Generation and characterization of humanized synergistic neutralizing antibodies against SARS-CoV-2

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Abstract
The emerging coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the causative agent of coronavirus disease 2019 (COVID-19), which has become a severe threat to global public health and local economies. In this study, several single-chain antibody fragments that bind to the receptor-binding domain (RBD) of the SARS-CoV-2 spike (S) protein were identified and used to construct human-mouse chimeric antibodies and humanized antibodies. These antibodies exhibited strong binding to RBD and neutralization activity towards a SARS-CoV-2 pseudovirus. Moreover, these antibodies recognize different RBD epitopes and exhibit synergistic neutralizing activity. These provide candidate to combination use or bispecific antibody to potential clinical therapy for COVID-19.

KEYWORDS
COVID-19, humanized neutralizing antibody, pseudovirus system, SARS-CoV-2, synergistic neutralizing activity

1 | INTRODUCTION

The emerging coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the causative agent of coronavirus disease 2019 (COVID-19) and has become a severe threat to global public health and local economies. As of the end of February 2022, this novel virus had led to over 426 million infections, with 5.91 million deaths worldwide. (https://www.cdc.gov/coronavirus/novel-coronavirus-2019.html).

Unfortunately, the number of SARS-CoV-2 infections continue to increase. It has been well-established that antibody-based passive immunotherapy is an effective approach to combat virus infection.1 Recent reports have demonstrated that the administration of convalescent plasma containing neutralizing antibodies can improve the clinical status of some critically ill patients2-5; however, the outcomes of passive plasma therapy are unpredictable, and the widespread clinical use of plasma therapy is unrealistic. Therefore, it is important to develop neutralizing antibodies that can be administered to enhance the prevention and treatment of COVID-19.

It has been well established that SARS-CoV-2 spike (S) protein played a critical role in the entry into host cells which is an important determinant of viral infection and pathogenesis. The S protein consists of two subunits: S1 and S2. In general, S1 protein-bonded cells expressing the viral receptor, angiotensin-converting enzyme 2 (ACE2), through the receptor-binding domain (RBD) and the RBD protein also harbored the binding sites for virus-neutralizing antibodies. Therefore the blocking of viral binding to the cell through RBD has become a major target for the design of the candidate vaccines and drugs.6

Currently, several research efforts have been devoted to the characterization of SARS-CoV-2 neutralizing mAbs specific to RBD proteins.7-9

The convalescent serum of recovered patients is optimal for the preparation of potent neutralizing monoclonal antibodies (mAbs). However, limitations of strict hospital management and inconvenient
transportation during home quarantine measures, made the collection of sufficient convalescent serum samples challenging. In this study, we constructed a murine phage antibody library against the RBD protein of SARS-CoV-2. Using antibody display technology, a panel of chimeric human-mouse neutralizing antibodies were generated and characterized. The results indicate that some mAbs exhibited favorable biological activity in vitro for the inhibition of viral entry into host cells and neutralizing SARS-CoV-2, providing the possibility for further therapeutic research.

2 | MATERIALS AND METHODS

2.1 | Plasmids, viruses, and cells

Phagemid pADSCFV-S was used to construct a single-chain antibody fragment (scFv) antibody phage library. The antibody eukaryotic expression vectors, pTSE-G1n and pTSE-K, which contain the constant region of the human heavy chain and light chain, respectively, were used to create the complete antibody molecules as previously described. A plasmid encoding an Env-defective, luciferase-expressing HIV-1 genome was kindly provided by Prof. Lihua Hou. pCAGGS-WSS was constructed to encode the wild-type SARS-CoV-2 S protein (deleting the 19AA of the C-terminal), which was constructed for preparation of the pseudovirus.

The Vero E6 cell line was propagated in Modified Eagle's Medium (MEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; HyClone); HEK293T and Huh (MEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; HyClone); HEK293T and Huh (MEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; HyClone). All the cells were incubated at 37°C in a 5% CO2 incubator.

SARS-CoV-2 was isolated from the lung lavage fluid of an infected patient. All research involving live SARS-CoV-2 was performed in a bio-safety level 3 (BSL-3) containment laboratory at Beijing Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, China. All work with the pseudo-virus was performed under bio-safety level 2 (BSL2) conditions.

