Comprehensive engineering of a therapeutic neutralizing antibody targeting SARS-CoV-2 spike protein to neutralize escape variants

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ABSTRACT

The emergence of escape variants of SARS-CoV-2 carrying mutations in the spike protein poses a challenge for therapeutic antibodies. Here, we show that through the comprehensive engineering of the variable region of the neutralizing monoclonal antibody 5A6, the engineered antibody, 5A6CCS1, is able to neutralize SARS-CoV-2 variants that escaped neutralization by the original 5A6 antibody. In addition to the improved affinity against variants, 5A6CCS1 was also optimized to achieve high solubility and low viscosity, enabling a high concentration formulation for subcutaneous injection. In cynomolgus monkeys, 5A6CCS1 showed a long plasma half-life and good subcutaneous bioavailability through engineering of the variable and constant region. These data demonstrate that 5A6CCS1 is a promising antibody for development against SARS-CoV-2 and highlight the importance of antibody engineering as a potential method to counteract escape variants.

Introduction

The use of therapeutic antibodies to treat moderately or severely ill patients with COVID-19 has been successful at reducing viral load with a good safety profile. As the spike protein of SARS-CoV-2 plays a key role in viral attachment, fusion, and entry, antibodies targeting the spike have proven to be effective in slowing and preventing viral infection. Within the spike protein, the receptor-binding domain (RBD) mediates viral attachment to host cells expressing the angiotensin-converting enzyme 2 (ACE2) receptor. Owing to this key mechanism for viral attachment, all therapeutic antibodies currently authorized for emergency use by the US Food and Drug Administration (FDA) are directed against the RBD. Similarly, mRNA vaccines encoding the spike protein have been highly effective in eliciting protective antibody responses in vaccinated individuals.

However, an increasing number of SARS-CoV-2 variants carrying mutations within the RBD have emerged. Some of these variants contain mutations that significantly reduce neutralization by therapeutic antibodies. For example, the Beta variant (B.1.351) and Gamma variant (P.1) have become resistant to bamlanivimab and etesevimab, resulting in a pause by the FDA in the distribution of bamlanivimab/etesevimab as monoclonal antibody (mAb) therapies for patients with COVID-19. Of particular concern is the Omicron variant (B.1.529), which was reported to escape the majority of existing therapeutic antibodies due to a large number of mutations on its RBD. Despite the emergence of escape variants, mAbs remain an important therapeutic modality for the treatment and prevention of COVID-19. There is therefore a pressing need to rapidly identify new antibodies that can effectively neutralize escape variants. Moreover, as new variants will continually emerge, the challenge presented is not so much the ability to identify new clinical antibodies, but rather the ease and speed with which this can be done.

The typical screening for new drug candidates against the escape variants requires more than one round of an extensive screening process. Large pools of diverse antibodies are progressively narrowed down using a set of criteria, such as neutralizing ability, binding affinity, and expression titer. Furthermore, the selected antibody should fit into the current cocktail therapy strategy and be compatible with another neutralizing antibody.

In contrast, the strategy of protein engineering we demonstrate here is a reverse approach of the traditional antibody selection process. Starting from a single antibody candidate, 5A6, more than 2000 variants were made by systematically mutating the complementary-determining regions (CDRs) and frameworks of the variable regions. Variants were evaluated in a battery of assays and promising substitutions were selected and combined. These mutations improved the binding...
affinity to the SARS-CoV-2 Spike RBD, the pharmacokinetics, and physicochemical properties while minimizing the probability of immunogenicity. The heavy chain constant region was also engineered to prolong the half-life of the antibody and ameliorate its binding to FcR receptors.

By comprehensively engineering the antibody, we not only rescued its neutralizing efficacy against escape mutants but simultaneously optimized the antibody to attain drug-like characteristics. This methodology preserved the inherent epitope of the parent antibody while screening for the stringent physicochemical properties that are required for large-scale manufacturing and therapeutic use. By this process, 5A6 was engineered into the final candidate 5A6CCS1, which was significantly improved in terms of escape variant neutralization, physicochemical properties, and pharmacokinetics. 5A6CCS1 exhibited a long half-life in human FcRn transgenic mice and cynomolgus monkeys, and showed good manufacturability. Furthermore, it can be formulated at a high concentration for subcutaneous injection, which is better for making medical treatment more accessible during this pandemic. These data highlight how antibody engineering can be effectively used to improve existing antibody drugs so that they neutralize the emerging escape mutants, bypassing the tedious and repetitive process of re-screening for neutralizing antibodies.

Results

Antibody engineering of the anti-SARS-CoV-2 antibody

We previously reported the discovery of 5A6, as a SARS-CoV-2 neutralizing antibody that trapped the SARS-CoV-2 Spike trimer in a pre-fusion state, thereby preventing membrane fusion and syncytium formation. However, the antigen binding fragment (Fab) of 5A6 showed weak affinity to the RBD of the SARS-CoV-2 spike protein and exhibited modest neutralization of the SARS-CoV-2 virus. Most critically, 5A6 was not able to neutralize the mutant V483A pseudovirus, raising the possibility that it may fail to neutralize other mutants as well.

Antibody engineering of 5A6 was performed with the aim to not only increase neutralization activity but also to achieve drug-like characteristics. Both the variable region and the constant region of 5A6 were engineered, taking into account multiple parameters, such as physicochemical properties, pharmacokinetics, and manufacturability. The flow of engineering is illustrated in Figure 1a.

To improve the binding affinity toward the SARS-CoV-2 RBD, we comprehensively engineered the variable regions of 5A6. Each position of the CDRs and the part of framework with the potential to affect the binding affinity in the light and heavy chain were systematically substituted with the 18 other amino acids, excluding the original amino acid and cysteine. All variants were generated and evaluated in IgG1 format during the engineering process. Of the approximate 1200 mutations tested, a total of 37 single mutations across 13 positions were identified to enhance the binding affinity compared with 5A6 (Figure 1b). Before starting work on combinations, the antibodies with these 37 single mutations were assessed by size-exclusion chromatography (SEC). The mutations that showed an increase in high molecular weight species or a late column retention time, which is an indication of nonspecific binding, were excluded for combination. To further reduce the number of positions for combination, mutations that showed a marginal increment in binding affinity were deprioritized. Finally, 29 mutations were shortlisted for combination, of which 23 were from the heavy chain and 6 were from the light chain.

