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BRIEF REPORT

A novel biparatopic hybrid antibody-ACE2 fusion that blocks SARS-CoV-2 infection: implications for therapy

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ABSTRACT

In the absence of a proven effective vaccine preventing infection by SARS-CoV-2, or a proven drug to treat COVID-19, the positive results of passive immune therapy using convalescent serum provide a strong lead. We have developed a new class of tetravalent, biparatopic therapy, 89C8-ACE2. It combines the specificity of a monoclonal antibody (89C8) that recognizes the relatively conserved N-terminal domain of the viral Spike (S) glycoprotein, and the ectodomain of ACE2, which binds to the receptor-binding domain of S. This molecule shows exceptional performance *in vitro*, inhibiting the interaction of recombinant S1 to ACE2 and transduction of ACE2-overexpressing cells by S-pseudotyped lentivirus with IC50s substantially below 100 pM, and with potency approximately 100-fold greater than ACE2-Fc itself. Moreover, 89C8-ACE2 was able to neutralize authentic viral infection in a standard 96-h co-incubation assay at low nanomolar concentrations, making this class of molecule a promising lead for therapeutic applications.

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Biparatopic; neutralizing antibody; antibody-ACE2 fusion; COVID-19; SARS-CoV-2

Introduction

The COVID-19 pandemic is the biggest global health threat in the past century. As of May 2020, studies on at least 120 possible COVID-19 vaccines were already underway, but whether a safe and effective product could be developed was still unclear. Historically, scientists have never been able to develop a medically proven vaccine against any strain of coronavirus. Natural immunity to coronaviruses seems short-lived, and studies have suggested the possibility of re-infection after initial exposure to SARS-CoV.[1,2] However, even if the development of effective vaccines proved possible, it may still require years to produce and administer the products to the masses. Thus, especially during a novel outbreak, there is an urgent need for the discovery of other therapeutic options. The administration of convalescent plasma collected from recovered patients has shown adequate safety and provided protection from clinical complications, including fever and respiratory symptoms in severe COVID-19 patients.[3–5] However, the success of convalescent plasma therapy hinges on many factors, including the availability of donors, concentration of anti-SARS-CoV-2 neutralizing antibodies, and the safe preparation of serum to eliminate risks of viral transmission via transfusion.

During disease outbreaks, approaches to isolate neutralizing antibodies (nAbs) from recovered patients have proven successful. Since the beginning of 2020, similar campaigns from industry (e.g., Eli Lilly & Co, Regeneron) and academia (e.g., Tsinghua University, Peking University, Oxford University) to discover and develop nAbs have been launched for COVID-19. The monovalent affinities of these neutralizing antibodies toward the viral spike protein (S1) are usually relatively weak. This has made it necessary to screen large numbers of antibodies to select potent neutralizing antibodies, or apply computational approaches for *in silico* affinity maturation to improve binding affinities. To boost neutralization activity, a cocktail approach of combining two or more neutralizing antibodies has been sought, but at the cost of increased manufacturing burden. An alternative approach to block viral infection is to target the viral entry receptor, ACE2.[6] Recombinant ACE2 has been shown to reduce viral infection and growth in cell cultures, including organoids, by acting as a decoy for SARS-CoV-2.[7] The fusion of ACE2 to an Fc domain, producing a recombinant bivalent ACE2, could extend its physiological half-life and offer avidity toward viral S1, and thus increase the potency of blocking viral entry.[6,8] Here we describe the design and discovery of a biparatopic construct, in which a nAb (89C8) that binds to the N-terminal domain (NTD) of S1 is fused to recombinant ACE2 (89C8-

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ACE2). 89C8-ACE2 offers superior binding affinity to viral S1 protein with potent neutralizing activity as demonstrated by pseudotype and authentic virus infectivity assays. This design may also offer neutralizing capacity toward different strains of coronaviruses by avoiding the potential loss of binding due to mutations in the receptor binding domain (RBD) of S1 protein,[9] and offers insight into a universal therapeutic design that could be adopted for the treatment of other infectious diseases.

