1 **Single-component, self-assembling, protein nanoparticles presenting the receptor**

2 **binding domain and stabilized spike as SARS-CoV-2 vaccine candidates**

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18 **KEYWORDS**

19 Coronavirus disease 2019 (COVID-19); heptad repeat 2 (HR2); self-assembling protein

20 nanoparticle (SApNP); severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); spike

21 (S) protein; vaccine.

22   **ABSTRACT (150 words)**

23   Vaccination against SARS-CoV-2 provides an effective tool to combat the COIVD-19 pandemic.

24   Here, we combined antigen optimization and nanoparticle display to develop vaccine candidates

25   for SARS-CoV-2. We first displayed the receptor-binding domain (RBD) on three self-

26   assembling protein nanoparticle (SApNP) platforms using the SpyTag/SpyCatcher system. We

27   then identified heptad repeat 2 (HR2) in S2 as the cause of spike metastability, designed an HR2-

28   deleted glycine-capped spike (S2GΔHR2), and displayed S2GΔHR2 on SApNPs. An antibody

29   column specific for the RBD enabled tag-free vaccine purification. In mice, the 24-meric RBD-

30   ferritin SApNP elicited a more potent neutralizing antibody (NAb) response than the RBD alone

31   and the spike with two stabilizing proline mutations in S2 (S2P). S2GΔHR2 elicited two-fold-

32   higher NAb titers than S2P, while S2GΔHR2 SApNPs derived from multilayered E2p and I3-

33   01v9 60-mers elicited up to 10-fold higher NAb titers. The S2GΔHR2-presenting I3-01v9

34   SApNP also induced critically needed T-cell immunity, thereby providing a promising vaccine

35   candidate.

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37   **ONE-SENTENCE SUMMARY (125 characters)**

38   The SARS-CoV-2 receptor binding domain and S2GΔHR2 spike elicited potent immune

39   responses when displayed on protein nanoparticles as vaccine candidates.

INTRODUCTION

Three coronaviruses (CoVs) have caused widespread outbreaks in humans, including severe acute respiratory syndrome CoV-1 (SARS-CoV-1), Middle East respiratory syndrome CoV (MERS-CoV), and SARS-CoV-2, which is the causative agent of COVID-19 (*1-3*) and has resulted in more than 1.9 million deaths worldwide (*4*). Enormous efforts are being undertaken to develop effective therapeutics and prophylactics for SARS-CoV-2. Small molecules that can block the host receptor, angiotensin-converting enzyme 2 (ACE2), and the transmembrane protease serine 2 (TMPRSS2) (*5*), which is required to process the spike protein, are being considered as treatments in addition to other interventions (*6*). While the immunology underlying COVID-19 is still being intensively studied (*6-8*), various vaccine candidates are now in clinical development (*9-12*). Inactivated virus vaccines have exhibited robust neutralizing antibody (NAb) responses in animals (*13, 14*), whereas viral vector vaccines based on human adenovirus (Ad5 and Ad26) and chimpanzee ChAdOx1 have been evaluated in nonhuman primates (NHPs) and human trials (*15-18*). Both DNA (*19-21*) and mRNA (*22, 23*) vaccines have been rapidly developed, with moderate NAb titers observed for the mRNA vaccine in medium and high dose groups (*22*). A recombinant spike protein adjuvanted with lipid nanoparticles (NPs), NVX-CoV2373, elicited high NAb titers in a human trial that were on average four-fold greater than in convalescent patients (*24, 25*). Efficacy was recently reported for a vector vaccine (AZD1222: 70.4%) (*26*) and two mRNA vaccines (mRNA-1273: 94.1% and BNT162b2: 95%) (*27, 28*). In December 2020, the U.S. Food and Drug Administration (FDA) issued the emergency use authorization (EUA) for the two mRNA vaccines.

The SARS-CoV-2 spike protein is a trimer of S1-S2 heterodimers. The S1 subunit contains a receptor-binding domain (RBD) that binds to ACE2 on host cells to initiate infection.

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63   The S2 subunit consists of a fusion peptide (FP) and heptad repeat regions 1 and 2 (HR1 and

64   HR2). Upon endocytosis of the virion, the S1 subunit is cleaved off to facilitate FP insertion into

65   the host cell membrane, while the remaining S2 refolds to bring HR1 and HR2 together to fuse

66   the viral and host cell membranes (*29*). The spike protein harbors all NAb epitopes and is the

67   main target for vaccine development against SARS-associated CoVs (*30*). Convalescent plasma

68   (CP) has been used to treat COVID-19 patients with severe conditions (*31*), highlighting the

69   importance of NAbs in protection (*32*). Due to moderate sequence conservation of the RBDs

70   (~73%), some previously identified NAbs targeting the SARS-CoV-1 RBD have been shown to

71   bind and cross-neutralize SARS-CoV-2 (*33, 34*). Using single-cell technologies and the SARS-

72   CoV-2 RBD or spike as bait, potent NAbs have now been isolated from COVID-19 patients (*35-*

73   *41*). Camelid-derived single-chain NAbs have also been obtained by panning naïve or immune

74   llama single-chain antibody (VHH) libraries (*42, 43*). Structures of the SARS-CoV-2 spike and

75   RBD in unliganded (*44, 45*), ACE2-bound (*46-48*), and antibody-bound (*49-51*) states

76   determined by x-ray crystallography and cryo-electron microscopy (cryo-EM) have paved the

77   way for rational vaccine design. Cryo-EM and cryo-electron tomography (ET) have revealed the

78   inherent spike metastability and the co-existence of pre/post-fusion spikes on virions (*52*). A

79   double-proline mutation (*52*) has been used in most soluble spike (S2P) constructs and all but

80   inactivated vaccines, although a HexaPro version with greater yield and stability is now available

81   (*53*). Cryo-ET has also uncovered a dynamic, triple-hinged HR2 stalk that facilitates viral entry

82   and immune evasion (*54-56*).

83   In this study, we designed and optimized SARS-CoV-2 antigens for multivalent display

84   on self-assembling protein nanoparticles (SApNPs) (*57-59*), including a ferritin (FR) 24-mer and

85   two 60-mers (E2p and I3-01) containing an inner layer of locking domains (LD) and a cluster of

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86   T-cell epitopes (*60*). To facilitate tag-free vaccine purification, we developed an immunoaffinity

87   column based on antibody CR3022 that binds to both SARS-CoV-1/2 RBDs (*34, 50*). We first

88   designed a scaffolded RBD trimer construct to mimic the "RBD-up" spike conformation. The

89   SARS-CoV-1/2 RBDs were attached to SApNPs using the SpyTag/SpyCatcher system (*61*),

90   providing a robust strategy for developing RBD-based nanoparticle vaccines. We then probed

91   the spike metastability by comparing two uncleaved spike antigens, S2P (K986P/V987P) and

92   S2G (K986G/V987G). The SARS-CoV-2 S2G spike exhibited abnormal behavior, suggesting

93   that an unidentified facet of the spike can promote conformational change and block antibody

94   access to the RBD. An HR2-deleted spike, S2GΔHR2, produced high-purity trimers, suggesting

95   that the HR2 stalk may be a trigger of spike metastability consistent with recent findings (*54-56*).

96   We next displayed S2GΔHR2 on three SApNPs by gene fusion, resulting in spike SApNPs with

97   high yield, purity, and antigenicity. In mouse immunization, the S2P spike protein elicited the

98   lowest level of NAb response. In contrast, the scaffolded RBD trimer registered two-to-three-

99   fold higher NAb titers, with another five-fold increase in NAb titer achieved by multivalent

100   display on FR. S2GΔHR2 elicited up to seven-fold higher NAb titers, while the large,

101   multilayered S2GΔHR2 E2p and I3-01 SApNPs induced up-to-10-fold higher NAb titers than

102   S2P. Further analysis indicated that the S2GΔHR2-presenting I3-01v9 SApNP can elicit a strong

103   Th1 response as well as other types of T-cell responses needed for protective cellular immunity.

104   Our study thus identifies the HR2 stalk as a major source of spike metastability, validates an

105   HR2-deleted spike design, and provides a set of RBD- and spike-based virus-like particles

106   (VLPs) as effective protein vaccine candidates against SARS-CoV-2.

107   **RESULTS**

108   **Rational design of scaffolded RBD trimers and RBD-presenting SApNPs**

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109   RBD binding to the ACE2 receptor initiates the membrane fusion process (*5*). The crystal

110   structure of SARS-CoV-2 RBD/ACE2 complex revealed the atomic details of receptor

111   recognition (*62*). The SARS-CoV-2 RBD has been used as bait to isolate monoclonal antibodies

112   (mAbs) from patient samples (*35-41*). For SARS-CoV-1 and MERS-CoV, RBD-based vaccines

113   have induced potent NAbs that effectively block viral entry (*30*). Therefore, the RBD represents

114   a major target for humoral responses following infection and can be used to develop epitope-

115   focused vaccines.

116   We first hypothesized that RBD attached to a trimeric scaffold could mimic the "RBD-

117   up" spike conformation and elicit NAbs that block ACE2 binding. To test this possibility, we

118   designed a fusion construct containing SARS-CoV-1/2 RBD, a short 5-aa G_4S linker (with a 2-aa

119   restriction site), and a trimeric viral capsid protein, SHP (PDB: 1TD0) (**Fig. 1A**). Structural

120   modeling showed that the three tethered RBDs form a triangle of 92 Å (measured at L492),

121   which is 14 and 18 Å wider than the SARS-CoV-1 "two-RBD-up" spike (PDB: 6CRX, measured

122   at L478) (*63*) and the MERS-CoV "all-RBD-up" spike (PDB: 5X59, measured for L506) (*64*),

123   respectively, allowing NAb access to each RBD. We then developed an immunoaffinity

124   chromatography (IAC) column to facilitate tag-free purification. Previously, NAb-derived IAC

125   columns have been used to purify HIV-1 Env trimers/NPs (*58, 59, 65, 66*), hepatitis C virus

126   (HCV) E2 cores/NPs (*57*), and Ebola virus (EBOV) GP trimers/NPs (*60*). Tian et al. reported

127   that a SARS-CoV-1 NAb, CR3022, can bind SARS-CoV-2 RBD (*34*). The SARS-CoV-2

128   RBD/CR3022 structure revealed a conserved cryptic epitope that is shared by the two SARS-

129   CoVs, suggesting that transient breathing motions of the spike protein enabled CR3022 binding

130   to the RBD (*50*). Here, we examined the utility of CR3022 in IAC columns. The SARS-CoV-1/2

131   RBD-5GS-1TD0 constructs were transiently expressed in 100-ml ExpiCHO cells and purified on

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132   a CR3022 column prior to size-exclusion chromatography (SEC) using a Superdex 200 10/300

133   GL column. While the SARS-CoV-1 RBD construct showed both aggregate (~8.6 ml) and trimer

134   (~12.7 ml) peaks in the SEC profile, the SARS-CoV-2 RBD construct produced a single, pure

135   trimer peak at ~12.8 ml (**Fig. 1B**). In sodium dodecyl sulfate-polyacrylamide gel electrophoresis

136   (SDS-PAGE), a monomer band of ~37 kD and a trimer band of ~100 kD were observed under

137   reducing and non-reducing conditions, respectively (**fig. S1A**). Antigenicity was assessed for the

138   two scaffolded RBD trimers in enzyme-linked immunosorbent assay (ELISA) after CR3022/SEC

139   purification (**Fig. 1C**). RBD-specific NAbs targeting SARS-CoV-1 (CR3022 (*67*), m396 (*68*),

140   80R (*69*), and S230 (*70*)) and SARS-CoV-2 (B38 (*38*), CB6 (*37*), S309 from a SARS survivor

141   (*33*), and P2B-2F6 (*36*)), were tested in ELISA. Overall, similar half maximal effective

142   concentration (EC_{50}) values were observed for the two RBD trimers binding to their respective

143   NAbs (**Fig. 1C**). The SARS-CoV-1 RBD trimer showed greater affinity for CR3022 than its

144   SARS-CoV-2 counterpart with a 1.3-fold difference in EC_{50} values, consistent with previous

145   findings (*34, 50*). Of the SARS-CoV-2 NAbs, B38 yielded a similar EC_{50} value to CR3022,

146   which can bind but not neutralize SARS-CoV-2. Antibody binding kinetics was measured by

147   biolayer interferometry (BLI) (**Fig. 1D** and **fig. 1B**). Overall, all tested mAbs exhibited a fast on-

148   rate, but with visible differences in their off-rates. B38 showed a faster off-rate than other SARS-

149   CoV-2 NAbs, while CR3022, the mAb used to purify SARS-CoV-1/2 RBD proteins, exhibited a

150   comparable kinetic profile.

