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A tetrameric ACE2 protein broadly neutralizes SARS-CoV-2 spike variants of concern with elevated potency

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ABSTRACT

The SARS-CoV-2 receptor angiotensin converting enzyme 2 (ACE2) was previously engineered into a high affinity tetravalent format (ACE2-Fc-TD) that is a potential decoy protein in SARS-CoV-2 infection.We report that this protein shows greatly enhanced binding to SARS-CoV-2 spike proteins of the SARS-CoV-2 variants of concern B.1.1.7 (alpha variant, originally isolated in the United Kingdom) and B.1.351 (beta variant, originally isolated in South Africa) with picomolar compared with nanomolar Kd values. In addition, ACE2-Fc-TD displays greater neutralization of SARS-CoV-2 pseudotype viruses compared to a dimeric ACE2-Fc, with enhanced activity on variant B.1.351. This tetrameric decoy protein would be a valuable addition to SARS-CoV-2 therapeutic approaches, especially where vaccination cannot be used but also should there be any future coronavirus pandemics.

The global pandemic caused by SARS-CoV-2 continues and the development of efficacious vaccines (Folegatti et al., 2020; Polack et al., 2020; Wang et al., 2021) which raise an immune response against the viral spike protein are a major milestone in preventing the onset of the disease. In addition, a large number of neutralizing antibodies have been rapidly developed (Brouwer et al., 2020; Y. L. Cao et al., 2020; DeFrancesco, 2020) and some have entered clinical use for treating patients with COVID-19 (Weinreich et al., 2021). SARS-CoV-2 infects epithelial cells by binding to the angiotensin converting enzyme 2 (ACE-2) receptor as does SARS-CoV-1 (Kuba et al., 2005) and clinically applied monoclonal antibodies (Chen et al., 2021; Weinreich et al., 2021) and vaccinations (Folegatti et al., 2020; Wang et al., 2021; Xie et al., 2021) have been a dramatic success in virus neutralization. However, there has been a recent surge in the evolution of SARS-CoV-2 variants with spike mutations (so-called variants of concern, VOC) that appear more transmissible and accordingly have become dominant in some regions (Davies et al., 2021). The VOC B1.1.7 (Alpha, first identified in the UK) and B.1.351 (Beta, first identified in South Africa) have multiple mutations (**B.1.1.7** S: del69–70 HV, del144 Y, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H; **B.1.351** S: L18F, D80A, D215G, R2461I, K417N, E484K, N501Y, D614G, A701V (Galloway et al., 2021; Gomez et al., 2021; Plante et al., 2021) of which the N501Y change increases affinity to ACE2 (Ali et al., 2021; Luan et al., 2021) while E484K may be partly responsible for reduced or escaped binding to anti-SARS-CoV-2 antibodies (Jangra et al., 2021; Nonaka et al., 2021). The B.1.617 lineage (Delta, first identified in India) which has L452R and D614G mutations, has been recently been designated as a VOC by the World Health Organization (2021). Further, B1.526 (first identified in New York) and B.1427/429 (first identified in California) have L452R and Y453F mutations that also increase binding affinity to viral receptor ACE2 (Bayarri-Olmos et al., 2021; Deng et al., 2021; Tchesnokova et al., 2021). Therefore, there is fear that VOC may reduce the efficacy of the anti-SARS-CoV-2 antibodies and vaccines against the first identified strain, Wuhan Hu-1 (herein referred to as the Wuhan strain) or eventually evade them completely. The Emergency Use Authorization of monoclonal antibody therapies targeted against the SARS-CoV-2 S1 protein (e.g. Casirivimab, Imdevimab, Bamlanivimab and Etesevimab ("An EUA for bamlanivimab - a monoclonal antibody for COVID-19,"

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2020; "An EUA for bamlanivimab and etesevimab for COVID-19," 2021; "An EUA for casirivimab and imdevimab for COVID-19," 2020) offer protection to high-risk patients with mild-to-moderate COVID-19 however their efficacy is also compromised by SARS-CoV-2 S1 variants (Starr et al., 2021).