Results

Antibody selection

In this study, we aimed to isolate antibodies against SARS-CoV-2. We first collected human peripheral venous blood samples from 10 donors at the Fifth Affiliated Hospital, Sun Yat-Sen University. Using biolayer interferometry (BLI), serum samples from 3 to 10 donors displayed a strong reaction to SARS-CoV-2 S protein compared with the equivalent samples obtained from healthy donor controls (Supplemental Figure 1). Antibody libraries were constructed from B cells for yeast display screening. Three libraries ($>10^8$ unique sequences each) of individual donors were constructed separately to minimize heavy/light-chain mispairing. S1-specific Fabs that were displayed on yeast cells were selected using S1-protein-coated magnetic beads and subsequently sorted by fluorescence-activated cell sorting (FACS). A schematic diagram showing this workflow is illustrated in Figure 1.

A total of 473 individual clones were picked for sequencing, and 115 unique, paired Fab sequences were obtained. Of these, 50 unique Fab sequences were sub-cloned into a eukaryotic expression vector for the generation of monoclonal antibody (mAb) protein for subsequent testing. These 50 antibodies were tested for binding to HEK293 cells expressing the full length S1 protein of SARS-CoV-2, followed by further characterization. Further considerations of lead selection included thermal stability, nonspecific off-target binding and a faster intrinsic association constant toward S1 protein.

Design and construction of the biparatopic molecule

Next, our goal was to produce an anti-S1-recombinant ACE-2 fusion protein with biparatopic properties to provide superior binding affinity toward S1. Thus, we tested whether our antibodies could block the interaction between S1 and ACE2, with preference for the screening of non-blocking antibodies. One candidate, named 89C8, was chosen as the lead due to its faster association constant, lack of binding toward untransfected HEK293 cells, and a superior Fab Tm (82°C by differential scanning fluorimetry).

A tetravalent, biparatopic molecule was engineered with ACE2 fused with a stable $(G_4S)G$ linker to the heavy-chain C-terminal domain of 89C8 (Figure 2a). Alternative constructs with ACE2 fused to the N-terminus of either the LC or HC were also generated and included for comparison. We examined the binding of C-terminal and N-terminal ACE2 constructs to SARS-CoV-2 S1 in an Octet-based binding assay.[10] Interestingly, only the C-terminal constructs showed strong binding, whereas none of the N-terminal constructs could show any binding to viral S1. 89C8 alone showed fairly strong monovalent binding to S1, with a relatively slow dissociation rate of ~2E-04 S^{-1} (Figure 2b). ACE2-Fc exhibited a fast on/fast off profile, with a monovalent binding affinity of ~50 nM (Figure 2c). For the biparatopic molecule 89C8-ACE2, the monovalent dissociation was slower by a factor of 10 (Figure 2d). To determine the avidity of 89C8-ACE2, recombinant S1 protein was first biotinylated and immobilized on a streptavidin biosensor, then tested for binding against 89C8-ACE2. The biparatopic molecule tightly bound to S1 with a strong multivalent affinity (Figure 2e). Compared to the monovalent antibody molecule, our multivalent design may provide stronger binding affinity and possess potential for longer-lasting protection from infection. Proper negative controls were performed to confirm the lack of