2.2 | Construction of a mouse scFv-phage antibody immune library

Mice immunizations were performed in accordance with institutional regulations and guidelines. Five 6–8-week-old, specific pathogen-free BALB/c female mice (purchased from Beijing Laboratory Animal Center), were intraperitoneally administered 20 µg recombinant SARS-CoV-2 RBD protein (Sino Biological Inc.) diluted in phosphate-buffered saline (PBS), followed by two similar immunizations 2 and 4 weeks later. An enzyme-linked immuno-sorbent assay (ELISA) was applied to determine the titer of the antibody in mice. The mice with the highest and specific activity against the RBD protein were selected for the construction of a mouse scFv-phage antibody immune library. A final booster dose of 20 µg RBD protein was administered 3 days before the spleen was removed from the hyper-immunized mice. Serum collected from the immunized mice was used as a positive control.

The total RNA was extracted from the spleen tissue of the hyper-immunized mice using an RNA isolation kit (Omega). Variable light (VL) and variable heavy (VH) chain genes were amplified by RT-PCR and fused to the scFv gene using overlay-extended PCR. The scFv gene repertoire was digested with Not I/Sfi I and inserted into a phage display vector (pADSCFV-S). Competent Escherichia coli TG1 cells were transformed with the ligation mixture by electroporation. The final scFv antibody gene libraries were identified, aliquoted, and stored at −80°C. The resultant recombinant phage library was produced by the addition of a wild-type M13K07 helper phage.

2.3 | Phage scFv clone selection of the SARS-CoV-2 RBD protein

Selection of scFv phage clones from the library was accomplished via four successive rounds of affinity enrichment with the SARS-CoV-2 RBD protein. Both the input and output phages in each cycle were titrated on TYE plates. The general binding specificity of the selective phage clones was detected by a phage-ELISA. The clones which had an optical density (OD) value greater than or equal to 2.0 was selected to operate a DNA sequence analysis. The VH and VL sequences of the scFv was determined using the IMGT database (http://www.imgt.org/IMGTlec/).

2.4 | Human-mouse chimeric immunoglobulin G (IgG), humanized IgG, and purification

VH and VL chain regions from the 12 selected scFv clones were amplified by PCR and recloned into the pTSE-G1n and pTSE-K plasmids for IgG1 heavy chain and light chain expression to express human-mouse chimeric IgG. To construct humanized IgG, the VH and VL amino acid sequences were analyzed on the website http://www.abysis.org/abysis/; the amino acid residues with frequency less than 0.1 in the framework were replaced by high frequency in Homo sapiens to increase the degree of humanization according to the Z-score. The space structure of the humanized IgG was constructed with Swissmodel, and the accessible surface area of amino acid residue solution was analyzed to determine which amino acid residues could be humanized.

For antibody purification, FreeStyle™ 293-F cells were transfected with equal quantities of heavy chain and light chain expression plasmids using FectoPRO transfection reagent (Polyplus-Transfection) for complete antibody expression. Four days posttransfection, the harvested supernatants were clarified by centrifugation, filtered through a 0.22 µm filter membrane, and loaded onto a HiTrap MabSelect Xtra Column (GE Healthcare). The successfully expressed and purified antibodies were then buffer-exchanged in PBS, sterilized by filtration.
through a 0.22-μm filter (Millipore), and stored at a concentration of 1 mg/ml at 4°C for further research.

### 2.5 | ELISA assay

The wells of ELISA plates (9018; Costar) were coated with 200 ng antigen and incubated overnight at 4°C. The wells were subsequently blocked with 200 μl of 5% (w/v) skimmed milk dissolved in PBS for 2 h at 37°C, followed by the addition of 200 μl antibody, and another 2 h incubation at 37°C. The plates were washed three times with PBS-Tween (0.1% v/v), goat anti-mouse horseradish peroxidase-conjugated IgG antibody (1:5000, v/v) was added, and the plates were incubated for 1 h at 37°C. Finally, three washes in PBS-Tween were performed, and detection at 492 and 630 nm was performed using o-phenylenediamine (OPD) chromogen substrate.

### 2.6 | KD analysis

KD of monoclonal antibodies was measured by ForteBio® Octet QK® System (Pall ForteBio Corporation), which based on biolayer interferometry (BLI). The purified antibodies were diluted to 200 nM by HBS-EP buffer and then fixed on Anti-hIgG Fc Capture (AHC) biosensors. After a 1 min baseline with HBS-EP, the biosensors were immersed in sample wells containing a series of gradient diluted SARS-CoV-2 RBD proteins for association, and then the dissociation step was performed. The KD were calculated in Data Analysis Software 7.0 (Pall ForteBio Corporation) using the 1:1 binding model.

