

1     **Mechanism of a COVID-19 nanoparticle vaccine candidate that elicits a broadly**  
2                   **neutralizing antibody response to SARS-CoV-2 variants**

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4             Yi-Nan Zhang<sup>1</sup>, Jennifer Paynter<sup>1</sup>, Cindy Sou<sup>1</sup>, Tatiana Fourfouris<sup>1</sup>, Ying Wang<sup>3,4</sup>, Ciril  
5                   Abraham<sup>3</sup>, Timothy Ngo<sup>1</sup>, Yi Zhang<sup>3,4</sup>, Linling He<sup>1</sup>, and Jiang Zhu<sup>1,2,\*</sup>

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7     <sup>1</sup>Department of Integrative Structural and Computational Biology, <sup>2</sup>Department of Immunology  
8     and Microbiology, The Scripps Research Institute, La Jolla, California 92037, USA

9     <sup>3</sup>Fels Institute for Cancer Research and Molecular Biology, and <sup>4</sup>Department of Microbiology  
10    and Immunology, Temple University, Philadelphia, Pennsylvania 19140, USA.

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12    \*Corresponding author

13    JZ: Phone +1 (858) 784-8157; Email: [jiang@scripps.edu](mailto:jiang@scripps.edu)

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15    **KEYWORDS**

16    Ancestral strain; broadly neutralizing antibody (bNAb); coronavirus disease 2019 (COVID-19);  
17    severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); self-assembling protein  
18    nanoparticle (SApNP); vaccine; variant of concern (VOC).

19 **ABSTRACT (150 words)**

20 Vaccines that induce potent neutralizing antibody (NAb) responses against emerging variants of  
21 severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are essential for combating the  
22 coronavirus disease 2019 (COVID-19) pandemic. We demonstrated that mouse plasma induced  
23 by self-assembling protein nanoparticles (SAPNPs) that present 20 rationally designed  
24 S2GΔHR2 spikes of the ancestral Wuhan-Hu-1 strain can neutralize the B.1.1.7, B.1.351, P.1,  
25 and B.1.617 variants with the same potency. The adjuvant effect on vaccine-induced immunity  
26 was investigated by testing 16 formulations for the multilayered I3-01v9 SAPNP. Using single-  
27 cell sorting, monoclonal antibodies (mAbs) with diverse neutralization breadth and potency were  
28 isolated from mice immunized with the receptor binding domain (RBD), S2GΔHR2 spike, and  
29 SAPNP vaccines. The mechanism of vaccine-induced immunity was examined in mice.  
30 Compared with the soluble spike, the I3-01v9 SAPNP showed 6-fold longer retention, 4-fold  
31 greater presentation on follicular dendritic cell dendrites, and 5-fold stronger germinal center  
32 reactions in lymph node follicles.

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

36 With a well-defined mechanism, spike nanoparticle vaccines can effectively counter SARS-  
37 CoV-2 variants.

## 38 INTRODUCTION

39 The COVID-19 pandemic has led to more than 188 million infection cases and 4 million deaths  
40 globally. Antibody responses to SARS-CoV-2 spike antigens can be sustained for several months  
41 in most COVID-19 patients after infection (1-4). However, recently identified variants of  
42 concern (VOCs) exhibit higher transmissibility and resistance to prior immunity as SARS-CoV-2  
43 continues to adapt to the human host (5, 6). One such variant, B.1.1.7 (WHO classification:  
44 Alpha), emerged from southeast England in October 2020 and accounted for two-thirds of new  
45 infections in London in December 2020, with a higher transmission rate (43-90%) and risk of  
46 mortality (32-104%) than previously circulating strains (7, 8). Other variants, such as B.1.351  
47 (Beta) and P.1 (Gamma), also became prevalent in three provinces in South Africa and Manaus,  
48 Brazil, respectively (6, 9, 10). The B.1.617.2 (Delta) variant, which was initially identified in  
49 India, is becoming a dominant strain in many countries (11, 12) and responsible for the majority  
50 of new COVID-19 cases. This variant was found to be ~60% more transmissible than the highly  
51 infectious B.1.1.7 variant (12). The rise of SARS-CoV-2 VOCs and their rapid spread worldwide  
52 result in more infection cases, hospitalizations, and potentially more deaths, further straining  
53 healthcare resources (10).

54 To date, eight COVID-19 vaccines have been approved for emergency use in humans,  
55 with more than 90 candidates assessed in various phases of clinical trials (13). With the  
56 exception of inactivated whole-virion vaccines, diverse platforms have been used to deliver the  
57 recombinant SARS-CoV-2 spike, such as mRNA-encapsulating liposomes (e.g., BNT162b2 and  
58 mRNA-1273), adenovirus vectors (e.g., ChAdOx1 nCoV-19 [AZD1222], CTII-nCoV, Sputnik  
59 V, and Ad26.COV2.S), and micelle-attached spikes (e.g., NVX-CoV2373). These vaccines  
60 demonstrated 65-96% efficacy in Phase 3 trials, with lower morbidity and mortality associated

61 with COVID-19 disease (14-19). However, a notable loss of vaccine efficacy against new SARS-  
62 CoV-2 variants was reported, likely caused by spike mutations in the receptor-binding domain  
63 (RBD; e.g., K417N, E484K, and N501Y), N-terminal domain (NTD; e.g., L18F, D80A, D215G,  
64 and  $\Delta$ 242-244), and other regions that are critical to spike stability and function (e.g., D614G and  
65 P681R) (6, 11, 20-25). Among circulating VOCs, the B.1.351 lineage appeared to be most  
66 resistant to neutralization by convalescent plasma (9.4-fold) and vaccine sera (10.3- to 12.4-fold)  
67 (26), whereas a lesser degree of reduction was observed for an early variant, B.1.1.7 (27-29).  
68 Based on these findings, it was suggested that vaccines would need to be updated periodically to  
69 maintain protection against rapidly evolving SARS-CoV-2 (30-32). However, in a recent study,  
70 convalescent sera from B.1.351 or P.1-infected individuals showed a more visible reduction of  
71 B.1.617.2 neutralization than convalescent sera from individuals infected with early pandemic  
72 strains (33). Together, these issues raise the concern that herd immunity may be difficult to  
73 achieve, highlighting the necessity of developing vaccines that can elicit a broadly neutralizing  
74 antibody (bNAb) response to current and emerging variants (25, 31). As previously reported (34-  
75 38), the production of a bNAb response relies on long-lived germinal center (GC) reactions to  
76 activate precursor B cells, stimulate affinity maturation, and form long-term immune memory. In  
77 particular, antigen retention and presentation within lymph node follicles are key to the induction  
78 of long-lived GC reactions (34, 36, 39) and should be considered in the development of bNAb-  
79 producing vaccines (40).

80 We previously investigated the cause of SARS-CoV-2 spike metastability and rationally  
81 designed the S2G $\Delta$ HR2 spike, which was displayed on three self-assembling protein  
82 nanoparticle (SApNP) platforms, including ferritin (FR) 24-mer and multilayered E2p and I3-  
83 01v9 60-mers, as COVID-19 vaccine candidates (41). In the present study, we investigated the

84 vaccine-induced NAb response to SARS-CoV-2 VOCs and mechanism by which SApNP  
85 vaccines (e.g., I3-01v9) generate such a response. We first examined the neutralizing activity of  
86 mouse plasma from our previous study (41) against four representative SARS-CoV-2 variants,  
87 B.1.1.7, B.1.351, P.1, and B.1.617<sub>Rec</sub>, which was derived from an early analysis of the B.1.617  
88 lineage (11) and shares key spike mutations with VOC B.1.617.2. Mouse plasma induced by the  
89 S2GΔHR2 spike-presenting I3-01v9 SApNP potently neutralized all four variants with  
90 comparable titers to the wildtype strain, Wuhan-Hu-1. When a different injection route was  
91 tested in mouse immunization, E2p and I3-01v9 SApNPs sustained neutralizing titers against the  
92 four variants, even at a low dosage of 3.3 μg, whereas a significant reduction of plasma  
93 neutralization was observed for the soluble spike. Next, we examined the adjuvant effect on  
94 vaccine-induced humoral and T-cell responses for the I3-01v9 SApNP. While detectable plasma  
95 neutralization was observed for the non-adjuvanted I3-01v9 group, conventional adjuvants, such  
96 as aluminum hydroxide (AH) and phosphate (AP), boosted the titers by 8.6- to 11.3-fold (or 9.6  
97 to 12.3 times). Adjuvants that target the stimulator of interferon genes (STING) and Toll-like  
98 receptor 9 (TLR9) pathways enhanced neutralization by 21- to 35-fold, alone or combined with  
99 AP, in addition to a Th1-biased cellular response. We then performed antigen-specific single-cell  
100 sorting and isolated 20 monoclonal antibodies (mAbs) from RBD, spike, and I3-01v9 SApNP-  
101 immunized mice. These mAbs were derived from diverse B cell lineages, of which some  
102 neutralized the wildtype Wuhan-Hu-1 strain and four variants with equivalent potency. Lastly,  
103 we investigated how SApNPs behave in lymph nodes and induce GCs by characterizing vaccine  
104 delivery and immunological responses at the intraorgan, intracellular, and intercellular levels in  
105 mice. The I3-01v9 SApNP showed 6-fold longer retention, 4-fold greater presentation on  
106 follicular dendritic cell (DC) dendrites, and 5-fold higher GC reactions than the soluble spike.

107 Intact SApNPs in lymph node tissues could be visualized by transmission electron microscopy  
108 (TEM). Our study thus demonstrates that a spike-presenting SApNP vaccine derived from the  
109 “ancestral” SARS-CoV-2 strain may confer broad protection against emerging variants.

## 110 **RESULTS**

### 111 **Spike and SApNP vaccine-induced neutralizing responses to SARS-CoV-2 variants.**

112 We previously demonstrated that the rationally designed S2GΔHR2 spike was more  
113 immunogenic than the S2P spike (42), and SApNPs displaying 8-20 spikes outperformed soluble  
114 spikes in NAb elicitation (41) (**Fig. 1A**). Notably, the I3-01v9 SApNP that presents 20  
115 S2GΔHR2 spikes induced a potent NAb response to both SARS-CoV-1 and SARS-CoV-2, as  
116 well as critically needed T-cell responses (41). Because SARS-CoV-1 shares only modest  
117 sequence similarity (~73% in the RBD) with SARS-CoV-2, we hypothesized that our vaccines  
118 would protect against emerging variants that are much more closely related to the ancestral  
119 SARS-CoV-2 strain, Wuhan-Hu-1.

120 We first assessed the neutralizing activity of polyclonal plasma induced by various spike  
121 and SApNP vaccine formulations from our previous study (41) against the wildtype SARS-CoV-  
122 2 strain, Wuhan-Hu-1, as a baseline for comparison (**Fig. 1B**). Mouse plasma collected at week 5  
123 after two intraperitoneal (i.p.) injections of adjuvanted vaccine antigens (50 μg) was analyzed in  
124 pseudoparticle (pp) neutralization assays (43). The soluble S2P<sub>ECTO</sub> spike elicited the lowest 50%  
125 inhibitory dilution (ID<sub>50</sub>) titers, whereas the soluble S2GΔHR2 spike increased neutralization  
126 with a 7.1-fold higher average ID<sub>50</sub> titer, which did not reach statistical significance because of  
127 within-group variation. All three spike-presenting SApNPs elicited superior neutralizing  
128 responses than the soluble S2P<sub>ECTO</sub> spike (41). Notably, the I3-01v9 SApNP achieved the highest  
129 potency, with an average ID<sub>50</sub> titer of 2090, which was 8.1-fold higher than the soluble S2P<sub>ECTO</sub>

130 spike. Despite differences in ID<sub>50</sub> titers, the overall pattern remained the same as reported in our  
131 previous study (41). The differences might be attributable to the inherent variation of  
132 pseudovirus assays (43, 44). We then assessed plasma neutralization against four major SARS-  
133 CoV-2 variants (**Fig. 1C, fig. S1A, B**). The I3-01v9 SApNP induced a stronger neutralizing  
134 response against variants, with 0.5-fold (B.1.1.7), 0.8-fold (B.1.351), 1.8-fold (P.1), and 1.0-fold  
135 (B.1.617<sub>Rec</sub>) higher (or 1.5-2.8 times) ID<sub>50</sub> titers compared with the wildtype strain (**Fig. 1C**).  
136 Altogether, these results confirmed our hypothesis and highlighted the advantages of spike-  
137 presenting SApNPs.

138         Next, we examined the influence of injection dosage and route on the plasma neutralizing  
139 response to various SARS-CoV-2 strains. To this end, we performed a mouse study, in which  
140 three groups of mice were immunized with 5, 15, and 45 µg of the I3-01v9 SApNP three times  
141 via i.p. injection. Remarkably, all four variants were neutralized by mouse plasma with  
142 comparable ID<sub>50</sub> titers observed across dose groups (**fig. S1C, D**). To examine whether routes of  
143 injection affect the plasma neutralizing response against variants, we performed another mouse  
144 study, in which a low dose (3.3 µg) of adjuvanted antigen was intradermally administered into  
145 four footpads (i.e., 0.8 µg/footpad). At week 5, the large (~55-60 nm) E2p and I3-01v9 SApNPs  
146 that present 20 S2GΔHR2 spikes yielded higher ID<sub>50</sub> titers against the wildtype strain than the  
147 soluble S2GΔHR2 spike (**Fig. 1D, fig. S1E, F**), whereas a notable reduction of ID<sub>50</sub> titers against  
148 the variants was noted for mouse plasma from the S2GΔHR2 group (**Fig. 1E, fig. S1E, F**),  
149 suggesting that multivalent display is critical for eliciting a broad neutralizing response. Overall,  
150 the E2p and I3-01v9 SApNP groups exhibited similar or slightly stronger plasma neutralization  
151 against the four variants relative to the wildtype strain, Wuhan-Hu-1 (**Fig. 1E**). Lastly, we  
152 assessed longevity of the low-dose vaccination-induced neutralizing response by testing week-26

153 plasma against Wuhan-Hu-1 (**Fig. 1F, fig. S1G, H**). It is noteworthy that ID<sub>50</sub> titers at week 26  
154 were at the same level as week 5, suggesting a long-lasting protective humoral immunity. In our  
155 previous study, a panel of human NAbS was used to evaluate antigenicity of the stabilized  
156 S2GΔHR2 spike and SApNPs and validate the SARS-CoV-2-pp neutralization assays (41). Here,  
157 this antibody panel was tested against SARS-CoV-2-pps that carry spikes of the wildtype strain  
158 and the four variants (**Fig. 1G, fig. S1I**). Lower potency against the B.1.351 and P.1 variants,  
159 measured by the 50% inhibitory concentration (IC<sub>50</sub>), was observed for all human NAbS, with the  
160 exception of NAb S309, which was identified from a SARS-CoV-1 patient (45). This finding is  
161 consistent with recent reports on convalescent patient plasma (26-28). Interestingly, most human  
162 NAbS remained effective against B.1.617<sub>Rec</sub> showing a similar pattern to the wildtype Wuhan-  
163 Hu-1 strain and B.1.1.7 variant, consistent with the results of a recent cohort analysis of  
164 convalescent sera from individuals infected with early VOCs against a rising B.1.617 (33). As a  
165 negative control, mouse plasma induced by the S2GΔHR2-presenting I3-01v9 SApNP was tested  
166 against pseudoviruses carrying the murine leukemia virus (MLV) envelope glycoprotein (Env),  
167 or MLV-pps. Nonspecific MLV-pp neutralization was not detected for plasma samples produced  
168 in two independent immunization experiments (**fig. S1J, K**).

