Molecular engineering of a cryptic epitope in Spike RBD improves manufacturability and neutralizing breadth against SARS-CoV-2 variants

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Abbreviations:
RBD, Receptor Binding Domain
RBM, Receptor Binding Motif
RBD-J, RBD-L452K-F490W
RBD-J6, RBD-S383D-L452K-F490W-L518D
ACE2, angiotensin converting enzyme 2
SMNP, saponin monophosphoryl lipid A nanoparticles

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1. Introduction

The global distribution of COVID-19 vaccines continues to lag in low- and middle-income countries (LMICs), which have struggled to acquire and distribute doses due to high prices and logistical distribution requirements such as cold chains[1]. Vaccines that effectively prevent severe COVID-19 symptoms and death are broadly
available in high-income countries[2], but SARS-CoV-2 variants of concern (VOCs) continue to emerge with increased transmissibility and the ability to escape known neutralizing antibodies[3–6]. Effective and accessible vaccines or boosters are still important, especially ones that can be manufactured at low costs, that can be stored at non-freezing or ambient temperatures, and could offer protection against VOCs.

Protein subunit-based vaccines typically can tolerate higher temperatures than mRNA vaccines for storage and transport and have been proven effective for prophylactic prevention of SARS-CoV-2[7,8]. While the SARS-CoV-2 spike (S) protein antigen elicits strong neutralizing responses when administered with adjuvants, the full trimeric form of the S protein remains difficult to manufacture[9,10]. Smaller subunit antigens such as the S protein receptor binding domain (RBD) also elicit protective immunity against SARS-CoV-2 in animal models and humans[11–15]. The RBD is also currently produced in microbial hosts with existing manufacturing infrastructure in LMICs[16,17].

Improving the productivity of microbial fermentation can further lower the cost of manufacturing subunit vaccines[18]. Enhancements to stabilize subunit proteins can improve manufacturing yields[19] and reduce costs associated with storage and distribution[20]. We previously used molecular engineering to improve the production of RBD in yeast, as well as its biophysical stability[11]. Here, we performed further molecular engineering of the SARS-CoV-2 RBD. We modified a hydrophobic region that is buried in the closed-form S protein, but is exposed on the RBD antigen itself. These changes led to a three-fold improvement in secreted titer and increased biophysical stability. The alterations described here did not significantly alter the antigenicity or immunogenicity of the RBD when displayed on a virus-like particle (VLP). VLP-RBD conjugates elicited immunity in mice against and protection against SARS-CoV-2 Alpha and Beta variants.

2. Results

2.1. Mutation of the RBD core hydrophobic patch to improve expression

We previously identified an exposed hydrophobic patch in the receptor binding motif (RBM) of the RBD that may cause both intracellular and extracellular aggregation of the RBD[11]. Two mutations to the RBM conferred a significant improvement to the production of secreted protein from yeast and the biophysical stability of the protein. These point mutations (L452K and F490W) were selected from the highly conserved sarbecovirus genetic background to retain binding to the human ACE2 receptor and minimize effects on antigenicity.

Here, we sought to apply similar rational engineering to a second predicted hydrophobic patch on the RBD core[21], near the C-terminus. This hydrophobic patch is not predicted to be exposed in the native S protein with the RBD in the “down” conformation (Fig. 1a). This patch is, however, exposed when the RBD is expressed independently as a soluble monomer. We hypothesized that, similar to the hydrophobic patch found in the RBM, the RBD core hydrophobic patch may reduce the solubility, stability, and efficient secretion of the RBD[22]. We sought here to eliminate or reduce this hydrophobic patch by rational substitutions of amino acids similar to our previous engineering efforts[11,16].

We selected 21 mutations from a previously published deep mutational scanning analysis that appeared to boost expression in yeast while retaining binding to ACE2[23]. We tested each mutation individually with a 201 amino-acid RBD (amino acids 332–532 in the Spike protein). Each mutant RBD also included the mutation L452K, which, in our previous work, improved the expression and stability of the RBD[11]. We transformed each RBD variant into yeast and assessed the secretion of the RBD (Supplementary Fig. S1). As expected, the RBD variant with only the previously described L452K mutation was secreted with almost 60% higher titer than the Wuhan-Hu-1 RBD. Among RBD variants with additional mutations to RBD-L452K, we observed up to a ~4x improvement in specific productivity over the Wuhan-Hu-1 RBD. Interestingly, we found that three of the mutants that most improved secretion included mutations to an aspartic acid (S383D, R408D, and L518D) at locations in or near the RBD core hydrophobic patch (Supplementary Fig. S1). This result suggested that mutation of a heavily hydrophobic region may improve the secretion of this protein. Indeed, solvent-accessible hydrophobic regions can be destabilizing[24,25], and partially unfolded complexes can promote aggregation[26]. Notably, aspartic acid substitutions have been shown to improve the solubility of antibody complementarity-determining regions (CDRs)[27,28].