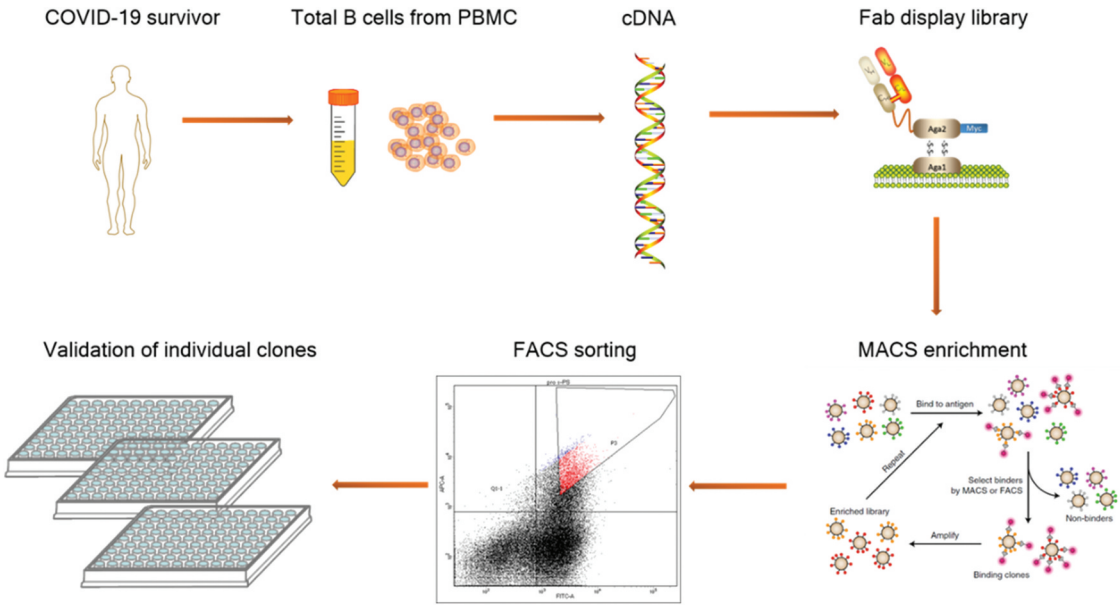

Figure 1. A schematic diagram showing the workflow of antibody discovery.

