High activity of an affinity-matured ACE2 decoy against Omicron SARS-CoV-2 and pre-emergent coronaviruses

Running Title: ACE2 decoy maintains activity against emerging coronavirus variants

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Abstract

The viral genome of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and particularly its cell-binding spike protein gene, has undergone rapid evolution during the coronavirus disease 2019 (COVID-19) pandemic. Variants including Omicron now seriously threaten the efficacy of therapeutic monoclonal antibodies and vaccines that target the spike protein. Viral evolution over a much longer timescale has generated a wide range of genetically distinct *sarbecoviruses* in animal populations, including the pandemic viruses SARS-CoV-2 and SARS-CoV-1. The genetic diversity and widespread zoonotic potential of this group complicates current attempts to develop drugs in preparation for the next *sarbecovirus* pandemic. Receptor-based decoy inhibitors can target a wide range of viral strains with a common receptor and may have intrinsic resistance to escape mutant generation and antigenic drift. We previously generated an affinity-matured decoy inhibitor based on the receptor target of the SARS-CoV-2 spike protein, angiotensin-converting enzyme 2 (ACE2), and deployed it in an adeno-associated viral vector (rAAV) for intranasal delivery and passive prophylaxis against COVID-19. Here, we demonstrate the exceptional binding and neutralizing potency of this ACE2 decoy against SARS-CoV-2 variants including Omicron, as well as binding to diverse ACE2-dependent coronaviruses. We also discuss a strategy of decoy-based treatment and passive protection to mitigate the ongoing COVID-19 pandemic and future airway virus threats.

Author Summary

Viral sequences can change dramatically during pandemics lasting multiple years. Likewise, evolution over centuries has generated genetically diverse virus families posing similar threats to humans. This variation presents a challenge to drug development, in both the breadth of achievable protection against related groups of viruses and the durability of therapeutic agents or vaccines during extended outbreaks. This phenomenon has played out dramatically during the coronavirus disease 2019 (COVID-19) pandemic. The highly divergent Omicron variant of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has upended previous gains won by vaccine and monoclonal antibody development. Moreover, ecological surveys have increasingly revealed a broad class of SARS-CoV-2-like viruses in animals, each poised to cause a future human pandemic. Here, we evaluate an alternative to antibody-based protection and prevention—a decoy molecule based on the SARS-CoV-2 receptor. Our engineered decoy has proven resistant to SARS-CoV-2 evolution during the ongoing COVID-19 pandemic and can neutralize all variants of concern, including Omicron. Furthermore, the decoy binds tightly to a broad class of *sarbecoviruses* related to pandemic SARS-CoV-2 and SARS-CoV-1, indicating that receptor decoys offer advantages over monoclonal antibodies and may be deployed during the COVID-19 pandemic and future coronavirus outbreaks to prevent and treat severe illness.

1. Introduction

Monoclonal antibody therapeutics with the ability to bind the spike protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and prevent cell entry have been critical tools in managing the coronavirus disease 2019 (COVID-19) pandemic (1, 2). These drugs prevent hospitalizations when applied early in the course of infection (3) and can provide critical passive protection for vulnerable populations of immunocompromised patients who cannot mount a protective response to vaccines (4). However, monoclonals have proven particularly susceptible to SARS-CoV-2 evolution (5). This susceptibility may arise because the spike epitopes most sensitive to neutralization have been under intense selection as the virus has made gains in transmissibility and its ability to evade human immunity (6). Furthermore, evidence suggests that single monoclonals applied in a therapeutic setting can rapidly give rise to escape mutants (7-10). Together, these findings call into question the ability of the antibody platform to keep pace with the course of the COVID-19 pandemic or to be of use in future pandemics caused by other coronaviruses.

