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Article

Identification of Novel Neutralizing Monoclonal Antibodies against SARS-CoV-2 Spike Glycoprotein

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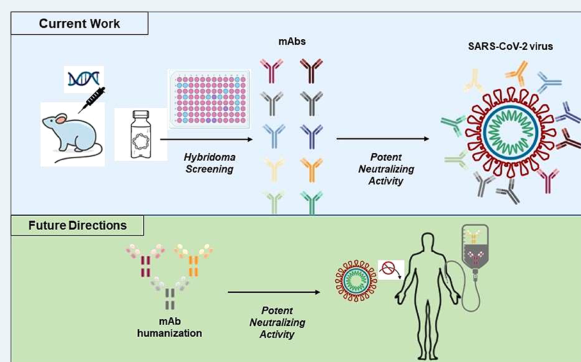

ABSTRACT: Coronavirus disease 2019 (COVID-19) is caused by the newly emerged human coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Due to the highly contagious nature of SARS-CoV-2, it has infected more than 137 million individuals and caused more than 2.9 million deaths globally as of April 13, 2021. There is an urgent need to develop effective novel therapeutic strategies to treat or prevent this infection. Toward this goal, we focused on the development of monoclonal antibodies (mAbs) directed against the SARS-CoV-2 spike glycoprotein (SARS-CoV-2 Spike) present on the surface of virus particles as well as virus-infected cells. We isolated anti-SARS-CoV-2 Spike mAbs from animals immunized with a DNA vaccine. We then selected a highly potent set of mAbs against SARS-CoV-2 Spike protein and evaluated each candidate for their expression, target binding affinity, and neutralization potential using complementary ACE2-blocking and pseudovirus neutralization assays. We identified a total of 10 antibodies, which specifically and strongly bound to SARS-CoV-2 Spike, blocked the receptor binding domain (RBD) and angiotensin-converting enzyme 2 (ACE2) interaction, and neutralized SARS-CoV-2. Furthermore, the glycomic profile of the antibodies suggested that they have high Fc-mediated effector functions. These antibodies should be further investigated for elucidating the neutralizing epitopes on Spike for the design of next-generation vaccines and for their potential in diagnostic as well as therapeutic utilities against SARS-CoV-2.

KEYWORDS: *COVID-19, SARS-CoV-2/Spike/RBD, synthetic DNA, mAbs, neutralizing antibodies*

Human coronaviruses (CoVs) are positive-stranded RNA viruses having the largest viral genome (27−32 kilobase pairs) identified to date. They are prime causes of illnesses related to the upper respiratory tract.[1,2] The members of this group infect the respiratory, gastrointestinal, hepatic, and central nervous systems of humans as well as birds, bats, mice, livestock, and different wild animals.[3] During late 2019, a novel human CoV, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in the city of Wuhan, China, and is currently causing a pandemic across the world.[4] In January 2020, the World Health Organization (WHO) identified this virus as the causative agent of the 2019 novel coronavirus infectious disease (COVID-19).[3,5] Notably, SARS-CoV-2 is the seventh coronavirus identified, causing infections in humans, after SARS-CoV, MERS-CoV, HKU1, NL63, OC43, and 229E.[6] Due to their alarming impacts on humans, SARS-CoV, MERS-CoV, and SARS-CoV-2 are recognized as highly pathogenic and lethal human CoVs.[7] SARS-CoV-2 exhibits genetic relatedness to SARS-CoV, which itself led to an epidemic with over 8000 confirmed cases in more than 25 countries globally.[8] The case fatality rates of SARS and MERS were reported to be 10 and 34%, respectively, whereas for SARS-CoV-2, it is comparatively lower, estimated at approximately 2%. However, due to its rapid establishment in the global population and much more rapid spread, the total number of infections and deaths due to SARS-CoV-2 is much higher.[9]

SARS-CoV-2 is a *Betacoronavirus* with 5′ cap structure and 3′ poly-A tail. The open reading frames near the 3′ terminus

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encode four main structural proteins, namely, spike (S), membrane (M), envelope (E), and nucleocapsid (N).[3,10] The spike glycoprotein (referred to herein as "Spike"), the clublike extensions projecting from the viral surface (which are of corona shape and hence the name coronavirus), facilitates the transfer of viral genetic material into a host cell by adhesion.[11] This occurs via interaction between a host receptor and the receptor-binding domain (RBD) present in the S1 subunit, followed by fusion of the viral and host membranes via the S2 subunit. Thus, Spike represents the most likely and important target for developing neutralizing antibodies (Nabs), virus attachment inhibitors, and vaccines.[12] Furthermore, angiotensin-converting enzyme 2 (ACE2) is known to be an important receptor for SARS-CoV. Notably, this receptor is also reported to play a key role in SARS-CoV-2 infections, and the overall ACE2-binding modes of both viruses have been found to be highly similar.[13] Hence, inhibiting the interaction between SARS-CoV-2 Spike and ACE2 might offer new avenues for preventing the viral spread.[14]

Different immunotherapeutic approaches were found to be successful in combating coronaviruses including SARS-CoV and MERS-CoV through utilization of vaccines and monoclonal antibodies (mAbs).[15] mAbs are regarded as a highly viable therapeutic regimen for different disease targets. As a matter of fact, more than 60 recombinant mAbs were developed and licensed for use in the last two decades for different disease conditions including infectious diseases.[16] Evidence suggests that they can also induce long-lasting protective antiviral immunity by recruiting the endogenous immune system of infected individuals during the period of therapy.[17] Several mAbs engaging the RBD or S2 subunit of SARS-CoV-2 Spike have been isolated and are being studied for their efficacy to develop antibody-based therapeutic interventions for the management of COVID-19.[18−26] Furthermore, human neutralizing mAbs with the ability to recognize the N-terminal domain (NTD) of SARS-CoV-2 Spike protein were also identified and characterized.[25,27] It is particularly pressing to rapidly identify and study high potency neutralizing antibodies which may have clinical value in disease therapy.

In this study, we aimed to generate and study mAbs targeting SARS-CoV-2 Spike. Toward this goal, we have identified and cloned a total of 10 different IgG mAbs obtained from mice immunized with a SARS-CoV-2 Spike DNA vaccine and boosted with RBD protein, providing a unique immunization platform for study. Proteins expressed by DNA vaccines largely exhibit their native conformations, which are thought to mimic natural viral antigens.[28,29] Then, we evaluated each antibody for expression, target binding affinity, and neutralization potential using complementary ACE2-blocking and pseudovirus neutralization assays. These mAbs were found to exhibit specific binding to SARS-CoV-2 Spike and block RBD/ACE2 interaction, and they were predicted to have high Fc-mediated effector functions and neutralization activity in the SARS-CoV-2 pseudotyped viral assay.

■ MATERIALS AND METHODS

Cell Culture. Human embryonic kidney 293T cells were obtained from the ATCC and CHO-ACE2 cell line (stably expresses ACE2 on the cell surface) was procured from the Creative Biolabs, USA. The cells were maintained in D10 media comprised of Dulbecco's modified Eagle's medium (Invitrogen Life Science Technologies, USA) with heat-inactivated fetal bovine serum (FBS, 10%), glutamine (3 mM), penicillin (100 U/ml), and streptomycin (100 U/ml). R10 media comprising RPMI1640 (Invitrogen Life Science Technologies, USA), heat-inactivated FBS (10%), glutamine (3 mM), penicillin (100 U/ml), and streptomycin (100 U/ml) was used for the mouse splenocyte cells. All the cells were maintained at 37°C and 5% CO_2.