169 Altogether, our results demonstrate that spike-presenting SApNPs are more advantageous  
170 than soluble spikes in eliciting a strong neutralizing response to diverse SARS-CoV-2 variants.  
171 In our previous study, soluble SARS-CoV-2 spikes induced a more effective neutralizing  
172 response to SARS-CoV-1 than a scaffolded SARS-CoV-2 RBD trimer (41). Recently, a two-  
173 component RBD-NP vaccine showed reduced serum neutralization of variants bearing the  
174 E484K mutation (46). It is plausible that both the nanoparticle (NP) platform (one-component



175 SApNP vs. two-component NP) and the antigen type (spike vs. RBD) contribute to vaccine  
176 breadth.

177 **Adjuvant effect on vaccine-induced neutralizing antibody and T-cell responses.**

178 Innate immunity plays an important role in regulating adaptive immunity, including humoral and  
179 cellular immune responses (47-49). Adjuvant-formulated vaccines have been shown to recruit  
180 and activate innate immune cells more effectively at injection sites and local lymph nodes (50-  
181 52). Among commonly used adjuvants, AH and AP create depots for the recruitment and  
182 activation of antigen-presenting cells (APCs) at injection sites and sentinel lymph nodes (53, 54),  
183 whereas oil-in-water emulsions such as MF59 promote antigen retention and APC stimulation in  
184 lymph nodes (55). Pattern recognition receptor (PRR) agonists (e.g., STING, TLR3, TLR4,  
185 TLR7/8, and TLR9 agonists) stimulate APCs at injection sites and nearby lymph nodes (47, 52,  
186 56-59). Macrophage inhibitors (e.g., clodronate liposomes, termed CL) directly stimulate B cells  
187 or inhibit antigen sequestration by subcapsular sinus macrophages, thus resulting in more  
188 effective GC simulation in lymph nodes (60). Adjuvant combinations may generate a synergistic  
189 immune response by simultaneously activating multiple pathways (52, 57).

190 To examine the effect of innate signaling pathways on SApNP-induced immune  
191 responses, we tested 16 adjuvant formulations in a systematic study (**Fig. 2A**), in which mice  
192 were immunized with the adjuvanted I3-01v9 SApNP (20 µg) via intradermal injections in four  
193 footpads (i.e., 5 µg/footpad). We first tested mouse plasma neutralization against the wildtype  
194 Wuhan-Hu-1 stain. Mouse plasma at week 2 after a single dose was analyzed in SARS-CoV-2-  
195 pp assays, with most groups showing negligible or borderline ID<sub>50</sub> titers (**fig. S2A, B**). We then  
196 analyzed mouse plasma at week 5 after two injections (**Fig. 2B, fig. S2C, D**). The non-  
197 adjuvanted group showed detectable neutralization after two doses, with an average ID<sub>50</sub> titer of

198 160, which was used as a baseline in this analysis. By comparison, conventional adjuvants, such  
199 as AH, AP, and AddaVax, increased ID<sub>50</sub> titers by 10.3-, 7.6-, and 12.5-fold, respectively. The  
200 macrophage inhibitor CL boosted plasma neutralization by merely 1.6-fold relative to the non-  
201 adjuvanted group. Adjuvants that target various PRRs exhibited differential effects on plasma  
202 neutralization, increasing ID<sub>50</sub> titers by 1.1- to 34.2-fold. Notably, STING and CpG (TLR9)  
203 substantially enhanced neutralizing titers, whereas TLR3, TLR4, and TLR7/8 agonists only  
204 exerted a modest effect. In most cases, adjuvants combined with AP further boosted plasma  
205 neutralizing activity. For example, when TLR4 and TLR7/8 agonists were mixed with AP, a 3.1-  
206 fold increase in ID<sub>50</sub> titers was observed, suggesting a synergistic effect of stimulating multiple  
207 immune pathways. Overall, STING and CpG, either alone or combined with AP, showed plasma  
208 neutralization superior to that of any other adjuvant or adjuvant mix, increasing ID<sub>50</sub> titers by 21  
209 to 34-fold compared with the non-adjuvanted group. This is consistent with the results of the S-  
210 Trimer (SCB-2019), which, when formulated with CpG 1018 (TLR9 agonist) and alum  
211 adjuvants, induced potent NAb responses in nonhuman primates and human trials (61, 62).  
212 Mouse plasma at week 8 showed further increases in ID<sub>50</sub> titers (1 to 3-fold) for most adjuvant  
213 groups (**Fig. 2C, fig. S2E, F**). Lastly, we examined mouse plasma at week 5 from the STING  
214 and CpG groups against the B.1.1.7, B.1.351, P.1, and B.1.617<sub>rec</sub> variants (**Fig. 2D, fig. S2G, H**).  
215 Both adjuvant groups exhibited potent neutralizing responses to the four variants, with ID<sub>50</sub> titers  
216 comparable to the wildtype strain.

217 We previously demonstrated that the AP-formulated I3-01v9 SApNP induces interferon- $\gamma$   
218 (IFN- $\gamma$ )-producing CD4<sup>+</sup> T helper 1 (Th1) cells and IFN- $\gamma$ /interleukin-4 (IL-4) double-positive  
219 memory CD4<sup>+</sup> T cells (41). Given the superior plasma neutralizing response observed for STING  
220 and CpG, we examined the impact of these two adjuvants on vaccine-induced T-cell responses.

221 IFN- $\gamma$ -producing CD4<sup>+</sup> Th cells are important for optimal antibody responses and the induction  
222 of cellular immunity to clear viruses (63-65). To assess the effect of STING and CpG on  
223 vaccine-induced Th cells, we isolated splenocytes from mice 8 weeks after vaccination and  
224 cultured them in the presence of BALB/c mouse DCs pulsed with the spike-presenting I3-01v9  
225 SApNP. Compared with the non-adjuvanted control, STING and CpG (TLR9) induced 3.7 and  
226 5.5-fold more IFN- $\gamma$ -producing CD4<sup>+</sup> Th1 cells, and 5.5 and 16-fold more IL-4-producing CD4<sup>+</sup>  
227 Th2 cells, respectively (**Fig. 2E, fig. S2I**). A visible but nonsignificant trend toward a higher  
228 frequency of both Th1 and Th2 cells was noted in mice immunized with the CpG-formulated I3-  
229 01v9 SApNP than other formulations. Nonetheless, both adjuvants induced more IFN- $\gamma$ -  
230 producing CD4<sup>+</sup> Th1 cells than IL-4-producing CD4<sup>+</sup> Th2 cells, suggesting a dominant Th1  
231 response in these mice. This is consistent with the results for the S-Trimer (SCB-2019), which,  
232 when formulated with the AS03 adjuvant or mixed CpG 1018/alum adjuvants, induced Th1-  
233 biased cellular responses in mice (61). STING and CpG also enhanced CD8<sup>+</sup> T-cell responses by  
234 6- and 10-fold, respectively, compared with the PBS control. Notably, this effect was more  
235 visible for CpG in terms of both the frequency and number of IFN- $\gamma$ -producing CD8<sup>+</sup> effector T  
236 cells (**Fig. 2F, fig. S2J**).

237 Our results demonstrate that the I3-01v9 SApNP itself is immunogenic, and adjuvants  
238 can further enhance vaccine-induced NAb responses in plasma by up to 35-fold. The I3-01v9  
239 SApNP, when formulated with the STING or TLR9 agonist, yielded the highest ID<sub>50</sub> titers with  
240 robust CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses, highlighting their potential as adjuvants in the  
241 development of more effective SARS-CoV-2 vaccines.

242 **Diverse variant-neutralizing mouse antibody lineages identified by single-cell analysis.**

243 Although plasma neutralization confirmed the effectiveness of our newly designed SARS-CoV-2  
244 vaccines (41) against variants, the nature of this response was unclear. It might result from  
245 multiple NAb lineages that each target a specific strain (non-overlapping), a few bNAb lineages  
246 that are each able to block multiple strains (overlapping), or a combination of both. Previously,  
247 we used antigen-specific single-cell sorting to identify potent mouse NAb elicited by an I3-01  
248 SApNP that presents 20 stabilized HIV-1 Env trimers (66). Here, we applied a similar strategy to  
249 decipher NAb responses induced by SARS-CoV-2 vaccines using mouse samples from our  
250 previous study (41), for which potent plasma neutralization against four variants has been  
251 verified (**Fig. 1C**).

252 Spleen samples from M4 in the spike group (S2GΔHR2-5GS-1TD0) and M2 in the spike-  
253 SApNP group (S2GΔHR2-10GS-I3-01v9-L7P), along with a control sample from M2 in the  
254 RBD (RBD-5GS-1TD0) group were analyzed. Two probes, RBD-5GS-foldon-Avi and  
255 S2GΔHR2-5GS-foldon-Avi, were produced, biotinylated, and purified to facilitate antigen-  
256 specific B-cell sorting (**fig. S3A, B**). Following antibody cloning, reconstituted mouse mAbs  
257 were tested for neutralizing activity against the wildtype strain, Wuhan-Hu-1, in SARS-CoV-2-  
258 pp assays. A total of 20 mAbs, four from the RBD group (**fig. S3C**), six from the spike group  
259 (**fig. S3D**), and 10 from the I3-01v9 SApNP group (**fig. S3E**), were found to be NAb. The  
260 genetic analysis of mAb sequences revealed some salient features of the vaccine-induced NAb  
261 response in mice (**Fig. 3A**). Overall, these mAbs evolved from diverse germline origins. The  
262 RBD-elicited mAbs appeared to use distinct germline variable (V) genes for both heavy chain  
263 (HC) and  $\kappa$ -light chain (KC), or  $V_H$  and  $V_K$ , respectively, whereas the spike and I3-01v9  
264 SApNP-elicited mAbs shared some common  $V_H$  genes, such as IGHV14-1/3 and IGHV1S81.  
265 This result was not unexpected because the RBD vaccine presents a structurally distinct antigen

266 to the immune system compared with the spike and I3-01v9 vaccines, which both present the  
267 S2GAHR2 spike. These mAbs showed low levels of somatic hypermutation (SHM) with respect  
268 to their germline genes. Heavy chain complementarity-determining region (HCDR3) loops  
269 ranged from 4 to 12 aa in length, whereas most KCs contained 9 aa KCDR3 loops. Collectively,  
270 diverse germline genes and HCDR3 loops, accompanied by low degrees of SHM, suggest that  
271 many antibody lineages must have been generated upon vaccination, and some could achieve  
272 neutralizing activity without an extensive maturation process.

273 We then examined the biological function of these mouse mAbs. Neutralizing activity  
274 was assessed in SARS-CoV-2-pp assays against the wildtype strain and four variants (**Fig. 3B**,  
275 **fig. S3F**). Overall, diverse yet consistent patterns were observed for the three sets of mAbs. Both  
276 the RBD vaccine (an RBD scaffold) and the two spike vaccines, albeit in different forms,  
277 appeared to elicit potent NAbs against the wildtype strain. MAbs TRBD-R-4G5, S2GD-S-2C10,  
278 and I3V9-R-1G9 showed similar  $IC_{50}$  values (0.02-0.03  $\mu\text{g/ml}$ ) against Wuhan-Hu-1, on par with  
279 the human NAbs CB6 (67) and CC12.1/3 (68) (**Fig. 1F**). All three vaccines elicited bNAbs  
280 responses, despite variation in potency for different mAbs against different strains. Notably,  
281 I3V9-R-1G9, which was isolated from an I3-01v9 SApNP-immunized mouse, demonstrated high  
282 potency across all four variants ( $IC_{50}$ : 0.01-0.02  $\mu\text{g/ml}$ ). This bNAbs provided evidence that  
283 individual bNAbs lineages may critically contribute to the plasma neutralization of diverse  
284 variants (**Fig. 1C**). All three vaccines generated NAbs that preferentially neutralize specific  
285 SARS-CoV-2 strains. For example, TRBD-R-4B6 was more effective against the wildtype strain  
286 and an early VOC, B.1.1.7, whereas S2GD-R-2E4 neutralized B.1.351, P.1, and B.1.617<sub>Rec</sub> with  
287 greater potency. Notably, more than 60% (13) of the mAbs exhibited different patterns in the  
288 neutralization of B.1.617<sub>Rec</sub> vs. VOCs B.1.351 and P.1, as indicated by the fold change in  $IC_{50}$ ,

289 suggesting that B.1.617 may represent a distinct SARS-CoV-2 lineage. Although RBD-isolated  
290 NAbs likely neutralized SARS-CoV-2 by blocking its receptor binding, those spike-isolated  
291 NAbs could target the RBD, N-terminal domain (NTD), or epitopes in the S2 subunit. Thus, we  
292 tested these mAbs in an enzyme-linked immunosorbent assay (ELISA) against the RBD  
293 monomer and S2GΔHR2-5GS-1TD0 spike, both based on the wildtype Wuhan-Hu-1 backbone  
294 (**Fig. 3C, fig. S3G, H**). Overall, all of the NAbs bound the RBD and spike with a half-maximal  
295 concentration ( $EC_{50}$ ) of 0.034  $\mu\text{g/ml}$  or lower, except for I3V9-R-2F2 (1.973  $\mu\text{g/ml}$  for the RBD  
296 and 2.051  $\mu\text{g/ml}$  for the spike). Most (15) NAbs showed greater binding affinity (or lower  $EC_{50}$   
297 values) for the spike, suggesting that the two arms of the immunoglobulin (Ig) can each interact  
298 with one RBD of the spike, resulting in an avidity effect. Notably, diverse binding patterns were  
299 observed for I3-01v9 SApNP-elicited NAbs. Although I3V9-S-1C9 and I3V9-S-1F5 bound to  
300 the spike more favorably than the RBD, as indicated by a 4.7 to 4.9-fold reduction of their  $EC_{50}$   
301 values, three NAbs from this group (I3V9-R-1G3, I3V9-R-1G9, and I3V9-R-2F10) preferred the  
302 RBD monomer over the spike. This preference might be explained by steric hindrance when  
303 these NAbs approach the RBDs on a trimeric spike at specific angles.