Receptor decoys may represent a mode of viral neutralization that is more resistant to continued viral evolution and escape-mutant generation (11). SARS-CoV-2 evolution has occurred in a way that retains tight binding to its primary cell entry receptor, angiotensin-converting enzyme 2 (ACE2) (12). We and others have developed affinity-matured, soluble ACE2 decoy molecules that potently neutralize SARS-CoV-2 (11, 13-18). Our soluble Fc-fused decoy, CDY14HL-Fc4, contains six amino acid substitutions that improve the neutralization of CoV-2 variants by 300-fold versus un-engineered ACE2 and an active site nutation to ablate its endogenous angiotensin-

cleaving activity. Furthermore, CDY14HL maintains tight binding or neutralizing activity for the distantly related *sarbecoviruses* WIV1-CoV, and SARS-CoV-1 despite being engineered for improved activity against SARS-CoV-2 (13). This property suggests that this decoy may be a useful tool to combat future pandemics from currently pre-emergent, ACE2-dependent coronaviruses.

Here, we evaluate the binding and neutralization activity of CDY14HL against a wide range of emerging SARS-CoV-2 variants, including Omicron, and pre-emergent ACE2-dependent coronaviruses. These studies suggest the broad utility of decoy-based viral entry inhibitors in combating current and future coronavirus pandemics.

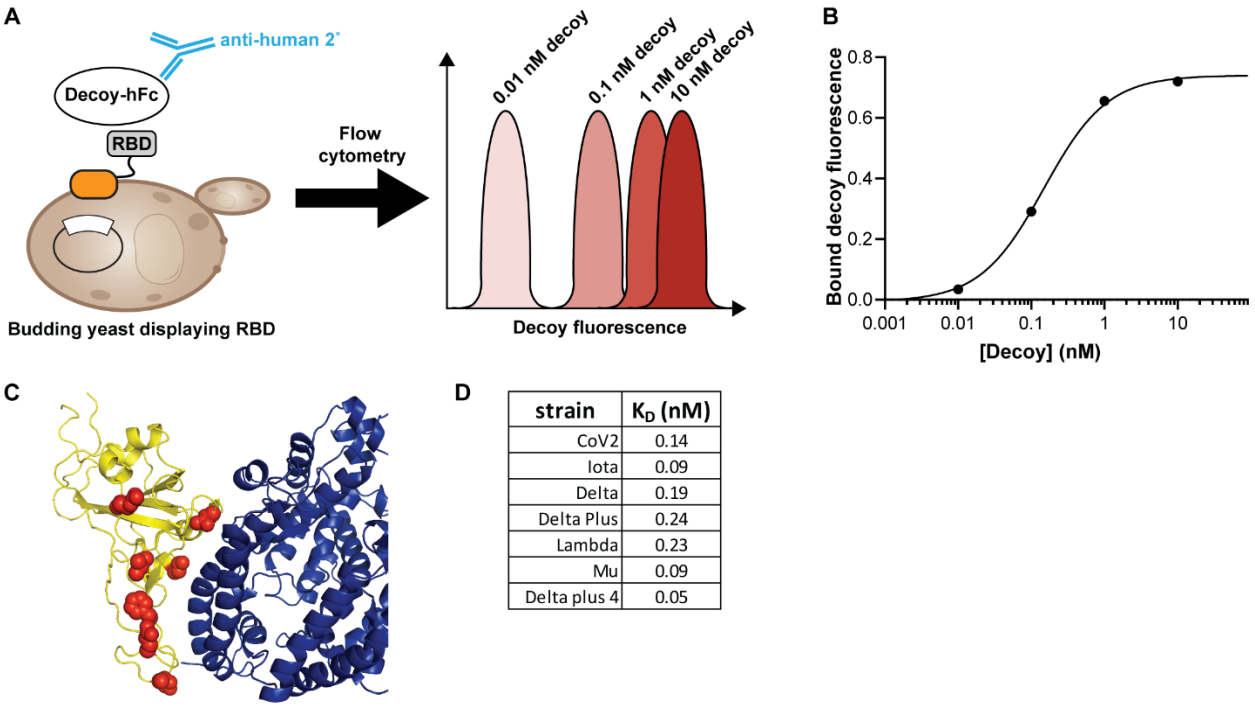
2. Results

2.1 CDY14HL maintains tight binding to diverse SARS-CoV-2 variants

We set out to evaluate the ability of our engineered ACE2 decoy to neutralize emerging SARS-CoV-2 strains. As a first step, we assessed binding to variant receptor-binding domains (RBDs) using a yeast display system (19) (**Figure 1A**). We incubated budding yeast displaying viral RBDs on the cell surface with CDY14HL-Fc fusion protein and then assessed decoy binding via flow cytometry by staining bound decoy with a fluorescent secondary antibody. CDY14HL-Fc bound the ancestral (Wuhan-Hu1) RBD with an apparent affinity of 0.14 nM (**Figure 1B**). This result is in good agreement with the picomolar binding affinity we previously measured for the engineered decoy:RBD interaction using surface plasmon resonance (13).

