Nanoparticle display of prefusion coronavirus spike elicits S1-focused cross-reactive protection across divergent subgroups

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ABSTRACT

Multivalent antigen display is a fast-growing area of interest towards broadly protective vaccines. Current nanoparticle-based vaccine candidates demonstrate the ability to confer antibody-mediated immunity against divergent strains of notably mutable viruses. In coronaviruses, this work is predominantly aimed at targeting conserved epitopes of the receptor-binding domain. However, targeting other conserved non-RBD epitopes could further limit the potential for antigenic escape. To further explore new potential targets, we engineered protein nanoparticles displaying CoV_S-2P trimers derived from MERS-CoV, SARS-CoV-1, SARS-CoV-2, hCoV-HKU1, and hCoV-OC43 and assessed their immunogenicity in mice. Monotypic SARS-1_S-2P nanoparticles elicited cross-neutralizing antibodies against MERS_S and protected against MERS-CoV challenge. MERS and SARS-I53_dn5 nanoparticles elicited S1-focused antibodies, revealing a conserved site on the NTD. Moreover, mosaic nanoparticles co-displaying distinct CoV_S-2P trimers elicited antibody responses to distant cross-group antigens while protecting against MERS challenge despite diminished valency of MERS_S-2P. Our findings will inform further efforts towards the development of pan-coronavirus vaccines.

INTRODUCTION

Coronaviruses (CoVs) comprise a broad family of viruses with diverse strains known to infect mammals and birds. Betacoronaviruses (β-CoVs) in particular thrive in animal reservoirs and represent a constant threat to human health. Inclusive of the most recently emerged β-CoV, SARS-CoV-2, there are seven CoVs known to infect humans (human CoVs or hCoVs); four of which circulate endemically\(^1,2\). Although endemic hCoV infections typically manifest as mild respiratory disease, zoonotic spillover of β-CoVs into human populations has been associated...
with high disease morbidity and mortality, economic burden, and widespread global epidemics\(^3\). Since the emergence of SARS-CoV-2 in late 2019, over 600 million infections and nearly 7 million deaths worldwide\(^4\) have been attributed to COVID-19, the disease caused by SARS-CoV-2, underscoring the need for broadly protective CoV vaccines. In addition to the four endemic human coronaviruses— hCoV-OC43, hCoV-HKU1, hCoV-229E, and hCoV-NL63—the emergence of the epidemic- and pandemic-causing SARS-CoV-1, MERS-CoV, and SARS-CoV-2 from zoonotic spillover events has resulted in outbreaks with substantial morbidity and mortality\(^2,5\). Evidenced by the ongoing pandemic, SARS-CoV-2 will continue to evolve and is likely to become the next endemic human coronavirus. The emergence of SARS-CoV-2 variants of concern (VOC), particularly the delta and omicron VOC (Pango naming of B.1.617.2 and B.1.1.529, respectively), highlights the threat of viral escape from antibodies induced by the currently available COVID-19 vaccines and the need for next-generation vaccines that are capable of inducing broadly protective immunity against a wide range of CoVs\(^6\). Recently, there has been documentation of broad cross-reactivity either deriving from natural infection\(^7\), or from immunogens delivering multiple sarbecoviral antigens\(^8,9,10\). There is much interest in designing immunogens to target antibody responses to domains of the S1 subunit at the apex of the spike (S) protein\(^11,12\)—particularly the Receptor Binding Domain (RBD), as this region is targeted by potently neutralizing antibodies (nAbs)\(^13\). However, as evidenced by the omicron VOC, the RBD is particularly susceptible to mutation and recombination leaving room for immune evasion via antigenic drift and shift. Others have previously shown elicitation of broad protection from influenza through the multivalent display of hemagglutinin on the I53_dn5 nanoparticle platform\(^14\). Applying lessons learned from these studies here, we describe nanoparticles that display spike trimer from diverse coronaviruses. We show that CoV prefusion-stabilized S (CoV_S-2P) trimers displayed on I53_dn5 self-assembling nanoparticles are able to elicit broadly cross-reactive and protective antibody responses. Furthermore, by co-displaying these
diverse spikes in a mosaic antigen array, we induce robust and protective immunity even at low valency of individual S proteins

RESULTS

**Immunogen design and characterization**

The introduction of two proline mutations (2P) at the apex of the central helix of a wide range of coronavirus spike proteins has previously been shown to stabilize the prefusion conformation and elicit potent antibody responses. We applied these stabilizing mutations to the S proteins of MERS-CoV, SARS-CoV-1, and SARS-CoV-2—the three epidemic and pandemic-causing β-hCoVs and adapted these antigens for display on the computationally designed two-component nanoparticle, I53_dn5. S-2P antigens were genetically fused to the trimeric component I53_dn5B and assembled in vitro by the addition of the pentameric component I53_dn5A to generate monotypic particles displaying 20 copies of the specified S-2P trimer. During size exclusion chromatography (SEC), peaks corresponding to S-2P_I53_dn5 nanoparticles indicated efficient assembly and formation (Fig. 1b). Purified S-2P trimers and nanoparticles were tested for antigenicity by ELISA using monoclonal antibodies specific to each CoV S. Antibody binding was comparable between soluble trimer and nanoparticle in each case indicating that antigenicity is similarly intact in each formulation (Fig. 1b). The purified nanoparticles were also imaged by negative stain electron microscopy (NS-EM) showing that the nanoparticles were well-assembled and homogeneous, displaying highly-ordered S proteins (Fig. 1d). These characterization data show that, we were able to efficiently express, and assemble antigenically intact nanoparticle immunogens.
Display of SARS-1_S-2P on I53_dn5 qualitatively alters antibody cross-reactivity and potency