Construction of SARS-CoV-2 Spike Synthetic DNA. The SARS-CoV-2 Spike plasmid DNA encoded construct was developed by the alignment of Spike protein sequences available in the PubMed database. The sequences corresponding to Spike (including the transmembrane domain) were genetically optimized, and the N-terminal IgE leader sequence was added to facilitate expression. The synthetic Spike construct was synthesized and then subcloned into a modified pMV101 expression vector with *BamH*1 and *Xho*1 restriction enzymes under the control of the cytomegalovirus immediate-early promoter as described previously.[30,31]

Generation and Evaluation of Anti-SARS-CoV-2 Hybridomas. Mice (BALB/c) were immunized with the synthetic sequence of SARS-CoV-2 Spike DNA on days 0 and 14 by intramuscular immunization followed by subcutaneous delivery of recombinant RBD protein (Genscript, USA) on day 28, as described.[23,32] One week after the final immunization, the immune sera from the immunized mice were collected and evaluated by enzyme-linked immunosorbent assay (ELISA) to detect the presence of antibodies targeting SARS-CoV-2 Spike. After confirmation, mouse splenocytes were used to generate hybridomas as described previously.[33] Subsequently, positive hybridoma clones were characterized by an ELISA, and those selected were further subcloned and expanded. The antibodies were purified from hybridoma supernatants and used for further studies.

Enzyme-Linked Immunosorbent Assay. ELISA assays were carried out for mAb characterization. MaxiSorp high-binding 96 well ELISA plates (ThermoFisher, USA) were coated with 1 μg/mL recombinant SARS-CoV-2 RBD (Sino Biological, USA) as well as full-length Spike overnight at 4 °C. Following blocking with 10% FBS in phosphate-buffered saline (PBS) for 1 h, the plates were incubated with serially diluted mAb clones using PBS with 1% FBS for 2 h. Then the samples were probed with anti-mouse IgG antibodies conjugated to horseradish peroxidase (HRP) (Sigma-Aldrich, USA) at a dilution of 1:20 000 for 1 h. Following this, tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, USA) was added to all the wells and incubated for 10 min. Then, 2 N H_2SO_4 was used to stop the reaction. The optical density was measured at 450 nm using an ELISA plate reader (Biotek, USA). Furthermore, end-point titers were determined at the highest dilution with S/N (Signal/Negative) ratio $\geq$2.1. The signal was designated as positive binding to SARS-CoV-2 RBD or full-length Spike compared to the signal of the negative control which was binding of an irrelevant mAb to the antigen.

Western Blot Analysis. A binding Western blot analysis was carried out to evaluate anti-SARS-CoV-2 mAb-binding specificity. Briefly, 2.5 μg of SARS-CoV-2 RBD (Sino Biological, USA) protein was run in 12% NuPAGE Novex polyacrylamide gels (Invitrogen Life Science Technologies, USA) and transferred to PVDF membranes (Invitrogen Life Science Technologies, USA). The membranes were blocked using Odyssey blocking buffer (LiCor BioSciences, USA) and then incubated with the supernatants from the mAb clones for

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overnight at 4 °C. After incubation, the membranes were washed with PBS containing 0.05% Tween 20 or PBST. Subsequently, the membranes were stained with IRDye800 goat anti-mouse secondary antibody (LI-COR Biosciences, USA) at room temperature and then again washed with PBST. Finally, the membranes were scanned using a LI-COR Odyssey CLx imager. Furthermore, for determining the heavy and light chain expressions of the mAb clones, this assay was carried out, in which 6 ng of each mAb clone was run in 12% NuPAGE Novex polyacrylamide gels (Invitrogen Life Science Technologies, USA) and subsequently probed with goat anti-mouse IgG secondary antibody (LiCor BioSciences, USA).

Surface Plasmon Resonance (SPR) Analysis of SARS-CoV-2 Spike Monoclonal Antibody Clones' Binding to RBD Protein. Binding of mAb clones to SARS-CoV-2 RBD protein was measured using a Biacore T200 surface plasmon resonance (SPR) system. SARS-CoV-2 RBD (Sino Biological Inc., USA) protein was immobilized with running buffer with 10 mM HEPES (pH 7.4), 150 mM NaCl, and 0.05% Tween20 using standard amine coupling procedures to a carboxymethyl dextran sensor chip (CMD200L, Xantec Bioanalytics, Germany). Briefly, the chip was first washed in a buffer with 0.1 M sodium borate (pH 9.0) and 1 M NaCl, followed by activation with EDC/NHS for 8 min using a running buffer of Milli-Q distilled water. After activation, each protein (10 μg/mL in 10 mM sodium acetate, pH 5) was added until the desired immobilization level was achieved. Approximately 2500 RU of SARS-CoV-2 RBD protein was immobilized on the flow cell; 5000 RU of bovine serum albumin (BSA) was also immobilized to another flow cell which served as the negative control. After immobilization, the remaining activated sites were blocked with 1 M ethanolamine (pH 8.5). The running buffer was then switched to buffer with 10 mM HEPES (pH 7.4), 150 mM NaCl, and 0.05% Tween20. Each IgG mAb clone was tested at three different concentrations in duplicate (0.13, 0.41, and 1.2 nM). Dilutions were prepared in running buffer. The association time was 300 s, and the dissociation time was 600 s with a flow rate of 30 μL/min and a measurement temperature of 20 °C. After each injection, the surface was regenerated by injecting 20 mM glycine (pH 2.0) for 60 s. Data were collected and analyzed using Biacore Evaluation Software. Subsequently, kinetic parameters for SARS-CoV-2 RBD binding to IgG mAb clones were determined using a Protein A/G coated carboxymethyldextran sensor chip (Xantec Bioanalytics, Germany) in a Biacore T200 SPR system. Approximately 400 RU of each mAb clone was captured on the chip surface for each concentration of antigen. SARS-CoV-2 RBD was tested at concentrations ranging from 0 to 100 nM, and the flow rate was 30 μL/min. The reference surface was coated with 400 RU of mouse IgG isotype control. The association time was 210 s, and the dissociation time was 900 s. After each concentration of antigen, the antibody−antigen complex was removed from the chip using 20 mM glycine (pH 2.0). Data are the mean of duplicate determinations, and kinetic parameters were determined using the 1:1 binding model in the Biacore T200 Evaluation software.

Glycan Analysis of Antibodies. Hybridoma supernatants (500 μL) were concentrated using Amicon Ultra-0·5 Centrifugal Filter Unit (Millipore Sigma, USA). Bulk IgG from five BALB/c mice and a human plasma sample (Innovative Research, USA) were used as controls. Total IgG was purified using Pierce Protein G Spin Plate for IgG Screening (ThermoFisher, USA), and IgGs were further concentrated using Amicon Ultra-0·5 Centrifugal Filter Unit (Millipore Sigma, USA). *N*-Glycans were released using peptide-*N*-glycosidase F (PNGase F) and labeled with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) using the GlycanAssure APTS Kit (ThermoFisher, USA), following the manufacturer's protocol. Labeled *N*-glycans were analyzed using the 3500 Genetic Analyzer capillary electrophoresis system. The relative abundance of IgG glycan structures was quantified by calculating the area under the curve of each glycan structure divided by the total glycans.