We next evaluated combinations of the three aspartic acid substitutions in the RBD core hydrophobic patch. These designs also included both of the mutations to the hydrophobic patch in the RBM from our previous work (L452K, F490W, dubbed RBD-J)[11,29]. We observed the greatest improvement in the secreted titer (60 mg/L to 173 mg/L) upon addition of the S383D and L518D mutations (Fig. 1b). We computationally evaluated this variant of RBD-J and observed a reduction of the predicted surface hydrophobicity, AggScore[30], from 157.6 to 128.1 (Fig. 1c). We concluded that this hybrid variant of RBD-J, S383D-L452K-F490W-L518D, denoted as RBD-J6, merited further characterization to assess its antigenicity and immunogenicity.

2.2. Biophysical characterization of engineered SARS-CoV-2 RBDs

We compared the biophysical properties of RBD-J and RBD-J6. First, we performed far-UV circular dichroism (CD) spectroscopy as a function of temperature and observed that the thermal melting temperature (Tm) value of RBD-J6 (63.0 ± 0.4 °C) was ~1.5 °C higher than that of RBD-J (61.6 ± 3.5 °C). This result suggested that the overall secondary structure of RBD-J6 was modestly more stable when compared to RBD-J (Fig. 1d). Next, we performed differential scanning calorimetry and observed that the Tm value of RBD-J6 (54.4 ± 0.1 °C) was also slightly higher than the RBD-J (53.8 ± 0.1 °C), a result consistent with the CD analysis, suggesting a small improvement to overall conformational stability of RBD-J6 (Fig. 1e). Lastly, we performed static light scattering as a function of temperature to assess the tendency of these two protein antigens to form aggregates. The thermal onset temperature (Tonset) value of RBD-J6 (70.0 ± 1.8 °C) was ~10 °C higher than that of RBD-J (60.0 ± 0.8 °C) (Fig. 1f), suggesting the colloidal stability of the RBD-J6 was enhanced. Taken together, these results indicate that mutation of solvent accessible hydrophobic regions can improve overall conformational stability of the antigen, and consequently, colloidal stability as well.

2.3. Antigenic characterization of engineered SARS-CoV-2 RBDs

Modification of the RBD to improve its physical qualities and stability as a vaccine candidate could potentially alter the antigenicity of the RBD. Unlike our previous engineering of the RBD, the two mutations introduced in RBD-J6 (S383D, L518D) are not native to the sarbecoviruses, and could affect the antigenicity of the RBD[31]. To assess the antigenicity of RBD-J6, and identify potential changes compared to RBD-J, we evaluated its binding to ACE2 and to several neutralizing antibodies targeting different epitopes of RBD[32,33]. We observed that RBD-J6 and RBD-J exhibited similar binding to ACE2 (Fig. 2a). RBD-J6 and RBD-J also exhibited similar binding to CB6[34] and CV30[35], two class I neutralizing
antibodies that target the RBM (Fig. 2b, c), and to S309, a class III neutralizing antibody that targets the proteoglycan site of the RBD.[36] We did observe, however, that RBD-J6 bound less strongly than RBD-J to CR3022 and EY6A, two class IV neutralizing antibodies that bind the RBD core near the C-terminus[33,34] (Fig. 2e, f), suggesting that the modified residues altered the RBD core epitope created when expressing the RBD domain alone.

We then evaluated how polyclonal antibodies raised in mice immunized with RBD-J bound to RBD-J6. In our previous study, we formulated RBD-J with different adjuvant combinations including alum alone, CpG1826 alone, alum + CpG1826, or a saponin-based adjuvant SMNP.[11,37] We found that antibodies in sera from mice immunized with alum alone, alum + CpG1826, or SMNP exhibited similar binding to both RBD-J and RBD-J6 (Fig. 2g). These results suggest that antibodies raised against the adjuvanted RBD-J may not target the RBD core epitope when delivered with these adjuvants. Indeed, most potent neutralizing antibodies raised against the RBD target the RBM.[38,39] Interestingly, we observed that antibodies raised against RBD-J formulated with CpG1826 alone exhibited less binding to RBD-J6. Indeed, we have hypothesized previously that CpG adjuvants may alter the structure and antigenicity of soluble RBD,[12] and have recently demonstrated that CpG and aluminum salt adjuvants destabilize formulated RBD-J during storage.[40]

Finally, we evaluated the binding to both engineered RBDs of antibodies from convalescent sera obtained from patients infected with SARS-CoV-2 Delta (B.1.617.2), including ones who had been vaccinated with approved mRNA COVID-19 vaccines before infection. The binding of these antibodies to either RBD-J or RBD-J6 was not significantly different, regardless of vaccination status.