151   We then hypothesized that the SpyTag/SpyCatcher (termed SPY) system could be used to

152   conjugate RBD to SApNPs to create multivalent RBD vaccines capable of eliciting a more

153   potent NAb response (**Fig. 1E**). The 13-aa SpyTag spontaneously reacts with the SpyCatcher

154   protein to form an irreversible isopeptide bond (*61*). The SPY system has been successfully used

155   to attach antigens to VLPs (*71*). Here, SpyTag was fused to the C terminus of RBD, while

156   SpyCatcher was fused to the N terminus of an SApNP subunit, both with a 5-aa G_4S linker. This

157   design was first tested for the 24-meric ferritin (FR). Here, we compared two production

158   strategies, co-expression of RBD-5GS-SpyTag and SpyCatcher-5GS-FR versus supernatant mix

159   after separate expression, both followed by purification on a CR3022 column. Protein obtained

160   from transient transfection in 50-ml ExpiCHO cells was analyzed by SEC on a Superose 6

161   10/300 GL column (**Fig. 1F**). Both production strategies produced a peak (12 ml) corresponding

162   to SApNPs. While the SARS-CoV-2 construct outperformed its SARS-CoV-1 counterpart in

163   particle yield (0.6-1.0 mg versus 0.3-0.5 mg after CR3022/SEC), the supernatant mix appeared to

164   be superior to co-expression for yield in both cases. The results thus suggest that both strategies

165   can be used to produce RBD SApNPs in Chinese hamster ovary (CHO) cells in an industrial

166   setting, as this mammalian expression system has been widely used to manufacture therapeutic

167   glycoproteins under good manufacturing practice (GMP) conditions (*72, 73*). Antigenicity was

168   assessed for SEC-purified RBD-5GS-SPY-5GS-FR SApNPs. In ELISA, RBD SApNPs showed

169   slightly improved mAb binding compared to the RBD trimers, as indicated by EC_{50} values (**Fig.**

170   **1G**). In BLI, a more pronounced effect of multivalent display on antigenicity was observed,

171   showing notably increased binding signals and plateaued dissociation (**Fig. 1H** and **fig. 1C**).

172   Structural integrity of various RBD SApNPs was analyzed by negative stain EM (nsEM) (**Figs.**

173   **1I** and **1J**). For SARS-CoV-1, a genetically fused RBD-10GS-FR construct produced very few,

174   albeit highly pure, SApNPs (**Fig. 1I**, left). In contrast, the RBD-5GS-SPY-5GS-FR construct

175   produced a high yield of SApNPs with visible surface decorations (**Fig. 1I**, right). For SARS-

176   CoV-2, the purified RBD-5GS-SPY-5GS-FR SApNPs, irrespective of the production strategy,

177   showed morphology of well-formed particles (**Fig. 1J**). Previously, we genetically fused a small

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178   LD protein and a T-cell epitope to each subunit of the E2p and I3-01v9 60-mers, resulting in

179   "multilayered" SApNPs (*60*). PADRE, a 13-aa pan-DR epitope that activates CD4$^+$ T cells (*74*),

180   was used to promote B cell development toward NAbs. Here, two of the best designs, E2p-LD4-

181   PADRE (or E2p-L4P) and I3-01v9-LD7-PADRE (or I3-01v9-L7P), were tested for their ability

182   to display SARS-CoV-1/2 RBDs. Following the strategy established for FR, SARS-CoV-1/2

183   RBDs were attached to the I3-01v9-L7P SApNP using the SPY system (**Fig. 1K**). Despite the

184   modest yield in ExpiCHO cells (**Fig. 1L**), large and pure particles were observed in the EM

185   images (**Fig. 1M**). However, some impurities were noted for the RBD-presenting E2p-L4P

186   SApNPs (**fig. S1D**). Nonetheless, we illustrated the utility of the SPY system for rapid

187   development of SARS-CoV-1/2 RBD vaccines based on three different SApNP platforms.

188   Recently, RBDs were displayed on various SApNPs using the SPY system as MERS-

189   CoV and SARS-CoV-2 vaccine candidates (*75, 76*). Walls et al. also reported a SARS-CoV-2

190   RBD vaccine candidate based on the two-component NP platform (*77*), which requires a

191   "connector" component to facilitate NP assembly (*78*), likely resulting in suboptimal stability.

192   Additionally, all these vaccine candidates require a complex production process involving two

193   expression systems, separate purification steps, and an *in vitro* assembly step before final

194   purification. In contrast, our SPY-linked RBD SApNPs are single-component by nature and can

195   be produced in CHO cells with a simple purification scheme, offering unique advantages in

196   stability and manufacturability.

Rational design of prefusion spike through minimizing metastability

198   In addition to the RBD, the SARS-CoV-1/2 spikes contain other NAb epitopes (*30*), which are

199   all presented in a trimeric context (**Fig. 2A**). A double-proline mutation (2P) between HR1 and

200   the central helix (CH) has been used to stabilize the MERS-CoV (*79*) and SARS-CoV-1 spikes

201   (*63*). A similar 2P mutation (K986P/V987P) was introduced into the SARS-CoV-2 spike (termed

202   S2P), which has been used to isolate and characterize NAbs (*33, 35, 40, 42-45, 49*) and is the

203   antigen in almost all vaccine candidates in clinical development (*11, 12*). However, a recent

204   cryo-EM study revealed an unexpected packing of S1 in the S2P spike, positioned ~12 Å

205   outwards, compared to the full-length native spike, as well as a more ordered FP proximal region

206   (FPPR) in S2 (*52*). New designs have been generated to control the spike conformation (*80*) or to

207   further stabilize it with more prolines (HexaPro) (*53*). Recent cryo-EM and cryo-ET studies

208   revealed that the SARS-CoV-2 spikes could adopt diverse orientations on native virions due to

209   the highly flexible HR2 stalk (*54-56*). Previously, we identified an HR1 bend as the cause of

210   HIV-1 Env metastability (*58, 81*) and examined the role of an equivalent HR1 bend and the HR2

211   stalk in EBOV GP metastability (*60*). This understanding of metastability proved critical for

212   designing stable trimers and trimer-presenting SApNP vaccines for both viruses (*58-60, 81*). It is

213   therefore imperative to explore the cause(s) of spike metastability to facilitate rational vaccine

214   design for SARS-CoV-2.

215   We first created uncleaved spike ectodomain (S_{ECTO}) constructs for SARS-CoV-1/2, both

216   containing the 2P mutation (K968P/V969P and K986P/V987P, respectively), a 5-aa G_4S linker, a

217   trimerization motif (PDB: 1TD0), and a C-terminal His_6 tag. The two constructs were transiently

218   expressed in 50-ml ExpiCHO cells followed by purification on either a Nickel column or a

219   CR3022 column. The $S2P_{ECTO}$-5GS-1TD0-His_6 protein was characterized by SEC on a Superose

220   6 10/300 GL column (**Fig. 2B**, panels 1 and 2). After the Nickel column, both $S2P_{ECTO}$

221   constructs showed a trimer peak (~12 ml) with the left and right shoulders indicative of

222   aggregate and dimer/monomer species, respectively. CR3022 purification resulted in a consistent

223   trimer peak, as well as reduction in dimer/monomer species. We then compared a pair of S_{ECTO}

224   constructs for SARS-CoV-1/2, both containing a double glycine (2G) mutation, K968G/V969G

225   and K986G/V987G, respectively. The 2G mutation had little effect on the SARS-CoV-1 spike

226   but produced abnormal SEC profiles and showed no yield for the SARS-CoV-2 spike after

227   purification by Nickel and CR3022 columns, respectively (**Fig. 2B**, panels 3 and 4). Lastly, we

228   tested a pair of S2G constructs without the HR2 stalk (E1150-Q1208), termed S2GΔHR2.

229   Deletion of the HR2 stalk restored the SARS-CoV-2 trimer peak and reduced aggregates for both

230   SARS-CoV-1/2, as shown by the SEC profiles upon CR3022 purification (**Fig. 2B**, panel 5).

231   Since the triple-hinged HR2 stalk can generate diverse spike orientations on native virions (*54-*

232   *56*), and the fusion core is formed by HR1 and HR2, we hypothesized that HR2 may be a key

233   determinant of SARS-CoV-2 spike metastability (**Fig. 2C**, left). It is plausible that the

234   interactions between HR1 and HR2 of two neighboring spikes may facilitate the pre-to-post-

235   fusion transition in addition to ACE2 binding and S1 dissociation. Given the extensive sequence

236   difference in HR1 (9 amino acids in total) compared to SARS-CoV-1 (**Fig. 2C**, right), we sought

237   to examine the contribution of HR1 to SARS-CoV-2 spike metastability with two HR1-swapped

238   (HR1$_S$) spike constructs. Interestingly, while HR1 swapping proved ineffective, deletion of the

239   HR2 stalk once again restored the trimer peak, suggesting a more important role for HR2 (**fig.**

240   **S2**, **A** to **C**). Therefore, S2GΔHR2 appeared to be a general spike design for SARS-CoV-1/2 and

241   perhaps other CoVs. Four separate production runs of SARS-CoV-2 S2GΔHR2-5GS-1TD0 in

242   300-ml ExpiCHO cells resulted in nearly identical SEC profiles with trimer yields of 0.8-1.0 mg

243   (**Fig. 2D**, left). Consistently, blue native polyacrylamide gel electrophoresis (BN-PAGE) showed

244   high trimer purity across SEC fractions (**Fig. 2D**, right). Antigenicity was assessed for

245   CR3022/SEC-purified SARS-CoV-2 S2P$_{ECTO}$ and S2GΔHR2 spike proteins. In ELISA, the

246   S2GΔHR2 spike showed slightly higher affinity for the five representative mAbs than did the

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247   S2P$_{ECTO}$ spike (**Fig. 2E**). When tested against three newly identified human NAbs, C105 (*49*)

248   and CC12.1/CC12.3 (*41*), the two spikes yielded similar EC$_{50}$ values (**fig. S2D**). In BLI, the

249   S2GΔHR2 spike showed higher binding signals than the S2P$_{ECTO}$ spike at the highest

250   concentration, while exhibiting similar binding kinetics (**Fig. 2F**). The use of NAb P2B-2F6 (*36*)

251   for spike purification resulted in higher trimer yield with similar purity to the CR3022 column

252   across SEC fractions (**fig. S2E**). Thermostability was assessed by differential scanning

253   calorimetry (DSC) for CR3022/SEC-purified SARS-CoV-2 S2P$_{ECTO}$ and S2GΔHR2 spikes (**fig.**

254   **S2F**). Upon the 2P-to-2G substitution and HR2 deletion, a small increase in thermal denaturation

255   midpoint (T$_m$) was observed (46.8 vs. 47.6 ℃), with notably higher onset temperature (T$_{on}$), 35.2

256   vs. 40.0 ℃, and narrower half width of the peak *(ΔT$_{1/2}$)*, 4.7 vs. 3.9 ℃. Altogether, we

257   demonstrated that the HR2 stalk is a major source of spike metastability and S2GΔHR2 presents

258   an alternative spike design to S2P, although more stabilizing mutations may be required to

259   achieve greater thermostability.