SARS-CoV-2 Surrogate Virus Neutralization Assay. A SARS-CoV-2 surrogate virus neutralization test kit (sVNT kit; Genscript, USA) was used for detecting the potential of the mAbs to neutralize SARS-CoV-2 RBD and ACE2 interaction. It is a species- and isotype-independent blocking ELISA detection tool which determines the circulating neutralizing antibodies against SARS-CoV-2 that can block protein−protein interaction between the RBD and human ACE2 receptor. Briefly, each mAb clone (500 ng/mL) and controls were preincubated with HRP-conjugated RBD (HRP-RBD) for 30 min at 37 °C to facilitate binding between mAb clones and HRP−RBD. Subsequently, the mixture was added to the capture plate precoated with human ACE2 receptor protein and incubated for 15 min at 37 °C. Following washing of the plate using wash solution, TMB substrate was added to all the wells and incubated for 15 min, and then stop solution was added to each well to quench the reaction. The absorbance was measured at 450 nm using an ELISA plate reader (Biotek, USA), and the inhibition values were determined. The cutoff value of 20 was considered based on a panel of confirmed COVID-19 immune sera and healthy control sera as recommended (Genscript, USA).

Competition ELISA. Competitive inhibition of SARS-CoV-2 Spike binding to ACE2 receptor in the presence of SARS-CoV-2 Spike mAb clones was evaluated by a competition ELISA as described.[34] First, 96-well half-area plates (Corning, USA) were coated at room temperature for 3 h with 1ug/mL SARS-CoV-2 S1+S2 ECD (Sino Biological, USA), followed by overnight blocking at 4 °C with blocking buffer containing 1× PBS, 5% skim milk, and 0.1% Tween-20. Plates were washed four times with wash buffer containing 1× PBS and 0.05% Tween-20. A huACE2-IgMu control (Sino Biological, USA), PBS buffer control, or mouse hybridoma gradient purification was serially diluted 3-fold with blocking buffer and incubated on the plate for 1 h at room temperature (starting concentration 100 μg/mL for protein control and 1:100 dilution for mAb clones). Plates were washed four times. Recombinant huACE2-IgHu was added at a constant concentration of 0.1 μg/mL to each of the wells and incubated for 1 h at room temperature. After washing four times, the plates were further incubated at room temperature for 1 h with goat anti-human IgG-Fc fragment cross-adsorbed antibody (Bethyl Laboratories, USA) at 1:10 000 dilution. This was followed by four washes and the addition of TMB substrate (ThermoFisher, USA). The plates were then quenched with 1 M H_2SO_4. The absorbances at 450 and 570 nm were recorded with a BioTek plate reader.

SARS-CoV-2 Pseudovirus Production and Neutralization Assays. The SARS-CoV-2 pseudovirus was produced by cotransfection of HEK293T cells with a 1:1 ratio of DNA plasmid encoding SARS-CoV-2 Spike (Genscript, USA) and backbone plasmid pNL4−3.Luc.R^-E^- (NIH AIDS Reagent)

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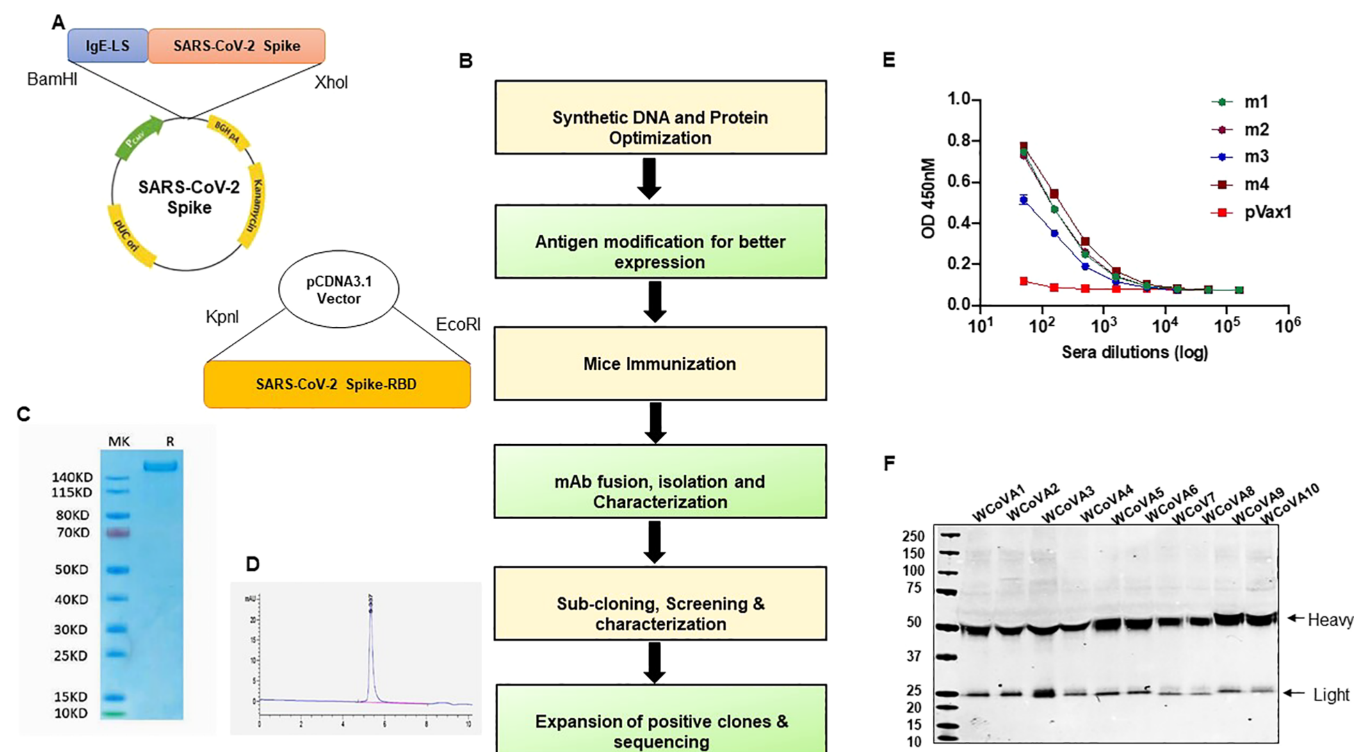

Figure 1. mAb development and expression of SARS-CoV-2 Spike full-length protein. (A) Schematic diagram of SARS-CoV-2 Spike inserts used to generate SARS-CoV-2 Spike DNA vaccine and recombinant SARS-CoV-2 Spike RBD protein synthesis. (B) BALB/c mice were immunized with a synthetic SARS-CoV-2-spike DNA as primer and boosted with SARS-CoV-2 RBD protein. One week after the final immunization, immune sera from the immunized mice were collected and then evaluated for the presence of antibodies against SARS-CoV-2 by ELISA. Upon confirmation, mouse splenocytes were harvested and fused to make hybridomas. Finally, positive hybridoma clones were characterized by ELISA, and those selected were subcloned and expanded for future characterization and analyses. (C) SARS-CoV-2 full-length Spike protein was expressed in a mammalian system with a Fc tag and Avi tag at the C-terminal and its size was confirmed in a Bis-Tris PAGE gel. (D) It exhibited >95% purity as shown by HPLC. (E) Validation of SARS-CoV-2 full-length protein's specificity by ELISA using the immune sera from SARS-CoV-2 Spike DNA-vaccinated mice ($n = 4$). In the case of all the mice sera samples, dose-dependent binding curves were obtained. m1−m4 designate 4 different mice for which binding curves were obtained. (F) Western blot analysis for IgG mAb clones (WCoVA1, WCoVA2, WCoVA3, WCoVA4, WCoVA5, WCoVA6, WCoVA7, WCoVA8, WCoVA9, and WCoVA10) for their expression of heavy and light chain by SDS-PAGE (12%) analysis.

using GeneJammer (Agilent, USA) in D10 medium. The supernatant containing pseudovirus was harvested at 48 h post-transfection and enriched with FBS to 12% total volume, steri-filtered, and stored at −80°C. The pseudovirus was titrated using the stable commercially available CHO-ACE2 cell line. For the neutralization assay, 10 000 CHO-ACE2 cells in 100 μL of D10 media were plated in 96-well plates and rested overnight at 37°C and 5% CO_2 prior to the neutralization assay. The following day, serially diluted samples were incubated with SARS-CoV-2 pseudovirus at room temperature for 90 min before the mixture was added to the already plated CHO-ACE2 cells. The cells were incubated at 37°C and 5% CO_2 for 72 h and subsequently harvested and lysed with BriteLite reagent (PerkinElmer, USA). Luminescence from the plates were recorded with a BioTek plate reader and used to compute percentage neutralization of the samples at each dilution.

