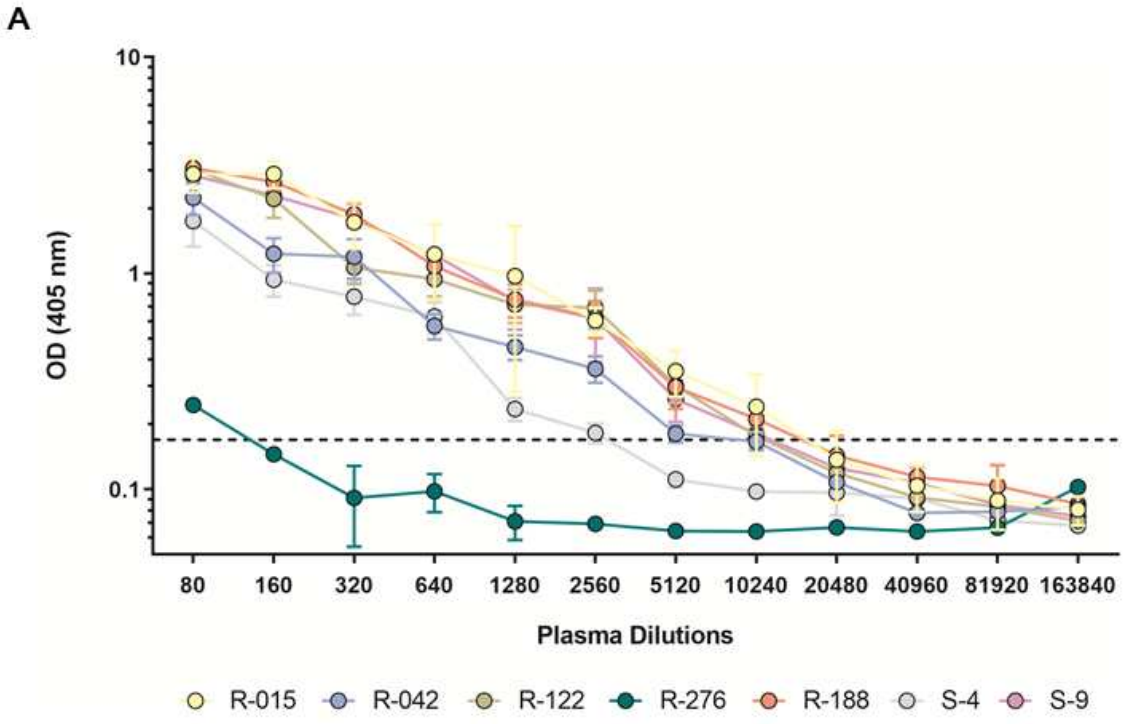
Table 1. SARS-CoV-2 convalescent donors plasma analyses. Plasma S-protein binding titers for each subject were measured by ELISA assays. Neutralization activity was detected by NOB and by neutralization of SARS-CoV-2 infection of Vero cells; ND = Not Done.

Subject ID	Plasma S-protein Binding (ELISA)	Plasma Neutralization of binding (NOB)	Plasma Virus Neutralization
R-015	10240	>540	320
R-042	5120	>540	640
R-122	10240	>540	640
R-276	80	180	10
R-188	10240	>540	>2560
S-4	2560	ND	ND
S-9	10240	ND	ND

Table 2. SARS-CoV-2 convalescent donors S-protein specific MBCs analyses. The Table reports the number of S-protein-specific MBCs that were sorted and screened (for binding by ELISA and for functionality by NOB and viral neutralization) for each subject enrolled in this study; ND = Not Done.

Subject ID	Antigen Specific MBCs (%)	S-protein⁺ MBCs Sorted	S-protein⁺ mAbs (ELISA)	Neutraliziation of binding (NOB)	SARS-CoV-2 Neutralization
R-015	1,01	230	18	3	0
R-042	0,27	39	3	1	0
R-122	0,61	5	2	0	0
R-276	0,27	66	4	2	0
R-188	0,17	324	72	8	4
S-4	1,41	367	158	60	13
S-9	0,87	136	53	ND	ND
	Total	1,167	318	74	17

FIGURES



B

	R-015	R-042	R-122	R-276	R-188	Cell Control	Virus Control
10	-	-	-	-	-	-	+
20	-	-	-	+	-	-	+
40	-	-	-	+	-	-	+
80	-	-	-	+	-	-	+
160	-	-	-	+	-	-	+
320	-	-	-	+	-	-	+
640	+	-	-	+	-	-	+
1280	+	+	+	+	-	-	+
2560	+	+	+	+	-	-	+