260   **Rational design of single-component self-assembling spike nanoparticles**

261   Although it was possible to conjugate trimeric SARS-CoV-2 spikes to an SApNP using the SPY

262   system (*82*), the random, irreversible linking will likely result in irregular display with

263   unoccupied but spatially occluded anchoring sites on the surface. The SPY system is perhaps

264   more suitable for small individual antigens (e.g. the RBD). Using the gene fusion approach, we

265   previously designed single-component SApNPs displaying stabilized HIV-1 Env trimers (*58, 59*)

266   and optimized HCV E2 cores (*57*). Recently, we reengineered E2p and I3-01v9 60-mers to

267   incorporate an inner layer of LDs to stabilize the non-covalently formed NP shell, in addition to

268   a cluster of T-cell epitopes (*60*). This multilayered design may be essential to the stability of

269   resulting SApNP vaccines when they are used to display large, complex viral antigens such as

270   the SARS-CoV-2 spike.

271   Native SARS-CoV-2 virions present both pre- and post-fusion spikes (*52, 54, 55*) (**Fig.**

272   **3A**, top), while our vaccine strategy aims to develop single-component SApNPs that each

273   present 8 or 20 stable prefusion S2GΔHR2 spikes to the immune system (**Fig. 3A**, bottom). As

274   demonstrated in our previous studies (*58, 59*), different linker lengths may be needed to connect

275   a trimer to the SApNP surface, as the spacing between the N termini of NP subunits around each

276   three-fold axis varies: FR (20 Å), E2p (9 Å), and I3-01v9 (50.5 Å). Based on this consideration,

277   we displayed the S2GΔHR2 spike on FR with a 5-aa G_4S linker, on E2p with a 5-aa G_4S linker,

278   and on I3-01v9 with a 10-aa $(G_4S)_2$ linker, resulting in SApNPs with diameters of 47.9, 55.9, and

279   59.3 nm, respectively (**Fig. 3B**). The multilayered E2p-L4P and I3-01v9-L7P (*60*), which were

280   validated for presenting RBDs (**Fig. 1**, **K** to **L**; **fig. S1D**), were used here as NP carriers of the

281   S2GΔHR2 spike. Together, three S2GΔHR2 SApNP constructs were transiently expressed in

282   400-ml ExpiCHO cells, followed by CR3022 purification and SEC on a Superose 6 10/300 GL

283   column (**Fig. 3C**). Three production runs for each of the three constructs generated highly

284   consistent SEC profiles, despite the variation of low molecular weight (m.w.) impurities

285   observed for the FR and E2p-L4P. After CR3022 and SEC purification, we obtained 0.3-0.4, 0.5-

286   1.0, and 0.8-1.2 mg protein for S2GΔHR2-5GS-FR, S2GΔHR2-5GS-E2p-L4P, and S2GΔHR2-

287   10GS-I3-01v9-L7P, respectively. Overall, the I3-01v9-derived S2GΔHR2 SApNP appeared to

288   perform best in terms of particle yield, purity, and stability. The structural integrity was analyzed

289   by nsEM, which showed well-formed particles of 45-65 nm with recognizable protrusions on the

290   surface (**Fig. 3D**). The varying shape of these profusions may correspond to S2GΔHR2 spikes

291   with "open" RBDs, in contrast to an array of "closed" HIV-1 and EBOV trimers on the SApNP

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292   surface (*58-60*). Such open conformation may facilitate induction of a strong RBD-specific NAb

293   response *in vivo*. Antigenicity of S2GΔHR2 SApNPs was assessed using the same panel of

294   mAbs. In ELISA, three SApNPs showed slightly improved binding to some, but not all, mAbs

295   compared to the individual spike (**Fig. 3E**). In BLI, we observed a clear correlation between peak

296   mAb binding signal and antigen valency, with E2p/I3-01v9 > FR > spike (**Fig. 3F**). Multivalent

297   display on the two 60-mers substantially increased mAb binding compared to the FR 24-mer. In

298   previous studies, we observed a similar correlation for HIV-1 Env trimer and HCV E2 core

299   versus their SApNPs (*57, 58*). In summary, these VLP-size SApNPs with 8 or 20 spikes on the

300   surface provide promising vaccine candidates for *in vivo* evaluation.

301   **SARS-CoV-1/2 vaccine-induced binding antibody response**

302   Selected SARS-CoV-1/2 RBD- and spike-based vaccine constructs were assessed in BALB/c

303   mice (**Fig. 4A**). We adopted a similar immunization protocol to be consistent with our previous

304   studies on HIV-1, HCV, and EBOV SApNPs (*57, 58, 60*). Briefly, groups of five mice were

305   immunized four times at three-week intervals. All antigens (50 μg per dose) were formulated

306   with AddaVax, an oil-in-water emulsion adjuvant, except for I3-01v9, which was mixed with

307   aluminum phosphate (AP) (*83*). We first analyzed the binding antibody (bAb) responses, as

308   measured by EC_{50} titers, in the two SARS-CoV-2 RBD vaccine groups (**Fig. 4B** and **fig. S4**).

309   The SPY-linked RBD SApNP (RBD-5GS-SPY-5GS-FR) elicited significantly higher bAb titers

310   than the scaffolded RBD trimer (RBD-5GS-1TD0) at w2 and w5, irrespective of the coating

311   antigen, and yielded a significant *P* value at w8 when the RBD was coated. Compared to the

312    $S2P_{ECTO}$ spike ($S2P_{ECTO}$-5GS-1TD0), the RBD SApNP elicited significantly higher bAb titers

313   against the RBD at w2, w5, and w8 (**Fig. 4B**, right), demonstrating a strong epitope-focusing

314   effect. Mouse sera bound the SARS-CoV-1 spike with lower EC_{50} titers than the SARS-CoV-2

315   spike but with similar patterns (**fig. S4A**). We then analyzed the bAb responses induced by

316   SARS-CoV-2 spikes $S2P_{ECTO}$-5GS-1TD0 and S2GΔHR2-5GS-1TD0, as well as three SApNPs

317   each displaying 8 or 20 S2GΔHR2 spikes (**Fig. 4C** and **fig. S5**). The S2GΔHR2 spike showed

318   two-three-fold higher average EC_{50} titers than the $S2P_{ECTO}$ spike irrespective of the coating

319   antigen (of note, to facilitate a fair comparison, mouse sera from these two groups were tested

320   against their respective spike antigens). Three SApNPs exhibited different temporal patterns

321   depending on the coating antigen. In terms of spike-specific response, the I3-01v9 group

322   registered a steady increase in average EC_{50} titer over time, showing the highest bAb titers at w2

323   and w8 and significantly outperforming the $S2P_{ECTO}$ group at all time points. The I3-01v9 group

324   also yielded higher EC_{50} titers than the S2GΔHR2 group throughout, although not with

325   significant *P* values. The FR SApNP group exhibited a similar temporal pattern with lower EC_{50}

326   titers, which were still significantly higher than the $S2P_{ECTO}$ group. Among the three SApNPs,

327   E2p exhibited the lowest average EC_{50} titer at w2 and reached the highest at w5, which then

328   decreased slightly at w8. In terms of RBD-specific response, the five groups showed a clear

329   ranking based on their average EC_{50} titers, which remained consistent across time points. At w2,

330   I3-01v9 showed an average EC_{50} titer of 175, whereas all other spike-based vaccines induced

331   little RBD-specific bAb response. At w5 and w8, S2GΔHR2 elicited higher bAb titers (on

332   average by two-fold) than $S2P_{ECTO}$, while all three SApNPs outperformed the individual

333   S2GΔHR2 spike with a ranking of average EC_{50} titers correlated with their size (FR < E2p < I3-

334   01v9). Sera reacted with the SARS-CoV-1 spike similarly, albeit at a lower level (**fig. S5A**).

335   Lastly, we compared the bAb responses induced by three SARS-CoV-1 vaccines, $S2P_{ECTO}$ spike

336   ($S2P_{ECTO}$-5GS-1TD0), scaffolded RBD trimer (RBD-5GS-1TD0), and SPY-linked RBD SApNP

337   (RBD-5GS-SPY-5GS-FR) (**Fig. 4D** and **fig. S6**). Based on average EC_{50} titers, the SARS-CoV-1

338   S2P$_{ECTO}$ spike appeared to have elicited a more robust bAb response than the SARS-CoV-2

339   S2GΔHR2 spike, whereas the SARS-CoV-1 RBD SApNP was less advantageous than its SARS-

340   COV-2 counterpart. Serum reactivity with the SARS-CoV-2 S2P$_{ECTO}$ spike was observed for all

341   three SARS-CoV-1 vaccine groups (**fig. S6A**). In summary, RBD SApNPs can elicit higher titers

342   of RBD-specific bAbs than the scaffolded RBD trimer and S2P$_{ECTO}$ spike, albeit at different

343   levels for the two SARS-CoVs. The S2GΔHR2 spike is more immunogenic than the S2P$_{ECTO}$

344   spike, showing on average two-fold higher bAb titers. The S2GΔHR2-presenting E2p and I3-

345   01v9 SApNPs are the best performers among all the spike-based vaccines, consistent with our

346   previous findings for these two large SApNPs (*57, 58, 60*).