Statistical Analysis. Statistical analyses were carried out using Graph Pad Prism software by either Student's *t*-test or the nonparametric Spearman's correlation test for calculating the statistical significance. The data are represented as the mean ± SEM. *p*-value of <0.05 was considered significant for the tests.

RESULTS

Generation and Characterization of Antibodies Targeting SARS-CoV-2 Spike. Increasing lines of evidence suggest that NAbs could be important for treatment or as preventives for several infectious diseases including respiratory syncytial virus (RSV) and now human immunodeficiency virus (HIV).[35,36] Notably, SARS-CoV and MERS-CoV vaccine studies revealed strong polyclonal antibody responses in an *in vivo* setting, which result in the inhibition of viral entry, suggesting the potential of anti-Spike antibodies to inhibit the entry of SARS-CoV-2 coronavirus.[24,31,37] Recent animal studies of a subset of anti-SARS-CoV-2 antibodies could inhibit disease in small animals[38−40] and most recently infection in larger animals.[20]

In this study, BALB/c mice were immunized with synthetic DNA plasmid constructs encoding the consensus sequence of full-length SARS-CoV-2 Spike antigen[41] as prime and SARS-CoV-2 Spike−RBD recombinant protein as boost as described earlier.[33] The strategy and the steps used for immunization and follow up procedures are outlined in Figure 1A,B. Finally, B lymphocytes were isolated from the spleens of the immunized mouse and were used to generate hybridomas. For the analysis of sera from the immunized animals, a full-length spike protein expression construct was generated. SARS-CoV-2 Spike full-length protein was expressed using the mammalian cell system

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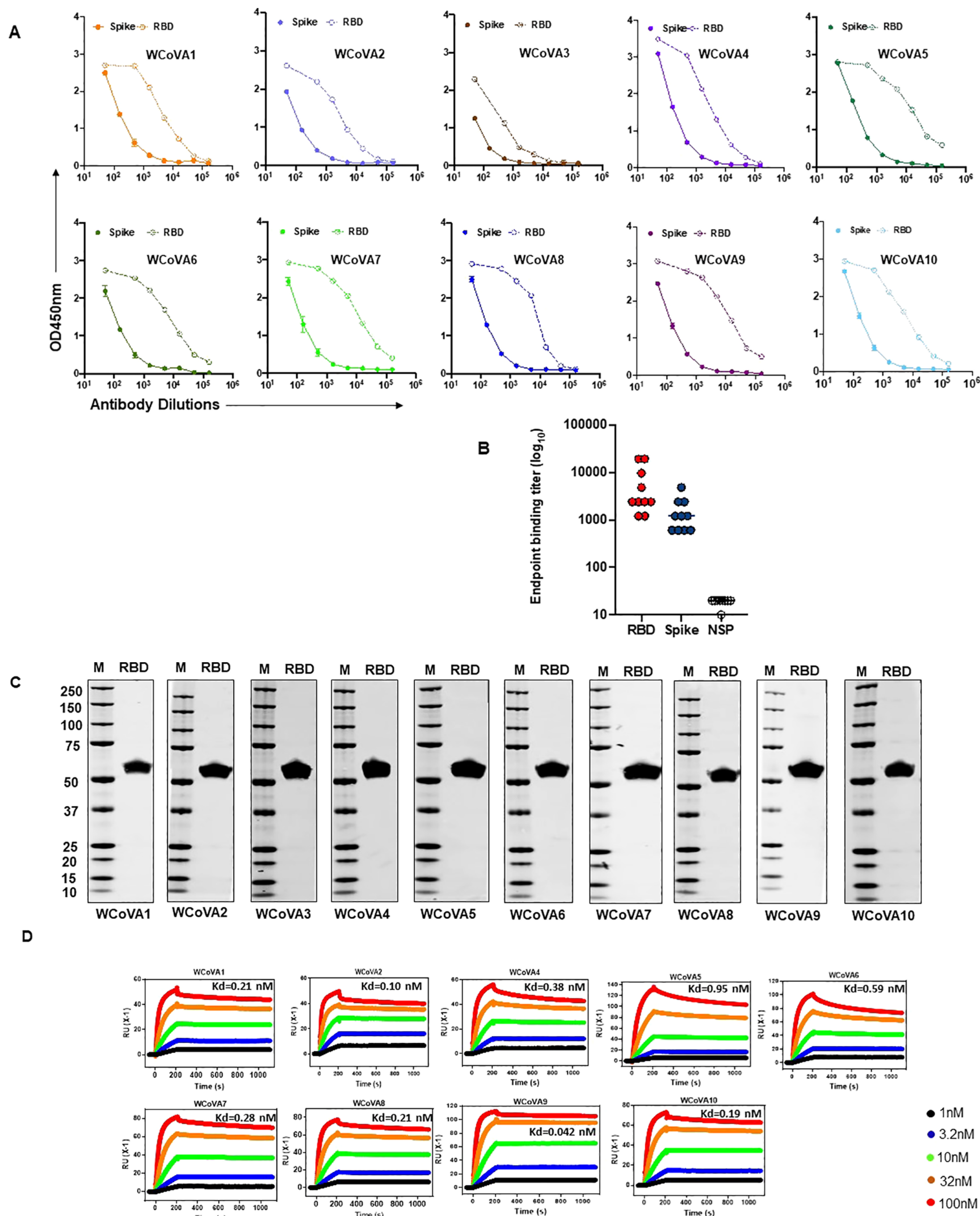

Figure 2. Characterization of SARS-CoV-2 Spike IgG mAbs. (A) Evaluation of IgG clones for their binding potential to SARS-CoV-2 full-length Spike and RBD. ELISA plates were coated with recombinant SARS-CoV-2 full-length Spike protein (1 μg/mL) as well as RBD (1 μg/mL, dashed line) and were tested with serially diluted mAb clones as indicated. (B) Serum IgG end point titers of the mAb clones to SARS-CoV-2 full-length Spike protein and RBD were determined using end-point titer ELISA. Recombinant Avi-Tag protein was used as binding control where no binding of IgG mAb clones was observed. (C) Binding specificity analysis of the mAb clones against SARS-CoV-2 RBD protein by Western blot. The antibodies were probed with goat anti-mouse IgG-IRDye CW-800 secondary antibody. (D) Binding kinetics of SARS-CoV-2 Spike mAbs and their target SARS-CoV-2 RBD by surface plasmon resonance analysis. Sensograms representing the responses of IgG mAb clones which exhibited robust binding to SARS-CoV-2 RBD, against time, showing the progress of their interaction.