Since our first description of CDY14HL (13), several SARS-CoV-2 variants of concern (VoCs) have emerged with far greater transmissibility and clinical sequelae than the original Wuhan strain (20); most of this evolution has occurred at the RBD:ACE2 interface (**Figure 1C**). We used the yeast display system to evaluate decoy binding to five of these VoCs. We included an additional RBD mutant not observed in natural SARS-CoV-2 isolates. This RBD sequence, "Delta 4+," contains four additional substitutions (K417N, N439K, E484K, and N501Y) derived from systematic analysis of RBD monoclonal antibody epitopes. These substitutions are hypothesized to hold maximum potential for antibody escape (21). Remarkably, CDY14HL maintained subnanomolar binding affinity for all VoC RBDs, including Iota, Delta +, Lambda, and Mu, and the "Delta 4+" RBD (**Figure 1D**). This finding is consistent with the broad

resistance of CDY14HL to SARS-CoV-2 variant evolution previously observed in binding and pseudotype neutralization studies (13).



strain	K_D (nM)
CoV2	0.14
Iota	0.09
Delta	0.19
Delta Plus	0.24
Lambda	0.23
Mu	0.09
Delta plus 4	0.05

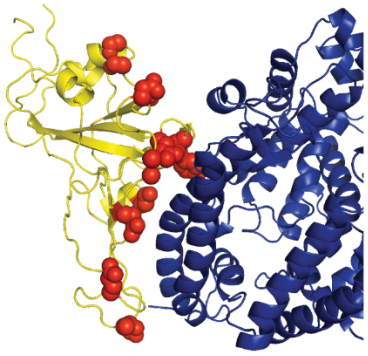
Fig 1. CDY14HL maintains tight binding to diverse SARS-CoV-2 variants

(A) Scheme for measuring decoy binding to yeast surface-expressed RBDs. (B) Representative decoy binding data for the RBD from the ancestral (Wuhan-Hu1) SARS-CoV-2 strain. (C) RBD amino acid positions mutated relative to the ancestral strain in the panel of SARS-CoV-2 variants are highlighted in red spheres on the yellow RBD ribbon diagram. These mutations cluster around the interface with ACE2 (blue ribbons), as shown by the coordinates of the complex (36) (D) Fitted values of the dissociation equilibrium constant (K_D) for SARS-CoV-2 variants.

2.2 CDY14HL maintains potent neutralization for diverse SARS-CoV-2 variants

We next investigated whether the broad decoy affinity for SARS-CoV-2 variants observed using the yeast display binding assay would translate to potent viral neutralization. We first examined the Omicron VoC. Unlike previous variants, which contain one, two, or three RBD mutations, the Omicron RBD differs from the ancestral strain by 15 amino acids (**Figure 2A**) (22). This level of mutation has caused a reduction in the efficacy of first-generation vaccines and most of the monoclonal antibodies developed for therapeutic and passive prophylaxis applications (23-28). We used a lentivirus harboring a luciferase reporter gene and pseudotyped with the SARS-CoV-2 spike protein from the Omicron strain to measure the neutralization potency (half-maximal inhibitory concentration [IC50]) of purified CDY14HL-Fc decoy (**Figure 2B**). CDY14HL-Fc neutralizes Omicron more potently than the ancestral strain (18 ng/ml vs. 35 ng/ml, **Figure 2C**). We extended this approach to include VoCs and variants of interest (VoI) not previously evaluated. CDY14HL neutralized all SARS-CoV-2 strain pseudotypes tested, including Lambda, Kappa, Delta, Delta +, Mu, and Zeta, with IC50 values near or below the potency of the ancestral strain, Wuhan, against which it was engineered (**Figure 2C**).