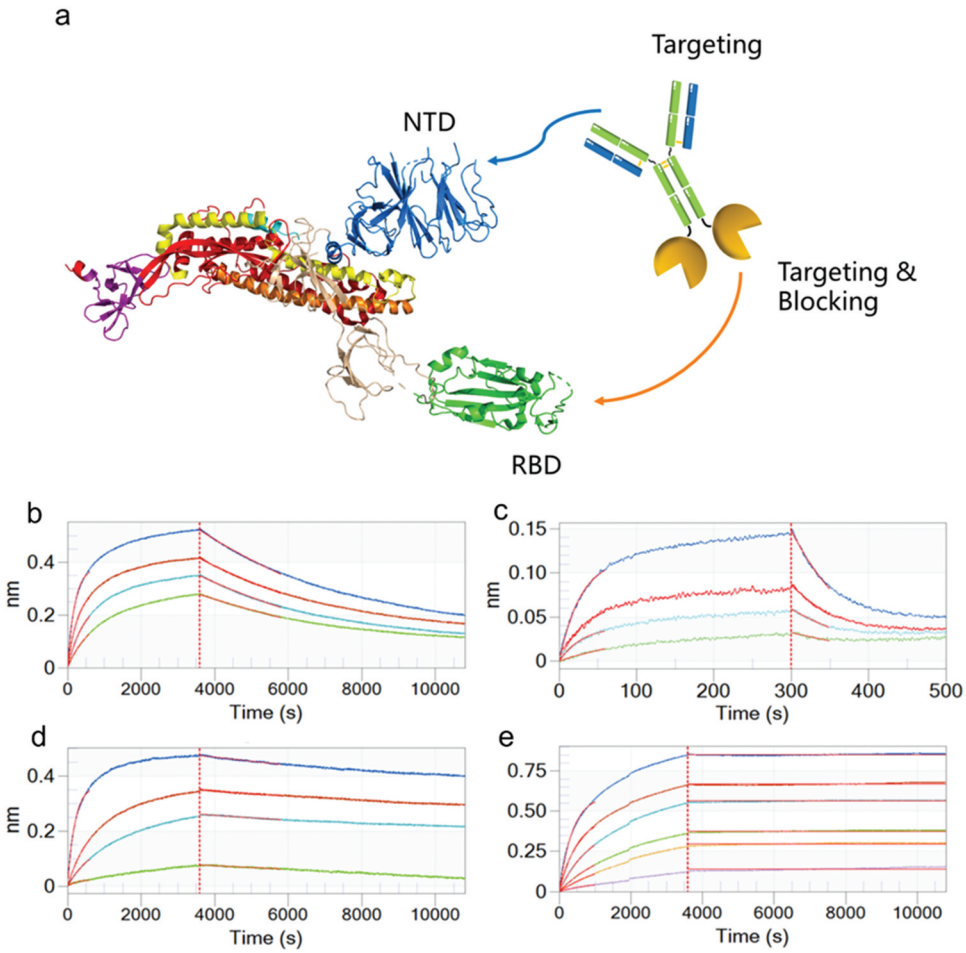

Figure 2. 2a) schematic illustration of the biparatopic 89C8-ACE2 antibody-fusion design; BLI binding kinetics of 89C8 (2b), ACE2-Fc (2 c) and 89C8-ACE2 (2d) binding to the spike protein S1 (monovalent K_D); 2e) 89C8-ACE2 binding to the spike protein in the avid format. In 2b-d, 2-fold dilutions of S1 protein, starting at 100 nM, were used. In 2e, dilution series of 89C8-ACE2 with a starting concentration of 20 nM, with 2-fold dilutions, were used.

nonspecific interactions between S1 protein with the sensors or between the test antibodies and bovine serum albumin.

To identify the site of the SARS-CoV-2 S protein bound by 89C8, a binding assay was performed using recombinant NTD and RBD of SARS-CoV-2 S1. 89C8 binds specifically to the NTD, but not RBD of S1 protein (Supplemental Figure 2), thus explaining the absence of competition between 89C8 and ACE2 for binding to SARS-CoV-2 S protein. ACE2-Fc fusion protein is a natural blocker of the S1/ACE2 interaction, but its blocking activity may be relatively weak due to its low monovalent binding affinity. A cell-based blocking assay was used to investigate the blocking activity of 89C8-ACE2 (Figure 3a). 89C8 mAb and ACE2-Fc were also included as controls for comparison. ACE2-Fc was able to block the binding of soluble SARS-CoV-2 S1 with ACE2 receptor transiently expressed on Chinese hamster ovary (CHO) cells with an IC50 of 5 nM. Compared to ACE2-Fc, 89C8-ACE2 enhanced the potency over a 100-fold with an IC50 of 55 pM, whereas 89C8 did not have such blocking activity.

Biparatopic 89C8-ACE2 is a potent neutralizer against SARS-CoV-2 infection

The neutralization potency of 89C8-ACE2 against SARS-CoV -2 infection was first assessed using an S-pseudotyped lentiviral reporter neutralization assay (Figure 3b). hACE2-overexpressing HEK293T cells were challenged with pseudotype virus encoding a luciferase reporter gene. Both 89C8-ACE2 and ACE2-Fc neutralized 100% of the input pseudotype virus, whereas 89C8 alone reached no more than 90% neutralization. Reciprocal IC50 neutralization titers (i.e., the concentration resulting in 50% reduction of infectivity) for 89C8-ACE2 against the pseudovirus was found to be 29 pM, which is much more potent than ACE2-Fc and 89C8, with IC50 values of 1.5 nM and 4.5 nM, respectively.

In the presence of 89C8-ACE2, infection of VeroE6 cells with authentic SARS-CoV-2 (Figure 3c) was neutralized with a PRNT50 and PRNT90 of 1.69 nM (95% confidence limits, 1.35–2.1 nM) and 6.23 nM (95% confidence limits 3.85–-10.9 nM), respectively, in a plaque reduction neutralization test. 89C8-ACE2 was superior compared to its parental molecule ACE2-Fc, which had PRNT 50 and PRNT 90 of 3.66 nM (95% confidence limits 2.28–6.05 nM) and 33.3 nM (95% confidence limits13.9–96 nM), respectively. However, 89C8 only showed very weak neutralization activity, below the confidence level for deducing a reliable PRNT50 value. Intriguingly, the neutralizing potential of 89C8-ACE2 was substantially higher than that of a potent neutralizing nanobody fusion, VHH72-Fc (15.7 nM), which binds to RBD-S1

