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Research Article

Isolation of a human monoclonal antibody specific for the receptor binding domain of SARS-CoV-2 using a competitive phage biopanning strategy

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Abstract

The infection of the novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused more than 200 000 deaths, but no vaccine or therapeutic monoclonal antibody is currently available. SARS-CoV-2 relies on its spike protein, in particular the receptor-binding domain (RBD), to bind human cell receptor angiotensin-converting enzyme 2 (ACE2) for viral entry, and thus targeting RBD holds the promise for preventing SARS-CoV-2 infection. In this work, a competitive biopanning strategy of a phage display antibody library was applied to screen blocking antibodies against RBD. High-affinity antibodies were enriched after the first round using a standard panning process in which RBD-His was immobilized as a bait. At the next two rounds, immobilized ACE2-Fc and free RBD-His were mixed with the enriched phage antibodies. Antibodies binding to RBD at epitopes different from ACE2-binding site were captured by the immobilized ACE2-Fc, forming a "sandwich" complex. Only antibodies competed with ACE2 can bind to the free RBD-His in the supernatant and be subsequently separated by the nickel-nitrilotriacetic acid magnetic beads. rRBD-15 from the competitive biopanning of our synthetic antibody library, Lib AB1, was produced as the full-length IgG1 format. It was proved to competitively block the binding of RBD to ACE2 and potently inhibit SARS-CoV-2 pseudovirus infection with IC_{50} values of 12 nM. Nevertheless, rRBD-16 from the standard biopanning can only bind to RBD *in vitro*, but not have the blocking or neutralization activity. Our strategy can efficiently isolate the blocking antibodies of RBD, and it would speed up the discovery of neutralizing antibodies against SARS-CoV-2.

Statement of Significance: The authors developed a competitive phage screening strategy to isolate a high-affinity human antibody that binds the SARS-CoV-2 receptor binding domain and inhibits its interaction with human angiotensin-converting enzyme 2.

KEYWORDS: SARS-CoV-2; monoclonal antibody; phage display; synthetic library; biopanning

INTRODUCTION

The recent outbreak of a novel coronavirus disease (COVID-19) has emerged from a public health emergency of international concern to global pandemic. Its pathogen, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a newly identified β-coronavirus. Coronavirus got the family name from the spike (S) protein on the viral particle. The highly glycosylated S protein stays compact in a trimeric state, recognizes receptor on the host cell membrane and undergoes a series of conformation changes, proteolysis events and membrane fusion to complete viral entry. For vaccines, clinical diagnosis, early

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prevention and medication, the S protein is the most significant target.

The primary sequences of S protein between SARS-CoV and SARS-CoV-2 share about 76% identities and 86% similarities, which indicates high possibility of structural homology and similar infection pathway. SARS-CoV and SARS-CoV-2 recognize the same host cell receptor angiotensin-converting enzyme 2 (ACE2) for mediating viral entry into host cells. It was reported that SARS-CoV S protein trimer bound to ACE2 at 1:1 in ratio [1,2]. Before infection, receptor-binding domain (RBD) of each SARS-CoV S monomer was partially buried in the inactive "down" conformation and not able to bind ACE2 due to steric clash. Once infection started, one RBD monomer turned "up" to expose enough space to ACE2, inducing further conformational open and loose for proteolysis [1,3]. Atomic-level structural analysis suggested that the spatial interaction and interface between SARS-CoV-2 RBD and ACE2 was mostly in accordance with the SARS-CoV case [4]. Besides, a cryoelectron microscopy (Cryo-EM) structure of SARS-CoV-2 S protein trimer published recently showed that one of the three RBDs was in "up" conformation and naturally exposed the whole interaction interface [5], while the classic closed symmetric trimer still existed [6]. That might explain why SARS-CoV-2 is much more contagious and problematic than SARS-CoV worldwide.

