


A novel plant-made monoclonal antibody enhances the synergetic potency of an antibody cocktail against the SARS-CoV-2 Omicron variant

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Received 25 August 2022;

revised 6 November 2022;

accepted 12 November 2022.

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Keywords: COVID-19, Monoclonal antibody (mAb), Variants of Concern, Plant-made antibody, Neutralization synergy, Antibody cocktail.

Summary

This study describes a novel, neutralizing monoclonal antibody (mAb), 11D7, discovered by mouse immunization and hybridoma generation, against the parental Wuhan-Hu-1 RBD of SARS-CoV-2. We further developed this mAb into a chimeric human IgG and recombinantly expressed it in plants to produce a mAb with human-like, highly homogenous N-linked glycans that has potential to impart greater potency and safety as a therapeutic. The epitope of 11D7 was mapped by competitive binding with well-characterized mAbs, suggesting that it is a Class 4 RBD-binding mAb that binds to the RBD outside the ACE2 binding site. Of note, 11D7 maintains recognition against the B.1.1.529 (Omicron) RBD, as well neutralizing activity. We also provide evidence that this novel mAb may be useful in providing additional synergy to established antibody cocktails, such as Evusheld™ containing the antibodies tixagevimab and cilgavimab, against the Omicron variant. Taken together, 11D7 is a unique mAb that neutralizes SARS-CoV-2 through a mechanism that is not typical among developed therapeutic mAbs and by being produced in *ΔXFT Nicotiana benthamiana* plants, highlights the potential of plants to be an economic and safety-friendly alternative platform for generating mAbs to address the evolving SARS-CoV-2 crisis.

Introduction

Coronavirus disease 2019 (COVID-19) is caused by infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Azkur *et al.*, 2020; Chen *et al.*, 2020; Chou *et al.*, 2022; Cotugno *et al.*, 2021). Vaccine development against SARS-CoV-2 proceeded at an unprecedented pace and has resulted in multiple effective products, even against emerging variants (Carreño *et al.*, 2021; Garcia-Beltran *et al.*, 2022; Hager *et al.*, 2022; Heath *et al.*, 2021; El Sahly *et al.*, 2021; Thomas *et al.*, 2021). However, immunocompromised and elderly individuals may still benefit from the continued development of prophylactic and therapeutic monoclonal antibodies (mAbs), given that these subpopulations may not develop a robust and durable response from vaccination or prior infection (Brockman *et al.*, 2022; Obeid *et al.*, 2022; Ryan *et al.*, 2022).

The cellular receptor mediating SARS-CoV-2 infection in humans is angiotensin-converting enzyme 2 (ACE2) (Qiao *et al.*, 2020; Schurink *et al.*, 2022; Q. Wang *et al.*, 2020). The spike (S) protein of the virus interacts with ACE2 and can be broadly broken down into two domains. S1 contains the N-terminal domain (NTD) and the receptor-binding domain (RBD), while S2 houses the fusion peptide, necessary for the structural rearrangements leading to membrane fusion between

virus and host (Lan *et al.*, 2020; Xu *et al.*, 2021). The RBD is flexible, being found mostly in an 'up and open' or a 'down and closed' conformation, with a transitional conformation between the two also being observed (Pramanick *et al.*, 2021; Walls *et al.*, 2020). It is upon the binding of ACE2 with one 'up and open' RBD, that structural changes in the S protein result in viral entry (Cai *et al.*, 2020). The interaction between ACE2 and RBD makes the RBD a dominant target for antibodies with neutralizing capability and indeed, accounts for most of the neutralizing antibodies in a polyclonal response (Greaney *et al.*, 2021; Piccoli *et al.*, 2020; Schmidt *et al.*, 2021).

Monoclonal antibodies are useful therapeutics for preventing viral disease by neutralizing viral particles, accomplishing the same results as the polyclonal response to immunization or natural infection (Both *et al.*, 2013). Many groups have identified and developed potentially neutralizing mAbs against SARS-CoV-2 in the last 2 years, with some being advanced to clinical use via Emergency Use Authorization (EUA) (Dong *et al.*, 2021; Loo *et al.*, 2022; Rogers *et al.*, 2020; Shi *et al.*, 2020; Wu *et al.*, 2020; Zost *et al.*, 2020). The mechanism by which many mAbs interfere with viral infection has been elucidated by structural studies and can be broadly categorized into four classes based on the mAb's epitope and which RBD conformation it recognizes (Barnes *et al.*, 2020; Greaney *et al.*, 2021; Gruell *et al.*, 2022). Class 1

mAbs overlap with the ACE2 binding site of the RBD in the 'up and open' conformation, while Class 2 mAbs overlap with the ACE2 binding site in both 'up and open' and 'down and closed' conformations of the RBD. By directly interfering with viral binding to ACE2, these classes tend to be potent neutralizers. Class 3 mAbs also bind to both conformations of the RBD, with the epitopes laying outside of the ACE2 binding site, yet many mAbs of this class are also potent neutralizers. Class 4 mAbs bind further away from the ACE2 binding site and are generally less potent neutralizers (Huo *et al.*, 2020). Characterizing mAbs using these classes helps inform which mAbs may be useful in mAb cocktails, where inclusion of multiple classes with non-overlapping epitopes can reduce immune evasion (Ku *et al.*, 2021). Indeed, the urgent need for continued development of mAb cocktails is driven by the emergence of variants of concern accumulating mutations in the RBD, causing changes in binding to ACE2 (Ozono *et al.*, 2021; Wang *et al.*, 2022). This will potentially reduce neutralizing potency of host antibody response against the new variants (Greaney *et al.*, 2021; Hoffmann *et al.*, 2021) and, in some cases, abolish the utility of previously potent therapeutic mAbs (Chen *et al.*, 2021; Starr *et al.*, 2021). Several mAbs that received EUA from the Food and Drug Administration (FDA) have been amended or revoked as they lose therapeutic activity against emerging variants, specifically the B.1.1.529 (Omicron) and other variants of this lineage (Focosi *et al.*, 2022). This necessitates the development of mAbs that have potential synergy in cocktails to help reduce immune escape by SARS-CoV-2.

Discovery of new mAbs against antigens of interest can be accomplished in several ways. Single-cell antigen-specific sorting and sequencing (Gilchuk *et al.*, 2020) and phage display techniques (Kumar *et al.*, 2019) are among the leading technologies used for mAb generation and screening. The method of mouse immunization followed by hybridoma generation (Köhler and Milstein, 1975; Mitra and Tomar, 2021) is the classic method of obtaining full-length, monoclonal IgGs. The hybridoma method has benefits over single-cell and phage techniques, in that it does not require sophisticated instrumentation and preserves the process of *in vivo* affinity maturation, respectively. Moreover, variable region gene sequences of mAbs from lead hybridoma candidates can be rescued and humanized for recombinant production.

Plants are a versatile system for recombinant protein production and have made substantial contributions in developing countermeasures against the current COVID-19 pandemic. For example, a plant-based vaccine based on virus-like particles (VLP) displaying a modified S protein of SARS-CoV-2 was developed. Results from clinical trials demonstrated that this vaccine was safe and had 78.8% efficacy against moderate-to-severe disease caused by the homologous strain and 69.5% overall efficacy against five circulating variants of concern (VOCs) (Hager *et al.*, 2022). This plant-made vaccine is currently licensed for human use by Canada Health (Health Canada, 2022). Several other plant-made COVID-19 vaccine candidates are currently being evaluated in human clinical trials (Stander *et al.*, 2022). Plant-made ACE2 has also been explored as a prophylactic/therapeutic to prevent or treat SARS-CoV-2 infection (Siriwattananon *et al.*, 2021). An innovative approach of using ACE2 is the use of an ACE2-formulated chewing gum to trap SARS-CoV-2 in the saliva, blocking transmission of the virus among individuals in the population (Daniell, 2022). Studies have shown that

chewing gums containing plant-made cholera-toxin B (CTB)-ACE2 fusion protein significantly reduced salivary SARS-CoV-2 load *in vitro* (Daniell, 2022). This approach is being evaluated in a Phase III clinical trial and will have a broad impact on the prevention of other respiratory viral diseases, as well as on the development of orally deliverable drug platforms that do not require cold chain.

Plants have also been demonstrated as a favourable system for mAb development and production. Traditional mammalian expression platforms for mAb production require sterile facilities and can be cost-intensive. However, producing mAbs in plants has potential to significantly reduce overall costs, increase safety by reducing the possibility of introducing human pathogens during production and increase efficacy by allowing for facile and homogeneous manipulation of N-linked glycans (Chen, 2016, 2022; Loos and Steinkellner, 2014; Nandi *et al.*, 2016). In addition, transient production of mAbs in plants is accomplished through simple cloning of mAb genes and agroinfiltration, resulting in rapid, high-level accumulation of recombinant mAbs within a short period of time (Chen *et al.*, 2013; Leuzinger *et al.*, 2013). Many examples of mAbs produced in plants also show that plant-made pharmaceuticals have potential to be viable alternatives to mammalian-made counterparts (Jugler *et al.*, 2021; Kallolimath *et al.*, 2021; Lai *et al.*, 2010; Rattanapisit *et al.*, 2020; Shanmugaraj *et al.*, 2020; Sun *et al.*, 2021, 2023).

In the current study, we describe the development of an original mAb against the RBD of SARS-CoV-2 by hybridoma generation, followed by chimerization and expression in plants. We also provide evidence that this mAb, namely 11D7, neutralizes variants of concern, including B.1.617.2 (Delta) and B.1.1.529 (Omicron). 11D7 has a unique RBD-binding site that is not overlapping with those of several other EUA-authorized, SARS-CoV-2-neutralizing mAbs in Classes 1, 2 and 3 and displays neutralizing synergy with these classes of therapeutic mAbs in neutralizing the Omicron and other variants. This study highlights the utility of non-ACE2-competing mAbs in forming effective cocktails and of plant-based expression systems in developing novel mAbs against the ever-evolving SARS-CoV-2 pandemic.