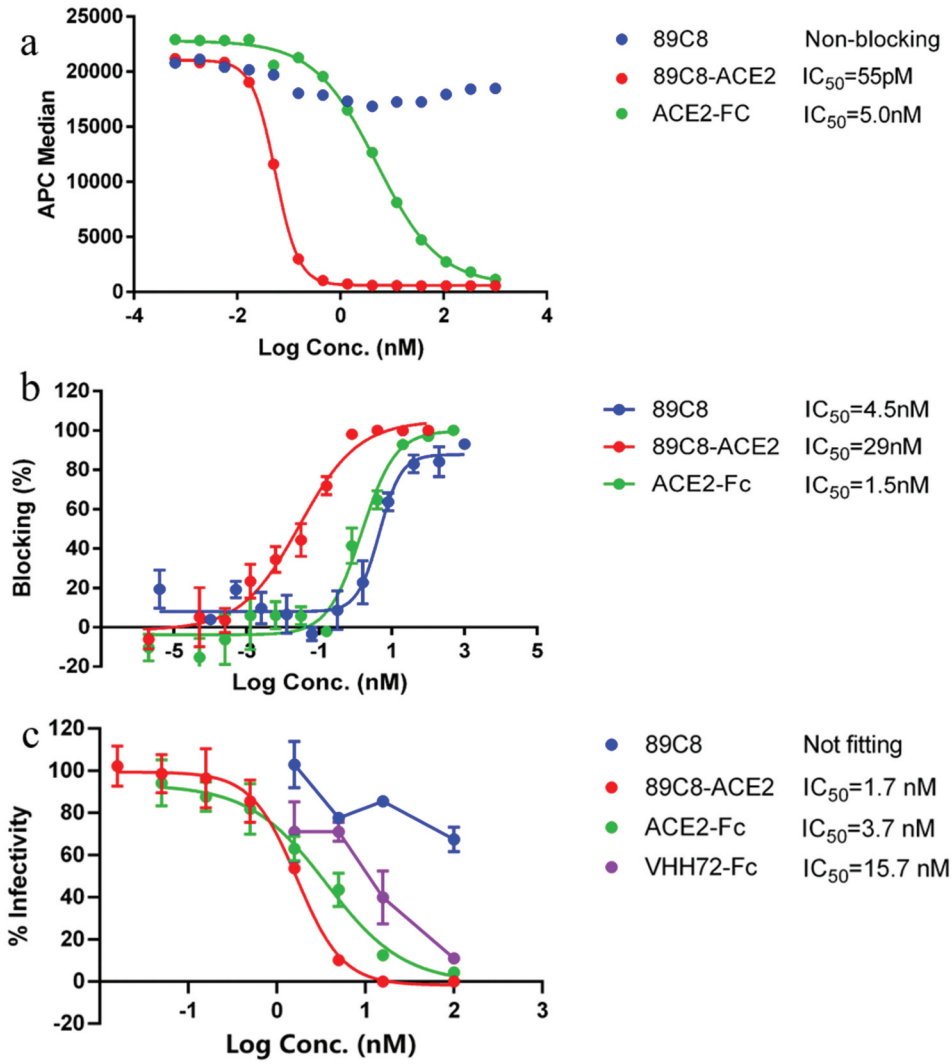

Figure 3. Blocking and neutralization experiments by 89C8, ACE2-Fc and 89 C8-ACE2 between 3a) Spike protein S1 (m-Fc format) with CHO cells overexpressing ACE2; 3b) pseudovirus with HEK293 cells overexpressing ACE2; 3 c) Live virus (Australia/Vic1/2020) plaque reduction neutralization test with Vero cells, VHH72-Fc was included as a positive control.

with broad neutralization activity against betacoronaviruses,[11] revealing the superior neutralizing activity of 89C8-ACE2. ACE2-Fc showed stronger than expected neutralization activity, likely due to the presence of aggregates (~40%) in the new batch of material obtained from vendor, which was absent from the in-house material used during pseudovirus assay.