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Table 1. Binding Kinetics of IgG mAb Clones and Their Target SARS-CoV-2 RBD Analyzed by SPR through Immobilization of SARS-CoV-2 RBD Using Standard Amine Coupling

clone	K_{on} ($\times 10^5$ M^{-1} s^{-1})	K_{off} ($\times 10^{-4} s^{-1}$)	$t_{1/2}$ (min)	K_D (nM)	R_{max} (RU)	mean capture level	expected R_{max}	% active IgG (1:1)
WCoVA1	3.7	0.78	148	0.21	45	405	81	56
WCoVA2	7	0.72	160	0.10	40	445	89	45
WCoVA4	4.1	1.6	72	0.38	47	438	88	54
WCoVA5	2.1	2	58	0.95	125	408	82	153
WCoVA6	3.7	2.2	53	0.59	86	458	92	94
WCoVA7	3.3	0.93	124	0.28	74	380	76	97
WCoVA8	3.9	0.82	140	0.21	69	250	50	138
WCoVA9	4.7	0.19	607	0.042	104	390	78	133
WCoVA10	3.6	0.70	165	0.19	66	380	76	87

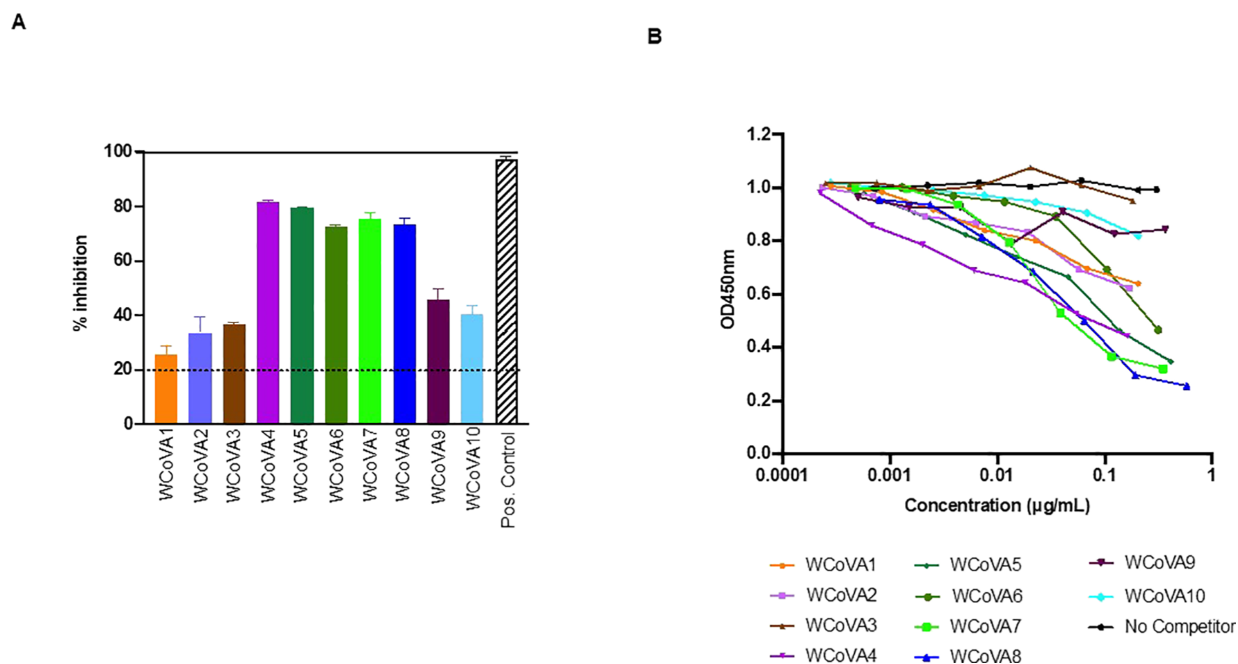

Figure 3. SARS-CoV-2- Spike mAbs compete with ACE2 receptor for SARS-CoV-2 Spike protein binding. (A) Serial dilutions of SARS-CoV-2 Spike IgG mAbs were added to SARS-CoV-2-coated wells prior to the addition of ACE2 protein. ACE2 binding to SARS-CoV-2 Spike mAbs was measured; competition was displayed as SARS-CoV-2 Spike IgG mAbs against ACE2 receptor binding to SARS-CoV-2 Spike protein. (B) Competition exhibited by SARS-CoV-2 Spike mAb clones against ACE2 receptor binding to Spike protein. Curves were normalized to the average absorbance of the buffer control (no competitor control or maximum absorbance). Each sample was run in duplicate.

with a Fc tag and an Avi tag at the C-terminus, and its size and purity were verified by SDS-PAGE and HPLC techniques (Figure 1C–D). As expected, the full-length spike protein (160 kDa) revealed a clear band at the correct position on Bis-Tris PAGE gel. This protein was also found to be of high purity as suggested by the sharp single peak obtained from HPLC. Furthermore, the protein specificity was confirmed by ELISA using immune sera from mice vaccinated with SARS-CoV-2 Spike DNA (Figure 1E). Hybridomas were then screened in order to determine the clones with ability to produce antibodies with the highest affinity against SARS-CoV-2 Spike protein. This screening led to the identification of 10 unique IgG mAbs (WCoVA1, WCoVA2, WCoVA3, WCoVA4, WCoVA5, WCoVA6, WCoVA7 WCoVA8, WCoVA9, and WCoVA10). The isotype analysis of the mAbs revealed that WCoVA1, WCoVA2, WCoVA3, WCoVA4, WCoVA7, and WCoVA8 clones exhibited an IgG1 heavy chain, whereas WCoVA5, WCoVA6, WCoVA9, and WCoVA10 clones expressed an IgG2a heavy chain. The individual hybridoma clones were further evaluated for their binding and specificity.

Binding and Specificity Analysis of SARS-CoV-2 Spike Monoclonal Antibodies. The antibody specificity was confirmed by Western blot analysis for heavy and light chain expression (Figure 1F). Furthermore, we investigated the ability of these mAbs to bind to full-length SARS-CoV-2 Spike as well as the RBD through an indirect ELISA. The results showed that all the mAbs specifically and strongly bound to both full-length Spike as well as the RBD antigen, whereas no binding was observed to the nonspecific (NSP) control. Figure 2A shows a dose-dependent binding curve for IgG clones represented by the average ELISA signals plotted versus different dilutions of mAb clones. All these clones showed high end-point titers (Figure 2B). The RBD protein is smaller than the full-length Spike antigen, so the higher binding observed in the case of the RBD compared to that of full-length Spike is likely an assay related difference, not a functional difference. Furthermore, the binding specificity of mAb clones were also confirmed by Western blot analysis. For this analysis, SARS-CoV-2 RBD protein was loaded and subsequently probed with equal concentrations of mAb clones as mentioned in the

