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Elicitation of broadly protective sarbecovirus immunity by receptor-binding domain nanoparticle vaccines

Graphical abstract

Highlights
- RBD-NP enables dose-sparing and protects mice after only one dose
- RBD-NP elicits diverse polyclonal antibody responses in non-human primates
- RBD-NP and HexaPro confer similar resilience to antigenic drift
- Mosaic and cocktail RBD-NPs protect against heterotypic SARS-CoV challenge

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In brief
A clinical stage multivalent SARS-CoV-2 spike receptor-binding domain nanoparticle (RBD-NP) vaccine is protective in mice after a single immunization and elicits strong antibody responses across circulating mutants and some sarbecoviruses. Multivalent sarbecovirus RBD-NP vaccines elicit heterotypic protection against sarbecoviruses.
Elicitation of broadly protective sarbecovirus immunity by receptor-binding domain nanoparticle vaccines

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https://doi.org/10.1016/j.cell.2021.09.015

SUMMARY

Understanding vaccine-elicited protection against SARS-CoV-2 variants and other sarbecoviruses is key for guiding public health policies. We show that a clinical stage multivalent SARS-CoV-2 spike receptor-binding domain nanoparticle (RBD-NP) vaccine protects mice from SARS-CoV-2 challenge after a single immunization, indicating a potential dose-sparing strategy. We benchmarked serum neutralizing activity elicited by RBD-NPs in non-human primates against a lead prefusion-stabilized SARS-CoV-2 spike (HexaPro) using a panel of circulating mutants. Polyclonal antibodies elicited by both vaccines are similarly resilient to many RBD residue substitutions tested, although mutations at and surrounding position 484 have negative consequences for neutralization. Mosaic and cocktail nanoparticle immunogens displaying multiple sarbecovirus RBDs elicit broad neutralizing activity in mice and protect mice against SARS-CoV challenge even in the absence of SARS-CoV RBD in the vaccine. This study provides proof of principle that multivalent sarbecovirus RBD-NPs induce heterotypic protection and motivates advancing such broadly protective sarbecovirus vaccines to the clinic.
INTRODUCTION

The emergence of SARS-CoV-2 in late 2019 resulted in the COVID-19 pandemic that brought the world to a standstill. Moreover, the recurrent spillovers of coronaviruses in humans along with detection of SARS-CoV-2, SARS-CoV-, and MERS-CoV-related coronaviruses in bats suggest that future zoonotic transmission events may continue to occur (Menachery et al., 2015, 2016; Zhou et al., 2020a). SARS-CoV-2 infects host cells through the attachment of the viral transmembrane spike (S) glycoprotein to angiotensin-converting enzyme 2 (ACE2), followed by fusion of the viral and host membranes (Hoffmann et al., 2020; Lan et al., 2020; Letko et al., 2020; Shang et al., 2020; Walls et al., 2020a; Wang et al., 2020; Wrapp et al., 2020; Yan et al., 2020; Zhou et al., 2020b). The SARS-CoV-2 S protein is the primary target of neutralizing antibodies (Abs), and the immunodominant receptor-binding domain (RBD) accounts for more than 90% of the neutralizing activity in COVID-19 convalescent sera and vaccinated individuals (Grenaney et al., 2021a, 2021b; Piccoli et al., 2020). Numerous monoclonal Abs (mAbs) recognizing distinct antigenic sites on the RBD were isolated and shown to neutralize viral entry and protect small animals and non-human primates (NHPs) from SARS-CoV-2 challenge (Barnes et al., 2020; Baum et al., 2020a; Brouwer et al., 2020; Piccoli et al., 2020; Pinto et al., 2020; Rogers et al., 2020; Starr et al., 2021; Tortorici et al., 2020; Wec et al., 2020; Zost et al., 2020). As a result, SARS-CoV-2 S is the focus of nucleic acid, vectored, and protein subunit vaccines currently being developed and deployed (Corbett et al., 2020a, 2020b; Jackson et al., 2020; Mercado et al., 2020; Polack et al., 2020; Tostanoski et al., 2020; Yu et al., 2020).

Worldwide sequencing of SARS-CoV-2 clinical isolates has led to the identification of numerous mutations in the >1,500,000 genome sequences available to date (https://www.gisaid.org). The SARS-CoV-2 S D614G mutation has become globally dominant and is associated with enhanced viral transmission and replication but does not significantly affect Ab-mediated neutralization (Hou et al., 2020; Korber et al., 2020; Plante et al., 2020; Yurkovetskiy et al., 2020). Conversely, some mutations found in circulating SARS-CoV-2 isolates were shown to promote escape from mAbs and to reduce neutralization by immune sera (Baum et al., 2020b; Collier et al., 2021; Li et al., 2020; McCallum et al., 2021a, 2021b; Wang et al., 2021a; Weisblum et al., 2020; Wibmer et al., 2021). As a result, formulation of mAb cocktails or the use of mAbs targeting conserved epitopes and neutralizing a broader spectrum of circulating SARS-CoV-2 variants emerged as a promising strategy to overcome this issue (Baum et al., 2020b; Cathcart et al., 2021; Dong et al., 2021; Greaney et al., 2020; Jette et al., 2021; Martinez et al., 2021a; Pinto et al., 2020; Tortorici et al., 2020, 2021). The recent emergence of several variants and variants of concern (VOCs) with numerous S mutations is especially worrisome, including B.1.1.7 (alpha), B.1.351 (beta), B.1.427/B.1.429 (epsilon), P.1 (gamma), and B.1.617.2 (delta) that originated in the UK, South Africa, the USA, Brazil, and India, respectively (Davies et al., 2020; Deng et al., 2021; Faria et al., 2021; McCallum et al., 2021b; Tegally et al., 2020). Some of these mutations lead to significant reductions in the neutralization potency of N-terminal domain (NTD)- and RBD-specific mAbs, convalescent sera, and Pfizer/BioNTech BNT162b2- or Moderna mRNA-1273-elicited sera (Collier et al., 2021; McCallum et al., 2021a, 2021b; Wang et al., 2021a).

We recently described a multivalent subunit vaccine displaying the SARS-CoV-2 RBD nanoparticle (RBD-NP) in a highly immunogenic array using a computationally designed self-assembling protein nanoparticle (Bale et al., 2016; Walls et al., 2020b). Vaccination with RBD-NP resulted in 10-fold higher neutralizing Ab titers in mice than the prefusion-stabilized S2P trimer (which is used in most current vaccines) despite a 5-fold lower dose and protected mice against mouse-adapted SARS-CoV-2 (SARS-CoV-2-MA) challenge (Dinnon et al., 2020; Walls et al., 2020b). We demonstrated that the RBD-NP vaccine elicited robust neutralizing Ab and CD4 T cell responses in NHPs when formulated with several clinic-ready adjuvants and conferred protection against SARS-CoV-2 infection in the nose, pharynges, and bronchioles (Arunachalam et al., 2021). The RBD-NP vaccine is currently being evaluated in two phase I/II clinical trials (ClinicalTrials.gov: NCT04742738 and NCT04750343) and recently received funding from the coalition for epidemic preparedness innovations for phase III clinical trials.

Although the S fusion machinery (S2 subunit) has higher sequence conservation than the RBD (Pinto et al., 2021; Sauer et al., 2021; Tortorici and Veesler, 2019; Walls et al., 2016, 2020a), the breadth of neutralization and protection provided by RBD-based vaccines remains unknown. The isolation of RBD-specific cross-reactive mAbs neutralizing SARS-CoV-2 and SARS-CoV suggests that RBD-based vaccines could, in principle, elicit Abs that neutralize distantly related sarbecoviruses, which have future pandemic potential (Cathcart et al., 2021; Jette et al., 2021; Martinez et al., 2021a; Pinto et al., 2020; Rappazzo et al., 2021; Starr et al., 2021; Tortorici et al., 2021; Wec et al., 2020). RBD-based vaccines are also unaffected by S mutations outside of the RBD, especially in the highly variable NTD that is heavily mutated in many VOCs (Andreano et al., 2020; Avanzato et al., 2020; Choi et al., 2020; Collier et al., 2021; Wang et al., 2021a; McCallum et al., 2021a, 2021b; McCarthy et al., 2021). Here, we explored dose-sparing strategies for the RBD-NP vaccine and evaluated the impact of genetic diversity among SARS-CoV-2 clinical isolates and sarbecoviruses on vaccine-elicited Abs. We further designed mosaic and cocktail sarbecovirus RBD-NPs that elicit broad and protective Ab responses against heterologous sarbecovirus challenge, which could represent the next generation of pan-sarbecovirus vaccines.

RESULTS

Dose-sparing RBD-NP vaccination protects mice against SARS-CoV-2 challenge

Considering the unprecedented need for rapid global distribution of SARS-CoV-2 vaccines, we investigated the ability of RBD-NP to induce neutralizing and protective Ab titers at lower doses than previously evaluated. Specifically, we set out to test whether vaccine-elicited neutralizing Ab titers are altered by lowering the immunogen dose 10-fold (two 0.1 μg immunizations, RBD antigen dose) or reducing the number of doses...
(a single 1 μg immunization, RBD antigen dose) compared to our initial work (Walls et al., 2020b). To test this, BALB/c cByJ mice were immunized intramuscularly at week 0 with either 0.1 μg (ten mice) or 1 μg (two groups of eight mice or sixteen mice total) of AddaVax-adjuvanted RBD-NP. Two weeks post-prime, serum binding and neutralizing Ab titers were roughly comparable for the two vaccine doses (neutralization geometric mean titer [GMT] ~2–4 × 10^2) measured using a mouse leukemia virus (MLV) pseudotyping system (Millet and Whittaker, 2016; Walls et al., 2020a) (Figures 1A and 1B; Data S1). Three weeks post-prime, we boosted the mice that were immunized with 0.1 μg of the vaccine and eight of the sixteen mice immunized with 1 μg of the vaccine with the same respective dose. Two weeks post-boost, serum binding and neutralizing Ab titers were comparable for the 0.1 μg and 1 μg groups (neutralization GMT ~2–4 × 10^2) (Figures 1C and 1D; Data S1), suggesting that a 10-fold reduction in the RBD-NP dose does not affect serum neutralizing responses. Furthermore, we observed that the magnitude of binding and neutralizing Ab titers increased over time for the mice that received a single immunization (neutralization GMT 2–3 × 10^3). These results suggest that lowering the vaccine dose or reducing the number of immunizations represent possible dose-sparing strategies that did not compromise elicitation of high levels of neutralizing Ab titers using RBD-NPs.

To further evaluate vaccine efficacy, eight mice that received one dose and eight mice that received two doses of the 1-μg RBD-NP vaccine along with six unvaccinated mice were challenged ten weeks post-prime with 10^5 plaque-forming units (PFUs) of mouse-adapted SARS-CoV-2 MA10 (Leist et al., 2020). The SARS-CoV-2 MA10 model causes weight loss and lung pathology when 10^5 PFUs (the highest titer tested [Leist et al., 2020]) are used in young BALB/c mice. The mice were followed for four days to assess protection from disease. All mice in the RBD-NP-vaccinated groups were protected from weight loss throughout the duration of the experiment regardless of the number of doses, whereas control mice lost ~10% of their weight by day four (Figure 1E). Analysis of lung pathology and viral titers in lung tissues indicated that the vaccinated mice were not affected by SARS-CoV-2 MA10 challenge, whereas the control mice showed lung discoloration four days post-infection and a high viral load (~1 × 10^4 PFUs) (Figures 1F and 1G). These results indicate that one or two immunizations with 1 μg RBD-NPs results in protection against SARS-CoV-2 MA10-induced disease. Furthermore, mice vaccinated twice with a 10-fold lower dose of RBD-antigen (0.1 μg) had higher
serum neutralizing Ab titers than mice receiving a single 1-μg dose, suggesting that they would likely also be protected since neutralizing Ab titers are a correlate of protection against SARS-CoV-2 (Arunachalam et al., 2021; McMahan et al., 2021). These data suggest that multiple RBD-NP dose-sparing regimens may be possible.

RBD-NP vaccination induces Abs targeting diverse antigenic sites in NHPs

To characterize the epitopes targeted by RBD-NP-elicited polyclonal Abs, we used deep mutational scanning (DMS) with sera obtained 56 days post-prime from one of two pigtail macaques immunized at days 0 and 28 with a dose of AddaVax-adjuvanted RBD-NP containing 88 μg of the SARS-CoV-2 RBD (with a linker of twelve glycine/serine (GS) residues between the RBD and the NP) (Walls et al., 2020b)( Figures 2A and 2B). These experiments rely on yeast surface display of RBD libraries covering nearly all possible amino acid mutations coupled with fluorescence-activated cell sorting (FACS) to identify RBD mutants with attenuated Ab binding compared to the wild-type (Wuhan-Hu-1) SARS-CoV-2 RBD (Greaney et al., 2020; Starr et al., 2020a). No single mutation had more than a marginal effect on serum Ab (IgG/IgM/IgA) binding, indicating broad targeting of distinct RBD epitopes, whereas several previously described COVID-19 human convalescent plasmas (HCPs) analyzed by DMS displayed greater sensitivity to individual mutations and are shown for comparison (Greaney et al., 2021b)( Figures 2B and S1; Table S1). These results show that RBD-NP vaccination elicits highly diverse polyclonal Ab responses that target multiple distinct antigenic sites and are more resilient to escape mutations reducing polyclonal Ab binding than HCP (Barnes et al., 2020; Greaney et al., 2021b; Piccoli et al., 2020; Starr et al., 2021).