Fig. 1. Molecular engineering of RBD core hydrophobic hotspot improves manufacturability. (a) Structural rendering of SARS-CoV-2 Spike trimer (PDB ID:7DK3). Amino acids included in RBM hydrophobic patch (red). Amino acids included in RBD core hydrophobic patch (blue). (b) Titer of mutated RBD secretion in 3 mL plate cultures, measured by reverse-phase liquid chromatography. (c) Structural rendering of RBD-J and RBD-J6 with predicted hydrophobic patches (red). (d) Molar ellipticity at 230 nm as a function of increasing temperature of purified RBD-J and RBD-J6 as measured by Far-UV circular dichroism. Errors bars represent standard deviation of three independent measurements. (e) Normalized DSC thermograms of purified RBD-J and RBD-J6. (f) Static light scattering as a function of increasing temperature of purified RBD-J and RBD-J6. The data in e-f are the mean of two independent experiments and the error bars in f represent the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
These data, together, suggest that the S383D and L518D RBD mutations do not impact binding to a breadth of human antibodies raised in response to infection or vaccination where native S protein is present.

2.4. Expression and antigenicity of RBD-J6 with mutations from circulating VOCs

Emerging variants of concern (VOCs) of SARS-CoV-2 have spurred the development of updated vaccine candidates that incorporate new viral mutations. In this study, we focused on two SARS-CoV-2 variants Alpha (B.1.1.7) and Beta (B.1.351). Several natural RBD mutations, including N501Y (Alpha and Beta), K417N (Beta), and E484K (Beta), reportedly increase virus transmissibility [41,42] and evasion of neutralizing antibodies [43,44]. We sought to determine if the benefits in biophysical stability and manufacturability that we observed by mutation of the RBD core hydrophobic patch in RBD-J6 would also benefit RBD antigens that include mutations from the Alpha and Beta variants.

We added the three mutations found in the RBD of the Beta variant to RBD-J6 (hereafter, RBD-J6 β). We first evaluated the secreted expression of RBD-J6 β in yeast and observed 30% lower titers compared to RBD-J6. This reduced titer of the β version of RBD-J6 agrees with our previous report on the expression of the Wuhan RBD and its B.1.351 variant in yeast [16]. This reduced expression reduction also coincides with an increase of the AggScore from 128.1 to 216.5 when incorporating Beta variant mutations to RBD-J6 consistent with our hypotheses on effects of hydrophobicity on RBD stability.

We evaluated binding of RBD-J6 β to ACE2 and observed similar binding as RBD-J6 (Supplementary Fig. S2). Previous studies report that these mutations found in the RBD from SARS-CoV-2 Alpha and Beta increase affinity to ACE2 [45], but we did not observe this difference in combination with the RBD-J6 mutations. Both RBD-J6 and RBD-J6 β had similar binding to S309, CR3022 and EY6A antibodies as well (Supplementary Fig. S2). We observed that RBD-J6 β bound less strongly than RBD-J6 to the neutralizing antibodies CB6 and CV30, which target the RBM (Supplementary Fig. S2). This result suggests that these amino acids, K417, E484 and N501, are targeted by ACE2-blocking antibodies raised against the original Wuhan variant, and that the Beta variant mutations may reduce recognition of certain epitopes. With the exception of the class IV antibodies, RBD-J β exhibited a comparable binding profile to RBD-J6 β. Lastly, RBD-J β and RBD-J6 β were bound comparably by the same human convalescent sera from Delta SARS-CoV-2 breakthrough infections (Supplementary Fig. S2, p = 0.3214, Paired t-test).