SARS-CoV-1/2 vaccine-induced NAb response

348   One major goal in COVID-19 vaccine development is to generate a potent NAb response that

349   can protect against SARS-CoV-2 infection. Pseudoparticle (SARS-CoV-1/2-pp) neutralization

350   assays (*84*) were used to evaluate serum NAb responses elicited by different vaccines. We first

351   analyzed the NAb responses, as measured by 50% inhibitory dilution (ID$_{50}$) titers, in the two

352   SARS-CoV-2 RBD vaccine groups (**Fig. 5A** and **fig. S7**). The SPY-linked RBD SApNP elicited

353   a NAb response against autologous SARS-CoV-2 as early as w2, albeit with low titers, and

354   retained its advantage at w5 and w8, suggesting that such RBD SApNP vaccines can elicit a

355   rapid NAb response. The scaffolded RBD trimer group showed higher average ID$_{50}$ titers than

356   the S2P$_{ECTO}$ spike group at both w5 and w8. A somewhat different pattern was observed in the

357   SARS-CoV-1-pp assay. At w2, no vaccine group showed detectable heterologous NAb response.

358   At w5 and w8, the S2P$_{ECTO}$ spike elicited a more potent anti-SARS-CoV-1 NAb response than

359   the scaffolded RBD trimer, suggesting that non-RBD epitopes on the spike may contribute to

360   cross-neutralization. We then analyzed the NAb responses induced by five spike-based vaccines

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361   (**Fig. 5B** and **fig. S8**). In terms of autologous neutralization, no spike-based vaccine elicited any

362   NAb response that blocks SARS-CoV-2-pps after one dose, but a consistent pattern of serum

363   neutralization was observed at w5 and w8 (**Fig. 5B**, upper panel). Specifically, the S2P$_{ECTO}$ spike

364   showed the lowest average ID$_{50}$ titers, 879 and 2481 at w5 and w8, respectively, whereas the

365   S2GΔHR2 spike induced a stronger NAb response with 2.8-6.7-fold higher average ID$_{50}$ titers,

366   which did not reach $P \leq 0.05$ due to within-group variation. Nonetheless, this result confirmed

367   the beneficial effect of the 2G mutation and the HR2 stalk deletion on NAb elicitation. Among

368   the three SApNPs, E2p was the best performer at w5, showing an average ID$_{50}$ titer of 8435 that

369   is 9.6-fold higher than S2P$_{ECTO}$ and 1.4-fold higher than S2GΔHR2, while I3-01v9 showed the

370   most potent NAb response at w8 with an average ID$_{50}$ titer of 17351 that is about 7-fold and 2.5-

371   fold higher than S2P$_{ECTO}$ and S2GΔHR2, respectively. A similar temporal pattern was observed

372   in the heterologous SARS-CoV-1-pp assay (**Fig. 5B**, lower panel). It is worth noting that the

373   multilayered I3-01v9 SApNP elicited a SARS-CoV-1 NAb response with an average ID$_{50}$ titer of

374   351 at w2, whereas all other groups showed no detectable serum neutralization. These results

375   suggest that a well-designed SARS-CoV-2 S2GΔHR2 SApNP vaccine may provide protection

376   against both SARS-CoVs. Lastly, we analyzed the NAb responses induced by three SARS-CoV-

377   1 vaccines (**Fig. 5C** and **fig. S9**). In the autologous SARS-CoV-1-pp assay, the S2P$_{ECTO}$ spike

378   and the RBD SApNP induced significantly higher NAb titers than the scaffolded RBD trimer at

379   w2 and w5, and all three vaccine groups showed similar ID$_{50}$ titers at w8. However, heterologous

380   SARS-CoV-2 neutralization was below or at the baseline level for three SARS-CoV-1 vaccines

381   at w2, w5, and w8. In this study, the pseudovirus neutralization assay was validated using a panel

382   of known SARS-CoV-1/2 NAbs (**fig. S9C**). As a negative control, the w8 mouse sera were tested

383   against pseudoparticles bearing the murine leukemia virus (MLV) Env, MLV-pps, showing no

384   detectable reactivity (**fig. S9D**). In summary, these results demonstrate an advantage in NAb

385   elicitation by the SARS-CoV-2 S2GΔHR2 spike and its SApNPs compared to the widely used

386   S2P$_{ECTO}$ spike. Although SARS-CoV-2 RBD- and S2GΔHR2-presenting SApNPs are both

387   effective at eliciting an autologous NAb response, the latter can induce high NAb titers to SARS-

388   CoV-1 and may provide broader protection against SARS-associated CoVs.

SARS-CoV-2 vaccine-induced T-cell response

390   While humoral immunity is required to block host-virus interaction and prevent viral infection,

391   cellular immunity is essential for eliminating infected host cells to control viral infection (*85-88*).

392   Emerging evidence indicates that an early T-cell response (*89, 90*), as well as T-cell memory

393   (*91*), is critical for protection against SARS-CoV-2. However, COVID-19 vaccines must induce

394   a CD4$^+$ T helper 1 (Th1), but not Th2-type, T-cell response, as the latter has been implicated in

395   vaccine-associated enhancement of respiratory disease (VAERD) (*10*). In addition, T follicular

396   helper cells (Tfh) play an important role in the maturation and production of NAbs. Therefore,

397   understanding T-cell responses is crucial for development of an effective and safe COVID-19

398   vaccine.

399   Interferon (IFN)-γ-producing Th1 cells are important for generating an optimal antibody

400   response and for induction of cellular immunity (*85-87*). We first examined various SARS-CoV-

401   2 vaccines on induction of CD4$^+$ Th1 responses specific to the vaccine antigen at w11, two

402   weeks after the fourth immunization, when memory T cells had already developed in spleen (*88*).

403   Mouse splenocytes from the S2P$_{ECTO}$ group and two S2GΔHR2 SApNP groups (E2p and I3-

404   01v9) were analyzed by flow cytometry using naïve samples as a negative control. The I3-01v9

405   group induced about 1.5- and 2.3-fold higher frequency of IFN-γ-producing CD4$^+$ Th1 cells than

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406   the $S2P_{ECTO}$ and E2p groups, respectively (**Fig. 6A**). Notably, after re-stimulation with the

407   respective antigens for as few as 4 hours, both SApNP groups produced ~two-fold higher

408   frequency of CD107a-producing cytolytic $CD4^+$ T cells than the $S2P_{ECTO}$ and naïve groups (**Fig.**

409   **6B**). IFN-γ/IL-4 (interleukin-4) double-positive cells are memory $CD4^+$ T cells that have

410   acquired the ability to produce IL-4 while still retaining the ability to produce IFN-γ under Th1

411   conditions (*92*). It appeared that I3-01v9 induced three- and five-fold more IFN-γ/IL-4 double-

412   positive memory $CD4^+$ T cells than $S2P_{ECTO}$ and E2p (**Fig. 6A**). These results suggest that I3-

413   01v9 can induce both $CD4^+$ Th1 cells and IFN-γ/IL-4 double-positive memory $CD4^+$ T cells. In

414   addition, I3-01v9 induced more IFN-γ/GM-CSF (granulocyte-macrophage colony-stimulating

415   factor) double-positive $CD8^+$ effector T cells than $S2P_{ECTO}$ and E2p (**Fig. 6C**), suggesting that

416   protective $CD8^+$ T cells were also generated in mice immunized with I3-01v9. Of note, $CD8^+$ T

417   cells derived from mice immunized with I3-01v9, rather than those with $S2P_{ECTO}$ and E2p,

418   acquired the ability to rapidly produce IFN-γ upon antigen re-stimulation (**Fig. 6D**), suggesting

419   generation of I3-01v9-responsive effector/memory T cells. Our results indicate that the

420   S2GΔHR2 I3-01v9 SApNP can induce robust T-cell responses consisting of $CD4^+$ Th1 cells,

421   IFN-γ/IL-4 double-positive memory $CD4^+$ T cells, and effector $CD8^+$ T cells, thus providing

422   protective cellular immunity in addition to a potent NAb response. Since T cell immunity against

423   the SApNP backbone cannot be ruled out, a more detailed T-cell analysis using soluble spikes,

424   spike peptides, and naked SApNPs for re-stimulation may be warranted.

425   **DISCUSSION**

426   COVID-19 is the first worldwide pandemic of this scale since the infamous Spanish influenza

427   over a century ago (*93*), which caused ~50 million deaths worldwide and remains a painful

428   reminder of our vulnerability to a new virus without a protective vaccine. The rapid spread of

19

429 SARS-CoV-2 therefore demanded rapid vaccine development (*10*). Operation Warp Speed

430 (OWS) was launched in May 2020 with the initial goal to deliver 300 million doses of safe and

431 effective vaccines by January 2021 (*94*), although that goal has not yet been realized.

432 Nevertheless, in December 2020, while other vaccine candidates were still being evaluated in

433 clinical trials, two OWS-supported mRNA vaccines were approved for EUA, marking an

434 important turning point in the battle against the pandemic. However, vaccine development during

435 a pandemic against a new virus poses unique challenges, one of which is how to balance public

436 health needs versus scientific rigor (*95-97*). The global vaccine campaign also provides a unique

437 opportunity to compare different vaccine design strategies and platforms – especially new ones –

438 against a common target. While mRNA and viral vector vaccines remain the front runners,

439 protein-based vaccines are highlighted in a recent review with the prediction that they will

440 eventually reach a larger fraction of the global population (*98*). As the NAb titers induced by the

441 first-generation nucleic acid vaccines wane over time, effective protein vaccines will be needed

442 to sustain long-term immunity against SARS-CoV-2.

443 Here, we approached SARS-CoV-2 vaccine with a rational design strategy and set out to

444 develop protein nanoparticle vaccines that can be used alone or as a booster vaccine. To this end,

445 a panel of vaccine candidates have been generated based on three SApNP platforms with

446 different *in vitro* and *in vivo* attributes (**table S1**). Our comparative analysis of these vaccine

447 constructs has offered some valuable insights. First, the choice of antigen is critical to the

448 success of SARS-CoV-2 vaccine irrespective of the delivery platform. Most vaccine antigens

449 including OWS's vaccine candidates are based on S2P, which produces a spike structure that

450 differs in detail from the full-length wildtype spike, e.g. in FPPR of S2 and in the relative

451 dispositions of the S1 domains (*52*). These differences may complicate interpretation of vaccine

452   outcome. S2P and other empirical spike designs (*53*) have attempted to constrain the spike

453   conformation and increase trimer yield. However, as we previously demonstrated for HIV-1 Env

454   and EBOV GP (*58, 60, 81*), it is important to identify and eliminate (if possible) the cause of

455   spike metastability. During antigen screening, we found that deletion of the HR2 stalk with a 2P-

456   to-2G substitution renders a more stable spike, which is consistent with recent reports on a highly

457   flexible HR2 stalk in the native spikes on SARS-CoV-2 virions (*54-56*). Thus, S2GΔHR2 would

458   seem to represent a major advance in spike design. Second, single-component SApNPs provide a

459   powerful platform for vaccine development against diverse viral pathogens (*57, 58, 60*). Here,

460   S2GΔHR2 was genetically fused, rather than chemically linked, to three SApNPs, including two

461   multilayered 60-meric carriers with enhanced stability and an embedded T-help signal. Such

462   protein vaccines should be more effective in eliciting a potent NAb response and less likely to

463   induce adverse responses (*98, 99*). An epitope-focused vaccine strategy was also explored by

464   designing scaffolded RBD trimers and SPY-linked RBD SApNPs. Third, to achieve high

465   efficacy and ensure safety, vaccine-induced NAb and T-cell responses must be evaluated in

466   animals prior to clinical trials. Indeed, in our mouse study, S2GΔHR2 appeared to be more

467   effective than S2P in NAb elicitation, both alone and displayed on SApNPs. Of note, the

468   multilayered S2GΔHR2 I3-01v9 SApNP elicited not only high NAb titers but also desired T-cell

469   responses. In addition to CD4$^+$ Th1 cells and memory CD4$^+$ T cells, this SApNP also induced

470   CD107a-producing cytolytic CD4$^+$ T cells, which may directly kill infected host cells, and GM-

471   CSF-producing CD8$^+$ effector T cells, which may promote the generation of macrophages and

472   functional dendritic cells (DCs) to facilitate the clearance of infected cells. It is also worth noting

473   that the NAb response to the large SApNPs plateaued after three doses, suggesting that the

474   number of injections and dosage can be reduced without diminishing vaccine-induced responses.

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475   Lastly, expression of vaccine antigens in CHO cells followed by purification using an antibody

476   column, such as CR3022, would allow rapid and industrial-scale vaccine production. In

477   summary, our study provides promising COVID-19 vaccine candidates for evaluation in clinical

478   trials.