As it was not feasible to generate all possible combinations from the 29 mutations, we adopted a stepwise approach where combinations are incrementally introduced. This strategy is shown in Figure 1c. In the first round of combination, only two mutations were combined. We selectively picked the individual mutations that conferred a large increase of affinity improvement and generated 59 double combination variants. In addition to binding affinity, the physicochemical properties of each variant were assessed by SEC, thermal shift assay (TSA), extracellular matrix (ECM) binding, affinity-capture self-interaction nanoparticle spectroscopy (AC-SINS) and hydrophobic interaction chromatography (HIC). Combination variants that showed additive or synergistic effect in binding affinity as well as acceptable physicochemical properties were shortlisted for further combinations. Conversely, combinations were discarded when there was little improvement in binding affinity or when the affinity improvement was associated with poor physicochemical behavior (Figure 1d). This process was repeated iteratively for nine cycles, with as many as 100 combination variants generated in one cycle until a final combination variant was obtained.

The frameworks of 5A6 that were obtained from the naïve human Fab phage display library were replaced with human germline frameworks, as these pose a lower immunogenicity risk than non-germline frameworks. The selected human germline sequences for the heavy chain of framework 1, 2, 3, and 4 were IGHV3-64*04, IGHV3-30*01, IGHV3-48*03, and JH1, respectively, and those for the light chain of framework 1, 2, 3, and 4 were IGKV1D-39*01, IGKV3-20*01, GKV1D-39*01, JK4, respectively. The replacement frameworks did not affect the binding affinity against SARS-CoV-2 RBD (data not shown).

To optimize the antibody isoelectric point (pI), we then used the final combination variant obtained from affinity maturation as a template and introduced negatively charged amino acids into the variable region at positions that did not compromise antibody binding (Figure 1b-c). This was performed in order to extend the antibody half-life of 5A6. These positions were previously identified during the systematic mutagenesis of the variable region using the 18 other amino acids. After several rounds of combination for pI engineering, we selected the top 30 variants to assess their binding to mutant RBD. The final two variants were selected for further analysis. The additional assays included pharmacokinetic assessment in human FcRn transgenic mice and cynomolgus monkeys, in vitro pseudovirus neutralization, hamster infection study, immunogenicity assessment, and manufacturability assessment. For these final two variants, the heavy constant region IgG1 was
replaced with SG1095ACT3, which is a combination of mutations of our proprietary Fc technology SG1095 and ACT3. The SG1095 contains a set of mutations to reduce the binding affinity against human FcγRs while maintaining C1q binding to avoid the risk of antibody-dependent enhancement of infection. The ACT3 contains a set of mutations to increase FcRn binding within acidic endosomes for a longer plasma half-life of the antibody. At the end of this evaluation process, we picked the final candidate, 5A6CCS1-SG1095ACT3.

**Generation of strong affinity and neutralizing ability of 5A6CCS1**

Compared to the parental 5A6, 5A6CCS1 had more than 1000-fold improved binding affinity against the RBD and trimeric Spike proteins of SARS-CoV-2, as shown in Table 1. 5A6CCS1 achieved a much slower dissociation rate than 5A6, whereas the association rate was comparable between the two antibodies. The dissociation rate of 5A6CCS1 binding toward the RBD and the trimeric spike proteins of SARS-CoV-2 reached the limitation of kinetic fitting.
measured with the Biacore 8 K instrument under the current conditions. The binding sensogram is shown in Supplementary Figure 1.

Next, we compared the binding of 5A6CCS1 and 5A6 against a panel of SARS-CoV-2 RBD mutants using surface plasmon resonance. These RBD mutants represent a stretch of amino acids near the binding epitope of 5A6. The panel also includes mutations seen in widely circulating variants of concern, such as the N501Y mutation in the Alpha variant and K417N in the Beta variant. As shown in Figure 2, the parental 5A6 antibody was observed to be sensitive to many mutations along the RBD, namely, N481D, G482S, V483A, E484K, E484Q, G485S, F486S, and F490S. In particular, binding to the E484K and F490S mutants was severely affected; the binding response was barely detectable under the current assay conditions. In contrast, affinity-improved 5A6CCS1 rescued the binding to these mutations, although its binding to E484K RBD was decreased by 30% compared to the wildtype (WT) RBD.

To confirm if this improved affinity can be translated to enhanced neutralization of the virus, we evaluated the neutralization potential of 5A6CCS1 against pseudoviruses expressing WT or mutant SARS-CoV-2 Spike glycoprotein tagged with a luciferase reporter. Pseudovirus neutralization was evaluated using Chinese hamster ovary (CHO) cells stably expressing human ACE2 (CHO-ACE2). The following mutant pseudoviruses with various single amino acid mutations were generated: K417N, V483A, E484K, N501Y, and D614G. In addition, pseudoviruses for variants of concern (VOCs) (Alpha, Beta, Gamma, Delta, Omicron) and variants of interest (VOIs) (Kappa, and Epsilon) were also generated. As shown in Figure 3a, the parental 5A6 antibody was not able to neutralize the V483A and E484K single mutant pseudviruses. Poor neutralization was also observed in Beta, Gamma, Kappa, and Omicron variants. In contrast, the affinity-improved 5A6CCS1 was able to neutralize V483A, E484K, and all the VOCs and VOIs generated for the pseudovirus neutralization assay.

We also compared the ability of 5A6CCS1 with etesevimab and bamlanivimab to neutralize the D614G and Delta variant pseudoviruses. As shown in Figure 3b, 5A6CCS1 had comparable neutralization to etesevimab and bamlanivimab against the D614G variant. For the Delta variant, 5A6CCS1 and etesevimab showed similar neutralization, whereas bamlanivimab did not exhibit any neutralizing activity.

**Characterization of physicochemical properties of 5A6 antibodies**

Next, we evaluated the physicochemical properties of 5A6 and 5A6CCS1 in various assays to assess its drug-likeness as the clinical candidate. As shown in Table 2, the engineered variant, 5A6CCS1 showed good physicochemical properties in SEC, AC-SINS, ECM, HIC, and TSA. In particular, 5A6CCS1 showed low aggregation tendency and achieved a main peak in SEC analysis of 98.3%, compared to 75.9% in 5A6.