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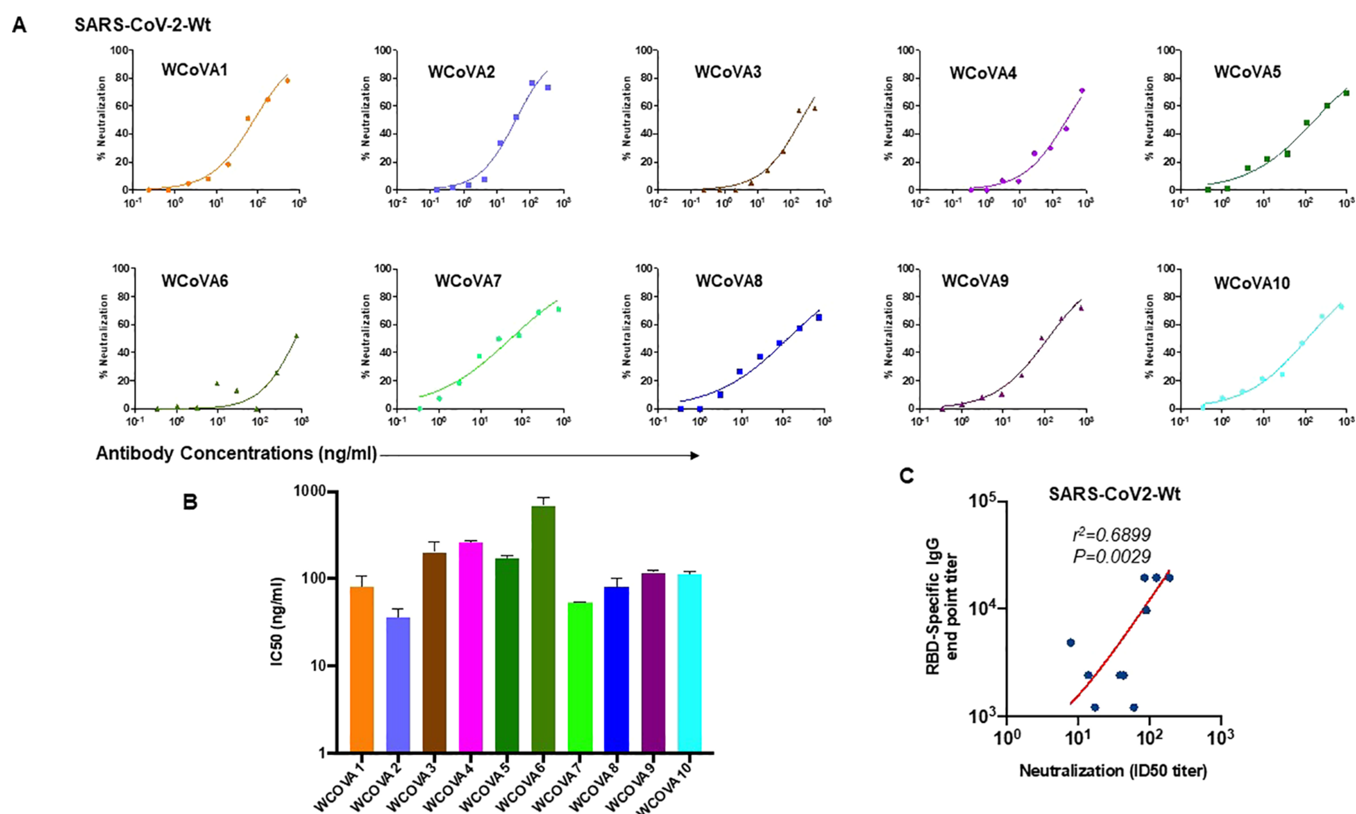

Figure 4. Neutralization efficacy of SARS-CoV-2 Spike mAbs against SARS-CoV-2 pseudovirus assay. The functionalities of SARS-CoV-2 Spike IgG mAbs were evaluated using a pseudovirus neutralization assay. (A) Neutralization of SARS-CoV-2 pseudovirus by SARS-CoV-2 Spike IgG mAb clones represented in terms of percent neutralization. (B) IC_{50} values of the SARS-CoV-2 Spike IgG mAb clones are represented in the bar graph. (C) Correlation between ELISA titers and viral neutralization titers. Statistical analysis was carried out in GraphPad Prism.

"Materials and Methods" section. All the antibodies bound specifically to SARS-CoV-2 RBD protein (Figure 2C).

Kinetic Analysis of SARS-CoV-2 Spike Antibodies and Their Target by SPR Analysis. SPR binding analysis is an important tool for mAb−antigen binding characterization. The binding kinetics of the mAb clones and their target, the RBD region of SARS-CoV-2 Spike, were analyzed by SPR. Initially, SARS-CoV-2 RBD was immobilized on a carboxymethyl dextran sensor chip via amine coupling. We used recombinant ACE2 to confirm that the SARS-CoV-2 RBD was functional after immobilization. The mAb clones were tested at 0.13, 0.41, and 1.3 nM concentrations. We observed that 9 out of 10 clones showed dose-dependent binding to SARS-CoV-2 RBD (Figure S1). These nine mAb clones were then further characterized to determine the kinetic parameters for the interaction. For this experiment, the mAb clones were immobilized on a Protein A/G sensor chip which allows for an estimation of the active antibody immobilized on the chip surface. The target immobilization level for the antibodies was 400 RU; given the MW ratio between the antibody and SARS-CoV-2 RBD, this would result in a theoretical R_{max} of 80 RU. The sensograms for these clones are shown in Figure 2D, and the binding kinetic values for all nine IgG mAb clones are summarized in Table 1. Very low K_D values (<1 nM) were obtained in case of all nine IgG mAb clones which strongly indicates a high affinity interaction with SARS-CoV-2 RBD protein. WCoVA9 exhibited both the highest affinity as well as the slowest dissociation rate. Three of the clones (WCoVA5, WCoVA8, and WCoVA9) had R_{max} values greater than 100% of the expected R_{max}. This suggests that these antibodies may bind to two molecules of SARS-CoV-2 RBD per antibody molecule, as previously reported by others.[42] Three of the clones (WCoVA1, WCoVA2, and WCoVA4) had R_{max} values around 50% of the expected R_{max}.

ACE2 Inhibition by SARS-CoV-2 Spike mAbs. The ACE2-binding ridge in the SARS-CoV-2 RBD possess a compact conformation.[43] NAbs with the ability to target the SARS-CoV-2 RBD have the potential to block ACE2 binding by the virus or to otherwise prevent viral entry and possibly protect cells therapeutically from infection. Therefore, therapeutically active mAbs targeting the interaction between SARS-CoV-2 Spike and the ACE2 receptor is of interest for screening the hybridomas. Nevertheless, antibodies with the ability to bind the RBD without blocking ACE2−RBD interaction are also reported to cause neutralization of SARS-CoV-2. Furthermore, neutralization antibodies to the NTD have also been evaluated, and some of them possess similar neutralization efficacy as the RBD-targeting antibodies.[44,45] Hence, we next evaluated whether these mAbs can block the interaction between the SARS-CoV-2 RBD and ACE2 with the help of a blocking ELISA. The results showed that the mAb clones could block SARS-CoV-2 RBD−ACE2 interaction with variable efficiency (Figure 3A). A total of five clones (WCoVA4, WCoVA5, WCoVA6, WCoVA7, and WCoVA8) were able to cause more than 70% inhibition of SARS-CoV-2 RBD and ACE2 interaction in this assay. Furthermore, we observed competitive inhibition of SARS-CoV-2 Spike binding to the ACE2 receptor in the presence of the mAbs using ELISA. In particular, clones WCoVA4, WCoVA5, WCoVA7,