2.5. Vaccination of mice with RBD-J6 β and challenge with SARS-CoV-2 Alpha and Beta

We next sought to compare the immunogenicity and protection of the RBD-J β and RBD-J6 β. We previously reported a vaccine design in which an RBD antigen was displayed on the surface
of Hepatitis B surface antigen virus-like particles (HBsAg VLPs)\[12\]. Importantly, pre-existing immunity against the HBsAg elicited by an approved Hepatitis B vaccine, EngerixB\[8\], had no effect on the antigen-specific antibody levels elicited by an HBsAg-displayed antigen in mice\[46\]. This result supports the potential to develop multiple vaccine candidates based on the HBsAg VLP platform. In this design, attachment of the RBD to the HBsAg VLP was mediated by spontaneous conjugation of SpyTag and SpyCatcher peptides \[47\]. In addition to the demonstrated immunogenicity of this vaccine candidate in rhesus macaques\[12\], the modular design of this system could allow integration of new RBD antigens such as RBD-J6 to improve stability and manufacturability, or RBD-J6 β to address new VOCs such as Beta. We chose, therefore, to evaluate the immunogenicity of RBD-J β and RBD-J6 β conjugated with HBsAg VLPs. RBD-J β and RBD-J6 β were expressed in yeast, and each purified RBD antigen was conjugated onto HBsAg VLPs (Supplementary Fig. S3). We evaluated the antigenicity of each vaccine and observed similar binding and avidity to ACE2 as well as S309 antibody for both VLP – RBD-J β conjugate and VLP – RBD-J6 β conjugate (Supplementary Fig. S3).

We previously evaluated both monomeric RBD and multimeric VLP – RBD vaccine candidates with different adjuvants\[11,12\]. We sought here to evaluate the impact of the RBD-J6 mutations on the immunogenicity of RBD-based vaccines and minimize potential confounding effects of adjuvant-antigen interactions\[40\]. For these reasons, we formulated 10μg of VLP – RBD conjugate adjuvanted with 100μg of alum-only to compare the potential intrinsic differences from the altered sequences. We immunized K18-hACE2 transgenic mice\[48–50\] intramuscularly with three doses of the alum adjuvanted VLP – RBD conjugate at weeks 0, 3 and 5. As a positive control, a cohort of mice were immunized with two doses of 3 μg of Comirnaty mRNA (Pfizer-BioNTech) at weeks 0 and 3. At weeks 2, 5, and 7, we characterized the serological response against RBDS from several SARS-CoV-2 VOCs (Fig. 3a). After one dose (week 2), we observed significantly higher RBD-specific IgG titers across multiple VOCs in sera from mice immunized with the VLP – RBD-J6 β conjugate compared to sera from mice immunized with the VLP – RBD-J β conjugate (Fig. 3b, Supplementary Table 1). After three doses (week 7), the VLP – RBD-J6 β conjugate also elicited a significantly higher RBD-specific IgG response across multiple VOCs compared to the VLP – RBD-J β conjugate. Antibody titters elicited by the VLP – RBD-J6 β were not significantly different to titers elicited by two doses of Comirnaty\[8\] (Fig. 3c, Supplementary Table 2).

Next, at week 7, we challenged K18-hACE2 mice that received RBD-J β and RBD-J6 β vaccines with SARS-CoV-2 Alpha or Beta. We monitored weight loss and temperature for 11 days following challenge (Supplementary Fig. S4, S5). Four out of five mice immunized with VLP – RBD-J β or VLP – RBD-J6 β survived challenges from Alpha or Beta SARS-CoV-2, while most non-vaccinated mice were euthanized after becoming morbid following the Alpha (5/5) or Beta (4/5) challenges, respectively (Fig. 3d, e). All mice immunized with two doses of Comirnaty\[8\] survived Alpha or Beta challenges. We evaluated the titers of viral RNA in the lung and brain of challenged mice. For both the Alpha and Beta challenge, we observed significantly reduced viral RNA in the lung and brain cells in mice immunized with either the VLP – RBD-J β or RBD-J6 β conjugates compared to the unvaccinated and challenged control group (Fig. 4). All detected viral RNA in mice immunized with VLP – RBD-J β or VLP – RBD-J6 β were statistically comparable to titers observed in mice that were immunized with Comirnaty\[8\], except in the lungs of mice challenged with SARS-CoV-2 Alpha. A histopathological analysis of pulmonary tissue showed reduced chronic and acute inflammation throughout the lung parenchyma after SARS-CoV-2 Alpha or Beta challenges compared to unvaccinated, challenged mice (Supplementary Fig. S6).

Finally, we evaluated neutralizing activity of sera from mice challenged with Alpha and Beta variants against RBDS from SARS-CoV-2 VOC (in this assay, higher neutralizing activity is represented as lower area under the curve (AUC) of a dose–response curve between serum dilutions and electrochemiluminescence). No significant differences were observed in the sera neutralizing activity from mice immunized with either the VLP – RBD-J β or VLP – RBD-J6 β conjugates, and Comirnaty\[8\], after challenge with the Alpha variant (Fig. 3f). We also observed no significant differences in the neutralizing activity of sera from mice immunized with the VLP – RBD-J6 β conjugate and Comirnaty\[8\], after challenge with the Beta variant suggesting that the VLP – RBD-J6 β conjugate induces similar neutralizing potency across VOC RBDS compared to Comirnaty\[8\] (Fig. 3g). Sera from mice immunized with the VLP – RBD-J β conjugate and challenged with Beta, however, exhibited significantly lower neutralization of Wuhan, Alpha and Delta variants compared to sera from mice immunized with Comirnaty\[8\]. Lastly, we confirmed that the challenge itself did not induce a neutralizing response in mice (Supplementary Fig. S7).