A



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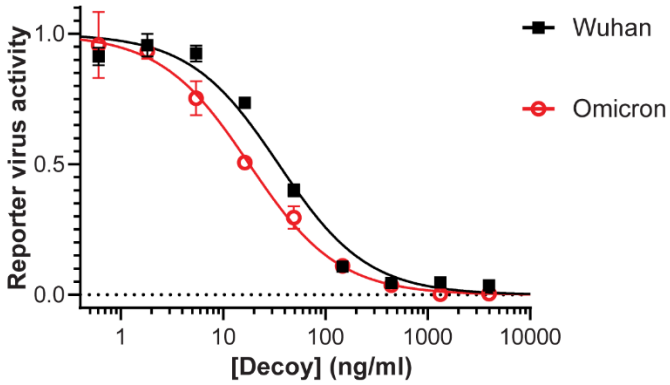

C

RBD mutations	CoV-2 strain	IC50 (ng/ml)
Reference	ancestral (Wuhan-Hu1)	35
L452Q F490S	lambda	11
L452R E484Q	kappa	18
L452R T478K	delta	14
K417N L452R T478K	delta plus	10
G339D S371L S373P S375F K417N N440K G446S S477N T478K E484A Q493K G496S Q498R N501Y Y505H	omicron	18
R346K E484K N501Y	mu	21
E484K	zeta	37

Fig 2. CDY14HL maintains potent neutralization for diverse SARS-CoV-2 variants

(A) RBD amino acid positions mutated relative to the ancestral strain in the Omicron variant of SARS-CoV-2 are highlighted in red spheres on the yellow RBD ribbon diagram. These mutations cluster around the interface with ACE2 (blue ribbons), as shown by the coordinates of the complex(36). (B) Viral neutralization assay using lentiviruses pseudotyped with the ancestral (Wuhan-Hu1) or Omicron variant spike protein. (C) Table of CDY14HL neutralization IC50 values collected for SARS-CoV-2 variant pseudotypes along with the RBD mutations of each variant.

2.3 CDY14HL binds diverse ACE2-dependent CoVs

Next, we evaluated the ability of CDY14HL to bind diverse RBDs from coronaviruses with pandemic potential. We identified 10 *sarbecoviruses* isolated from bats in southern China and Laos (12, 29) that are thought to utilize ACE2 as a receptor (**Figure 3A**). We cloned synthetic RBD genes into the yeast display format for binding analysis. Additionally, we included the RBD from the human coronavirus NL63, an alpha-CoV with a genetically distinct RBD that has been shown to use ACE2 for cell entry (30). We determined the binding affinities of yeast-displayed RBD to CDY14HL-Fc by flow cytometry. Remarkably, the decoy bound to all but one strain with subnanomolar affinity (**Figure 3B**). Research has recently shown that the weakest decoy binder in the group, RATG13, also binds ACE2 more weakly than other members of the clade (31). This behavior suggests that affinity for the decoy and the endogenous ACE2 receptor are closely linked, as we have previously observed (13).

These binding data are in broad agreement with our previous work demonstrating tight decoy binding to RBDs from SARS-CoV-1 and WIV1-CoV [(13). In the case of SARS-CoV-1, both decoy and wild-type (wt) ACE2 (32) binding were weaker than for SARS-CoV-2, although the decoy more potently neutralized SARS-CoV-1 than SARS-CoV-2 (18 ng/ml vs. 37 ng/ml) (13). Thus, decoy affinity measurements alone are not perfectly predictive of neutralizing potency. Instead, the competitive binding of the viral spike for the decoy versus ACE2 receptors may better predict neutralization.

To assess this possibility, we employed a competitive binding assay between the decoy and ACE2 receptor in the yeast system. We incubated RBD yeast with a low concentration of decoy (1 nM of CDY14HL-hFc; 95 ng/ml) along with a 100-fold molar excess of wt-ACE2 (100 nM of wt-ACE2-mFc, with a mouse Fc fusion to distinguish it from the decoy). We assessed the level of decoy binding retained in the presence of receptor competition by flow cytometry and compared these values across the set of RBDs (**Figure 3C**). The positive control, the RBD from the well-neutralized ancestral SARS-CoV-2 strain, retained 32% of decoy binding in the presence of saturating levels of wt-ACE2-mFc (**Figure 3D**). Similar to SARS-CoV-2, all *sarbecovirus* RBDs retained >30% binding in the competition assay. Together with the observed subnanomolar binding affinity, these data predict broad and potent neutralization of ACE2-dependent beta-CoVs. The lone alpha-CoV in our study, NL63, retained a lower fraction of decoy binding in the competition assay (23%, **Figure 3D**). Further study is needed to determine whether this result indicates a lower neutralizing potency of the decoy for the genetically distinct ACE2-dependent alpha-CoVs.