To measure the magnitude of vaccine-elicited Abs against distinct RBD antigenic sites, we used quantitative competition ELISAs with human ACE2-Fc (hACE2-Fc), which binds to the receptor-binding motif (RBM) corresponding to antigenic sites Ia and Ib, as well as with structurally characterized mAbs recognizing antigenic sites II (CR3022) and IV (S309) (Huo et al., 2020; Piccoli et al., 2020; Pinto et al., 2020; Yuan et al., 2020)( Figures 2C–2E; Data S1). These experiments

Figure 2. RBD-NP vaccination elicits high titers of Abs targeting diverse RBD antigenic sites in NHPs

(A) NHP study designs.
(B) Effect of RBD mutations on polyclonal Ab binding measured by DMS analysis of serum obtained 8 weeks post-prime from an immunized pigtail macaque (n = 1) compared to a previously reported DMS measurement from a representative COVID-19 HCP sample (reproduced here for comparison; Greaney et al., 2021b). The line plots on the left show the summed effect of all

mutations at a site in the RBD on serum or plasma binding, with RBD residues on the x axis and Ab escape on the y axis (Table S1). Due to the use of sample-specific FACS gates (Figure S1), the y axes are scaled independently. Sites in the logo plots are colored dark blue if located in the receptor-binding ridge or cyan if located in the RBD 443–450 loop. Larger values indicate more Ab escape.

(C–E) Competition ELISA between 0.2-nM hACE2-Fc (LOD of 2 × 10⁻⁷) (C), 2-nM CR3022 mAb (LOD of 2 × 10⁻⁷) (D), or 0.01-nM S309 mAb (LOD of 4 × 10⁻⁷) (E), and RBD-NP-elicited sera in pigtail macaques (n = 2), RBD-NP-elicited sera in rhesus macaques (n = 5), or HexaPro S-elicited sera in rhesus macaques (n = 5) at various time points following vaccination, benchmarked against HCP (n = 4) (Table S2). Each plot shows the magnitude of inhibition of hACE2/mAb binding to immobilized SARS-CoV-2 S2P, expressed as reciprocal serum dilution blocking 50% of the maximum binding response. Statistical significance was determined by Kruskal-Wallis test and shown only when significant. **p < 0.01. LODs are shown as gray horizontal dotted lines. Raw data curves shown in Data S1.
used sera from the aforementioned pigtail macaques (RBD GMT EC_{90} 5 × 10^5) followed for 168 days and sera from 5 rhesus macaques immunized twice on days 0 and 21 with AS03-adjuvanted RBD-NPs containing 25 μg of the SARS-CoV-2 RBD (with a 16-GS linker between the RBD and the NP; RBD GMT EC_{90} 1 × 10^5) and followed for 98 days (Arunachalam et al., 2021; Walls et al., 2020b; Figures 2A and S2A). These two sets of RBD-NP sera were compared to sera from 5 rhesus macaques immunized twice on days 0 and 21 with 12.5 μg of AS03-adjuvanted prefusion-stabilized HexaPro S trimer (RBD EC_{90} 1 × 10^5) and followed for 42 days (Arunachalam et al., 2021; Hsieh et al., 2020), as well as a panel of COVID-19 HCP (Table S2) (RBD EC_{90} 2 × 10^3) (Figures 2A and S2A). All NHP sera post boost had high titers of Abs targeting sites Ia and Ib in the immunodominant RBM (day 42 hACE2 competition RBD-NP GMT 3 × 10^3 and HexaPro GMT 7 × 10^3) (Figure 2C; Data S1), a correlate of neutralization potency (Piccoli et al., 2020), in agreement with the potent immunogenicity and protective efficacy of RBD-NP and HexaPro S in NHPs (Arunachalam et al., 2021). We also observed strong Ab responses against antigenic sites II (RBD-NP day 42 GMT 4 × 10^2 and HexaPro GMT 4 × 10^2) with CR3022 competition and IV (RBD-NP day 42 GMT 2.5 × 10^3 and HexaPro GMT 6.5 × 10^3) (Figures 2D and 2E; Data S1), which comprise conserved sarbecovirus epitopes recognized by broadly neutralizing sarbecovirus mAbs such as S2X259 (site II) and S309 (site IV) (Piccoli et al., 2020; Pinto et al., 2020; Tortorici et al., 2021). Ab responses were durable against all 3 antigenic sites regardless of dose or adjuvant, with a decrease of competition titers by roughly half an order to one order of magnitude 168 days (pigtail macaque) or 98 days (rhesus macaque) post-prime. RBD-NP elicited much higher peak binding titers toward all RBD antigenic sites evaluated compared to HexaPro S and HCP, showdown the potency, durability, and multi-specificity of Ab responses induced by multivalent display of the RBD, possibly as a result of enhanced accessibility of the antigen.

**RBD-NP vaccine elicits potent neutralizing Ab responses in NHPs against a panel of SARS-CoV-2 S variants**

To assess the neutralization breadth of RBD-NP- and HexaPro S-elicited Abs in NHPs at peak titers (42 days post-prime), we evaluated serum neutralizing activity against a panel of pseudo-typed viruses comprising wild-type (D614G) SARS-CoV-2 S and twelve single-residue SARS-CoV-2 RBD mutants detected in clinical isolates (N439K, L452R, Y453F, L455F, S477N, E484A/K/Q, F486L, S494P, N501Y, and G504D) as well as the B.1.1.7 (H69-V70 deletion, Y144 deletion, E444K, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H); B.1.1.7 + E484K (H69-V70 deletion, Y144 deletion, E444K, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H); B.1.351 (L18F, D80A, D215G, L242-L244 deletion, K417N, E444K, N501Y, D614G, H655Y, T1027I); and P.1 (L18F, T20N, P26S, D138Y, R190S, K417T, E444K, N501Y, D614G, H655Y, T1027I). Several single-residue RBD mutations did not affect neutralizing titers compared to wild-type D614G SARS-CoV-2 S, for which GMTs of 1.8 × 10^3 and 2.4 × 10^3 were determined with RBD-NP- and HexaPro S-elicited sera, respectively, using a vesicular stomatitis virus (VSV) pseudotyped virus (Figure 3A; Data S1). For example, the N439K- (GMT 2.5 × 10^3 for RBD-NP and 2.7 × 10^3 for HexaPro S) and mink-associated Y453F (GMT 4 × 10^3 for RBD-NP and 5 × 10^3 for HexaPro S) mutation did not dampen the neutralization potency of either RBD-NP- or HexaPro S-elicited sera (Figure 3A; Data S1). However, the N501Y substitution (present in the B.1.1.7, P.1, and B.1.351 lineages) reduced the neutralization potency of RBD-NP- (GMT 5 × 10^2) and HexaPro S (GMT 8 × 10^2-) elicited sera 3-to-4-fold and the L452R mutation (present in the B.1.427/B.1.429, B.1.617.1, and B.1.617.22 variant) led to a 3.4-to-4.4-fold drop in neutralization potency (GMT 4 × 10^2 for RBD-NP and 7 × 10^2 for HexaPro S). These substitutions have been associated with the loss of binding and neutralization for vaccine-elicited Abs and mAbs (Collier et al., 2021; McCallum et al., 2021b; Wang et al., 2021b; Thomson et al., 2021; Wang et al., 2021c). The E484A mutation reduced serum neutralization activity up to 2-fold (GMT 1.1x10^3 for RBD-NP and 1.1x10^3 for HexaPro S) whereas E484K, present in B.1.351 and P1 (GMT 2 × 10^2 for RBD-NP and 2 × 10^2 for HexaPro S), and E484Q, found in B.1.617.1 (GMT 5 × 10^2 for RBD-NP and 3.6 × 10^2 for HexaPro S), decreased it 9-fold (E484K) and 4-fold (E484Q) for RBD-NP and 12-fold (484K) and 7-fold (484Q) for HexaPro S compared to D614G SARS-CoV-2 S). These experiments suggest that a substantial fraction of the neutralizing activity elicited by both RBD-NP and HexaPro S is focused on the RBM, especially near position 484, as further supported by the ~5-fold decrease in neutralization resulting from the F486L (GMT 3.7 × 10^2 for RBD-NP and 5 × 10^2 for HexaPro S) substitution.

To understand the impact of the full constellation of mutations present in the S proteins of the aforementioned VOCs, we evaluated RBD-NP- and HexaPro S-elicited serum neutralizing activity against corresponding HIV and VSV pseudotyped variants (D614G GMT RBD-NP HIV 5 × 10^3 and VSV RBD-NP 6 × 10^2, respectively, and D614G GMT HexaPro S HIV 3.5 × 10^2 and VSV 1.2 × 10^3) (Figures 3B and 3C). Although we did not observe major reductions in neutralization titers (up to to 2-fold) toward the B.1.1.7 VOC (GMT RBD-NP HIV 2.5 × 10^2 and VSV 4.7 × 10^2 and HexaPro S HIV 1.8 × 10^2, VSV 8.8 × 10^2) (Figures 3B and 3C), addition of the E484K mutation to the B.1.1.7 background reduced neutralization 4-to-6-fold for RBD-NP- (GMT HIV 8.8 × 10^2) and HexaPro S-elicited sera (GMT HIV 8.6 × 10^2), confirming the importance of the 484 position (Figure 3B). Neutralization of B.1.351 was 10-fold lower using HIV pseudovirus (GMT RBD-NP 5 × 10^2 and HexaPro S 3.6x10^2) (Figure 3B) and ~3-fold lower using VSV pseudovirus (GMT RBD-NP 2 × 10^2 and HexaPro S 4x10^2) (Figure 3C) for both RBD-NP- and HexaPro-elicited sera, in agreement with authentic virus neutralization data (Arunachalam et al., 2021). Interestingly, RBD-specific binding Ab titers did not differ significantly between the B.1.351 RBD (GMT RBD-NP 6.5 × 10^3 and HexaPro S 3 × 10^3) and Wuhan-Hu-1 RBD (GMT RBD-NP 1.4 × 10^3 and HexaPro S 1.7 × 10^4) (Figure S2A), indicating that neutralizing activity is accounted for by a portion of total binding Abs. Reductions in neutralizing Ab titers were also observed against P.1 S VSV pseudoviruses, with a 6-fold drop for RBD-NP (GMT 1 × 10^3) and 8-fold dampening for HexaPro S (GMT 1.5 × 10^3) (Figure 3C). Furthermore, we observed a 10-fold reduction in plasma...
neutralizing potency for individuals who received two doses of the Pfizer-BioNTech BNT162b2 mRNA vaccine against B.1.351 S pseudotyped virus (GMT 6 × 10^3) relative to the wild-type (D614G) SARS-CoV-2 S (GMT 6 × 10^2) pseudotyped virus (Figure 3B; Data S1). These findings show that, as is the case for many convalescent individuals (Greaney et al., 2021b; Liu et al., 2021; Piccoli et al., 2020), an important fraction of vaccine-elicited neutralizing Abs in NHPs and humans is focused on the RBM (specifically around position 484), independently of the immunogen (RBD-NP, HexaPro S, or 2P-stabilized S [Pallesen et al., 2017]) or the vaccine modality (protein subunit or mRNA). However, we note that neutralization assays may underestimate the contribution of NTD-specific or non-RBM RBD-targeted neutralizing Abs (Lempp et al., 2021; McCallum et al., 2021a; Suryadevara et al., 2021), and protection from challenge will be the ultimate readout.

To further investigate the relationships between neutralizing Ab titers and emerging SARS-CoV-2 variants, we immunized the two pigtail macaques a third time with RBD-NP, formulated with an oil-in-water (O/W) emulsion-based adjuvant, 168 days after the primary immunization (Figure 2A). This boost induced potent serum neutralizing activity against wild-type (D614G) SARS-CoV-2 S, B.1.1.7, and B.1.351 S in rhesus macaques (gray) with an LOD of 2.5 × 10^5. Neutralization performed twice and a representative shown.

Statistical significance was determined by Kruskal-Wallis test and shown in Table S3. LODs are shown as gray horizontal dotted lines. Raw data curves shown in Data S1 and GMTs in Table S4. The various pseudovirus backbones were benchmarked against NIBSC standard and are shown in Table S5.

Figure 3. RBD-NP and HexaPro S elicit Abs with similar neutralization breadth toward SARS-CoV-2 variants

(A) Neutralizing Ab titers against wild-type (D614G) SARS-CoV-2 S and B.1.1.7 S (GMT 6 × 10^3) pseudotyped virus with an LOD of 3.3 × 10^3. Neutralization performed once.