### 2.7 | Competitive binding assay between RBD and MAbs

Competitive binding experiments were performed using a ForteBio® Octet QK® System (Pall ForteBio Corporation) to determine whether the neutralizing antibodies blocked the binding between RBD and ACE2. Purified ACE2 in HBS-EP buffer (Cytiva) at 300 nM was loaded on an individual biosensor followed by a 1 min wash in HBS-EP buffer. Afterward, a 10 min association was performed in the sample plate with either 150 nM RBD or RBD combined with MAbs. The results were analyzed using Data Analysis Software 7.0 (Pall ForteBio Corporation).

### 2.8 | Competitive binding assay between MAbs

The competitive binding experiment was performed using a ForteBio® Octet QK® System (Pall ForteBio Corporation) to determine whether the binding region of the humanized antibodies on the RBD surface were the same. Purified SFC3 (200 nM) in HBS-EP buffer was loaded on an individual Anti-hIgG Fc Capture (AHC) biosensors followed by a 1 min wash to 1000 rpm in HBS-EP buffer. Afterward, a 10 min association was performed in the sample plate with 400 nM RBD or HBS-EP buffer as a control. Next, a reassociation step was executed in a new sample plate containing either 200 nM purified antibodies, SFC1, HSA-1F, or no correlation antibody. Background binding of RBD to sensor was performed for quality control by detecting the binding to biosensor loaded with control human IgG. The reverse experiment with SFC1, HSA-1F captured on AHC biosensors was performed in the same manner. The results were analyzed using Data Analysis Software 7.0 (Pall ForteBio Corporation).

### 2.9 | Pseudovirus-based neutralization assay (PBNA)

A human immunodeficiency virus (HIV) pseudovirus system was employed to produce SARS-CoV-2 pseudovirus particles, which was expected to present the SARS-CoV-2 S protein (NC_045512.2, Genbank) on the surface of the HIV-based virions. Briefly, 30 ml FreeStyle™293-F cells (1 × 10⁷/ml) were cotransfected with 18 μg of the pNL4.3-Luc-R′E– plasmid and 6 μg of pcAGGS-WSS. After 72 h, the supernatants of the transfected cells were harvested, titered, aliquoted, and stored at −80°C.

In the pseudovirus neutralization assay, the assessment of neutralization ability of the test antibody was based on the detection of a change in luciferase gene expression in the pseudovirus. The pseudovirus was preincubated with serially diluted antibodies in PBS at 37°C for 1 h before it was added to 96-well culture plates containing HuH7 cells in triplicate. After 48 h, the cells were lysed with 40 μl Passive Lysis Buffer. After a 10 min incubation at room temperature, the lysates were transferred to white solid 96-well plates for luminescence detection using a microplate luminometer (PerkinElmer). The 50% inhibitory dilution (IC₅₀) was defined as the serum dilution at which the relative light units (RLUs) were reduced by 50% compared with the positive virus control wells. In addition, the neutralization of the Omicron variant (B.1.1.529.1) by the combination of antibodies (SFC3 + HSA-1F) was detected by Vazyme.

### 2.10 | Virus micro-neutralization assay

A standard virus neutralization assay approved by the national control authority was performed as follows. A panel of tested antibodies was serially diluted and mixed with 100 TCID₅₀/well SARS-CoV-2 (Wuhan-hu-1 strain). The antibody-virus mixtures were incubated for 60 min and then added to 96-well plates containing confluent monolayers of Vero-E6 cells in triplicate. Following a 1 h adsorption at 37°C, the supernatants were removed and replaced with 200 μl/well cell culture medium. The plates were then incubated at 37°C with 5% CO₂ for 3 days. The cells were stained with crystal violet and absorbance at 570 nm/630 nm were measured. Neutralization was defined as percent reduction compared to the positive controls. Neutralization titers were calculated using nonlinear regression analysis in GraphPad Prism 5.0.
2.11 Statistical analysis

All experiments were repeated for two or three times with three duplicates, except for the virus micro-neutralization assay. Data are presented as the means ± standard deviation (SD). Statistical significance was determined using GraphPad Prism 5.0 software. The affinity graph was performed, and the IC₅₀ was determined using GraphPad Prism 5.0 software.