We hypothesized that multivalent display of CoV S-2P trimers would elicit a more robust antibody response than soluble S-2P, improving potency of potentially cross-reactive antibodies specific to broadly conserved yet potentially subdominant epitopes. To test this, we immunized BALBc/J mice twice with 10 µg of SARS-1_S-2P as a soluble trimer fused to the foldon trimerization domain or displayed on I53_dn5 nanoparticles, as well as MERS_S-2P or influenza HA (H1) displayed on I53_dn5, with Sigma adjuvant system (SAS) at weeks 0 and 3, then assessed serological responses at week 5. Sera were screened for cross-reactive IgG to SARS-1_S-2P, SARS-2_S-2P, and MERS_S-2P. Immunization with SARS-1_S-2P induced higher antibody titers against SARS-1_S-2P, SARS-2_S-2P, and MERS_S-2P than those elicited by SARS-1_I53_dn5, (Fig. 2a-c). Sera were then used to assess neutralization of SARS-1, SARS-
2, and MERS pseudoviruses. Both SARS-1_S-2P and SARS-1_I53_dn5 elicited comparable strain-matched neutralizing antibodies against SARS-1, but neither neutralized SARS-2.

Interestingly, SARS-1_I53_dn5-elicited antibodies were able to neutralize MERS pseudovirus at levels similar to MERS_I53_dn5 (Fig. 2f).

To assess the quality of the vaccine-matched and cross-reactive antibody responses, we generated correlation plots of neutralizing-to-binding antibody titers for SARS-1, SARS-2, and MERS, in which the slope indicates the ratio of neutralizing to binding activity. Because SARS-1_S-2P-elicited antibodies binding SARS-1_S-2P exceeded the limit of detection, a reliable SARS-1 correlation plot could not be generated; however, particle assembly reduced SARS-1_S-2P binding titers while neutralization remained intact. This suggests that particle assembly improved SARS-1 neutralization potency (Fig. 2g). SARS-2 correlation plots could not be generated due to the lack of detectable neutralizing antibodies (Fig. 2h). MERS correlation plots comparing quality of MERS-binding and neutralizing antibodies elicited by SARS-1_S-2P, SARS-1_I53_dn5 and MERS_I53_dn5 indicate that while the overall magnitude of MERS-neutralizing and binding antibody titers elicited from MERS_I53_dn5 nanoparticles was higher, SARS-1_I53_dn5 nanoparticles elicited more potently neutralizing antibodies with higher neutralization-to-binding ratios (Fig. 2i). To further validate the SARS-1_I53_dn5 immunogen, we performed an antigenicity ELISA with SARS-specific (S652-27) and MERS-specific (JC57-13) monoclonal antibodies (mAb) to SARS-1_I53_dn5. mAb S652-27 bound SARS-1_I53_dn5, but neither JC57-13 nor influenza-HA-specific mAb CR6261 bound indicating that SARS-1_I53_dn5 immunogens were free of MERS spike contamination (Supplementary Fig. 1).

We also performed a dose escalation study in which C57BL/6 mice were immunized at weeks 0 and 3 with 0.1, 1.0, or 10µg of SARS-1_S-2P or SARS-1_I53_dn5. ELISA binding assays revealed that SARS-1_I53_dn5-elicited antibodies able to bind MERS_I53_dn5 appeared to be dose-dependent and were only evident at 10µg (Supplementary Fig. 2). Sera from the 10µg dose of this study as well as from 2-3 independent studies also using C57BL/6
mice immunized under the same timing regimen with 10μg of SARS-1_S-2P, SARS-1_I53_dn5, or MERS_I53_dn5 were assessed for binding and neutralization. Data from these assays were aggregated and used to generate neutralization-to-binding correlation plots (supplementary Fig. 3a-i). Notably, in C57BL/6 mice, particle assembly of SARS-1_S-2P reduced cross-binding to SARS-2_S-2P while binding to MERS-S-2P increased (Supplementary Fig. 3a-b). MERS cross-neutralization from SARS-1_I53_dn5 nanoparticles was also found in C57BL/6 mice (Supplementary Fig. 3f). Soluble SARS-1_S-2P and SARS-1_I53_dn5 nanoparticles elicited similar quality antibodies against SARS_S, as indicated by their slopes, and neither group elicited neutralizing antibodies against SARS-2 resulting in a horizontal slope (Supplementary Fig. 3g). Again, SARS-2 correlation plots could not be generated due to the lack of detectable neutralizing antibodies (Supplementary Fig. 3h). MERS correlation plots generated from C57BL/6 mice also show higher overall magnitude of binding and neutralizing antibody responses elicited by MERS_I53_dn5 compared to SARS-1_I53_dn5, but comparable correlations of neutralization-to-binding (Supplementary Fig. 3i). Together, these data indicate that while SARS-1_I53_dn5 elicited MERS-reactive antibodies that were relatively low-binding, they were still able to potently neutralize MERS pseudovirus at a similar or greater level than MERS_I53_dn5 in two genetically distinct mouse strains.
Fig. 2: Assembly of SARS-1_S-2P on dn5 elicits potent cross-neutralizing antibodies.

a-f Groups of 10 female BALB/c were immunized at weeks 0 and 3 with 10 µg of SARS-1_S-2P as a soluble trimer or displayed on I53_dn5 particles, MERS_S-2P trimer or H1 trimer displayed on I53_dn5 nanoparticles with SAS adjuvant and bled at week 5 for serology. Control mice were immunized with H1_dn5.