479   **MATERIALS AND METHODS**

480   **Design, expression, and purification of SARS-CoV-1/2 RBD and spike antigens**

481   The spike (S) genes of the SARS-CoV-1 isolate Tor2 (GenBank accession #: NC_004718) and

482   the SARS-CoV-2 isolate Wuhan-Hu-1 (GenBank accession #: MN908947) were used to design

483   all the RBD and spike constructs following codon-optimization for expression in mammalian

484   cells. The RBD sequence is defined as P317-D518 and P330-N532 for SARS-CoV-1 and 2,

485   respectively. The S_{ECTO} sequence is defined as M1-Q1190 and M1-Q1208 for SARS-CoV-1 and

486   2, respectively. To remove the S1/S2 cleavage site, an R667G mutation and a ^{682}GSAGSV^{687}

487   modification were introduced in the SARS-CoV-1 and 2 spikes, respectively. The 2P (or 2G)

488   mutation was made to K968/V969 and K986/V987 in the SARS-CoV-1 and 2 spikes,

489   respectively. The SARS-CoV-2 C-terminal region (E1150-Q1208) containing the HR2 stalk was

490   removed from S2G$_{ECTO}$, resulting in an HR2-deleted spike construct, termed S2GΔHR2. The

491   viral capsid protein SHP (PDB: 1TD0) was used as a trimerization motif in spike constructs for

492   immunization, whereas the foldon domain from the bacteriophage T4 fibritin (PDB: 1RFO) was

493   used in coating spike antigens for ELISA to mask the 1TD0-derived antibody response. All

494   constructs were transiently expressed in ExpiCHO cells (Thermo Fisher). Briefly, ExpiCHO

495   cells were thawed and incubated with ExpiCHO^{TM} Expression Medium (Thermo Fisher) in a

496   shaker incubator at 37 °C, 135 rpm and 8% CO_2. When the cells reached a density of 10×10^6 ml$^-$

497   ^{1}, ExpiCHO^{TM} Expression Medium was added to reduce cell density to 6×10^6 ml^{-1} for

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498 transfection. The ExpiFectamine^{TM} CHO/plasmid DNA complexes were prepared for 100-ml

499 transfection in ExpiCHO cells following the manufacturer's instructions. For a given construct,

500 100 μg of plasmid and 320 μl of ExpiFectamine^{TM} CHO reagent were mixed in 7.7 ml of cold

501 OptiPRO™ medium (Thermo Fisher). After the first feed on day one, ExpiCHO cells were

502 cultured in a shaker incubator at 33 °C, 115 rpm and 8% CO_2 following the Max Titer protocol

503 with an additional feed on day five (Thermo Fisher). Culture supernatants were harvested 13 to

504 14 days after transfection, clarified by centrifugation at 4000 rpm for 25 min, and filtered using a

505 0.45 μm filter (Thermo Fisher). The CR3022 antibody column was used to extract SARS-CoV-

506 1/2 antigens from the supernatants, which was followed by SEC on a Superdex 200 10/300 GL

507 column (for scaffolded RBD trimers) or a Superose 6 10/300 GL column (for SPY-linked RBD

508 SApNPs, spikes, and S2GΔHR2 SApNPs). A human NAb, P2B-2F6 (*36*), was also used to pack

509 antibody columns for purification of the SARS-CoV-2 S2GΔHR2 spike, which was followed by

510 SEC on a HiLoad 16/600 Superose 6 column. For comparison, His$_6$-tagged S_{ECTO}-5GS-1TD0

511 spike protein was extracted from the supernatants using an immobilized Ni Sepharose^{TM} Excel

512 column (GE Healthcare) and eluted with 500 mM Imidazole prior to SEC. Protein concentration

513 was determined using UV_{280} absorbance with theoretical extinction coefficients.

514 **Blue native polyacrylamide gel electrophoresis**

515 SARS-CoV-2 spikes and spike-presenting SApNPs were analyzed by blue native polyacrylamide

516 gel electrophoresis (*BN-PAGE*) and stained with Coomassie blue. The proteins were mixed with

517 sample buffer and G250 loading dye and added to a 4-12% Bis-Tris NativePAGE^{TM} gel (Life

518 Technologies). BN-PAGE gels were run for 2 to 2.5 hours at 150 V using the NativePAGE^{TM}

519 running buffer (Life Technologies) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay

Each well of a Costar^{TM} 96-well assay plate (Corning) was first coated with 50 µl PBS containing 0.2 µg of the appropriate antigens. The plates were incubated overnight at 4 °C, and then washed five times with wash buffer containing PBS and 0.05% (v/v) Tween 20. Each well was then coated with 150 µl of a blocking buffer consisting of PBS and 40 mg ml^{-1} blotting-grade blocker (Bio-Rad). The plates were incubated with the blocking buffer for 1 hour at room temperature, and then washed five times with wash buffer. For antigen binding, antibodies (in the immunoglobulin G (IgG) form) were diluted in the blocking buffer to a maximum concentration of 5 µg ml^{-1} followed by a 10-fold dilution series. For each antibody dilution, a total of 50 µl volume was added to the appropriate wells. For mouse sample analysis, plasma was diluted by 20-fold in the blocking buffer and subjected to a 10-fold dilution series. For each sample dilution, a total of 50 µl volume was added to the wells. Each plate was incubated for 1 hour at room temperature, and then washed 5 times with PBS containing 0.05% Tween 20. For antibody binding, a 1:5000 dilution of goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories, Inc), or for mouse sample analysis, a 1:3000 dilution of horseradish peroxidase (HRP)-labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories), was then made in the wash buffer (PBS containing 0.05% Tween 20), with 50 µl of this diluted secondary antibody added to each well. The plates were incubated with the secondary antibody for 1 hour at room temperature, and then washed 6 times with PBS containing 0.05% Tween 20. Finally, the wells were developed with 50 µl of TMB (Life Sciences) for 3-5 min before stopping the reaction with 50 µl of 2 N sulfuric acid. The resulting plate readouts were measured at a wavelength of 450 nm. Of note, the w2 serum binding did not reach the plateau (or saturation) to allow for accurate determination of EC$_{50}$ titers. Nonetheless, the EC$_{50}$ values at w2 were derived

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543   by setting the lower/upper constraints of OD_{450} at 0.0/3.2 to facilitate the comparison of different

544   vaccine groups at the first time point.

545   **Bio-layer interferometry**

546   The kinetics of SARS-CoV-1/2 vaccine antigens, RBD versus RBD-presenting SApNPs as well

547   as spike versus spike-presenting SApNPs, binding to a panel of known antibodies (in the IgG

548   form) was measured using an Octet RED96 instrument (FortéBio, Pall Life Sciences). All assays

549   were performed with agitation set to 1000 rpm in FortéBio 1× kinetic buffer. The final volume

550   for all the solutions was 200 μl per well. Assays were performed at 30 °C in solid black 96-well

551   plates (Geiger Bio-One). For all antigens except for S2GΔHR2 SApNPs, 5 μg ml^{-1} of antibody in

552   1× kinetic buffer was loaded onto the surface of anti-human Fc Capture Biosensors (AHC) for

553   300 s. For S2GΔHR2 SApNPs, anti-human Fc Quantitation Biosensors (AHQ) were used. A 60 s

554   biosensor baseline step was applied prior to the analysis of the association of the antibody on the

555   biosensor to the antigen in solution for 200 s. A two-fold concentration gradient of antigen,

556   starting at 950 nM for scaffolded RBD trimers, 37 nM for SPY-linked RBD FR SApNP (RBD-

557   5GS-SPY-5GS-FR), 150 nM for soluble spikes, and 9/3.5/3.5 nM for S2GΔHR2 presented on

558   FR/E2p/I3-01v9 SApNPs, was used in a titration series of six. The dissociation of the interaction

559   was followed for 300 s. Correction of baseline drift was performed by subtracting the mean value

560   of shifts recorded for a sensor loaded with antibody but not incubated with antigen, and for a

561   sensor without antibody but incubated with antigen. Octet data were processed by FortéBio's

562   data acquisition software v.8.1. Experimental data were fitted with the binding equations

563   describing a 2:1 interaction to achieve optimal fitting. Of note, the S2GΔHR2 spike was also

564   measured using AHQ biosensors to facilitate comparison of mAb binding with the S2GΔHR2-

565   presenting SApNPs.

25

Differential scanning calorimetry (DSC)

Thermal melting curves of SARS-CoV-2 S2$_{PECTO}$-5GS-1TD0 and S2GΔHR2-5GS-1TD0 spike proteins following CR3022 and SEC purification were obtained from a MicroCal PEAQ-DSC Man instrument (Malvern). The purified spike trimer protein produced from ExpiCHO cells were buffer exchanged into 1×PBS and concentrated to 0.8 μM before analysis by the instrument. Melting was probed at a scan rate of 60 °C·h^{-1} from 20 °C to 100 °C. Data processing, including buffer correction, normalization, and baseline subtraction, was conducted using the MicroCal PEAQ-DSC software. Gaussian fitting was performed using the Origin 9.0 software.

Electron microscopy (EM) assessment of nanoparticle constructs

The EM analysis of various RBD and S2GΔHR2-presenting SApNPs was performed by the Core Microscopy Facility at The Scripps Research Institute. All SApNP samples were prepared at the concentration of 0.01-0.05 mg/ml. Carbon-coated copper grids (400 mesh) were glow-discharged and 8 µL of each sample was adsorbed for 2 min. Excess sample was wicked away and grids were negatively stained with 2% uranyl formate for 2 min. Excess stain was wicked away and the grids were allowed to dry. Samples were analyzed at 80 kV with a Talos L120C transmission electron microscope (Thermo Fisher) and images were acquired with a CETA 16M CMOS camera.

Animal immunization and sample collection

Similar immunization protocols have been reported in our previous vaccine studies (*57, 58, 60*). Briefly, the Institutional Animal Care and Use Committee (IACUC) guidelines were followed with animal subjects tested in the immunization study. Eight-week-old BALB/c mice were purchased from The Jackson Laboratory and housed in ventilated cages in environmentally

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588   controlled rooms at The Scripps Research Institute, in compliance with an approved IACUC

589   protocol and AAALAC (Association for Assessment and Accreditation of Laboratory Animal

590   Care) International guidelines. Mice were immunized at weeks 0, 3, 6, and 9 with 200 μl of

591   antigen/adjuvant mix containing 50 μg of vaccine antigen and 100 μl of adjuvant, AddaVax or

592   Adju-Phos (InvivoGen), via the intraperitoneal (i.p.) route. Blood was collected two weeks after

593   each immunization. All bleeds were performed through the retro-orbital sinus using heparinized

594   capillary tubes into EDTA-coated tubes. Samples were spun at 1200 RPM for 10 min to separate

595   plasma (top layer) and the rest of the whole blood layer. Upon heat inactivation at 56 °C for 30

596   min, the plasma was spun at 2000 RPM for 10 min to remove precipitates. The rest of the whole

597   blood layer was diluted with an equal volume of PBS and then overlaid on 4.5 ml of Ficoll in a

598   15 ml SepMate^{TM} tube (STEMCELL Technologies) and spun at 1200 RPM for 10 min at 20 °C

599   to separate peripheral blood mononuclear cells (PBMCs). Cells were washed once in PBS and

600   then resuspended in 1 ml of ACK Red Blood Cell lysis buffer (Lonza). After washing with PBS,

601   PBMCs were resuspended in 2 ml of Bambanker Freezing Media (Lymphotec). Spleens were

602   harvested at w11 and ground against a 70-μm cell strainer (BD Falcon) to release the splenocytes

603   into a cell suspension. Splenocytes were centrifuged, washed in PBS, treated with 5 ml of ACK

604   lysing buffer (Lonza), and frozen with 3ml of Bambanker freezing media. Plasma was used for

605   ELISA and neutralization assays to determine binding and neutralizing antibody responses in

606   mouse serum.