To assess the ability to formulate 5A6CCS1 as a subcutaneous drug, we evaluated the developability of 5A6CCS1 at high concentration. The viscosity of 5A6CCS1 at high concentration in a formulation buffer at pH 6.0 was evaluated. 5A6CCS1 showed good solubility at a high concentration at 208 mg/mL and the viscosity reading was 21.49 mPa.

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**Table 1. Binding affinity of 5A6 & 5A6CCS1 against RBD proteins, trimeric spike proteins, and D614G trimeric spike proteins of SARS-CoV-2.**

| Antibody | SARS-CoV-2 S protein RBD<sup>a</sup> | Trimeric SARS-CoV-2 S protein<sup>b</sup> | Trimeric SARS-CoV-2 S protein (D614G)<sup>b</sup> |
|----------|-----------------------------------|-----------------------------------|-----------------------------------|
|          | ka (1/Ms) | kd (1/s) | KD (M) | ka (1/Ms) | kd (1/s) | KD (M) | ka (1/Ms) | kd (1/s) | KD (M) |
| 5A6      | 1.65E+06  | 7.01E-02 | 4.35E-08 | 1.76E+06  | 9.04E-04 | 5.13E-10 | 2.25E+06  | 7.22E-04 | 3.21E-10 |
| 5A6CCS1  | 1.45E+06  | 5.72E-08 | #       | 3.95E-14 | 1.57E+06 | 1.65E-06 | #       | 1.05E-12 | 1.95E+06 | 2.98E-07 | 1.53E-13 |

<sup>a</sup>kinetic constant kd is approaching the limit that can be measured by the instrument.

<sup>b</sup>Antibody was captured on a protein A/G sensor surface and different concentrations of specific antigen were injected.

<sup>c</sup>Antigen was captured on an anti-His tag sensor surface and different concentrations of each antibody were injected. The KD values represent apparent KD.

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**Figure 2.** Biacore binding analysis of 5A6 & 5A6CC1 binding to the RBD mutants.
We conducted a heat acceleration stability study of 5A6CCS1 at 150 mg/mL in a formulation buffer at pH 6.0 for 2 weeks and 4 weeks at 5 °C, 25 °C and 40 °C. There was no increment of high molecular weight (HMW) components at 150 mg/mL at all three temperatures after 4 weeks. The results of the developability assessment of 5A6CCS1 are shown in Figure 4.

In addition, we obtained an antibody titer of approximately 500 mg/L of 5A6CCS1 by transient expression in Expi293 cells. These data demonstrated that 5A6CCS1 has the requisite drug-like properties for development as a subcutaneous drug.

**Pharmacokinetics of 5A6 antibodies in human FcRn transgenic mice and cynomolgus monkey**

Three antibodies, 5A6-SG1095, 5A6CCS1-SG1095, and 5A6CCS1-SG1095ACT3, were injected at doses of 2 mg/kg into human FcRn transgenic mice in the presence of intravenous immune globulin (IVIG) (Figure 5). Blood samples were collected until day 28 and antibody concentration was measured. The parental 5A6-SG1095 showed fast clearance.
As we previously demonstrated a correlation between high antibody isoelectric point (pI) and fast antibody CL, the variable region of 5A6CCS1 was engineered to lower the pI. By lowering pI from 9.22 to 7.83, the clearance of antibody was improved from 15.2 mL/day/kg to 8.25 mL/day/kg.

To further enhance the pharmacokinetics, we used the ACT3 that contains mutations to enhance the affinity toward human FcRn. The clearance of the antibody was further reduced to 3.57 mL/day/kg. As summarized in Table 3, the clearance of 5A6CCS1-SG1095 and 5A6CCS1-SG1095ACT3 was improved by 1.8-fold and 4.3-fold, respectively, compared to 5A6-SG1095, demonstrating the effectiveness of lowering the antibody pI in combination with enhancing affinity to human FcRn.

Finally, the pharmacokinetics of 5A6CCS1-SG1095ACT3 was assessed in cynomolgus monkeys at an intravenous dose of 0.6 mg/kg and 6 mg/kg, and the antibody concentration was monitored until day 70. 5A6CCS1-SG1095ACT3 showed linear pharmacokinetics and slow clearance (Figure 6 and Table 4). The bioavailability of 5A6CCS1-SG1095ACT3 was estimated to be 99.9% when subcutaneously administered at a dose of 0.6 mg/kg.

Table 3. PK data in hFcRn transgenic mice. Area under the curve (AUC), half-life (T½), and total clearance (CL) of 5A6-SG1095, 5A6CCS1-SG1095, and 5A6CCS1-SG1095ACT3 in human FcRn transgenic mice administered with IVIG after intravenous injection.

| Antibody               | Dosage (mg/kg) | AUCINF (day·ug/mL) | CL (mL/day/kg) | T½ (day) | Vss (mL/day) |
|------------------------|----------------|--------------------|----------------|----------|--------------|
| 5A6CCS1-SG1095ACT3     | 2.0            | 564                | 3.57           | 16.7     | 83.4         |
| 5A6CCS1-SG1095         | 2.0            | 245                | 8.25           | 10.8     | 104          |
| 5A6-SG1095             | 2.0            | 136                | 15.2           | 8.24     | 138          |

Figure 4. Developability assessment of 5A6CCS1. Viscosity measurement of 5A6CCS1 in a formulation buffer at pH 6.0 (a). HMW content analyzed by SEC for storage of 5A6CCS1 formulated at 150 mg/mL in a formulation buffer incubated at 5 °C, 25 °C, and 40 °C (b).