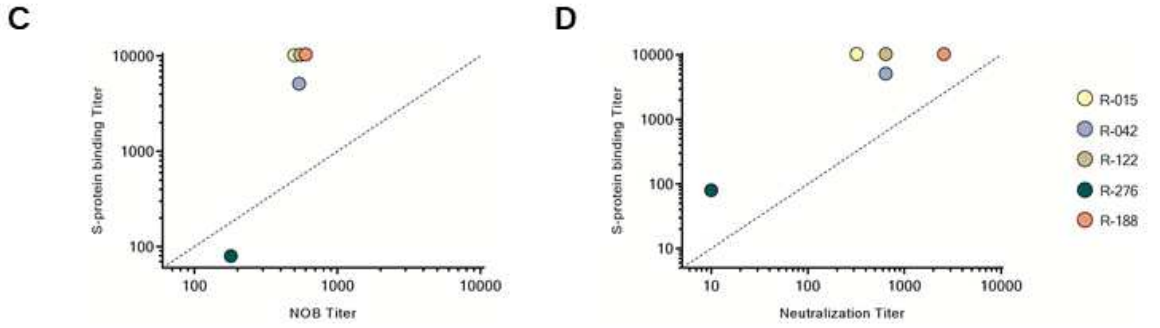


Fig. 1. S-protein binding and neutralization titration of SARS-CoV-2 convalescent donors plasma. (A) Plasma samples were two-fold diluted starting at 1:80 to test their ability to bind the S-protein trimer in its prefusion state by ELISA. Results were considered as positive when the OD_{405} value was at least two times higher than the blank. (B) Plasma samples were two-fold diluted starting at 1:10 to test their ability to neutralize SARS-CoV-2 *in vitro*. Results were considered as positive when no cytopathic effect (-) was observed on Vero E6 cells . (C) The graph shows on the Y axis the Log_{10} S-protein binding titer and on the X axis the Log_{10} NOB titer of plasma collected from Covid-19 convalescent patients. Donors R-015 and R-122 are not visible in the graph as their data overlap with those of donor R-188. (D) The graph shows on the Y axis the Log_{10} S-protein binding titer and on the X axis the Log_{10} neutralization titer of plasma collected from Covid-19 convalescent patients.