Delivered, soluble ACE2 receptor proteins represent a potential variant-proof therapy by working as virus-binding decoys because the SARS CoV-2 lifecycle requires ACE2 receptor binding (Hoffmann et al., 2020). In addition, if a SARS-CoV-2 S1 mutation increases ACE2 binding affinity, potentially increasing viral entry and infectivity, then binding affinity to ACE2 receptor decoy is also expected to increase. The soluble ACE2 receptor alone is not suitable for therapeutic use against SARS-CoV-2 due to short systemic half-life (Haschke et al., 2013) and low affinity of the monovalent receptor (Linsky et al., 2020). Both of these properties have been improved by generating ACE2 fusion to human immunoglobulin crystallizable fragment (Fc) domain (Ferrari et al., 2021; Lei et al., 2020). An alternative approach to the problem of short serum half-life is to avoid systemic administration altogether. This has been demonstrated through the generation of small ACE2 derived "miniproteins" which may be administered intranasally allowing access to the lung, a major site of COVID-19 pathogenesis (L. Y. Cao et al., 2020; Case et al., 2021). Sole use of inhalational therapeutics however will not address the multiorgan tropism of SARS-CoV-2 and the use of truncated ACE2 peptides may allow future mutational escape. We have recently described an engineered ACE2 fused to Fc and the p53 tetramerization domain (Miller et al., 2021). This tetravalent ACE2-Fc-TD protein, termed a quad, displayed effective *in vitro* neutralization of pseudotyped virus displaying spike protein of the original Wuhan SARS-CoV-2 strain. We now show that the ACE2-Fc-TD decoy Quad protein not only maintains efficacious binding to SARS-CoV-2 variant spike proteins but it does so with significantly enhanced potency unlike monoclonal antibodies in clinical use. This emphasizes the importance of increased valency and avidity in molecular interactions and potential for use of multimeric ACE2 as a variant-proof COVID-19 therapeutic decoy protein.

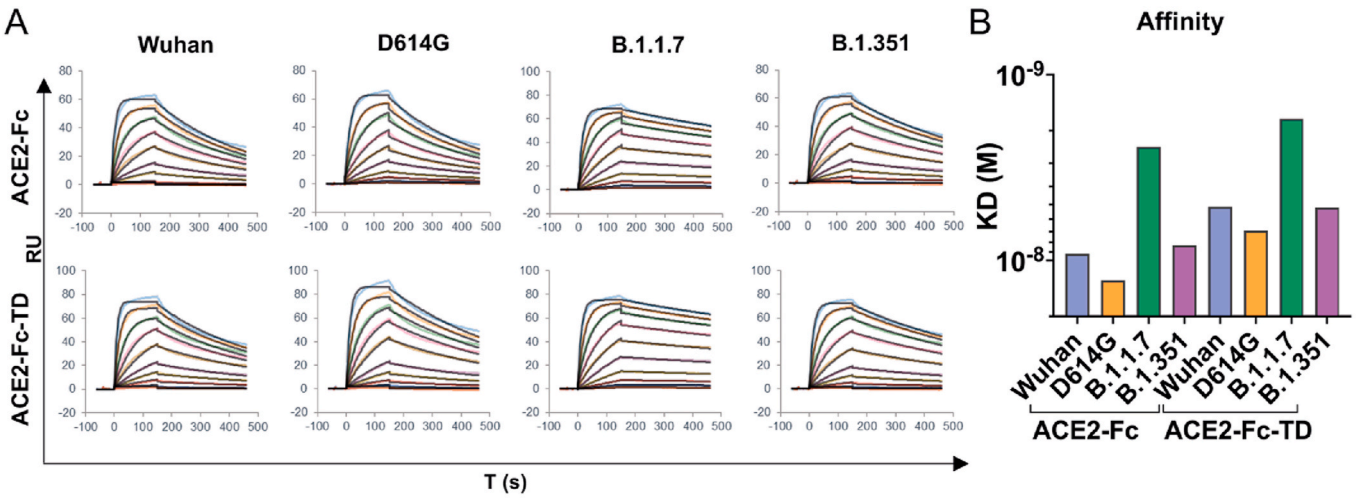
Kinetic binding parameters of the tetrameric ACE2-Fc-TD with viral spike proteins were determined alongside a commercially-sourced bivalent ACE2-Fc protein (Acro Biosystems, AC2-H5257). Alongside the Wuhan, B.1.1.7 and B.1.351 spike proteins, a version with the D614G mutation alone was also included since this mutation stabilizes spike trimers, increasing the number available to facilitate infection (Fernandez, 2020; Zhang et al., 2021) and increasing viral load in the upper respiratory tract (Ozono et al., 2021; Plante et al., 2021). This mutation is found in all variants tested here. Initially binding was assessed following capture of ACE2-Fc or ACE2-Fc-TD and using the spike protein as the analyte (Fig. 1A). This setup effectively disregards the valency of the ACE2 constructs and measures the intrinsic 1:1 affinity of interaction between the spike paratope and ACE2 epitope for each construct. As previously reported for the bivalent ACE2-Fc (Ferrari et al., 2021), the affinity of the B.1.1.7 spike for the ACE2 proteins was greater than that of the Wuhan or D614G spikes (Fig. 1 A & B, Table 1), as might be expected given their greater transmissibility. In a different experimental protocol, spike protein was captured on the chip at 3 densities: 40, 130 and 230 RU, designated low, mid and high respectively. ACE2 constructs were then used as the analyte. In this setup the valency of the ACE2 constructs can contribute to the affinity of spike interaction through avidity effects. Again, the same trend of increased affinity interactions is observed with the B.1.1.7 and B.1.351 VOC versus the Wuhan or D614G spikes (Fig. 1C), although this was particularly apparent for the affinity of interaction of the tetravalent ACE2-Fc-TD especially at high spike densities (Fig. 1D, Table 2). The B.1.1.7 variant again had the highest affinity for the ACE2 constructs.