No effective cure or vaccine is currently available for COVID-19. Based on structure information above, blocking SARS-CoV-2 RBD is a rational therapeutic approach. Here we developed a competitive biopanning strategy to efficiently isolate blocking antibodies from phage display antibody libraries. Several high-affinity antibodies targeting SARS-CoV-2 RBD and blocking its binding to ACE2 were isolated, and the top 1 lead exhibited a neutralization activity of SARS-CoV-2 pseudotyped vesicular stomatitis virus infection.

MATERIALS AND METHODS

Recombinant proteins

ACE2-His was purchased from Novoprotein (Shanghai, China). ACE2-hFc and SARS-CoV-2 RBD-His were purchased from Sino Biological (Beijing, China). SARS-CoV-2 RBD-mFc was expressed using ABLINK Biotech's HEK 293F expression system.

A phage display antibody library

A synthetic human Fab antibody library AB1 (LibAB1) was constructed according to a procedure previously described [7]. Human germline immunoglobulin variable segments VH3-30 and VL1-16 were employed as templates, the complementarity-determining regions L3 (CDR-L3) and H3 (CDR-H3) were diversified by the designed mutagenic oligonucleotides. The oligonucleotides were synthesized using the trimer phosphoramidites mix Z (Glen Research) containing codons for 12 amino acids in the following molar ratios: 20% each Y, S &G, 6% each T & A, and 4% each P, H, R, F, W, V & L. The number of positions denoted by Z in CDR-L3 (QQ (Z)n PLT) and -H3 (AR (Z) n (A/G/D/Y) FDY) was varied from 3 to 12 and 8 to 12, respectively. The library size is estimated to be 1×10^{12}.

Standard biopanning

Antibodies against RBD were screened at the first round using a standard biopanning protocol [8]. Briefly, RBD-His was coated on 96-well Maxisorp plates at 4 °C overnight. After the coating buffer was decanted, the plate was blocked with 1% polyvinyl alcohol (PVA) at room temperature for 1 h. 100 μL of phage libraries (10^{13} pfu/mL) was added per well for 2-h binding. After washing eight times with PT buffer (0.05% Tween-20 in phosphate-buffered saline (PBS)), bound phages were eluted with 100 mM HCl (100 μL per well), followed by 5-min incubation. The eluent was transferred into a 1.5 mL microfuge tube and neutralized with 1 M Tris-HCl (pH 8.0). Half the neutralized phage solution was mixed with 1 mL of actively growing *E. coli* NEB 5-alpha F′ (OD600 = 0.8) in ×2 YT media containing 10 μg/mL tetracycline and incubated at 37°C for 1 h. 1×10^{10} pfu of M13K07 helper phages were added next and incubated for another 1 h. The infected bacteria were amplified in 50 mL ×2 YT medium containing 50 μg/mL carbenicillin and 25 μg/mL kanamycin, shaking at 200 rpm and growing overnight at 37°C. The next day, phages were harvested in precipitant with PEG/NaCl solution and resuspended in PBS buffer for the following rounds of panning.

Competitive biopanning

After the first round of the standard biopanning, a competitive biopanning protocol that included steps of competitive binding, magnetic separation, elution and amplification (Fig. 1) was applied to isolate the epitope-specific antibodies. Briefly, 100 μL of ACE2-hFc protein (5 μg/mL) was coated on the 96-well Maxisorp plates. The wells were washed and blocked with 1% PVA, and then the mixture of antibody library (1×10^{10} pfu per well) and free RBD-His protein (100 ng per well) was added. After a 2-h competitive binding, the supernatant was transferred into a 1.5 mL microfuge tube containing the pre-washed nickel-nitrilotriacetic acid (Ni-NTA) magnetic beads (GenScript) and incubated on a shaker at room temperature for 1 h. Beads were collected using the magnetic separation rack and washed by the PT buffer for eight times. Bound phages were eluted with 100 mM HCl (100 μL per tube) after 5-min incubation. Beads were collected using the magnetic separation rack and the supernatant was transferred into a tube for neutralization. Half the neutralized phage solution was mixed with 1 mL of actively growing NEB alpha F′ cells and amplified as the standard biopanning protocol. 10 μL of the bacterial culture before infection with helper phages was taken, diluted, and grown on the LB plates containing 50 μg/mL carbenicillin at 37°C overnight. The single clones were picked up next day for the phage enzyme-linked immunosorbent assay (ELISA).