Results

Expression of a novel Anti-SARS-CoV-2 RBD monoclonal antibody in *Nicotiana benthamiana*

BALB/c mice were immunized with recombinant Wuhan-Hu-1 RBD (BEI Resources, NR-52309) on a prime and boost regimen followed by splenocyte isolation and hybridoma generation. Hybridomas were screened for reactivity to the immunogen (Wuhan-Hu-1 RBD). Several strongly reactive antibodies were identified, and one, 11D7, was chosen for variable region gene rescue. The murine variable regions of the light and heavy chains were grafted onto human kappa and human gamma constant regions, respectively. The resulting chimeric mAb was codon-adapted for plant-based expression and transiently expressed in Δ XFT *N. benthamiana* mutant, carrying N-glycans lacking plant-specific xylose and fucose residues (Strasser *et al.*, 2008). Plant-made 11D7 (p11D7) reached peak expression of 131 μ g of mAb per gram of fresh leaf weight (FLW) 5 days after transgene delivery (Figure 1). When total plant protein extract containing the p11D7 mAb was subjected to Protein A affinity chromatography, the recombinant mAb was homogeneously purified in a manner similar to a mammalian-made mAb purified by the same

method (Figure 2a). Furthermore, the bands observed from SDS-PAGE analysis were confirmed to be the expected heterotetrameric IgG (Figure 2b) and its constitutive light chain (Figure 2c) and heavy chain (Figure 2d). We next investigated the glycosylation status of p11D7 and 11D7 produced in mouse hybridoma (m11D7) by mass spectrometry analysis. Our p11D7 was found to carry 91.1% bi- or mono-antennary N-acetylglucosamine (GnGn, MGn), mammalian-like glycans typical for mAbs produced in Δ XFT plants, compared with the more heterogeneous, mouse hybridoma-produced m11D7 (Table 1).

p11D7 binds to the RBD of the B.1.1.529 (Omicron) variant

We next assessed the functionality of the mAb by testing the binding to the antigen of interest, the SARS-CoV-2 RBD. ELISA analysis with the original WA1/2020 strain RBD showed specific binding of p11D7 in a dose-dependent manner, as expected

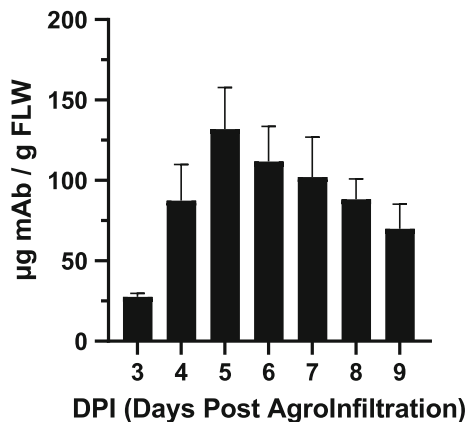


Figure 1 Recombinant expression of 11D7 in Δ XFT *Nicotiana benthamiana*. Δ XFT *N. benthamiana* leaves were infiltrated with 11D7 gene constructs and total soluble leaf proteins were extracted 3–9 days post agroinfiltration (DPI). The expression levels of p11D7 were analysed by a sandwich ELISA that detects only fully assembled IgG. Mean \pm SEM are shown from two independent experiments performed in technical duplicates.

(Figure 3a). Furthermore, a dissociation constant (KD) for p11D7 of 0.15 nM was determined from the ELISA curve, suggesting that p11D7 has a relatively high affinity for the WA1/2020 RBD. However, given the continuing emergence of variants of SARS-CoV-2 with novel mutations in the RBD that may impact mAb binding, we also tested the binding of p11D7 to the RBD of the B.1.1.529 (Omicron) variant of concern to assess the practical utility of this mAb against emerging variants. We observed specific binding of p11D7 to the Omicron RBD at higher concentrations than that of the WA1/2020, indicating a reduced affinity (Figure 3b). By contrast, a Class 1 (CB6) and a Class 4 (CR3022) mAb against the RBD of the WA1/2020 variant showed no binding activity to the Omicron RBD (Figure 3b). As expected, the IgG isotype negative control did not show specific binding to the RBD (Figure 3a). In spite of the reduced affinity, our novel p11D7 mAb still retained recognition of the Omicron RBD, suggesting it may be able to neutralize emerging variants of concern.

p11D7 neutralizes multiple variants of SARS-CoV-2

After validating the binding of the chimeric p11D7 to its target antigen, the neutralizing potency of the mAb was assessed. Utilizing authentic SARS-CoV-2 in a foci-forming assay (FFA), a neutralization curve generated against the ancestral WA1/2020 strain resulted in a half-maximal inhibitory concentration (IC₅₀) of 25.37 μ g/mL (Figure 4a). The ongoing emergence of new variants of concern also urged us to test p11D7 against the B.1.617.2 (Delta) variant, where an IC₅₀ of 59.52 μ g/mL was observed (Figure 4b), and the B.1.1.529 (Omicron) variant, giving an IC₅₀ of 948.7 μ g/mL (Figure 4c). The overall neutralization data showed that the neutralizing ability of p11D7 decreased with the accumulation of mutations in the variants derived from the ancestral strain, but nevertheless still neutralizes variants of concern of SARS-CoV-2.

Synergy of therapeutic mAbs with p11D7 in neutralizing omicron

Next, we investigated whether our novel p11D7 mAb could be categorized into a mAb class against the RBD of SARS-CoV-2 as described previously (Barnes *et al.*, 2020; Greaney *et al.*, 2021) by

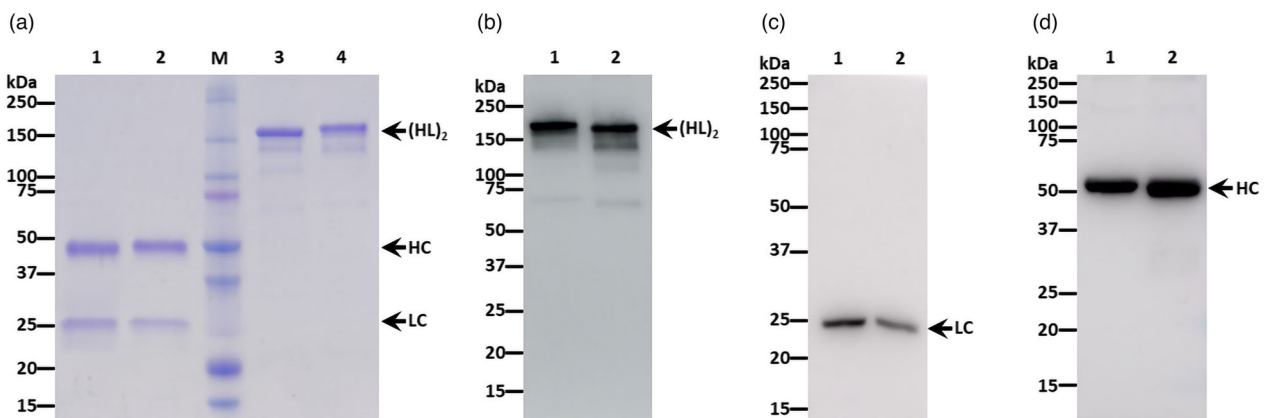


Figure 2 Biochemical characterization of p11D7. Protein A-purified, p11D7 and an IgG isotype control were subjected to SDS-PAGE under reducing (a, Lanes 1 and 2) or non-reducing conditions (a, Lanes 3 and 4) and total protein content was stained with Coomassie blue. In parallel, SDS-PAGE-separated proteins under non-reducing (b) or reducing conditions (c and d) were transferred to PVDF and probed for human kappa light (b and c) or for human gamma chain (d). Lanes 1 and 3: IgG isotype control. Lanes 2 and 4: p11D7. HC: heavy chain. LC: light chain. (HL)₂: assembled heterotetrameric form of IgG. One representative result of multiple experiments is shown.

Table 1 Glycan analysis of m11D7 and p11D7

Major N-glycan species	Schematic presentation	m11D7 (%)	p11D7 (%)
GnGn/MGn		5.7	91.1
AGn/AA		12.3	
GnGnF ₆ /MGnF ₆		25.7	
AMF ₆		3.3	
AGnF ₆		33.4	
AAF ₆		19.5	
Man ₇₋₉		0.1	8.9

Heavy chains of either hybridoma-made 11D7 (m11D7) or 11D7 produced in Δ XFT *Nicotiana benthamiana* plants (p11D7) were extracted after SDS-PAGE, trypsin digested and analysed by LC-ESI-MS. Glycopeptide peaks were identified using FreeStyle 1.8 and assigned percentages based on approximate molar ratios from the peak heights. Consortium for Functional Glycomics nomenclature was used.

Mannose; N-acetylglucosamine; Fucose; Galactose.

performing a competitive binding assay with well-characterized mAbs of all RBD-binding classes. In an ELISA assay using the WA1/2020 RBD and p11D7 conjugated to horseradish peroxidase (HRP), we observed that CR3022 binding to the RBD abolished the ability of p11D7 to bind to the RBD, while several other EUA therapeutic mAbs (CB6, tixagevimab and cilgavimab) did not inhibit the binding of p11D7 to the RBD (Figure 5). This was the expected outcome for CR3022 and CB6, given that we have

previously provided evidence of this (Jugler *et al.*, 2022), validating that p11D7 has at least a partially overlapping epitope on the RBD with CR3022 (a Class 4 mAb) but not with CB6 (also known as etesevimab, a Class 1 mAb). Similarly, the two mAbs, tixagevimab (also known as COV2-2196, a Class 1/2 mAb) and cilgavimab (COV2-2130, a Class 3 mAb) did not compete with the binding of p11D7 to the RBD. The unique binding site of p11D7 encouraged us to evaluate potential synergy of p11D7 with anti-RBD mAbs in other classes as a dual and triple mAb cocktails. After empirically determining the IC₅₀ and IC₂₅ for p11D7, tixagevimab and cilgavimab in the FFA, we combined these mAbs at the IC₅₀ and IC₂₅ concentration and measured per cent neutralization of the mAb cocktail against the B.1.1.529 (Omicron) variant. We also calculated the predicted per cent neutralization for the cocktail by importing the individually measured per cent neutralizations of each mAb into SynergyFinder and analysed them with the highest single agent (HSA) and the Loewe models. The calculated values from the models indicate the per cent neutralization where there is no synergistic interaction between mAbs in the cocktail. In all the combinations, the cocktails' observed per cent neutralization is consistently higher than those calculated by prediction models. Specifically, we observed strong neutralization synergy for the p11D7 + tixagevimab combination and for the tixagevimab + cilgavimab combination at their respective IC₅₀'s (Table 2), and with the highest per cent neutralization for the triple mAb cocktail at both IC₅₀'s and IC₂₅'s (Table 2 and Table 3). This indicates that there is neutralizing synergy between p11D7 and tixagevimab and cilgavimab, both as dual combinations, as well as a triple mAb cocktail.