304 Lastly, we characterized these mouse NAbs in antigen-specific B-cell repertoires by next-  
305 generation sequencing (NGS), as previously demonstrated for NAbs isolated from HIV-1  
306 SApNP-immunized mice and rabbits (66). Using the same RBD and spike probes (**fig. S3A**),  
307 ~1500 splenic B cells were bulk-sorted from each of the three mice that were analyzed by single-  
308 cell sorting for mAb isolation (**fig. S4A**). Unbiased mouse antibody HC and KC libraries were  
309 constructed and sequenced on an Ion S5 platform, which yielded up to 4 million raw reads (**fig.**  
310 **S4B**). The antibody NGS data were then processed using a mouse antibodyomics pipeline (69) to  
311 remove low-quality reads, resulting in 0.11-0.41 full-length HCs and KCs (**fig. S4B**).

312 Quantitative profiles of critical antibody properties, such as germline gene usage, the degree of  
313 SHM, and CDR3 loop length, were determined for the RBD and spike-specific B-cell  
314 populations (**fig. S4C**). All 20 single-cell-sorted mouse NAb could well fall in the range of  
315 these repertoire profiles, but some  $V_H/V_K$  genes that accounted for large portions of antigen-  
316 specific B cells, such as IGHV9 and IGHV5, were not used by any NAb, suggesting that they  
317 might give rise to non-neutralizing binding antibodies. Two-dimensional (2D)  
318 divergence/identity plots were generated to visualize these NAb in the context of NGS-derived  
319 B-cell repertoires (**Fig. 3D, fig. S4D-F**). Somatic variants were identified for each NAb by  
320 searching for sequences of the same  $V_H/V_K$  gene with a CDR3 identity cutoff of 90% (or 85%  
321 for evolutionarily more remote variants). For the most potent NAb, TRBD-R-4G5, from an  
322 RBD-immunized mouse (M2), 34 HC variants were identified that overlapped with an “island”  
323 of high sequence similarity to TRBD-R-4G5 on the plot, whereas more KC variants (1183) were  
324 found, likely due to the lack of diversity in the KCDR3 region. A similar pattern was observed  
325 for the potent bNAb, I3V9-R-1G9, from an SApNP-immunized mouse (M2). By comparison,  
326 fewer putative somatic variants were identified for other NAb in the antigen-specific B-cell  
327 repertoires regardless of the sorting probe used (**fig. S4D-F**), suggesting that these NAb either  
328 were from less prevalent lineages or were generated in response to a previous injection (each  
329 mouse received four doses) (41). Similar observations were reported for the vaccination of  
330 nonhuman primates and humans in longitudinal repertoire analyses of single-cell-sorted NAb  
331 (70, 71).

332 Single-cell isolation identified a panel of mouse mAb with different neutralization  
333 breadth and potency against the wildtype SARS-CoV-2 strain and four major variants. The  
334 ELISA analysis suggested that the I3-01v9 SApNP can elicit NAb with more diverse angles of

335 approach to their epitopes than the RBD and soluble spike vaccine. Structural analysis by  
336 crystallography and EM will provide a more detailed understanding of epitope recognition by  
337 these mouse mAbs.

### 338 **Distribution and trafficking of I3-01v9 SApNP in mouse lymph node.**

339 After validating these vaccines against variants at both the plasma and mAb levels, we studied *in*  
340 *vivo* behaviors of the S2GΔHR2 spike and two large 60-meric SApNPs to understand why  
341 SApNPs outperform soluble spikes in bNAb elicitation. In principle, these SApNPs need to be  
342 transported to lymph nodes, retained, and presented to various immune cell populations to induce  
343 robust innate and adaptive immune responses. Here, we first examined the transport and  
344 distribution of I3-01v9 SApNPs in mouse lymph nodes via footpad injections (10 μg/footpad).  
345 The mice were sacrificed 12 h after single-dose (**Fig. 4A**) and prime-boost (**Fig. 4B**) regimens.  
346 The axillary, brachial, and popliteal sentinel lymph nodes were isolated for histological analysis.  
347 The lymph node tissues were stained with the human anti-spike antibody P2B-2F6 (72) to  
348 characterize SARS-CoV-2 spikes presented on the I3-01v9 SApNPs. Consistent with our  
349 previous study (73), SApNPs accumulated in lymph node follicles, regardless of the number of  
350 doses. SApNPs were sequestered in the center of lymph node follicles after a single dose (**Fig.**  
351 **4A**, images on the left, schematics on the right) but were located along the outer layer of  
352 expanded lymph node follicles after the second injection due to preexisting humoral immunity  
353 (i.e., GC reactions) that was induced by the first dose (**Fig. 4B**, images on the left, schematics on  
354 the right). Overall, the majority of SApNPs accumulated in lymph node follicles, but their  
355 distribution differed slightly, depending on the doses.

356 In this context, we examined patterns of trafficking and lymph node follicle retention for  
357 soluble S2GΔHR2 spike *vs.* the S2GΔHR2-presenting E2p and I3-01v9 SApNPs. To facilitate



358 this analysis, the mice were sacrificed 2 h to 8 weeks after a single dose (**Fig. 4C**) and 2 h to 5  
359 weeks after the boost (**Fig. 4D**). The injection dose was normalized to the total amount of protein  
360 (10  $\mu$ g) per injection into each footpad (40  $\mu$ g/mouse). As shown in **Fig. 4C**, the S2G $\Delta$ HR2  
361 spikes that trafficked into lymph node follicles at 2 h cleared within 48 h. In contrast, the two  
362 large SApNPs accumulated in the subcapsular sinus at 2 h and then trafficked into follicles 12 h  
363 after the single-dose injection. Remarkably, I3-01v9 SApNPs remained detectable in lymph node  
364 follicles after 2 weeks, suggesting 6-fold longer retention than the S2G $\Delta$ HR2 spike (**Fig. 4C**).  
365 The results for these protein nanoparticles are thus consistent with the pattern of size dependency  
366 that was observed for ovalbumin-conjugated gold nanoparticles in our previous study (73), in  
367 which small (5-15 nm) nanoparticles cleared shortly after the injection, whereas large (50-100  
368 nm) nanoparticles were retained in lymph node follicles for weeks. Similar patterns of antigen  
369 retention were observed after the second injection, although the boost appeared to exert a more  
370 positive effect on the soluble spike, which could be detected in lymph node follicles at 48 h (**Fig.**  
371 **4D**). Nonetheless, prolonged retention was observed for both E2p and I3-01v9 SApNPs 2 weeks  
372 after the boost injection. Overall, the multivalent display of S2G $\Delta$ HR2 spikes on the I3-01v9  
373 SApNP resulted in 325- and 4-fold greater accumulation in lymph node follicles compared with  
374 the soluble spike 48 h after the single-dose (**Fig. 4E**) and prime-boost (**Fig. 4F**) injections,  
375 respectively. These findings reveal the advantage of a leading vaccine candidate identified in our  
376 previous study, S2G $\Delta$ HR2-10GS-I3-01v9-L7P (41), in terms of spike antigen retention in lymph  
377 node follicles.

### 378 **Retention and presentation of I3-01v9 SApNP on follicular dendritic cell dendrites.**

379 Antigen retention and presentation in lymph node follicles are prerequisites to the stimulation of  
380 robust B cell responses and GC reactions (34, 36). Resident cells spatially rearrange antigens and

381 present them to B cells. Follicular dendritic cells (FDCs) are resident stromal cells in follicles  
382 and retain soluble antigens, immune complexes, virus-like particles (VLPs), viruses, and bacteria  
383 (73-76). FDCs are also key to GC initiation, maintenance, and B-cell affinity maturation (37, 77,  
384 78). Here, we hypothesized that FDCs comprise the major cell population in lymph node follicles  
385 that retain SARS-CoV-2 spikes and spike-presenting SApNPs. To test this hypothesis, we  
386 administered vaccines via footpad injections and collected mouse lymph nodes at the peak of  
387 accumulation (12 h) after single-dose (**Fig. 5A**) and prime-boost (**Fig. 5B**) injections. Lymph  
388 node tissue samples were stained with the anti-spike antibody P2B-2F6 (72) for the S2GΔHR2  
389 spike, as well as anti-CD21 and anti-CD169 antibodies for FDCs and subcapsular sinus  
390 macrophages, respectively. The spike and SApNP (E2p or I3-01v9) signals colocalized with  
391 FDC (CD21<sup>+</sup>) networks in lymph node follicles (**Fig. 5A, B**). This result confirmed the critical  
392 role of FDC networks in mediating vaccine retention in lymph node follicles.

393 The induction of potent bNAbs responses by spike-presenting SApNPs in mice suggests  
394 the effective activation of naïve B cells and subsequent recalls by crosslinking B cell receptors  
395 (76, 79, 80). We visualized the interface between FDC networks and B cells to better understand  
396 how FDC networks present SApNPs to engage B cells. Briefly, fresh lymph nodes were isolated  
397 and directly immersed in fixative. The processed tissue samples were sectioned and stained on  
398 copper grids for TEM analysis. We first determined whether SApNPs, such as the S2GΔHR2-  
399 presenting I3-01v9 SApNP, remain intact *in vivo* (**fig. S5**). Mouse lymph nodes were isolated 2 h  
400 after the injection of a high dose (50 μg) of the non-adjuvanted I3-01v9 SApNP. The TEM  
401 images revealed that round-shape granules corresponding to intact SApNP aligned on the  
402 macrophage surface or inside endolysosomes of the macrophage in a lymph node (**fig. S5**). We  
403 next studied the relative location between FDCs and I3-01v9 SApNPs and how FDCs present

404 SApNPs to B cells. Mouse lymph nodes were collected 2, 12, and 48 h after a single-dose (50  
405  $\mu\text{g}$ ) and 12 h after the boost of the I3-01v9 SApNP vaccine. The FDCs exhibited the  
406 characteristic morphology of long dendrites that surrounded and interacted with B cells in lymph  
407 node follicles (**Fig. 5C, fig. S6**). Few I3-01v9 SApNPs were observed on FDC dendrites at 2 h  
408 (**fig. S6D**), whereas notably more nanoparticles migrated to and aligned on FDC dendrites at 12  
409 and 48 h (**Fig. 5C, fig. S6A-C**, yellow arrows). The TEM images indicated that FDCs can  
410 present many SApNPs to neighboring B cells in this “hugging mode”, in which their long  
411 dendrites brace B cells to maximize interactions between multivalently displayed spikes and B  
412 cell receptors. These results demonstrated the intrinsic nature of FDCs as a reservoir for the  
413 sequestration, retention, and presentation of virus-like particles, or SApNPs with similar  
414 molecular traits, to initiate GC reactions.

#### 415 **Robust germinal center reactions induced by spike-presenting SApNPs.**

416 Long-lived GC reactions induce immune stimulation for B-cell selection and affinity maturation,  
417 as well as production of immune memory and bNAb responses (34, 35, 40). Here, we  
418 investigated whether the prolonged retention of S2G $\Delta$ HR2-presenting E2p and I3-01v9 SApNPs  
419 induces more robust GCs in lymph node follicles than the soluble S2G $\Delta$ HR2 spike.  
420 Immunohistological analysis was performed to characterize GC B cells (GL7<sup>+</sup>) and T follicular  
421 helper (T<sub>fh</sub>) cells (CD4<sup>+</sup>Bcl6<sup>+</sup>). For the I3-01v9 SApNP, 2 weeks after immunization, we  
422 observed robust GCs in lymph node B cell follicles (B220<sup>+</sup>) with well-formed dark zone (DZ)  
423 and light zone (LZ) compartments, which contain GC B cells, FDCs, and T<sub>fh</sub> cells (35, 81-83)  
424 (**Fig. 6A**). We then extended the analysis to the S2G $\Delta$ HR2 spike and spike-presenting SApNPs  
425 2, 5, and 8 weeks after the single-dose injection (**Fig. 6B, fig. S7A-C**) and 2 and 5 weeks after  
426 the boost (**Fig. 6C, fig. S7D, E**). Two metrics, the GC/FDC ratio (i.e., whether GC formation is

427 associated with an FDC network, %) and GC size (i.e., occupied area), were used. Overall, the  
428 soluble spike and both large SApNPs induced robust GCs 2 weeks after immunization (**Fig. 6B,**  
429 **fig. S7A**). The E2p and I3-01v9 SApNPs that present 20 spikes induced robust, long-lived GCs,  
430 whereas the spike alone failed to sustain robust GCs at week 8 with either the single-dose (**Fig.**  
431 **6B, D**) or prime-boost (**Fig. 6C, E**) injections. The I3-01v9 SApNP generated larger GCs than  
432 the soluble spike, 2.0-fold larger after the single dose (**Fig. 6B, D**) and 2.4-fold larger after the  
433 boost (**Fig. 6C, E**), measured at week 8.