Phage ELISA

Single clones were inoculated into 400 μL ×2 YT medium containing 50 μg/mL carbenicillin, 25 μg/mL kanamycin

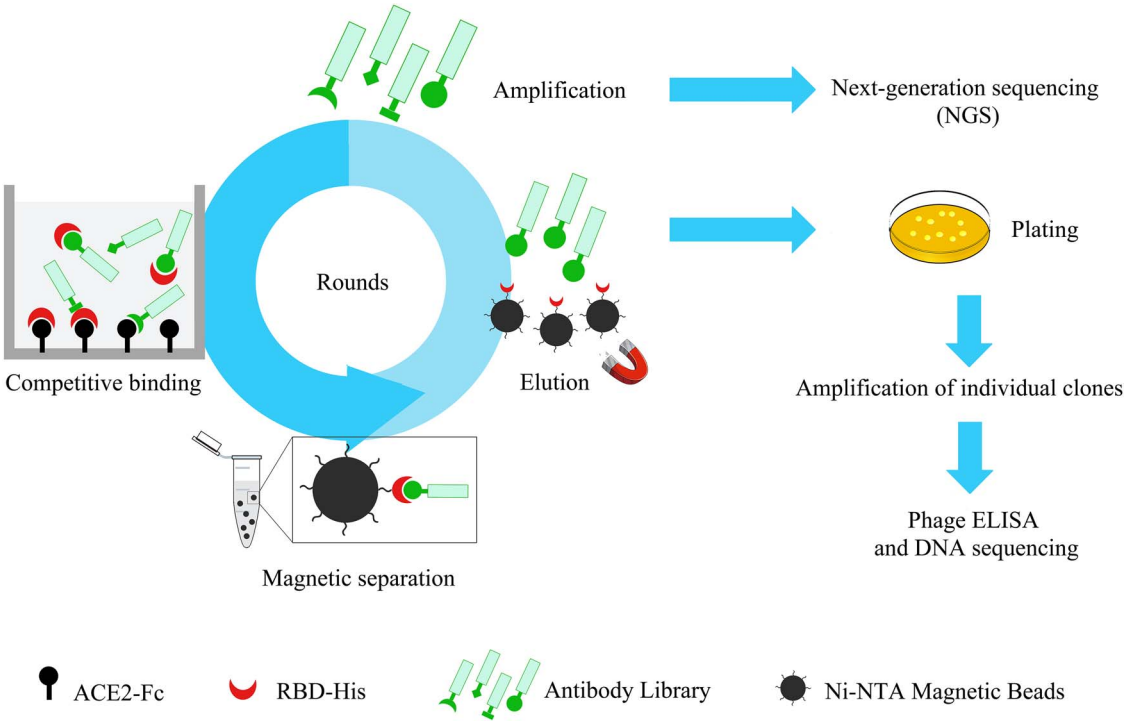

Figure 1. Schematic presentation of a competitive biopanning strategy. A specific binder of target protein was added during the binding step for the selection of blocking antibodies. In this work, the immobilized ACE2-hFc captured RBD-His and the antibodies binding RBD at different epitopes, forming a complex like a "sandwich." However, when an antibody recognized the same or similar epitopes within RBD as the ACE2 did, it could block RBD-ACE2 interaction. The antibodies would bind to the free RBD-His in the supernatant and be subsequently separated by the Ni-NTA magnetic beads.

and 10^{10} pfu/mL helper phages in 96-deep-well plates and incubated overnight at 37°C and 250 rpm. The plates were centrifuged at 4000 rpm and the supernatant was applied for phage ELISA. The 96-well Maxisorp plates were coated overnight at 4°C with RBD-mFc (1 μg/mL, 100 μL per well). After blocking with 1% PVA, plates were incubated with 50 μL bacterial supernatant containing phages for 2 h at room temperature. After six times of wash with PT, bound phages were detected using a horseradish peroxidase (HRP) conjugated anti-M13 antibody (Sino biological) and tetramethylbenzidine as substrate. Absorption at 450 nm was measured.