Figure 5. Pharmacokinetics of 5A6-SG1095, of 5A6CCS1-SG1095, and 5A6CCS1-SG1095 in human FcRn transgenic mice. Time profiles of plasma concentration of 5A6-SG1095, of 5A6AM1-SG1095, and 5A6CCS1-SG1095 after intravenous injection at 2 mg/mL. Each data point represents the mean ± s.d (n = 3).
**Therapeutic efficacy of 5A6CCS1-SG1095ACT3 against SARS-CoV-2 infection**

To confirm that the engineered 5A6CCS1 antibody had neutralizing potency against the live SARS-CoV-2 virus, we tested the efficacy of the antibody in a hamster model of SARS-CoV-2 infection. Hamsters were intranasally infected with $1 \times 10^5$ pfu of SARS-CoV-2 (D614G) virus, strain Slovakia/SK-BMC5/2020, and 6 hours later the 5A6CCS1 antibody was administered intraperitoneally. As a control antibody, IC17-hlgG1, which binds to an irrelevant antigen (KLH), was administered. The hamsters were sacrificed 4 days later, and lungs were harvested to determine the viral load. As shown in Figure 7, 5A6CCS1 significantly reduced the lung viral load as determined by qRT-PCR analysis of the SARS-CoV-2 RNA-dependent RNA polymerase gene and the ORF1ab gene.

In summary, the anti-SARS-CoV-2 Spike protein mAb, 5A6CCS1-SG1095ACT3, shows strong affinity against various SARS-CoV-2 mutant variants and successfully neutralizes virus infection. It also has excellent physicochemical properties, a long half-life, and good bioavailability. These properties enable less frequent dosage with subcutaneous injection and are expected to be effective in the prevention and treatment of COVID-19.

**Discussion**

We report an antibody engineering strategy to simultaneously improve virus neutralization and drug-likeness, which we demonstrated by generating a potent neutralizing IgG antibody, 5A6CCS1, against the RBD of the SARS-CoV-2 Spike. During the process of antibody engineering, it is common that mutations that improve binding inadvertently compromise the physicochemical properties of the antibody, affecting its drug-likeness and manufacturability. During the antibody engineering process of 5A6, many promising variants with potent neutralizing activities showed poor physicochemical properties, for example, precipitating during antibody purification, binding strongly to ECM, or having a high aggregation tendency (data not shown). Although assays to evaluate the physicochemical properties of antibodies have been developed, no single assay is able to assess multiple parameters, such as self-

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**Figure 6.** Pharmacokinetics of 5A6CCS1-SG1095ACT3 in cynomolgus monkeys. Time profiles of plasma concentration of 5A6CCS1-SG1095ACT3 after intravenous injection at 0.6 and 6 mg/kg (a) and intravenous or subcutaneous injection at 0.6 mg/kg (b). Each data point represents the mean ± s.d (n = 2).

**Figure 7.** Therapeutic efficacy of treatment with 5A6CCS1-SG1095ACT3 in a live SARS-CoV-2 virus inoculated Hamster model. Lung viral load was determined by qPCR of the SARS-CoV-2 RNA dependent RNA polymerase (RdRp) and ORF1ab genes. Data are presented as geometric mean, n = 5 per group. *** p < .001, 1-way ANOVA with Dunnett’s multiple comparison test against control group.

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**Table 4.** PK data in Cynomolgus monkey. AUC, $T_{1/2}$, and CL of 5A6CCS1-SG1095ACT3 in cynomolgus monkey after intravenous or subcutaneous injection.

| Dosage (mg/kg) | Dosing route | $AUC_{tot}$ (day*ug/mL) | CL (mL/day/kg) | $T_{1/2}$ (day) | $V_{ss}$ (mL/day) | Bioavailability (%) |
|---------------|--------------|-------------------------|----------------|----------------|------------------|-------------------|
| 0.6           | IV           | 226                     | 2.67           | 25.2           | 92.1             | NA                |
| 0.6           | SC           | 226                     | 2.71           | 28.3           | 107              | 99.9              |
| 6             | IV           | 2918                    | 2.10           | 43.4           | 100              | NA                |
association, hydrophobicity profile, charge interaction, stability, and viscosity. Moreover, though algorithms to predict drug-like physicochemical properties have been reported, they are currently unable to substitute for wet lab experiments. As such, we propose that multidimensional evaluation of antibodies should be done in parallel with affinity maturation. One possible method to achieve this is by a comprehensive mutagenesis workflow, as we have described in Figure 1.

Results from a Phase 3 prevention trial showing that subcutaneous injection of anti-SARS-CoV-2 antibodies prevented symptomatic and asymptomatic infection of household contacts was recently reported. Given the pandemic setting, subcutaneous delivery of drugs is not only convenient but also important to reduce patient load on hospitals and treatment centers. 5A6CCS1 was engineered to have physicochemical properties enabling high concentration formulation for subcutaneous delivery. The small injection volume requires 5A6CCS1 to be stable and have low propensity for aggregation at higher concentration while simultaneously having a viscosity below 20 centipoise (cP) to enable the use of a pre-filled syringe or auto injector. At 208 mg/mL, 5A6CCS1 had a viscosity of 21.49 mPa.s. At 150 mg/mL, which is within a typical concentration range for subcutaneous therapeutic drugs, 5A6CCS1 showed excellent stability and solubility. The expression titer for 5A6CCS1 was approximately 500 mg/L using transient expression in Expi293 cells. As it was reported that optimizing an antibody’s physicochemical properties enhances its biosynthesis in CHO cells, one reason for the higher yields of optimized 5A6CCS1 could be its superior biophysical properties, since we carefully selected mutations that do not affect expression titer and physicochemical properties during the engineering process.

In most antibody engineering campaigns, affinity improvement is conducted against one antigen. However, the large diversity of SARS-CoV-2 mutant RBDs presents a formidable technical challenge, as strong binding to multiple RBD mutants is required. In this study, we used the WT RBD for affinity improvement during the comprehensive mutagenesis of 5A6 CDRs. The binding affinity to mutant RBD was only evaluated at the late stages of optimization, after several rounds of iterations had been conducted. Interestingly, by improving the binding affinity to the WT RBD, significant gains in affinity were also seen in the RBD mutants tested, even toward the E484K and F490S mutations. This observation is consistent with recent reports. While comparing the B cells sampled from patients 1 month and 6 months after infection, Muecksch et al. observed that somatic mutation-mediated affinity matured SARS-CoV-2 neutralizing antibodies had neutralization potency against escape mutants. The E484K mutation in Beta and Gamma variants is particularly challenging because it introduces a reversal in the electrostatic charge of the side chain on top of steric clashes with antibodies. Several therapeutic antibodies have impaired binding to SARS-CoV-2 strains containing this mutation. Although we showed that 5A6CCS1 was able to neutralize the E484K and triple mutant, a 10-fold higher antibody concentration was required compared to the WT virus. Nonetheless, we hypothesize that re-engineering of CCS1 using the Beta and Gamma variant RBD can be performed to identify antibodies with improved neutralization.