Discussion

The continuing pandemic caused by SARS-CoV-2 urges the ongoing development of novel mAb therapeutics using platforms that have potential to optimize utility and safety. This study describes a novel, SARS-CoV-2-neutralizing mAb discovered in a traditional hybridoma system and further developed in a plant-based expression system. Initial screening of mAbs secreted by hybridomas generated from RBD-immunized mice was performed by ELISA with both biotinylated and non-biotinylated RBD. This ensured that RBD-binding mAbs were not overlooked whose epitope may have been excluded by direct adhering of antigen to the plate. Of several positive hits from the screening process, one

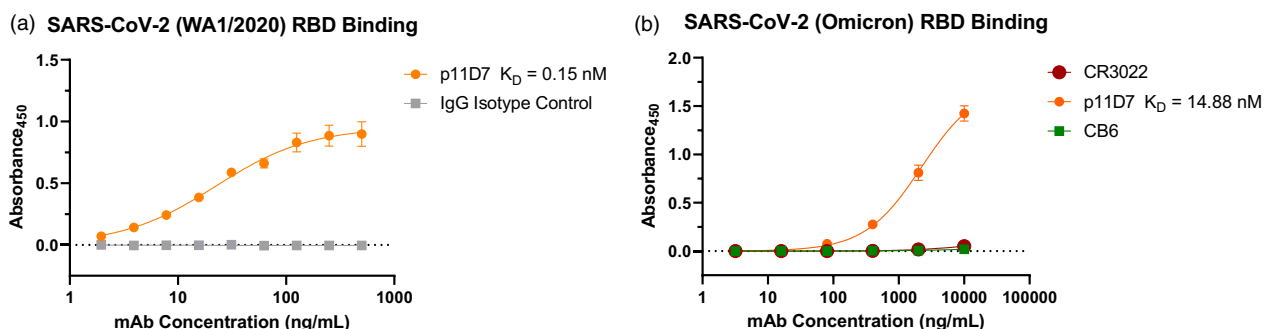


Figure 3 p11D7 recognition of RBD from various SARS-CoV-2 variants. Various dilutions of p11D7 and reference mAbs were incubated with the WA1/2020 RBD (a) or the B.1.1.529 (Omicron) RBD (b) that was immobilized on ELISA plates. Specific binding of the RBD-mAb complex was detected with a horseradish peroxidase (HRP)—conjugated secondary antibody. The absorbance at 450 nm is plotted and is representative of at least two independent experiments performed with technical duplicates. Error bars represent SEM.

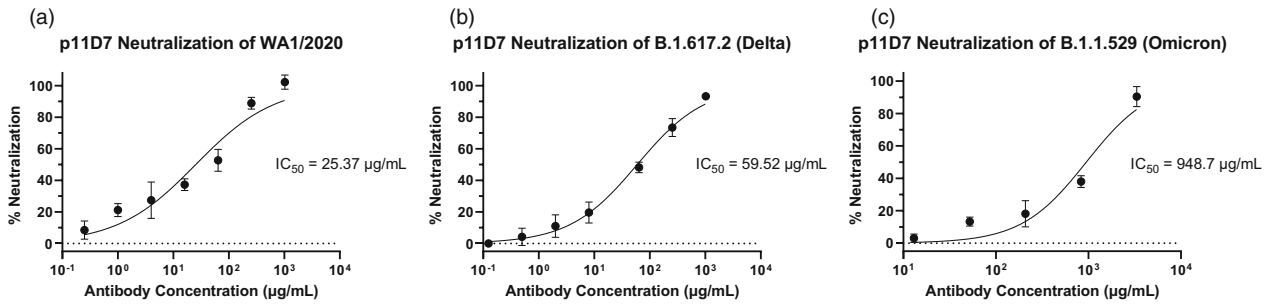


Figure 4 Neutralization of SARS-CoV-2 variants by p11D7. Serial dilutions of p11D7 were mixed with SARS-CoV-2 WA1/2020 (a), Delta (b), or Omicron (c) before adding to Vero E6 (a and b) or Vero-hACE2-TMPRSS2 (c) cells. Cells were then fixed, permeabilized and stained for SARS-CoV-2 spike protein (a and b) or nucleocapsid protein (c). Foci were counted, per cent neutralization determined, and IC_{50} calculated using GraphPad Prism 9. Error bars are SD and curves represent at least two independent experiments performed in technical triplicates.

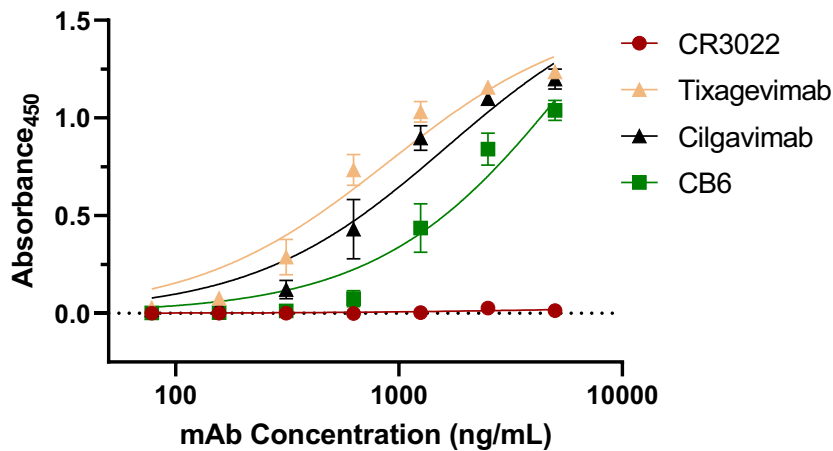


Figure 5 Competitive binding between p11D7 and other anti-RBD mAbs to the WA1/2020 RBD. Dilutions of each reference mAb were coated on 96-well plates followed by addition of 2 μ g/mL of the WA1/2020 RBD. After washing, the plates were incubated with HRP-conjugated p11D7 to detect either non-competitive binding (detection of a signal) or overlapping binding (absence of a signal) of p11D7 with each reference mAb to RBD. Error bars represent SD and at least two independent experiments with technical triplicates were performed.

Table 2 Neutralizing synergy of p11D7 with plant-made tixagevimab and plant-made cilgavimab against the B.1.1.529 variant

mAb Combination	Observed Neutralization (%)	HSA Predicted Neutralization (%)	Loewe Predicted Neutralization (%)
p11D7 + tixagevimab	91.97 \pm 2.60	35.58 \pm 7.15	40.89 \pm 11.29
p11D7 + cilgavimab	68.21 \pm 13.60	38.31 \pm 12.57	48.98 \pm 1.90
tixagevimab + cilgavimab	89.63 \pm 2.26	47.50 \pm 3.38	50.34 \pm 0.55
p11D7 + tixagevimab + cilgavimab	96.59 \pm 0.64	47.85 \pm 3.04	66.22 \pm 5.40

Per cent neutralization was obtained via FFA for each individual and combination of mAbs with each mAb at the concentration that corresponds to the IC_{50} . The per cent neutralization data from each individual mAb was used to calculate the predicted per cent neutralization of the cocktail with the highest single agent (HSA) and Loewe models in [SynergyFinder.org](https://www.synergyfinder.org/), assuming there is no neutralization synergy between mAbs in the cocktail. If the empirically determined observed per cent neutralization value of the cocktail is higher than that predicted by the models, it indicates synergistic interaction of the mAbs in the cocktail. At least two independent experiments with technical triplets were performed for each mAb and mAb combination with SD presented.

mAb, 11D7, was chosen for further development and recombinant expression in plants due to its ability to efficiently neutralize the WA1/2020 strain in the FFA. The chimeric 11D7 transiently expressed in Δ XFT *N. benthamiana* plants peaked within a week after agroinfiltration to approximately 131 μ g of p11D7 per gram of FLW. This level of expression is similar to other mAbs recently produced by our group using the same expression vector based on the geminiviral bean yellow dwarf virus (Jugler *et al.*, 2022)

and allowed accumulation of milligram levels necessary for *in vitro* and future *in vivo* studies. However, this level of expression is lower than those of mAbs we produced using more optimized geminiviral vectors (Diamos *et al.*, 2020) or other plant-made proteins such as griffithsin (GRFT) (Fuqua *et al.*, 2015) that have shown potent anti-viral activity against SARS-CoV-2 (Alsaïdi *et al.*, 2021; Palmer, 2022). The yield of p11D7 can be increased by using optimized versions of expression vectors (Diamos

Table 3 Neutralizing synergy of p11D7 with plant-made tixagevimab and cilgavimab at IC₂₅ concentrations against the B.1.1.529 variant

mAb Combination	Observed Neutralization (%)	HSA Predicted Neutralization (%)	Loewe Predicted Neutralization (%)
p11D7 + tixagevimab	43.96 ± 9.59	18.84 ± 3.96	26.08 ± 4.84
p11D7 + cilgavimab	34.76 ± 27.73	29.18 ± 14.99	48.90 ± 1.98
tixagevimab + cilgavimab	65.03 ± 2.62	35.14 ± 9.02	38.33 ± 7.02
p11D7 + tixagevimab + cilgavimab	93.92 ± 1.88	35.14 ± 9.02	50.45 ± 7.46

