SHORT REPORTS

Characterization of a broadly cross reactive tetravalent human monoclonal antibody, recognizing conformational epitopes in receptor binding domain of SARS-CoV-2

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Abstract

We used human semi-synthetic phage antibody gene libraries to select anti-SARS-CoV-2 RBD scFv antibody fragment and subsequent characterization of this novel tetravalent monoclonal antibody targeting conformational epitopes in the receptor binding domain of SARS-CoV-2. Binding studies suggest that II62 tetravalent antibody cross-reacts with RBD protein of SARS-CoV2 and its different variants of concerns. The epitope mapping data reveals that II62 tetravalent antibody targets an epitope that does not directly interferes with RBD: ACE2 interaction. Neutralization studies with live authentic SARS-CoV2 virus suggests that increase in valency of II62 mAb from monovalent to tetravalent doesn't perturbate virus interactions with the ACE2 expressing host cells in cytopathic effect-based (CPE) assay.

Keywords SARS-CoV2 · Cross-reactive · Non-neutralizing antibodies · Tetravalent · scFv-Fc-scFv · RBD

Introduction

Coronavirus pandemic preparedness mainly involves effective respond towards the viruses as soon as it appears leading its containment or minimizing their spread. Alternatively, discovery of neutralizing antibodies (nAbs) against coronaviruses, and by utilizing the molecular knowledge of their epitopes leads towards rational designing of pan-coronavirus vaccines (Dai et al. 2020). Receptor Binding Domain (RBD) is a highly immunogenic component of spike protein which is recognized by the majority of nAbs and is, therefore, a major target of current nAb-based vaccine design efforts (Cao et al. 2020, p. 2; Robbiani et al. 2020; Bertoglio et al.

2021). As the SARS-CoV-2 virus continues to mutate and spread rapidly, studies show that convalescent individual develops potent nAbs targeting towards epitopes that overlap with ACE2 binding site, these epitopes tend to be under heavy immune selection pressure at the population level, contributing to the selection of SARS-CoV-2 neutralization escape variants (Wibmer et al. 2021; Wang et al. 2021 p. 351). Such mutations can reduce the efficacy of vaccine-induced antibody responses in humans.

The effects of avidity can boost an antibody's neutralizing potency against a virus by orders of magnitude by enhancing their capacity to bind physically related antigens (e.g., distinct spikes on the surface of the same virus) at the same time (Icenogle et al. 1983; Wu et al. 2005). The antigen sites must be dense enough for avidity to occur, so that once the first Fab has attached, the second Fab may join its partner before the first Fab dissociates (Klein et al. 2009, p. 10). The avidity-based neutralization is impacted by the presence of functional spike/antigen molecule present on the viral surface. We recently isolated a SARS-CoV-2 RBD directed scFv antibody, II62; from a naïve semi-synthetic library (Parray et al. 2020). Here, we have designed and characterized tetravalent form of II62 scFv (scFv-Fc-scFv). Biochemical characterization of II62 antibody suggests that,

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it targets a conformational epitope on RBD, the targeted epitope does not directly interfere with the RBD:ACE2 interaction. Our binding data suggests that II62 tetravalent mAb broadly cross-reacts with the RBD protein towards variants of concerns of SARS-CoV2.

Results and discussion

Neutralizing antibodies target the exposed spike glycoprotein on the viral surface, along with the receptor binding domain (RBD)., being the most common target. As a result, immunogens derived from RBDs are promising vaccine candidates. It's crucial to explore the mechanisms of action of various antibodies, including non-neutralizing ones, that targets RBD, while evaluating the effect SARS-CoV2 vaccines against new emerging mutant variants.