As the Omicron variant emerged after this work had been submitted for possible publication, we had a unique opportunity to test in an unbiased way if the affinity engineered 5A6CCS1 indeed had broad cross-reactivity and could neutralize a newly discovered variant. Interestingly, 5A6CCS1 was able to neutralize the Omicron variant pseudovirus. Although a high dose of antibody was required for complete neutralization, this is expected as the RBD of the Omicron variant is highly mutated. Our results thus demonstrate the utility of the antibody engineering process to create potent antibodies that are more resilient against mutations.

Antibody affinity improvement can be conducted using antibody-antigen structures derived from X-ray crystalization, nuclear magnetic resonance, or cryo-electron microscopy. Rationally designed mutations are introduced to improve the spatial interaction between the antibody and antigen. Although this targeted mutagenesis approach is effective, it is completely dependent on the availability of high-resolution structure information. The ever-growing diversity of SARS-CoV-2 mutants presents a logistical challenge for structure-based engineering. Library-based approaches such as phage or ribosome display techniques are also commonly used to conduct affinity maturation. Although hits with significant improvement in affinity can be efficiently identified, a major drawback is that these hits typically contain multiple mutations. As each mutation’s effect on binding affinity and physiochemistry is not known, it is difficult to rationally combine mutations among the hits for further optimization. In contrast, a systematic and comprehensive position-by-position mutation provides a large panel of data to efficiently identify the best combination of mutations to achieve drug-likeness.

In summary, we have demonstrated that antibody engineering is a potential method to counteract escape variants and generate drug-like candidates. Antibody engineering permits multiple properties to be simultaneously evaluated and optimized in a systemic approach, therefore potentially speeding up the process of drug candidate screening. The application of this antibody engineering method goes beyond SARS-CoV-2 and can be broadly applied to any target, creating the possibility for faster and more efficient drug development programs.

Materials and methods

Antibody generation

The polynucleotides encoding variable regions of the heavy and light chain were synthesized by GenScript Inc. and were cloned into expression vectors containing a polynucleotide encoding the heavy chain constant region or the light chain constant region sequence, respectively. Recombinant antibodies were expressed transiently using the Expi293 Expression System (Thermo Fisher) according to the manufacturer’s instructions. For high throughput antibody generation during the antibody engineering process, antibodies in normal IgG1 format were purified with protein A (GE Healthcare) from supernatant of transient expression. An additional purification step was performed to further evaluate the top two selected candidates by using SEC to remove HMW and low molecular weight components. The heavy chain constant region for the
Biacore binding assays

The binding affinities of clone 5A6 and 5A6CCS1 toward recombinant SARS-CoV-2 S protein RBD were measured using Biacore 8 K instrument (GE Healthcare). Antibodies in IgG1 format were captured onto the Protein A/G that was immobilized on a CM4 sensor chip using amine coupling kit (GE Healthcare). Recombinant SARS-CoV-2 S protein WT-RBD (Acrobiosystems, #SPD-C52H3) were injected at different concentrations (6.25 nM – 100 nM, two-fold serial dilution). All antibodies and analytes were prepared in ACES pH 7.4 containing 20 mM ACES, 150 mM NaCl, 0.05% Tween 20, 0.005% NaN3. The sensor surface was regenerated each cycle with 10 mM Gly-HCl pH 1.5. Binding kinetics and affinity were determined by processing and fitting the data to 1:1 binding model using Biacore T200 Evaluation software, version 2.0 (GE Healthcare).

Antibody neutralization assay with SARS-CoV-2 spike glycoprotein pseudovirus

To evaluate the neutralization potential of antibodies, pseudoviruses expressing WT or mutant SARS-CoV-2 spike glycoprotein tagged with a luciferase reporter were generated. Neutralization of pseudovirus was evaluated using CHO cells stably expressing human ACE2 (CHO-ACE2, a kind gift from Professor Yee-Joo Tan, Department of Microbiology, NUS & IMCB, A*STAR, Singapore).22 Pseudotyped viral particles expressing SARS-CoV-2 Spike protein were produced as we in our previous study.10 Briefly, 30 million 293 T cells were transfected with 12 μg pMDLg/pRRE (a gift from Didier Trono, Addgene #12251), 6 μg pRSV-Rev (a gift from Didier Trono, Addgene #12253), 24 μg pHIV-Luc-ZsGreen (a gift from Bryan Welm, Addgene #39196) and 12 μg pTT5Lnx-CoV-SP (expressing SARS-CoV-2 Spike protein, GenBank: YP_009724390.1, a kind gift from DSO National Laboratories) using Lipofectamine 2000 transfection reagent (Invitrogen, Cat#11668-019). The transfected cells were cultured in a 37°C incubator for 3 days. Viral supernatant was harvested, centrifuged, and filtered through a 0.45 μm filter unit (Sartorius, Cat#16555). Lenti-X p24 rapid titre kit (Takara Bio, Cat#632200) was used to quantify the viral titers. pTT5Lnx-CoV-SP plasmids with different mutations were generated using QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent, Cat#210513) and were used to generate mutant pseudoviruses. CHO-ACE2 cells were seeded at a density of 3.2 × 10^4 cells in 100 μL of complete medium without Geneticin in 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates (Corning, #3904). Serially diluted IgGs were incubated in a 96-well flat-bottom cell culture plate (Costar, #3596) with an equal volume of pseudovirus (6 ng of p24) at the final volume of 50 μL at 37°C for one hour, and the mixture was added to the monolayer of pre-seeded CHO ACE2 cells in duplicate. After one hour of pseudovirus infection at 37°C, 150 μL of culture medium was added to each well and the cells were further incubated for another 48 hours. Upon removal of culture medium, cells were washed with sterile phosphate-buffered saline, and then lysed in 20 μL of 1x Passive lysis buffer (Promega, E1941) with gentle shaking at 400 rpm at 37°C for 30 minutes. Luciferase activity was then assessed using the Luciferase Assay System (Promega, E1510) on a Promega GloMax Luminometer. The relative luciferase units (RLU) were converted to percent neutralization and plotted with a non-linear regression curve fit using PRISM (Graphpad).

dtop two selected candidates was replaced with SG1095ACT3. The purified antibodies were kept in a formulation containing 20 mM His, 150 mM Arg, 162.1 mM Asp, 20 mM Met, pH6.0.