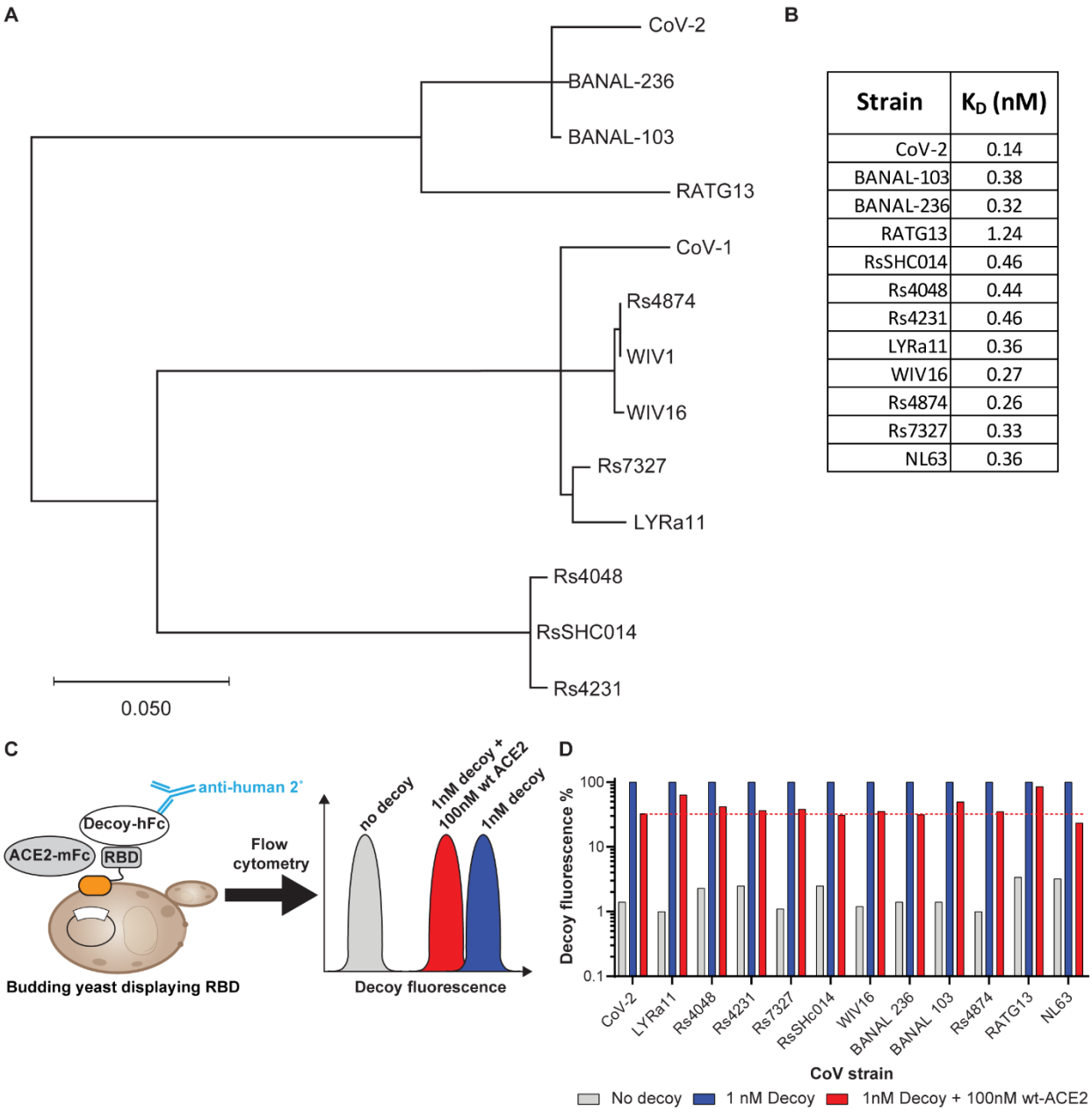

Fig 3. CDY14HL binds diverse ACE2-dependent CoVs

(A) Phylogenetic tree of *sarbecovirus* RBDs created using the maximum likelihood method. (B) Table of dissociation equilibrium constants for the decoy interacting with various CoV RBDs. (C) Schematic for measuring the competition between decoy and endogenous ACE2 receptor using yeast-displayed RBDs. (D) Relative levels of decoy binding to diverse RBDs under several conditions, as assessed via the yeast display system.

3. Discussion

We previously reported the development of an affinity-matured, soluble ACE2 decoy, termed CDY14HL-Fc. This decoy binds and neutralizes SARS-CoV-2 strains from the early pandemic as well as the related pandemic *sarbecovirus*, SARS-CoV-1 (13). In this study, we have shown that the affinity-matured decoy retains broad neutralizing activity against every SARS-CoV-2 variant tested, including Delta, Delta +, and Omicron.

Our original strategy for deploying the decoy was developed in the context of preventing SARS-CoV-2 infection. We accomplished this aim through the creation of an adeno-associated virus (AAV) vector expressing the decoy that is administered via nasal administration to engineer proximal airway cells to express neutralizing levels of the decoy at the airway surface (i.e., the virus' entry point). This approach could be particularly useful for immunocompromised patients who do not generate protective immunity following active vaccination. We are also developing the decoy as a therapeutic protein for treatment, or possibly prevention in high-risk groups, following parenteral administration.

The relentless emergence of new, highly transmissible SARS-CoV-2 variants in the current pandemic reminds us of our vulnerability to the power of zoonosis and the intense selection pressures experienced by pandemic viruses to evolve into more pathogenic and/or transmissible variants. This experience suggests the importance of proactively developing countermeasures against future pandemics, which will likely be caused by a coronavirus based on the recent history of SARS, Middle East respiratory syndrome, and COVID-19. Indeed, CoVs constitute a