3 RESULTS

3.1 Panning and screening of anti-RBD scFv clones with an ELISA

Five mice were intraperitoneally immunized with four sequential injections of SARS-CoV-2 RBD protein. Following the third immunization, the anti-RBD antibody in sera of immunized mice reached at least 1:16 000. The total RNA extracted from the spleen cells of the hyper-immunized mice was successfully used in RT-PCR reactions to amplify the VH and VL genes. The practical size of the resultant scFv antibody gene library was estimated to be 6.6 × 10⁷. The scFv proteins incorporated on the surface of recombinant M13 phage particles with the helper phage infection resulted in a combined phage library of 2 × 10¹¹ plaque-forming unit/ml (PFU/ml).

SARS-CoV-2 RBD protein (Sino Biological Inc.) was immobilized in microtiter plates and used for antibody selection. Four rounds of bio-panning were performed to enrich for specific binding of the recombinant phage particles. With each round of panning, the stringency of the washing steps increases and thereby the affinity of the scFv clones towards the RBD protein improves proportionately. The enrichment of high-affinity scFv phage particles was performed using a phage ELISA. A total of 288 out of 384 clones were associated with OD values greater than 2.0 by ELISA, demonstrating a high binding affinity with the RBD proteins. All 288 clones were sequenced and analyzed. A total of 12 unique antibody sequences were obtained, revealing a favored selection and strong enrichment of specific anti-RBD scFv antibodies in the panning system.

3.2 Production, binding, and neutralization of chimeric antibodies

The VH and VL of the selective scFv clones were amplified and cloned into the pTSE-G1n and pTSE-K antibody expression vectors, respectively, to express as human-mouse chimeric IgG1. Upon cotransfection into the FreeStyle™293-F cells with each pair of the modified antibody plasmids, chimeric antibodies were obtained and purified for subsequent experiments. First, ELISA assay was performed to test whether 12 fully human IgG1 molecules could bind the SARS-CoV-2 RBD protein. As shown in Figure 1A, three chimeric IgGs with relatively high affinity were obtained (only antibodies C3, C11, and A-1F were shown in detail, and the other results can be seen in Supporting Information: Figure1). Furthermore, we used an HIV-based system to produce a pseudovirus with the SARS-CoV-2 S protein by cotransfection into FreeStyle™293-F cells with pNL4.3-Luc-R’E⁻ and pcAGGS-WSS. The pseudovirus was successfully packaged at titers of approximately 5 × 10⁴/ml. A PBNA was performed to assess the neutralization ability of the antibodies. As shown in Figure 1B, all three antibodies could completely inhibit SARS-CoV-2 pseudovirus infection. mhC11, mhC3, and mhA-1F could neutralize pseudovirus infection with 50% neutralization at 1.260, 4.041, and 2.266 nM, respectively.

3.3 Production, binding, and neutralization of humanized antibodies

To reduce human anti-mouse antibody (HAMA) reactivity, human-mouse chimeric antibodies were humanized using the website, http://www.abysis.org/abysis/, and Swissmodel (The data of Z-score, the simulation diagram of the spatial trend of carbon atoms were shown in

**Figure 1** The binding and neutralization of chimeric antibodies. (A) An enzyme-linked immunosorbent assay (ELISA) analysis of the binding between receptor-binding domain (RBD) and chimeric antibodies mhC3, mhC11, and mhA-1F. (B) Pseudovirus-based neutralization assay of the chimeric antibodies, mhC3, mhC11, and mhA-1F. Data were obtained from three separate experiments and shown as the mean ± SD, and the IC₅₀ value was obtained via nonlinear regression.
Supporting Information: Figures 2 and 3). Through sequence analysis and substitution of noncritical amino acids, humanization of the three antibody strains was successfully completed, and the degree of humanization was above 96% (Supporting Information: Table 1). Humanized VH and VL were also amplified and cloned into pTSE-G1n and pTSE-K antibody expression vectors, and cotransfected into FreeStyle™-293-F cells to express humanized IgG1 SFC3, SFC11, and HSA-1F. Compared with human-mouse chimeric mAbs, the humanized mAbs were able to bind to the RBD protein with similar binding activity by ELISA (Figure 2A). To further analyze the binding ability of antibodies, the determination of KD was performed by biolayer interferometry. The results are shown in Supporting Information: Figure 4, the binding of antibodies to SARS-CoV-2 RBD showed remarkable binding kinetics. Compared with the other two antibodies, SFC11 decreased the most gently in the dissociation phase (Supporting Information: Figure 4B). Apparently, considering the KD of the three antibodies, SFC11 bound to RBD much more strongly than SFC3 and HSA-1F (Table 1).