da-c Serum was screened for binding by ELISA to SARS-1_ , SARS-2_ , and MERS_S-2P. d-f Serum was then assessed for its capacity to neutralize SARS-1, SARS-2, and MERS pseudoviruses. g-i To plot the potency of neutralizing antibodies, correlation plots of binding (x-axis) to neutralization (y-axis) where the slope (neutralization/binding) indicates the ratio of neutralizing to binding antibody titers were generated. b-g Boxes and horizontal bars denote the interquartile range (IQR) and medians, respectively. Whisker endpoints are equal to the minimum and maximum values. Statistical analysis was performed using non-parametric Kruskal-Wallis test with Dunn's multiple comparisons. *P<0.05, **P < 0.01, ****P < 0.0001.
Cross-reactive antibodies target the S1 domain of spike

We hypothesized that an antibody derived from a rare B cell population specific for a conserved site of vulnerability on the MERS-spike could be responsible for the potent cross-neutralization despite lower binding capacity. To understand the domain specificity of cross-reactive antibodies elicited by SARS-I53_dn5 to MERS-S-2P, we performed S domain-specific depletion assays where sera from mice immunized with SARS-I53_dn5 were depleted with either the full-length MERS-S-2P trimer or its subdomains: S1, S2 (stabilized stem), or RBD.

Depleted sera were then tested for residual binding to SARS-1_S-2P, SARS-2_S-2P, and MERS_S-2P. Depletion with MERS_S-2P or _S1 effectively eliminated binding to MERS_S-2P, while binding remained intact after depletion with MERS_S2 (Fig. 3a-c). This indicates that cross-reactive antibodies induced by immunization with SARS_I53_dn5 to SARS-2_S-2P and MERS_S-2P are specific to the S1 subunit. Notably, depletion with MERS RBD did not result in a statistically significant loss in MERS-binding IgG titers (Fig. 3c), suggesting that there may be potent and highly conserved epitopes elsewhere in S1, such as in the N-terminal domain (NTD), that may be responsible for the observed cross-neutralization. Interestingly, when sera depleted of MERS-specific antibodies were assayed for binding to HKU1_S-2P, binding patterns were the same as for non-depleted sera; MERS_S1 depletion did not reduce binding to HKU1_S-2P as it did against SARS-2_S-2P and MERS_S-2P, indicating that the shared epitopes responsible for cross-reactivity elicited by SARS-1_I53_dn5 against SARS-2_S-2P and MERS_S-2P are distinct from the cross-reactive epitopes between SARS-1_S-2P and HKU1_S-2P (Supplementary Fig. 4).

We hypothesized that after nanoparticle assembly, crowding of the CoV spike antigens on the nanoparticle surface sterically restricts B cell receptor (BCR) access to the S2 subdomain, and focuses antibody responses to S1. To investigate this in the context of MERS S, we immunized mice once with 10 µg of either MERS_S-2P, MERS_I53_dn5, or bare I53_dn5 as a control and tested for antibody binding to MERS_S-2P and MERS_SS at week 3. Both
soluble MERS_S-2P and MERS_I53_dn5 nanoparticles elicited antibodies that bound to MERS_S-2P (Supplementary Fig. 5a). Interestingly, while MERS_I53_dn5 elicited greater binding antibody titers to full-length MERS_S-2P (Supplementary Fig. 5a), only the soluble MERS_S-2P immunogen was able to elicit binding antibodies to the MERS S2 domain (Supplementary Fig. 5b), suggesting that access to S2 is restricted on I53_dn5 and may bias towards selection of B cells specific to the S1 domain. To see if antibodies cross-reactive to S1 could be visualized by EM, sera from mice immunized with SARS-1_I53_dn5 were pooled to isolate IgG, which was then digested with papain to generate antigen-binding fragments (Fabs). We assembled immunocomplexes of SARS-1_I53_dn5-elicited polyclonal Fabs bound to MERS_S-2P in a 75:1 ratio and imaged them with negative stain EM. Both representative images and 2D class averages show Fabs bound to the apex and side of the MERS-S1 (Fig. 3d-e). We next generated an NS-EM 3D reconstruction of the complex with the polyclonal Fabs and overlayed it with the structure of MERS_S-2P (PDB: 5X5F). The apex-binding Fab appeared to target a similar epitope at a similar angle as the previously characterized MERS-specific, NTD-binding mAb, G2 (PDB: 6PXH), while only a partial density could be generated for the side-binding Fab (Fig. 3f-g). Together, these data indicate that assembly of SARS-1_S-2P on I53_dn5 restricts BCR access to the S2 domain and focuses B cell selection and antibody responses to S1. The potential avidity bonus from the multivalent display may provide an advantage to unique, potentially low-affinity, yet cross-reactive B cells specific for vulnerable sites on S1.
Fig. 3: SARS_dn5 elicits two distinct antibody populations targeting the S1 domain of MERS_S-2P.

a-c To elucidate cross-reactive domain specificity, SARS-1_dn5 sera was depleted with MERS_S-2P and its domains, S1, SS, and RBD, then screened for residual binding to SARS-1_S-2P, SARS-2_S-2P, and MERS_S-2P. d-g Mice immunized with SARS-1_dn5 were terminally bled and serum was pooled, IgG-purified and digested to Fabs. Immunocomplexes of SARS-1_dn5-elicited Fabs bound to MERS_S-2P were imaged with negative stain EM. d-e Squares are a magnified views of Fabs bound within the image. Scale bars correspond to 100 nm (representative image) and 20 nm (2D classes). Arrows point to Fabs bound to the top or side of MERS_S-2P. f-g 3D map reconstruction was generated from NSEM and overlayed with structures of MERS_S-2P, and MERS-specific mAb G2 a-c Boxes and horizontal bars denote the IQR and medians, respectively. Whisker endpoints are equal to the minimum and maximum values. Circles denote each individual animal. Statistical analysis was performed using non-parametric Kruskal-Wallis test with Dunn’s multiple comparisons. **P < 0.01, ***P < 0.001, ****P < 0.0001.
Display of diverse CoV spikes on I53_dn5 elicits cross-reactive antibodies