434 We further characterized GC reactions by flow cytometry. Fresh mouse lymph nodes  
435 were disaggregated into a single cell suspension and stained with an antibody cocktail to quantify  
436 GC B cells and T<sub>fh</sub> cells (**fig. S8A**). The results were consistent with the immunohistological  
437 analysis, in which all spike-based vaccine antigens, including the S2GΔHR2 spike and SApNPs,  
438 showed robust GCs at week 2 after the injection that declined over time, as measured at weeks 5  
439 and 8 (**Fig. 6F**). The E2p and I3-01v9 SApNPs generated a larger population of GC B cells than  
440 both the S2P<sub>ECTO</sub> and S2GΔHR2 spikes at week 2 (**fig. S8B, C**). Although the boost dose had  
441 little impact on the frequency of GC B cells and T<sub>fh</sub> cells, it appeared to extend GC formation  
442 within lymph nodes (**Fig. 6F, G**), which may promote B cell development toward bNAbs.  
443 Notably, the GC B cell and T<sub>fh</sub> cell populations elicited by the soluble S2GΔHR2 spike were  
444 barely detectable 5 weeks after immunization (**Fig. 6F, G**). This result was reminiscent of a  
445 recent study of an mRNA vaccine, in which GC reactions diminished to baseline levels at week 4  
446 after a single-dose injection (84). The S2GΔHR2-presenting I3-01v9 SApNP generated 3.7/5.2-  
447 fold more GC B cells and 3.7/4.4-fold more T<sub>fh</sub> cells than the soluble S2GΔHR2 spike at week 8  
448 after one/two-dose immunization (**Fig. 6F, G**). Therefore, SApNPs that were retained on FDC  
449 dendrites could present NAb epitopes to enable more effective B cell recognition than the soluble

450 spike, and consequently induce more robust and long-lived GCs in lymph nodes. Patterns of  
451 trafficking and retention may be specific to antigen size, as shown previously (73) and in the  
452 present study (**Figs. 4 and 5**), but GC reactions are largely determined by vaccine adjuvants. This  
453 effect was briefly demonstrated for the E2p and I3-01v9 SApNPs, which were previously  
454 formulated with the AddaVax and AP adjuvants (41). At week 2 after a single-dose injection, the  
455 adjuvanted SApNPs induced stronger GC reactions than the non-adjuvanted groups (**fig. S9**).  
456 This result can also explain the differences in plasma neutralization between the adjuvanted and  
457 non-adjuvanted I3-01v9 SApNPs (**Fig. 2**).

458 NGS has been used to assess vaccine-draining lymph node B-cell responses (85). Here,  
459 we characterized lymph node B cells at the repertoire level for three groups of mice immunized  
460 with two doses (3.3  $\mu$ g each) of the S2G $\Delta$ HR2 spike, E2p, and I301v9 SApNPs via footpad  
461 injections. At this dosage, the spike showed less effective plasma neutralization of variants than  
462 the large SApNPs (**Fig. 1E**). Given their differences in retention, presentation, and GC reaction  
463 (**Figs. 4-6**), they were expected to yield different lymph node B-cell profiles. Interestingly,  
464 antigen-specific sorting identified more spike-targeting lymph node B cells from the I3-01v9  
465 SApNP group than both the spike and E2p SApNP groups (**fig. S10A**). The antibody NGS data  
466 were processed by the mouse antibodyomics pipeline (69) (**fig. S10B**) to derive quantitative B-  
467 cell profiles (**fig. S10C-E**). Compared with the spike, the I3-01v9 SApNP appeared to activate  
468 fewer  $V_H/V_K$  genes (**fig. S10F**, left two), while generating a larger population of spike-specific  
469 lymph node B cells (**fig. S10A**). The three vaccine groups exhibited a similar degree of SHM for  
470  $V_H$  genes, with the I3-01v9 SApNP showing the highest SHM for  $V_K$  genes (**fig. S10F**, middle  
471 two). A highly uniform HCDR3 loop length distribution (~10 aa) was observed for mice in the  
472 I3-01v9 SApNP group with little variation, as measured by the root-mean-square fluctuation

473 (RMSF) (**fig. S10F**, right two). In our previous studies (86, 87), a similar approach was applied  
474 to assess hepatitis C virus (HCV) and Ebola virus (EBOV) vaccine-induced B cell responses in  
475 the spleen, a major lymphoid organ (88), after mice received four i.p. injections. We observed  
476 distinct B-cell profiles associated with the viral antigen and NP platform (86, 87). Here, the  
477 lymph node B-cell profiles appeared to be rather different, revealing the complex inner workings  
478 of another primary site for vaccine-induced immunity. Notably, I3-01v9 SANP exhibited more  
479 “focused” B-cell activation and development in vaccine-draining lymph nodes, as indicated by  
480 fewer activated germline genes and a narrower HCDR3 length distribution. More in-depth  
481 studies are needed to investigate the effect of injection route, adjuvant, and lymphoid organ, in  
482 addition to viral antigen and NP platform, on the resulting B-cell profiles. Single-cell immune  
483 profiling and antibody isolation (89) may provide further insights into the clonality of vaccine-  
484 induced B-cell lineages within lymph nodes.

## 485 **DISCUSSION**

486 To end the COVID-19 pandemic, vaccines need to effectively block current and emerging  
487 SARS-CoV-2 variants that evade NAb responses by mutating key epitopes on the viral spike  
488 (31). To overcome this challenge, some suggested that COVID-19 vaccines need to be updated  
489 on a regular basis (30-32), whereas others developed mosaic or cocktail vaccines for related  
490 sarbecoviruses (46, 90). These vaccine strategies need to be evaluated for long-term protection,  
491 because SARS-CoV-2 is evolving rapidly and may acquire new mutations to evade vaccine-  
492 induced immunity (e.g., B.1.617) (11). In our previous study (41), the spike-presenting SApNPs  
493 induced a potent NAb response to SARS-CoV-1, which is evolutionarily much more distant to  
494 the wildtype SARS-CoV-2 strain, Wuhan-Hu-1, than all its circulating variants. Emerging data  
495 from human serum analysis suggested that vaccines derived from early pandemic strains may

496 provide broad protection against current variants (33). Based on these findings, we hypothesized  
497 that SApNPs presenting stabilized ancestral Wuhan-Hu-1 spikes may provide an effective  
498 vaccine against SARS-CoV-2 variants. In the present study, we sought to confirm this hypothesis  
499 by testing four major variants and, if proven true, investigate the mechanism underlying such a  
500 broadly protective vaccine.

501 We explored several critical aspects related to the vaccine response, with a focus on the  
502 lead candidate identified in our previous study, S2GΔHR2-10GS-I3-01v9-L7P (41). We first  
503 tested vaccine-induced mouse plasma, which represents a polyclonal response, against four  
504 SARS-CoV-2 variants. Mouse plasma generated previously (41) and in new studies using  
505 different regimens (e.g., injection route, dosage, and adjuvant) potently neutralized the variants.  
506 Notably, SApNPs retained their high ID<sub>50</sub> titers at a dosage as low as 3.3 μg, whereas  
507 formulations with the STING and TLR9 agonists further enhanced the I3-01v9 SApNP-induced  
508 neutralizing response. While plasma neutralization data may be interpreted with caution due to  
509 assay variation (44), single-cell-sorted mAbs provided unambiguous evidence of the vaccine-  
510 induced bNAb response. Our results revealed that a plethora of NAb lineages were generated  
511 upon vaccination, with I3-01v9 SApNP being the most effective at eliciting bNAbs.  
512 Additionally, our results confirmed the necessity of a prime-boost strategy for eliciting a potent  
513 NAb response, regardless of the regimen (e.g., injection route, dosage, and adjuvant). Such an  
514 NAb response, once generated, can persist for an extended period of time post vaccination.  
515 Although SARS-CoV-2 challenge in relevant animal models gives more accurate assessment of  
516 vaccine protection (91), NAb titers have been found to be highly predictive of immune  
517 protection from symptomatic infections in a large cohort study (92). Protein vaccines, despite the  
518 well-established records of safety and effectiveness, have yet to be deployed to mitigate the

519 COVID-19 pandemic (93-95). One protein vaccine, NVX-CoV2373 (micelle-attached spikes  
520 formulated with the Matrix-M<sup>TM</sup> adjuvant), showed ~90% efficacy in human trials (19). Our  
521 study indicates that SApNPs displaying 20 stabilized spikes provide a promising protein vaccine  
522 candidate that can be used either alone or as a booster for nucleic acid (e.g., mRNA and viral  
523 vector) vaccines in the battle against emerging SARS-CoV-2 variants (11).

524 We explored the mechanism of SApNP vs. spike vaccines following the previously used  
525 strategy to analyze the *in vivo* behaviors of antigen-attached gold nanoparticles (73). In principle,  
526 SApNP vaccines must induce long-lasting GCs to facilitate the development of bNAbs. Effective  
527 vaccine retention and presentation are critical for inducing and sustaining GC reactions, which in  
528 turn promote the proliferation and affinity maturation of antigen-specific B cells. Indeed, we  
529 found that the I3-01v9 SApNP, our leading vaccine candidate (41), elicited 6-fold longer  
530 retention and 4-fold greater accumulation in lymph node follicles than the stabilized S2GΔHR2  
531 spike alone with a prime-boost regimen. This can be attributed to the intrinsic physiological  
532 properties of lymph nodes that mediate vaccine trafficking and retention in follicles in a size-  
533 dependent manner, which would favor retaining large (> 50 nm) virus-like particles (73-75, 80,  
534 96). Supporting this notion are the TEM images of retained SApNPs aligned on long FDC  
535 dendrites, suggesting that such protein nanoparticles can present spike antigens to B cells for  
536 rapid initiation and then sustain GC reactions in lymph node follicles for an extended period of  
537 time. Specifically, the I3-01v9 SApNP generated 2.4-fold larger GCs and greater numbers of GC  
538 B cells (5.2-fold) and T<sub>fh</sub> cells (4.4-fold) than the soluble S2GΔHR2 spike with the prime-boost  
539 regimen. These findings provide quantitative evidence that spike-presenting SApNPs are  
540 uniquely suited for inducing long-lived robust GCs in lymph node follicles. Our analyses thus



541 shed light on the mechanism by which the I3-01v9 SApNP can elicit a more effective bNAb  
542 response than the soluble spike.

543 Rational design of next-generation COVID-19 vaccines requires an in-depth  
544 understanding of bNAb elicitation (31). Superior NAb (but not necessarily bNAb) responses  
545 have been reported for several vaccine candidates that employ particulate display (90, 97-104).  
546 The I3-01v9 SApNP elicited a potent bNAb response to four variants, overcoming a major  
547 challenge facing the current COVID-19 vaccines. Mechanistic studies of vaccine trafficking,  
548 retention, presentation, and GC reactions provided valuable insights into the spike and SApNP-  
549 induced immunity (95, 105, 106). Such knowledge, if can be obtained for other vaccine  
550 platforms (e.g., inactivated whole virions, mRNAs, and viral vectors) will facilitate rational  
551 selection of the most effective vaccine candidates to mitigate the pandemic and ultimately stop  
552 the spread of SARS-CoV-2.

## 553 **MATERIALS AND METHODS**

### 554 **SARS-CoV-2 spike and SApNP vaccine antigens**

555 The design, expression, and purification of a stabilized SARS-CoV-2 spike, S2GΔHR2, and  
556 three SApNPs that present either 8 or 20 S2GΔHR2 spikes were described in our recent study  
557 (41). Briefly, the spike gene of the SARS-CoV-2 isolate Wuhan-Hu-1 (GenBank accession no.  
558 MN908947) was modified to include the mutations <sup>682</sup>GSAGSV<sup>687</sup> and K986G/V987G, in  
559 addition to truncation of the HR2 stalk (ΔE1150-Q1208). The viral capsid protein SHP (Protein  
560 Data Bank: 1TD0) was added as a C-terminal trimerization motif to stabilize the S2GΔHR2  
561 trimer, resulting in a soluble S2GΔHR2-5GS-1TD0 spike (41). The S2GΔHR2 spike was  
562 genetically fused to ferritin (FR), multilayered E2p, and multilayered I3-01v9 with 5GS, 5GS,  
563 and 10GS linkers, respectively, resulting in three S2GΔHR2-presenting SApNPs (41). An

564 S2P<sub>ECTO</sub>-5GS-1TD0 spike construct that contained the mutations <sup>682</sup>GSAGSV<sup>687</sup> and  
565 K986G/V987G but without HR2 deletion (41) was included for comparison. All vaccine  
566 antigens were transiently expressed in ExpiCHO cells and purified by a CR3022 antibody  
567 column and size-exclusion chromatography (SEC) as described previously (41). Briefly,  
568 ExpiCHO cells were thawed and incubated with ExpiCHO<sup>TM</sup> Expression Medium (Thermo  
569 Fisher) in a shaker incubator at 37 °C at 135 rotations per minute (rpm) with 8% CO<sub>2</sub>. When the  
570 cells reached a density of 10×10<sup>6</sup> ml<sup>-1</sup>, ExpiCHO<sup>TM</sup> Expression Medium was added to reduce  
571 cell density to 6×10<sup>6</sup> ml<sup>-1</sup> for transfection. The ExpiFectamine<sup>TM</sup> CHO/plasmid DNA complexes  
572 were prepared for 100-ml transfection in ExpiCHO cells according to the manufacturer's  
573 instructions. For a given construct, 100 µg of plasmid and 320 µl of ExpiFectamine<sup>TM</sup> CHO  
574 reagent were mixed in 7.7 ml of cold OptiPRO<sup>TM</sup> medium (Thermo Fisher). After the first feed  
575 on day 1, ExpiCHO cells were cultured in a shaker incubator at 33 °C at 115 rpm with 8% CO<sub>2</sub>  
576 according to the Max Titer protocol with an additional feed on day 5 (Thermo Fisher). Culture  
577 supernatants were harvested 13-14 days after transfection, clarified by centrifugation at 4000  
578 rpm for 25 min, and filtered using a 0.45 µm filter (Thermo Fisher). The CR3022 antibody  
579 column was used to extract SARS-CoV-2 antigens from the supernatants, followed by SEC on a  
580 Superdex 200 10/300 GL column (for scaffolded RBD trimers), a Superose 6 16/600 GL column  
581 (for the S2GAHR2 spike, with and without Avi-tag), or a Superose 6 10/300 GL column (for  
582 SApNPs). Protein concentration was determined using UV<sub>280</sub> absorbance with theoretical  
583 extinction coefficients.