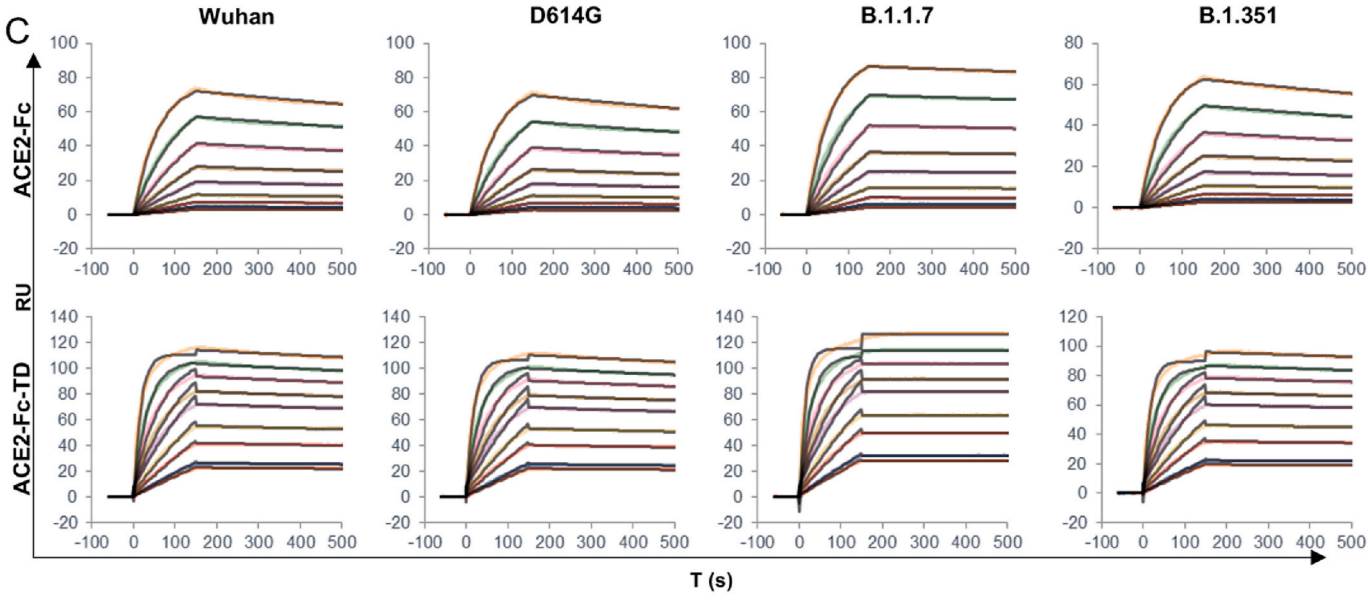
Binding of the ACE2-Fc-TD protein shows increased affinity for the B.1.1.7 and B.1.351 spike VOC compared to the Wuhan spike protein. We determined if this interaction enhancement was reproduced in the ability of ACE2-Fc-TD to neutralize virus infection through membrane-bound ACE2 and comparing this with the dimeric ACE2-Fc. The infection of the four pseudotyped viruses was assayed in the presence of increasing amounts of the dimeric ACE2-Fc or ACE2-Fc-TD tetrameric quad protein with a normalized viral input (Fig. 2A–D). The ACE2-Fc-TD quad protein was more potent in virus neutralization than ACE2-Fc for the Wuhan virus as previously shown (Miller et al., 2021) but also for neutralizing the VOC B.1.1.7 and B.1.351 as well as the D614G pseudotyped virus. Comparing the IC_{50} for virus neutralization demonstrates a significant improved potency of the tetrameric ACE2 compared to the dimeric form (Fig. 2E).