Antibody sequences for LY-CoV555 and JS-016, were obtained from the International Immunogenetics Information System (www.imgt.org) with seq IDs INN11876 and INN11873, respectively. Recombinant antibodies were expressed transiently using the Exp293 Expression System (Thermo Fisher) according to the manufacturer’s instructions.

Then, clone 5A6 and 5A6CCS1 were injected at different concentration (6.25 nM – 100 nM, two-fold serial dilution). All antibodies and analytes were prepared in ACES pH 7.4 containing 20 mM ACES, 150 mM NaCl, 0.05% Tween 20, 0.005% NaN3. The sensor surface was regenerated each cycle with 10 mM Gly-HCl pH 1.5. Binding kinetics and affinity were determined by processing and fitting the data to 1:1 binding model using Biacore T200 Evaluation software, version 2.0 (GE Healthcare).
PK study using human FcRn transgenic mice
Antibodies were intravenously administered at 2 mg/kg to 6-weeks old male human FcRn transgenic mice (B6.mFcRn2/2. hFcRn Tg line 32+/+ mouse, Jackson Laboratories) with a single intravenous injection at 1 g/kg of hlgG (Hizentra, CSL Behring) to mimic endogenous human IgGs. Blood samples were collected at 5 min, 7 hr, 1, 2, 3, 7, 14, 21, and 28 days after administration. The plasma antibody concentration was determined using coating Multi-Array 96-well plate (Meso Scale Discovery) with SARS-CoV-2 S Protein RBD, His Tag (Acro Biosystems), and detected with SULFO-TAG labeled anti-delta-GK antibody (in-house).

PK study using cynomolgus monkey
Antibodies were intravenously or subcutaneously administered at 0.6 or 6 mg/kg into 3–5 years old male cynomolgus monkeys. Blood samples were collected at 2, 7 hours, 1, 2, 3, 4, 7, 10, 14, 21, 28, 35, 42, 49, 56, 63, and 70 days after administration. For animals that received intravenously administered antibodies, blood samples were additionally collected at 5 min after administration. The plasma antibody concentration was determined using coating 384-well High Bind plate (Meso Scale Discovery) with Goat anti-Human IgG (Southern Biotech, #2049-01), and detected with Goat anti-Human Kappa Light Chain biotin (Immuno-Biological Laboratories, #17249) and SULFO-TAG Labeled Streptavidin (Meso Scale Discovery, #R32AD).

Antibody treatment of golden syrian hamsters infected with SARS-CoV-2
Female Golden Syrian Hamsters (Janvier labs, France), 6–8 weeks old, were weighed and allocated into seven homogenous groups of five animals for each study. Hamsters were anesthetized using isoflurane and infected intranasally with 70 μL of virus suspension containing 1 × 10^5 pfu of SARS-CoV-2 (D614G) virus, strain Slovakia/SK-BMCS/2020 (European Virus Archive global). Six hours after infection, hamsters received an intraperitoneal injection of antibodies at the indicated dose. Hamsters were sacrificed 4 days after infection. Hamsters were anesthetized using a cocktail of Zoletil (30 mg/kg) and Xylazine (10 mg/kg) injected intraperitoneally, followed by gentle cervical dislocation, thoracotomy, and terminal blood sampling. The left lungs were placed in RNA later overnight at 4 °C then stored at −80 °C until RNA extraction for quantification of viral load by qRT-PCR.

Virus load detection by qPCR
Viral load determination in lungs by qRT-PCR Extraction of viral RNA was performed using QIAamp viral RNA mini kit (Qiagen), and RT-PCR was performed using SuperScript III One-Step qRT-PCR Kit1 (Life Technologies, #11732). SARS-CoV-2 RNA was quantitated using IP2 and IP4 primers that detect the RNA-dependent RNA polymerase gene (RdRp) and ORF1ab gene detection primers. RdRp gene IP2 primers: IP2-12669Fw 5’-ATGAGCTTAGTCTGTGTTGIP2-12759Rv 5’-CTCCCTTTGTTGGTGTTGTG. IP2 probe sequence: IP2-12696bProbe(+). IP4-1459Rtw 5’-GGTCTCTGTTATCTTCTTCTTCTTTTGIP4-14146Rv 5’-CTGTCAGTGGTTATAATAGGIP4 probe sequence: IP4-14084Probe(+). TCATACATTTCGCTTGGTA [5] [H] [3] BHQ-1. RdRp gene IP4 primers: IP4-14059Fw 5’-GGTCTCTGTTATCTTCTTCTTCTTTTGIP4-14146Rv 5’-CTGTCAGTGGTTATAATAGGIP4 probe sequence: IP4-14084Probe(+). TCATACATTTCGCTTGGTA [5] [H] [3] BHQ-1. ORF1ab gene sequence: AAGGATCAGTGCCAAGCTCGTGCAGTGTTG, IP2-12669Fw 5’-ATGAGCTTAGTCTGTGTTGIP2-12759Rv 5’-CTCCCTTTGTTGGTGTTGTG. IP2 probe sequence: IP2-12696bProbe(+). IP4-1459Rtw 5’-GGTCTCTGTTATCTTCTTCTTCTTTTGIP4-14146Rv 5’-CTGTCAGTGGTTATAATAGGIP4 probe sequence: IP4-14084Probe(+). TCATACATTTCGCTTGGTA [5] [H] [3] BHQ-1.

Statistical analysis
Statistical analysis was performed using PRISM (Graphpad).

Heat accelerated stability study
Samples were concentrated to 150 mg/mL using the Amicon Ultra 4 mL 30 K (Millipore # UFC803024) in a formulation buffer (20 mM His, 150 mM Arg, 162.1 mM Asp, 20 mM Met, pH6.0). Samples were kept at three temperatures: 5 °C, 25 °C, and 40 °C. Time points were taken at day 14, and 28. Samples were then analyzed by SEC (Tosoh Bioscience # A00166) with running buffer containing 50 mM sodium phosphate, 300 mM sodium chloride, pH 7.0.