FFA data for each individual and combination of mAbs were performed at the IC₂₅ and per cent neutralization was analysed with the highest single agent (HSA) and Loewe models in [SynergyFinder.org](https://www.synergyfinder.org/). Predicted values represent the expected per cent neutralization if no synergy between mAbs was occurring, while the observed per cent neutralization was the empirically determined value of the combination at the IC₂₅. An observed value higher than a predicted value is inferred to have synergetic interaction. At least two independent experiments with technical triplets were performed with SD values presented.

et al., 2020) and/or via co-expression with chaperons (Margolin *et al.*, 2020) to make it cost-effective for commercial production. Our result also showed that recombinant p11D7 assembled correctly into an IgG, as determined by the Western blot analysis, without any observable degradation or truncation of the heterotetrameric protein. The correct assembly was further validated by observing specific binding of the mAb to its target antigen, the WA1/2020 RBD. The dissociation constant determined from the ELISA was 0.15 nM, suggesting that our novel mAb has a relatively high affinity for the SARS-CoV-2 RBD as this is similar to the dissociation constant for other neutralizing mAbs against SARS-CoV-2, CA1 and CB6, produced in plants (Jugler *et al.*, 2022). Interestingly, although the affinity is close to that of these neutralizing mAbs, the IC₅₀ for p11D7 in neutralizing the WA1/2020 strain, is much higher at 25.37 µg/mL, indicating that p11D7 is not a potently neutralizing mAb by itself. However, taken together with its unique binding site on the RBD, we speculate that p11D7 may neutralize viral particles by a mechanism other than interfering with ACE2 binding. For example, its binding may change the overall conformation of the S protein and hinder the post-binding steps required for viral entry.

The continuous emergence of viral variants with accumulating mutations in the spike protein must be considered when developing therapeutic mAbs. The S protein that is responsible for host cell entry is under high selective pressure due to its wide use as a vaccine and therapeutic target. The variant of concern that emerged at the end of 2021, B.1.1.529, colloquially termed Omicron, has overtaken all previous variants, with the parental Omicron variant having accumulated 15 mutations in the RBD region, a prominent neutralizing target (Hu *et al.*, 2022; Kuchipudi *et al.*, 2022). As a result, it is important that novel mAbs being developed for therapeutic purposes retain recognition to circulating variants that have outcompeted prior variants. We assessed the capability of p11D7 to bind to the B.1.1.529 RBD and observed specific binding of p11D7, with an approximately 100-fold reduction in affinity compared with the WA1/2020 RBD. Of note, CR3022, a Class 4 non-neutralizing mAb against SARS-CoV-2 (Barnes *et al.*, 2020), derived from the original SARS-CoV outbreak (ter Meulen *et al.*, 2006) did not recognize the Omicron RBD. This is of particular interest because we validated in this study that p11D7 has an overlapping epitope with CR3022 (Jugler *et al.*, 2022), strongly suggesting that p11D7 may itself be a Class 4 RBD-binding mAb. However, even with an overlapping epitope, the undetermined epitope of p11D7 appears to be more conserved on the RBD than that of CR3022, due to retained binding with accumulating RBD mutations. Furthermore, we show that p11D7 retained neutralizing capacity against the B.1.617.2 (Delta) variant, albeit with an approximately 2.3-fold

reduction in the IC₅₀, as well as the Omicron variant with a further approximately 15.9-fold reduction in IC₅₀ when compared to the Delta variant. Ultimately, this is a 37.4-fold reduction in neutralizing ability from the WA1/2020 strain to the Omicron variant that emerged nearly 2 years later. The evidence that p11D7 retains recognition of the Omicron RBD at all is significant, given that many other more potent neutralizing mAbs that were developed and used clinically now do not bind or neutralize what is now the dominant variant of SARS-CoV-2 (Planas *et al.*, 2021; VanBlargan *et al.*, 2022). From a structural and mechanistic perspective, this is not surprising, given that many of the anti-RBD mAbs receiving an EUA early belong to Class 1 or 2, with epitopes on the RBD that overlap with the ACE2 binding site, making them particularly sensitive to mutations that impact ACE2 binding (Deshpande *et al.*, 2021). However, Class 4 mAbs bind to epitopes on the RBD outside and distant from the ACE2 binding site (Deshpande *et al.*, 2021), suggesting they have a neutralization mechanism that is different from ACE2-competition like Class 1 and 2 mAbs. In addition, the Class 4 mAbs tend to have more conserved epitopes on the RBD, making them more resistant to mutations that occur more often within or near the ACE2 binding site (Gruell *et al.*, 2022a). This suggests that p11D7, which overlaps with a Class 4 mAb, is a good candidate to complement other classes of mAbs in a cocktail to provide synergistic potency against multiple variants. Hence, we sought to use it as a cocktail member with other, more potent mAbs that are still active against the Omicron variant. Our plant-made mAbs derived from the parental mAbs COV2-2130 (commercialized into cilgavimab or AZD1061) and COV2-2196 (commercialized into tixagevimab or AZD8895) (Dong *et al.*, 2021; Zost *et al.*, 2020), which are well-characterized mAbs designated to be Class 3 and Class 1/2, respectively (Barnes *et al.*, 2020; Greaney *et al.*, 2021), do not interfere with p11D7 binding to the WA1/2020 RBD, as determined by a competitive ELISA. We also performed the competition assay with the Class 1 CB6, knowing that it should not interfere with p11D7 binding to the RBD (Jugler *et al.*, 2022). These observations further support the hypothesis that p11D7 is likely a Class 4 mAb against the RBD and that p11D7, not having overlapping epitopes with cilgavimab and tixagevimab, would be a complementary cocktail partner for these two mAbs that are still functionally active against Omicron variants (Planas *et al.*, 2021; VanBlargan *et al.*, 2022).

The synergy analysis performed with dual and triple combinations of p11D7 with plant-made cilgavimab and tixagevimab at IC₅₀ concentration, do indeed suggest that there is neutralizing synergy of both these mAbs with p11D7 against the Omicron variant. Synergy was also observed at the lower IC₂₅ concentration as dual combinations of p11D7 with tixagevimab and

tixagevimab with cilgavimab, and as a triple combination. Notably, the triple mAb combination showed a greater increase in neutralizing activity than all of the dual combinations, especially at low mAb concentrations. Interestingly, the combination of cilgavimab and tixagevimab, used clinically as Evusheld™, still retains neutralizing potency against highly divergent SARS-CoV-2 variants, such as BA.4/5, highlighting the continuing clinical utility of this mAb cocktail (Wang *et al.*, 2022; Yamasoba *et al.*, 2022). Previously, we have demonstrated that m11D7 has synergy with the Class 1 EUA mAb, etesevimab (CB6) in neutralizing the WA1/2020 strain (Jugler *et al.*, 2022). Combined with the results reported in this study, the cumulative observations suggest that p11D7 has potential to enhance the neutralizing potency of partner mAbs against multiple variants of concern in a cocktail setting. Therefore, efforts to continue to evaluate p11D7 against emerging variants will inform whether this unique mAb may have potential to increase the neutralizing synergy of an already effective mAb cocktail by a different mechanism to reduce the occurrence of viral immune escape.

We further developed this mAb by generating it as a murine/human chimeric IgG in plants to take advantage of the benefits mAb production in plants offers. Specifically, plant-based production utilizes inexpensive facilities and growth media for easily scalable mAb generation (Chen and Davis, 2016), as well as potential economic advantages in upstream production (Buyel *et al.*, 2017; Nandi *et al.*, 2016). This, in combination with homogenous N-linked glycosylation achieved with Δ XFT *N. benthamiana* plants, allows for development of mAbs with potentially greater safety and efficacy at lower costs than mAbs produced in typical mammalian systems (Dent *et al.*, 2016; Kallolimath *et al.*, 2021; Lai *et al.*, 2010; Strasser *et al.*, 2008; Sun *et al.*, 2021). Indeed, the glycan analysis performed on p11D7 confirmed that 91.1% of the recombinant mAb carried the mammalian-like glycoform of GnGn or a partially processed MGn, the human-like intermediate for more complex N-linked glycans (Chen, 2016). This is regarded as necessary by regulatory agencies for potential human therapeutics made in plants to eliminate immunogenicity of plant-specific glycans (Schähs *et al.*, 2007). In addition, the N-linked glycan pattern of IgGs located in the constant heavy 2 (CH2) region at asparagine 297 (N297) is necessary for Fc effector functions mediated through Fc gamma receptors (Fc γ Rs) (Jefferis and Lund, 2002). Fc effector functions are responsible for pathogen clearance through cell-mediated mechanisms, as well as enhancing antigen presentation (Lu *et al.*, 2017). It has also been demonstrated that Fc function is necessary for more favourable therapeutic protection against SARS-CoV-2 (Winkler *et al.*, 2021). The lack of core-fucose residues is of interest here, in that fucosylated IgGs weaken antibody-dependent cellular cytotoxicity (ADCC) activity by decreasing affinity to the Fc γ R11a (Wang *et al.*, 2018). Furthermore, ADCC activity of IgGs against SARS-CoV-2 has been observed in plasma containing both neutralizing and non-neutralizing IgGs (Tso *et al.*, 2021), indicating that there may be a protective role for weakly neutralizing or non-neutralizing mAbs that still bind to SARS-CoV-2 through an Fc-mediated mechanism. Although p11D7 is not as potent a neutralizer compared with other therapeutically developed mAbs for SARS-CoV-2 (Gruell *et al.*, 2022b; Jugler *et al.*, 2022; Shi *et al.*, 2020; VanBlargan *et al.*, 2022; Wu *et al.*, 2020; Zost *et al.*, 2020), the plant-made mAb developed in this study, has a highly homogeneous (91.1%) glycan population without detectable core fucose, compared with the 81.9% of the hybridoma-made mAb that

contains core fucose. This suggests that p11D7 may have increased *in vivo* efficacy by means other than neutralizing potency, namely through Fc-mediated mechanisms such as ADCC, and will be the focus of future studies.