(B) Neutralizing Ab titers against wild-type (D614G) SARS-CoV-2 S, B.1.1.7 S, B.1.351 S, or P.1 S, determined using RBD-NP-elicited sera in rhesus macaques (blue) or HexaPro S-elicited sera in rhesus macaques (gray) with an LOD of 3.3 × 10^3. Neutralization performed twice and a representative shown.

(C) Neutralizing Ab titers against wild-type (D614G) SARS-CoV-2 S, B.1.1.7 S, B.1.351 S, or P.1 S, determined using HexaPro S-elicited sera in rhesus macaques (gray) or plasma from individuals who received two doses of Pfizer mRNA vaccine (open circles) with an LOD of 3.3 × 10^3. Neutralization performed twice and a representative shown.

(D) Neutralizing Ab titers against wild-type (D614G) SARS-CoV-2 S, B.1.1.7, and B.1.351 S in rhesus macaques (gray) with an LOD of 3.3 × 10^3. Neutralization performed twice and a representative shown.

(E) Neutralizing Ab titers against wild-type (D614G) SARS-CoV-2 S, B.1.1.7, and B.1.351 S in rhesus macaques (gray) with an LOD of 3.3 × 10^3. Neutralization performed twice and a representative shown.

(F) Neutralizing Ab titers against wild-type (D614G) SARS-CoV-2 S, B.1.1.7, and B.1.351 S in rhesus macaques (gray) with an LOD of 3.3 × 10^3. Neutralization performed twice and a representative shown.

Neutralization performed twice and a representative shown. Statistical significance was determined by Kruskal-Wallis test and shown in Table S3.
SARS-CoV-2 (GMT $2 \times 10^3$) (Figure 3D) as well as the B.1.1.7 (GMT $2 \times 10^3$) and B.1.351 (GMT $8 \times 10^3$) VOC pseudoviruses, suggesting that an overall increase in neutralizing Ab titers may be a suitable strategy to cope with emerging variants. These results are consistent with recent studies demonstrating that boosting COVID-19 convalescent individuals with a single mRNA vaccination elicited high (neutralizing) Ab titers, including against the B.1.351 variant (Abu Jabal et al., 2021; Krämer et al., 2021; Stamatakis et al., 2021), and suggest that a third vaccination of naive individuals could be a suitable strategy to limit the impact of emerging variants. This strategy is currently being evaluated for immunocompromised patients in several countries.

**RBD-NP vaccine elicits cross-reactive sarbecovirus polyclonal Abs in NHPs**

As RBD-NP- and HexaPro S-elicited polyclonal Abs exhibited similar resilience to a range of mutations, we next investigated cross-reactivity with a panel of sarbecovirus RDVs. RBD-NP-elicited polyclonal Abs purified from pigtail macaque serum obtained 70 days post-prime strongly cross-reacted with the SARS-CoV-2-related Pangolin-GD and RaTG13 RDVs and bound more weakly to distantly related RmYN02, SARS-CoV-WIV16, and ZXC21 RDVs (Figures S2B and S2C). Measurement of Ab binding titers using the SARS-CoV and SARS-CoV-2 S2P ectodomain trimers by ELISA showed that RBD-NPs and HexaPro S induced similar levels of cross-reactive Abs against each antigen, with responses roughly two orders of magnitude higher against SARS-CoV-2 S2P (detection antigen matching immunogen) compared to SARS-CoV S2P (Figure S2D; Data S1). The two immunogens also elicited similar peak levels of SARS-CoV S ACE2-competing Abs (GMT $2 \times 10^2$ RBD-NP and $5 \times 10^2$ HexaPro) suggesting that cross-neutralizing Abs might have been induced to similar extents between the two vaccines (Figure S2E).

Motivated by the cross-reactivity of RBD-NP-elicited polyclonal Abs with various sarbecovirus RDVs and the correlation between ACE2 competition and serum neutralization titers (Piccoli et al., 2020), we evaluated neutralization of a panel of HIV (D614G GMT 5.5 $\times 10^2$) and VSV (D614G GMT 6 $\times 10^2$) pseudoviruses harboring sarbecovirus S glycoproteins. RBD-NP-elicited sera efficiently neutralized pseudotyped viruses harboring the S glycoprotein of the Pangolin-GD isolate (GMT 6.6 $\times 10^2$ and 7.7 $\times 10^2$) and RaTG13 (GMT VSV 6 $\times 10^2$) (Figures 3E and 3F) (Lam et al., 2020; Zhou et al., 2020b), in agreement with the close phylogenetic relationship of their RDVs with that of SARS-CoV-2 S (Figure S2B). HexaPro S-elicited NHP sera also efficiently neutralized Pangolin-GD S (GMT HIV 4.7 $\times 10^2$ and VSV 9 $\times 10^2$) and RaTG13 S pseudotypes (GMT VSV 4.7 $\times 10^2$) (Figures 3E and 3F). Furthermore, we observed that both RBD-NP- and HexaPro S-elicited polyclonal Abs that weakly neutralized HIV pseudovirus carrying SARS-CoV S (RBD-NP GMT 6 $\times 10^2$ and HexaPro GMT 1 $\times 10^2$) (Figures 3E and 3F). Collectively, these data demonstrate that both immunogens elicited comparable neutralization breadth and potency against the pseudoviruses tested, reinforcing the notion that most sarbecovirus S-directed neutralizing Abs target the RBD (Greaney et al., 2021a, 2021b; Piccoli et al., 2020).

### Design, assembly, and characterization of mosaic and cocktail sarbecovirus RBD-NPs

We and others have recently evaluated nanoparticle immunogens that display multiple antigenic variants of viral glycoproteins as a potential route toward broadly protective vaccines (Boyoglu-Barnum et al., 2021; Cohen et al., 2021a, 2021b; Kane-kiyo et al., 2019). Given the large number of coronaviruses circulating in zoonotic reservoirs, such vaccines could be important for understanding vaccine-induced neutralization/protection breadth and preventing future pandemics (Menachery et al., 2015, 2016). We expressed and purified four RDVs from the S proteins of SARS-CoV-2, SARS-CoV, and the bat coronaviruses WIV1 and RaTG13 genetically fused to the IS3-50A trimer. An equimolar mixture of four of these proteins was added to the IS3-50B pentamer to assemble a mosaic RBD-NP (mRBD-NP) displaying the four RDVs on the same nanoparticle (Figure 4A and S3A). We also assembled a trivalent mosaic RBD-NP designated “drop out” lacking the SARS-CoV RBD (mRBD-NP-DO), as well as cocktail immunogens with three (cRBD-NP-DO; lacking the SARS-CoV RBD) or four (cRBD-NP-DO) independently assembled nanoparticles, each displaying a single type of the aforementioned RDVs, mixed after independent assembly. Finally, we made a bivalent mosaic RBD-NP co-displaying the SARS-CoV and SARS-CoV-2 RDVs (Figure S3A) to directly confirm co-display on the nanoparticles using a sandwich binding assay. All of the nanoparticle immunogens formed the intended icosahedral architecture and retained native antigenicity, as shown by SDS-PAGE, dynamic light scattering, negative staining electron microscopy, and binding to hACE2-Fc (Figures S3B–S3E). We found that the vaccine candidates were stable for at least 4 weeks at several temperatures except the highest temperature evaluated (37°C), at which we observed a decrease in hACE2 recognition over time beginning after 7 to 14 days, presumably due to aggregation (Figures 4B–4E). Following immobilization using the SARS-CoV-2-specific mAb S2H14 (Piccoli et al., 2020), the quadrivalent, trivalent, and bivalent mRBD-NPs all bound the Fab of the SARS-CoV-specific Ab S230 (Piccoli et al., 2020; Rockx et al., 2008; Walls et al., 2019), confirming co-display, whereas the monovalent SARS-CoV-2 RBD-NP did not (Figure S3G). We determined that the reactivity of the trivalent mRBD-NP derived from the inclusion of the WIV1 RBD in this vaccine, as the monovalent WIV1 RBD-NP also bound the S230 Fab after immobilization with hACE2 (as expected [Menachery et al., 2016]), whereas the monovalent SARS-CoV-2 and RaTG13 RBD-NPs did not (Figure S3H). Collectively, these data demonstrate that all of the nanoparticles were stable and (co-)displayed the various RDVs as intended.

**Cocktail and mosaic RBD-NP vaccines elicit cross-reactive and broadly neutralizing sarbecovirus Abs**

The mosaic and cocktail nanoparticle immunogens were compared to monovalent RBD-NP vaccines and a non-assembling control vaccine comprising all four RBD-IS3-50A trimeric components and a non-assembling IS3-50B pentamer (Figure S3F) in an immunization study in BALB/c cByJ mice (Figures 4A and S3A). All immunizations comprised 1 μg of total RBD antigen, such that ∼0.25 μg of each RBD was given in each administration of the quadrivalent vaccines.
Figure 4. *In vitro* assembly and accelerated stability studies of mosaic and cocktail nanoparticle immunogens

(A) Schematic of *in vitro* assembly of mRBD-NPs and cRBD-NPs.

(B–E) The physical and antigenic stability of mRBD-NP, cRBD-NP, and (SARS-CoV-2) RBD-NP samples incubated at four different temperatures was followed for four weeks.

(B) The ratio of UV/vis absorbance at 320 nm/280 nm is a measure of turbidity (proxy for aggregation).

(C) Hydrodynamic diameter of the nanoparticles measured using dynamic light scattering.

(D) hACE2-Fc binding measured by comparing the peak amplitude of hACE2-Fc binding for each sample to a reference sample stored at < −70°C using biolayer interferometry.

(E) Electron micrographs of negatively stained samples after incubation for 28 days at the indicated temperatures. Scale bar, 50 nm.
and ~0.33 μg of each RBD in the trivalent vaccines. After two immunizations, all four mosaic or cocktail RBD-NP vaccines elicited strong binding (GMT ~1–5 × 10^3) (Figure S4A; Data S1) and potent serum neutralizing (GMT 2–8 × 10^3; Figure 5A; Data S1) Ab titers against wild-type (D614G) SARS-CoV-2 S pseudovirus. Competition ELISAs using hACE2, CR3022, S309, and S2X259 demonstrated that all mosaic and cocktail vaccine candidates elicited robust Ab titers targeting all antigenic sites tested, showcasing the diversity of RBD-specific Abs elicited (Figures S4B–S4E; Data S1). The neutralizing SARS-CoV-2 S (D614G) Ab responses were slightly higher for mRBD-NPs and slightly lower for cRBD-NPs than that of the monovalent RBD-NP (GMT 3 × 10^3) (Figure 5A), similar to the aforementioned low-dose RBD-NP immunization study (Figure 1D) and suggesting that the dose of strain-matched antigen in the multivalent vaccines is not a limiting factor of the magnitude of neutralizing Ab responses. The neutralizing activity against SARS-CoV-2 elicited by the other monovalent RBD-NPs and the non-assembling control were ~2 orders of magnitude lower (GMT < 5 × 10^1) than for the monovalent RBD-NP, indicating poor elicitation of cross-neutralizing Abs by these heterologous monovalent RBD-NPs.

Figure 5. Mosaic and cocktail RBD-NP vaccines elicit neutralizing Abs against multiple sarbecoviruses
(A) Neutralizing Ab titers in mice (n = 8) against wild-type (D614G) SARS-CoV-2 S MLV pseudovirus five weeks post-prime elicited by monovalent, mosaic, and cocktail RBD-NPs with an LOD of 1 × 10^1.
(B) Neutralizing Ab titers in mice against SARS-CoV S MLV pseudovirus five weeks post-prime elicited by monovalent, mosaic, and cocktail RBD-NPs with an LOD of 1 × 10^1.
(C) Neutralizing Ab titers in mice against SHC014 VSV pseudovirus five weeks post-prime elicited by monovalent, mosaic, and cocktail RBD-NPs with an LOD of 1.7 × 10^1.
Raw data curves shown in Data S1. Statistical significance was determined by Kruskal-Wallis test and shown only when significant. **p<0.01. LOD is shown as a gray horizontal dotted line in (C).
Figure A: Graph showing % starting body weight against dpi for different groups.

Figure B: Scatter plot showing Congestion Score for different groups.

Figure C: Box plot comparing PFU/lobe across groups.

Figure D: Scatter plot showing mean eosinophils/HPF for different groups.