3. Discussion

We previously described engineering the SARS-CoV-2 RBD to improve manufacturability, stability, and immunogenicity. Specifically, we reduced the predicted hydrophobicity of the receptor binding motif (RBM) in the engineered variant RBD-J11\[11\]. Here, we report a second-generation engineered variant, RBD-J6 (RBD-S383D-L452K-F490W-L518D), which further improves manufacturability and stability over the first-generation variant, RBD-J (RBD-L452K-F490W-L518D), due to two mutations that reduce the predicted hydrophobicity of a second hydrophobic patch on the core of the RBD subunit. Notably, in situ, this second hydrophobic patch is primarily exposed when the full S protein RBD is in the “up” position (Fig. 1a), and residues like S383 form hydrogen bonds with surrounding S trimer domains\[51,52\]. This report agrees with other studies that have also introduced mutations near the RBD core, leading to increased production and stability \[53,54\]. These results and the general strategy used may inform approaches to engineer other vaccine antigens and therapeutic proteins based on protein subunits that create new surface exposed hydrophobic patches when truncated from their full-length protein. Monoclonal antibodies are often designed and selected for reduced surface hydrophobicity during development, which has been shown to be a critical factor associated with clinical success\[55,56\].

We also evaluated the antigenicity of the RBD-J6 β variant and observed that binding to the target receptor ACE2 and several known neutralizing antibodies was not impacted by the addition of mutations in the RBD core hydrophobic patch relative to RBD-J β. Binding was only disrupted, however, for antibodies that targeted the mutated epitope. Notably, these antibodies that target the class IV epitope do not block binding of the RBD to the ACE2 receptor. These results suggest that the overall secondary and tertiary structure of the protein was not measurably impacted by the additional mutations. When we tested the RBD-J6 β antigen in a transgenic mouse model, we observed higher titers of antibodies that bind to several RBD variants compared to the RBD-J β antigen (Fig. 3). Likewise, the RBD-J6 β antigen elicited improved neutralizing activity of several SARS-CoV-2 VOC RBDS in comparison to the RBD-J β antigen (Fig. 3). This result demonstrates that the immunogenicity and antigenicity of the RBD-J β antigen are sufficient, despite modification of an epitope for previously identified neutralizing antibodies\[52\]. We hypothesize, furthermore, that the apparent breadth of antibody binding across RBD variants conferred by immunization with the RBD-J6 β antigen may be due to focusing of the immune response on other, possibly broadly neu-
The breadth that could be conferred by focusing on highly conserved epitopes is an active field of research with respect to SARS-CoV antigens and other pathogens [58–60]. We acknowledge that one limitation of the current study is that there was no direct comparison of the engineered variants of RBD to their corresponding ancestral sequences (used in current vaccines). While the results here emphasize the influence of the engineering changes in RBD-J6 β to the RBD-J β, this additional comparison to the ancestral sequences may have also informed the potential benefits of breadth afforded from the mutations intrinsic to the beta variant itself.

Over two rounds of rational engineering, we have now achieved a >10-fold increase in expression titer over the sequences for the ancestral RBD, and overall improvements to its biophysical stability [11]. Surprisingly, both rounds of rational engineering also improved the potency and breadth of the immune response to the RBD antigen compared to, first the native ancestral sequence [11], and now to RBD-J. Together, these improvements suggest a correlation exists among protein structure, biophysical properties, and antigenic response. Indeed, stabilization of protein-based vaccines has been reported to enhance MHC presentation and TH1 responses [61].

Mice challenged with Alpha and Beta variants of SARS-CoV-2 after immunization with protein subunit VLP-RBD conjugates had nearly complete protection, and the neutralizing activity elicited by three doses of the VLP – RBD-J6 β conjugate was comparable to two doses of Comirnaty®. Recent literature has demonstrated that individuals immunized with currently approved mRNA vaccines show a marked decline in anti-RBD IgG titers after several months [62,63], suggesting that vaccine boosters may be needed to maintain long-term immunity [64]. Several studies have reported using RBD as a booster for Spike protein primed
animals[65,66]. Future studies are merited to evaluate the use of this VLP-RBD candidate or other multimeric versions as a 1st or 2nd booster to an mRNA vaccination. We acknowledge that further optimization of a vaccine candidate based on the engineered immunogens tested here could benefit from the inclusion of one or more adjuvants, including CpG, SMNP, or others for enhancing immunogenicity [11,29,67].