The ACE2-Fc-TD quad protein retains neutralization potency across the Wuhan (Fig. 2A), D614G alone (2B), B.1.1.7 (2C) and B.1.351 variants (2D). Thus, despite the differences detected in the SPR assay, the ACE2-Fc-TD has comparable enhanced neutralization potencies across the Wuhan, D614G and B.1.1.7 but shows a 2.2-fold increased neutralization for the B.1.351 pseudotype (IC_{50} 0.11 nM compared to 0.24 nM for the Wuhan pseudovirus) (Fig. 2E). It is interesting to note that the ACE2-Fc bivalent molecule shows an increased neutralization for both B.1.1.7 and B.1.351 VOC, compared to the Wuhan pseudotype but the ACE2-Fc-TD tetramer only has increased neutralization for the B.1.351, possibly due to different spike protein densities and to the different modality in which the bivalent and tetramer molecules may interact with viruses. Despite these differences, the ACE2-Fc-TD has 38- and 88-fold enhancement in neutralization potency of the B.1.1.7 and B.1.351 VOC respectively compared to the dimeric ACE2-Fc.

The emergence of SARS-CoV-2 variants of concern places constraints on the use of monoclonal antibodies for treating COVID-19. Their narrow breadth of epitope specificity makes monoclonal antibodies particularly vulnerable to escape S1 variants with amino acid changes or deletions affecting the antigenic site (McCarthy et al., 2021; Starr et al., 2021; Starr et al., 2021). The same concern surrounds vaccines designed on exposing the host humoral and cell-based immune response to spike protein antigens. Furthermore, vaccination is also not suitable or effective in some people such as those receiving B-cell depleting therapies for haematological malignancies or undergoing immuno-suppression like transplant patients, or those suffering from rheumatoid arthritis or multiple sclerosis (Baker et al., 2020; Mehta et al., 2020). The SARS-CoV-2 receptor ACE2 will not be subject to these issues since viral infection into epithelial cells requires ACE2 as the viral receptor.