Figures E, F, G, H, I: Images depicting histological sections for different groups.
Although ELISA binding titers were comparable across mosaic and cocktail groups against SARS-CoV S2P (GMT $3\times10^{10}$), the corresponding pseudovirus neutralization titers showed more nuanced patterns (Figures 5B and S4F; Data S1). Tetravalent mosaic and cocktail RBD-NPs elicited potent neutralizing activity (GMT $3\times10^{9}$) with magnitudes roughly comparable to that of the monovalent SARS-CoV-RBD-NP (GMT $5\times10^{8}$; Figure 5B). Strikingly, the trivalent nanoparticle immunogens (mRBD-NP-DO and cRBD-NP-DO)—which did not contain the SARS-CoV RBD—also elicited potent neutralization (GMT $1\times10^{8}$). This cross-neutralization likely arose from the inclusion of the WIV1 RBD (Figure S4G; Data S1) in the trivalent immunogens, as WIV1 cross-reacts with a SARS-CoV-specific mAb (Figure S3H) (Menachery et al., 2016), and the monovalent WIV1-RBD-NP induced similar levels of pseudovirus neutralization (GMT $2\times10^{8}$). The non-assembling control immunogen, which contains all four RBD-Is3-50A trimeric components, also elicited substantial neutralizing activity against SARS-CoV (GMT $1\times10^{9}$) but not against SARS-CoV-2. Furthermore, we show that sera elicited by the monovalent, mRBD-NPs, and cRBD-NPs neutralize SHC014 pseudotyped virus, a distantly related sarbecovirus sharing 76%, 76%, 82%, and 84% amino acid sequence identity with the SARS-CoV-2, RaTG13, SARS-CoV, and WIV1 RBDs, respectively (Figure 5C; Data S1). These data show that both mRBD-NPs and cRBD-NPs are promising vaccine candidates for eliciting broad sarbecovirus immunity, in agreement with previous findings using a different nanoparticle platform (Cohen et al., 2021a) or chimeric sarbecovirus S glycoproteins (Martinez et al., 2021b) and recent results obtained with influenza virus hemagglutinin nanoparticle vaccines (Boy-oglu-Barnum et al., 2021).

### Mosaic RBD-NPs protect mice against heterotypic SARS-CoV challenge

To gauge the ability of the multivalent RBD-NPs to confer protection against vaccine-matched and heterotypic sarbecoviruses, we challenged groups of eight mice with a high dose ($10^5$ PFUs) of the mouse-adapted SARS-CoV MA15 virus (Roberts et al., 2007). In agreement with the pseudovirus neutralization data, animals immunized with mRBD-NP, cRBD-NP, and cRBD-NP-DO were protected from weight loss (ranging between 1% and 5%) and serious lung pathology throughout the four days of the experiment (Figures 6A and 6B). Unvaccinated mice exhibited up to 15% average weight loss and signs of lung pathology (congestion score $\sim1.5$) that were only minorly seen in all other vaccinated groups (Figures 6A and 6B). mRBD-NP- and mRBD-NP-DO-vaccinated mice were completely protected from viral replication in the lungs (Figure 6C). Overall, the trivalent mRBD-NP-DO provided protection that was indistinguishable from the tetravalent mRBD-NP, despite lacking the SARS-CoV RBD. Five out of eight and six out of eight cRBD-NP- and cRBD-NP-DO-vaccinated mice were completely protected from viral replication in the lungs, respectively, with viral burden ranging between $10^6$ and $10^5$ PFUs/lobe for the remaining animals (compared to unvaccinated mice with $\sim10^6$ PFUs/lobe, Figure 6C). To assess whether the RBD-NP platform causes vaccine-induced immune pathology, which was previously described with double-inactivated SARS-CoV vaccines (Bolles et al., 2011), we investigated eosinophil infiltration and inflammation in the lungs following SARS-CoV MA15 challenge. Although infection induced inflammation and infiltration of eosinophils in the lungs of unvaccinated mice (Figures 6D, 6I, and SSA), no signs of eosinophilia were detected for any animals in the vaccinated groups using histological analysis of stained lung sections (Figures 6D–6I and SSA). These data show that mRBD-NP, mRBD-NP-DO, cRBD-NP, and cRBD-NP-DO protect against weight loss, lung injury, and viral replication following challenge, with no detectable vaccine-induced immune pathology.

To identify the gain in protective breadth provided by multivalent display of sarbecovirus RBD-NP, relative to monovalent RBD-NPs, we performed a second SARS-CoV MA15 challenge experiment including monovalent RBD-NP and SARS-CoV-RBD-NP (Figure S5B). In agreement with the pseudovirus neutralization data and first challenge (Figures 4B–4D and 6A–6C), animals immunized with the SARS-CoV-RBD-NP, mosaic, and cocktail RBD-NP formulations, as well as the non-assembling control immunogen, were protected from weight loss and serious lung pathology throughout the four days of the experiment. The animals receiving the monovalent RBD-NP vaccine experienced up to 12% average weight loss, whereas the unvaccinated mice exhibited further (up to 15%) average weight loss and signs of lung pathology (congestion score $\sim2$) that were only minorly seen in all other vaccinated groups (Figures S5B and S5C). All mice vaccinated with SARS-CoV-RBD-NP, RBD-NP, and mRBD-NP were protected from viral replication in the lungs, whereas we detected $\sim10^{2.5}$ and $10^6$ PFUs/lobe for half of the mice receiving the non-assembling control immunogen and unvaccinated mice, respectively (Figure SSD). These results provide proof-of-principle that mosaic and cocktail RBD nanoparticle vaccines elicit broad protection against heterotypic sarbecovirus challenge and could represent the next generation of vaccines developed in anticipation of future spillovers.

### Figure 6. Mosaic and cocktail RBD-NP vaccines protect against heterotypic SARS-CoV-MA15 challenge in 15-week-old BALB/c cByJ mice

(A) Weight loss following SARS-CoV MA15 challenge up to 4 days post infection ($n=8$). Unvaccinated animals are shown as black circles.

(B) Congestion score following SARS-CoV MA15 infection with a score of 0 indicating unchanged lung color and 4 indicating a darkened and diseased lung ($n=8$).

(C) Viral titers in mice lungs (expressed in PFUs per lobe) following challenge ($n=8$) with an LOD of $9\times10^1$. LOD is shown as a gray horizontal dotted line. Statistical significance was determined by Kruskal-Wallis and shown when significant. **$p<0.01$.

(D) Mean eosinophils per high power field (HPF) per sample run over 10 HPF per lung stained with congo red. Significance was determined using one-way ANOVA and shown when significant.

(E–I) Histological analysis of stained lung sections for mRBD-NP (E), mRBD-NP-DO (F), cRBD-NP (G), cRBD-NP-DO (H), and unvaccinated mice (I). Arrowheads indicate eosinophils. Scale bar, 20 μm.
The data presented here show that two ultra-low dose immunizations or a single immunization with RBD-NP produces potent neutralizing Ab responses in mice. The latter strategy confers protection against SARS-CoV-2 MA10 challenge, suggesting that the nanoparticle platform could enable dose-sparing regimens to achieve global vaccination, especially given the high shelf stability of this vaccine (Wals et al., 2020b). NHP vaccination with RBD-NP was concurrently shown to also elicit CD4 T cell responses and to protect from viral replication (Arunachalam et al., 2021). Moreover, RBD-NP vaccination, which is currently under phase I/II evaluation in the clinic, elicits diverse Ab responses neutralizing a broad spectrum of SARS-CoV-2 variants detected in clinical isolates with similar potency to those elicited by HexaPro S vaccination. Several RBM mutations, including at position E484, however, lead to reductions in neutralizing activity elicited by either RBD-NP or HexaPro S in NHPs. Although both RBD-NP- and HexaPro S-elicited sera robustly neutralize the B.1.1.7/E484K variants, this does not contain the E484 substitution, neutralization of the B.1.351, P.1, and B.1.1.7/E484K variants was dampened similarly to sera from individuals vaccinated twice with the Pfizer-BioNTech BNT162b2 mRNA. These findings are in agreement with reports showing that the serum neutralizing activity against the B.1.351 variant from mRNA-1273-vaccinated individuals was comparably reduced (Wu et al., 2021), as was also the case for neutralization of authentic SARS-CoV-2 B.1.351 by HCP (Wibmer et al., 2021). Collectively, these data indicate that a significant fraction of vaccine-elicited neutralizing activity is directed to the RBM, which is the target of potent neutralizing Abs, irrespective of the antigen design strategy (RBD- or S-based), the vaccine modality (protein subunit or mRNA), or species (NHPs or humans). We show that receiving a third immunization, even of an unaltered vaccine, improves the neutralizing Ab responses to VOCs, which is reminiscent of what has been shown in vaccinated humans previously infected with SARS-CoV-2 (Abu Jabal et al., 2021; Krammer et al., 2021; Stamatakis et al., 2021), suggesting that further booster immunizations may limit the impact of VOCs without vaccine update.

The ongoing global spread of SARS-CoV-2 and the circulation of a large number of sarbecoviruses in bats (Menachery et al., 2015, 2016) and other animal reservoirs strongly motivate the development of vaccines that protect against a broad spectrum of coronaviruses. We observed that vaccination of NHPs with RBD-NPs or HexaPro S induced comparable but moderate neutralization breadth against genetically distinct sarbecoviruses. We demonstrate here that co-display (mRBD-NP) and co-immunization (cRBD-NP) of multivalently arrayed sarbecovirus RBDS elicit robust neutralizing Ab responses against SARS-CoV-2, SARS-CoV, and SHCo14 pseudotyped viruses and outperform their monovalent vaccine counterparts. The observation that mosaic and cocktail RBD-NPs elicit greater titers of Abs competing with the broadly neutralizing sarbecovirus mAb S2X259 than monovalent vaccines suggest that this vaccine design strategy could enhance neutralization breadth. Accordingly, mice vaccinated with multivalent vaccines were protected from disease upon SARS-CoV MA15 challenge, including with formulations that did not include the SARS-CoV RBD, whereas RBD-NP-vaccinated mice experienced weight loss. Previous studies showed that double-inactivated SARS-CoV vaccines could lead to eosinophil infiltration in the lungs and subsequent immune pathology (Bolles et al., 2011). Our data show that none of the multivalent vaccine candidates evaluated here induced eosinophilia whereas SARS-CoV MA15 challenge did, pointing to the safety and efficacy of these immunogens. Our data highlight the potential of this approach to achieve broad sarbecovirus immunity, overcoming both the emergence of SARS-CoV-2 variants and putative future zoonosis of genetically distinct sarbecoviruses not included in the vaccine. Based on the RBD-centric nature of neutralizing Ab responses resulting from infection and vaccination (Dejnirattisai et al., 2021; Greaney et al., 2021a, 2021b; Piccoli et al., 2020), irrespective of immunogen format or vaccine modality, and the enhanced elicitation of Abs targeting all RBD antigenic sites for multivalent RBD-NPs compared to S-based vaccines, this study paves the way for advancing RBD pan-sarbecovirus subunit vaccines to the clinic.

**Limitations of study**

Due to the cost of challenge and BSL-3 vivarium limitations, the mice vaccinated with a low dose of RBD-NP were not challenged, and therefore we can only infer the likelihood of protection from their sera’s ability to neutralize SARS-CoV-2 pseudovirus better than other mice (i.e., those given a single vaccine dose) that were challenged. Neutralization is a correlate of protection in NHPs (Arunachalam et al., 2021), but we recognize that neutralization assays using HEK-ACE2 cell lines may underestimate the effects of non-RBM targeting Abs (Lempp et al., 2021; McCallum et al., 2021a; Suryadevara et al., 2021). Due to the limitations of the number of animals and the cost of challenge, we cannot challenge with all desired point mutant or variant strains and must use neutralization as the best correlate for interpreting the expected challenge outcome.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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Supplemental information can be found online at https://doi.org/10.1016/j.cell.2021.09.015.

ACKNOWLEDGMENTS

We thank Helen Chu and Marion Pepper for coordination of HCP samples and Ratika Krishnamurty for program management. The authors thank Kathryn Guerrero, Brieann Brown, Solomon Wangari, Joel Ahrens, Naoto Iwayama, and William Garrison for their technical assistance with the pigtail macaque study. This study was supported by the Bill and Melinda Gates Foundation (INV-017592 to J.S.M. and OPP1156262 to N.P.K. and D.V.); generous gifts from the Audacious Project, Jodi Green and Mike Halperin, and the Hanauer Foundation; and may hold shares in Vir Biotechnology. The other authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| CR3022              | ter Meulen et al., 2006 | N/A |
| S309                | Pinto et al., 2020 | N/A |
| S2X259              | Tortorici et al., 2021 | N/A |
| S2H14               | Piccoli et al., 2020 | N/A |
| S230                | Rockx et al., 2008 | N/A |
| Goat anti-mouse HRP | Invitrogen | Cat #626520; Lot #TG275230; AB_2533947 |
| Goat anti-human HRP | Invitrogen | Cat #A18817; Lot #65-180-071919; AB_2535594 |
| Streptavidin-HRP    | Thermo Scientific | Cat #N100 |
| CD45/LCA-A700-C61   | BD Biosciences | Cat#5605010 |
| CD11c-BV501         | Biolegend | Cat#117338; AB_2562016 |
| SiglecF-BV650       | BD Biosciences | Cat#740557; AB_2740258 |
| II-1-Hybridoma      | ATCC | CRL-2700 |
| **Biological samples** | | |
| BALB/c mice         | Jackson Laboratory | Cat#000651 |
| Pigtail macaques (Macaca nemestrina) | WANPRC | |
| Rhesus macaques (Macaca mulatta) | NIRC | |
| 20/130 COVID-19 plasma | NIBSC | Sample#20/130 |
| VSV (G*D-luciferase) | Kaname et al., 2010 | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| AddaVax adjuvant    | InvivoGen | Cat# vac-adx-10 |
| AS03                | GSK | N/A |
| O/W                 | VFI | N/A |
| TMB                 | SeraCare | Cat# 5120-0083 |
| Thrombin            | Sigma | Cat# T9326-150UN |
| Immobilized Papain  | ThermoScientific | Cat# 20341 |
| Lys-C-endoproteinase | NEB | Cat# P6109S |
| EZ-Link Sulfo-NHS-LC Biotinylation Kit | Thermo Fisher Scientific | Cat#21435 |
| **Experimental models: Cell lines** | | |
| Expi 293F           | ThermoFisher | Cat #A14527 |
| HEK-ACE2 adherent   | BEI (Gift from Bloom lab) | Sample#NR-52511 |
| HEK293T/17 Adherent | ATCC | Cat# CRL-11268 |
| **Recombinant DNA** | | |
| HexaPro S           | Hsieh et al., 2020 | N/A |
| RBD-16GS-50A        | GenScript | Walls et al., 2020b | N/A |
| RBD-12GS-50A        | GenScript | Walls et al., 2020b | N/A |
| SARS-COV-RBD-16GS-50A | GenScript | N/A |
| WV11-RBD-16GS-50A   | GenScript | N/A |
| RaTG13-RBD-16GS-50A | GenScript | N/A |
| SARS-CoV-2 S-2P trimer | GenScript | Walls et al., 2020a | Vector# BEI NR-52421 |
| SARS-CoV S-2P trimer | GeneArt | Walls et al., 2019 | N/A |
| SARS-CoV-2 RBD      | GenScript | Walls et al., 2020a | Vector# BEI NR-52422 |
| SARS-CoV RBD        | GenScript | N/A |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**  
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David Veesler (dveesler@uw.edu).