Conclusion

To our knowledge, this is the most comprehensive study describing a novel mAb produced in plants that binds to a unique epitope on the RBD, exhibiting neutralizing potency against multiple variants of concern by itself, and shows synergy in neutralizing the Omicron variant of SARS-CoV-2. As variants continue to emerge, there is a need to proceed to develop therapeutic mAbs that maintain antigen recognition, as well as continue to neutralize the virus, which can be most efficiently accomplished with synergizing antibody cocktails. Our study specifically highlights the utility of mAbs that do not directly hinder ACE2 binding to the RBD in forming effective cocktail components, as well as plants as a mAb expression platform and the potential of this platform to contribute to therapeutic development against the ever-evolving SARS-CoV-2 pandemic.

Methods

Generation of anti-RBD monoclonal antibodies and variable gene rescue

Generation of the hybridoma as the source of the mAb 11D7 was described previously (Jugler *et al.*, 2022). Briefly, BALB/c mice were immunized subcutaneously with RBD protein (NR-52309 obtained from BEI) under protocol #22-1881 T approved by the Institutional Animal Care and Use Committee (IACUC) of Arizona State University. Splenocytes were isolated to establish hybridomas using a standard method (Köhler and Milstein, 1975). Hybridomas were screened for reactivity to both biotinylated and non-biotinylated RBD (Wuhan-Hu-1 RBD) to ensure the recovery of all RBD-binding mAbs including those with epitopes that may be excluded by direct adhering of antigen to the ELISA plate (Jugler *et al.*, 2022). cDNA was generated from cells of the 11D7 hybridoma using a RNeasy kit for total RNA isolation (Qiagen), followed by first-strand generation by the Reverse Transcription System (Promega). The coding sequences of mAb variable regions were generated by PCR using degenerate primers (Wang *et al.*, 2000), cloned into the CloneJET PCR Cloning Kit (Thermo Scientific), and sequenced by Sanger sequencing as described previously (Jugler *et al.*, 2022).

Variable gene plant codon adaptation and plant expression

Variable region sequences rescued and determined from the 11D7 hybridoma and the variable regions for two mAbs, COV2-2130 (cilgavimab) and COV2-2196 (tixagevimab) from the literature (Dong *et al.*, 2021; Loo *et al.*, 2022; Zost *et al.*, 2020) were codon-adapted for plant-based expression using GeneDesigner2.0. and synthesized by Integrated DNA Technologies (IDT). Synthesized fragments were further grafted onto a human kappa constant sequence that was codon-adapted for plant expression of the light chain and a human gamma constant sequence codon-adapted for plant expression of the heavy chain. Subsequent light and heavy chain genes were inserted into bean yellow dwarf virus geminiviral vector and transiently expressed in *N. benthamiana* plants by agroinfiltration (Chen *et al.*, 2013; Jugler *et al.*, 2021, 2022; Leuzinger *et al.*, 2013).

Antibody extraction and purification

The extraction and purification of mAbs from plants followed our published protocols (Jugler *et al.*, 2020; Lai *et al.*, 2010). Briefly, *N. benthamiana* leaves expressing mAbs were harvested 5 days after infiltration and homogenized in extraction buffer (1X PBS pH 5.2 with 10 mg/mL Na-L-ascorbate, 2 mM PMSF and 1 mM EDTA) and filtered through cheesecloth. Total plant protein extract was then centrifuged several times and incubated at least 4 h at 4 °C at pH 5.2 to precipitate host proteins. Further centrifugation was performed, and the clarified protein extract was filtered through a 0.2-micron vacuum filter. Protein extract containing recombinant mAbs was then subjected to gravity flow Protein A purification prior to further analysis.

SDS-PAGE and western blot analysis

SDS-PAGE and Western blot analysis were performed as described previously (Hurtado *et al.*, 2020). Briefly, purified mAbs were subjected to SDS-PAGE analysis under reducing and non-reducing conditions on 4%–20% acrylamide gels and total protein content was stained with Coomassie Blue R-250. For Western blots, proteins were separated on 12% acrylamide gels under reducing conditions or on 4%–20% acrylamide gels under non-reducing conditions. After separation, proteins were transferred to PVDF membranes, blocked with 5% milk in 1X PBST and probed with goat anti-human kappa chain conjugated to horseradish peroxidase (HRP) (Southern Biotech) or goat anti-human IgG-HRP (Southern Biotech). Pierce West Pico Western blotting substrate (Thermo Scientific) was added to the membranes and images were taken with an Amersham ImageQuant instrument.

p11D7 temporal expression in plants

Expression of 11D7 in plants was performed utilizing an ELISA assay as described previously (Dent *et al.*, 2016). Briefly, 96-well plates were coated with goat anti-human IgG (Southern Biotech) at 2 µg/mL overnight at 4 °C and blocked with 5% milk in 1X PBST. All wash steps between incubations were performed three times with 1X PBST. *N. benthamiana* leaves expressing recombinant p11D7 were harvested at 24-h intervals ranging from 3 to 9 days post agroinfiltration. Leaves were homogenized as described above and the extract clarified by centrifugation. After blocking, various dilutions of each time point was added to the plate for 1 h at 37 °C, alongside a control IgG of known concentration. Recombinant, p11D7 was then detected by adding goat anti-human kappa chain-HRP diluted 1:4000 in 5% milk in 1X PBST. TMB substrate (SeraCare Life Sciences Inc.) was then added and developed for 5 min before using a 1 M H₂SO₄ stop solution. Absorbance was read at 450 nm and GraphPad Prism was used to calculate microgram of recombinant 11D7 per gram of fresh leaf weight.

Glycan analysis

The N-glycosylation profiles of purified plant-made 11D7 and hybridoma-made 11D7 were determined by mass spectrometry (MS) as described previously (Esqueda and Chen, 2023; Kallolmath *et al.*, 2021; Sun *et al.*, 2021). In brief, heavy chains were extracted from an SDS-PAGE, trypsin digested and analysed with an LC-ESI-MS system (Orbitrap Exploris 480, Thermo Scientific). Glyco-peptides were identified as sets of peaks consisting of the peptide and the attached N-glycan moiety, varying in the number

of N-acetylhexosamine (HexNAc) units, hexose (mannose, galactose, glucose, etc.), deoxyhexose (fucose) and pentose (xylose) residues. Using FreeStyle 1.8 (Thermo Scientific), manual glycopeptide searches were performed, and deconvolution was done using the Extract function. Heights of peaks roughly reflect the molar ratios of the glycoforms. Glycan nomenclature according to Consortium for Functional Glycomics (<http://www.functionalglycomics.org>) was used.

RBD-specific binding of mAbs

Indirect ELISAs were used to show specific binding to the WA1/2020 or the B.1.1.529 RBD using a previously described method (He *et al.*, 2021). Briefly, 2 µg/mL (WA1/2020) or 5 µg/mL (B.1.1.529) of RBD was coated on 96-well plates overnight at 4 °C and blocked with 5% milk in 1X PBST. All wash steps between incubations were performed three times with 1X PBST. Dilutions of mAbs were made in 5% milk in 1X PBST and incubated 1 h at 37 °C, followed by detection with goat anti-human IgG-HRP. TMB substrate was then added, and the plate developed for 5 min before using a 1 M H₂SO₄ stop solution. Absorbance was read at 450 nm and GraphPad Prism was used to generate graphs and calculate approximate dissociation constants (K_D) of p11D7 with one-site specific-binding model.

Competitive ELISA

An ELISA was used to assess RBD-binding competition between 11D7 and other RBD-binding mAbs as previously described (Jugler *et al.*, 2022). Dilutions of each mAb (all produced in plants) were coated on 96-well plates overnight at 4 °C and blocked with 5% milk in 1X PBST. All wash steps between incubations were performed three times with 1X PBST. The WA1/2020 RBD was incubated at 2 µg/mL for 1 h at 37 °C. Plant-made 11D7 was conjugated to HRP using EZ Link Plus Activated Peroxidase Kit (Thermo Scientific) and added to the plate at a 1:1000 dilution. TMB substrate was then added, and the plate developed for 5 min before using a 1 M H₂SO₄ stop solution. Absorbance was read at 450 nm and GraphPad Prism was used to generate binding curves.

Focus-forming assay

The focus-forming assay was used to assess neutralization of mAbs as described previously (Case *et al.*, 2020; Jugler *et al.*, 2022), with some modifications. Briefly, 25 000 Vero E6 cells or Vero-hACE2-TMPRSS2 cells (for infection with Omicron) were plated in 100 µL of DMEM +10% FBS 1 day prior to the assay in 96-well clear, flat bottom plates. On the day of the assay, mAbs were diluted to various concentrations and SARS-CoV-2 stocks were diluted to 2000 plaque forming units (PFU) and mixed with the mAb dilutions for 1 h at 37 °C prior to adding to the plated cells for an additional hour at 37 °C. Dilutions were performed in DMEM +2% FBS. A 100 µL per well MEM: methylcellulose overlay was then added and infected cells were incubated for 24 h (WA1/2020 and Omicron) or 40 h (Delta). The overlay was then removed, and cells were fixed with 4% paraformaldehyde. Cells were further washed six times with 0.1% saponin and 0.1% BSA in 1X PBS. Cells infected with WA1/2020 or Delta strains were stained with plant-made CR3022 at 2 µg/mL followed by detection with goat anti-human IgG-HRP (Sigma). Omicron-infected cells were stained with a SARS-CoV-2 nucleocapsid antibody (GeneTex) and goat anti-rabbit IgG-HRP (Sigma). KPL TrueBlue substrate (SeraCare Life Sciences Inc.) was

added, and foci were quantified with an AID Spot Reader. Data were analysed with GraphPad Prism 9 and per cent neutralization was calculated as (average number of foci in virus-only wells—number of foci in antibody-treated well)/average number of foci in virus-only wells. Each antibody was tested in triplicate and at least two independent experiments were performed.