Accordingly, we have characterized the binding and pseudotyped virus neutralization capacity of a novel tetrameric form of ACE2-Fc-TD (Miller et al., 2021). The binding properties of this protein was analyzed with recombinant spike proteins corresponding to those from the original SARS-CoV-2 Wuhan isolate and from B.1.1.7 (Alpha) and B.1.351 (Beta). The binding data show that ACE2-Fc-TD is a potent protein that neutralizes all the pseudotypes analyzed (original isolate and two VOC). This illustrates the inherent value of the decoy receptor strategy in maintaining neutralization of the more infectious variants by both dimeric ACE2-Fc and tetrameric ACE2-Fc-TD. The importance of ACE2 multimerization for binding potency is demonstrated by the ACE2-Fc-TD displaying high affinities for all the spike proteins. The translation of this potency to pseudotyped virus neutralization further articulates the importance of valency and avidity in improving interaction of the receptor protein and the viral spike protein. These observations show that the increased valency of ACE2-Fc-TD generate a potent inhibitor of two known VOC pseudotyped viruses. It is projected the ACE2-Fc-TD would also have a similar increased neutralization potency against other emerging highly transmissible VOC, such as the more recent B.1.617 (Delta) variant originating from India which has the D614G mutation that we show in three variant pseudotyped viruses. Therefore, based on the concept of mandatory interaction of SARS-CoV-2 with ACE2 receptor for COVID-19, our data encourages the need for further development of tetravalent ACE2-Fc-TD for clinical application. This could be applied against current and future SARS-CoV-2 variants in those individuals not able to receive an appropriate SARS-CoV-2 vaccine, either due to their





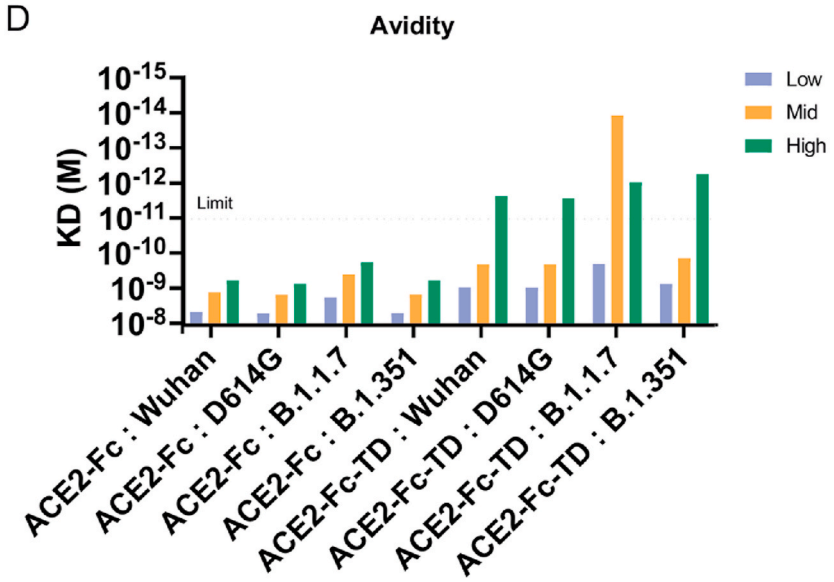


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Fig. 1. Biophysical characterization of ACE2-Fc-TD binding to recombinant SARS-CoV-2 variant spike proteins.
A. Representative sensorgrams of binding between chip-captured ACE2-Fc or ACE2-Fc-TD (70–80 RU) and Wuhan or variant spike proteins (D614G, B.1.1.7 and B.1.351) used as analyte at a concentration of 250 nM with 2-fold serial dilutions.
B. Affinity of ACE2 constructs for Wuhan and variant spike proteins as measured using the ACE2-Fc or ACE2-Fc-TD coated chip SPR.
C. Representative sensorgrams of binding between chip-captured Wuhan or variant spike proteins (D614G, B.1.1.7 and B.1.351) at a S1 coating density of 130 RU, and using ACE2 proteins as the analyte at concentrations of 100 nM with 2-fold serial dilutions.
D. Valency of the ACE2 construct can affect binding parameters with this protocol. Avidity of ACE2 constructs for Wuhan and variant spike S1 proteins was determined by immobilizing spike proteins on the SPR chip at low, mid or high densities (40, 130 and 230 RU respectively) and using ACE2 proteins as the analyte.

Table 1
Binding parameters of recombinant SARS-CoV-2 variant spike proteins to chip-captured ACE2-Fc-TD or ACE2-Fc.