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| WIV1 RBD           | GenScript | N/A        |
| RaTG13 RBD         | GenScript | N/A        |
| SARS-CoV-2 S full-length D614G (YP 009724390.1) | Crawford et al., 2020 | Vector# BEI NR-52514 |
| SHCo14 S full-length (AGZ48806.1) | GenScript | N/A        |
| SARS-CoV S full-length (YP 009825051.1) | GeneArt (Walls et al., 2020) | N/A        |
| RaTG13 S full-length (QHR63300.2) | GenScript | N/A        |
| WIV1 full-length S (AGZ48831.1) | GenScript | N/A        |
| PangolinGD full-length S (QLR06867.1) | GenScript | N/A        |
| Murine leukemia virus gag-pol | Millet and Whittaker 2016 | N/A        |
| pTG-Luciferase     | Millet and Whittaker 2016 | N/A        |
| HIV Hgpm2          | Crawford et al., 2020 | Vector# BEI NR-52517 |
| HIV luciferase     | Crawford et al., 2020 | Vector# BEI NR-52516 |
| HIV tat1b          | Crawford et al., 2020 | Vector# BEI NR-52518 |
| HIV Rev1b          | Crawford et al., 2020 | Vector# BEI NR-52519 |

**Software and algorithms**

- UCSF ChimeraX: Goddard et al., 2018  
  [https://www.rbvi.ucsf.edu/chimerax/](https://www.rbvi.ucsf.edu/chimerax/)
- Prism: Graphpad  
  [https://www.graphpad.com/scientific-software/prism/](https://www.graphpad.com/scientific-software/prism/)
- FlowJo v10: FlowJo  
  [https://www.flowjo.com](https://www.flowjo.com)
- Dms-variants package: Greaney et al., 2020  
  [https://jbloomlab.github.io/dms_variants/dms_variants.globalepistasis.html](https://jbloomlab.github.io/dms_variants/dms_variants.globalepistasis.html)

**Other**

- Octet Biosensors: protein A: Sartorius (FortéBio)  
  Cat# 18-5010
- Octet Biosensors: ARG2: Sartorius (FortéBio)  
  Cat# 18-5092
- EM supplies 300 mesh grids: Ted Pella  
  Cat# 01843-F
- Filter paper: Cytiva  
  Cat# 1004047
- Uranyl formate: SPI Chem  
  Cat# 02545-AA
- Unis Capillary Cassettes: Unchained Labs  
  Cat# 201-1010
- Protein A resin: Cytiva  
  Cat# 17549802
- Superdex 200 Increase SEC column: Cytiva  
  Cat# 28-9909-44
- Superose 6 Increase SEC column: Cytiva  
  Cat# 29091996
- Talon resin: TaKaRa  
  Cat# 635652
- Excel resin: Cytiva  
  Cat# 17371203
- Patterson Veterinary, Isoflurane, USP: Patterson  
  Cat# 07-893-1389
- EndoSafe LAL Test Cartridges: Charles River Labs  
  Cat # PTS20005F
- Lemo21(DE3): New England BioLabs  
  Cat#C2528J
- Isopropyl-B-D-thiogalactoside (IPTG): Sigma-Aldrich  
  Cat#6758
- Kanamycin Sulfate: Sigma-Aldrich  
  Cat#K1876
- HiLoad S200 pg: Cytiva  
  Cat#28989336
- Ni Sepharose 6 FF: Cytiva  
  Cat#17531808
- HisTrap FF: Cytiva  
  Cat#17525501
**Materials availability**

All reagents will be made available on request after completion of a Materials Transfer Agreement.

**Data and code availability**

All data supporting the findings of this study are found within the paper and its Supplemental information, and are available from the Lead Contact author upon request. Additional supplemental items are available from Mendeley Data at https://doi.org/10.17632/5k989kb8t7.1.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell lines**

Expi293F (derived from 293 cells which are female) cells are derived from the HEK293F cell line (Life Technologies). Expi293F cells were grown in Expi293 Expression Medium (Life Technologies), cultured at 36.5°C with 8% CO₂ and shaking at 150 rpm. HEK293T/17 is a female human embryonic kidney cell line (ATCC). The HEK-ACE2 (derived from HEK293T cells which are female) adherent cell line was obtained through BEI Resources, NIAID, NIH: Human Embryonic Kidney Cells (HEK293T) Expressing Human Angiotensin-Converting Enzyme 2, HEK293T-hACE2 Cell Line, NR-52511. All adherent cells were cultured at 37°C with 8% CO₂ in flasks with DMEM + 10% FBS (Hyclone) + 1% penicillin-streptomycin. Cell lines other than Expi293F were not tested for mycoplasma contamination nor authenticated.

**Mice**

Female BALB/c mice (Stock # 000651, BALB/c cByJ mice) four weeks old were obtained from Jackson Laboratory, Bar Harbor, Maine, and maintained at the Comparative Medicine Facility at the University of Washington, Seattle, WA, accredited by the American Association for the Accreditation of Laboratory Animal Care International (AAALAC). Animal procedures were performed under the approvals of the Institutional Animal Care and Use Committee (IACUC) of University of Washington, Seattle, WA, and University of North Carolina, Chapel Hill, NC.

**Pigtail macaques**

Two adult male Pigtail macaques (*Macaca nemestrina*) were immunized in this study. All animals were housed at the Washington National Primate Research Center (WaNPRC), an AAALAC International accredited institution. All experiments were approved by The University of Washington’s IACUC. Animals were singly housed in comfortable, clean, adequately-sized cages with ambient temperatures between 72–82°F. Animals received environmental enrichment for the duration of the study including grooming contact, perches, toys, foraging experiences and access to additional environment enrichment devices. Water was available through automatic watering devices and animals were fed a commercial monkey chow, supplemented daily with fruits and vegetables. Throughout the study, animals were checked twice daily by husbandry staff.

**Rhesus macaques**

Male Rhesus macaques (Macaca mulatta) of Indian origin, aged 3–7 years were assigned to the study (Arunachalam et al., 2021). Animals were distributed between the groups such that the age and weight distribution were comparable across the groups. Animals were housed and maintained at the New Iberia Research Center (NIRC) of the University of Louisiana at Lafayette, an AAALAC International accredited institution, in accordance with the rules and regulations of the Guide for the Care and Use of Laboratory Animal Resources. The entire study (protocol 2020-8808-15) was reviewed and approved by the University of Louisiana at Lafayette IACUC. All animals were negative for SIV, simian T cell leukemia virus, and simian retrovirus.

**Convalescent human sera**

Samples collected between 1–60 days post infection from individuals who tested positive for SARS-CoV-2 by PCR were profiled for anti-SARS-CoV-2 S antibody responses and those with anti-S Ab responses were maintained in the cohort (Walls et al., 2020b). Individuals were enrolled as part of the HAARVI study at the University of Washington in Seattle, WA. Baseline sociodemographic and clinical data for these individuals are summarized in Table S1. This study was approved by the University of Washington Human Subjects Division Institutional Review Board (STUDY00000959 and STUDY00003376). All experiments were performed in at least two replicates. One sample is the 20/130 COVID-19 plasma from NIBSC (https://www.nibsc.org/documents/ifu/20-130.pdf).

**Pfizer vaccinated human sera**

Blood samples were collected from participants who had received both doses of the Pfizer mRNA vaccine and were 7–30 days post second vaccine dose. Clinical data for these individuals are summarized in Table S1. Individuals were enrolled in the UWARN: COVID-19 in WA study at the University of Washington in Seattle, WA. This study was approved by the University of Washington Human Subjects Division Institutional Review Board (STUDY00010350).
METHOD DETAILS

Plasmid construction
The SARS-CoV-2–RBD-Avi construct was synthesized by GenScript into pcDNA3.1− with an N-terminal mu-phosphatase signal peptide and a C-terminal octa-histidine tag, flexible linker, and avi tag (GHHHHHHHGGSSGLNDIFEAQKIEWHE). The boundaries of the construct are N-terminal FPPN331 and C-terminal KKST531 (Walls et al., 2020a). The GD-Pangolin (326–527), WIV1 (316–518), RaTG13 (359–562), RmYN02 (307–492), and ZXC21 (323–507) were synthesized by GenScript into vector pcDNA3.1− or CMVR with a preceding mu-phosphatase signal peptide and a C-terminal octahistidine tag. SARS-CoV-1 (306–575) was subcloned from a GenArt synthesized SARS-CoV-1 Spike ectodomain. The SARS-CoV S2P ((Pallesen et al., 2017)) was synthesized by GeneArt and placed into a modified pOPING vector with its original N-terminal mu-phosphatase signal peptide, and an engineered C-terminal extension: SG-RENLYFQG (TEV protease site), GGGSG-YIPEAPRDGQAYVRKDGWLLSTFL (folding trimerization motif), G-HHHHHH (hexa-histidine tag), just upstream of the predicted transmembrane region (YIK). The SARS-CoV-2 S2P ectodomain trimer (GenBank: YP_009724390.1, BEI NR-52420) was synthesized by GenScript into pCMV with an N-terminal mu-phosphatase signal peptide and a C-terminal TEV cleavage site (GSGRENLYFQG), T4 fibritin foldon (GGGSGYIPEAPRDGQAYVRKDGWLLSTPL), and octa-histidine tag (GHHHHHHHH) (Walls et al., 2020a). The construct contains the 2P mutations (proline substitutions at residues 986 and 987; and an 682SGAG685 substitution at the furin cleavage site). The SARS-CoV-2 RBD was genetically fused to the N terminus of the trimeric IS3-50A nanoparticle component using 12 or 16 glycine and serine residues. RBD-12GS-IS3-50A fusions were synthesized and cloned by GenScript into pCMV. The RBD-16GS-IS3-50A fusion was cloned into pCMV/R using the Xba1 and AvrII restriction sites and Gibson assembly (Gibson et al., 2009). All RBD-bearing components contained an N-terminal mu-phosphatase signal peptide and a C-terminal octa-histidine tag. Human ACE2 ectodomain was genetically fused to a sequence encoding a thrombin cleavage site and a human Fc fragment at the C-terminal end. hACE2-Fc was synthesized and cloned by GenScript with a BM40 signal peptide. Genes encoding CR3022 heavy and light chains were ordered from GenScript and cloned into pCMV/R. S309 construct as previously described (Pinto et al., 2020). SARS-CoV-2 HexaPro construct is as previously described (Hsieh et al., 2020) and placed into CMVR with an octa-histag.

Transient transfection
Proteins were produced using endotoxin free DNA in Expi293F cells grown in suspension using Expi293F expression medium (Life Technologies) at 33°C, 70% humidity, 8% CO2 rotating at 150 rpm. The cultures were transfected using PEI-MAX (Polyscience) with cells grown to a density of 3.0 million cells per mL and cultivated for 3 days. Supernatants were clarified by centrifugation (5 min at 4000 rcf), addition of PDADMAC solution to a final concentration of 0.0375% (Sigma Aldrich, #409014), and a second spin (5 min at 4000 rcf).