Further, a PBN assay was performed to assess the neutralization ability of the antibodies. While the humanized mAbs also inhibited pseudovirus infection, the neutralization ability was slightly lower compared with the human-mouse chimeric mAbs. In addition, SFC3, SFC11, and HSA-1F could neutralize SARS-CoV-2 pseudovirus infection with 50% neutralization at 8.193, 20.81, and 9.638 nM (Figure 2B).

### 3.4 Humanized mAbs bind to RBD through distinct regions

Since the S1 protein-bonded cells express the viral receptor ACE2 through RBD, we investigated whether the neutralizing antibodies can inhibit RBD and ACE2 binding. Figure 3 shows that while SFC3 and HSA-1F were able to block the binding between RBD and ACE2, the SFC11 neutralizing antibody could not. This finding indicates that SFC11 may have a different epitope. Furthermore, a competitive binding assay was performed to confirm if any of the three mAbs competitively bound RBD. As shown in Figure 4A, even when the binding between SFC3 and RBD reached saturation, SFC11 and HSA-1F still combine with RBD to increase the signal. Moreover, the reverse was also observed (Figure 4B). These results reveal that three antibodies did not bind to RBD competitively. Interestingly, we found that the binding of SFC11 to RBD was reduced when SFC3 was former immobilized in biosensors compared to SFC11 being immobilized as former. We speculated that the reason may be that the binding of SFC3 to RBD would cause a certain steric hindrance to the binding of SFC11.

Previously, Tian et al. divided the neutralizing antibody sites on the RBD into four main regions (Supporting Information: Figure 5). To further confirm the binding region to RBD, we performed competitive binding between CB6 (Class 1), REGN10933 (Class 1), P2B-2F6 (Class 2), REGN10987 (Class 3), S2A4 (Class 4), and MW05 with our antibodies (Supporting Information: Figure 6). The summary of the results was shown in Figure 4D. SFC3 only had no competition with S2A4 (Class 4). We speculated that SFC3 had partial epitope overlap with these reference antibodies, or that SFC3 had strong steric hindrance, as in Figure 4B. It was very clear that HSA-1F only competed with antibodies in Class 1, and its binding site to RBD would be like with antibodies in Class 1. Interestingly, there was no competitive binding between antibody SFC11 and antibody of Class 1–Class 4, but only with antibody MW05. These suggested that the binding site of SFC11 to RBD may partially overlap with the MW05 but has no overlapping epitope with the reference antibodies in Classes 1–4. In conclusion, the three

### TABLE 1 Dissociation constant of antibody.

| Antibody | Mean Kon (10^5 M s^-1) | Kdis (10^-2 s^-1) | KD (10^-7 M) | χ^2 |
|----------|------------------------|-------------------|-------------|-----|
| SFC3     | 1.19                   | 3.16              | 2.66        | 0.02 |
| SFC11    | 1.39                   | 0.02              | 0.02        | 0.03 |
| HSA-1F   | 0.2                    | 3.04              | 14.2        | 0.01 |

Note: All data were calculated by 1:1 binding model in Analysis Software 7.0.

Abbreviations: Kdis, dissociation constant; KD, equilibrium dissociation constant; KD, Kdis/Kon; Kon, association constant.
mAbs recognized different epitopes and had great potential to construct antibody cocktails.

### 3.5 The antibodies demonstrate synergistic neutralizing activity

Since the three antibodies targeted different regions of the RBD, we next tested whether the combined antibodies (1:1 ratio) could enhance their neutralizing activity with a PBNA. The results shown in Figure 5A show that mhA-1F combined with mhC3 or mhC11 could enhance the neutralization activity compared with Figure 1B. However, mhC3 combined with mhC11 could not enhance the neutralization activity. For humanized mAbs, synergistic neutralizing activity was also observed. As shown in Figure 5B, HSA-1F combined with SFC3 or SFC11 could both enhance the neutralization activity, especially when HSA-1F was combined with SFC3, compared with Figure 2B. However, the combination neutralization activity of three antibodies was much lower than HSA-1F combined with SFC3. So, the combination of three antibodies was not considered in the following test. We speculate that mhA-1F and HSA-1F may play an important role in the synergistic neutralization activity.