Clone	Spike S1	1:1 binding ka (1/Ms)	kd (1/s)	KD (M)	Half-life (ln2/kd)
ACE2-Fc-TD	Wuhan	5.21E+05	2.67E-03	5.13E-09	2.58E+02
	D614G	3.05E+05	2.11E-03	6.91E-09	3.27E+02
	B.1.1.7	3.35E+05	5.80E-04	1.73E-09	1.19E+03
	B.1.351	2.93E+05	1.52E-03	5.18E-09	4.54E+02
ACE2-Fc	Wuhan	2.88E+05	2.65E-03	9.21E-09	2.60E+02
	D614G	2.56E+05	3.27E-03	1.28E-08	2.11E+02
	B.1.1.7	2.93E+05	7.19E-04	2.45E-09	9.60E+02
	B.1.351	2.23E+05	1.85E-03	8.28E-09	3.73E+02

Table 2
Binding parameters of chip-captured recombinant SARS-CoV-2 variant spike proteins to ACE2-Fc-TD or ACE2-Fc.

Clone	Antigen density	Spike S1	1:1 binding ka (1/Ms)	kd (1/s)	KD (M)	Half-life (ln2/kd)
ACE2-Fc-TD	Low density	Wuhan	7.35E+05	6.22E-04	8.46E-10	1.11E+03
		D614G	7.31E+05	6.32E-04	8.65E-10	1.10E+03
		B.1.1.7	8.43E+05	1.58E-04	1.87E-10	4.40E+03
		B.1.351	7.67E+05	5.26E-04	6.85E-10	1.32E+03
	Mid density	Wuhan	6.73E+05	1.30E-04	1.93E-10	5.34E+03
		D614G	6.65E+05	1.29E-04	1.95E-10	5.35E+03
		B.1.1.7	8.46E+05	*1.00E-08*[a]	*1.18E-14*[a]	6.90E+07
		B.1.351	7.42E+05	9.58E-05	1.29E-10	7.24E+03
	High density	Wuhan	5.58E+05	1.27E-06	2.27E-12	5.48E+05
		D614G	5.53E+05	1.46E-06	2.64E-12	4.74E+05
		B.1.1.7	8.42E+05	*7.66E-07*[a]	*9.10E-13*[a]	9.05E+05
		B.1.351	6.58E+05	*3.56E-07*[a]	*5.41E-13*[a]	1.95E+06
ACE2-Fc	Low density	Wuhan	2.63E+05	1.12E-03	4.25E-09	6.20E+02
		D614G	2.55E+05	1.20E-03	4.70E-09	5.79E+02
		B.1.1.7	1.96E+05	3.22E-04	1.64E-09	2.15E+03
		B.1.351	2.30E+05	1.05E-03	4.57E-09	6.58E+02
	Mid density	Wuhan	2.13E+05	2.52E-04	1.18E-09	2.75E+03
		D614G	2.05E+05	2.82E-04	1.37E-09	2.46E+03
		B.1.1.7	2.13E+05	8.01E-05	3.76E-10	8.65E+03
		B.1.351	1.96E+05	2.67E-04	1.36E-09	2.59E+03
	High density	Wuhan	1.75E+05	9.74E-05	5.55E-10	7.11E+03
		D614G	1.70E+05	1.14E-04	6.73E-10	6.06E+03
		B.1.1.7	2.10E+05	3.56E-05	1.69E-10	1.95E+04
		B.1.351	1.99E+05	1.09E-04	5.50E-10	6.34E+03

[a] *Outside instrument limits. kd limited to 1E-08.*

health status or the origin of viral variants outpacing vaccine development. Certainly, as more infectious SARS-CoV-2 variants become dominant in circulation, multimeric ACE2 decoys could be an important part of the medical armory as a single-agent therapy to neutralize VOC without the need to establish complex cocktails of neutralizing antibodies. Multimeric ACE2 decoys could be applied in clinical settings similar to those of neutralizing antibodies, namely in prevention of symptomatic infection or in the early stages of symptomatic infection (Taylor et al., 2021), but without the reduced efficacy due to the circulation of VOCs. Finally, if new human-tropic coronaviruses arise in future (which is not an unlikely scenario given the spontaneous appearance of MERS-CoV, SARS-CoV and SARS-CoV-2 in the human population), multimeric ACE2 decoys could become front line, off-the-shelf therapeutics for immediate use during which time appropriate specific vaccines are developed and become available.