Microbial protein expression and purification
The IS3-50A and IS3-50B.4PT1 proteins (Bale et al., 2016) were expressed in Lemo21(DE3) (NEB) in LB (10 g Tryptone, 5 g Yeast Extract, 10 g NaCl) grown in 2 L baffled shake flasks or a 10 L BioFlo 320 Fermenter (Eppendorf). Cells were grown at 37°C to an OD600 ~0.8, and then induced with 1 mM IPTG. Expression temperature was reduced to 18°C and the cells shaked for ~16 h. The cells were harvested and lysed by microfluidization using a Microfluidics M110P at 18,000 psi in 50 mM Tris, 500 mM NaCl, 30 mM imidazole, 1 mM PMSF, 0.75% CHAPS. Lysates were clarified by centrifugation at 24,000 g for 30 min and applied to a 2.6 x 10 cm Ni Sepharose 6 FF column (Cytaba) for purification by IMAC on an AKTA Avant150 FPLC system (Cytaba). Protein of interest was eluted over a linear gradient of 30 mM to 500 mM imidazole in a background of 50 mM Tris pH 8, 500 mM NaCl, 0.75% CHAPS buffer. Peak fractions were pooled, concentrated in 10K MWCO centrifugal filters (Millipore), sterile filtered (0.22 μm) and applied to either a Superdex 200 Increase 10/300, or HiLoad S200 pg GL SEC column (Cytaba) using 50 mM Tris pH 8, 500 mM NaCl, 0.75% CHAPS buffer. IS3-50A elutes at ~0.6 column volume (CV). IS3-50B.4PT1 elutes at ~0.45 CV. After sizing, bacterial-derived components were tested to confirm removal of endotoxin before using for nanoparticle assembly.

Protein purification
Proteins containing His tags were purified from clarified supernatants via a batch bind method where each clarified supernatant was supplemented with 1 M Tris-HCl pH 8.0 to a final concentration of 45 mM and 5 M NaCl to a final concentration of ~310 mM. Talon cobalt affinity resin (Takara) was added to the treated supernatants and allowed to incubate for 15 min with gentle shaking. Resin was collected using vacuum filtration with a 0.2 μm filter and transferred to a gravity column. The resin was washed with 20 mM Tris pH 8.0, 300 mM NaCl, and the protein was eluted with 3 column volumes of 20 mM Tris pH 8.0, 300 mM NaCl, 300 mM imidazole. The batch bind process was then repeated and the first and second elutions combined. SDS-PAGE was used to assess purity. RBD-IS3-50A fusion protein IMAC elutions were concentrated to > 1 mg/mL and subjected to three rounds of dialysis into 50 mM Tris pH 7.4, 185 mM NaCl, 100 mM Arginine, 4.5% glycerol, and 0.75% w/v 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) in a hydrated 10K molecular weight cutoff dialysis cassette (Thermo Scientific). S2P and HexaPro IMAC elution fractions were concentrated to ~1 mg/mL and dialyzed three times into 50 mM Tris pH 8, 150 mM NaCl, 0.25% L-Histidine in a hydrated 10K molecular weight cutoff dialysis cassette (Thermo Scientific).
Clarified supernatants of cells expressing monoclonal antibodies and human ACE2-Fc were purified using a MabSelect PrismA 2.6 × 5 cm column (Cytiva) on an AKTA Avant150 FPLC (Cytiva). Bound antibodies were washed with five column volumes of 20 mM NaPO₄, 150 mM NaCl pH 7.2, then five column volumes of 20 mM NaPO₄, 1 M NaCl pH 7.4 and eluted with three column volumes of 100 mM glycine at pH 3.0. The eluate was neutralized with 2 M Trizma base to 50 mM final concentration. SDS-PAGE was used to assess purity.

Recombinant S309 was expressed as a Fab in expiCHO cells transiently co-transfected with plasmids expressing the heavy and light chain, as described above (see Transient transfection) (Pinto et al., 2020). The protein was affinity-purified using a HiTrap Protein A Mab select Xtra column (Cytiva) followed by desalting against 20 mM NaPO₄, 150 mM NaCl pH 7.2 using a HiTrap Fast desalting column (Cytiva). The protein was sterilized with a 0.22 µm filter and stored at 4°C until use.

**In vitro nanoparticle assembly and purification**

Total protein concentration of purified individual nanoparticle components was determined by measuring absorbance at 280 nm using a UV/vis spectrophotometer (Agilent Cary 8454) and calculated extinction coefficients. The assembly steps were performed at room temperature with addition in the following order: RBD-IS3-50A trimeric fusion protein, followed by additional buffer (50 mM Tris pH 7.4, 185 mM NaCl, 100 mM Arginine, 4.5% glycerol, and 0.75% w/v CHAPS) as needed to achieve desired final concentration, and finally IS3-50B.4PT1 pentameric component (in 50 mM Tris pH 8, 500 mM NaCl, 0.75% w/v CHAPS), with a molar ratio of RBD-IS3-50A:IS3-50B.4PT1 of 1:1:1. All RBD-IS3-50 in vitro assemblies were incubated at 2-8°C with gentle rocking for at least 30 min before subsequent purification by SEC in order to remove residual unassembled component. Different columns were utilized depending on purpose: Superose 6 Increase 10/300 GL column was used analytically for nanoparticle size estimation, a Superdex 200 Increase 10/300 GL column used for small-scale pilot assemblies, and a HiLoad 26/600 Superdex 200 pg column used for nanoparticle production. Assembled particles were purified in 50 mM Tris pH 7.4, 185 mM NaCl, 100 mM Arginine, 4.5% glycerol, and 0.75% w/v CHAPS, and elute at ~11 mL on the Superose 6 column and in the void volume of Superdex 200 columns. Assembled nanoparticles were sterile filtered (0.22 µm) immediately prior to column application and following pooling of fractions.

**Endotoxin measurements**

Endotoxin levels in protein samples were measured using the EndoSafe Nexgen-MCS System (Charles River). Samples were diluted 1:50 or 1:100 in Endotoxin-free LAL reagent water, and applied into wells of an EndoSafe LAL reagent cartridge. Charles River Endo-Scan-V software was used to analyze endotoxin content, automatically back-calculating for the dilution factor. Endotoxin values were reported as EU/mL which were then converted to EU/mg based on UV/vis measurements. Our threshold for samples suitable for immunization was < 50 EU/mg.

**Pigtail macaque immunization**

Two Pigtail macaques were immunized with 250 µg of RBD-12GS-IS3-50 nanoparticle (88 µg RBD antigen) with AddaVax at day 0, day 28, and O/W at day 168. Blood was collected every 14 days post-prime. Blood was collected in serum collection tubes and allowed to clot at room temperature. Serum was isolated after a 15 min spin at 1455 x g for 15 min and stored at −80°C until use. Prior to vaccination or blood collection, animals were sedated with an intramuscular injection (10 mg/kg) of ketamine (Ketaset®; Henry Schein). Prior to inoculation, immunogen suspensions were gently mixed 1:1 vol/vol with AddaVax adjuvant for immunizations 1 and 2 and O/W for immunization 3 (VFI) to reach a final concentration of 0.250 mg/mL antigen. The vaccine was delivered intramuscularly into both quadriceps muscles with 1 mL per injection site on days 0, 28, and 168. All injection sites were shaved prior to injection. Animals were observed daily for general health (activity and appetite, urine/feces output) and for evidence of reactogenicity at the vaccine inoculation site (swelling, erythema, and pruritus) for up to 1 week following vaccination. They also received physical exams including temperature and weight measurements at each study time point. None of the animals became severely ill during the course of the study nor required euthanasia.

**Rhesus macaque immunization**

Adapted from Arunachalam et al. (2021). AS03 was kindly provided by GSK Vaccines. AS03 is an oil-in-water emulsion that contains 11.86 mg a-tocopherol, 10.69 mg squalene, and 4.86 mg polysorbate 80 (Tween-80) in PBS. For each dose, RBD-NP was diluted to 50 µg/ml (RBD component) in 250 µL of Tris-buffered saline (TBS) and mixed with an equal volume of AS03. The dose of AS03 was 50% v/v (equivalent of one human dose). Soluble HexaPro was diluted to 50 µg/ml in 250 µL of Tris-buffered saline (TBS) and mixed with an equal volume of AS03. All immunizations were administered via the intramuscular route in right forelimbs. The volume of each dose was 0.5 ml.

**Deep mutational scanning**

All mutations that escape serum antibody binding were mapped via a deep mutational scanning approach (Greaney et al., 2020, 2021b). We used previously described yeast-display RBD mutant libraries (Greaney et al., 2020; Starr et al., 2020a). Briefly, duplicate mutant libraries were constructed in the spike receptor binding domain (RBD) from SARS-CoV-2 (isolate Wuhu-Hu-1, GenBank: MN908947, residues N331-T531) and contain 3,804 of the 3,819 possible amino-acid mutations, with > 95% present as single mutants. Each RBD variant was linked to a unique 16-nucleotide barcode sequence to facilitate downstream sequencing. As previously
described, libraries were sorted for RBD expression and ACE2 binding to eliminate RBD variants that are completely misfolded or non-functional (i.e., lacking modest ACE2 binding affinity) (Greaney et al., 2020). Antibody escape mapping experiments were performed in biological duplicate using two independent mutant RBD libraries, with minor modifications from Greaney et al. (2020), and exactly as described in Greaney et al. (2021b). The antibody escape mapping for the vaccinated NHP serum was performed in this study; the antibody escape mapping from convalescent human plasma was performed in Greaney et al. (2021b). Briefly, mutant yeast libraries induced to express RBD were washed and incubated with plasma or serum at a range of dilutions for 1 h at room temperature with gentle agitation. For each sample, we chose a sub-saturating dilution such that the amount of fluorescent signal due to plasma antibody binding to RBD was approximately equal across samples. A 1:1000 dilution was used for the vaccinated NHP serum, and the exact dilutions of human convalescent plasma are reported in Greaney et al. (2021b). After the antibody incubations, the libraries were secondarily labeled with 1:100 FITC-conjugated anti-MYC antibody (Immunology Consultants Lab, CYMC-45F) to label for RBD expression and 1:200 Alexa-647-conjugated goat anti-human-IgA+IgG+IgM (Jackson ImmunoResearch 109-605-064) to label for bound serum or plasma antibodies. A flow cytometric selection gate was drawn to capture approximately 5% of the RBD mutant libraries with the lowest amount of plasma binding for their degree of RBD expression. For each sample, approximately 10 million RBD+ cells were processed on the cytometer. Antibody-escaped cells were grown overnight in SD-CAA (6.7g/L Yeast Nitrogen Base, 5.0g/L Casamino acids, 1.065 g/L MES acid, and 2% w/v dextrose) to expand cells prior to plasmid extraction.

Plasmid samples were prepared from pre-selection and overnight cultures of antibody-escaped cells (Zymoprep Yeast Plasmid Miniprep II) as previously described (Greaney et al., 2020). The 16-nucleotide barcode sequences identifying each RBD variant were amplified by PCR and sequenced on an Illumina HiSeq 2500 with 50 bp single-end reads as described in Greaney et al. (2020 and Starr et al. (2020a)). Escape fractions were computed as described in Greaney et al. (2020), and exactly as described in Greaney et al. (2021b). We used the dms_variants package (https://jbloomlab.github.io/dms_variants/, version 0.8.2) to process Illumina sequences into counts of each barcoded RBD variant in each pre-sort and antibody-escape population using the barcode/RBD look-up table from Starr et al. (2021).

For each serum selection, we computed the “escape fraction” for each barcoded variant using the deep sequencing counts for each variant in the original and serum-escape populations and the total fraction of the library that escaped antibody binding via the formula provided in Greaney et al. (2020). These escape fractions represent the estimated fraction of cells expressing that specific variant that fall in the antibody escape bin, such that a value of 0 means the variant is always bound by serum and a value of 1 means that it always escapes serum binding. We then applied a computational filter to remove variants with low sequencing counts or highly deleterious mutations that might cause antibody escape simply by leading to poor expression of properly folded RBD on the yeast cell surface (Greaney et al., 2020; Starr et al., 2020a). Specifically, we removed variants that had (or contained mutations with) ACE2 binding scores <-2.35 or expression scores <-1, using the variant- and mutation-level deep mutational scanning scores from Starr et al. (2020a). Note that these filtering criteria are slightly more stringent than those used in Greaney et al. (2020) but are identical to those used in Greaney et al. (2021b) and Starr et al. (2020b).

We next deconvolved variant-level escape scores into escape fraction estimates for single mutations using global epistasis models (Otwinowski et al., 2018) implemented in the dms_variants package, as detailed at (https://jbloomlab.github.io/dms_variants/dms_variants.globalepistasis.html) and described in Greaney et al. (2020). The reported escape fractions throughout the paper are the average across the libraries (correlations shown in Figure S2); these scores are also in Table S1 and at https://github.com/jbloomlab/SARS-CoV-2-RBD_MAP_RBD-nano-vax-NHP1/blob/main/results/supp_data/NHP_CCS_raw_data.csv. Sites of strong escape from each antibody were determined heuristically as sites whose summed mutational escape scores were at least 10 times the median sitewise sum of selection, and within 10-fold of the sitewise sum of the most strongly selected site. Sites shown in Figures 2 and S2A are the sites of strong escape for any of the three human convalescent plasma, plus sites 417, 452, and 501 due to their prevalence in circulating SARS-CoV-2 variants. For each plasma, the y axis is scaled to be the greatest of (a) the maximum site-wise escape metric observed for that plasma, (b) 20x the median site-wise escape fraction observed across all sites for that plasma, or (c) an absolute value of 1.0 (to appropriately scale plasma that are not “noisy” but for which no mutation has a strong effect on plasma binding). Full documentation of the computational analysis is at https://github.com/jbloomlab/SARS-CoV-2-RBD_MAP_RBD-nano-vax-NHP1. These results are also available in a zoomable, interactive form at https://jbloomlab.github.io/SARS-CoV-2-RBD_MAP_RBD-nano-vax-NHP1/.