We next sought to profile the breadth of cross-reactive antibody responses elicited from additional β-CoV S-2P antigens displayed on I53_dn5 as well as a mosaic nanoparticle assembled to co-display each of the β-CoV_S-2P antigens on I53_dn5. HKU1_ and OC43_ I53_dn5 nanoparticles were generated as above (Supplementary Fig. 6) and BALB/c mice were immunized at weeks 0 and 3 with 10 µg of either MERS_, SARS-1_, SARS-2_, HKU1_, OC43_, or β-CoV mosaic on I53_dn5 nanoparticles and bled to assess antibody binding to the S-2P proteins derived from MERS, SARS-1, SARS-2, HKU1, OC43, as well as two α-hCoVs, hCoV-229E and hCoV-NL63 (Fig. 4a). H1_I53_dn5-immunized mice served as negative controls (Fig. 4b). As shown above, SARS-1_I53_dn5 nanoparticles elicited cross-reactive antibody responses to MERS_S-2P and SARS-2_S-2P, however binding was also detected against OC43_S-2P and HKU1_S-2P (Fig. 4c). SARS-2_I53_dn5 particles also resulted in broad binding, extending to MERS_, SARS-1_, OC43_, and HKU1_S-2P (Fig. 4d). MERS_I53_dn5 particles elicited cross-binding antibodies against SARS-1_, SARS-2_, OC43_, and HKU1_S-2P (Fig. 4e). HKU1_I53_dn5 elicited cross-binding to MERS_, SARS-1_, SARS-2_, and OC43_S-2P (Fig. 4f). OC43_I53_dn5 elicited cross-reactive antibody titers to MERS_S-2P, SARS-1_, SARS-2_, HKU1_, and even α-CoV-derived 229E_S-2P (Fig. 4g). We also immunized C57BL/6 mice with these groups and found similarly broad, yet lower magnitude cross-reactivity (Supplementary Fig. 7a-f). Mice immunized with a β-CoV mosaic nanoparticle co-displaying MERS_, SARS-1_, SARS-2_, HKU1_, and OC43_S-2P showed comparably high antibody responses to their respective corresponding monotypic I53_dn5 nanoparticle (Fig. 4h). Notably, the β-CoV mosaic nanoparticle also elicited cross-binding antibodies to 229E_S-2P indicating that despite reduced valency of OC43_S-2P per particle, the mosaic was able to retain its contribution of cross-reactive breadth\textsuperscript{14}. 
**SARS-1_ and β-CoV mosaic_I53_dn5 assemblies protect against MERS-CoV challenge**

To determine the protective efficacy of CoV S-2P immunogens using the I53_dn5 platform, we immunized 288/330+/− mice twice with 10 µg of either MERS_I53_dn5, SARS-1_I53_dn5, SARS-2_I53_dn5, HKU1_I53_dn5, or OC43_I53_dn5 to compare antibody responses elicited from co-display and monotypic display of each spike. Control mice were immunized with H1_I53_dn5. Mice were bled at week 5 for serology. Sera was screened by ELISA for IgG binding to each strain. Boxes and horizontal bars denote the IQR and medians, respectively. Whisker endpoints are equal to the minimum and maximum values. Circles denote each individual animal.

**Fig. 4: β-mosaic particles elicit broad antibody responses.**

- **a** Groups of 10 female BALB/c mice were immunized twice with β-CoV mosaic_I53_dn5, MERS_I53_dn5, SARS-1_I53_dn5, SARS-2_I53_dn5, HKU1_I53_dn5, or OC43_I53_dn5 to compare antibody responses elicited from co-display and monotypic display of each spike. Control mice were immunized with H1_I53_dn5. Mice were bled at week 5 for serology. **b-h** Sera was screened by ELISA for IgG binding to each strain. **a-h** Boxes and horizontal bars denote the IQR and medians, respectively. Whisker endpoints are equal to the minimum and maximum values. Circles denote each individual animal.
induced cross-subgroup protection at a level approaching that of MERS_I53_dn5, with both
groups sustaining body weight throughout the course of challenge. These findings are
consistent with our serology data showing that SARS-1_I53_dn5 elicits MERS-binding and
neutralizing antibodies that correlate similarly to those elicited by MERS_I53_dn5
(Supplementary Fig. 3b, f, and i). β-CoV mosaic nanoparticles with decreased MERS_S-2P
valency elicited protective responses to a similar degree as MERS_I53_dn5, as discerned by
weight loss over 5 days post MERS-CoV challenge (Fig. 5a). Lungs were harvested 5 days post
challenge to obtain discoloration scores and viral titers. Mice immunized with the β-CoV mosaic
nanoparticles had a lung discoloration score of 0 for 3 out of the 4 mice (Fig. 5b) and lung viral
titers at similarly low levels to mice immunized with MERS_I53_dn5 nanoparticles (Fig. 5c),
again indicating that the mosaic nanoparticle with a lower MERS_S-2P molar content elicited
levels of protection against MERS-CoV challenge comparable to MERS_I53_dn5. Additionally,
for SARS-1_I53_dn5 and SARS-2_I53_dn5, lung discoloration scores were lower relative to
their S-2P trimer-immunized counterparts (Fig. 5b), though viral loads in soluble S-2P-
immunized groups were only marginally higher than the I53_dn5 comparators (Fig. 5c).
Nonetheless, these trends indicate that display of heterotypic hCoV_S-2P on the I53_dn5
platform for SARS-1_S-2P and SARS-2_S-2P augments baseline immunogenicity and cross-
reactivity to MERS-CoV S-2P.
Fig. 5: β-CoV mosaic particles protect against lethal MERS-CoV challenge.