In this study, we designed a tetravalent mAb targeting the RBD of SARS-CoV2 from previously characterized RBD directed antibody II62 (Parray et al. 2020). The II62 tetravalent (tetravalent scFv-Fc-scFv, Fig. 1A) construct was transiently expressed in Expi293F cells and purified as soluble protein using protein G affinity column, with > 95% purity and yields of 30–55 mg/L. The purified II62 tetravalent migrated as a single protein band on SDS-PAGE around the estimated molecular weight of ~ 70 kDa (Fig. 1B). The tetravalent forms of II62 antibody showed specific binding to RBD and spike protein in ELISA, Western blot and did not show any reactivity with BSA and milk coated wells (Fig. 1C, D). The II62 tetravalent antibody showed nanomolar affinity to RBD protein in Bio-layer interferometry (Fig. 2A). We further elucidated that II62 tetravalent antibody binds to conformational epitopes in RBD protein and specifically interacts
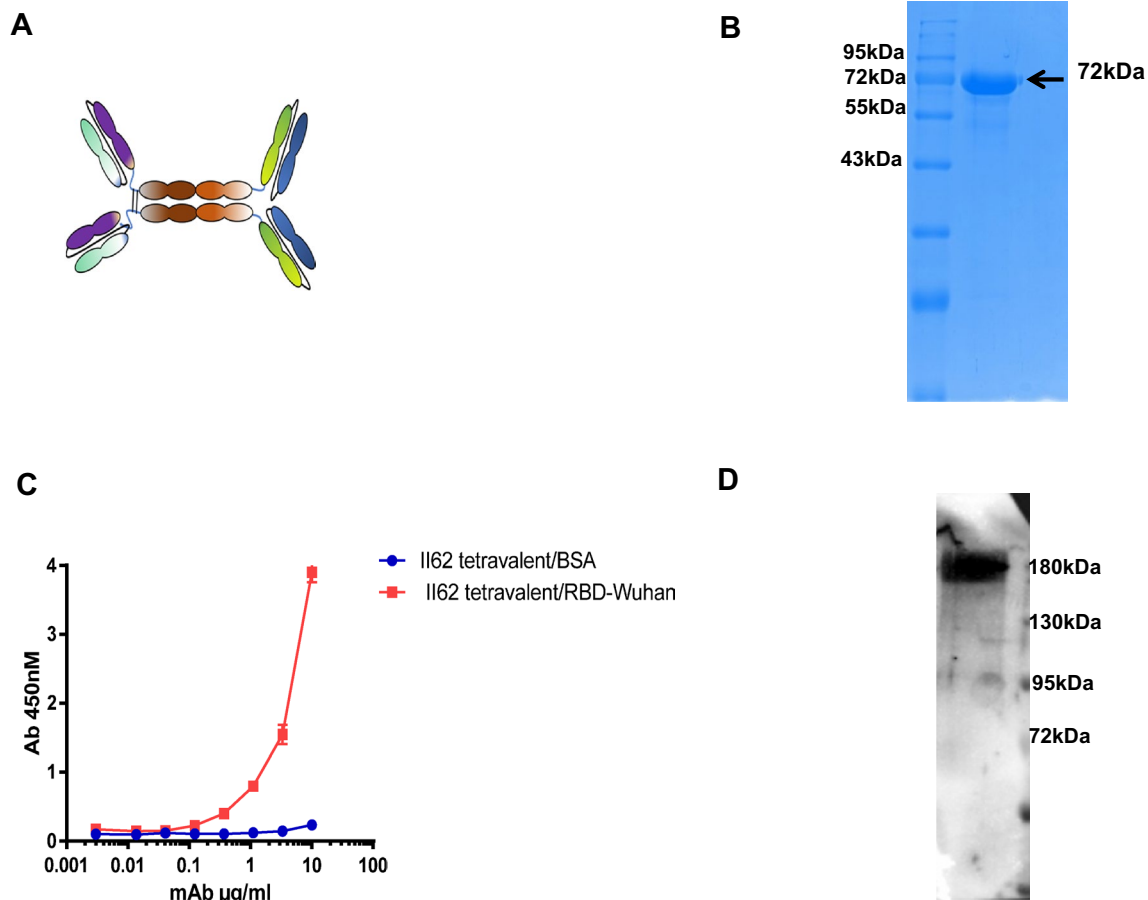