#### 584 **Animal immunization and sample collection**

585 Similar immunization protocols were reported in our previous vaccine studies (41, 86, 87).  
586 Briefly, Institutional Animal Care and Use Committee (IACUC) guidelines were followed for all

587 of the animal studies. BALB/c mice (6 weeks old) were purchased from the Jackson Laboratory  
588 and kept in ventilated cages in environmentally controlled rooms at The Scripps Research  
589 Institute. The mouse studies were conducted according to Association for the Assessment and  
590 Accreditation of Laboratory Animal Care guidelines, and the protocols were approved by the  
591 IACUC. For the immunogenicity study, the mice were intraperitoneally immunized at weeks 0  
592 and 3 with 200  $\mu$ l of antigen/adjuvant mix containing 5-50  $\mu$ g of vaccine antigen and 100  $\mu$ l of  
593 adjuvant (41), or intradermally immunized at weeks 0 and 3 with 80  $\mu$ l of antigen/adjuvant mix  
594 containing 3.3  $\mu$ g of vaccine antigen and 40  $\mu$ l of adjuvant. The intradermal (i.d.) immunization  
595 was done through injections into four footpads, each with 20  $\mu$ l of antigen/adjuvant mix. For the  
596 mechanistic study of vaccine trafficking, retention, and induced GCs, the mice were immunized  
597 at weeks 0 and 3 with 80  $\mu$ l of antigen/adjuvant mix containing 40  $\mu$ g of vaccine antigen per  
598 mouse. To visualize the I3-01v9 SApNPs in lymph node tissues using TEM, each mouse was  
599 immunized at weeks 0 and 3 with 140  $\mu$ l of antigen/adjuvant mix containing 100  $\mu$ g of vaccine  
600 antigen (40  $\mu$ l of adjuvant) into the two hind footpads. Vaccines were intradermally administered  
601 into mouse footpads using a 29-gauge insulin needle under 3% isoflurane anesthesia with  
602 oxygen. Blood was drawn from the maxillary/facial vein into an ethylenediaminetetraacetic acid  
603 (EDTA)-coated tube 2 weeks after each immunization. Plasma was isolated from blood after  
604 centrifugation at 14000 rpm for 10 min. Plasma was heat-inactivated at 56°C for 30 min, with  
605 the supernatant collected after centrifugation at 8000 rpm for 10 min. Plasma was used in  
606 pseudovirus neutralization assays to determine the vaccine-induced NAb responses. The axillary,  
607 brachial, and popliteal sentinel lymph nodes were collected at the end timepoint for further  
608 analysis.

## 609 **Experimental adjuvants and formulation**

610 The adjuvants squalene-oil-in-water (AddaVax), aluminum hydroxide (AH), aluminum  
611 phosphate (AP), 2'3'-c-di-AM(PS)<sub>2</sub> (Rp,Rp) (STING ligand), monophosphoryl lipid A from S.  
612 minnesota (MPLA-SM) R595 (TLR4 agonist), imidazoquinoline compound R848 (TLR7/8  
613 agonist), and CpG ODN 1826, Class B (murine) (TLR9 agonist) were purchased from  
614 InvivoGen. PIKA, a TLR3 agonist with enhanced T cell and antibody responses reported for a  
615 Phase I rabies vaccine trial (107), was used as an adjuvant. PIKA was generously provided by  
616 Yisheng Biopharma and included in this study as an adjuvant that activates the TLR3 pathway.  
617 Macrophage inhibitors clodronate liposomes (Liposoma BV, catalog no. CP-005-005) were used  
618 to eliminate subcapsular sinus macrophages in lymph nodes to promote more robust B-cell  
619 activation. Mouse immunization was performed to examine the effects of 16 adjuvants or  
620 adjuvant combinations on the I3-01v9 SApNP-induced immune response with respect to the non-  
621 adjuvanted vaccine (PBS instead of an adjuvant). Vaccine antigen and adjuvants were mixed  
622 thoroughly 10 min before immunization. Each mouse was intradermally immunized at weeks 0,  
623 3, and 6 with 120-140  $\mu$ l of antigen/adjuvant mix containing 20  $\mu$ g of vaccine antigen (I3-01v9  
624 SApNP) and 80-100  $\mu$ l of adjuvant, which was evenly split and injected into four footpads.  
625 Mouse blood was isolated at weeks 5 and 8 after two and three intradermal injections,  
626 respectively. Spleens and lymph nodes were harvested at week 8 for immunological analyses.  
627 Spleen samples were ground through a 70  $\mu$ m cell strainer to release splenocytes into a cell  
628 suspension. Splenocytes were spun down at 400  $\times$  g for 10 min, washed with PBS, and treated  
629 with the ammonium-chloride-potassium (ACK) lysing buffer (Lonza). Splenocytes were then  
630 frozen with 3 ml of Bambanker freezing media.

### 631 **SARS-CoV-2 pseudovirus neutralization assay**

632 The SARS-CoV-2-pp neutralization assays were described in our previous study (41). Briefly,  
633 SARS-CoV-2-pps were generated by the co-transfection of HEK293T cells with the HIV-1  
634 pNL4-3.lucR-E- plasmid (obtained from the National Institutes of Health AIDS reagent program;  
635 <https://www.aidsreagent.org/>) and the expression plasmid encoding the *S* gene of five SARS-  
636 CoV-2 strains, including the wildtype Wuhan-Hu-1 strain (GenBank accession no. MN908947),  
637 three VOCs (GISAID accession no. EPI\_ISL\_601443, EPI\_ISL\_678597, and EPI\_ISL\_792680  
638 for B.1.1.7, B.1.351, and P.1, respectively), and B.1.617<sub>Rec</sub>, a reconstituted strain based on an  
639 early analysis of the B.1.617 lineage (11). The HEK293T-hACE2 cell line (catalog no. NR-  
640 52511) and pcDNA3.1(-) vector containing the *S* gene of the wildtype Wuhan-Hu-1 strain  
641 (catalog no. NR52420) were requested from the BEI Resources (<https://www.beiresources.org/>)  
642 on September 23, 2020 and used in the pseudovirus neutralization assays (43). Based on  
643 sequence alignment, spike mutations were incorporated into the *S* gene of the Wuhan-Hu-1 strain  
644 (catalog no. NR52420) to create respective expression plasmids for B.1.1.7, B.1.351, P.1, and  
645 B.1.617<sub>Rec</sub>. For B.1.617<sub>Rec</sub>, G142D, L452R, E484Q, D614G and P681R were included as  
646 representative spike mutations in this SARS-CoV-2 lineage (11). SARS-CoV-2-pp neutralization  
647 by immunized mouse plasma and human or mouse mAbs was performed according to our  
648 previously described protocol (41). Using the same co-transfection expression system as  
649 described above for the SARS-CoV-2-pps, we produced pseudoviruses carrying the murine  
650 leukemia virus (MLV) Env, MLV-pps, for use as a negative control (41). Percent neutralization  
651 data were analyzed using GraphPad Prism 9.1.2 software. ID<sub>50</sub>/IC<sub>50</sub> values were calculated using  
652 constraints for percent neutralization (0-100%), whereas unconstrained neutralization plots are  
653 shown in **Fig. 1** and **figs. S1-S3**.

#### 654 **Enzyme-linked immunosorbent assay**

655 Each well of a Costar<sup>TM</sup> 96-well assay plate (Corning) was first coated with 50  $\mu$ l of PBS  
656 containing 0.2  $\mu$ g of the appropriate antigens. The plates were incubated overnight at 4 °C, and  
657 then washed five times with wash buffer containing PBS and 0.05% (v/v) Tween 20. Each well  
658 was then coated with 150  $\mu$ l of blocking buffer consisting of PBS and 40 mg/ml blotting-grade  
659 blocker (Bio-Rad). The plates were incubated with blocking buffer for 1 h at room temperature,  
660 and then washed five times with wash buffer. Mouse mAbs, in the immunoglobulin G (IgG)  
661 form, were diluted in blocking buffer to a maximum concentration of 10  $\mu$ g/ml followed by a 10-  
662 fold dilution series. For each dilution, a total volume of 50  $\mu$ l was added to the appropriate wells.  
663 Each plate was incubated for 1 h at room temperature and then washed five times with PBS  
664 containing 0.05% Tween 20. A 1:5000 dilution of horseradish peroxidase (HRP)-conjugated goat  
665 anti-human IgG antibody (Jackson ImmunoResearch Laboratories) was then made in wash  
666 buffer (PBS containing 0.05% Tween 20), with 50  $\mu$ l of this diluted secondary antibody added to  
667 each well. The plates were incubated with the secondary antibody for 1 h at room temperature  
668 and then washed six times with PBS containing 0.05% Tween 20. Finally, the wells were  
669 developed with 50  $\mu$ l of TMB (Life Sciences) for 3-5 min before stopping the reaction with 50  $\mu$ l  
670 of 2 N sulfuric acid. The resulting plate readouts were measured at a wavelength of 450 nm. The  
671 ELISA data were analyzed to calculate EC<sub>50</sub> values using GraphPad Prism 9.1.2 software.

## 672 **Histology, immunostaining, and imaging**

673 The mice were sacrificed 2 h to 8 weeks after a single-dose immunization and 2 h to 5 weeks  
674 after the boost immunization. The axillary, brachial, and popliteal sentinel lymph nodes were  
675 isolated for histological analysis. Fresh lymph nodes were rapidly merged into frozen section  
676 compound (VWR International, catalog no. 95057-838) in a plastic cryomold (Tissue-Tek at  
677 VWR, catalog no. 4565) using liquid nitrogen to preserve antigens on the cell membrane and

678 spike. Lymph node samples were stored at  $-80^{\circ}\text{C}$  and sent to the Centre for Phenogenomics  
679 (<http://phenogenomics.ca>) on dry ice for sample processing and imaging. Tissue sections (8  $\mu\text{m}$ )  
680 were cut on a cryostat (Cryostar NX70) and collected on charged slides. Sections were post-fixed  
681 in 10% neutral buffered formalin and permeabilized in PBS containing 0.5% Triton X-100  
682 before immunostaining. Protein Block (Agilent) was used to block nonspecific antibody binding  
683 before incubating the sections with primary antibody overnight at  $4^{\circ}\text{C}$ . After washing in TBST,  
684 the sections were incubated in fluorophore-conjugated secondary antibodies for 1 h at room  
685 temperature. Lymph node tissue sections were stained with human anti-spike antibody P2B-2F6  
686 (72) (1:50) and biotinylated goat anti-human secondary antibody (Abcam, catalog no. ab7152,  
687 1:300), followed by streptavidin-HRP reagent (Vectastain Elite ABC-HRP Kit, Vector, catalog  
688 no. PK-6100) and diaminobenzidine (DAB) (ImmPACT DAB, Vector, catalog no. SK-4105) to  
689 study the distribution and retention of the soluble S2G $\Delta$ HR2 spike alone and S2G $\Delta$ HR2 spike-  
690 presenting E2p and I3-01v9 SApNPs. For immunofluorescent staining, tissue sections were  
691 stained for FDCs using anti-CD21 antibody (Abcam, catalog no. ab75985, 1:1800) followed by  
692 anti-rabbit secondary antibody conjugated with Alexa Fluor 555 (Thermo Fisher, catalog no.  
693 A21428, 1:200), stained for B cells using anti-B220 antibody (eBioscience, catalog no. 14-0452-  
694 82, 1:100) followed by anti-rat secondary antibody conjugated with Alexa Fluor 674 (Thermo  
695 Fisher, catalog no. A21247, 1:200), and stained for subcapsular sinus macrophages using anti-  
696 sialoadhesin (CD169) antibody (Abcam, catalog no. ab53443, 1:600) followed by anti-rat  
697 secondary antibody conjugated with Alexa Fluor 488 (Abcam, catalog no. ab150165, 1:200).  
698 Germinal center B cells were labeled using rat anti-GL7 antibody (FITC; BioLegend, catalog no.  
699 144604, 1:250). T<sub>fh</sub> cells were labeled using anti-CD4 antibody (BioLegend, catalog no. 100402,  
700 1:100) followed by anti-rat secondary antibody conjugated with Alexa Fluor 488 (Abcam,

701 catalog no. ab150165, 1:1000) and Bcl6 antibody (Abcam, catalog no. ab220092, 1:300)  
702 followed by anti-rabbit secondary antibody conjugated with Alexa Fluor 555 (Thermo Fisher,  
703 catalog no. A21428, 1:1000). Nuclei were then counterstained with 4',6-diamidino-2-  
704 phenylindole (DAPI) (Sigma-Aldrich, catalog no. D9542, 100 ng/ml). The stained tissue sections  
705 were scanned using an Olympus VS-120 slide scanner and imaged using a Hamamatsu ORCA-  
706 R2 C10600 digital camera for all bright-field and fluorescent images. Bright-field images of  
707 stained S2GΔHR2 spike and S2GΔHR2 spike-presenting SApNPs in lymph node follicles and  
708 fluorescent images of GCs were quantified using ImageJ software (108).

### 709 **Electron microscopy analysis of protein nanoparticles and lymph node tissues**

710 Electron microscopy (EM) analysis was performed by the Core Microscopy Facility at The  
711 Scripps Research Institute. For the negative-staining EM analysis of protein nanoparticles, the  
712 S2GΔHR2-10GS-I3-01v9-L7P SApNP samples were prepared at a concentration of 0.01 mg/ml.  
713 Carbon-coated copper grids (400 mesh) were glow-discharged, and 10 μl of each sample was  
714 adsorbed for 2 min. Excess sample was wicked away and grids were negatively stained with 2%  
715 uranyl formate for 2 min. Excess stain was wicked away and the grids were allowed to dry. For  
716 the EM analysis of mouse tissues, the lymph nodes were dissected from each animal and  
717 immersed in oxygenated 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1M Na cacodylate  
718 buffer (pH 7.4) fixative overnight at 4°C . After washing in 0.1 M sodium cacodylate buffer, the  
719 tissue samples were post-fixed in buffered 1% osmium tetroxide and 1.5% potassium  
720 ferrocyanide for 1-1.5 h at 4°C, rinsed in the same buffer, and then stained *en bloc* with 0.5%  
721 uranyl acetate overnight at 4°C. The tissue samples were washed in double-distilled H<sub>2</sub>O and  
722 dehydrated through a graded series of ethanol followed by acetone, infiltrated with LX-112  
723 (Ladd) epoxy resin, and polymerized at 60°C. Ultrathin lymph node sections (at 70-nm



724 thickness) were prepared for imaging. Samples were analyzed at 80 kV with a Talos L120C  
725 transmission electron microscope (Thermo Fisher), and images were acquired with a CETA 16M  
726 CMOS camera.