When designing RBD-based vaccines, presentation of a variety of pan-sarbecovirus RBDs can elicit cross-neutralizing antibodies that target conserved epitopes, potentially withstanding the continuous mutation of the SARS-CoV-2 virus[68]. The ablation of a conserved RBD core epitope (like the one targeted by class IV antibodies in this RBD candidate) could also provide means of focusing an existing immune memory to more variable RBM epitopes with higher neutralizing rates[38,39].

To date, the optimal frequency of vaccine boosters for SARS-CoV-2 and, subsequently, the overall yearly global demand for vaccine doses are unknown[64]. Despite the increased access of boosters in developed countries, likely tens of billions of vaccine doses will need to be manufactured and distributed at low cost to maintain global long-term immunity, suppress the formation and spread of new VOCs, and reach all populations with limited access to healthcare[69,70]. We have demonstrated that the improved RBD antigen reported here is compatible with several vaccines designed for access in low- and middle-income countries[16]. We believe that the improved manufacturability, breadth, and stability of RBD-J6 could be incorporated into existing RBD-based vaccines like CorbeVax™[71], Soberana, Cuba’s RBD conjugate vaccine[72], and other candidates[73–75]. The enhancements to the production of RBD afforded by targeted protein engineering like those demonstrated here suggest another strategy towards developing accessible, low-cost, and effective RBD-based vaccines for sarbecoviruses[76].

4. Materials and methods

4.1. Strains

Recombinant genes for RBD variant expression were codon optimized for Komagataella phaffii expression, and synthesized cloned into a custom vector on a BioXP (Codex). Linear DNA was
purified, and constructs were transformed into a modified wild-type *K. phaffii* (NRRL Y-11430) as described previously[11,77].

4.2. Cultivations

Strains for titer evaluation of each RBD mutant were cultivated in 3 mL in 24-deep well plates as described previously[11]. Strains producing material for further analytical characterization and mice immunization were cultivated in 200 mL in 1L baffled flasks as described previously[11]. Cells were inoculated at 0.1 OD 600 growth for 24 h in complex media containing 4% glycerol, pelleted, resuspended, and grown for 24 h in complex media containing 40 g/L sorbitol and 1% methanol.

4.3. Protein purification

Purification of harvested cell culture supernatant containing the recombinant protein was performed using the downstream processing (DSP) module of InScyT as described previously[11,78]. The supernatant pH was adjusted inline to pH 5.0 with 100 mM citric acid. The supernatant was loaded into a 5 mL prepacked CMM HyperCel column (Sartorius AG, Gottingen, Germany). The column was re-equilibrated with 20 mM citric acid, pH 5.0, washed with 20 mM sodium phosphate pH 6.5, and elution was initiated with 20 mM sodium phosphate, 300 mM NaCl, pH 8.5. Eluate from column 1 above 20mAu was loaded into a 1 mL prepacked HyperCel STAR AX column (Sartorius AG, Gottingen, Germany). Flow-through material above 20mAu was collected.

4.4. Preparation of vaccine materials

Purified RBD-SpyTag was conjugated onto Hepatitis B surface antigen (HBsAg) SpyCatcher VLPs overnight at a 1.5:1 RBD to HBsAg molar ratio. Conjugated VLP-RBD was buffer exchanged and concentrated with 100 K molecular weight cutoff centrifugal filters. Materials were formulated with a 10 mM Histidine, 20 mM Sodium phosphate, 5 mM Tris, 37.5 mM NaCl, pH 7.4 buffer. VLP-RBD formulations were diluted to a final concentration of 100ug/mL. Alhydrogel (Invivogen) was added to a final concentration of 400ug/mL.

4.5. Reverse phase chromatography

Reverse phase high performance liquid chromatography (HPLC) for supernatant titer measurement was performed on an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA) using a PLRP-S column (2.1 x 150 mm, 300 Å, 3 μm) (Agilent Technologies, Santa Clara, CA) as described previously[11].

4.6. Far-UV circular dichroism (CD) spectroscopy

Far-UV CD spectroscopy was performed using a Chirascan-plus CD spectrometer (Applied Photophysics Ltd., Leatherhead, UK) equipped with a 6-cuvette position Peltier temperature controller (Quantum Northwest, Liberty Lake, WA) and a high-performance solid-state detector as described previously[11].