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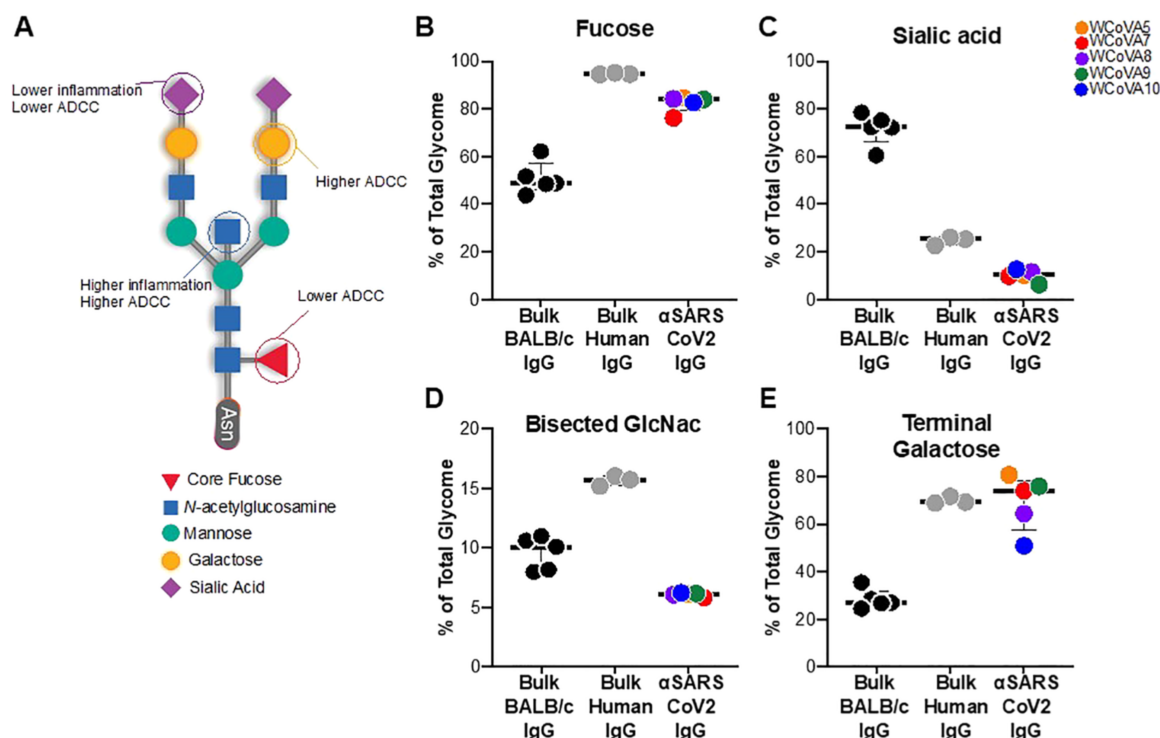

Figure 5. Glycomic analysis of SARS-CoV-2 antibodies reveals a profile compatible with higher Fc-mediated effector functions. (A) Schematic structure of antibody glycosylation highlighting several monosaccharides and their known impact on Fc-mediated effector functions. (B–E) Percentages of total core fucose (B), terminal sialic acid (C), bisected GlcNac (D), and terminal galactose (E), within the total glycome of five SARS-CoV2 antibodies, bulk IgG from five control BALB/c mice, and bulk IgG from a human control sample (ran in triplicate). Median and interquartile ranges are displayed.

and WCoVA8 exhibited high competition against ACE2 receptor (Figure 3B).

Neutralization of SARS-CoV-2 Pseudovirus by SARS-CoV-2 Spike Antibodies. NAbs possess the ability to inhibit viral infection through blockage of the replication cycle of virus.[46,47] We, therefore, next evaluated the SARS-CoV-2 Spike mAbs using a pseudovirus neutralization assay.[41] As expected, we observed pseudovirus neutralization by positive control ACE2-Ig but not in the case of negative control murine antibody TA99. Antibodies secreted by 10 out of 10 IgG mAb clones were capable of neutralizing more than 50% of the virus (Figure 4A). The 100% neutralization of SARS-CoV-2 pseudotyped virus was obtained in the cases of WCoVA7 and WCoVA9 mAb clones when they were evaluated using higher antibody concentrations (Figure S3). In addition, we also assessed the IC_{50} values of these clones which suggested they effectively blocked infection. mAb clones WCoVA1, WCoVA2, WCoVA7, WCoVA8, WCoVA9, and WCoVA10 were observed to be the most potent against the SARS-CoV2 pseudovirus (Figure 4B). Also, we observed a significant correlation between the total IgG end-point titers and the neutralization titers of these SARS-CoV-2 Spike mAbs (Figure 4C). These results suggested increased antiviral activity of these mAbs to be related to their increased binding affinity with RBD, but their detailed molecular interactions require further studies.

Glycomic Analysis of SARS-CoV-2 Antibodies. Non-neutralizing Fc-mediated effector functions of antibodies, including antibody-dependent cellular cytotoxicity (ADCC), play an important role in controlling viral infections.[48−56] Antibody glycosylation strongly impacts its effector functions. The presence of core fucose results in a weaker binding to Fcγ receptor IIIA and reduces ADCC.[57] Although core fucose has the most significant impact on ADCC, other glycomic features have also been shown to impact ADCC: Terminal sialic acid reduces ADCC,[58−60] bisecting GlcNAc induces both innate immune function and inflammation,[61−63] and terminal galactose induces ADCC (Figure 5A).[64] We examined the glycosylation of WCoVA5, WCoVA7, WCoVA8, WCoVA9, and WCoVA10. We found that all of these antibodies (especially WCoVA7) have lower levels of core fucose, lower levels of sialic acid, lower levels of bisecting GlcNac, and equivalent terminal galactose compared to bulk human IgG. These antibodies also have lower levels of sialic acid, lower levels of bisecting GlcNac, and higher levels of fucose and terminal galactose compared to bulk BALB/c IgG (Figure 5B–E). This glycomic profile suggests that these antibodies would exhibit high Fc-mediated effector functions (i.e., ADCC, antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC)).[65] Thus, examining the detailed Fc-mediated effector functions of these antibodies should be the subject of future studies.

DISCUSSION

A number of SARS-CoV-2 candidate vaccines are being developed across the world: Thirteen have been approved/authorized for use in different countries, some are under clinical evaluation, and many are in preclinical development. The different types of candidates include inactivated vaccines, DNA vaccines, RNA-based vaccines, protein subunit vaccines, live-attenuated vaccines, viruslike particle (VLP) vaccines, and nonreplicating and replicating viral vector vaccines.[66,67] Three vaccines, namely, BNT162b2, mRNA-1273, and Ad26.-

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Table 2. Characteristics of the Downselected SARS-CoV-2 Spike Monoclonal Antibodies[a]

antibody clones	spike binding ELISA	RBD binding ELISA	specificity by WB	SPR by Biacore	ACE2 blocking by surrogate virus neutralization assay	ACE2 blocking by competition ELISA	neutralization of SARS-CoV-2 pseudovirus
WCoVA1	+++	++	+++	++	+	++	+++
WCoVA2	++	++	+++	+++	+	++	+++
WCoVA3	+	+	+++	−	+	−	++
WCoVA4	+++	+++	+++	++	+++	+++	++
WCoVA5	+++	++	+++	+	+++	+++	++
WCoVA6	++	++	+++	+	+++	++	+
WCoVA7	++	+++	+++	++	+++	+++	+++
WCoVA8	++	+++	+++	++	+++	+++	+++
WCoVA9	++	+++	+++	+++	++	+	+++
WCoVA10	+++	+++	+++	+++	++	+	+++

[a]'+' indicates that the tested clones are positive with the respective assays. The number of '+' corresponds to a higher degree of positivity (higher binding, specificity, ACE2 blocking and neutralization), whereas '−' indicates that the tested clones are negative with the respective assays.