Viscosity measurement
Samples were concentrated to around 200 mg/mL using the Amicon Ultra 4 mL 30 K (Millipore # UFC803024) in a formulation buffer (20 mM His, 150 mM Arg, 162.1 mM Asp, 20 mM Met, pH6.0). The viscosity of mAb solutions was measured at 25 °C by an electromagnetic spinning (EMS) method using an EMS viscometer (Kyoto Electronics Manufacturing, Kyoto, Japan). An aluminum ball was put in a glass vial containing the sample and the viscosity of each sample solution was obtained at rotation speeds at 1000 rpm. The viscosity of the liquid sample can be calculated from the rotational speed of the aluminum ball measured by using the flash lamp and the CCD video camera.
Affinity-capture self-interaction nanoparticle spectroscopy

In AC-SINS assay, 0.1 mg/mL concentration of the test antibodies were added to gold nanoparticles (Ted Pella Inc. #15705) pre-coated with 80% of polyclonal goat anti-human IgG Fc (Jackson ImmunoResearch #109-005-098) as capturing and 20% of goat nonspecific antibody (Jackson ImmunoResearch #005-000-003). Test antibodies were incubated with the pre-coated gold nanoparticles for 2 hours at room temperature and the wavelength shift was measured using SpectraMax M2 (Molecular Devices).

Size exclusion chromatography

Test antibodies were injected to ACQUITY UPLC Protein BEH200, 1.7um, 46.6 mm × 150 mm column (Waters) with a linear gradient of mobile phase A (50 mM Phosphate, 300 mM NaCl, pH7.0) over 10 min at a flow rate of 0.3 mL/min with UV absorbance monitoring at 280 nm and 220 nm. Temperature for column compartment was set at 30 °C.

Hydrophobic interaction chromatography

Test antibodies were injected to TSKgel Ether-5PW 7.5 × 75 mm (TOSOH), connected to the Acquity HPLC (Waters). The column was equilibrated with 90% mobile phase A (20 mM Na-phosphate, 1.5 M (NH4)2SO4, pH7.0) and 10% mobile phase B (20 mM Na-phosphate, pH7.0). The samples were eluted using an inverted gradient from mobile phase A to mobile phase B over 23 min at a flow rate of 0.7 mL/min with UV absorbance monitoring at 280 nm. Temperature for column compartment was set at 25 °C.

Thermal shift assay

The Tm was determined using a Rotor-gene Q-5PLEX (QIAGEN). Briefly, 18 μL of 0.1 mg/mL sample was mixed with 2 μL of 100× SYPRO orange (Invitrogen, #S6651). The plate was ramped from 30 °C to 99 °C with 0.4 °C/step, and the temperature was held for 90s for the first step and 6s for subsequent step. The Tm was assigned using the first derivative of the raw data from the Rotor-Gene Q Software 2.3.5.1. In high throughput screening, the first peak of the melting curve (Tm1) was referenced for ranking among antibody variants.

Extracellular matrix binding assay

The binding to ECM was measured using electrochemiluminescence assay. BD Matrigel Basement Membrane Matrix (BD) was dispensed onto a MULTI-ARRAY 96-well Plate (Meso Scale Discovery) and incubated overnight at 4 °C. SULFO-Tag labeled goat anti-human antibody (Meso Scale Discovery, #R32AJ) was used as detection antibody. 3 ug/mL concentration of test antibodies diluted with ACES buffer pH 7.4 (20 mM ACES, 150 mM NaCl, 1.2 mM CaCl2, 0.01% Tween 20, and 0.1% bovine serum albumin) were dispensed onto ECM-coated plate and incubated for 1 hour at 30 °C with gentle shaking at 600 rpm. The signal was detected by Sector Imager 2400 (Meso Scale Discovery). The data were further processed to obtain the ratio of detected signal for each sample to that of tocilizumab (assay control).

Capillary isoelectric focusing

cIEF analyses of antibodies were performed with a Maurice system (Protein Simple). Briefly, 8.5 μL of 0.5 mg/mL concentration of antibody solutions were diluted with 41.5 μL of cIEF master mix solution containing methyl cellulose, urea, Pharmalyte 5–8 and 8–10.5, arginine, iminodiacetic acid, and PI markers. Samples were focused at 1500 volts for 1 minute, followed by 3000 volts for 6 minutes. The PI of each of the antibodies was analyzed using Compass for iCE software.

Abbreviations

ACE2  Angiotensin-converting enzyme 2
AC-SINS  Affinity-capture self-interaction nanoparticle spectroscopy
ADE  Antibody-dependent Enhancement
AUC  Area under the curve
CDR  Complementary-determining region
CHO  Chinese hamster ovary
CL  Clearance
COVID-19  Coronavirus disease 2019
ECM  Extracellular matrix
Fab  Fragment antigen binding
Fc  Fragment crystallizable
FcγR  Fc gamma receptor
FcRn  Neonatal Fc receptor
HEK  Human Embryonic Kidney
HIC  Hydrophobic interaction chromatography
HMW  High molecular weight
IgG  Immunoglobulin G
IVIG  Intravenous immune globulin
mAb  monoclonal antibody
PI  Isoelectric point
PK  Pharmacokinetic
RBD  Receptor binding domain
SARS-CoV-2  Severe acute respiratory syndrome coronavirus 2
SEC  Size-exclusion chromatography
TSA  Thermal shift assay
VOCs  Variants of concern
VOIs  Variants of interest
WT  Wildtype

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References
1. Weinreich DM, Sivapalasingam S, Norton T, Ali S, Gao H, Bhore R, Musser BJ, Soo Y, Rasool D, Im J, et al. REGN-COV2, a Neutralizing Antibody Cocktail, in Outpatients with Covid-19. N Engl J Med. 2020;383(4):238–51. doi:10.1056/NEJMoa2035002.

2. Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, Perez JL, Perez Marc G, Moreira ED, Zerbini C, et al. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. N Engl J Med. 2020;383(27):2603–15. doi:10.1056/NEJMc2034577.

3. Baden LR, El Sahly HM, Essink B, Kotloff K, Frey S, Novak R, Diemert D, Spector SA, Rouphael N, Creech CR, et al. Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. N Engl J Med. 2020;383(5):403–16. doi:10.1056/NEJMoA2035389.