1. Materials and methods

1.1. Recombinant protein preparation

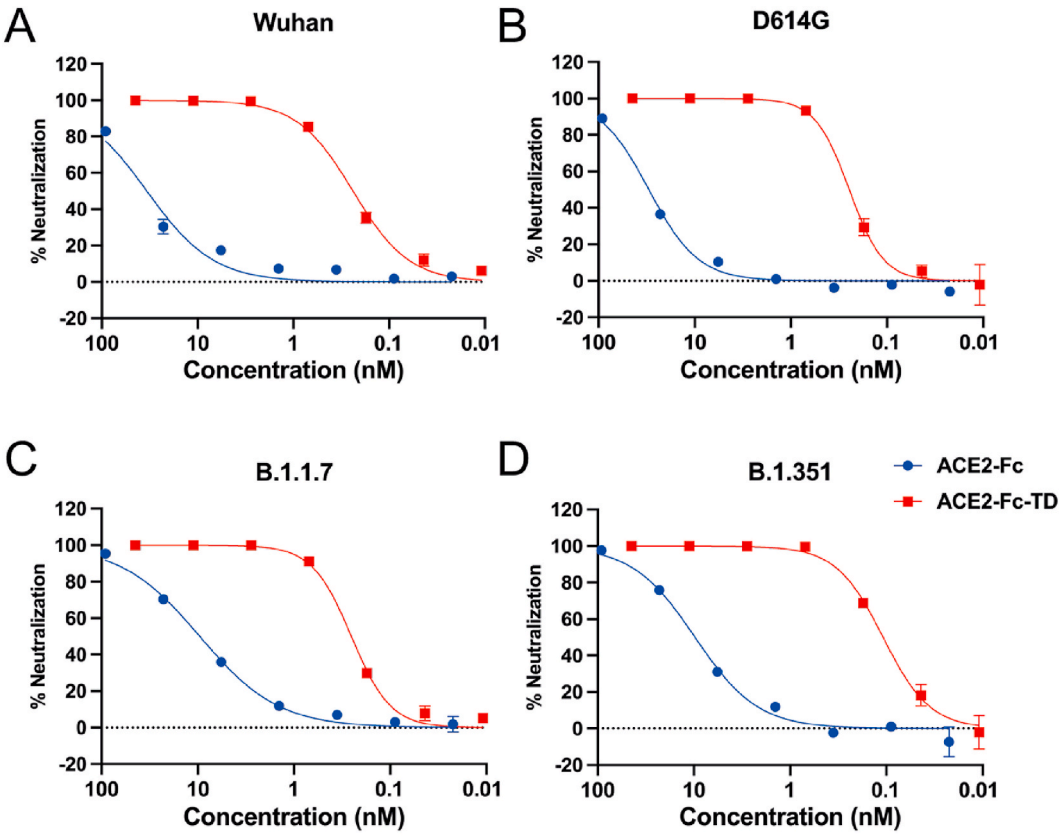
ACE-Fc-TD protein preparation was carried out as previously described (Miller et al., 2021) and also commercially sourced active ACE2-Fc was used as a control (ACRO biosystems - AC2-H5257).

SARS-CoV-2 S1 proteins were prepared as previously described (Ferrari et al., 2021). Briefly, SARS-CoV-2 S1 domains (aa1-681 from reference GenBank - QHD43416.1) of Wuhan, D614G, B.1.1.7 and B.1.351 variants were transiently expressed in Expi293 cells and purified via TALON metal affinity chromatography.