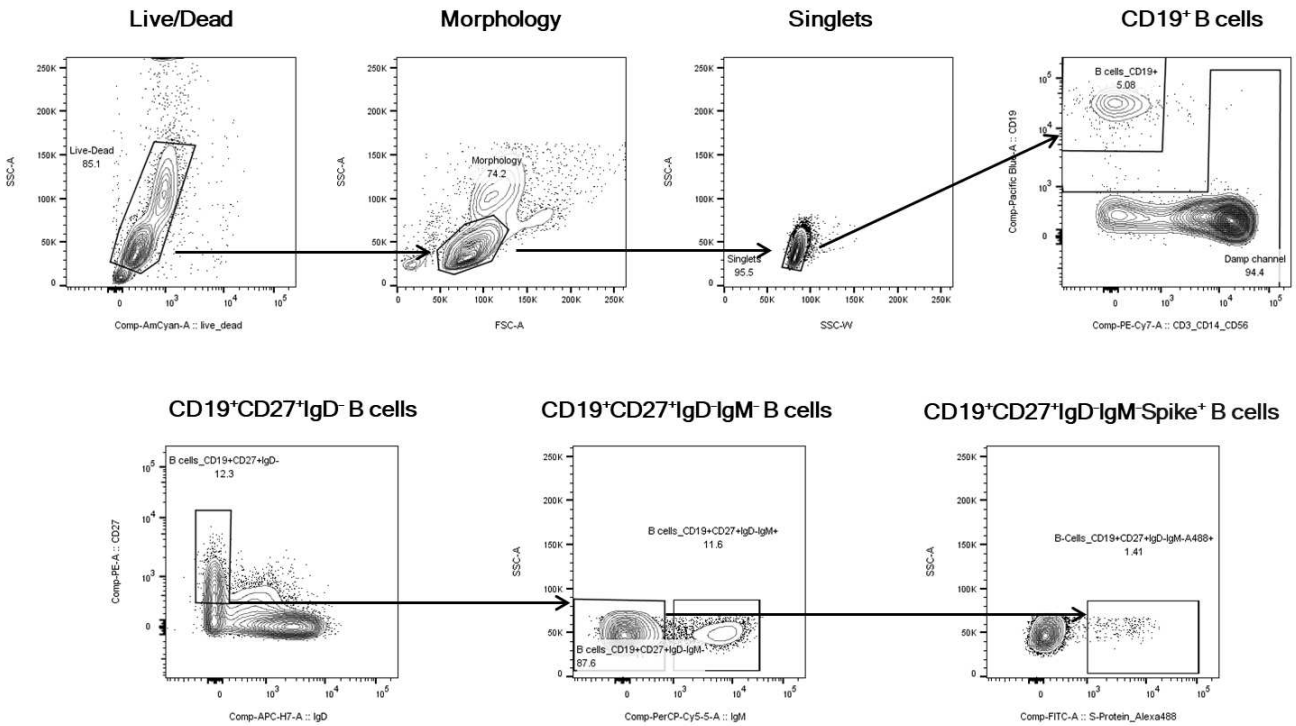


Fig. 2. Gating strategy for S-protein specific MBC single cell sorting. Starting from top left to the right panel, the gating strategy shows: Live/Dead; Morphology; Singlets; $CD19^+$ B cells; $CD19^+CD27^+IgD^-$; $CD19^+CD27^+IgD^-IgM^-$; $CD19^+CD27^+IgD^-IgM^-$S-protein$^+$B cells.