4. Planas D, Veyer B, Daidaluu A, Staropoli I, Guivel-Benhassine F, Rajah MM, Planche C, Porrot F, Robillard T, Puech N et al. Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. Nature. 2021;596(7871):276–80. doi:10.1038/s41586-021-03779-9.

5. Wang P, Nair MS, Liu L, Iketani S, Luo Y, Guo Y, Wang M, Yu J, Zhang B, Kwong PD, et al. Antibody resistance of SARS-CoV-2 variants B.1.351 and B.1.1.7. Nature. 2021;593(7857):130–35.

6. Falcone M, Tiseo G, Valorti B, Barbieri C, Occhini S, Mazzetti P, Vatteroni ML, Suardi LR, Riccardi N, and Pestillo M, et al. Efficacy of Bamlanivimab/Etesevimab and Casirivimab/Imdevimab in Preventing Progression to Severe COVID-19 and Role of Variants of Concern. Infect Dis Ther. 2021;10(4):2479-2488.

7. Cao Y, Wang J, Jian F, Xiao T, Song W, Yisimayi A, Huang W, Li Q, Wang P, An R, et al. Omicron escapes the majority of existing SARS-CoV-2 neutralizing antibodies. Nature. 2021. doi:10.1038/s41586-021-04385-3.

8. Asarnow D, Wang B, Lee WH, Hu Y, Huang CW, Faust B, Ng PML, Ngoi EZX, Bohn M, Bulkley D, et al. Structural insight into SARS-CoV-2 neutralizing antibodies and modulation of syncytia. Cell. 2021;184(12):3192–3204.e3116. doi:10.1016/j.cell.2021.04.033.

9. Wang B, Asarnow D, Lee W-H, Huang C-W, Faust B, Ng PML, Ngoi EZX, Bohn M, Bulkley D, and Pizzarro A, et al. Bivalent binding of a fully human IgG to the SARS-CoV-2 spike proteins reveals mechanisms of potent neutralization. bioRxiv. accessed. 2020. Jul. 15. [accessed 2020 Jul 15]. doi:10.1101/2020.07.14.203414. https://www.biorxiv.org/content/10.1101/2020.07.14.203414v1.full.

10. Igawa T, Tsunoda H, Tachibana T, Maeda A, Mimoto F, Moriyama C, Nanami M, Sekimori Y, Nabuchi Y, Aso Y, et al. Reduced elimination of IgG antibodies by engineering the variable region. Protein Eng Des Sel. 2020;23(5):385–92. doi:10.1093/protein/gzq009.

11. Maeda A, Iwayanagi Y, Hayara K, Tachibana T, Nakamura G, Nambu T, Esaki K, Hattori K, Igawa T. Identification of human IgG1 variant with enhanced FcRn binding and without increased binding to rheumatoid factor autoantibody. mAbs. 2017;9(5):844–53. doi:10.4161/19420862.2017.1314873.

12. Berteau C, Filippe-Santos O, Wang T, Roja HE, Granger C, Schwarzenbach F. Evaluation of the impact of viscosity, injection volume, and injection flow rate on subcutaneous injection tolerance. Med Devices (Auckl). 2015;8:473–84. doi:10.2147/MDER.S91019.

13. Zhang Y, Wu L, Gupta P, Desai AA, Smith MD, Rabia LA, Ludwig SD, Tesser PM. Physicochemical Rules for Identifying Monoclonal Antibodies with Drug-like Specificity. Mol Pharm. 2020;17(7):2555–69. doi:10.1021/acs.molpharmaceut.0c00257.

14. O’Brien MP, Forleo-Neto E, Musser BJ, Isa F, Chan K-C, Sarkar N, Bar KJ, Barnabas RV, Barouch DH, Cohen MS, et al. Subcutaneous REGN-COV Antibody Combination to Prevent Covid-19. N Engl J Med. 2021;385(13):1184–95. doi:10.1056/NEJMoA2109682.

15. Bauer J, Mathias S, Kube S, Otte K, Garidel P, Gamer M, Blech M, Fischer S, Karow-Zwick AR. Rational optimization of a monoclonal antibody improves the aggregation propensity and enhances the CMC properties along the entire pharmaceutical process chain. mAbs. 2020;12(1):1. doi:10.1080/19420862.2020.1787121.

16. Muecksf J, Weisblum Y, Barnes CO, Schmidt F, Sahafer-Babaej D, Wang Z, Lorenz JC, G AI F, AT D, KE H-T, et al. Affinity maturation of SARS-CoV-2 neutralizing antibodies confers potency, breadth, and resilience to viral escape mutations. Immunity. 2021;54(8):1853–1868.e1857. doi:10.1016/j.immuni.2021.07.008.

17. Sokal A, Barba-Spaeth G, Fernández I, Broketa M, Azzaoui I, de La Selle A, Vandenbergh B, Fourati S, Roeser A, Meola A, et al. mRNA vaccination of naive and COVID-19-recovered individuals elicits potent memory B cells that recognize SARS-CoV-2 variants. Immunity. 2021;54(12):2893–2907.e2895. doi:10.1016/j.immuni.2021.09.011.

18. Starr TN, Greeney AJ, Addetta A, Hannon WO, Choudhary MC, Dingens AS, Li JZ, Bloom JD. Prospective mapping of viral mutations that escape antibodies used to treat COVID-19. Science. 2021;371(6531):580–584. doi:10.1126/science.abh9302.

19. Groves MAT, Amanuel L, Campbell JL, Rees DG, Sridharan S, Finch DK, Lowe DC, Vaughan TJ. Antibody VH and VL recombination using phage and ribosome display technologies reveals distinct structural routes to affinity improvements with VH-VL interface residues providing important structural diversity. mAbs. 2014;6(4):236–45. doi:10.1089/mabs.27261.

20. Lip K-M, Shen S, Yang X, Keng C-T, Zhang A, Oh H-J, Li Z-H, Huang L-A, Chou C-F, Fielding BC, et al. Monoclonal Antibodies Targeting the HR2 Domain and the Region Immediately Upstream of the HR2 of the S Protein Neutralize In Vitro Infection of Severe Acute Respiratory Syndrome Coronavirus. Journal of Virology. 2006;80(2):941–50. doi:10.1128/JVI.80.2.941-950.2006.