Fig. 1 **A** Representative image of tetravalent form of II62 mAb (scFv-Fc-scFv). **B** SDS-PAGE analysis showing purified II62-tetravalent antibody. **C** Binding specificity of purified II62-teravalent antibody was tested in ELISA with increasing concentration against target antigen, RBD. Unrelated antigens used as experimental control showed no binding. **D** On a Western blot, His-tagged full length spike is identified using II62 tetravalent antibody and HRP conjugated anti-Fc antibody was used to develop the blot. All of the experiments were carried out in duplicates and at least twice
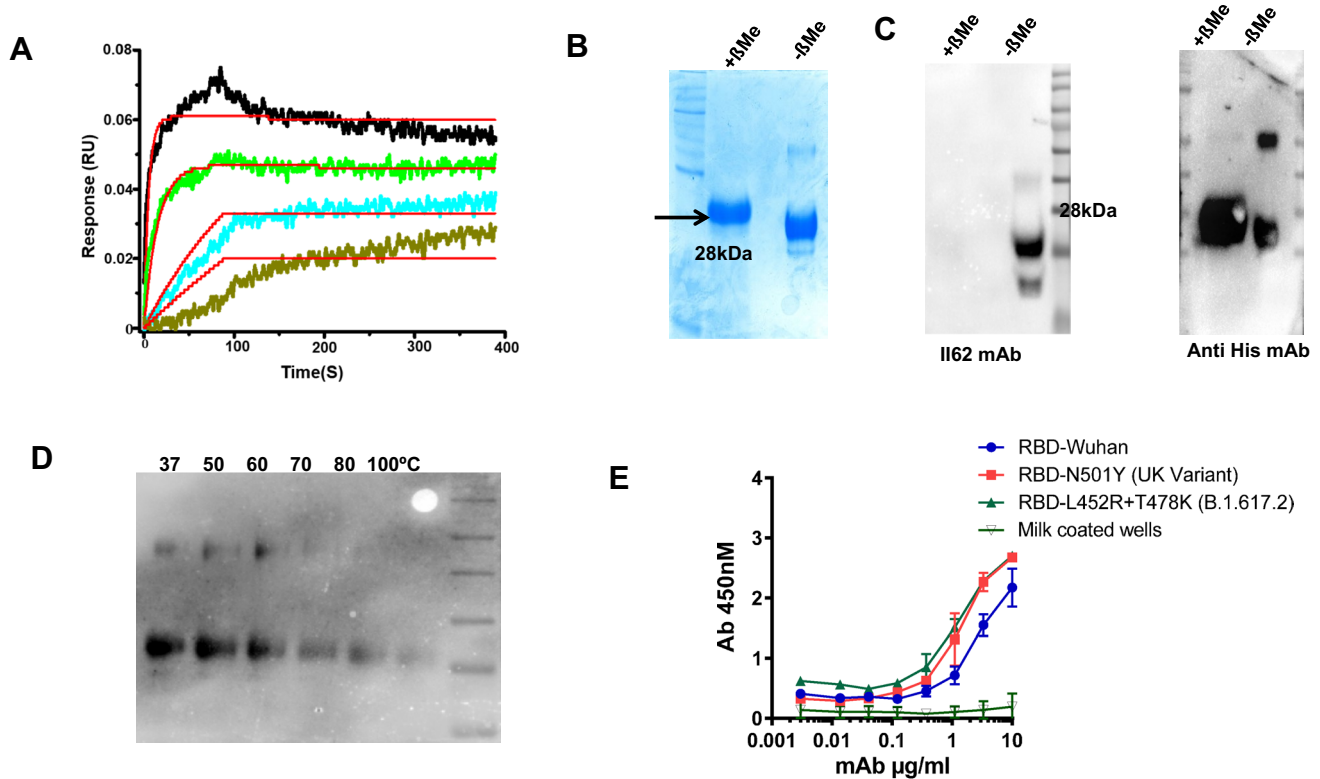
Fig. 2 A Biolayer interferometry (BLI) used to measure the binding kinetics of II62-tetravalent against RBD, **B** Representative images of purified RBD protein in denatured (100 °C in the presence of β mercapto-ethanol containing dye) and without denaturing conditions (No βME and No heat) (~28 kDa) in 12% SDS gel shows a slight differential pattern of bands. **C** In Lane 1 His-tag RBD protein in denatured (100 °C in presence of ß mercapto-ethanol containing dye) and Lane 2, non-denaturing conditions (No βME and No heat) was tested for its binding in Western blot using II62 tetravalent antibody using HRP conjugated anti-Fc antibody. The II62 tetravalent antibody specifically binds with the non-denatured form of the RBD protein. As an experimental control, in a parallel blot same amount of protein was loaded in similar conditions as mentioned above and blot was developed in presence of Anti-His Tag HRP antibody **D** To test the temperature mediated binding of II62-tetravalent antibody to RBD protein, we pre-treated RBD protein with different temperatures ranging from 37 to 100 °C for 20 min before running on polyacrylamide gel electrophoresis. The gel was transferred for western blot and probed with II62 tetravalent antibody followed by anti-Human-Fc-HRP. **E** Cross-reactive binding of II62-tetravalent antibody with different RBD protein corresponding to different VOCs were tested in ELISA