a-c 288/330+/+ mice were immunized twice with 10 µg of the specified nanoparticle or soluble trimer. Control mice were immunized with H1_dn5. 4 weeks post-boost, mice were challenged with a lethal dose, 5x10^5 plaque-forming units (p.f.u.) of maM35c4 MERS-CoV. a Following challenge, mice were monitored for weight loss. b-c 5 days post challenge, lung discoloration (scored as: 0=no discoloration, 4=severe discoloration in all lobes) in b and lung viral titers in c were assessed. In a-c, all groups were compared with H1_dn5 control mice by Kruskal–Wallis analysis of variance (ANOVA) with Dunn’s multiple comparisons test, in a for each day post challenge. *P<0.05, **P < 0.01. Data are GMT ± geometric s.d. in c or mean ± s.d. in a-b. In c, the dotted line represents the assay limit of detection.
Here, we adapt a previously described computationally designed protein nanoparticle platform\textsuperscript{14} to co-display CoV S-2P antigens. Our results demonstrate that the breadth and quality of cross-reactive antibodies elicited by monotypic nanoparticle display of hCoV S-2P is extensive. However, mosaic display of hCoV S-2P proteins on the I53_dn5 platform can achieve similar potency of antibody responses compared to their monotypic counterparts, despite the lower molarity of S-2P immunogens from each individual hCoV, without sacrificing breadth. Others have previously shown that in the context of influenza, mosaic display of diverse hemagglutinins (HA) on I53_dn5 elicits both potent head-directed antibody responses as well as cross-reactive responses against conserved epitopes on the stem, and achieved HA-stem-specific antibody-mediated protection against highly divergent heterosubtypic strains of influenza\textsuperscript{22}. In both that work and our present study, the two-component nature of the computationally designed nanoparticle scaffold facilitated co-display through simple mixing of independently purified subunits \textit{in vitro}\textsuperscript{18, 22, 23}.

There was a notable lack of cross-reactive antibody responses directed to the more conserved S2 subdomain when using SARS-1_I53_dn5 as an immunogen. However, SARS-1_I53_dn5 still induced cross-reactive binding and neutralizing responses to the S1 domain of MERS S. We also found a reduction in cross-reactive binding titers elicited by SARS-1_I53_dn5 against SARS-2_S-2P compared to those against MERS_S-2P, despite higher overall conservation and sequence homology between SARS-1_S-2P and SARS-2_S-2P. The regions with greatest sequence conservation are concentrated in the S2 subunit, where there is approximately 90\% amino acid identity between the SARS-1-CoV and SARS-CoV-2 spikes\textsuperscript{24}. However, our data suggest that display of CoV_S-2P on the I53_dn5 nanoparticle initially restricts BCR access to the S2, biasing towards selection of B cells that recognize shared epitopes on the S1 domain. This could be explained by the larger size of S (relative to HA) occupying more space on the nanoparticle scaffold and crowding out BCR access to S2\textsuperscript{25}, an effect that was confirmed in previous studies of two-component nanoparticle immunogens.
displaying HIV-1 Env trimers\textsuperscript{26,27}. Given the structural and mechanical homology between prefusion coronavirus spikes, we suspect that the apparent S2-occlusion/S1-targeting may also occur with other CoV-S\textsubscript{dn5} formulations, potentially eliciting potent S1-directed cross-neutralization in other coronaviruses as well—particularly in the $\beta$-mosaic that may exploit vulnerable S1 epitopes. However, we propose that it may be possible to improve access to S2 epitopes, with nanoparticle scaffolds that appropriately space the displayed spike antigens.

Notably, each monotypic CoV\textsubscript{S-2P} assembled on I53\textsubscript{dn5} elicited a distinct cross-reactive antibody profile to MERS\textsubscript{-}, SARS\textsubscript{-1-}, SARS\textsubscript{-2-}, OC43\textsubscript{-}, 229E\textsubscript{-}, NL63\textsubscript{-}, and HKU1\textsubscript{S-}2P. Qualitative differences between antibody responses elicited by I53\textsubscript{dn5} immunogens were further exemplified by the observation that while SARS\textsubscript{-1-}\textsubscript{I53\textsubscript{dn5}} and SARS\textsubscript{-2-}\textsubscript{I53\textsubscript{dn5}} elicited a similar cross-reactive profile and comparable MERS\textsubscript{S-2P}-reactive antibody binding titers (Fig. 4c-d and supplementary Fig. 7b-c), SARS\textsubscript{-1-}\textsubscript{I53\textsubscript{dn5}} protected mice from lethal MERS challenge while SARS\textsubscript{-2-}\textsubscript{I53\textsubscript{dn5}} did not (Fig. 5a). Additionally, the potency of cross-reactivity appeared to be unidirectional as immunization with MERS\textsubscript{I53\textsubscript{dn5}} elicited SARS\textsubscript{-1-}-binding, but non-neutralizing antibodies. Such instances may hint at the targeting of partially conserved epitopes of vulnerability as opposed to protection conferred merely through antibody quantity or affinity. Depletion data showed that the cross-reactive antibodies elicited by SARS\textsubscript{-1-}\textsubscript{I53\textsubscript{dn5}} to SARS\textsubscript{-2-} and MERS\textsubscript{S-2P} must bind the MERS S1 domain and likely bind shared epitopes.