Discussion

COVID-19 is a novel and rapidly evolving pandemic causing severe morbidity and mortality and huge economic losses worldwide. The winter season has been correlated with the outbreak of SARS-CoV and SARS-CoV-2,[12] and it is possible that COVID-19 may reemerge in the Northern hemisphere during the winter. No approved vaccines or targeted therapeutics are available for COVID-19, and researchers are exploring various medical interventions, including neutralizing antibodies. Development of nAbs targeting vulnerable sites on viral surface proteins is considered to be an effective approach to combat viral infection, including coronaviruses.[13]

Potent nAbs to SARS and MERS that often target S1 and S2 to disable binding between the RBD and receptor (targeting S1-RBD), interfere with the conformational change of S protein (targeting S1-NTD) or hinder S2-mediated membrane fusion (targeting S2),[14] and can thus block the infection of SARS-CoV -2 into host cells. Although S1-RBD is the most targeted domain for nAb development for SARS and COVID-19 (as RBD-specific antibodies have shown greater potency to neutralize infection with divergent virus strains), S1-RBD mutations with enhancing structural stability and ACE2 affinity were observed recently from circulating SARS-CoV-2 strains.[15] On the other hand, S1-NTD specific neutralizing antibodies have been shown to be potent in blocking SARS-CoV infection,[16] due to the high homology of NTD in SARS-CoV and SARS-CoV-2, and thus the NTD could be a common target for vulnerability.[17] ACE2 is the cell entry receptor for SARS-CoV-2. Studies have recently demonstrated that soluble ACE2 fused to Ig[16,18] or human recombinant soluble ACE2[7] can inhibit SARS-CoV-2 infection, suggesting that an ACE2 decoy could be a promising strategy for COVID-19 treatment.

Previous studies have also demonstrated that ACE2 is also playing a protective role during pulmonary edema, so ACE2 serves both as the entry receptor for SARS-CoV and to protect the lung from injury.[7,19] The severity of SARS could be partially explained by viral S binding to and depletion of ACE2,[18,20,21] causing a detrimental cycle of viral infection and loss of protection from local lung injury. Therefore, an ACE decoy that lures the virus to attach itself to the decoy rather than the target host cells may protect the host from lung injury.

Here, we designed and generated a novel biparatopic antibody combining the above-mentioned concepts by fusing two copies of ACE2 to the C-terminal domain of a neutralizing antibody (89C8) that targets S1-NTD. Our approach is different from isolation of neutralizing antibodies from recovered patients by single B cell cloning technology, which maintains the original HC and LC pairing. In our case, we built a recombinant Fab library for selection of higher affinity antibodies against the S1 protein. During the library building process, there is a LC and HC shuffling step, which gives the HC a chance to find a better LC pair, in terms of the binding affinity toward the S1 protein, at the cost of original pairing. The risk for the newly paired LC and HC combination is the potential general stickiness that is usually negatively selected away by natural biological processes. To exclude antibodies with such undesirable biophysical properties, we performed systematic stickiness tests, such as hydrophobic interaction chromatography, empty HEK293 cell binding, and thermal stability testing. 89C8 was chosen due to its high binding affinity, clean binding profile against blank HEK293 cells and good thermal stability. This antibody-ACE2 fusion design takes advantage of distinct neutralization mechanisms, via direct blocking and conformation-locking on the same S1 protein, and offers ultrahigh avidity. It was noted that 89C8-ACE2 binds to S1 with potent multivalent affinity and exhibits long-lasting effects. No detectable dissociation was observed for up to 2 hours. Furthermore, we confirmed the high blocking potency of 89C8-ACE2 toward SARS-CoV-2 S1 and pseudovirus-expressing S Ag to hACE2-expressing cells, with IC50s in the picomolar range. 89C8-ACE2 exhibits substantial neutralizing activities against pseudovirus expressing S Ag or live virus *in vitro*, with superior activity over its parental mAb or ACE2 decoy, as well as a known potent neutralizer, VHH72-Fc. VHH72-Fc possesses a broad range of neutralizing activity against beta-coronaviruses. Our data suggest that 89C8-ACE2 inhibits SARS-CoV-2 infection through a novel mechanism that takes advantage of added avidity effects via binding to both NTD-S1 and RBD-S1 simultaneously.