to non-reduced form of the RBD protein as shown in our western blot analysis. To prove the above findings, we run a polyacrylamide gel electrophoresis in reduced (heat and DTT denatured RBD) and non-reduced form of RBD (No heat and without DTT/βME treatment) protein. The II62 tetravalent antibody specifically binds with the non-reduced form of the RBD in Western blot and did not show reactivity with reduced form of protein (Fig. 2B and C). These results confirm that II62 antibody specifically binds to the native conformation of the RBD protein. To further validate the above findings, we run a temperature gradient polyacrylamide gel of RBD protein. Here, the RBD protein was pre-treated with different temperatures ranging from 37 to 100 °C for 20 min before running on polyacrylamide gel electrophoresis. The western blot result showed that as we increased the temperature the binding of II62 was decreased with the RBD protein (Fig. 2D).

We further tested the cross-reactive binding potential of II62 tetravalent antibody to different SARS-CoV-2 VOCs RBD in ELISA binding assay. The data showed that II62 tetravalent antibody showed cross-reactive binding with the RBD proteins of different VOCs (Fig. 2E). A recent study by Liu et al. suggests that avidity is the key for the cross-neutralization activity of mAb COVA1-16. The COVA1-16 Fab fragment doesn't neutralize SARS-CoV-2 viruses, however, when converted into bivalent form (IgG), it neutralized both SARS-CoV-1 and CoV-2 viruses. Similar phenomenon has also been reported previously for broadly neutralizing influenza antibodies to the hemagglutinin (HA) receptor binding site, where increase in valency was found to be directly proportional to the increase in neutralization breadth [10]. To examine, whether increase in valency from monovalent to tetravalent of II62 has any direct effect on neutralization potential. In our previous study, we reported that II62 scFv

does not neutralized authentic SARS-CoV2 wuhan strain at 50 µg/ml concentration. In contrast, we find that increase in valency of II62 antibody from 1 (scFv) to 4 (tetravalent) does not impair the neutralization potential against SARS-CoV-2 virus in CPE-based neutralization assay (Fig. S1). To further elucidate the mechanism for the non-neutralizing behaviour of II62 (tetravalent), we did RBD-ACE2 competition assay and we find that II62 tetra antibody did not compete with the RBD-ACE2 interaction (Fig. S2). These data further confirm that II62 mAb binds to an epitope in RBD protein that spans away from the ACE2 interaction zone and binding of tetravalent antibody does not even structurally occlude the RBD-ACE2 interaction. These results also explain why increase in valency from monovalent to tetravalent doesn't affect the neutralization potential of II62 antibody.