IgG expression and purification

VH and VL of the positive phage were subcloned, respectively, into the pFUSEss-CHIg-hG1 and pFUSEss-CLIg-hK (Invivogen). Antibodies were transiently expressed in FreeStyle™ HEK 293-F cells (Life Technologies) using 293fectin transfection reagent according to manufacturer's instructions. After transfection, cells were grown in the serum-free medium for an additional 5 days. The supernatant was collected and purified on a MabSelect Protein A column (GE healthcare). Eluted IgG was dialyzed against PBS and stored at −80°C.

Competitive blocking ELISA

Recombinant human ACE2-His (5 μg/mL, 100 μL per well) was coated on 96-well Maxisorp plates, followed by a pre-incubated mixture of the anti-RBD antibody titrated into a constant amount of RBD-mFc (1 μg/mL). RBD binding to ACE2 was detected using HRP-conjugated anti-mouse Fc antibody.

Pseudotyped virus neutralization assay

The neutralization effects of antibodies on SARS-CoV-2 pseudovirus were performed by the Genscript Inc. (Nanjing, China) under a research service contract. Briefly, 20 000 of the human ACE2-overexpressing Hela monoclonal cells were seeded into each well of a 96-well plate. SARS-CoV-2 pseudovirus and antibodies were incubated at ambient temperature for 1 h. The mixture was transferred into wells and incubated with cells at 37°C, 5% CO_2 for 24 h. The culture medium was freshly replaced and cells were incubated for another 24 h. The culture medium was removed and cells were rinsed with PBS. 50 μL lysis buffer was added and further incubated at ambient temperature for 40 min. 40 μL supernatant was transferred to a Sterile Un-Clear 96-well plate with the Bio-Glo luciferase substrate added, and the luminescence signal was measured with EnVision. The dose response curves were plotted with the relative luminescence unit against the antibody concentration. The assay results were processed by Microsoft Office Excel 2013 and GraphPad Prism 6.

RESULTS

High-affinity antibodies were identified by the phage ELISA

RBD had a high affinity to ACE2 with an EC_{50} of around 1 μg/mL (Supplementary Fig. S1). Thus 1 μg/mL of RBD

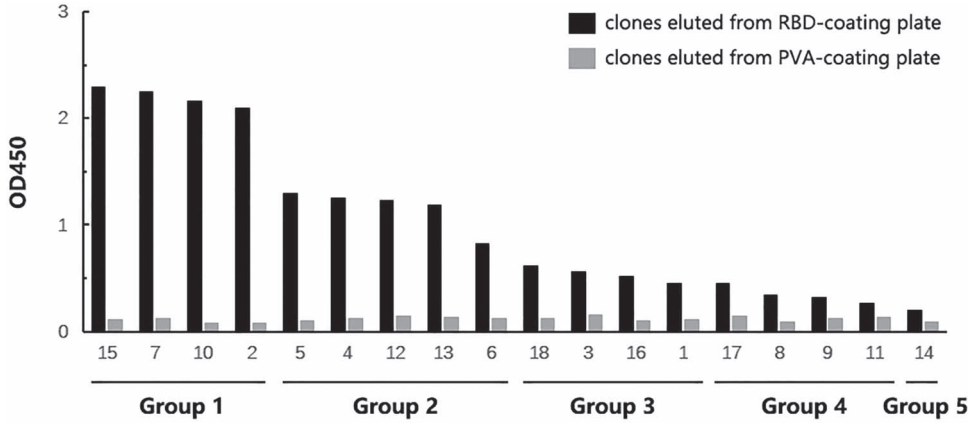

Figure 2. Identification of positive clones to immobilized antigen in competitive manner. Taken OD450 readings as measurement (*Y*-axis), data fluctuated within 20% were divided into each group. The numbers on *X*-axis were the primitive clone labels, and the highest-ranking one was renamed rRBD-15 in this work.