607   **SARS-CoV-1/2 pseudovirus neutralization assay**

608   Pseudoparticle (SARS-CoV-1/2-pp) neutralization assays were utilized to assess the neutralizing

609   activity of previously reported antibodies and vaccine-induced murine antibody response. SARS-

610   CoV-1/2-pps were generated by co-transfection of HEK293T cells with the HIV-1 pNL4-3.lucR-

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611   E- plasmid (obtained from the NIH AIDS reagent program: https://www.aidsreagent.org/) and

612   the expression plasmid encoding the S gene of SARS-CoV-1 isolate Tor2 (GenBank accession #:

613   NC_004718) and the SARS-CoV-2 isolate Wuhan-Hu-1 (GenBank accession #: MN908947) at a

614   4:1 ratio by lipofectamine 3000 (Thermo Fisher Scientific). After 48 to 72 hours, SARS-CoV-

615   1/2-pps were collected from the supernatant by centrifugation at 4000 rpm for 10 min, aliquoted,

616   and stored at -80 °C before use. The mAbs at a starting concentration of 0.1-10 μg/ml, or mouse

617   plasma at a starting dilution of 100-fold, were mixed with the supernatant containing SARS-

618   CoV-1/2-pps and incubated for 1 hour at 37°C in white solid-bottom 96-well plate (Corning). A

619   3-fold dilution series was used in the assay. The HEK293T-hACE2 cell line (catalogue#: NR-

620   52511) and the vector pcDNA3.1(-) containing the SARS-CoV-2 S gene (catalogue#: NR52420)

621   were obtained from BEI RESOURCES (https://www.beiresources.org/) and used in pseudovirus

622   neutralization assays (*84*). Briefly, HEK293T-hACE2 cells at 1×10^4 were added to each well and

623   the plate was incubated at 37°C for 48 hours. After incubation, overlying media was removed,

624   and cells were lysed. The firefly luciferase signal from infected cells was determined using the

625   Bright-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions.

626   Data were retrieved from a BioTek microplate reader with Gen 5 software, the average

627   background luminescence from a series of uninfected wells was subtracted from each well, and

628   neutralization curves were generated using GraphPad Prism 8.4.3, in which values from wells

629   were compared against a well containing SARS-CoV-1/2-pp only. The same HIV-1 vectors

630   pseudotyped with the murine leukemia virus (MLV) Env gene, termed MLV-pps, were produced

631   in HEK293T cells and included in the neutralization assays as a negative control. As the NAb

632   titers plateaued after w8, the w11 results were not shown in Fig. 5 but included in figs. S7-S9 for

633   comparison.

Dendritic cell (DC) production

634

635 Mouse bone marrow (BM) was cultured in RPMI 1640 medium containing 10% fetal bovine

636 serum and recombinant mouse Flt3L (50 ng/mL) and SCF (10 ng/ml) for 9 days as described

637 (*100*). To induce DC activation, immature DCs were incubated with lipopolysaccharide (LPS,

638 100 ng/mL), R848 (Resiquimod, 100 ng/mL) or CpG (ODN 1585, 1µM) overnight, which

639 activated Toll-like receptor (TLR)4 , TLR7/8 or TLR9 signaling, respectively. Cells were

640 harvested for experiments. pDCs were sorted to isolate CD11c$^+$B220$^+$ cells using FACS cell

641 sorter and magnetic beads (Miltenyi-Biotech, CA).

Antibodies and flow cytometry analysis

642

643 All antibodies used for immunofluorescence staining were purchased from eBioscience (San

644 Diego, CA), BioLegend (San Diego, CA) or BD Biosciences (San Jose, CA). Magnetic

645 microbead-conjugated Abs and streptavidin were purchased from Miltenyi-Biotech (Auburn,

646 CA). Recombinant human IL-2 protein was purchased from R&D Systems (Minneapolis, MN).

647 Recombinant mouse Flt3 ligand (Flt3L) and mouse SCF were purchased from Shenandoah

648 Biotech (Warwick, PA). Cells were stained with appropriate concentrations of mAbs. Dead cells

649 were excluded using Fixable Viability Dye from eBioscience (San Diego, CA). Flow cytometry

650 (FC) analyses were performed using LSRII (BD Bioscience, CA) and Canto cytometers (Becton

651 Dickinson, NJ). Cells were sorted on BD FACSAria II (BD Bioscience, CA).

T cell culture and activation

652

653 Splenic mononuclear cells from each group of immunized mice were cultured in the presence of

654 DCs pulsed with or without S2P$_{ECTO}$, multilayered S2GΔHR2-presenting E2P or I3-01v9 SApNP

29

655    $(1 \times 10^{-7}~\mu M)$ in complete IMDM medium containing IL-2 (5.0 ng/ml). Cells were collected 16

656   hours and 4 hours later for intracellular cytokine staining and flow cytometric analysis.

657   **Statistics**

658   In antibody analysis, comparison of different vaccine groups was performed in GraphPad Prism

659   8.4.3 using the two-tailed unpaired Student's *t* test. In T cell analysis, comparison of means was

660   done using the two-tailed unpaired Student's t test, ANOVA and then post-hoc *t* test. P values of

661   0.05 or less were considered significant.

662

663   **SUPPLEMENTARY MATERIALS**

664   Supplementary material for this article is available at http://xxx/xxx/xxx.

665   **fig. S1**. In-vitro characterization of SARS-CoV-1/2 RBD-based immunogens.

666   **fig. S2**. In-vitro characterization of SARS-CoV-2 spike antigens.

667   **fig. S3.** In-vitro characterization of SARS-CoV-2 S2GΔHR2 SApNPs.

668   **fig. S4.** SARS-CoV-2 RBD/RBD-SApNP vaccine-induced binding antibody responses.

669   **fig. S5.** SARS-CoV-2 spike/spike-SApNP vaccine-induced binding antibody responses.

670   **fig. S6.** SARS-CoV-1 spike/RBD/RBD-SApNP vaccine-induced binding antibody responses.

671   **fig. S7.** SARS-CoV-2 RBD/RBD-SApNP vaccine-induced neutralizing antibody responses.

672   **fig. S8.** SARS-CoV-2 spike/spike-SApNP vaccine-induced neutralizing antibody responses.

673   **fig. S9.** SARS-CoV-1 spike/RBD/RBD-SApNP vaccine-induced neutralizing antibody

674   responses.

675   **table. S1.** Key *in vitro* and *in vivo* characteristics of RBD and spike-based SARS-CoV-2

676   vaccines.

677   References

30

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890

893 **Author contributions:** Project design by L.H., Y.Z., I.A.W. and J.Z; construct design of RBD

894 and spike-based immungoens by L.H. and J.Z.; plasmid design and processing by L.H. and C.S.;

895 antigen production, purification and basic characterization by L.H., X.L., and T.N.; antibody

896 production and column packing by L.H., X.L., and T.N.; antibody-antigen ELISA and BLI by

897 L.H. and C.S.; DSC by L.H. and J.Z.; negative-stain EM by L.H. and J.Z.; plasma-antigen

898   ELISA by L.H., X.L., and C.S.; antibody and mouse plasma neutralization by L.H. and X.L.;

899   vaccine-induced T-cell response analysis by Y.W., C.A., and Y.Z.; Manuscript written by L.H.,

900   Y.Z., I.A.W. and J.Z. All authors were asked to comment on the manuscript. The TSRI

901   manuscript number is 30028. **Competing interests:** Authors declare no competing interests.

902   **Data and materials availability:** All data are available in the main text or in the supplementary

903   materials. Additional data related to this paper may be requested from the corresponding author.

904

905   **Figure legends**

906   **Fig. 1. Rational design of SARS-CoV-1/2 RBD-based vaccines.** **(A)** Structural model of RBD-

907   5GS-1TD0 in an extended RBD-up conformation (top view and side view). 1TD0 is a

908   trimerization scaffold of viral origin. **(B)** SEC profiles of SARS-CoV-1/2 scaffolded RBD

909   trimers following ExpiCHO expression and CR3022 purification. **(C)** ELISA binding of SARS-

910   CoV-1/2 scaffolded RBD trimers to a panel of mAbs. EC_{50} (μg/ml) values are labeled. **(D)** Octet

911   binding of the SARS-CoV-2 scaffolded RBD trimer to five mAbs. Sensorgrams were obtained

912   from an Octet RED96 instrument at six antigen concentrations from 950 to 29.5 nM by twofold

913   dilutions. **(E)** Diagram of conjugating RBD to the 24-meric FR SApNP using the

914   SpyTag/SpyCatcher (SPY) system. **(F)** SEC profiles of SARS-CoV-1/2 RBD-5GS-SPY-5GS-FR

915   SApNPs produced in ExpiCHO by co-expression (black line) and supernatant mix (red line). **(G)**

916   ELISA binding of SARS-CoV-1/2 RBD-FR SApNPs to a panel of mAbs. EC_{50} (μg/ml) values

917   are labeled. **(H)** Octet binding of the SARS-CoV-2 RBD-FR SApNP to five mAbs. Sensorgrams

918   were obtained from an Octet RED96 instrument at six antigen concentrations from 37 to 1.1 nM

919   by twofold dilutions. **(I)** EM images of SARS-CoV-1 RBD-10GS-FR (gene fusion) and RBD-

920   5GS-SPY-5GS-FR (co-expression). **(J)** EM images of SARS-CoV-2 RBD-5GS-SPY-5GS-FR

921   produced by co-expression and supernatant mix. **(K)** Diagram of conjugating RBD to the 60-

922   meric multilayered I3-01v9 SApNP using the SPY system. Locking domains (LD) and T-helper

923   epitopes within the multilayered SApNP are depicted. **(L)** and **(M)** SEC profiles and EM images

924   of SARS-CoV-1/2 RBD-5GS-SPY-5GS-I3-01v9-LD7-PADRE (or I3-01v9-L7P) SApNPs

925   produced by supernatant mix. SEC profiles of scaffolded RBD trimers in (B) and SPY-linked

926   RBD SApNPs in (F) and (L) were obtained from a Superdex 200 10/300 GL column and a

927   Superose 6 10/300 GL column, respectively.

928   **Fig. 2. Rational design of SARS-CoV-2 spike antigens. (A)** Structural model of prefusion S

929   spike linked to the C-terminal trimerization domain (1TD0) with a 5GS linker in transparent

930   molecular surface. The approximate position for the unstructured HR2 stalk, or in this case a 5-

931   aa G_4S linker, is highlighted with a dashed line box. **(B)** SEC profiles of SARS-CoV-1 (top) and

932   SARS-CoV-2 (bottom) spikes. Left to right panels: $S2P_{ECTO}$-5GS-1TD0 purified on Nickel and

933   CR3022 columns (1 and 2), $S2G_{ECTO}$-5GS-1TD0 on Nickel and CR3022 columns (3 and 4), and

934   S2GΔHR2-5GS-1TD0 on a CR3022 column (5). All spike constructs tested in (B) contain a C-

935   terminal His_6 tag to facilitate Nickel column purification. Results from three separate production

936   runs are shown for SARS-CoV-2 $S2G_{ECTO}$-5GS-1TD0. **(C)** Schematic representation of a full-

937   length SARS-CoV-2 spike on the virus membrane surface in the presence of host ACE2 and the

938   HR2 region from a neighboring spike (left), and sequence alignment of SARS-CoV-1/2 HR1 and

939   HR2 regions (right, top and bottom). The HR1 and HR2 segments that form a six-helix bundle in

940   the post-fusion state are highlighted in green and brown shade, respectively. **(D)** Left: SEC

941   profiles of S2GΔHR2-5GS-1TD0, showing results from four separate production runs. Right:

942   BN-PAGE analysis of $S2P_{ECTO}$-5GS-1TD0 and S2GΔHR2-5GS-1TD0. SEC fractions (12.5-

943   14.0) are shown for S2GΔHR2-5GS-1TD0 on the gel. SEC profiles in (B) and (D) were obtained

944   from a Superose 6 10/300 GL column. **(E)** ELISA binding of two SARS-CoV-2 spikes

945   (S2P$_{ECTO}$-5GS-1TD0 and S2GΔHR2-5GS-1TD0) to five mAbs. EC$_{50}$ (µg/ml) values are labeled.