major fraction of pre-zoonotic viruses ranked by multiple genetic and environmental factors for pandemic potential (33). This threat compelled us to evaluate the competitive binding of our ACE2 decoy to spike proteins from a variety of animal coronaviruses with zoonotic potential, particularly *sarbecoviruses* that use ACE2 as a receptor. We were delighted to find that the decoy retained very high binding activity against spike proteins from every pre-emergent strain that was studied.

COVID-19 has illustrated how powerful the drive for viral fitness can be in circumventing immunity generated from previous infection, vaccines, and antibody therapeutics. This rapid evolution is substantially amplified in the setting of a global pandemic caused by a highly transmissible virus. The use of a decoy protein based on a soluble version of a viral receptor holds the promise of significantly restricting viral escape, as any mutation that diminishes decoy binding will likely also diminish receptor binding and thus viral fitness. We are quickly moving this ACE2 decoy into the clinic in the AAV platform as well as a protein therapeutic as a possible solution to COVID-19 variants and to prepare for future coronavirus outbreaks.

4. Materials and Methods

4.1 CoV pseudotyped lentiviral neutralization assay

Replication-incompetent lentiviruses pseudotyped with CoV spike proteins and packaging for a Renilla luciferase reporter gene were purchased from Integral Molecular: RVP-701L Wuhan (lot CL-114B), RVP-763L Delta (lot CL-267A), RVP-736L Zeta (lot CL-255A), RVP-730L Kappa (lot CL-247A), RVP-768L Omicron (lot CL-297A), RVP-767L Mu (lot CL-274A), RVP-766L Lambda (lot CL-259A), and RVP-765L Delta + (lot CL-258A). We performed neutralization assays using human embryonic kidney 293T cells overexpressing ACE2 (Integral Molecular) as previously described (13).