ELISA

For anti-S2P ELISA, 25 μL of 2 μg/mL S2P was plated onto 384-well Nunc Maxisorp (ThermoFisher) plates in PBS and sealed overnight at RT. The next day plates were washed 4 x in Tris Buffered Saline Tween (TBST) using a plate washer (BioTek) and blocked with SuperBlock (ThermoFisher) for 1 h at 37°C. Plates were washed 4 x in TBST and 1:5 serial dilutions of mouse, NHP, or human sera were made in 25 μL TBST and incubated at 37°C for 1 h. Plates were washed 4 x in TBST, then anti-mouse (Invitrogen), anti-NHP (AlphaDiagnostics), or anti-human (Invitrogen) horseradish peroxidase-conjugated antibodies were diluted 1:5,000 and 25 μL added to each well and incubated at 37°C for 1 h. Plates were washed 4 x in TBST and 25 μL of TMB (SeraCare) was added to every well for Cell 184, 5432–5447.e1–e9, October 14, 2021 e6
~5 min at room temperature. The reaction was quenched with the addition of 25 μL of 1 N HCl. Plates were immediately read at 450 nm on a BioTek plate reader and data plotted and fit in Prism (GraphPad) using nonlinear regression sigmoidal, 4PL. X is log(concentration) to determine EC50 values from curve fits.

**Competition ELISA of NHP sera with hACE2, CR3022 IgG, and S309 IgG for immobilized SARS-CoV-2 S2P**

384-well Maxisorp plates (Thermo Fisher) were coated overnight at room temperature with 3 μg/mL of SARS-CoV-2 S2P (Pallesen et al., 2017) in 20mM Tris pH 8 and 150mM NaCl. Plates were slapped dry and blocked with Blocker Casein in TBS (Thermo Fisher) for one hour at 37°C. Plates were slapped dry and NHP sera was serially diluted 1:4 in TBST with an initial dilution of 1:4 for hACE2 competition or 1:2 for antibody competition. Random primary amine biotinylated (Thermo Fisher) hACE2-Fc, CR3022 (Yuan et al., 2020), or S309 (Pinto et al., 2020) were added, bringing the concentration of each well to the EC50 values of 0.2nM, 2nm, and 0.01nM, respectively. Plates were left for one hour at 37°C, then washed 4x with TBST using a 405 TS Microplate Washer (BioTek) followed by addition of 1:500 streptavidin-HRP (Thermo Fisher) for one hour at 37°C. Plates were washed 4x and TMB Microwell Peroxidase (Seracare) was added. The reaction was quenched after 1-2 minutes with 1 N HCl and the A450 of each well was read using a BioTek plate reader (BioTek). Data plotted and fit in Prism (GraphPad) using nonlinear regression sigmoidal, 4PL. X is log(concentration) to determine EC50 values from curve fits with upper and lower constraints determined by uncompeted ELISA per antigen.

**Competition ELISA of mouse sera and immobilized hACE2 or mAbs with SARS-CoV-2 or SARS-CoV S2P**

384-well Maxisorp plates (Thermo Fisher) were coated overnight at room temperature with 3 μg/mL of hACE2-Fc, CR3022 (Yuan et al., 2020), or S309 (Pinto et al., 2020) in 20mM Tris pH 8 and 150mM NaCl. Plates were slapped dry and blocked with Blocker Casein in TBS (Thermo Fisher) for one hour at 37°C. Plates were slapped dry and a 30-minute pre-incubated 1:5 serial dilution of mouse sera in TBST, with in initial dilution of 1:50 for hACE2-Fc competition or 1:10 for antibody competition, and a constant concentration of biotinylated (Avidity) SARS-CoV-2 S2P or SARS-CoV 2P at their EC50 values were added. Spike concentrations were 0.63nM, 5.98nM, and 0.22nM of SARS-CoV-2 S2P or 4.11nM, 2.89nM, and 0.19nM of SARS-CoV S2P for immobilized hACE2, CR3022, and S309, respectively. Plates were left for one hour at 37°C, then washed 4x with TBST using a 405 TS Microplate Washer (BioTek) followed by addition of 1:500 streptavidin-HRP (Thermo Fisher) for one hour at 37°C. Plates were washed 4x and TMB Microwell Peroxidase (Seracare) was added. The reaction was quenched after 1-2 minutes with 1 N HCl and the A450 of each well was read using a BioTek plate reader (BioTek). Data plotted and fit in Prism (GraphPad) using nonlinear regression sigmoidal, 4PL. X is log(concentration) to determine EC50 values from curve fits with upper and lower constraints determined by uncompeted ELISA per antigen.

**Pseudovirus production**

MLV-based D614G SARS-CoV-2 S and SARS-CoV S pseudotypes were prepared as previously described (Millet and Whittaker, 2016; Walls et al., 2020a, 2020b). Briefly, HEK293T cells were co-transfected using Lipofectamine 2000 (Life Technologies) with an S-encoding plasmid, an MLV Gag-Pol packaging construct, and the MLV transfer vector encoding a luciferase reporter according to the manufacturer’s instructions. Cells were washed 3 x with Opti-MEM and incubated for 5 h at 37°C with transfection medium. DMEM containing 10% FBS was added for 60 h. The supernatants were harvested by spinning at 2,500 g, filtered through a 0.45 μm filter, concentrated with a 100 kDa membrane for 10 min at 2,500 g and then aliquoted and stored at –80°C.

HIV-based pseudotypes were prepared as described previously (Crawford et al., 2020). Briefly, HEK293T cells were cotransfected using Lipofectamine 2000 (Life Technologies) with an S-encoding plasmid (D614G SARS-CoV-2 S (YP 009724390.1), Pangolin-Guangdong S (QLR06867.1), SARS-CoV-S (YP 009825051.1), P1, B.1.351 S, B.1.1.7, and B.1.1.7+E484K S) in an HIV Gag-Pol, Tat, Rev1B packaging construct, and the HIV transfer vector encoding a luciferase reporter according to the manufacturer’s instructions. Cells were washed 3 x with Opti-MEM and incubated for 5 h at 37°C with transfection medium. DMEM containing 10% FBS was added for 60 h. The supernatants were harvested by spinning at 2,500 g, filtered through a 0.45 μm filter, concentrated with a 100 kDa membrane for 10 min at 2,500 g and then aliquoted and stored at –80°C.

D614G SARS-CoV-2 S (YP 009724390.1), D614G SARS-CoV-2 point mutants, SHC014 (AGZ48806.1), RaTG13 S (QHR63300.2), Pangolin-Guangdong S (QLR06867.1), SARS-CoV S (YP 009825051.1), WIV1 S (AGZ48831.1), B.1.351 S, P1, and B.1.1.7 S pseudotyped VSV viruses were prepared as described previously (McCallum et al., 2021a; Sauer et al., 2021). Briefly, 293T cells in DMEM supplemented with 10% FBS, 1% PenStrep seeded in 10-cm dishes were transfected with the plasmid encoding for the corresponding S glycoprotein using lipofectamine 2000 (Life Technologies) following manufacturer’s indications. One day post-transfection, cells were infected with VSV(G*-D614G-luciferase) and after 2 h were washed five times with DMEM before adding medium supplemented with anti-VSV-G antibody (11- mouse hybridoma supernatant, CRL-2700, ATCC). Virus pseudotypes were harvested 18-24 h post-inoculation, clarified by centrifugation at 2,500 x g for 5 min, filtered through a 0.45 μm cut off membrane, concentrated 10 times with a 30 kDa cut off membrane, aliquoted and stored at –80°C.

**Pseudovirus neutralization**

HEK293-hACE2 cells (Crawford et al., 2020) were cultured in DMEM with 10% FBS (Hyclone) and 1% PenStrep with 8% CO2 in a 37°C incubator (ThermoFisher). One day prior to infection, 40 μL of poly-lysine (Sigma) was placed into 96-well plates and incubated with rotation for 5 min. Poly-lysine was removed, plates were dried for 5 min then washed 1 x with water prior to plating with 40,000
cells. The following day, cells were checked to be at 80% confluence. In an empty half-area 96-well plate a 1:3 serial dilution of sera was made in DMEM and diluted pseudovirus was then added to the serial dilution and incubated at room temperature for 30-60 min. After incubation, the sera-virus mixture was added to the cells at 37°C and 2 hours post-infection, 40 μL 20% FBS-2% PenStrep DMEM was added. After 17-20 hours (VSV) or 48 hours (HIV, MLV) 40 μL well of One-Glo-EX substrate (Promega) was added to the cells and incubated in the dark for 5-10 min prior reading on a BioTek plate reader. Measurements were done in at least duplicate. Relative luciferase units were plotted and normalized in Prism (GraphPad). Nonlinear regression of log(inhibitor) versus normalized response was used to determine IC₅₀ values from curve fits. Kruskal Wallis tests were used to compare two groups to determine whether they were statistically different.

**Sarbecovirus biolayer interferometry binding analysis**

Purification of Fabs from NHP serum was adapted from Boyoglu-Barnum et al. (2021). Briefly, 1 mL of day 70 serum was diluted to 10 mL with PBS and incubated with 1 mL of 3 PBS-washed protein A beads (GenScript) with agitation overnight at 37°C. The next day beads were thoroughly washed with PBS using a gravity flow column and bound Abs were eluted with 0.1 M glycine pH 3.5 into 1M Tris-HCl (pH 8.0) to a final concentration of 100 mM. Serum and early washes that flowed through were re-bound to beads overnight again for a second, repeat elution. IgGs were concentrated (Amicon 30 kDa) and buffer exchanged into PBS. 2 x digestion buffer (40 mM sodium phosphate pH 6.5, 20 mM EDTA, 40 mM cysteine) was added to concentrated and pooled IgGs. 500 μL of resuspended immobilized papain resin (ThermoFisher Scientific) freshly washed in 1 x digestion buffer (20 mM sodium phosphate, 10 mM EDTA, 20 mM cysteine, pH 6.5) was added to purified IgGs in 2 x digestion buffer and samples were agitated for 5 h at 37°C. The supernatant was separated from resin and resin washes were collected and pooled with the resin flow through. Pooled supernatants were sterile-filtered at 0.22 μm and applied 6 x to PBS-washed protein A beads in a gravity flow column. The column was eluted as described above and the papain procedure repeated overnight with undigested IgGs to increase yield. The protein A flow throughs were pooled, concentrated (using an Amicon 10 kDa) and buffer exchanged into PBS. Purity was checked by SDS-PAGE. Assays were performed and analyzed using biolayer interferometry on an Octet Red 96 System (Pall ForteBio/Sartorius) at ambient temperature with shaking at 1000 rpm. Different Sarbeco RBDs were purified like in Walls et al., 2020 and were diluted with different acetate buffers and applied to a black 96-well Greiner Bio-one microplate at 200μL per well. GD-Pangolin RBD was diluted in pH 6 buffer to 5 μg/mL, RmNY02 were diluted in pH 5 to 25 μg/mL, WIV16 was diluted in pH 5 to 10 μg/mL, SARS-CoV-2 was diluted in pH 6 to 5 μg/mL, RaTG13 was diluted in pH 6 to 10 μg/mL. RaTG13 was diluted in pH 6 to 10 μg/mL, SARS-CoV was diluted in pH 6 to 50 μg/mL, and finally ZXC21 was diluted in pH 5 to 10μg/mL. AR2G biosensors (ForteBio/Sartorius) following 600 s hydration were normalized in water for 180 s. Then tips were NHS-EDC activated for 300 s and the different sarbecovirus RBDs were loaded up to a 1.50 nm threshold for up to 600 s. Immobilized RBDs on the tips were quenched for 300 s in ethanolamine and dipped into kinetics buffer for a 60 s baseline. The association step was performed by dipping the mobilized RBDs into diluted purified polyclonal pigtail macaque IgGs for 600 s. Dissociation was measured by inserting the biosensors in kinetics buffer for 600 s. The data were baseline subtracted and the plots fitted using the Pall ForteBio/Sartorius analysis software (version 12.0).

**Cocktail and mosaic bio-layer interferometry (antigenicity)**

Binding of hACE2-Fc to monovalent RBD-I53-50 nanoparticles, mosaic-RBD-I53-50 nanoparticles, and cocktail of RBD-nanoparticles was analyzed for antigenicity experiments and real-time stability studies using an Octet Red 96 System (Pall ForteBio/Sartorius) at ambient temperature with shaking at 1000 rpm. Protein samples were diluted to 100 nM in Kinetics buffer (1× HEPES-EP+ (Pall Forté Bio), 0.05% nonfat milk, and 0.02% sodium azide). Buffer, receptor, and analyte were then applied to a black 96-well Greiner Bio-one microplate at 200 μL per well. Protein A biosensors (ForteBio/Sartorius) were first hydrated for 10 minutes in Kinetics buffer, then dipped into hACE2-Fc diluted to 10 μg/mL in Kinetics buffer in the immobilization step. After 150 s, the tips were transferred to kinetics buffer for 60 s to reach a baseline. The association step was performed by dipping the loaded biosensors into the immunogens for 300 s, and subsequent dissociation was performed by dipping the biosensors back into Kinetics buffer for an additional 300 s. The data were baseline subtracted prior for plotting using the FortéBio analysis software (version 12.0). Plots in Figure S3 show the 600 s of association and dissociation.