In our authentic virus neutralization test, we designed the experiment to mimic viral infection and antibody/drug neutralization similar to a physiological setting, where long-lasting protection is required. In other live virus blocking/neutralizing assays, the virus and drug complexes are allowed to interact with the cells for just 1 h before washing, followed by a short 8-h incubation. This may not reflect the real neutralizing power of the drug. The experimental differences also reveal the weak neutralizing activity of 89C8 alone, or in general NTD-targeting antibodies. These antibodies neutralize viral infection through locking of the NTD conformation, thus preventing downstream events from occurring. As these antibodies do not prevent the association between the S1 RBD and cell surface ACE2, the antibody:NTD complex needs to be maintained tightly. On the other hand, for direct blockers, even after antibody dissociation, the S1 protein still requires virus particles to migrate into close proximity to ACE2-expressing cells, allowing formation of new S1:drug complexes with circulating free drug in serum. This maintains the neutralization complexes distal from ACE-2-expressing tissues.

In summary, this type of biparatopic hybrid antibody-receptor design, either alone or in combination, may offer the potential to treat COVID-19, or can be adopted for other emerging diseases. Antibody combinations targeting distinct epitopes may act synergistically, resulting in reduction of the dosage required for therapeutic potency as well as the risk of immune escape through mutational changes.

Materials and methods

Yeast display library construction and antibody selection

Human peripheral blood samples were collected from recovered COVID-19 patients at the Fifth Affiliated Hospital, Sun Yat-Sen University according to approved protocols and with informed consent from patients. Blood was drawn roughly 10 days after fully recovery of a patient with two RNA test negative results to confirm absence of virus. Blood from two healthy donors were included as negative controls. Mononuclear cells were isolated from the blood by Ficoll-Paque (GE Healthcare) density gradient centrifugation according to the manufacturer's instructions. Total cellular RNA was isolated from cells using TRIZOL reagent (Invitrogen). cDNA was synthesized using SuperScript™ IV First-Strand Synthesis System (Invitrogen) primed with oligo (dT). Sequences of the antibody variable region were amplified using PCR. The light chain library was constructed by co-transforming linearized pFabVk vector and light chain PCR product by yeast gap repair. The Fab library was then constructed by co-transforming linearized pFabVH vector and heavy chain PCR product into the light chain library. Dual selection using Ura- and Trp- was performed to enrich the positive clones. Expression of Fab on the cell surface was induced in G/RCAA (substituting 2 g/L each of galactose and raffinose for the dextrose in SDCAA medium), and Fab-expressing cells were sorted by MACS (magnetic bead aided cell sorting, Miltenyi, Germany) then 4X by FACS (BD, AriaIII) using biotinylated SARS-CoV2 spike 1 protein (Supplemental Figure 3).

Recombinant antibody expression

The sequence of antibody 89C8 is listed in patent application 2020104153228. The extracellular domain of ACE2 (the peptidase domain only) was linked to the C-terminal of the heavy chain for 89C8 through a $(G_4S)_4G$ linker for the construction of 89C8-ACE2. The cDNAs encoding the variable regions of heavy (human IgG1 heavy chain) and light (kappa light chain constant regions) chains were cloned into expression plasmids (pCDNA3.1). The heavy- and light-chain encoding plasmids were transiently co-transfected into Expi293 cells (Life

technologies) using polyethylenimine. Transfected cells were incubated for 7 days at 37°C. IgG in the supernatants was purified by Protein A magnetic beads (Genscript) according to manufacturer's instructions with high purity (Supplemental Figure 4) by SEC.

K_D determination

Binding affinities of IgGs (including 89C-ACE2 and ACE2-Fc) and S1 protein were determined by BLI using an Octet 384 (Pall Life Sciences). IgGs were loaded onto AHC sensors prior to exposure to SARS-CoV-2 S1 antigen in solution (300s or 3600s) for association and then dissociation in a buffer solution (300s or 7200s). Data acquisition was performed at 30°C. Data were analyzed using the ForteBio Data Analysis Software. The data were fit to a 1:1 binding model to calculate an association and dissociation rate, and K_D was calculated using the ratio kd/ka.