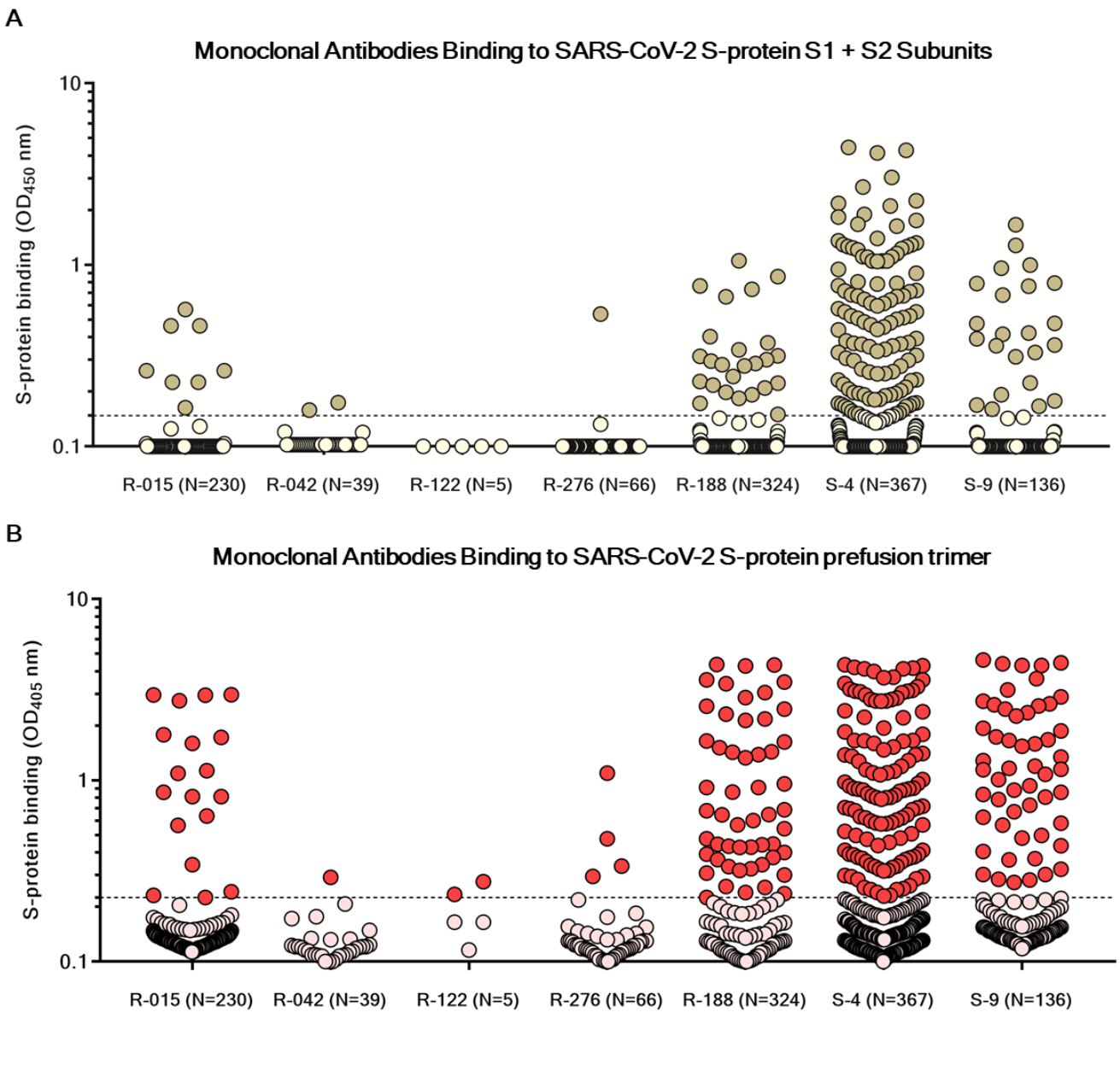


Fig. 3. Identification of SARS-CoV-2 S-protein specific mAbs isolated from convalescent donors. (A) The graph shows supernatants tested for binding to the SARS-CoV-2 S-protein S1 + S2 subunits. Threshold of positivity has been set as two times the value of the blank (dotted line) Darker dots represent mAbs which bind to the S1 + S2 while light yellow dots represent mAbs which do not bind. The total number (N) of single cell sorted B cell supernatants screened for binding is also shown for each donor. (B) The graph shows supernatants tested for binding to the SARS-CoV-2 S-protein stabilized in its prefusion conformation. Threshold of positivity has been set as two times the value of the blank (dotted line). Red dots represent mAbs which bind to the S-

499 protein while pink dots represent mAbs which do not bind. The total number (N) of single cell
500 sorted B cell supernatants screened for binding is also shown for each donor.