### 727 **Lymph node disaggregation, cell staining, and flow cytometry**

728 Germinal center reactions, including the percentage of GC B cells (GL7<sup>+</sup>B220<sup>+</sup>) and T<sub>fh</sub> cells  
729 (CD3<sup>+</sup>CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>), and the number of GC B cells and T<sub>fh</sub> cells were studied by flow  
730 cytometry (**fig. S5A**). The mice were sacrificed 2, 5, and 8 weeks after a single-dose  
731 immunization and 2 and 5 weeks after the boost immunization. Fresh axillary, brachial, and  
732 popliteal sentinel lymph nodes were collected and mechanically disaggregated. These lymph  
733 node samples were merged in enzyme digestion solution containing 958 µl of Hanks' balanced  
734 salt solution (HBSS) buffer (Thermo Fisher Scientific, catalog no. 14185052), 40 µl of 10 mg/ml  
735 collagenase IV (Sigma-Aldrich, catalog no. C5138), and 2 µl of 10 mg/ml of DNase (Roche,  
736 catalog no. 10104159001) in an Eppendorf tube. After incubation at 37°C for 30 min, lymph  
737 node samples were filtered through a 70 µm cell strainer and spun down at 400 × g for 10 min.  
738 The supernatant was discarded, and the cell pellet was resuspended in HBSS blocking solution  
739 containing 0.5% (w/v) bovine serum albumin and 2 mM EDTA. The nonspecific binding of Fc  
740 receptors was blocked using anti-CD16/32 antibody (BioLegend, catalog no. 101302) on ice for  
741 30 min. Cocktail antibodies, Zombie NIR live/dead stain (BioLegend, catalog no. 423106),  
742 Brilliant Violet 510 anti-mouse/human CD45R/B220 antibody (BioLegend, catalog no. 103247),  
743 FITC anti-mouse CD3 antibody (BioLegend, catalog no. 100204), Alexa Fluor 700 anti-mouse  
744 CD4 antibody (BioLegend, catalog no. 100536), PE anti-mouse/human GL7 antibody  
745 (BioLegend, catalog no. 144608), Brilliant Violet 605 anti-mouse CD95 (Fas) antibody  
746 (BioLegend, catalog no. 152612), Brilliant Violet 421 anti-mouse CD185 (CXCR5) antibody

747 (BioLegend, catalog no. 145511), and PE/Cyanine7 anti-mouse CD279 (PD-1) antibody  
748 (BioLegend, catalog no. 135216) were then mixed with the cells and placed on ice for 30 min.  
749 After washing cells with HBSS blocking solution after antibody staining, the samples were fixed  
750 using 1.6% paraformaldehyde (Thermo Fisher Scientific, catalog no. 28906) in HBSS on ice for  
751 30 min. The cell samples were stored in HBSS blocking solution for the flow cytometry study.  
752 Sample events were acquired by a 5-laser BD Biosciences LSR II analytical flow cytometer with  
753 BD FACS Diva 6 software at the Core Facility of The Scripps Research Institute. The data were  
754 further processed using FlowJo 10 software.

#### 755 **DC production, T cell culture, activation, and flow cytometry analysis**

756 Mouse bone marrow (BM) was cultured in RPMI 1640 medium containing 10% fetal bovine  
757 serum (FBS) and recombinant mouse Fms-like tyrosine kinase 3 ligand (Flt3L, 50 ng/ml) and  
758 stem cell factor (SCF, 10 ng/ml) for 9 days as previously described (109). To induce DC  
759 activation, immature DCs were incubated with lipopolysaccharide (LPS, 100 ng/ml) plus R848  
760 (Resiquimod, 100 ng/ml) overnight, which activated TLR4 or TLR7/8 signaling, respectively.  
761 Cells were harvested for the experiments. CD11c<sup>+</sup> DCs were sorted using magnetic beads  
762 (Miltenyi-Biotech, CA). Splenic mononuclear cells from each group of immunized mice were  
763 cultured in the presence of DCs pulsed with or without I3-01v9 SApNP ( $1 \times 10^{-7}$  mM) in  
764 complete IMDM medium containing IL-2 (5.0 ng/ml). Cells were collected 16 h later for  
765 intracellular cytokine staining and flow cytometry. All antibodies used for immunofluorescence  
766 staining were purchased from eBioscience (San Diego, CA), BioLegend (San Diego, CA) or BD  
767 Biosciences (San Jose, CA). Magnetic microbead-conjugated streptavidin was purchased from  
768 Miltenyi-Biotech (Auburn, CA). Recombinant human IL-2 protein was purchased from R&D  
769 Systems (Minneapolis, MN). Recombinant mouse Flt3 ligand (Flt3L) and mouse SCF were

770 purchased from Shenandoah Biotech (Warwick, PA). Cells were stained with appropriate  
771 concentrations of mAbs. Dead cells were excluded using Fixable Viability Dye (eBioscience,  
772 CA). Flow cytometry was performed using LSRII (BD Bioscience, CA).

773 **Bulk and single-cell sorting of SARS-CoV-2 antigen-specific mouse B cells.**

774 Spleens or lymph nodes were harvested from mice 15 days after the last immunization, and the  
775 cell suspension was prepared. Dead cells were excluded by staining with the Fixable Aqua Dead  
776 Cell Stain kit (Thermo Fisher, catalog no. L34957). Fc $\gamma$ III (CD16) and Fc $\gamma$ II (CD32) receptors  
777 were blocked by adding 20  $\mu$ l of 2.4G2 mAb (BD Pharmigen, catalog no. N553142). The cells  
778 were then incubated with 10  $\mu$ g of a biotinylated RBD-5GS-foldon-Avi trimer or biotinylated  
779 S2G $\Delta$ HR2-5GS-foldon-Avi spike. Briefly, the probes were generated by the biotinylation of  
780 Avi-tagged SARS-CoV-2 antigens using biotin ligase BirA according to the manufacturer's  
781 instructions (Avidity). Biotin excess was removed by SEC on either a Superdex 200 10/300  
782 column (GE Healthcare) for the RBD probe or a HiLoad Superose 6 16/600 column (GE  
783 Healthcare) for the spike probe. In the SEC profiles, the probe peak was well separated from the  
784 peak of biotin ligase (**fig. S3A**). Cells and biotinylated proteins were incubated for 5 min at 4 °C,  
785 followed by the addition of 2.5  $\mu$ l of anti-mouse IgG fluorescently labeled with FITC (Jackson  
786 ImmunoResearch catalog no. 115-095-071) and incubated for 15 min at 4 °C. Finally, 5  $\mu$ l of  
787 premium-grade allophycocyanin (APC)-labeled streptavidin was added to the cells and incubated  
788 for 15 min at 4 °C. In each step, the cells were washed with 0.5 ml of PBS and the sorting buffer  
789 (PBS with 2% FBS). FITC<sup>+</sup> APC<sup>+</sup> probe-specific B cells were sorted using MoFloAstrios EQ  
790 (Beckman Coulter). For bulk sorting, positive cells were sorted into an Eppendorf microtube  
791 with 20  $\mu$ l of lysis buffer. For single B-cell sorting, individual positive cells were sorted into the  
792 inner wells of a 96-well plate with 20  $\mu$ l of pre-reverse transcription (RT) lysis mix containing

793 0.1  $\mu$ l of NP40 (Sigma-Aldrich), 0.5  $\mu$ l of RNase Inhibitor (Thermo Fisher), 5  $\mu$ l of 5 $\times$  First  
794 Strand Buffer, and 1.25  $\mu$ l of DTT from the SuperScript IV kit (Invitrogen), with 13.15  $\mu$ l of  
795 H<sub>2</sub>O per well.

#### 796 **Antibody cloning from Env-specific single B cells and antibody production.**

797 The antibody cloning of SARS-CoV2-2 antigen-sorted single B cells was conducted as follows.  
798 A mix containing 3  $\mu$ l of Random Hexamers (GeneLink), 2  $\mu$ l of dNTPs, and 1  $\mu$ l of SuperScript  
799 IV enzyme (Thermo Fisher) was added to each well of a single-cell-sorted 96-well plate that  
800 underwent thermocycling according to the program outlined in the SuperScript IV protocol,  
801 resulting in 25  $\mu$ l of cDNA for each single cell. cDNA (5  $\mu$ l) was then added to a polymerase  
802 chain reaction (PCR) mix containing 12.5  $\mu$ l of 2 $\times$  Multiplex PCR mix (Qiagen), 9  $\mu$ l of H<sub>2</sub>O,  
803 0.5  $\mu$ l of forward primer mix, and 0.5  $\mu$ l of reverse mouse primer mix (*110*) for heavy and  $\kappa$ -  
804 light chains within each well. A second PCR reaction was then performed using 5  $\mu$ l of the first  
805 PCR as the template and respective mouse primers (*110*) according to the same recipe as the first  
806 PCR. The PCR products were run on 1% Agarose gel and those with correct heavy and light  
807 chain bands were then used for Gibson ligation (New England Biolabs), cloning into human IgG  
808 expression vectors, and transformation into competent cells. Mouse mAbs were expressed by the  
809 transient transfection of ExpiCHO cells (Thermo Fisher) with equal amounts of paired heavy and  
810  $\kappa$ -light chain plasmids. Antibody proteins were purified from the culture supernatant after 12-14  
811 days using Protein A bead columns (Thermo Fisher).

#### 812 **NGS and bioinformatics analysis of mouse B cells.**

813 Previously, a 5'-rapid amplification of cDNA ends (RACE)-PCR protocol was developed for the  
814 deep sequencing analysis of mouse B-cell repertoires (69). In the present study, this protocol was

815 applied to analyze bulk-sorted, RBD/spike-specific mouse B cells. Briefly, 5'-RACE cDNA was  
816 obtained from bulk-sorted B cells of each mouse with the SMART-Seq v4 Ultra Low Input RNA  
817 Kit for Sequencing (TaKaRa). The IgG PCRs were set up with Platinum *Taq* High-Fidelity DNA  
818 Polymerase (Life Technologies) in a total volume of 50  $\mu$ l, with 5  $\mu$ l of cDNA as the template, 1  
819  $\mu$ l of 5'-RACE primer, and 1  $\mu$ l of 10  $\mu$ M reverse primer. The 5'-RACE primer contained a  
820 PGM/S5 P1 adaptor, and the reverse primer contained a PGM/S5 A adaptor. The mouse 3'-C $\gamma$ 1-  
821 3/3'-C $\mu$  inner primers and 3'-mC $\kappa$  outer primer (110) were adapted as reverse primers for the 5'-  
822 RACE PCR processing of heavy and  $\kappa$ -light chains. A total of 25 cycles of PCR was performed  
823 and the expected PCR products (500-600 bp) were gel purified (Qiagen). NGS was performed on  
824 the Ion S5 GeneStudio system. Briefly, heavy and  $\kappa$ -light chain libraries from the same mouse  
825 were quantitated using a Qubit 2.0 Fluorometer with the Qubit dsDNA HS Assay Kit and then  
826 mixed at a 3:1 ratio before being pooled with antibody libraries of other mice at an equal ratio for  
827 sequencing. Template preparation and (Ion 530) chip loading were performed on Ion Chef using  
828 the Ion 520/530 Ext Kit, followed by sequencing on the Ion S5 system with default settings (86).  
829 The mouse Antibodyomics pipeline (69) was used to process raw NGS data, derive quantitative  
830 profiles for germline gene frequency, the degree of SHM, and CDR3 loop length distribution,  
831 and generate 2D divergence/identity plots to visualize mAbs in their respective repertoires (86).

### 832 **Statistical analysis**

833 Data were collected from 4-7 mice per group. All of the statistical analyses were performed and  
834 graphs were generated using GraphPad Prism 9.1.2 software. In the analysis of vaccine-induced  
835 plasma neutralization, different vaccine groups were compared using one-way analysis of  
836 variance (ANOVA), whereas for a given vaccine group ID<sub>50</sub> titers of the same plasma sample  
837 against different variants were compared using repeated measures one-way ANOVA. In both

838 cases, they were followed by Dunnett's multiple comparison *post hoc* test. For the vaccine  
839 accumulation and GC study, different vaccine groups were compared using one-way ANOVA,  
840 followed by Tukey's multiple comparison *post hoc* test. Statistical significance was indicated as  
841 the following: ns (not significant), \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

842

## 843 SUPPLEMENTARY MATERIALS

844 Supplementary material for this article is available at <http://xxx/xxx/xxx>.

845 **fig. S1.** Spike and spike-presenting SApNP vaccine-induced neutralizing antibody responses  
846 against the wildtype SARS-CoV-2 strain and four variants.

847 **fig. S2.** Immune responses against the wildtype SARS-CoV-2 strain and four variants induced by  
848 the I3-01v9 SApNP formulated with different adjuvants.

849 **fig. S3.** Single-cell isolation and functional evaluation of monoclonal neutralizing antibodies  
850 from mice immunized with the RBD, spike, and SApNP vaccines.

851 **fig. S4.** Unbiased repertoire analysis of bulk-sorted SARS-CoV-2 antigen-specific mouse splenic  
852 B cells and tracing of mouse neutralizing antibodies in the NGS-derived repertoires.

853 **fig. S5.** SARS-CoV-2 spike-presenting SApNP interaction with macrophages in a lymph node.

854 **fig. S6.** TEM images of SARS-CoV-2 spike-presenting I3-01v9 SApNP interaction with FDCs in  
855 a lymph node.

856 **fig. S7.** Immunohistological analysis of SARS-CoV-2 spike/spike-presenting SApNP vaccine-  
857 induced GCs.

858 **fig. S8.** Flow cytometry analysis of SARS-CoV-2 spike/spike-presenting SApNP vaccine-  
859 induced GCs.

860 **fig. S9.** Adjuvant effect on SARS-CoV-2 spike/spike-presenting SApNP vaccine-induced GCs.

861 **fig. S10.** NGS analysis of SARS-CoV-2 spike-specific lymph node (LN) B cells from mice  
862 immunized with the spike and SApNP vaccines.