EMPEM of SARS\textsubscript{-1-}\textsubscript{I53\textsubscript{dn5}}-derived IgG Fabs bound to MERS\textsubscript{S-2P} showed at least two cross-binding antibody species. The most easily identified appears to be a G2-like Ab that binds an epitope on the apex of the NTD\textsuperscript{20}. Notably, this epitope and Ab binding angle is also analogous to a conserved and previously described SARS\textsubscript{-2-} antigenic supersite\textsuperscript{28}. The second cross-reactive species is visible by 2D class average EMPEM images, which revealed a fab binding to the side of MERS\textsubscript{S-2P}. The 3D map reconstruction was unable to reveal a clear binding site or angle of this Fab, indicating flexibility or dynamic motion of the region to which
the Fab is bound. Diminished antibody binding from depleted sera, further suggests that this
species may bind motile MERS_RBD. While these SARS-1_I53_dn5-elicited antibodies failed to
neutralize SARS-2 pseudovirus, they did bind SARS-2_S-2P. This binding was abrogated when
depleted with MERS S1 suggesting that these antibodies likely bind shared epitopes. However,
MERS S1 depletion did not appear to impact binding to HKU1_S-2P, suggesting that any
epitopes responsible for cross-reactivity between SARS-1_, SARS-2_S-2P, and MERS_S-2P
are not shared with hCoV-HKU1 and are bound by different antibody populations
(Supplementary Fig. 2). These findings, in combination with the unidirectional cross-
neutralization of SARS-1_I53_dn5 to MERS_CoV (but not MERS_I53_dn5 to SARS-CoV-1),
hints at distinct structural requirements for epitope recognition by naïve B cells and the
subsequent induction of cross-neutralizing antibodies.

Other studies have reported intrasubgroup cross-reactivity through natural infection7 or
via nanoparticle-based immunogens using mosaic co-display of RBDs from diverse SARS-like
CoV S proteins9,10. We chose to display the full-length S ectodomain because it comprises the
full complement of epitopes exposed on the spike on the viral surface, including the highly
conserved S2 fusion machinery, and therefore provides a greater chance of identifying cross-
reactive epitopes between diverse CoVs. However, the finding that S2-specific antibodies were
not induced suggests that BCR access may have been limited to the apex of S. New designs
with fewer S-2P displayed or larger nanoparticles to change the geometry of antigen
presentation and improve access to S2 could increase the level cross-reactivity induced.
Additionally, employing an S2-only display of diverse hCoV spikes and employing our mosaic
approach may increase recognition of conserved epitopes on this subdomain. Nonetheless, our
results confirm the presence of conserved protective epitopes between divergent subgroups on
the NTD and potentially either RBD or other subdomains that can be targeted by next-
generation immunogens.
The β-CoV_I53_dn5 mosaic nanoparticle, which has a relatively low valency of MERS_S-2P trimers, induced protective immunity against MERS-CoV lethal challenge in mice, comparable to protection elicited by monotypic MERS_I53_dn5 (Fig. 6). Further studies will need to evaluate the extent to which this mosaic nanoparticle protects against other CoV challenges and establish the minimum valency of S-2P content needed to confer protection in both homotypic and heterotypic challenge models. Future research will also be needed to map additional cross-reactive epitopes and B cell responses following CoV_I53_dn5 immunization, and additional experimentation will be required to define the optimal spacing and composition of S-2P antigens to improve the breadth and potency of cross-reactive responses. Follow-up experiments should evaluate the range of protective immune responses against a wider variety of β-CoVs and more distantly related α-CoVs. Additionally, distinct sensitivities to SARS-CoV-2 variants between humans and mice have recently been shown, and this platform merits evaluation in additional animal models.

Typically, as breadth increases, neutralizing activity decreases, thus highlighting the importance of understanding orthogonal antibody functions. Studies from both vaccinated and convalescent individuals have shown that neutralizing activity is highly predictive of protection from SARS-CoV-2 infection, and that even low levels of neutralization are sufficient for protection. Therefore, more experiments should be conducted to determine the mosaic nanoparticle design that confers maximum breadth while still retaining protection and potency.

The ongoing COVID-19 pandemic, caused by SARS-CoV-2, highlights the need for protection against pre-emergent zoonotic CoV threats in addition to continually emerging SARS-CoV-2 VOC. The mosaic display of S-2P antigens on nanoparticles may serve as the basis for developing a pan-CoV vaccine.

**METHODS**

**Expression and purification of antigens and immunogens**
Soluble CoV_S-2P trimers with foldon, as well as SARS-1_I53_dn5b nanoparticle components were expressed in Expi293F expression system (Thermo Fisher Scientific) with Expifectamine transfection reagent according to the manufacturer’s protocol. Soluble SARS-1_S-2P was harvested by centrifugation at 3700-4000 RPM and clarified supernatant was filtered through 0.22-0.45µm vacuum filters. Protein was purified by tangential flow filtration, followed by strep tag purification and 3C cleavage of tags. SARS-1_I53_dn5b nanoparticle components were expressed in Expi293 cells (Thermo Fisher Scientific) and purified by nickel resin, size exclusion chromatography using a Superdex 200 increase, and Superose 6 increase (GE Healthcare) was used to purify assemble particles. Briefly, salt and pH of cleared transfection supernatants were adjusted with Tris-HCl (pH 8.0) buffer at a final concentration of 50 mM Tris-HCl, ~50 mM NaCl. Nickel resin was washed with several bed volumes of either 1x PBS or water 3 times and incubated with supernatant overnight at 4°C or 2 hours at room temperature. Resin-supernatant mixture was loaded onto a column. The column was washed twice with 5 times bed volume of wash buffer (500mM NaCl, 50mM Tris-HCl, 5mM imidazole) and eluted twice with 5 times bed volume elution buffer (500 mM NaCl 50 mM Tris-HCL, 300mM imidazole). Eluates were concentrated and filtered through 0.22um and purified by size exclusion chromatography using a Superose 6 increase (GE Healthcare). Fractions containing SARS-1_I53_dn5b components were pooled and concentrated for particle assemblies. In vitro assembly of particles was performed by addition and vigorous mixing by pipette of equimolar amounts of I53_dn5a and I53_dn5b components for 20 seconds at room temperature and left at rest to assemble for 30 minutes. Assemblies were then purified by size exclusion using Superose 6 increase (GE Healthcare) and fractions collected.