946   **(F)** Octet binding of two SARS-CoV-2 spikes (S2P$_{ECTO}$-5GS-1TD0 and S2GΔHR2-5GS-1TD0)

947   to five mAbs. Sensorgrams were obtained from an Octet RED96 instrument at six antigen

948   concentrations from 150 to 4.7 nM by twofold dilutions. The spike constructs tested in (D)-(F)

949   do not contain a C-terminal His$_6$ tag.

950   **Fig. 3. Rational design of SARS-CoV-2 spike-presenting nanoparticle vaccines. (A)**

951   Schematic representation of SARS-CoV-2 virion (top) and spike-presenting SApNP vaccine

952   (bottom). For the SARS-CoV-2 virion, pre/post-fusion S, nucleocapsid, and RNA viral genome

953   are shown, while for the vaccine, stabilized spike and multilayered SApNP composition are

954   depicted. **(B)** Colored surface models of SApNP carriers (top) and spike-presenting SApNP

955   vaccines (bottom). The three SApNP carriers used here are ferritin (FR) 24-mer and multilayered

956   E2p and I3-01v9 60-mers (the LD layer and PADRE cluster are not shown). Particle size is

957   indicated by diameter (in nanometers). **(C)** SEC profiles of three SApNPs presenting the SARS-

958   CoV-2 S2GΔHR2 spike, from left to right, S2GΔHR2-5GS-FR, S2GΔHR2-5GS-E2p-LD4-

959   PADRE (or E2p-L4P), and S2GΔHR2-10GS-I3-01v9-LD7-PADRE (or I3-01v9-L7P). SEC

960   profiles were obtained from a Superose 6 10/300 GL column, each showing results from three

961   separate production runs. **(D)** EM images of three SARS-CoV-2 S2GΔHR2 SApNPs. **(E)** ELISA

962   binding of three SARS-CoV-2 S2GΔHR2 SApNPs to five mAbs. EC$_{50}$ (µg/ml) values are

963   labeled. **(F)** Antigenic profiles of SARS-CoV-2 S2GΔHR2 spike and three SApNPs against five

964   mAbs. Sensorgrams were obtained from an Octet RED96 using six antigen concentrations (150-

965   4.6 nM for spike, 9-0.27 nM for FR SApNP, and 3.5-0.1 nM for E2p and I3-01v9 SApNPs,

966   respectively, all by twofold dilutions) and AHQ biosensors, as shown in fig. S3B. The peak

967 binding signals (nm) at the highest concentration are listed. Color coding indicates the signal

968 strength measured by Octet (green to red: low to high).

969 **Fig. 4. SARS-CoV-1/2 vaccine-induced binding antibody (bAb) responses in BALB/c mice.**

970 **(A)** Schematic representation of the mouse immunization protocol. **(B)** SARS-CoV-2 RBD and

971 RBD-SApNP vaccine-induced bAb responses. Coating antigens: SARS-CoV-2 S2GΔHR2-5GS-

972 foldon (left) and RBD (right). **(C)** SARS-CoV-2 S2GΔHR2 spike and S2GΔHR2-SApNP

973 vaccine-induced bAb responses. Coating antigens: SARS-CoV-2 S2GΔHR2-5GS-foldon (top)

974 and RBD (bottom). In (B) and (C), the SARS-CoV-2 S2P$_{ECTO}$-5GS-1TD0 group is included for

975 comparison, with S2P$_{ECTO}$-5GS-foldon used as the coating antigen. **(D)** SARS-CoV-1 RBD and

976 RBD-SApNP vaccine-induced bAb responses. Coating antigens: SARS-CoV-1 S2P$_{ECTO}$-5GS-

977 foldon (left) and RBD (right). In (D), the SARS-CoV-1 S2P$_{ECTO}$-5GS-1TD0 group is included

978 for comparison, with S2P$_{ECTO}$-5GS-foldon used as the coating antigen. In (B) to (D), EC$_{50}$ titers

979 derived from the ELISA binding of mouse plasma to coating antigens are plotted, with average

980 EC$_{50}$ values labeled on the plots. The *P* values were determined by an unpaired *t* test in

981 GraphPad Prism 8.4.3 with (*) indicating the level of statistical significance. Detailed ELISA

982 data are shown in figs. S4-S6.

983 **Fig. 5. SARS-CoV-1/2 vaccine-induced neutralizing antibody (NAb) responses in BALB/c**

984 **mice. (A)** SARS-CoV-2 RBD and RBD-SApNP vaccine-induced NAb responses to autologous

985 SARS-CoV-2 (left) and heterologous SARS-CoV-1 (right). **(B)** SARS-CoV-2 S2GΔHR2 spike

986 and S2GΔHR2-SApNP vaccine-induced NAb responses to SARS-CoV-2 (top) and SARS-CoV-

987 1 (bottom). In (A) and (B), the SARS-CoV-2 S2P$_{ECTO}$-5GS-1TD0 group is included for

988 comparison. **(C)** SARS-CoV-1 RBD and RBD-SApNP vaccine-induced NAb responses to

989   autologous SARS-CoV-1 (left) and heterologous SARS-CoV-2 (right). In (C), the SARS-CoV-1

990   S2P$_{ECTO}$-5GS-1TD0 group is included for comparison. In (A) to (C), ID$_{50}$ titers derived from the

991   SARS-CoV-1/2-pp neutralization assays are plotted, with average ID$_{50}$ values labeled on the

992   plots. The P values were determined by an unpaired t test in GraphPad Prism 8.4.3 with (*)

993   indicating the level of statistical significance. Detailed SARS-CoV-1/2-pp neutralization data are

994   shown in figs. S7-S9.

995   **Fig. 6. SARS-CoV-2 vaccine-induced T-cell responses in BALB/c mice.** Splenocytes from

996   mice (n=5 in each group) immunized with S2P$_{ECTO}$ spike, S2GΔHR2 E2p SApNP, and

997   S2GΔHR2 I3-01v9 SApNP, were isolated at w11 and cultured in the presence of IL-2 and

998   dendritic cells (DC) pulsed with S2P$_{ECTO}$ (1×10^{-7} μM), S2GΔHR2 E2p (1×10^{-7} μM), and

999   S2GΔHR2 I3-01v9 (1×10^{-7} μM), respectively. Splenocytes from 5 naïve mice were used as

1000   control samples and cultured in the presence of DCs without antigen pulsing. Cells were assessed

1001   after 16 hours (A, C) and 4 hours (B, D) of culture. **(A)** and **(B)**: Vaccine-induced CD4$^+$ T cell

1002   immunity. **(C)** and **(D)**: Vaccine-induced CD8$^+$ T cell immunity. Plots show the frequencies of

1003   cell fraction. The P values were determined by one-way ANOVA analysis. *, $P < 0.05$; **, $P <$

1004 0.01; ***, $P < 0.001$.

A RBD-5GS-1TD0

B
SARS-CoV-1 (100 ml ExpiCHO)
SARS-CoV-2 (100 ml ExpiCHO)

C
SARS-CoV-1
8.3E-3	
3.9E-3	
6.7E-3	
7.5E-3	

SARS-CoV-2
1.1E-2	
1.0E-2	
6.6E-3	
4.1E-3	
5.7E-3	

CR3022   M396   S309
80R   CB6   S230
B38   P2B-2F6

D
CR3022   B38   CB6   S309   P2B-2F6

950 nM
475 nM
237 nM
119 nM
59.5 nM
29.5 nM

E RBD-5GS-SpyTag/SpyCatcher-5GS-FR
"SPY"   24-meric
RBD
SpyCatcher
Ferritin (FR)
SpyTag

F
SARS-CoV-1 (50 ml ExpiCHO)
SARS-CoV-2 (50 ml ExpiCHO)
NP
Co-expression
Supernatant mix

G
SARS-CoV-1
4.8E-3
4.2E-3
5.6E-3
5.6E-3

SARS-CoV-2
3.3E-3
8.1E-3
2.6E-3
2.4E-3
3.2E-3

H
CR3022   B38   CB6   S309   P2B-2F6

37 nM
18.5 nM
9.2 nM
4.6 nM
2.3 nM
1.1 nM

I
RBD-10GS-FR (Gene fusion; No SPY conjugation)
RBD-5GS-SPY-5GS-FR (Co-expression)
50nm

J
RBD-5GS-SPY-5GS-FR (Co-expression)
RBD-5GS-SPY-5GS-FR (Supernatant mix)
50nm

K RBD-5GS-SPY-5GS-I3-01v9-LD7-PADRE
60-meric
RBD
SpyCatcher
Locking domain (LD)
SpyTag
T-helper cluster
I3-01v9

L
SARS-CoV-1 (100 ml ExpiCHO)
SARS-CoV-2 (100 ml ExpiCHO)
NP

M
SARS-CoV-1 (Supernatant mix)
SARS-CoV-2 (Supernatant mix)
50nm

A Central helix (CH) capping
Prefusion spike
Unstructured HR2 stalk and/or 5-aa linker
1TD0

B
S2P$_{ECTO}$-5GS-1TD0 (50 ml ExpiCHO)
Nickel column | CR3022 column
S2G$_{ECTO}$-5GS-1TD0 (50 ml ExpiCHO)
Nickel column | CR3022 column
S2GΔHR2-5GS-1TD0 (50 ml ExpiCHO)

SARS-CoV-1 (UV$_{280}$)
SARS-CoV-2 (UV$_{280}$)

#1 #2 #3

Volume (ml)

C
Cell membrane
ACE2 ACE2
RBD
NTD
HR1
HR2
HR2
Viral membrane

```
        910                  HR1
SARS-CoV-2  GVTQNVLYENQKLIANQFNSAIGKIQDSLSSTASALGKLQDVVNQNAQA
SARS-CoV-1  GVTQNVLYENQKLIANQFNKAISQIQESLTTTSTALGKLQDVVNQNAQA
            ***********  ****** .**..:*::.::*::***************
                                    985
SARS-CoV-2  LNTLVKQLSSNFGAISSVLNDILSRLD
SARS-CoV-1  LNTLVKQLSSNFGAISSVLNDILSRLD
            ***************************
        1163                 HR2          1211
SARS-CoV-2  DVDLGDISGINASVVNIQKEIDRLNEVAKNLNESIIDLQELGKYEQYIK
SARS-CoV-1  DVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELGKYEQYIK
            *********************************:***************
```