was applied in our competitive biopanning strategy to ensure the immobilized ACE2 can completely capture the "sandwich binding" complex. During the standard biopanning, phages were always applied at a concentration of 1×10^{12} pfu. However, we changed it to 1×10^{10} pfu per well during the competitive biopanning to reduce the non-specific binding of phages to magnetic beads. After two rounds of the competitive biopanning, 48 clones were randomly selected. Their properties of binding to RBD were measured using phage ELISA. Positive binding was defined as an OD450 reading two or more times higher than the negative control (PVA alone). A total of 18 clones showed positive signals (Fig. 2). After the DNA sequencing, these clones were summarized into five groups of unique antibodies.

Binding and blocking abilities of the top 1 lead against RBD

rRBD-15, the top1 lead with the highest OD450 reading isolated from the competitive biopanning, and rRBD-16, the top 1 lead isolated from the standard biopanning at round 3, were expressed as full-length IgG1 antibodies using the 293F expression system. Their binding and blocking abilities against RBD were compared. Both rRBD-15 and rRBD-16 had high affinities for RBD, with EC_{50} at 3.8 nM and 5.3 nM, respectively. Only rRBD-15 blocked the binding of RBD to ACE2 with an IC_{50} at 3.0 nM, while rRBD-16 did not.

Neutralization abilities of the top 1 leads against SARS-CoV-2 pseudovirus

As a positive control, the recombinant ACE2-hFc (100 μg/mL) totally inhibited the infection of ACE2-overexpressing Hela monoclonal cells with SARS-CoV-2 pseudovirus. The antibody rRBD-15 showed a significant neutralization activity against the SARS-CoV-2 pseudovirus with IC_{50} values of 12.2 nM. However, the antibody rRBD-16 had no neutralization effect of the pseudovirus and there were no significant differences between the highest concentration antibody group and the blank group without antibody addition.

DISCUSSION

RBD-ACE2 interaction initiated viral infection of both SARS-CoV and SARS-CoV-2. Their RBDs share high sequence identities (73%) and structure homology, so the well-established SARS-CoV antibodies were firstly assumed short-cut therapeutic candidates for SARS-CoV-2. However, the real scenario is much more problematic. Several independent peer-reviewed studies as well as preprinted ones have proved that all structurally known SARS-CoV specific antibodies, including S230, 80R, m396 and F26G19, have no cross-reactivity of SARS-CoV-2 [4,5,9]. The lack of cross-reactivity of current mAbs may result from complicated factors. These antibodies all compete with ACE2 to bind SARS-CoV RBD, but their epitopes only have limited overlaps of the whole ACE2-RBD interface. Several key residue mutations from SARS-CoV S to SARS-CoV-2 S do not alter the binding of ACE2, but slight changes could be enough to break antibody recognition. CR3022 is a special case with 86% conserved key residues in the epitope between SARS-CoV-2 and SARS-CoV. Its cross-reactivity was remarkable, but just one site loss of N-glycan results in 1–2 magnitude reduction of binding affinity to SARS-CoV-2 RBD [9]. In humans, RBD-specific monoclonal antibodies derived from COVID-19 recovered individuals indicated similar patterns of no cross-reactivities with either SARS-CoV or MERS-CoV [10]. The findings using polyclonal antibodies are ambiguous. Sera from SARS-CoV S-immunized mice, not rabbits or SARS recovered patients, showed modest neutralization activity against SARS-CoV-2 [6,11], while sera from COVID-19 recovered patients had no effect on SARS pseudovirus [11].