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1124

## 1125 **Figure Legends**

1126 **Fig. 1. SApNP vaccines induce broadly neutralizing plasma responses to four**  
1127 **representative SARS-CoV-2 variants.** (A) Molecular surface representations of two spike  
1128 (S2P<sub>ECTO</sub>-5GS-1TD0 and S2G $\Delta$ HR2-5GS-1TD0) and three spike-SApNP (S2G $\Delta$ HR2-5GS-  
1129 ferritin [FR], S2G $\Delta$ HR2-5GS-E2p-LD4-PADRE [E2p-L4P], and S2G $\Delta$ HR2-10GS-I3-01v9-  
1130 LD7-PADRE [I3-01v9-L7P]) vaccines. Representative EM image of S2G $\Delta$ HR2-10GS- I3-01v9-  
1131 L7P SApNPs is shown on the right. (B) Neutralization of the wildtype Wuhan-Hu-1 strain by  
1132 mouse plasma induced by five different vaccines at week 5 after two intraperitoneal (i.p.)  
1133 injections ( $n = 5$  mice/group). ID<sub>50</sub> titers derived from SARS-CoV-2-pp neutralization assays are  
1134 plotted, with average ID<sub>50</sub> values labeled on the plots. (C) Mouse plasma neutralization against  
1135 Wuhan-Hu-1 and the B.1.1.7, B.1.351, P.1, and B.1.617<sub>Rec</sub> variants at week 5 after two i.p.  
1136 injections of the adjuvanted S2G $\Delta$ HR2-10GS-I3-01v9-L7P vaccine (Left panels 1-5: percent  
1137 neutralization plots; Right panel: ID<sub>50</sub> plot). In (B) and (C), the plasma samples were generated  
1138 in our previous study (41), in which mice were immunized with 50  $\mu$ g of adjuvanted vaccine  
1139 antigen. (D) Neutralization of mouse plasma against the wildtype Wuhan-Hu-1 strain induced by  
1140 the S2G $\Delta$ HR2 spike and two large SApNPs at week 5. Vaccines were administered via  
1141 intradermal (i.d.) footpad injections (0.8  $\mu$ g/injection, for a total of 3.3  $\mu$ g/mouse). (E) Mouse

1142 plasma neutralization against Wuhan-Hu-1 strain and the B.1.1.7, B.1.351, P.1, and B.1.617<sub>Rec</sub>  
1143 variants at week 5 after two i.d. footpad injections. (F) Neutralization of mouse plasma against  
1144 Wuhan-Hu-1 induced by the S2GΔHR2 spike and two large SApNPs at week 26. In (B)-(F), the  
1145 ID<sub>50</sub> values are plotted as mean ± SEM. The data were analyzed using one-way ANOVA for  
1146 comparison between different vaccine groups or repeated measures ANOVA for comparison of  
1147 ID<sub>50</sub> titers from the same plasma sample against different SARS-Cov-2 strains. Dunnett's  
1148 multiple comparison *post hoc* test was performed. ns (not significant), \*\**p* < 0.01, \*\*\*\**p* <  
1149 0.0001. (G) Neutralization of five SARS-CoV-2 strains by eight human monoclonal antibodies.  
1150 The IC<sub>50</sub> values were calculated with the % neutralization range constrained within 0.0-100.0%  
1151 and color-coded (white: IC<sub>50</sub> > 10 µg/ml; green to red: low to high).

1152 **Fig. 2. Adjuvants enhance the I3-01v9 SApNP vaccine -induced plasma neutralization of**  
1153 **both the wildtype strain and four variants.** (A) Schematic representation of mouse  
1154 immunization with the I3-01v9 SApNP with diverse adjuvant formulations and functional  
1155 assessment by SARS-CoV-2-pp neutralization assays and T-cell analysis. Conventional  
1156 adjuvants, STING/TLR agonists, macrophage inhibitors, and adjuvant combinations were  
1157 compared to non-adjuvanted control (PBS). (B, C) Mouse plasma neutralization against the  
1158 wildtype SARS-CoV-2 strain, Wuhan-Hu-1, at weeks 5 and 8 after two and three footpad  
1159 injections, respectively. ID<sub>50</sub> titers derived from SARS-CoV-2-pp neutralization assays are  
1160 plotted, with average ID<sub>50</sub> values labeled on the plots. (D) Neutralization against four variants by  
1161 mouse plasma from STING (top) and CpG (bottom)-formulated vaccine groups. ID<sub>50</sub> titers  
1162 derived from SARS-CoV-2-pp neutralization assays are plotted. Neutralization data were  
1163 analyzed using either one-way ANOVA (B-C) or repeated measures one-way ANOVA (D) to  
1164 compare ID<sub>50</sub> titers. Dunnett's multiple comparison *post hoc* test was performed. Splenic

1165 mononuclear cells derived from mice in the STING and CpG groups ( $n = 5$  mice/group) at week  
1166 8 were cultured in the presence of BALB/C DCs pulsed with I3-01v9 SApNP ( $1 \times 10^{-7}$  mM).  
1167 Cells were harvested 16 h following re-activation. (E) Production of IFN- $\gamma$ -producing Th1 CD4<sup>+</sup>  
1168 T cells and IL-4-producing Th2 CD4<sup>+</sup> T cells. (F) IFN- $\gamma$ -producing CD8<sup>+</sup> effector T cells. T-cell  
1169 responses were analyzed using one-way ANOVA followed by Tukey's multiple comparison *post*  
1170 *hoc* test. ns (not significant), \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

1171 **Fig. 3. Single-cell isolation identifies vaccine-elicited mouse neutralizing antibody lineages**  
1172 **with diverse breadth and potency.** (A) Genetic analysis of 20 mouse antibodies identified from  
1173 M2 in the RBD-5GS-1TD0 trimer group (four), M4 in the S2G $\Delta$ HR2-5GS-1TD0 spike group  
1174 (six), and M2 in the S2G $\Delta$ HR2-10GS-I3-01v9-L7P SApNP group (ten). Antibodies isolated by  
1175 the RBD and spike probes are highlighted in light gray and orange shade, respectively. (B)  
1176 Neutralization of five SARS-CoV-2 strains by 10 RBD and spike-elicited mouse antibodies (left)  
1177 and 10 SApNP-elicited mouse antibodies (right). The IC<sub>50</sub> values were calculated with the  
1178 %neutralization range constrained within 0.0-100.0% and color-coded (white: IC<sub>50</sub> > 100  $\mu$ g/ml;  
1179 green to red: low to high). (C) EC<sub>50</sub> ( $\mu$ g/ml) values of 20 mouse antibodies binding to the two  
1180 SARS-CoV-2 antigens, the RBD monomer and S2G $\Delta$ HR2-5GS-1TD0 spike, both with the  
1181 Wuhuan-Hu-1 backbone. Antigen binding was measured by ELISA in duplicate, with mean  
1182 value and standard deviation (SD) shown as black and red lines, respectively. (D) Divergence-  
1183 identity analysis of selected mouse NAb in the context of RBD/spike-specific splenic B cells.  
1184 HCs and KCs are plotted as a function of sequence identity to the template and sequence  
1185 divergence from putative germline genes. Color coding denotes sequence density. The template  
1186 and sequences identified based on the V gene assignment and a CDR3 identity of 90%/85% or  
1187 greater to the template are shown as black and orange/magenta dots on the 2D plots, with the



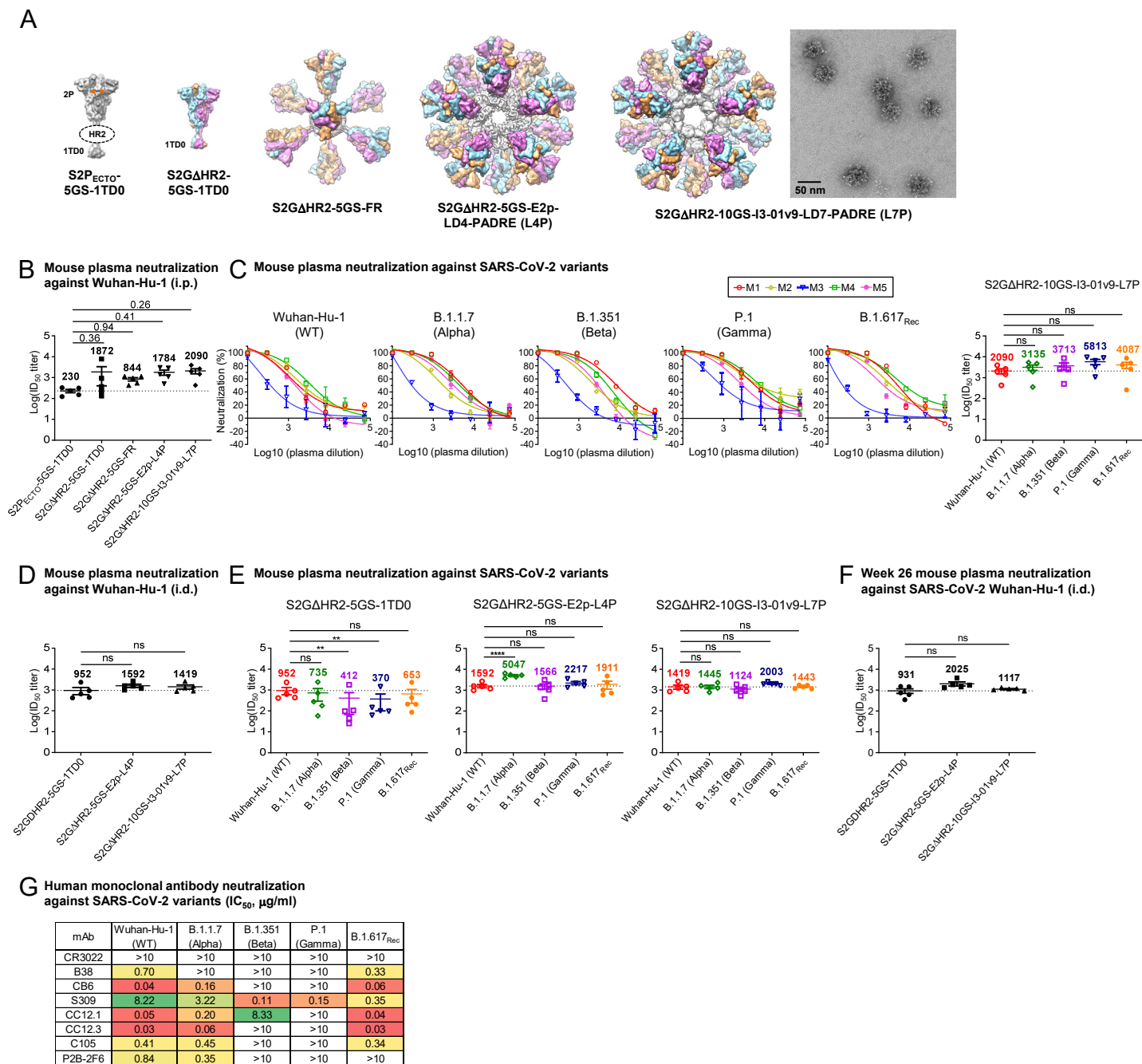
1188 number of related sequences labeled accordingly. The 2D plots for other NABs are shown in **fig.**  
1189 **S4D-F.**

1190 **Fig. 4. SARS-CoV-2 SApNP vaccines induce long-term lymph node follicle retention. (A, B)**  
1191 S2GΔHR2-presenting I3-01v9 SApNP vaccine distribution in a lymph node 12 h after (A) a  
1192 single-dose or (B) prime-boost footpad injections (10 μg/footpad, 40 μg/mouse). A schematic  
1193 illustration of SApNPs in lymph node follicles is shown. (C, D) Histological images of the  
1194 S2GΔHR2 spike and S2GΔHR2-presenting E2p and I3-01 SApNP vaccine trafficking and  
1195 retention in lymph node follicles 2 h to 8 weeks after (C) single-dose or (D) prime-boost  
1196 injections, with a scale bar of 50 μm shown for each image. (E, F) Quantification of vaccine  
1197 accumulation in lymph node follicles 48 h after (E) a single-dose or (F) prime-boost injections.  
1198 Data were collected from more than 10 lymph node follicles ( $n = 3-4$  mice/group). The data  
1199 points are expressed as mean ± SD. The data were analyzed using one-way ANOVA followed by  
1200 Tukey's multiple comparison *post hoc* test.  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ .

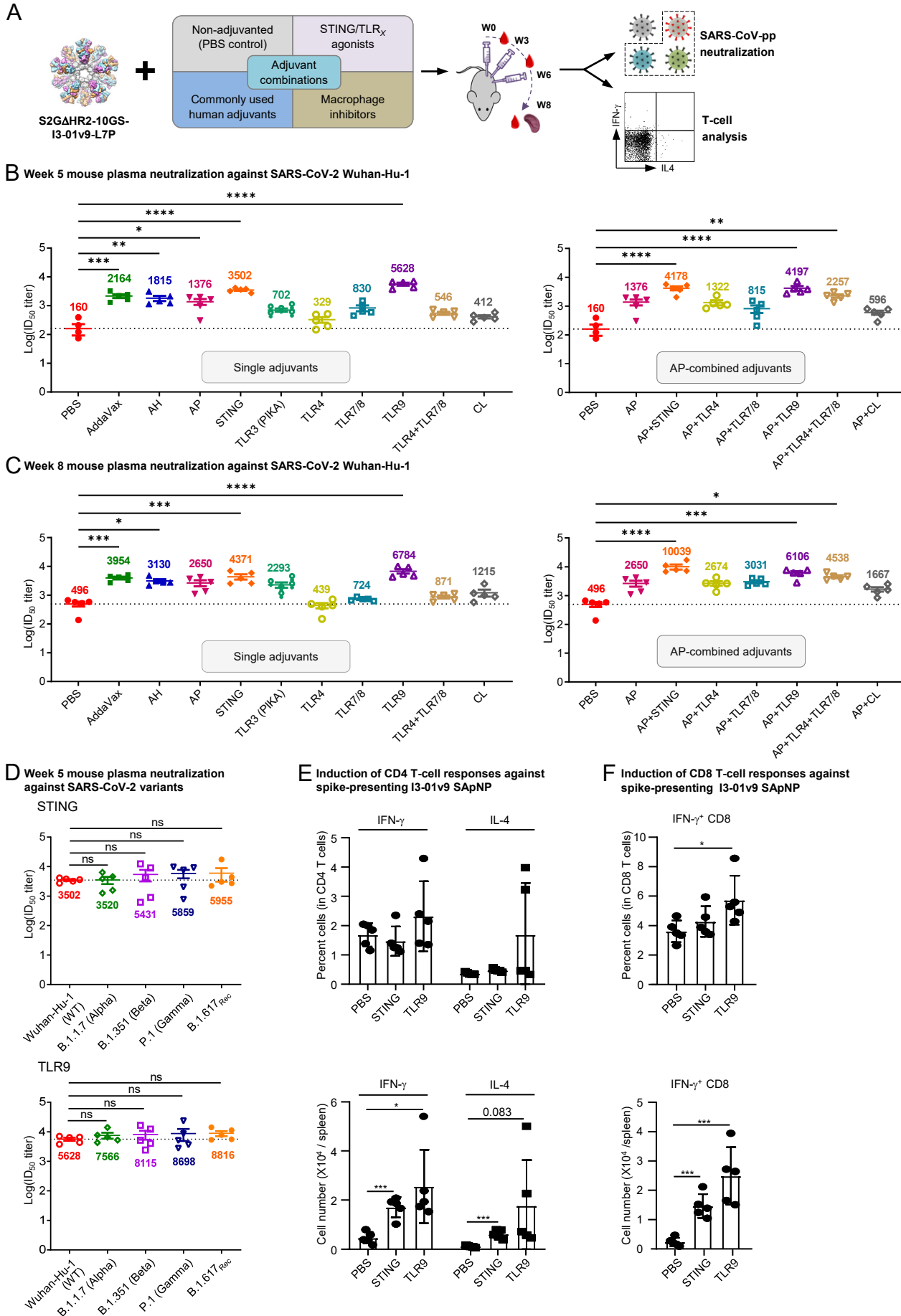
1201 **Fig. 5. SARS-CoV-2 SApNP vaccines interact with follicular dendritic cells (FDCs) and are**  
1202 **presented on FDC dendrites to B cells. (A, B)** S2GΔHR2 spike and S2GΔHR2-presenting E2p  
1203 and I3-01 SApNP vaccine interaction with FDC networks in lymph node follicles 12 h after (A)  
1204 a single-dose or (B) prime-boost injections (10 μg/footpad, 40 μg/mouse). Vaccine antigens (the  
1205 S2GΔHR2 spike and S2GΔHR2-presenting E2p and I3-01 SApNPs) colocalized with FDC  
1206 networks. Immunostaining is color-coded (Green: CD21; Red: CD169; White: anti-spike), with  
1207 scale bars of 500 μm and 100 μm shown for a complete lymph node and an enlarged image of a  
1208 follicle, respectively. (C) Representative TEM images of an FDC surrounded by multiple B cells.  
1209 S2GΔHR2-presenting I3-01 SApNPs (yellow arrows) presented on FDC dendrites.