4.7. Static light scattering (SLS)

SLS measurements as a function of temperature were made in triplicate using a dual emission PTI QM-40 Spectrofluorometer (Horiba Scientific Northampton, UK) equipped with a 4-position cell holder Peltier temperature control device, a high-power continuous 75 W short-arc Xe lamp (Ushio), and a Hamamatsu R1527 photomultiplier tube as described previously[11].

4.8. Differential scanning calorimetry (DSC)

DSC was performed in duplicate using an auto-VP capillary differential scanning calorimeter (MicroCal/GE Health Sciences, Pittsburgh, PA) equipped with Tantalum sample and reference cells pressurized at ~ 60 psi with nitrogen as described previously[11].

4.9. Biolayer interferometry (BLI)

Biolayer interferometry was performed using the Octet Red96 with Protein A (ProA) biosensors (Sartorius ForteBio, Fremont, CA), which were hydrated for 15 min in kinetics buffer prior to each run. Kinetics buffer comprising 1X PBS pH 7.2, 0.5% BSA, and 0.05% Tween 20 was used for all dilutions, baseline, and dissociation steps. ACE2-Fc was loaded at a concentration of 10 μg/mL. Antibodies CB6, CV30, S309, CR3022, EY6A were loaded at a concentration of 2μg/mL. Samples were loaded in a 96-well black microplate (Greiner Bio-One, Monroe, NC) at starting concentrations of 15 μg/mL for ACE2-Fc binding and 10 μg/mL for antibody binding. Association and dissociation were measured at 1000 rpm for 300 and 600 s, respectively. Binding affinity was calculated using the Octet Data Analysis software v1.0 (Falk ForteBio), using reference subtraction, baseline alignment, inter-step correction, Savitzky-Golay filtering, and a global 1:1 binding model.

4.10. Animal welfare, Biosafety and ethics statements

B6.Cg-Tg(K18-ACE2)2Prlnm/J mouse vaccine and SARS-CoV-2 challenge studies were executed under IACUC protocol number 2009036460. All mice were humanely euthanized based on the disease scoring system[50], and no deaths occurred in the cage. All SARS-CoV-2 challenge studies were conducted in the West Virginia University Biosafety Laboratory Level 3 facility under the IBC protocol number 20–04–01. SARS-CoV-2 samples were either inactivated with 1% Triton per volume or Trizol before exiting high containment.

4.11. Mice immunization

Four week old B6.Cg-Tg(K18-ACE2)2Prlnm/J mice were purchased from Jackson Laboratory (stock no: 034860). Mice were first primed with the VLP-RBD vaccine at 9 weeks old, and boosted 3 and 5 weeks after. Mice immunized with Comirnaty were primed at 9 weeks old and boosted 3 weeks later. Mice were immunized intramuscularly with 50μL of vaccine formulation.

4.12. MSD serological assay for IgG titer measurement

Anti-RBD IgG was measured in mice serum on a MSD QuickPlex SQ120 following the SARS-CoV-2 Plate 11 Multi-Spot 96-well, 10 spot plate manufacturer’s protocol. Sera from unvaccinated mice collected at weeks 2 and 7 was diluted 1:1000. Sera from vaccinated mice collected two weeks post prime (week 2) was diluted 1:4000–1:512000, and vaccinated sera collected two weeks post 2nd booster (week 7) was diluted 1:32000–1:409600.

4.13. SARS-CoV-2 challenged serum was analyzed using the SARS-CoV-2 Plate 11 Multi-Spot 96-well, 10 spot plate following the manufacturer protocol (catalog #: K15458U-2) on the MSD QuickPlex SQ120. The 10 spots contained the following RBD antigens, common designations, and lineages: 1) Epsilon - L452R (B.1.427; B.1.429; B.1.526.1) 2) Beta - K417N, E484K, N501Y (B.1.351; B.1.351.1) 3) Eta, Iota, Zeta - E484K (B.1.525; B.1.526; B.1.618; B.1.711; 4) Gamma - K417T, E484K, N501Y (P.1) 5) New York -
4.14. SARS-CoV-2 propagation and mouse challenge

Alpha (NR-54000) and Beta (NR-54008) SARS-CoV-2 variants were obtained from BEI Resources. Alpha and Beta VOC were propagated in Vero E6 cells (ATCC-CRL-1586) and re-sequenced before use in mouse challenge. K18-hACE2 mice were anesthetized using an intraperitoneal injection of ketamine (Patterson Veterinary 07–803-6637, 80 mg/kg) / xylazine (07–808-1947, 8.3 mg/kg) and were intranasally challenged with 50 µL of 104 PFU/dose of Alpha or Beta variant, 25 µL per nare. Mice were monitored until awake.