**Antibody expression and purification**

Paired VH and VL in a 1:1 ratio were co-transfected transiently into FreeStyle293F cells as previously described. The supernatant was harvested six days post-transfection and IgGs were
purified with Protein A agarose (Thermo Fisher Scientific). IgGs were eluted with 100 mM glycine, pH 3 into 1/10th volume 1 M Tris-HCl pH 8.0. IgGs were then buffer exchanged into PBS pH 7.4. Fabs were generated by digesting the IgGs with HRV 3C protease at 4°C. Fc was removed by passing digests over fresh Protein A agarose, leaving the Fab in the flowthrough, which was further purified by SEC using a Superdex 200 increase 10/300 column (GE Healthcare) in PBS buffer, pH 7.4.

Antigenicity testing by enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was used to measure antibody binding to each immunogen. 96-well ELISA plates were coated with 1 µg/mL nanoparticles or CoV_S-2P. Plates were incubated at 4°C overnight and blocked with PBS containing 5% skim milk at room temperature for 1 hour. Monoclonal antibodies were serially diluted in four-fold steps, then added to the plates and incubated at room temperature for 1 hour. Horseradish peroxidase (HRP)-conjugated anti-human IgG (Southern Biotech) was added and incubated at 37°C for 1 hour, followed by 3,3',5',5'-Tetramethylbenzidine (TMB; Sigma-Aldrich, St. Louis, MO) HRP substrate, and the signal that developed after the addition of 1 M sulfuric acid was measured by absorbance at 450 nm.

Negative stain electron microscopy

Proteins were diluted with buffer containing 10 mM HEPES, pH 7.0, and 150 mM NaCl to a concentration of approximately 0.05 mg/ml and adsorbed to a freshly glow-discharged carbon-coated grid. The grid was washed three times with the same buffer and stained with 0.7% uranyl formate. Images were collected at a nominal magnification of 57,000 (pixel size: 2.53 Å) using EPU software on a Thermo Fisher Scientific Talos F200C electron microscope equipped with a 4k x 4k Ceta camera or at a nominal magnification of 50,000 (pixel size: 4.4 Å) using SerialEM on an FEI T20 electron microscope equipped with a 2k x 2k Eagle camera. Both microscopes
were operated at 200 kV. Particles were picked automatically using EMAN2\textsuperscript{32} or in-house written software (YT, unpublished). Reference-free 2D classifications were performed using RELION\textsuperscript{33}. To obtain the 3D NS-EM map of the immunocomplex of SARS-1\_I53\_dn5-derived Fab and MERS\_S-2P, micrographs were collected at 0- and 30-degree tilt using the same microscope settings. RELION 3.1 was used to isolate the population of particles representing the immunocomplex using 2D and 3D classification and for the refinement of the final dataset containing 3927 particles. The resolution, determined at the FSC threshold of 0.5, was 26 Å.

**Animal studies**

All mouse experiments were carried out in compliance with all pertinent US National Institutes of Health regulations and approval from the Animal Care and Use Committee (ACUC) of the Vaccine Research Center, from Institutional Animal Care and Use Committee at University of North Carolina at Chapel Hill to guidelines outlined by the Association for the Assessment and Accreditation of Laboratory Animal Care and the U.S. Department of Agriculture. All infection experiments were done in animal biosafety level 3 (BSL-3) facilities at the University of North Carolina at Chapel Hill. For immunogenicity studies, female BALB/cJ or C57BL/6 mice aged 6- to 8-weeks (Jackson Laboratory) were used. Per the experimental design schema outlined, mice were inoculated intramuscularly with protein immunogens adjuvanted with SAS as previously described\textsuperscript{15} and bled for serological assays. For challenge studies to evaluate CoV\_I53\_dn5 vaccines, 16- to 20-week-old male and female 288/330\(^{+/+}\) mice\textsuperscript{21} were immunized, bled, and challenged, as detailed in Fig. 6a. Mice were challenged with 5 \times 10^5 PFU of a mouse-adapted MERS-CoV EMC derivative, mAM35c4\textsuperscript{34}. On days 3 and 5 post-challenge, lungs were collected from selected mice to assess viral titers and discoloration, using previously published methods. Briefly, caudal right lung lobes were harvested for analysis of viral load by plaque assay. Lung lobes were homogenized in 1mL of PBS and glass beads. For 288/330\(^{+/+}\) mice infected with MERS-CoV, mice were monitored up to 5 days post-challenge, and were
euthanized and lungs were collected to investigate lung discoloration, and viral burden. Sample size for animal experiments was determined on the basis of criteria set by institutional ACUC. Experiments were neither randomized nor blinded.