**Sandwich bio-layer interferometry (mosaic display antigenicity)**

Binding of hACE2-Fc or S2H14 mAb and S230 Fab to WIV1-RBD-I53-50, RaTG13-RBD-I53-50, SARS-CoV-SARS-CoV2-RBD-I53-50, SARS-CoV2-I53-50, and mosaic-RBD-I53-50 nanoparticles were analyzed for co-display of RBDs using an Octet Red 96 System (Pall ForteBio/Sartorius) at ambient temperature with shaking at 1000 rpm. Nanoparticles were diluted to 100 nM in Kinetics buffer. Kinetics buffer, mAb, nanoparticles and Fab were then applied to a black 96-well Greiner Bio-one microplate at 200 μL per well. Protein A biosensors (ForteBio/Sartorius) were first hydrated for 10 minutes in Kinetics buffer, then dipped into hACE2-Fc or S2H14 mAb diluted to 10 μg/mL in Kinetics buffer in the immobilization step. After 150 s, the tips were transferred to Kinetics buffer for 60 s to reach a baseline. The receptor or mAb was then loaded with nanoparticle by dipping the loaded biosensors into the immunogens for 300 s, and subsequent baseline was performed by dipping the biosensors back into the Kinetics buffer for an additional 60 s. Association of S230 Fab diluted to 100 nM in Kinetics buffer was then measured for 300 s and subsequent dissociation in Kinetics buffer of S230 Fab for 300 s. The data were baseline subtracted prior for plotting using the FortéBio analysis software (version 12.0). Plots in Figure S3 exclude the initial mAb loading and the first baseline.
Cocktail and mosaic negative stain electron microscopy

Monovalent RBD-I53-50 nanoparticles, mosaic-RBD-I53-50 nanoparticles, and cocktail of RBD-nanoparticles were first diluted to 75 μg/mL in 50 mM Tris pH 7.4, 185 mM NaCl, 100 mM Arginine, 4.5% v/v Glycerol, 0.75% w/v CHAPS prior to application of 3 μL of sample onto freshly glow-discharged 300-mesh copper grids. Sample was incubated on the grid for 1 minute before the grid was dipped into a 50 μL droplet of water and excess liquid blotted away with filter paper (Whatman). The grids were then dipped into 6 μL of 0.75% w/v uranyl formate stain. Stain was blotted off with filter paper, then the grids were dipped into another 6 μL of stain and incubated for ~70 s. Finally, the stain was blotted away and the grids were allowed to dry for 1 minute. Prepared grids were imaged in a Talos model L120C electron microscope at 57,000 × (nanoparticles).

Cocktail and mosaic dynamic light scattering

Dynamic Light Scattering (DLS) was used to measure hydrodynamic diameter (Dh) and % Polydispersity (%Pd) of monovalent RBD-I53-50 nanoparticles, mosaic-RBD-I53-50 nanoparticles, and cocktail of RBD-nanoparticles on an UNcle Nano-DSF (UNchained Laboratories). Sample was applied to a 8.8 L quartz capillary cassette (UNi, UNchained Laboratories) and measured with 10 acquisitions of 5 s each, using auto-attenuation of the laser. Increased viscosity due to 4.5% v/v glycerol in the RBD nanoparticle buffer was accounted for by the UNcle Client software in Dh measurements.

Mouse immunizations and challenge

At six weeks of age, 8 female BALB/c mice per dosing group were vaccinated with a prime immunization, and three weeks later mice were boosted with a second vaccination (IACUC protocol 4470.01). Prior to inoculation, immunogen suspensions were gently mixed 1:1 vol/vol with AddaVax adjuvant (Invivogen, San Diego, CA) to reach a final concentration of 0.01 mg/mL antigen. Mice were injected intramuscularly into the gastrocnemius muscle of each hind leg using a 27-gauge needle (BD, San Diego, CA) with 50 μL per injection site (100 μL total) of immunogen under isoflurane anesthesia. To obtain sera all mice were bled two weeks after prime and boost immunizations. Blood was collected via submental venous puncture and rested in 1.5 mL plastic Eppendorf tubes at room temperature for 30 min to allow for coagulation. Serum was separated from red blood cells via centrifugation at 2,000 g for 10 min. Complement factors and pathogens in isolated serum were heat-inactivated via incubation at 56 °C for 60 min. Serum was stored at 4 °C or −80 °C until use. The study was repeated twice. Five weeks post-boost, mice (aged 14 weeks) were exported from Comparative Medicine Facility at the University of Washington, Seattle, WA to an AAALAC accredited Animal Biosafety Level 3 (ABSL3) Laboratory at the University of North Carolina, Chapel Hill. After a 7-day acclimation time, mice were anesthetized with a mixture of ketamine/xylazine and challenged intranasally with 10^5 plaque-forming units (pfu) of mouse-adapted SARS-CoV-2 MA10 or SARS-CoV MA15 strain for the evaluation of vaccine efficacy (IACUC protocol 20-114.0). After infection, body weight and congestion score were monitored daily until the termination of the study two days post-infection, when lung and nasal turbinate tissues were harvested to evaluate viral load by plaque assay.

Histopathology of post challenge mouse lungs

For each mouse the left lung was incubated in formalin at 4 °C for at least 7 days to fix tissue and inactivate virus. The fixed tissue was processed and embedded in paraffin. 5 μm sections were cut and stained with either Congo red or hematoxylin and eosin (H&E). Eosinophils were enumerated by counting the Congo red positive leukocytes in 10 high power fields (400X final magnification) per mouse lung. Representative images were minimally and similarly adjusted with Adobe Photoshop 2020 to enhance contrast. Airway pathology was assessed in H&E stained sections to assess bronchial epithelial cell death (score: 0 = no dead cells, 1 = 1-5 dead cells, 2 = 6-10 dead cells, 3 = 11-20 dead cells and 4 = > 20 dead cells; scored for 10 400X fields per mouse lung), peribronchial inflammation (score 0 = none, 1 = 0%–25% circumference with > 1 leukocyte cell layer, 2 = 26%–50% circumference with > 1 leukocyte cell layer, and 3 = 50%–100% circumference with > 1 leukocyte cell layer; scored for 10 400X fields per mouse lung), and interstitial pneumonia (score = percentage of pulmonary alveolar parenchyma with septae expanded by leukocyte; scored for 10 100X fields).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical details of experiments can be found in the figure legends. For NHP experiments, 2-6 sera samples were used and experiments were done in at least duplicate unless mentioned. For mouse ELISAs, neutralization, and challenge experiments, sera from 6, 8, or 10 BALB/c animals were used and experiments were completed in at least duplicate unless mentioned. Geometric mean titers were calculated. Kruskal Wallis tests were performed to compare two groups to determine whether they were statistically different for ELISA and neutralization experiments. Significance is indicated with stars: *, p < 0.05; **** p < 0.0001 and non significant groups are not shown.
Figure S1. Effects of mutations on binding of HCP Abs to RBD and FACS gating strategy, related to Figure 2
(A) Correlation plots of site- and mutation-level escape for each of the two independent RBD mutant libraries for the Ab-escape map shown in Figure 2B. Site-level escape is the sum of the escape fractions for each mutation at a site. (B) Hierarchical FACS gating strategy used for selecting yeast cells expressing Ab-escape RBD variants. First, gates are selected to enrich for single cells (SSC-A versus FSC-A, and FSC-W versus FSC-H) that also express RBD (FITC-A versus FSC-A, cells in pink). Second, cells expressing RBD mutants with reduced polyclonal Ab binding, detected with an anti-IgA+IgG+IgM secondary Ab, were selected with a gate that captured the ~5% of cells with the lowest Ab binding (cells in blue).
Figure S2. Evaluation of vaccine-elicited binding and neutralizing Ab titers against SARS-CoV-2 variants and sarbecoviruses, related to Figure 3

(A) Wild-type (Wuhan-Hu-1) and B.1.351 SARS-CoV-2 RBD-specific Ab binding titers of RBD-NP-elicited sera in pigtail macaques (magenta, n = 2) and rhesus macaques (blue, n = 5), HexaPro S-elicited sera in rhesus macaques (gray, n = 5), or HCP (orange, n = 6, Table S1) analyzed by ELISA with an LOD of 1x10^2. (B) Cladogram based on sarbecovirus RBD amino acid sequences. (C) Biolayer interferometry analysis of binding of 1 μM purified polyclonal pigtail macaque IgGs (obtained 70 days post prime) to sarbecovirus RBDs immobilized at the surface of biosensors. (D) SARS-CoV-2 S2P (left) or SARS-CoV S2P (right) Ab binding titers of RBD-NP-elicited sera in pigtail macaques (magenta) or HexaPro S-elicited sera in rhesus macaques (gray) analyzed by ELISA with an LOD of 2.5x10^1. (E) Competition ELISA between 0.13 nM human ACE2-Fc and RBD-NP-elicited sera in pigtail macaques (magenta) and rhesus macaques (blue), or HexaPro S-elicited sera in rhesus macaques (gray) at various time points following vaccination, benchmarked against COVID-19 HCP with an LOD of 4x10^1, showing the magnitude of inhibition of ACE2 binding to immobilized SARS-CoV S2P expressed as reciprocal serum dilution blocking 50% of the maximum binding response. Statistical significance was determined by Kruskal-Wallis test and shown when significant. **, p < 0.01. All data repeated twice. LODs are shown as gray horizontal dotted lines. Raw data curves shown in Data S1.
Figure S3. In vitro characterization and confirmation of co-display of sarbecovirus RBD-NP immunogens, related to Figure 4

(A) Design models of the various vaccine candidates evaluated. Scale bars, 36 nm. (B) SDS-PAGE analysis of purified nanoparticles. DTT, dithiothreitol; F/T, freeze/thaw. (C) Dynamic light scattering. (D) Electron micrographs of negatively stained samples. Scale bars, 50 nm. (E) Binding of 100 nM SEC-purified nanoparticle immunogens and the non-assembling cocktail immunogen (which was not purified with SEC) to immobilized hACE2-Fc. (F) SEC chromatogram overlay of purified RBD-NP and non-assembling cocktail. (G-H) Sandwich biolayer interferometry. The SARS-CoV-2 S-specific mAb S2H14 immobilized on protein A biosensors was used to capture various nanoparticle immunogens from 300-480 s. The captured nanoparticles were subsequently exposed to a Fab derived from the SARS-CoV S-specific mAb S230 from 600-900 s (G). hACE2-Fc immobilized on protein A biosensors was used to capture various nanoparticle immunogens from 300-480 s. The captured nanoparticles were subsequently exposed to a Fab derived from the SARS-CoV S-specific mAb S230 from 600-900 s (H).
Figure S4. Serum Ab binding titers elicited by mosaic and cocktail RBD-NPs, related to Figure 5

(A) Ab binding titers to SARS-CoV-2 S2P at five weeks post prime analyzed by ELISA with an LOD of $1 \times 10^2$. (B–E) Titers of SARS-CoV-2 S-specific Abs competing with ACE2-Fc with an LOD of $5 \times 10^1$ (B), CR3022 with an LOD of $5 \times 10^1$ (C), S309 with an LOD of $1 \times 10^1$ (D), and S2X259 with an LOD of $1 \times 10^1$ (E) in immunized mouse sera analyzed by competition ELISA. (F) Ab binding titers to SARS-CoV S2P at week 5 analyzed by ELISA. (G–H) Ab binding titers to the WIV1 (G), and RaTG13 (H) RBDs analyzed by ELISA with an LOD of $1 \times 10^2$. Statistical significance was determined by Kruskal Wallis test and shown when significant. **$p < 0.01$. LODs are shown as gray horizontal dotted lines. Raw data curves shown in Data S1.
Figure S5. Monovalent, mosaic, and cocktail RBD-NPs protect against heterotypic SARS-CoV-MA15 challenge in 15-week-old BALB/c cByJ mice, related to Figure 6

(A) Normalized active inflammation following SARS-CoV MA 15 challenge shown in Figure 6 with venulitis, endarteritis, and interstitial pneumonitis shown as stacked bar graphs in dark gray, light gray, and black respectively. (B) Weight loss following SARS-CoV MA15 challenge (N = 6). Unvaccinated animals are shown as black circles. (C) Congestion score following SARS-CoV MA15 challenge with a score of 0 indicating unchanged lung color and 4 indicating a darkened and diseased lung (N = 6). (D) Viral titers in mice lungs (expressed in plaque forming units per lobe) following challenge (N = 8) with an LOD of 9x10^1. Statistical significance was determined by Kruskal Wallis test and shown when significant and **p < 0.01. LOD is shown as a gray horizontal dotted line.