Neutralization synergy

Neutralizing synergy of mAb combinations was performed as previously described (Jugler *et al.*, 2022). In brief, mAbs were diluted to their corresponding IC₂₅ and IC₅₀, followed by FFA experiments with mAbs alone, as dual combinations, or as a triple combination of three mAbs. Per cent neutralization was then calculated as described for the FFA, and data were imported into SynergyFinder and analysed with the highest single agent (HSA) or Loewe models to calculate predicted per cent neutralization at each concentration tested (Berenbaum, 1989; Ianevski *et al.*, 2020; Loewe, 1953). Predicted neutralization was then directly compared with empirically determined per cent neutralization of each combination and concentration. Synergy was inferred when the observed per cent neutralization was higher than that predicted by both models.

Acknowledgements

The authors thank Dr. K. Kibler for technical assistance, Dr. Y. Li for sharing SARS-CoV-2 B.1.1.529 stock and H. Lai for sharing the recombinant RBD proteins of various SARS-CoV-2 variants. We also thank Dr. D. Lake and F. Grill for their advice in gene rescue and contributions described in Jugler *et al.*, 2022, and Clemens Gruenwald-Gruber (Core Facility Mass Spectrometry, BOKU VIENNA) for MS analyses. This research was supported by a sponsored research agreement (FP00026198) funding to Q. Chen Lab via Arizona State University by the Halberd Corporation. The following reagent was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH: SARS-Related Coronavirus 2, Isolate USA-WA1/2020, NR-52281. The following reagent was obtained through BEI Resources, NIAID, NIH: SARS-Related Coronavirus 2, Isolate hCoV-19/USA/PHC658/2021 (Lineage B.1.617.2; Delta Variant), NR-55611, contributed by Dr. Richard Webby and Dr. Anami Patel. The following reagent was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH: SARS-Related Coronavirus 2, Isolate hCoV-19/USA/HI-CDC4359259-001/2021 (Lineage B.1.1.529; Omicron Variant), NR-56475, contributed by Centers for Disease Control.

Conflict of interest

M.F. is an employee of Halberd Corporation, a company that may have interest in 11D7 for possible commercial development.

Author Contributions

Q.C. conceptualized research. H.Y.S., C.J. and H.S. designed experiments. H.Y.S., K.N., C.J. and R.P. performed experiments and analysed data. M.F. provided funding resources. C.J. drafted the manuscript and Q.C. revised the paper with helpful suggestions by H.S., H.Y.S., R.P. and M.F. All authors have reviewed and agreed to the submitted version of the manuscript.

References

- Alsaïdi, S., Cornejal, N., Mahoney, O., Melo, C., Verma, N., Bonnaire, T., Chang, T. *et al.* (2021) Griffithsin and carrageenan combination results in antiviral synergy against SARS-CoV-1 and 2 in a pseudoviral model. *Mar. Drugs* **19**, 418.
- Azkar, A.K., Akdis, M., Azkur, D., Sokolowska, M., Veen, W., Brügggen, M., O'Mahony, L. *et al.* (2020) Immune response to SARS-CoV-2 and mechanisms of immunopathological changes in COVID-19. *Allergy* **75**, 1564–1581.
- Barnes, C.O., Jette, C.A., Abernathy, M.E., Dam, K.-M.A., Esswein, S.R., Gristick, H.B., Malyutin, A.G. *et al.* (2020) SARS-CoV-2 neutralizing antibody structures inform therapeutic strategies. *Nature* **588**, 682–687.
- Berenbaum, M.C. (1989) What is synergy? *Pharmacol. Rev.* **41**, 93–141.
- Both, L., Banyard, A.C., van Dolleweerd, C., Wright, E., Ma, J.K.C. and Fooks, A.R. (2013) Monoclonal antibodies for prophylactic and therapeutic use against viral infections. *Vaccine* **31**, 1553–1559.
- Brockman, M.A., Mwimanzu, F., Lapointe, H.R., Sang, Y., Agafitei, O., Cheung, P.K., Ennis, S. *et al.* (2022) Reduced magnitude and durability of humoral immune responses to COVID-19 mRNA vaccines among older adults. *J Infect Dis* **225**, 1129–1140.
- Buyel, J.F., Twyman, R.M. and Fischer, R. (2017) Very-large-scale production of antibodies in plants: The biologization of manufacturing. *Biotechnol. Adv.* **35**, 458–465.
- Cai, Y., Zhang, J., Xiao, T., Peng, H., Sterling, S.M., Walsh, R.M., Rawson, S. *et al.* (2020) Distinct conformational states of SARS-CoV-2 spike protein. *Science* **369**, 1586–1592.
- Carreño, J.M., Alshammari, H., Tcheou, J., Singh, G., Raskin, A., Kawabata, H., Sominsky, L. *et al.* (2021) Activity of convalescent and vaccine serum against SARS-CoV-2 Omicron. *Nature* **602**, 682–688.
- Case, J.B., Bailey, A.L., Kim, A.S., Chen, R.E. and Diamond, M.S. (2020) Growth, detection, quantification, and inactivation of SARS-CoV-2. *Virology* **548**, 39–48.
- Chen, G., Wu, D., Guo, W., Cao, Y., Huang, D., Wang, H., Wang, T. *et al.* (2020) Clinical and immunological features of severe and moderate coronavirus disease 2019. *J. Clin. Investig.* **130**, 2620–2629.
- Chen, Q. (2016) Glycoengineering of plants yields glycoproteins with polysialylation and other defined N-glycoforms. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 9404–9406.
- Chen, Q. (2022) Development of plant-made monoclonal antibodies against viral infections. *Curr. Opin. Virol.* **52**, 148–160.
- Chen, Q. and Davis, K.R. (2016) The potential of plants as a system for the development and production of human biologics [version 1; referees: 3 approved]. *F1000Research* **5**(F1000 Faculty Rev), 912.
- Chen, Q., Lai, H., Stahnke, J., Hurtado, J., Leuzinger, K. and Dent, M. (2013) Agroinfiltration as an effective and scalable strategy of gene delivery for production of pharmaceutical proteins. *Advanced Techniques in Biology & Medicine* **1**, 1–9. <https://doi.org/10.4172/atbm.1000103>
- Chen, R.E., Zhang, X., Case, J.B., Winkler, E.S., Liu, Y., VanBlargan, L.A., Liu, J. *et al.* (2021) Resistance of SARS-CoV-2 variants to neutralization by monoclonal and serum-derived polyclonal antibodies. *Nat. Med.* **27**, 717–726.
- Chou, J., Thomas, P.G. and Randolph, A.G. (2022) Immunology of SARS-CoV-2 infection in children. *Nat. Immunol.* **23**, 177–185.
- Cotugno, N., Ruggiero, A., Bonfante, F., Petrara, M.R., Zicari, S., Pascucci, G.R., Zangari, P. *et al.* (2021) Virological and immunological features of SARS-CoV-2-infected children who develop neutralizing antibodies. *Cell Rep.* **34**, 108852.
- Daniell, H. (2022) A phase 1/2, randomized double-blind placebo-controlled trial to test the safety and antiviral activity of ACE2 chewing gum on SARS-CoV-2 viral load (COVID 19). <https://clinicaltrials.gov/ct2/show/NCT05433181>. (Accessed Nov 28, 2022).
- Dent, M., Hurtado, J., Paul, A.M., Sun, H., Lai, H., Yang, M., Esqueda, A. *et al.* (2016) Plant-produced anti-dengue virus monoclonal antibodies exhibit reduced antibody-dependent enhancement of infection activity. *J. Gen. Virol.* **97**, 3280–3290.
- Deshpande, A., Harris, B.D., Martinez-Sobrido, L., Kobie, J.J. and Walter, M.R. (2021) Epitope classification and RBD binding properties of neutralizing antibodies against SARS-CoV-2 variants of concern. *Front. Immunol.* **12**, 1.