1210 **Fig. 6. SARS-CoV-2 SApNP vaccines induce robust long-lived germinal centers.** (A) Top:  
1211 Representative immunohistological images of germinal centers at week 2 after a single-dose  
1212 injection of the S2GΔHR2-presenting I3-01 SApNP vaccine (10 μg/injection, 40 μg/mouse).  
1213 Bottom: Germinal center B cells (GL7<sup>+</sup>, red) adjacent to FDCs (CD21<sup>+</sup>, green) in lymph node  
1214 follicles (left) and T<sub>fh</sub> cells in the light zone (LZ) of germinal centers (right). Scale bars of 500  
1215 μm and 50 μm are shown for a complete lymph node and an enlarged image of a follicle,  
1216 respectively. (B, C) Quantification of germinal center reactions using immunofluorescent  
1217 images: GC/FDC ratio and sizes of germinal centers 2, 5, and 8 weeks after (B) single-dose or  
1218 (C) prime-boost injections (*n* = 4-7 mice/group). The GC/FDC ratio is defined as whether the  
1219 germinal center formation is associated with an FDC network (%). (D, E) Representative  
1220 immunohistological images of germinal centers in mice immunized using S2GΔHR2 spike or  
1221 S2GΔHR2-presenting E2p and I3-01 SApNP vaccines at week 8 after (D) single-dose or (E)  
1222 prime-boost injections, with a scale bar of 50 μm shown for each image. (F, G) Quantification of  
1223 germinal center reactions using flow cytometry: percentage and number of germinal center B  
1224 cells and T<sub>fh</sub> cells 2, 5, and 8 weeks after (F) single-dose or (G) prime-boost injections. The data  
1225 points are shown as mean ± SD. The data were analyzed using one-way ANOVA followed by  
1226 Tukey's multiple comparison *post hoc* test for each timepoint. ns (not significant), \**p* < 0.05, \*\**p*  
1227 < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001.

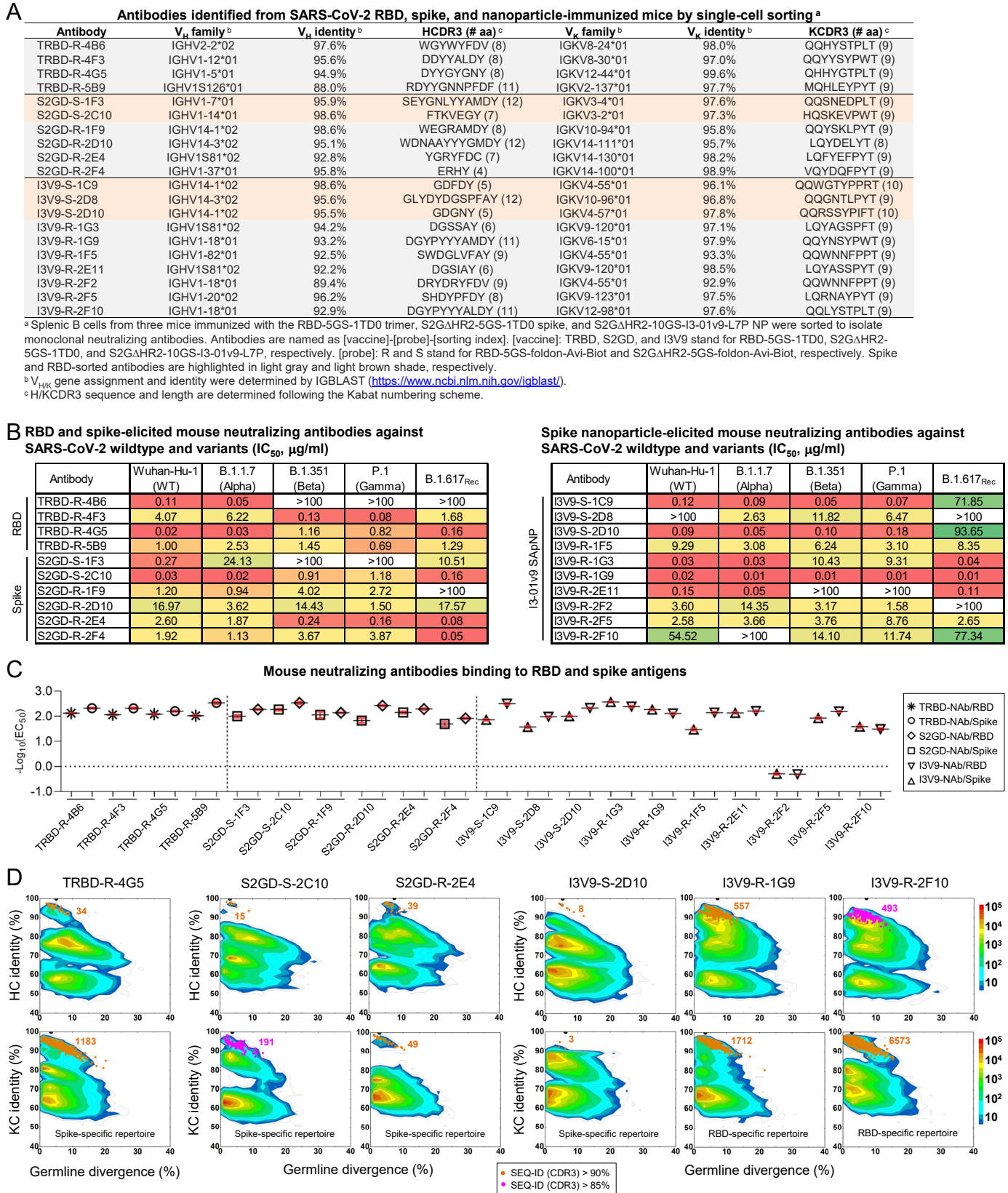
# Figure 1



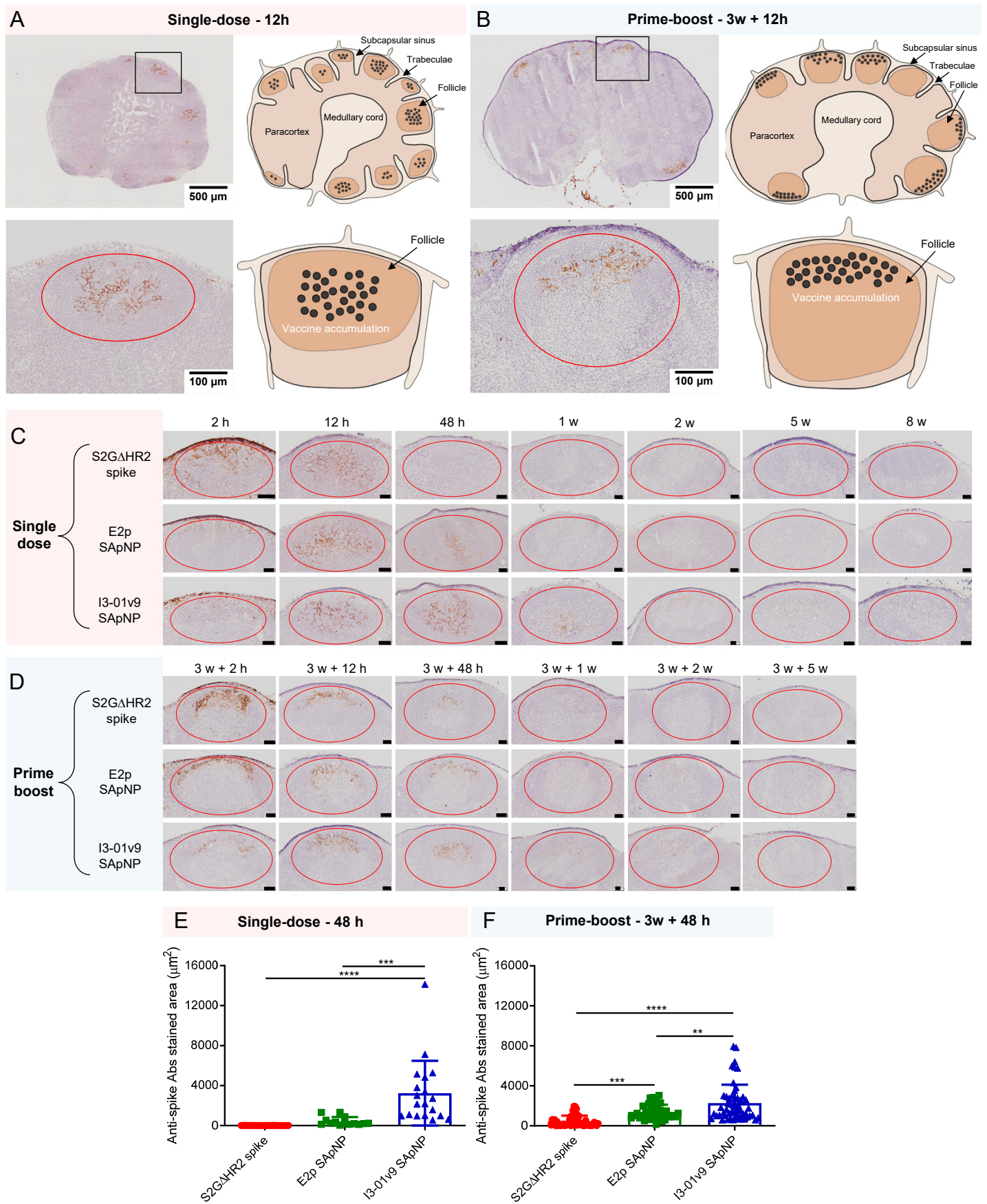
## Figure 2



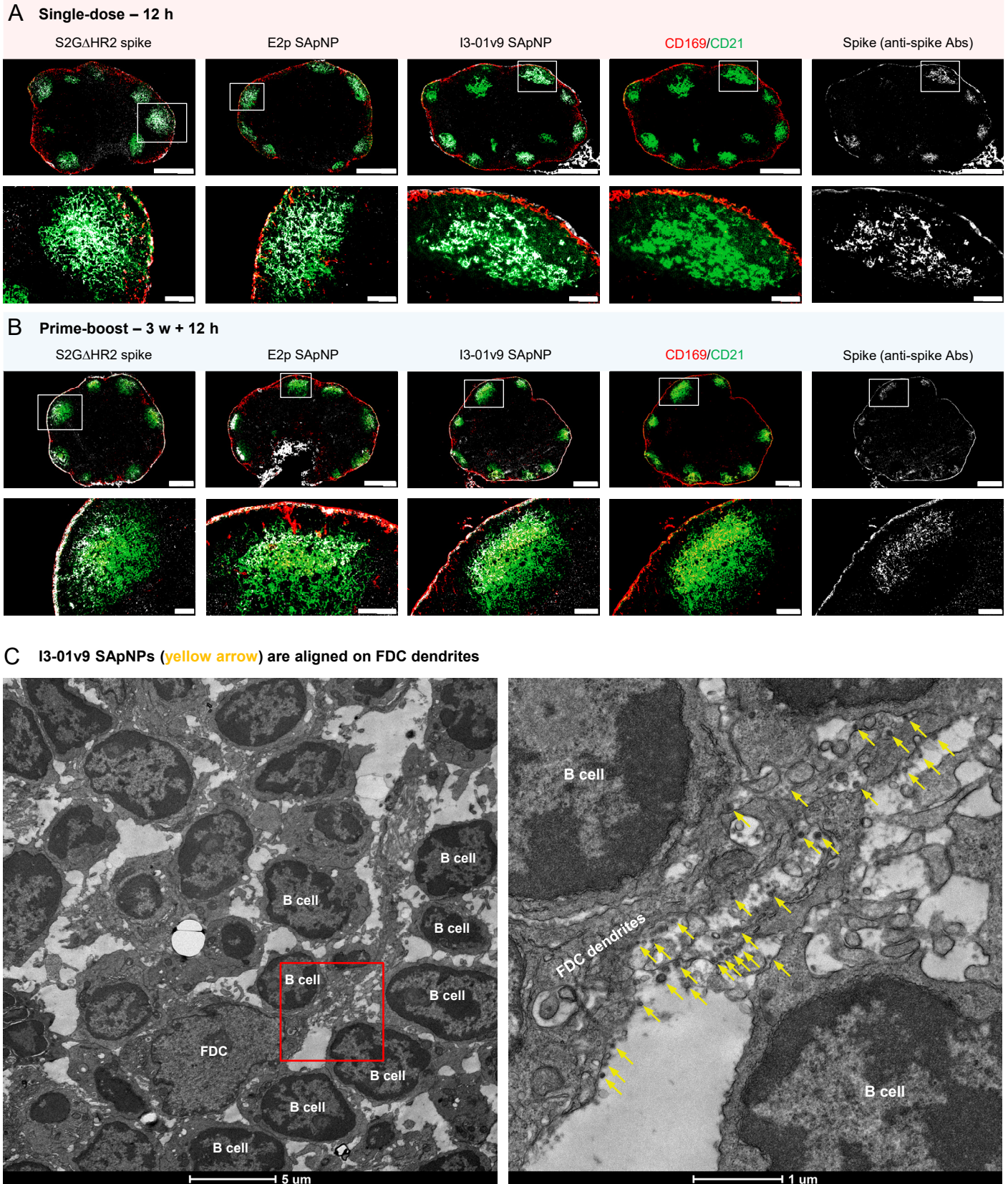
## Figure 3



## Figure 4



## Figure 5



## Figure 6