### 3.6 Neutralization of SARS-CoV-2 by synergistic neutralizing antibodies

To elucidate the inhibitory capacity of our anti-RBD antibodies against SARS-CoV-2, virus micro-neutralization assay was performed to test the ability of binding and neutralizing the SARS-CoV-2 epidemic strain (Wuhan-hu-1 strain). As shown in Figure 6A, both chimeric mhC3 and humanized SFC3 exhibited a potent viral neutralizing ability. Although HSA-1F could not neutralize SARS-CoV-2 infection with 100% neutralization at 250 nM, HSA-1F combined with SFC3 enhanced the neutralization activity.

Considering the mutation of SARS-CoV-2, several mutant strains pseudovirus were constructed using the HIV lentivirus vectors. The results of PBNA assay were shown in Figure 6B, the combination of SFC3 and HSA-1F could effectively neutralize D614G and N501Y mutant pseudovirus; the neutralization ability was slightly lower to neutralize E484K mutant pseudovirus; for too much mutant of Delta strain, the combination of SFC3 and HSA-1F could neutralize Delta mutant pseudovirus with high concentration. Importantly, the combination of SFC3 and HSA-1F could effectively neutralize Omicron variant pseudovirus at low concentrations. These results indicated that the combination of SFC3 and HSA-1F could effectively neutralize SARS-CoV-2.

### 4 DISCUSSION

Immediately following the COVID-19 outbreak, several research teams in multiple countries around the world rapidly responded. A variety of techniques were used to develop neutralizing antibodies against SARS-CoV-2, including single B cell antibody technology, immune library technology, and nano-antibody technology. Most of the reported neutralizing antibodies against SARS-CoV-2 could bind to RBD and blocked binding of RBD to ACE2 receptor, which is consistent with the findings of SARS-CoV-2 receptor studies. However, there were also several special neutralizing mAbs, such
(A) Graph showing Binding (aM) over Time (S) with various conditions labeled as 'Baseline', 'Loading', 'Association', and 'Re-association'.

(B) Similar graph as (A) with different conditions.

(C) Another graph similar to (A) with different conditions.

(D) Diagram illustrating sensor blockade with antibodies No.1Ab and No.2Ab, and a table summarizing their interactions with SFC3, SFC11, and HSA-1F:

| Antibody   | SFC3 | SFC11 | HSA-1F |
|------------|------|-------|--------|
| No.1Ab     | √    | ×     | ×      |
| No.2Ab     | ×    | √     | ×      |
| CB6        | √    | ×     | √      |
| I0933      | √    | ×     | √      |
| P2B-2F6    | √    | ×     | ×      |
| I0987      | √    | ×     | ×      |
| S2A4       | ×    | ×     | ×      |
| MW05       | √    | √     | ×      |

FIGURE 4 (See caption on next page)
as the 4A8 targeted N-terminal domain of the S protein (NTD). In the present study, C3 and A-1F both bound to RBD and blocked the binding of RBD to ACE2. Although C11 could not block the binding of RBD to ACE2, C11 still displayed good neutralizing activity.

It has been reported that there are four groups of epitopes associated with RBD. Through cross-competition experiments with a series of well-characterized antibodies, the possible regions of the three antibodies binding to the RBD were preliminarily determined. Although the specific binding epitopes of C3, C11, and A-1F were not well-established, ForteBio results indicated that the binding region of the three antibodies were different, and there was no binding competition between the three antibodies. This may also explain the synergistic effect of the three antibodies. In the future, we aim to develop antibody cocktails or bispecific antibodies using the three identified neutralizing antibodies to enhance the potential neutralization activity.

Several of the above antibodies had been approved for clinical use or entered clinical trials. Compared of the neutralizing activity, the combination of SFC3 and HSA-1F (IC50 values 0.18 μg/ml) had

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**FIGURE 4** Humanized mAbs bound to the RBD with different regions. (A–C) are binding kinetics processes that perform competition for binding to RBD by immobilizing different antibodies. (A) SFC3 was immobilized on the biosensor; (B) SFC11 was immobilized on the biosensor; (C) HSA-1F was immobilized on the biosensor. In the legend box, the samples were separated by slash “/” respectively to represent “Loading,” “Association,” and Reassociation stages of sample molecules. (D) was the identification of the binding regions to RBD of three mAbs by a BLI-based cross-competition assay. “√” means that the first antibody bound RBD competitively with the second antibody and “x” means that the first antibody was not competitive with the second antibody. mAbs, monoclonal antibodies; RBD, receptor-binding domain.