501

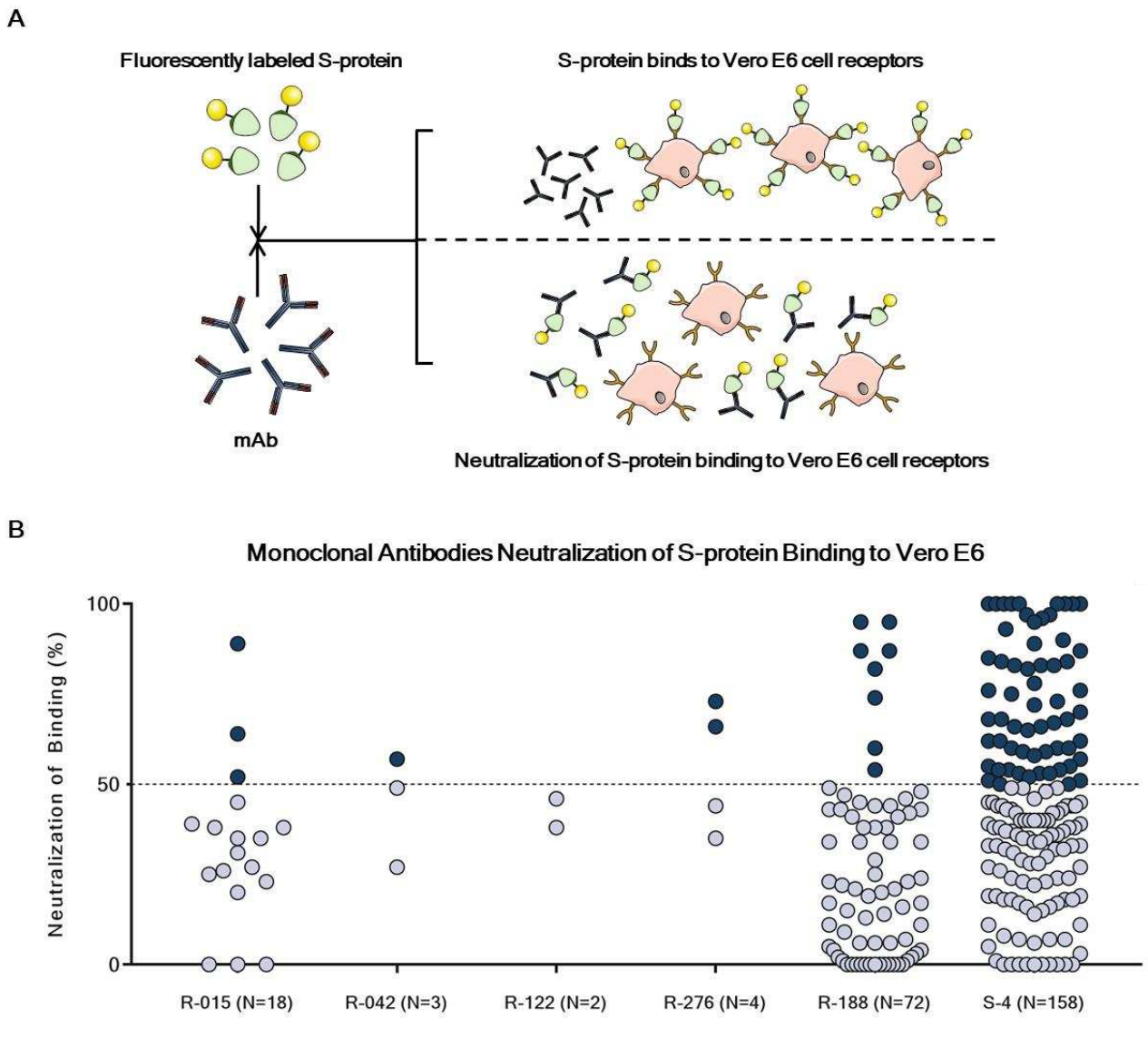


Fig. 4. Neutralization of S-protein binding to Vero E6 cell receptors by S-protein specific mAbs. (A) Schematic representation of the neutralization of binding (NOB) assay used to screen isolated S-protein specific mAbs for their ability to abrogate the interaction between SARS-CoV-2 and Vero E6 cell receptors. (B) The graph shows supernatants tested by NOB assay. Threshold of positivity has been set as 50% of binding neutralization (dotted line). Dark blue dots represent mAbs able to neutralize the binding between SARS-CoV-2 and receptors on Vero E6 cells, while light blue dots represent non-neutralizing mAbs. The total number (N) of S-protein specific supernatants screened by NOB assay is shown for each donor.

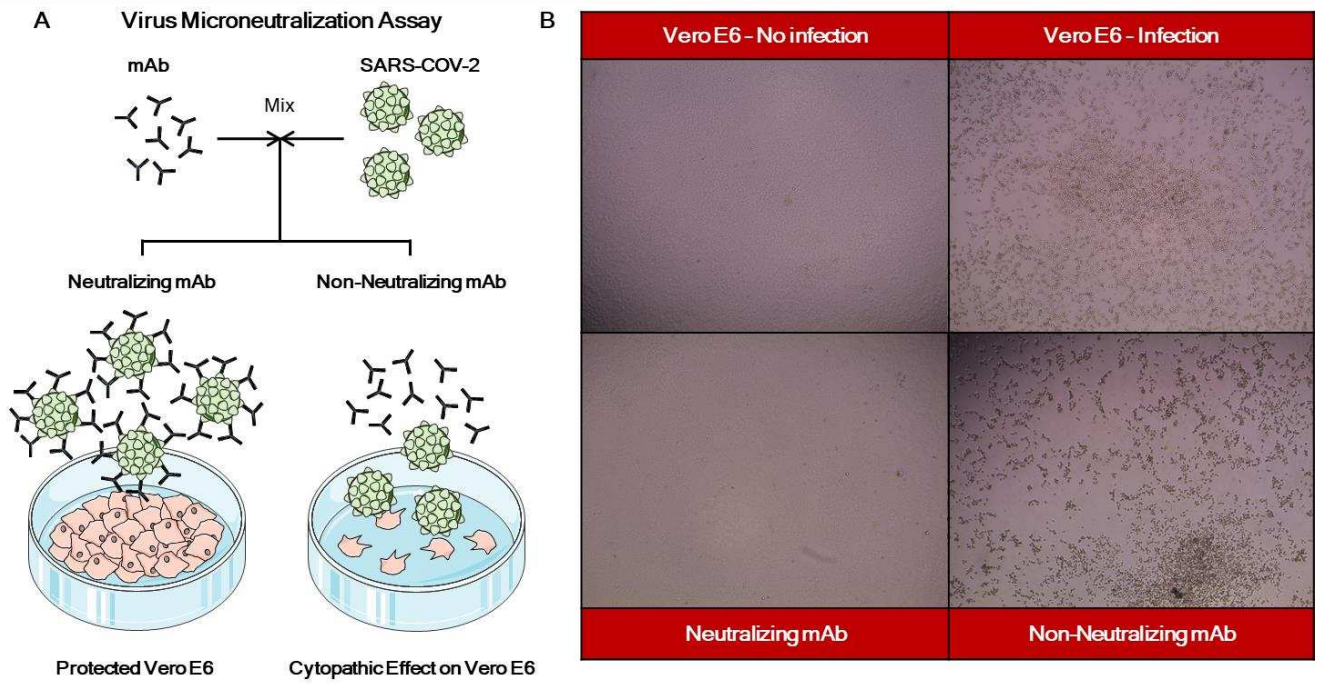


Fig. 5. SARS-CoV-2 neutralization assay for S-protein specific mAbs. (A) Schematic representation of the virus neutralization assay used in this study to assess functional activities of S-protein specific mAbs. (B) Representative microscope images showing the cytopathic effect of SARS-CoV-2 or the protective efficacy of the screened supernatants on Vero E6 cells.

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