- Diamos, A.G., Hunter, J.G.L., Pardhe, M.D., Rosenthal, S.H., Sun, H., Foster, B.C., DiPalma, M.P. et al. (2020) High level production of monoclonal antibodies using an optimized plant expression system. *Front. Bioeng. Biotechnol.* **7**, 472.
- Dong, J., Zost, S.J., Greaney, A.J., Starr, T.N., Dingens, A.S., Chen, E.C., Chen, R.E. et al. (2021) Genetic and structural basis for SARS-CoV-2 variant neutralization by a two-antibody cocktail. *Nat. Microbiol.* **6**, 1233–1244.
- Esqueda, A. and Chen, Q. (2023) Producing Biologics with Defined N-Glycosylation in Plants. In *Chemokine-Glycosaminoglycan Interactions: Methods and Protocols* (Lucas, A.R., ed), pp. 235–250. New York, NY: Springer US. [10.1007/978-1-0716-2835-5_17](https://doi.org/10.1007/978-1-0716-2835-5_17)
- Focosi, D., McConnell, S., Casadevall, A., Cappello, E., Valdiserra, G. and Tuccori, M. (2022) Monoclonal antibody therapies against SARS-CoV-2. *Lancet Infect. Dis.* **22**, e311–e326.
- Fuqua, J.L., Hamorsky, K., Khalsa, G., Matoba, N. and Palmer, K.E. (2015) Bulk production of the antiviral lectin griffithsin. *Plant Biotechnology* **13**, 1160–1168.
- Garcia-Beltran, W.F., Denis, K.J.S., Hoelzemer, A., Lam, E.C., Nitido, A.D., Sheehan, M.L., Berrios, C. et al. (2022) mRNA-based COVID-19 vaccine boosters induce neutralizing immunity against SARS-CoV-2 Omicron variant. *Cell* **185**, 457–466 e4.
- Gilchuk, P., Bombardi, R.G., Erasmus, J.H., Tan, Q., Nargi, R., Soto, C., Abbink, P. et al. (2020) Integrated pipeline for the accelerated discovery of antiviral antibody therapeutics. *Nature Biomedical Engineering* **4**, 1030–1043.
- Greaney, A.J., Loes, A.N., Crawford, K.H.D., Starr, T.N., Malone, K.D., Chu, H.Y. and Bloom, J.D. (2021) Comprehensive mapping of mutations in the SARS-CoV-2 receptor-binding domain that affect recognition by polyclonal human plasma antibodies. *Cell Host Microbe* **29**, 463–476.e6.
- Greaney, A.J., Starr, T.N., Barnes, C.O., Weisblum, Y., Schmidt, F., Caskey, M., Gaebler, C. et al. (2021) Mapping mutations to the SARS-CoV-2 RBD that escape binding by different classes of antibodies. *Nat. Commun.* **12**, 1–14.
- Gruell, H., Vanshylla, K., Weber, T., Barnes, C.O., Kreer, C. and Klein, F. (2022a) Antibody-mediated neutralization of SARS-CoV-2. *Immunity* **55**, 925.
- Hager, K.J., Marc, G.P., Gobeil, P., Diaz, R.S., Heizer, G., Llapur, C., Makarkov, A.I. et al. (2022) Efficacy and safety of a recombinant plant-based adjuvanted Covid-19 vaccine. *New England Journal of Medicine* **386**, 2084–2096.
- He, J., Lai, H., Esqueda, A. and Chen, Q. (2021) Plant-produced antigen displaying virus-like particles evokes potent antibody responses against west nile virus in mice. *Vaccine* **9**, 60.
- Health Canada. (2022). *Health Canada authorizes Medicago COVID-19 vaccine for adults 18 to 64 years of age*. <https://www.canada.ca/en/health-canada/news/2022/02/health-canada-authorizes-medicago-covid-19-vaccine-for-adults-18-to-64-years-of-age.html>. (Accessed October 25, 2022).
- Heath, P.T., Galiza, E.P., Baxter, D.N., Boffito, M., Browne, D., Burns, F., Chadwick, D.R. et al. (2021) Safety and efficacy of NVX-CoV2373 Covid-19 vaccine. *New England Journal of Medicine* **385**, 1172–1183.
- Hoffmann, M., Arora, P., Groß, R., Seidel, A., Hörnich, B.F., Hahn, A.S., Krüger, N. et al. (2021) SARS-CoV-2 variants B.1.351 and P.1 escape from neutralizing antibodies. *Cell* **184**, 2384–2393.e12.
- Hu, J., Peng, P., Cao, X., Wu, K., Chen, J., Wang, K., Tang, N. et al. (2022) Increased immune escape of the new SARS-CoV-2 variant of concern Omicron. *Cell. Mol. Immunol.* **19**, 293–295.
- Huo, J., Zhao, Y., Ren, J., Zhou, D., Duyvesteyn, H.M.E., Ginn, H.M., Carrique, L. et al. (2020) Neutralization of SARS-CoV-2 by destruction of the prefusion spike. *Cell Host Microbe* **28**, 445–454.e6.
- Hurtado, J., Acharya, D., Lai, H., Sun, H., Kallolimath, S., Steinkellner, H., Bai, F. et al. (2020) In vitro and in vivo efficacy of anti-chikungunya virus monoclonal antibodies produced in wild-type and glycoengineered Nicotiana benthamiana plants. *Plant Biotechnol. J.* **18**, 266–273.
- lanevski, A., Giri, A.K. and Aittokallio, T. (2020) SynergyFinder 2.0: visual analytics of multi-drug combination synergies. *Nucleic Acids Res.* **48**(W1), W488–W493.
- Jefferis, R. and Lund, J. (2002) Interaction sites on human IgG-Fc for FcγR: current models. *Immunol. Lett.* **82**, 57–65.
- Jugler, C., Grill, F.J., Eidenberger, L., Karr, T.L., Grys, T.E., Steinkellner, H., Lake, D.F. et al. (2022) Humanization and expression of igg and igm antibodies in plants as potential diagnostic reagents for valley fever. *Front. Plant Sci.* **13**, 925008.
- Jugler, C., Joensuu, J. and Chen, Q. (2020) Hydrophobin-protein A fusion protein produced in plants efficiently purified an anti-west nile virus monoclonal antibody from plant extracts via aqueous two-phase separation. *Int. J. Mol. Sci.* **21**, 2140.
- Jugler, C., Sun, H. and Chen, Q. (2021) SARS-CoV-2 spike protein-induced interleukin 6 signaling is blocked by a plant-produced anti-interleukin 6 receptor monoclonal antibody. *Vaccine* **9**, 1365.
- Jugler, C., Sun, H., Grill, F., Kibler, K., Esqueda, A., Lai, H., Li, Y. et al. (2022) Potential for a plant-made SARS-CoV-2 neutralizing monoclonal antibody as a synergetic cocktail component. *Vaccine* **10**, 772.
- Kallolimath, S., Sun, L., Palt, R., Stiasny, K., Mayrhofer, P., Gruber, C., Kogelmann, B. et al. (2021) Highly active engineered IgG3 antibodies against SARS-CoV-2. *Proc. Natl. Acad. Sci.* **118**, e2107249118.
- Köhler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–497.
- Ku, Z., Xie, X., Davidson, E., Ye, X., Su, H., Menachery, V.D., Li, Y. et al. (2021) Molecular determinants and mechanism for antibody cocktail preventing SARS-CoV-2 escape. *Nat. Commun.* **12**, 1–13.
- Kuchipudi, V., Kapur, V., Jacob, J., Ghimire, D., Han, Y. and Lu, M. (2022) Structural plasticity and immune evasion of SARS-CoV-2 spike variants. *Viruses* **14**, 1255.
- Kumar, R., Parry, H.A., Shrivastava, T., Sinha, S. and Luthra, K. (2019) Phage display antibody libraries: A robust approach for generation of recombinant human monoclonal antibodies. *Int. J. Biol. Macromol.* **135**, 907–918.
- Lai, H., Engle, M., Fuchs, A., Keller, T., Johnson, S., Gorlatov, S., Diamond, M.S. et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. *Proc. Natl. Acad. Sci.* **107**, 2419–2424.
- Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., Zhang, Q. et al. (2020) Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature* **581**, 215–220.
- Leuzinger, K., Dent, M., Hurtado, J., Stahnke, J., Lai, H., Zhou, X. and Chen, Q. (2013) Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins. *J. Vis. Exp.* **77**, e50521.
- Loewe, S. (1953) The problem of synergism and antagonism of combined drugs. *Arzneimittelforschung* **3**, 285–290.
- Loo, Y.-M., McTamney, P.M., Arends, R.H., Abram, M.E., Aksyuk, A.A., Diallo, S., Flores, D.J. et al. (2022) The SARS-CoV-2 monoclonal antibody combination, AZD7442, is protective in non-human primates and has an extended half-life in humans. *Sci. Transl. Med.* **14**, eab18124.
- Loos, A. and Steinkellner, H. (2014) Plant glyco-biotechnology on the way to synthetic biology. *In Frontiers in Plant Science* **5**, 523.
- Lu, L.L., Suscovich, T.J., Fortune, S.M. and Alter, G. (2017) Beyond binding: antibody effector functions in infectious diseases. *Nat. Rev. Immunol.* **18**, 46–61.
- Margolin, E., Oh, Y.J., Verbeek, M., Naude, J., Ponndorf, D., Meshcheriakova, Y.A., Peyret, H. et al. (2020) Co-expression of human calreticulin significantly improves the production of HIV gp140 and other viral glycoproteins in plants. *Plant Biotechnol. J.* **18**, 2109–2117.
- Mitra, S. and Tomar, P.C. (2021) Hybridoma technology; advancements, clinical significance, and future aspects. *Journal of Genetic Engineering & Biotechnology* **19**, 159.
- Nandi, S., Kwong, A.T., Holtz, B.R., Erwin, R.L., Marcel, S. and McDonald, K.A. (2016) Techno-economic analysis of a transient plant-based platform for monoclonal antibody production. *MAbs* **8**, 1456–1466.
- Obeid, M., Suffiotti, M., Pellaton, C., Bouchaab, H., Cairoli, A., Salvadé, V., Stenel, C. et al. (2022) Humoral Responses Against Variants of Concern by COVID-19 mRNA Vaccines in Immunocompromised Patients. *JAMA Oncol.* **8**, e220446.
- Ozono, S., Zhang, Y., Ode, H., Sano, K., Tan, T.S., Imai, K., Miyoshi, K. et al. (2021) SARS-CoV-2 D614G spike mutation increases entry efficiency with enhanced ACE2-binding affinity. *Nat. Commun.* **12**, 1–9.
- Palmer, K. E. (2022) *PREVENT-COVID-19: A Q-Griffithsin Intranasal Spray*, <https://clinicaltrials.gov/ct2/show/NCT05122260> (Accessed Oct 25, 2022).
- Piccoli, L., Park, Y.J., Tortorici, M.A., Czudnochowski, N., Walls, A.C., Beltramello, M., Silacci-Fregni, C. et al. (2020) Mapping neutralizing and immunodominant sites on the SARS-CoV-2 spike receptor-binding domain by structure-guided high-resolution serology. *Cell* **183**, 1024–1042.e21.