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**FIGURE 5** The antibodies had synergistic neutralizing activity. (A) Pseudovirus-based neutralization assay of chimeric antibody combination at a 1:1 ratio. (B) Pseudovirus-based neutralization assay of humanized antibodies combination at a 1:1 ratio. Data were obtained from three separate experiments and shown as the mean ± SD, and the IC50 value was obtained via nonlinear regression. The value of IC50 was the total antibodies concentration. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

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**FIGURE 6** Neutralization of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by synergistic neutralizing antibodies. (A) Virus microneutralization assay. Chimeric antibody, humanized antibody, and humanized antibody combination neutralized SARS-CoV-2. The Y value was the absorbance of OD570 nm minus OD 630 nm, and the detection values of chimeric antibodies and humanized antibodies are independent of each other. (B) Neutralizing activity of a combination of SFC3 and HSA-1F (1:1 ratio) against mutant pseudoviruses. The data showed the mean ± SD of two independent experiments and the IC50 value was obtained via nonlinear regression. The value of IC50 was the total antibodies concentration. Neutralization data for Omicron variant (B.1.1.529.1) from Vazyme. The data showed the mean ± SD, which was one of two independent experiments with three duplicates and the IC50 value was obtained via nonlinear regression.
lower inhibition of authentic SARS-CoV-2 than LY-CoV555 (AB169)\textsuperscript{19} and BRIL-196/BRIL-198 (P2B-1G5/P2C-1F11)\textsuperscript{7} with IC\textsubscript{50} values 0.02, 0.03 μg/ml; however, the combination of SFC3 and HSA-1F had higher inhibition than 47D1\textsuperscript{18} with IC\textsubscript{50} values 0.57 μg/ml. Interestingly, although D614G is one of several mutation sites in the Omicron variant, the neutralizing activity of SFC3 + HSA-1F on Omicron variant (B.1.1.529) was superior to that of the D614G mutation. Perhaps with the increase of the SARS-CoV-2 mutation, the spatial structure of the virus changes more and more, which also makes it possible to discover new neutralization sites that were originally present inside the virus. These indicated the combination of SFC3 and HSA-1F had potential value to develop. SARS-CoV-2 is an RNA virus with an unsegmented genome that is prone to mutations during the transmission and propagation processes. Several variants have been reported, including Alpha variant (B.1.1.7) in the United Kingdom, Beta variant (B.1.351) in South Africa, Gamma variant (P.1) in Japan, Delta variant (B.1.617.2) in India, and Omicron variant (B.1.1.529) in South Africa. Such variants may be resistant to vaccine-mediated immune protection, reduce the neutralizing activity of some mAbs, or increase the risk of developing secondary infections in previously infected people.\textsuperscript{20–22}

Moreover, viral mutations may lead to a reduction in the neutralizing activity of mAbs; however, bispecific antibodies may inhibit this decline. Thus, the development of bispecific antibodies using C3, C11, and A-1F may have significance for future SARS-CoV-2 therapeutics.

5 | CONCLUSION

In this study, we identified several humanized neutralizing antibodies specific to SARS-CoV-2 via the ability to bind RBD of the SARS-CoV-2 S protein. Three of these humanized antibodies exhibited strong binding and neutralization activity through different RBD regions. Moreover, these antibodies exhibit synergistic neutralizing activity.

AUTHOR CONTRIBUTIONS

Yunzhou Yu and Zhixin Yang conceived this study; Ying Huang, Jun Zhang, Peng Du, Jiansheng Lu, Qing Xie, and Rong Wang carried out the experiments and performed data analysis; Ying Huang drafted the manuscript; Rong Wang and Zhixin Yang wrote, edited, and reviewed the manuscript; Zhixin Yang acquired funding; Jiansheng Lu and Jun Zhang provided resources; Rong Wang, Ying Huang, Y.Zhixin Yang, and Zhixin Yang supervised the work.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study can be obtained from the corresponding authors upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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