Cell line generation and culture

HEK293T cells purchased from ATCC (CRL-3216TM) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) supplemented with GlutaMAX. Human ACE2 coding sequence (Uniport: Q9BYF1) was amplified (Genewiz) and cloned into pLVX-Puro vector. HEK293T cells were transfected with pLVX-Puro-hACE2 in addition to psPAX2 and pMD using Lipofectamine 3000 (Invitrogen). 24 h post transfection, media was replaced with complete media and further incubated 24 h before collection of supernatant. HKE293T or CHO-S were then infected with viral supernatant and selected using puromycin.

Cell-based blocking test

Gradient diluted antibodies or antibody-ACE2 fusion proteins were first pre-incubated with 0.1 nM S1-mFc (Sino Biological) at 37°C overnight, followed by incubation with CHO cells stably expressing ACE2 protein (CHO-ACE2) for 1 h at room temperature. The cells were washed and subsequently incubated with allophycocyanin (APC)-labeled goat anti-mouse IgG (Biolegend, catalog#405308) at 4°C for 30 min. APC fluorescence signals were determined using a Beckman flow cytometer and results were analyzed using GraphPad7 software.

Pseudotype virus-based neutralization assay

Pseudoviruses were purchased from National Institutes for Food and Drug Control (NIFDC) China. Spike genes (strain Wuhan-Hu-1, GeneBank: MN908947) were cloned into pcDNA3.1 after codon-optimization for human cell expression. The pseudoviruses were produced with VSV G backbone that packages expression cassettes for firefly luciferase.[22] HEK293T overexpressing hACE2 were seeded into the 96-well plates 16 h before infection. 325 TCID50 pseudovirus were incubated with equal volume of fivefold serially diluted antibodies overnight at 37°C to reach equilibrium. The mixture of pseudoviruses and the tested molecules/antibodies was added to the cells. After 24 h incubation at 37°C, cells were lysed and luminescence was measured with the Bio-Glo™ Luciferase Assay System (Promega). The IC50 values were calculated with non-linear regression log (inhibitor) vs. response (four parameters) using GraphPad Prism 7 (GraphPad Software, Inc.).

Authentic virus plaque reduction neutralization test

Plaque reduction neutralization tests were done essentially as originally described[23] with modifications.[24] Virus suspension (sequence-verified passage 4 of SARS-CoV-2 Victoria/01/2020) at appropriate concentrations in DMEM containing 1% FBS (D1; 100 µL) was mixed with test protein (antibody or recombinant chimeric protein, 100 µL) diluted in D1 at a final concentrations of 100 nM, 15.8 nM, 5 nM, 1.51 nM, 0.5 nM, 0.15 nM, 0.1 nM, and 0.018 nM, in triplicate, in wells of a 24-well tissue culture plate, and incubated at room temperature for 30 minutes. Thereafter, 0.5 mL of a single cell suspension of Vero E6 cells in D1 at 5 E5/mL was added and incubated for 2 h at 37°C before being overlain with 0.5 mL of D1 supplemented with carboxymethyl cellulose (1.5%). Cultures were incubated for a further 4 days at 37°C before plaques were revealed by staining the cell monolayers with amido black in acetic acid/methanol. The PRNT90 and PRNT50 values were calculated using the "FindECanything" non-linear curve fitting procedure of GraphPad Prism 8.4.1, with F set to 10 and 50, respectively.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Author contributions

M.X.N., Y.Z.J. and T.Y.Z. contributed to library construction, antibody selection and construction of antibody-fusion. L.Y. and C.L.D. contributed to pseudovirus neutralization assay. H.X. contributed to the collection of blood samples from COVID-19 recovered patients and isolation of B cells. L.S., J.W., T.A. and X.Y.D. contributed to manuscript writing. F.W. and Y.X. constructed library for selection. C.C. constructed ACE2 expressing CHO and HEK293 cell lines. W.C., J.W.C., G.W., S.X.D., Y.Y. contributed to the production and purification of transient material and generation of stable cell line and purification process development. P.T.L., Z.Y.F., F.W.H., W.L.P. contributed to the characterization and formulation of antibodies and antibody-fusion samples. J.G.J., K.M., T.K.T., R.P., T.A. and J.W. contributed to the generation of VHH72 and live virus neutralization test. S.J. and L.X.L. conceived the idea for the initiation of this project.

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