In general, structural and functional analysis suggests that targeting SARS-CoV-2 RBD could be a direct and promising therapeutic strategy, while focusing on previous SARS-CoV antibodies is not very ideal or efficient. No SARS-CoV-2 RBD-specific monoclonal antibody has been reported from human antibody libraries (up to 17 April 2020). In the meantime, SARS-CoV-2 spreads unexpectedly fast around the world, and a new study just shifted its basic reproductive number (R_0) from 2.2 to 5.7 [12]. A

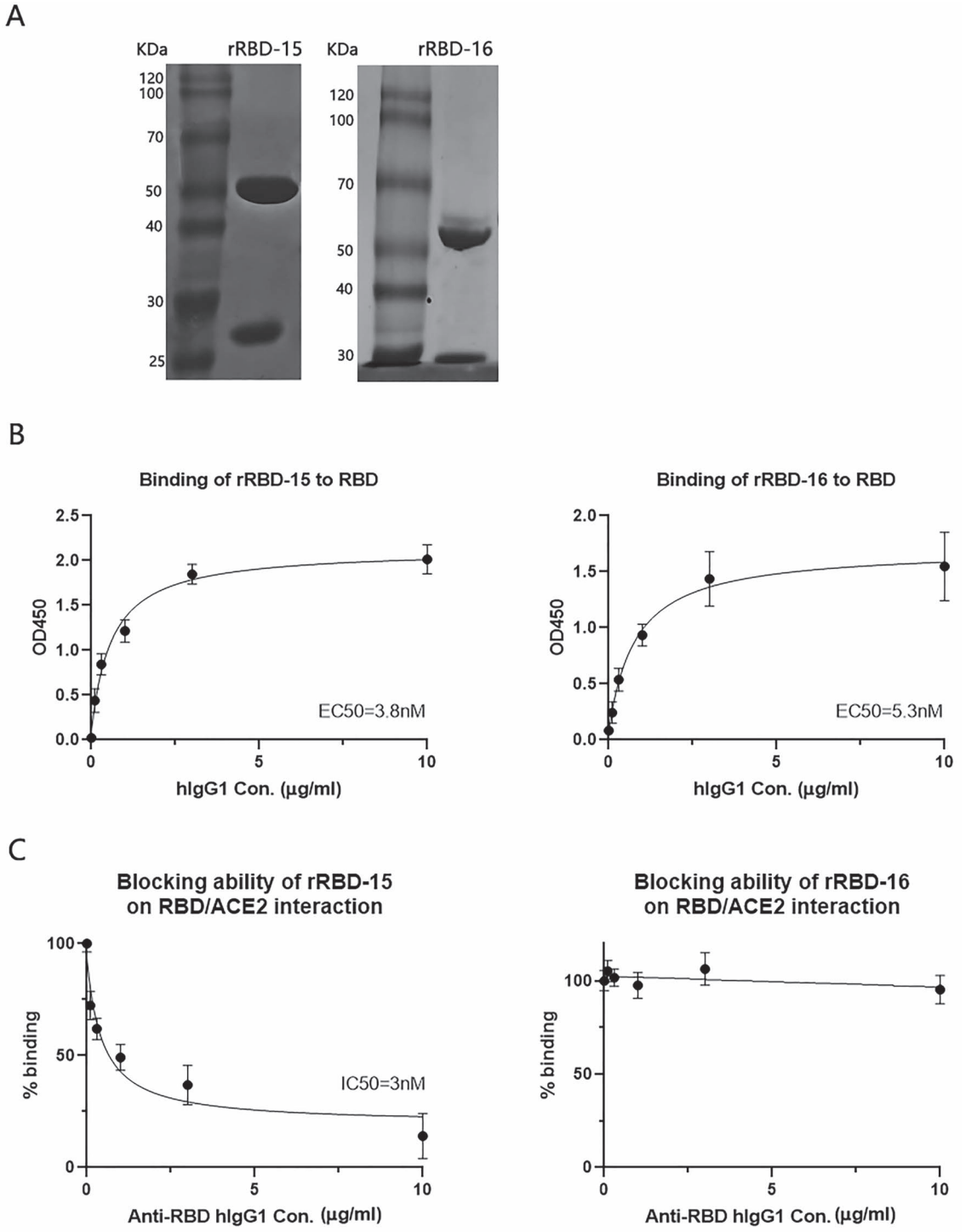

Figure 3. ELISA analysis of the full-length antibodies. (A) Molecular weights of purified soluble IgG antibodies were detected by SDS-PAGE and stained with typan blue. (B) The binding of antibodies to RBD-mFc protein was measured by ELISA. X-axis stood for human IgG1 concentration of different antibodies, and Y-axis stood for absorption at 450 nm. EC_{50} was calculated by curve fitting. (C) The activities of antibodies blocking the interaction between RBD-mFc and ACE2-His were measured by the competitive blocking ELISA. X-axis stood for RBD-binding human IgG1 concentration of different antibodies and Y-axis stood for percentage of uninterrupted RBD/ACE2 interaction.

rapid and effective method of obtaining the SARS-CoV-2 neutralizing antibodies is much required.