D
SARS-CoV-2 S2GΔHR2-5GS-1TD0 (300 ml ExpiCHO)
#1 #2 #3 #4
12.5 14
UV$_{280}$
Volume (ml)

S2P$_{ECTO}$-5GS-1TD0
S2ΔHR2-5GS-1TD0
12.5 Fractions 14
669
440
232

E
SARS-CoV-2
S2P$_{ECTO}$-5GS-1TD0 OD450
1.1E-2
3.6E-2
2.0E-3
2.4E-3
2.2E-3
Log$_{10}$ Ab (μg/ml)

SARS-CoV-2
S2GΔHR2-5GS-1TD0 OD450
8.9E-3
2.5E-2
1.4E-3
1.8E-3
1.4E-3
Log$_{10}$ Ab (μg/ml)

CR3022 S309
B38 P2B-2F6
CB6

F
CR3022 | B38 | CB6 | S309 | P2B-2F6
nm
Time (s)

150 nM 37.5 nM 9.5 nM
75 nM 19.0 nM 4.7 nM

A SARS-CoV-2 virion

Postfusion S2
Prefusion spike
Nucleocapsid
RNA viral genome

Vaccine

Stabilized prefusion spike
Locking domain (LD)
SApNP unit
T-helper cluster

B Ferritin (FR) · 12.2 nm · 47.9nm

E2p · 23.2 nm · 55.9nm

I3-01 · 25.0 nm · 59.3nm

C

S2GΔHR2-5GS-FR (400 ml ExpiCHO)
S2GΔHR2-5GS- E2p-LD4-PADRE(400 ml ExpiCHO)
S2GΔHR2-10GS-I3-01v9-LD7-PADRE (400 ml ExpiCHO)

#1 #2 #3

D

S2GΔHR2-5GS-FR
S2GΔHR2-5GS-E2p-LD4-PADRE
S2GΔHR2-10GS-I3-01v9-LD7-PADRE

50nm

E

S2GΔHR2-5GS-FR
1.4E-2, 6.5E-2, 1.8E-3, 3.2E-3, 1.9E-3

S2GΔHR2-5GS-E2p-LD4-PADRE
9.4E-3, 4.4E-2, 1.4E-3, 1.8E-3, 1.4E-3

S2GΔHR2-10GS-I3-01v9-LD7-PADRE
7.2E-3, 2.1E-2, 1.2E-3, 1.5E-3, 1.4E-3

CR3022, B38, CB6, S309, P2B-2F6

F

SARS-CoV-2 S constructs	CR3022	B38	CB6	S309	P2B-2F6
S2GΔHR2-5GS-1TD0	1.17	1.07	0.83	0.94	0.77
S2GΔHR2-5GS-FR	1.12	1.41	1.27	1.06	1.68
S2GΔHR2-5GS-E2p-LD4-PADRE	1.92	2.27	1.82	1.67	1.98
S2GΔHR2-10GS-I3-01v9-LD7-PADRE	1.93	1.99	1.93	1.73	1.92

A Immunization timeline. Immunization at Weeks 0, 3, 6, 9; spleen harvest at Week 11. Blood draws at Weeks 2, 5, 8, 11.

IMMUNIZATION
Immunogen: Protein (50 µg)
Adjuvant: Addvax (100 µl)
Adju-Phos (100µl)
Total volume: 200 µl
Route: Intraperitoneal (i.p.)

Mouse sera → (1) Antigen binding (2) PP neutralization

B SARS-CoV-2 RBD-based vaccine-induced binding antibody responses

Coated with SARS-CoV-2 spikes

	W2	W5	W8
S2P$_{ECTO}$-5GS-1TD0	405	28831	61563
RBD-5GS-1TD0	70	30547	225029
RBD-5GS-SPY-5GS-FR	3720	165841	285773

Coated with SARS-CoV-2 RBD

	W2	W5	W8
S2P$_{ECTO}$-5GS-1TD0	1453	1453	4818
RBD-5GS-1TD0		3678	25165
RBD-5GS-SPY-5GS-FR	754	72530	118671

SARS-CoV-2 vaccines
○ S2P$_{ECTO}$-5GS-1TD0
○ RBD-5GS-1TD0
○ RBD-5GS-SPY-5GS-FR

C SARS-CoV-2 spike-based vaccine-induced binding antibody responses

Coated with SARS-CoV-2 spikes

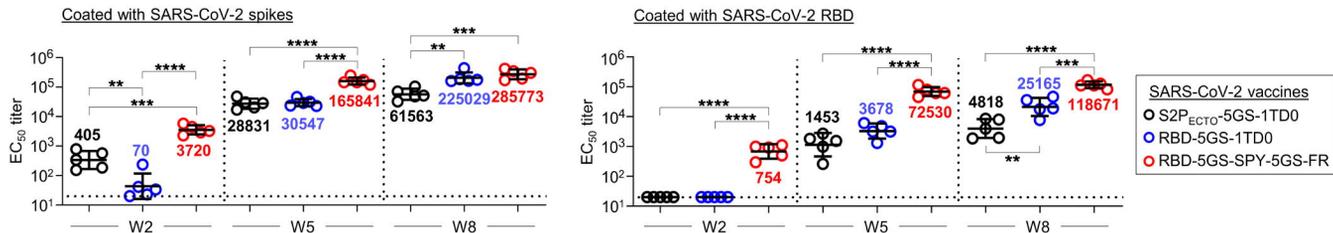|	W2	W5	W8
S2P$_{ECTO}$-5GS-1TD0	405	28831	61563
S2GΔHR2-5GS-1TD0	1215	58617	164083
S2GΔHR2-5GS-FR	2423	123421	153676
S2GΔHR2-5GS-E2p-L4P	612	231415	215456
S2GΔHR2-10GS-I3-01v9-L7P	3939	132619	297892

Coated with SARS-CoV-2 RBD

	W2	W5	W8
S2P$_{ECTO}$-5GS-1TD0		1453	4818
S2GΔHR2-5GS-1TD0		2637	10800
S2GΔHR2-5GS-FR		3494	11405
S2GΔHR2-5GS-E2p-L4P		9486	17358
S2GΔHR2-10GS-I3-01v9-L7P	175	13680	36457

SARS-CoV-2 vaccines
○ S2P$_{ECTO}$-5GS-1TD0
○ S2GΔHR2-5GS-1TD0
○ S2GΔHR2-5GS-FR
○ S2GΔHR2-5GS-E2p-L4P
○ S2GΔHR2-10GS-I3-01v9-L7P

D SARS-CoV-1 vaccine-induced binding antibody responses

Coated with SARS-CoV-1 spike

	W2	W5	W8
S2P$_{ECTO}$-5GS-1TD0	2230	141652	394945
RBD-5GS-1TD0		30115	266411
RBD-5GS-SPY-5GS-FR	1593	90323	164347

Coated with SARS-CoV-1 RBD

	W2	W5	W8
S2P$_{ECTO}$-5GS-1TD0	77	10003	19364
RBD-5GS-1TD0		1731	19571
RBD-5GS-SPY-5GS-FR	89	9639	21194

SARS-CoV-1 vaccines
○ S2P$_{ECTO}$-5GS-1TD0
○ RBD-5GS-1TD0
○ RBD-5GS-SPY-5GS-FR

A SARS-CoV-2 RBD-based vaccine-induced neutralizing antibody responses

SARS-CoV-2-pp

SARS-CoV-1-pp

SARS-CoV-2 vaccines
- ○ S2P$_{ECTO}$-5GS-1TD0
- ○ RBD-5GS-1TD0
- ○ RBD-5GS-SPY-5GS-FR

B SARS-CoV-2 spike-based vaccine-induced neutralizing antibody responses

SARS-CoV-2-pp

SARS-CoV-1-pp

SARS-CoV-2 vaccines
- ○ S2P$_{ECTO}$-5GS-1TD0
- ○ S2GΔHR2-5GS-1TD0
- ○ S2GΔHR2-5GS-FR
- ○ S2GΔHR2-5GS-E2p-L4P
- ○ S2GΔHR2-10GS-I3-01v9-L7P

C SARS-CoV-1 vaccine-induced neutralizing antibody responses

SARS-CoV-1-pp

SARS-CoV-2-pp

SARS-CoV-1 vaccines
- ○ S2P$_{ECTO}$-5GS-1TD0
- ○ RBD-5GS-1TD0
- ○ RBD-5GS-SPY-5GS-FR

A Induction of Th1 and memory responses

Gated on CD4+ TCR-β+ T cells

IFN-γ vs IL-4 (16 hours)

Naïve: 13, 1.6, 1.6
S2P_ECTO: 8.7, 0.6, 1.6
E2p: 15, 0.7, 2.1
I3-01v9: 30, 4, 1.7

IFN-γ+ (Th1)

** 0.0097
*** 0.0005
* 0.0379

IFN-γ+IL-4+ (Memory T cell)

* 0.0187
* 0.0271

B Induction of cytolytic CD4+ T cells

Gated on CD4+ TCR-β+ T cells

IFN-γ vs CD107a (4 hours)

Naïve: 10, 0.6
S2P_ECTO: 5.4, 0.9
E2p: 10, 1.2
I3-01v9: 21, 1.4

CD107a+ Cytolytic CD4+ T cells

** 0.0042
*** 0.00024

IFN-γ+ (Th1)

C Induction of recall response of CD8+ T cells

Gated on CD8+ TCR-β+ T cells

IFN-γ vs GM-CSF (16 hours)

Naïve: 8.8, 0.3, 2.7
S2P_ECTO: 8.6, 0.2, 1.9
E2p: 9.5, 0.3, 0.8
I3-01v9: 13, 6.7, 24

IFN-γ+GM-CSF+

* 0.0456
* 0.0437
* 0.0414

IFN-γ−GM-CSF+

* 0.0132
* 0.0157
** 0.0086
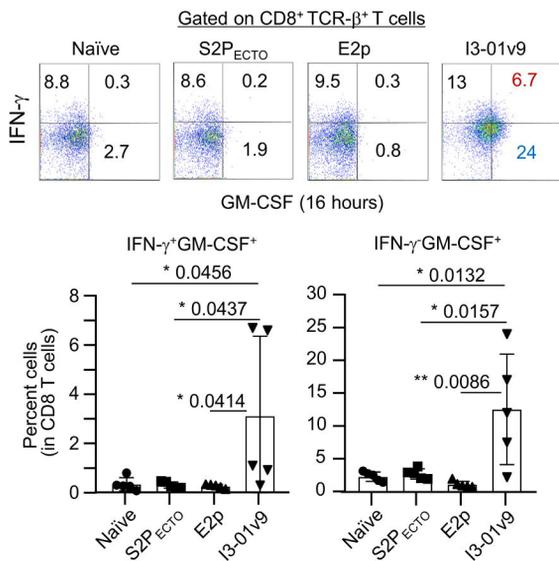
D Induction of rapid CD8+ T cell recall response

Gated on CD8+ TCR-β+ T cells

IFN-γ vs CD107a (4 hours)

Naïve: 5.5, 2.2, 2.6
S2P_ECTO: 3.3, 2.9, 1.2
E2p: 4.5, 3.5, 2.1
I3-01v9: 13, 2, 1.3

IFN-γ+ CD8

* 0.0247

IFN-γ+ CD107a+