4.2 Recombinant protein production

To generate wt-ACE2-Fc for competitive binding assays, we cloned human ACE2 (1–615) fused to a C-terminal mouse IgG2a Fc into pcDNA3.1. We transfected the plasmid into Expi293 cells for expression. The supernatant was collected and exchanged to 0.1 M sodium phosphate, pH 7.2 and 150 mM NaCl buffer for purification on Protein A Sepharose 4B (ThermoFisher). The protein was eluted in 0.1 M citric acid, pH 3.0 and neutralized in 1 M Tris, pH 9.0 before a final buffer exchange to 25 mM HEPES pH 7.2 and 150 mM NaCl by size-exclusion chromatography with Superose 6 resin (Cytiva). For these studies, we cloned the engineered CDY14HL 1–615 fragment in front of the human IgG1 Fc domain for expression and purification. We previously characterized a decoy fusion to human IgG4 Fc (13), but found that Fc1 and Fc4 decoy fusions behave similarly with respect to binding and neutralization (e.g., the IC50 values against Wuhan-Hu1 pseudotypes were 37 ng/ml and 35 ng/ml for CDY14HL-Fc4 and CDY14HL-Fc1, respectively).

4.3 Phylogenic tree construction

The RBD sequences of the CoVs were taken from spike protein coding sequences downloaded from the National Center for Biotechnology Information (NCBI). Using MEGA X (34), we aligned the amino acid sequences in ClustalW and constructed a phylogenic tree using maximum likelihood analysis.

4.4 Yeast display binding assays

The nucleic acid sequences of the CoV RBDs were taken from NCBI: CoV-2 (NC_045512.2), NL63 (AY567487), LYRa11 (KF569996), Rs4048 (KY417144), Rs4231 (KY417146), Rs7327 (KY417151), RsSHC014 (KC881005), WIV16 (KT444582.1), BANAL-236 (MZ937003), BANAL-103 (MZ937001), Rs4874 (KY417150), and RaTG13 (MN996532.2). We cloned the RBDs into a plasmid between an upstream Aga2 gene and a downstream hemagglutinin (HA) epitope tag with flexible GSG linkers. The plasmid has a low-copy centromeric origin similar to that of pTCON2 (35). Plasmids were transformed into EBY100 using the Frozen-EZ Yeast Transformation II Kit (Zymo). We grew colonies in SD-trp media before induction in log phase for 24 hr at 30˚C in SG-CAA (35). For competition or direct binding assays, we incubated the yeast with CDY14HL-Fc1 with or without wt-ACE-mFc for 30 min at 25˚C before staining the sample with goat anti-human fluorescein isothiocyanate (FITC; ThermoFisher A18812) and rabbit anti-HA-PE (Cell Signaling Technology 14904S). We used phosphate-buffered saline with 0.1% bovine serum albumin for all staining and washes. For the titration of CDY14HL-Fc1, we incubated the yeast with 1:10 dilution series of CDY14HL-Fc1 at 25˚C for 6 hr. The yeast were analyzed on an ACEA NovoCyte flow cytometer. We determined the level of CDY14HL-Fc1

298 binding by taking the mean FITC signal for 500 RBD+ yeast cells collected for each condition.
299 We fitted the decoy concentration versus the decoy binding signal in GraphPad Prism using a
300 three-parameter fit to the binding isotherm.
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302 **Data Availability Statement**

303 All data discussed in the manuscript are available in the main text.
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Author Contributions

J.J.S. – conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, supervision, validation, visualization, writing-original, writing-review, and edits. S.L. – data curation, formal analysis, investigation, methodology, resources, validation, visualization, writing-original, writing-review, and edits. J.M.W. – conceptualization, funding acquisition, writing-original, writing-review, and edits.

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Conflict of Interest Statement

J.M.W. is a paid advisor to and holds equity in Scout Bio and Passage Bio. He also holds equity in the G2 Bio-associated asset companies and iECURE. He has sponsored research agreements with Amicus Therapeutics, Biogen, Elaaj Bio, FA212, G2 Bio, G2 Bio-associated asset companies, iECURE, Janssen, Passage Bio, and Scout Bio, which are licensees of Penn technology. J.M.W. and J.J.S. are inventors on patents that have been licensed to various biopharmaceutical companies and for which they may receive payments.

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