The II62 mAb targeting epitopes does not depend on direct blocking of RBD-ACE2 binding interface, other than 417 no escape mutants have been reported for mAbs targeting away from receptor binding motif (RBM). A similar class of antibody, CR3022 which potently neutralize CoV-1 via destruction of prefusion spike conformation and at present with the available escape mutant data, CR3022 binding has been found to be unaffected. The another mAb CR3014 isolated from the same patient immune phage display library, targets the interface of ACE2- RBD binding complex and escape mutations could be readily generated for CR3014. The combination of two or more non-competing human mAbs that recognize different parts of the spike surface can theoretically monitor immune escape and expand the spectrum of defence. At the same time, synergy can allow a lower total dose of antibody to be administered for passive immune prophylaxis of SARS-CoV2 infection that recognize different parts of the SARS-CoV surface. Further structural analysis of II62 mAb in complex with RBD will guide the design of broad-spectrum vaccines and will give mechanistic view on its broad reactivity. It will be intriguing to combine these tetravalent antibodies with other mAbs that specifically disrupt ACE2 interaction by targeting the receptor binding motif (RBM) of the SARS-CoV-2 RBD protein, where antibody valency may have a direct influence on inhibition.

Materials and methods

Expression and purification of antibody and structural protein

Here, we constructed tetravalent II62 scFv-Fc-scFv antibodies with two N*co*I + N*ot*I compatible cloning sites flanking the Fc gene fragment as described by Pohl et al. (2012). The II62 tetravalent antibody was purified using a protein G affinity column after transient transfection of plasmid in

Expi293F cells. Similarly, mammalian cell codon optimized nucleic sequence of SARS-CoV-2-RBD–His and spike was expressed and produced in Expi 293 F mammalian expression system. The cells were transiently transfected with the expressing plasmids. The supernatant was collected after 5–6 days, and the soluble protein was purified using Ni-NTA affinity chromatography with Ni^{2+} ions immobilised on a resin by covalent attachment to nitrilotriacetic acid (NTA) (QIAGEN, Germany), as described (Parray et al. 2020; Perween et al. 2021).

ELISA

ELISA plates were coated with equimolar amounts of antigens (RBD-His and spike protein). II62 tetravalent antibody was added in threefold serial dilutions starting from 20 µg/ml. For Fc bearing antibody formats (scFv-Fc, IgG1and tetravalent), HRP-conjugated goat anti-human secondary antibody (Jackson Immunoresearch) was used. In case of scFv antibody, HRP conjugated Protein-L was used as secondary antibody. Standard protocols of blocking and washing of ELISA plates were followed as described previously (Kumar et al. 2012, 2019).

Biolayer interferometry binding assay

Binding assays were carried out using an Octet Red instrument (Forte'Bio) using biolayer interferometry (BLI), as previously mentioned by Parray et al. (2020). Briefly, anti-human Fc sensors (ForteBio Inc.) were used to capture the RBD-Fc or the scFv-Fc, tetravalent and IgG1 antibody formats at10 µg/ml in $1 \times$ kinetics buffer ($1 \times$ PBS, pH 7.4, 0.01% BSA and 0.002% Tween 20) and incubated with the indicated concentrations of RBD and Spike proteins. Associations and dissociations have been reported, depending on the analyte. Data was analysed using the software ForteBio Data Analysis.

Viral neutralization assay

For the plaque-based neutralization titer assay, 100 µg/ml of CR3022 mAb formats (concentration range of 50 µg/mL–1.53 ng/mL) was incubated with 100 PFU of the CoV 2 Wuhan-1 strain for 2 h at 37 °C. The virus and antibodies were then added to a 24-well plate seeded with Vero E6 cells. After 2-h incubation at 37 °C, cells were overlaid with 3 ml of 0.8% carboxy methyl cellulose in 2% medium. Plates were incubated for 2 days at 37 °C and cells were fixed in 4% *para*formaldeyhe stained with crystal violet 1 h, and plaques were counted.

Declarations

Conflict of interest All authors report that there are no conflicts of interest.

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