COV2.S, have been authorized for emergency use by the FDA. Two-dose regimens of BNT162b2 or mRNA-1273 have been reported to confer significant protection against COVID-19.[68,69] Furthermore, a single dose of Ad26.COV2.S, a recombinant, replication-incompetent adenovirus serotype 26 (Ad26) vector encoding a stabilized full-length SARS-CoV-2 Spike protein, is also highly protective.[70]

Despite the approval of several vaccine candidates, antibodies are being used for therapeutic treatment of infected individuals. Due to the development of viral escape mutants, additional antibody therapeutics may be important. Additionally, immunocompromised patients or individuals in high-age groups may not adequately mount protective immune responses to vaccination.[71] Thus, the continued development of effective therapeutics for treating COVID-19 is highly imperative. Many different potent neutralizing mAbs have been isolated using a wide array of approaches such as immortalized Epstein−Barr virus (EBV) memory B cells, antibody isolation from mouse hybridomas, phage display libraries, direct cloning of Ig-encoding genes from isolated B cells, microculture, and supernatant screening of sorted memory B cells.[72] As of April 13, 2021, more than 2000 SARS-CoV-2-targeting mAbs have been unveiled in various studies: 1150 are shown to target the RBD, whereas 585 of them are reported to exhibit a neutralization effect according to the reports of CoV-AbDab: The Coronavirus Antibody Database.[73] Studies have also supported the emergency use implementation for several NAb candidates and NAb cocktails.[71] The investigation cocktail of Regeneron Pharmaceuticals, Inc., REGN-COV2 (combination of casirivimab, REGN10933, and imdevimab, REGN10987) in mild to moderate nonhospitalized COVID-19 patients in phase I−III randomized clinical trials has been awarded an authorization for emergency use due to its ability to reduce viraemia and alleviate symptoms.[74] The BLAZE-1 trial by Eli Lilly and Company assessed the safety and potency of LY-CoV555 (bamlanivimab) and LY-CoV016. The results revealed that LY-CoV555, which functions through neutralization by IgG1 monoclonal Ab against SARS-CoV-2 Spike, ameliorated viral clearance at an earlier time point than the placebo, together with hospitalization rate and emergency room visits.[74]

However, the limited efficacy of the available NAbs as well as the rapid spread of new SARS-CoV-2 variants complicate the treatment strategies and stresses the requisite for continuing the development of new antibodies.[71] In this study, we have developed and successfully cloned 10 IgG mAbs specific to SARS-CoV-2 Spike protein. mAbs which target the vulnerable sites on viral surface proteins are increasingly known as a promising class of drugs against infectious diseases and have shown therapeutic efficacy for a number of viruses.[75] In line with this, identifying and cloning mAbs which can specifically target surface viral proteins to block viral entry into host cells seems to be a highly attractive approach for the prevention and treatment of SARS-CoV-2.[76] The spike protein of SARS-CoV-2 undergoes major conformational alterations, exposing the RBD and important residues for receptor binding in order to engage the host cell receptor ACE2. The binding of the RBD to ACE2 receptor protein leads to the detachment of S1 from S2, ultimately resulting in virus−host membrane fusion mediated by S2 and the entry of virus. Thus, the role of spike protein in the infection process of SARS-CoV-2 is highly critical and hence qualifies as a target for developing effective mAbs.[76] In order to be effective, the antibodies should meet several characteristics including specificity, high-affinity binding to antigen, and the ability to compete with the spike protein binding to receptor ACE2, thus blocking the infection of cells by the virus. All 10 mAbs were found to have specific and strong binding to SARS-CoV-2 RBD. Furthermore, they also caused blocking of the interaction between the SARS-CoV-2 RBD and the ACE2 receptor. In addition, the glycomic profile of the antibodies (especially WCoVA7) suggested they have high Fc-mediated effector functions. Therefore, the Fc-mediated effector functions of these antibodies need to be examined in more details. In addition, all 10 IgG mAb clones exhibited neutralization of SARS-CoV-2 Spike protein pseudotyped virus infection. The low IC_{50} values of WCoVA1, WCoVA2, WCoVA7, WCoVA8, WCoVA9, and WCoVA10 (<150 ng/mL) in terms of neutralization efficacy indicate their potency against SARS-CoV-2. Furthermore, antibody−antigen docking studies revealed the binding of WCoVA7 to the RBD and WCoVA9 to both the NTD and RBD regions (Figure S2). Altogether, these anti-SARS-CoV-2 Spike−ACE2 blocking mAbs hold significant potential and should be evaluated and explored further as possible therapeutic/prophylactic tools against SARS-CoV-2 and perhaps other similar coronaviruses (Table 2). For validation, clones WCoVA7 and WCoVA9 were recombinantly expressed using a pCDNA3.4 mammalian expression vector (unpublished data). The DNA-expressed antibodies neutralized SARS-CoV-2 pseudotyped virus with IC_{50} values of 97 ng/mL (WCoVA7) and 244 ng/mL (WCoVA9), respectively.

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The identification and characterization of new monoclonal antibodies against the Spike protein of SARS-CoV-2 add a valuable set of agents with possible therapeutic potential. Of the 10 mAbs described, all of them exhibit specificity and high affinity toward the RBD of the Spike protein. Hence, monoclonal antibodies have advantages over the use of polyclonal sera from convalescent patients recovering from COVID-19. On the basis of overall efficacy including Fc-mediated effector function, blockage of ACE2 and RBD interaction, and neutralization potential, WCoVA7 can be downselected for further studies, including the design of DNA-encoded monoclonal antibodies for additional studies. While the mAbs are independent from one another, the epitope recognized by the antibodies is not clear. The functional characteristics of the antibodies show their ability to neutralize SARS-CoV-2 Spike protein pseudotyped virus which is a surrogate measure of their effect on the virus. The antibodies should be evaluated in the infection studies with SARS-CoV-2 virus. The development of unique and specific mAbs against the Spike antigen and epitopes would enable the use of a "cocktail" (mixture of specific biologically active mAbs) to simultaneously engage multiple neutralizing epitopes on virions for enhanced therapeutic potency. Furthermore, the development of these additional biologically active anti-SARS CoV-2 neutralizing mAbs may provide novel epitopes for active immunization for prophylactic purposes. Finally, these reagents may be useful for the development of SARS-CoV-2-specific immune diagnostic assays.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.1c00058.

Data showing the binding of IgG mAbs to SARS-CoV-2 RBD protein by SPR analysis. Computational characterization of antibody−antigen docking. Neutralization efficacy of two most potent SARS-CoV2 Spike antibodies evaluated using higher antibody concentrations. (PDF)

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Notes

The authors declare the following competing financial interest(s): K.M. and D.B.W. are named inventors of the

PCT/US2020/63/068,868 patent application titled Antibody Constructs for use Against SARS-CoV-2. K.M. discloses grant funding, industry collaborations, speaking honoraria, and fees for consulting from Inovio Pharmaceuticals related to DNA and DMAb vaccine development. He has a patent application for DNA vaccine development and delivery of DNA-encoded monoclonal antibodies pending to Inovio Pharmaceuticals. Remuneration includes direct payments. D.B.W. discloses the following paid associations with commercial partners: GeneOne (Consultant), Geneos (Advisory Board), AstraZeneca (Advisory Board, Speaker), Inovio (BOD, SRA, Stock), Pfizer (Speaker), Merck (Speaker), Sanofi (Advisory Board), and BBI (Advisory Board). S.R., T.S., and L.H. are employees of Inovio Pharmaceuticals and as such receive salary and benefits, including ownership of stock and stock options. The other authors declare no competing financial interests. The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript. No writing assistance was utilized in the production of this manuscript.

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