1.2. Surface plasmon resonance

1:1 kinetic assay for ACE2-Fc and ACE2-Fc-TD on S1 spike protein domains from Wuhan, D614G, B.1.1.7 and B.1.351 strains was performed as previously described (Ferrari et al., 2021). Briefly, ACE2-Fc and ACE2-Fc-TD proteins were captured on a Protein A Series S chip (Cytiva - 29127555) to a density of 70–80 RU using a Biacore 8K instrument (GE Healthcare). S1 proteins were used as analyte and injected over the flow cells for 150s at 30 μl/min with 300s dissociation time, at a concentration of 250 nM with 2-fold serial dilutions. Since a monovalent interaction was sought, sensograms were fitted with a 1:1 Langmuir binding model.

For avidity interaction analysis, S1 spike protein domains from Wuhan, D614G, B.1.1.7 and B.1.351 strains were captured on a Series S CM5 chip functionalized with anti-His capture kit (Cytiva – 28995056)



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Pseudotyped virus	ACE2-Fc IC_{50} (nM)	ACE2-Fc-TD IC_{50} (nM)	Fold change ACE2-Fc-TD from ACE2-Fc
Wuhan	34.00	0.24	141
D614G	30.01	0.25	120
B.1.1.7	9.55	0.25	38
B.1.351	9.74	0.11	88

Fig. 2. Pseudotyped virus neutralization by tetrameric ACE2-Fc-TD.
In vitro neutralization of Wuhan (panel A) and variant D614G (panel B), B.1.1.7 (panel C) and B.1.351 (panel D) pseudotyped virus infection by ACE2-Fc and ACE2-Fc-TD (Mean ± SEM) (n = 2). (E) Tabulation of the concentrations required for 50 % neutralization (IC_{50}) of infection by pseudotyped virus bearing either Wuhan, D614G, B.1.1.7 or B.1.351 spike S1 proteins.

to a density of 40 (low), 130 (mid) and 230 RU (high) on flow cells 2, 3 and 4, respectively. Flow cell 1 was maintained empty for reference subtraction. ACE2-Fc and ACE2-Fc-TD were used as analyte and injected over the flow cells at 30 µl/min for 150s with 300s dissociation, at concentrations of 100 nM with 2-fold serial dilutions. A 0 concentration of buffer alone was used as double reference subtraction. Sensograms were fit to a 1:1 Langmuir binding model.

1.3. Viral vector production

Lentiviral vectors pseudotyped with SARS-CoV-2 spike glycoprotein from Wuhan, D614G, B.1.1.7 and B.1.351 variants were prepared from HEK293T transfection as previously described (Ferrari et al., 2021). Functional infectious viral titres (IU) were determined via flow cytometry (Fortessa X-20 cell analyser) on HEK293T cells expressing human ACE2 and TMPRSS2. Physical particle numbers were determined using QuickTiter Lentivirus Titer kit (Cell biolabs – VPK-107-T).

1.4. Pseudotyped virus neutralization assay

Pseudotyped virus neutralization was performed as previously described (Ferrari et al., 2021). Briefly, ACE2-Fc and ACE2-Fc-TD were incubated 1:1 with pseudotype vectors, normalized to the physical particles of 1×10^5 IU of WT Wuhan vector, for 1 h at 37 °C. Protein/virus mix was then added to $3x10^4$ HEK293T cells/well engineered to express human ACE2 and TMPRSS2 in the presence of 8 µg/ml of polybrene, and incubated for 72 h at 37 °C. Viral titers were quantified by flow cytometry (Fortessa X-20 cell analyser) and infectivity expressed as a percentage of viral titers in the PBS only control. Data was analyzed using GraphPad Prism v8 and data fit with non linear regression curves to obtain half-maximal inhibitory concentration (IC_{50}).

Author contributions

Originators of project: THR, AL, HA. Participated in research design: AL, MF, EM, GM, SO, HA, THR. Conducted experiments: AL, FTI, ZA, EB, AM. Performed data analysis: All authors. Wrote or contributed to the writing of the manuscript: All authors.

Additional information

Materials and methods appear in supplementary Information to this paper.

Declaration of competing interest

HA & THR are stake holders in Quadrucept Bio. FTI, ZA, MF and SO are employees of Autolus Therapeutics. None of the other authors have any conflicts of interest to declare.

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