Immunogenicity testing by enzyme-linked immunosorbent assay (ELISA)

Binding of immunoglobulin G (IgG) levels to screened antigens were examined as follows: 96-well enzyme-linked immunosorbent assay (ELISA) plates were coated with 1 μg/mL of CoV_S-2P antigen. Plates were incubated at 4°C overnight and blocked with PBS containing 5% skim milk at room temperature for 1 hour. Sera fractions from the immunized mice, serially diluted in four-fold steps and competed with the addition of 50 μg/mL of foldon. Foldon-serum dilutions were then added and the plates incubated at room temperature for 1 hour. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Southern Biotech, Birmingham, AL) was added and incubated at room temperature for 1 hour, followed by 3,3′,5′,5′- Tetramethylbenzidine (TMB; Sigma-Aldrich, St. Louis, MO) HRP substrate, and the yellow color that developed after the addition of 1M H2SO4 was measured by absorbance at 450 nm.

Depletion assay

Magnetic HIS dynabeads (Thermo Fisher Scientific) were used to deplete mouse sera of antibodies with specificity to MERS domains, S1, S2, and RBD according to manufacturer’s protocol. 5 μL sera was added to molar equivalents of depleting protein (calculated from 50 μg/ml foldon) in 445 μL of PBS and incubated for 1 hour at room temperature. Magnetic beads were washed three times in PBS, resuspended, and 50 μL was added to each serum-protein mixture and incubated at room temperature for 30 minutes. Beads were separated from solution by magnetic strip and after 5 minutes the supernatant was collected and used for future assays.

Lentiviral pseudovirus preparation
Pseudotyped lentiviral reporter viruses were produced as previously described (Corbett et al., 2020; Wu et al., 2021). Briefly, HEK293T/17 cells (ATCC CRL-11268) were co-transfected with pCMV-ΔR8.2 (lentiviral backbone) and pHR'-CMV-Luc (reporter genome) plasmids along with plasmids encoding desired S protein from Wuhan-Hu-1 strain (GenBank no. MN908947.3) with a p.Asp614Gly mutation (D614G), MERS England1 strain, and SARS-CoV-1 Urbani strain and human transmembrane serine protease 2 (TMPRSS2) by the Fugene6 transfection method (Promega). After overnight incubation, dishes were washed, and replenished with fresh medium. Forty-eight hours later, supernatants were harvested, filtered through a 0.45 µm, aliquoted, and frozen at −80 °C until use. Each pseudovirus stock was titrated prior to use in neutralization assays.

MERS-CoV pseudovirus neutralization assay

MERS-CoV pseudovirus neutralizing activity was tested as previously described. In brief, Huh7.5 cells were seeded at 10^4 cells/well in 96-well black/white Isoplates (PerkinElmer) the day before infection. Sera samples were serially diluted (1:40, 4-fold dilutions, 8x) in DMEM (Gibco) + 1% penicillin/streptomycin. Dilutions were mixed with a pseudotyped lentivirus expressing the MERS-CoV England1 spike, which was previously titrated to 10^4 relative luciferase units (RLU), and incubated for 30 minutes at RT. Sera-pseudovirus mixtures were added, in duplicate, to cells and incubated at 37°C and 5% CO_2 for 2-h. 100 µl of DMEM (Gibco) + 10% FBS + 1% penicillin/streptomycin (D10) media supplemented with 2 mM glutamine was then added, and incubated under the same conditions for 72-h. Cells were then lysed, and luciferase substrate (Promega) was added. Luciferase activity was measured in RLU at 570 nm, using a SpectraMaxL (Molecular Devices). Considering uninfected cells as 100% neutralization and cells transduced with pseudovirus alone as 0% neutralization, averages of duplicates were normalized, and sigmoidal curves were generated. 50% neutralizing antibody titers (ID_{50}) titers
were generated by fitting normalized values to a log(agonist) vs. normalized-response (variable slope) nonlinear regression model in Prism v9 (GraphPad).

SARS-CoV-2 and SARS-CoV-1 Neutralization Assay

SARS-CoV-1 and SARS-CoV-2 pseudovirus neutralizing activity was assessed similarly to MERS-CoV pseudovirus neutralizing activity. 24-h prior to infection, HEK293T cells stably overexpressing a humanized ACE2 receptor were seeded in 96-well black/white Isoplates (PerkinElmer) at 5000 cells/well and incubated in 37°C and 5% CO₂. Sera samples were diluted identically as for the MERS-CoV pseudovirus assay, mixed with appropriately titrated pseudotyped lentivirus reporter expressing either the SARS-CoV-1 Urbani spike or the SARS-CoV-2 Wuhan-1 Spike encoding the D614G mutation, and incubated for 45 minutes at 37°C and 5% CO₂. Sera-pseudovirus mixtures were then added to cells in triplicate and incubated at the above conditions for 72-h. Cells were lysed and luciferase substrate (Promega) was added. RLU readings and 50% neutralizing antibody titers (ID₅₀) were determined using the same methodology as for the MERS-CoV pseudovirus neutralization assay.

Statistics

In box plots, boxes and horizontal bars denote the interquartile range and medians, which represent data outside the 25th-75th percentile range. Whisker endpoints are equal to the minimum and maximum values. Geometric means or arithmetic means are represented by the heights of bars, or symbols, and error bars represent the corresponding s.d. Dotted lines indicate assay limits of detection. Figure legends detail all quantification and statistical analyses, inclusive of animal numbers (n), dispersion and precision measures, and statistical tests performed. To compare more than two experimental groups, Kruskal–Wallis ANOVA with Dunn’s multiple comparisons tests were applied assuming a non-Gaussian distribution. Reciprocal endpoint and reciprocal ID₅₀ titers were transformed so that all values were on a
log_{10} scale prior to statistical analyses (Fig. 2, 3, 5c). Statistical analyses were performed using GraphPad Prism v9.2.0. Significance is denoted by asterisks defined as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Data Availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions
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Competing Interests
The authors declare the existence of financial and non-financial competing interest.
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