4.15. Disease monitoring of SARS-CoV-2 challenged mice

Challenged K18-hACE2 mice were evaluated daily through both in-person health assessments in the BSL3 and SwifTAG Systems video monitoring for 11 days. Disease assessments of the mice were scored based on five criteria: 1) weight loss (scale 0–5), 2) appearance (scale 0–2), 3) activity (scale 0–3), 4) eye closure (scale 0–2), and 5) respiration (scale 0–2) [50]. All five criteria were scored based off a scaling system where 0 represents no symptoms and the highest number on the scale denotes the most severe disease phenotypes. Additive disease scores of the five criteria were assigned to each mouse after evaluation. Mice that scored an additive disease score of 5 or above among all 5 criteria, or weight loss of 20% or greater during the disease assessment required immediate euthanasia. Cumulative disease scoring was calculated by adding the disease scores of each mouse from each group. Morbid mice that were euthanized during the study, before day 11, retained their disease score for the remainder of the experiment [79].

4.16. Euthanasia and tissue collection

Challenged mice that were assigned a disease score of 5 or above or reached the end of the experiment were euthanized with an IP injection of Euthasol (390 mg/kg) (Pentobarbital) followed by secondary measure of euthanasia with cardiac puncture. Blood from cardiac puncture was collected in BD Microtainer gold serum separator tubes (BD 365967), centrifuged at 15,000 × g for 5 min and serum was collected for downstream analysis. Lungs were separated into right and left lobes. Right lobe of the lung was homogenized in 1 mL of PBS in gentleMACS C tubes (order number: 130–096-334) using the m_lung_02 program on the gentleMACS Dissociator. 300 µL of lung homogenate was added to 1000 µL of TRI Reagent (Zymo research) for downstream RNA purification and 300 µL of lung homogenate was centrifuged at 15,000 × g for 5 min and the lung supernatant was collected for downstream analyses. Brain was excised from the skull and was homogenized in 1 mL PBS in gentleMACS C tubes using the same setting as lung on the gentleMACS Dissociator. 500 µL of TRI Reagent was added to 1000 µL of brain homogenate for RNA purification.

4.17. SARS-CoV-2 viral RNA analysis of lung and brain by q-RT-PCR

RNA purification of the lung and brain were performed using the Direct-zol RNA miniprep kit (Zymo Research R2053) following the manufacturer protocol. SARS-CoV-2 copy numbers were assessed through qPCR using the Applied Biosystems TaqMan RNA to CT One Step Kit (Ref: 4392938), as described previously [79].

4.18. Lung histopathology

Left lobes of lungs were fixed in 10 mL of 10% neutral buffered formalin. Fixed lungs were paraffin embedded into 5 µm sections. Sections were stained with hematoxylin and eosin (H&E) and were analyzed by iHisto. Lungs were scored by a pathologist for chronic and acute inflammation in the lung parenchyma, blood vessels, and airways, as described previously [79].

5. Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Author contributions

S.R.A., N.C.D., and J.C.L. conceived and planned experiments. N.C.D. and R.S.J. generated and characterized yeast strains. S.R.A. and C.A.N performed HPLC assays. S.R.A. designed and performed protein purifications. S.B., K.K. and O. K. performed biophysical characterization. S.R.A performed antigenic characterization by BLI, H.D.R, M.P.R, R.R.L, U.S.S, S.B., R.C., G.N., and M.R. produced, purified and validated the HBsAg VLP material. N.C.D. and S.R.A. formulated and analyzed samples for animal studies. T.Y.W., B.P., R., K.S.L., H.A., and M.B. designed and performed mice immunizations, challenge and monitoring. T.Y.W. performed RBD ELISA assays. S.R.A., N.C.D., T.Y.W. and J.C.L. wrote the manuscript. J.C.L., F.H.D., D.B.V., S.B.J., J.R.B., S.B., and H.K. designed the experimental strategy and reviewed analyses of all authors reviewed the manuscript.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Sergio Andre Rodriguez-Aponte, Neil Chandra Dalvie, and J. Christopher Love have filed a patent related to the RBD-J sequence. J. Christopher Love has interests in Sunflower Therapeutics PBC, Honeycomb Biotechnologies, OneCyte Biotechnologies, QuantumCyte, and Repligen. J.C.L.’s interests are reviewed and managed under MIT’s policies for potential conflicts of interest. Harish D. Rao, Meghraj P. Rajurkar, Rakesh R. Lothe, Umesh S. Shaligram, Saurabh Batwal, Rahul Chandrasekaran, Gaurav Nagar are employees of Serum Institute of India Pvt. Ltd. Sumi Biswas is an employee of SpyBiotech Limited.

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