Naïve antibody libraries derived from natural immune systems have their capacity limits, while synthetic libraries with higher diversity have more opportunities to isolate binders especially for novel infectious antigens. In contrast to a naïve antibody library of 10^8–10^9 diversity, a synthetic library with additional artificial randomization

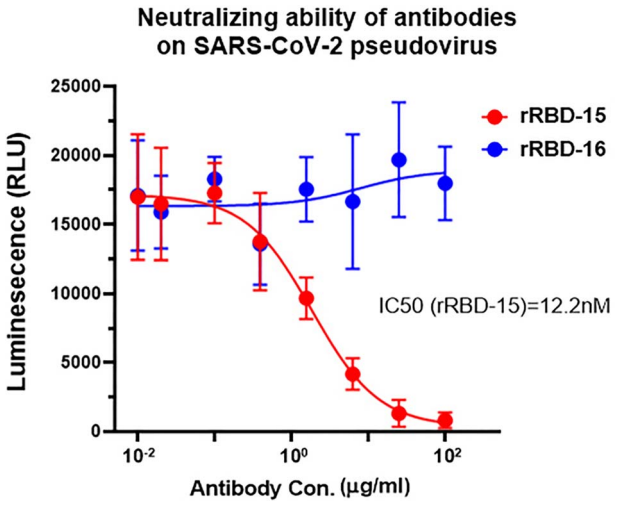

Figure 4. Two SARS-CoV-2 RBD-specific antibodies selected from different strategies showed different neutralization activities. Luminescence signal on Y-axis indicated relative proportions of pseudovirus entry into target cells. The antibody rRBD-15 competed with ACE2 could neutralize SARS-CoV-2 pseudovirus, but rRBD-16 could not.

on CDRs can reach diversity as high as 10^{12}–10^{13}. When the recombinant RBD and ACE2 proteins were ready, it took 3 weeks to isolate, produce and verify the antibodies in this study. Using the standard biopanning method, we enriched RBD-specific phages from our synthetic Lib AB1, but not from our naïve antibody libraries (data not shown). Unfortunately, the top1 lead rRBD-16 from the standard biopanning of Lib AB1 could not block the RBD-ACE2 interaction (Fig. 4), although it bound to RBD with an EC_{50} of 5.3 nM (Fig. 3).

The clinical potential and applications of an antibody often depends on its binding epitopes of the target protein. A high-affinity antibody against the target protein can be screened from a phage display antibody library using the standard biopanning process, but its binding epitopes are identified by some extra steps, such as epitope mapping and competitive ELISA. We therefore developed a new competitive biopanning strategy to efficiently isolate isotype-specific antibodies from libraries. As expected, the top1 lead rRBD-15 successfully bind to RBD in compete with ACE2 both in solution and in pseudovirus, and its binding affinity is quite high in 1–10 nM differing from measuring methods. Further experimental plan is scheduled to verify the neutralization effect in the live viruses and animal models, together with cross-reactivity assays of other disease-related coronaviruses.

In conclusion, our strategic discovery of human monoclonal antibodies against SARS-CoV-2 RBD may fill the blanks of antibody-related pharmaceutical development and shed light on new treatments in need of global health concerns.

AUTHORS' CONTRIBUTIONS

X.Z. and J. Liu designed the experiments and wrote manuscript; L.L., J. Lin, B.L., Y.K., S. Zeng, J.D. and H.X. accomplished the experiments; X.L., T.Z. and S. Zhang participated in experiment design and discussion.

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Conflict of interest statement. The authors declared no conflicts of interest.

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