- Planas, D., Saunders, N., Maes, P., Guivel-Benhassine, F., Planchais, C., Buchrieser, J., Bolland, W.H. *et al.* (2021) Considerable escape of SARS-CoV-2 Omicron to antibody neutralization. *Nature* **602**, 671–675.
- Pramanick, I., Sengupta, N., Mishra, S., Pandey, S., Girish, N., Das, A. and Dutta, S. (2021) Conformational flexibility and structural variability of SARS-CoV-2 S protein. *Structure* **29**, 834–845 e5.
- Qiao, Y., Wang, X.M., Mannan, R., Pitchaiya, S., Zhang, Y., Wotring, J.W., Xiao, L. *et al.* (2020) Targeting transcriptional regulation of SARS-CoV-2 entry factors ACE2 and TMPRSS2. *Proc. Natl. Acad. Sci. U. S. A.* **118**, e2021450118. [10.1073/PNAS.2021450118/SUPPL_FILE/PNAS.2021450118.SAPP.PDF](https://doi.org/10.1073/PNAS.2021450118/SUPPL_FILE/PNAS.2021450118.SAPP.PDF)
- Rattanapisit, K., Shanmugaraj, B., Manopwisedjaroen, S., Purwono, P.B., Siri Wattananon, K., Khorattanakulchai, N., Hanittinan, O. *et al.* (2020) Rapid production of SARS-CoV-2 receptor binding domain (RBD) and spike specific monoclonal antibody CR3022 in *Nicotiana benthamiana*. *Sci. Rep.* **10**, 1–11.
- Rogers, T.F., Zhao, F., Huang, D., Beutler, N., Burns, A., He, W.T., Limbo, O. *et al.* (2020) Isolation of potent SARS-CoV-2 neutralizing antibodies and protection from disease in a small animal model. *Science* **369**, 956–963.
- Ryan, A., Lee, Y.B., Wong, S.Y., Yi, L., Chai, A., Lee, S.C., Lee, M.X. *et al.* (2022) Efficacy of covid-19 vaccines in immunocompromised patients: systematic review and meta-analysis. *BMJ* **376**, e068632.
- El Sahly, H.M., Baden, L.R., Essink, B., Doblecki-Lewis, S., Martin, J.M., Anderson, E.J., Campbell, T.B. *et al.* (2021) Efficacy of the mRNA-1273 SARS-CoV-2 vaccine at completion of blinded phase. *New England Journal of Medicine* **385**, 1774–1785.
- Schähs, M., Strasser, R., Stadlmann, J., Kunert, R., Rademacher, T. and Steinkellner, H. (2007) Production of a monoclonal antibody in plants with a humanized N-glycosylation pattern. *Plant Biotechnol. J.* **5**, 657–663.
- Schmidt, F., Weisblum, Y., Rutkowska, M., Poston, D., da Silva, J., Zhang, F., Bednarski, E. *et al.* (2021) High genetic barrier to SARS-CoV-2 polyclonal neutralizing antibody escape. *Nature* **600**, 512–516.
- Schurink, B., Roos, E., Vos, W., Breur, M., van der Valk, P. and Bugiani, M. (2022) ACE2 protein expression during childhood, adolescence, and early adulthood. *Pediatr. Dev. Pathol.* **25**, 404–408.
- Shanmugaraj, B., Rattanapisit, K., Manopwisedjaroen, S., Thitithyanont, A. and Phoolcharoen, W. (2020) Monoclonal antibodies B38 and H4 produced in *Nicotiana benthamiana* neutralize SARS-CoV-2 in vitro. *Front. Plant Sci.* **11**, 589995.
- Shi, R., Shan, C., Duan, X., Chen, Z., Liu, P., Song, J., Song, T. *et al.* (2020) A human neutralizing antibody targets the receptor-binding site of SARS-CoV-2. *Nature* **584**, 120–124.
- Siri Wattananon, K., Manopwisedjaroen, S., Kanjanasirirat, P., Purwono, P.B., Rattanapisit, K., Shanmugaraj, B., Smith, D.R. *et al.* (2021) Development of plant-produced recombinant ACE2-Fc fusion protein as a potential therapeutic agent against SARS-CoV-2. *Front. Plant Sci.* **11**, 604663.
- Stander, J., Mbewana, S. and Meyers, A.E. (2022) Plant-derived human vaccines: Recent developments. *BioDrugs* **36**, 573–589.
- Starr, T.N., Greaney, A.J., Addetia, A., Hannon, W.W., Choudhary, M.C., Dingens, A.S., Li, J.Z. *et al.* (2021) Prospective mapping of viral mutations that escape antibodies used to treat COVID-19. *Science* **371**, 850–854.
- Strasser, R., Stadlmann, J., Schähs, M., Stiegler, G., Quendler, H., Mach, L., Glössl, J. *et al.* (2008) Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. *Plant Biotechnol. J.* **6**, 392–402.
- Sun, H., Lecio, J., & Chen, Q. (2023). Development of antibody-based therapeutics against West Nile virus in plants. In: *West Nile Virus: Methods and Protocols* (Bai, F. ed) pp. 211–225. New York, NY: Springer US. [10.1007/978-1-0716-2760-0_19](https://doi.org/10.1007/978-1-0716-2760-0_19)
- Sun, L., Kallolmath, S., Palt, R., Stiasny, K., Mayrhofer, P., Maresch, D., Eidenberger, L. *et al.* (2021) Increased in vitro neutralizing activity of SARS-CoV-2 IgA1 dimers compared to monomers and IgG. *Proc. Natl. Acad. Sci.* **118**, e2107148118.
- ter Meulen, J., van den Brink, E.N., Poon, L.L.M., Marissen, W.E., Leung, C.S.W., Cox, F., Cheung, C.Y. *et al.* (2006) Human monoclonal antibody combination against SARS coronavirus: Synergy and coverage of escape mutants. *PLoS Med.* **3**, e237.
- Thomas, S. J., Edson D. Moreira, Jr., Kitchin, N., Absalon, J., Gurtman, A., Lockhart, S., Perez, J. L., Marc, G. P., Polack, F. P., Zerbini, C., Bailey, R., Swanson, K. A., Xu, X., Roychoudhury, S., Koury, K., Bouguermouh, S., Kalina, W. V., Cooper, D., Robert W. Frenck, Jr, Jansen, K. U. (2021). Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine through 6 months. *New England Journal of Medicine*, **385**, 1761-1773.
- Tso, F.Y., Lidenge, S.J., Poppe, L.K., Peña, P.B., Privatt, S.R., Bennett, S.J., Ngowi, J.R. *et al.* (2021) Presence of antibody-dependent cellular cytotoxicity (ADCC) against SARS-CoV-2 in COVID-19 plasma. *PLOS ONE* **16**, e0247640.
- VanBlargan, L.A., Errico, J.M., Halfmann, P.J., Zost, S.J., Crowe, J.E., Purcell, L.A., Kawaoka, Y. *et al.* (2022) An infectious SARS-CoV-2 B.1.1.529 Omicron virus escapes neutralization by therapeutic monoclonal antibodies. *Nat. Med.* **28**, 490–495.
- Walls, A.C., Park, Y.J., Tortorici, M.A., Wall, A., McGuire, A.T. and Velesler, D. (2020) Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* **181**, 281–292.e6.
- Wang, Q., Chung, C.Y., Chough, S. and Betenbaugh, M.J. (2018) Antibody glycoengineering strategies in mammalian cells. *Biotechnol. Bioeng.* **115**, 1378–1393.
- Wang, Q., Guo, Y., Iketani, S., Nair, M.S., Li, Z., Mohri, H., Wang, M. *et al.* (2022) Antibody evasion by SARS-CoV-2 Omicron subvariants BA.2.12.1, BA.4 and BA.5. *Nature* **608**, 1–6.
- Wang, Z., Raifu, M., Howard, M., Smith, L., Hansen, D., Goldsby, R. and Ratner, D. (2000) Universal PCR amplification of mouse immunoglobulin gene variable regions: the design of degenerate primers and an assessment of the effect of DNA polymerase 3' to 5' exonuclease activity. *J. Immunol. Methods* **233**, 167–177. [https://doi.org/10.1016/S0022-1759\(99\)00184-2](https://doi.org/10.1016/S0022-1759(99)00184-2)
- Wang, Q., Zhang, Y., Wu, L., Niu, S., Song, C., Zhang, Z., Lu, G. *et al.* (2020) Structural and functional basis of SARS-CoV-2 entry by using human ACE2. *Cell* **181**, 894–904.e9.
- Wang, Y., Liu, C., Zhang, C., Wang, Y., Hong, Q., Xu, S., Li, Z. *et al.* (2022) Structural basis for SARS-CoV-2 Delta variant recognition of ACE2 receptor and broadly neutralizing antibodies. *Nat. Commun.* **13**, 1–12.
- Winkler, E.S., Gilchuk, P., Yu, J., Bailey, A.L., Chen, R.E., Chong, Z., Zost, S.J. *et al.* (2021) Human neutralizing antibodies against SARS-CoV-2 require intact Fc effector functions for optimal therapeutic protection. *Cell* **184**, 1804–1820.e16.
- Wu, Y., Wang, F., Shen, C., Peng, W., Li, D., Zhao, C., Li, Z. *et al.* (2020) A noncompeting pair of human neutralizing antibodies block COVID-19 virus binding to its receptor ACE2. *Science* **368**, 1274–1278.
- Xu, C., Wang, Y., Liu, C., Zhang, C., Han, W., Hong, X., Wang, Y. *et al.* (2021) Conformational dynamics of SARS-CoV-2 trimeric spike glycoprotein in complex with receptor ACE2 revealed by cryo-EM. *Science Advances* **7**, eabe5575. https://doi.org/10.1126/SCIADV.ABE5575/SUPPL_FILE/ABE5575_SM.PDF
- Yamasoba, D., Kosugi, Y., Kimura, I., Fujita, S., Uriu, K., Ito, J. and Sato, K. (2022) Neutralisation sensitivity of SARS-CoV-2 omicron subvariants to therapeutic monoclonal antibodies. *Lancet Infect. Dis.* **22**, 942–943.
- Zost, S.J., Gilchuk, P., Case, J.B., Binshtein, E., Chen, R.E., Nkolola, J.P., Schäfer, A. *et al.* (2020) Potently neutralizing and protective human antibodies against SARS-CoV-2. *Nature* **584**, 443–449.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Neutralization potency (IC₉₀) of p11D7 against various SARS-CoV-2 variants. Serial dilutions of p11D7 were mixed with the indicated SARS-CoV-2 variant before adding to Vero E6 (WA1/2020 and Delta) or Vero-hACE2-TMPRSS2 (Omicron) cells. Cells were then fixed, permeabilized and stained for SARS-CoV-2 protein. Foci were counted, per cent neutralization determined and IC₉₀ calculated using GraphPad Prism 9. At least two independent experiments were performed with technical triplicates.