

1 **Vaccine-mediated protection against merbecovirus and sarbecovirus challenge in mice**

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47 **SUMMARY**

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49       The emergence of three distinct highly pathogenic human coronaviruses – SARS-CoV in  
50 2003, MERS-CoV in 2012, and SARS-CoV-2 in 2019 – underlines the need to develop broadly  
51 active vaccines against the *Merbecovirus* and *Sarbecovirus* betacoronavirus subgenera. While  
52 SARS-CoV-2 vaccines are highly protective against severe COVID-19 disease, they do not  
53 protect against other sarbecoviruses or merbecoviruses. Here, we vaccinate mice with a trivalent  
54 sortase-conjugate nanoparticle (scNP) vaccine containing the SARS-CoV-2, RsSHC014, and  
55 MERS-CoV receptor binding domains (RBDs), which elicited live-virus neutralizing antibody  
56 responses and broad protection. Specifically, a monovalent SARS-CoV-2 RBD scNP vaccine  
57 only protected against sarbecovirus challenge, whereas the trivalent RBD scNP vaccine protected  
58 against both merbecovirus and sarbecovirus challenge in highly pathogenic and lethal mouse  
59 models. Moreover, the trivalent RBD scNP elicited serum neutralizing antibodies against SARS-  
60 CoV, MERS-CoV and SARS-CoV-2 BA.1 live viruses. Our findings show that a trivalent RBD  
61 nanoparticle vaccine displaying merbecovirus and sarbecovirus immunogens elicits immunity  
62 that broadly protects mice against disease. This study demonstrates proof-of-concept for a single  
63 pan-betacoronavirus vaccine to protect against three highly pathogenic human coronaviruses  
64 spanning two betacoronavirus subgenera.

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66 **Keywords:** bat coronavirus, MERS-CoV, nanoparticle, neutralization, receptor binding domain,  
67 SARS-CoV, SARS-CoV-2, universal vaccine.

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## 70 INTRODUCTION

71 The emergence of SARS-CoV in 2003, MERS-CoV in 2012, and SARS-CoV-2 in 2019  
72 into naïve human populations underlines the spillover potential of coronaviruses. SARS-CoV-2  
73 causes coronavirus disease of 2019 (COVID-19) <sup>1</sup>. The COVID-19 pandemic has had a  
74 devastating impact on human health and the world economy. SARS-CoV, SARS-CoV-2, and  
75 several zoonotic, pre-emergent SARS- and SARS2-related bat coronaviruses belong to the  
76 *Betacoronavirus* genus and *Sarbecovirus* subgenus and are classified as Group 2b coronaviruses  
77 <sup>2-4</sup>. Similarly, MERS-CoV and MERS-related bat zoonotic viruses also belong to the  
78 *Betacoronavirus* genus and *Merbecovirus* subgenus and are classified as Group 2c coronaviruses  
79 <sup>2,3</sup>. Given that in the last two decades, one merbecovirus and two sarbecoviruses have emerged  
80 into humans, the development of countermeasures against these important groups of viruses –  
81 including universal coronavirus vaccines –is a global health priority.

82 Several pan-betacoronavirus vaccine approaches have shown early promise in animal  
83 models <sup>5-8</sup>. Sortase-conjugated ferritin nanoparticles (scNPs) bearing the SARS-CoV-2 receptor  
84 binding domain (RBD) elicited neutralizing antibodies against bat SARS-related viruses and  
85 protected non-human primates (NHP) against SARS-CoV-2 challenge <sup>9</sup>. Moreover, monovalent  
86 SARS-CoV-2 RBD scNP vaccines elicit neutralizing antibodies against all tested SARS-CoV-2  
87 variants including D614G, Beta, Delta, Omicron BA.1, BA.2, BA.2.12.1, and BA.4/BA.5 <sup>10</sup>.  
88 Similar approaches with RBD nanoparticle vaccines also protect against sarbecovirus challenge  
89 in mice <sup>8</sup>. Chimeric spike antigens delivered as multiplexed mRNA-LNP vaccines similarly  
90 protected mice from genetically divergent bat zoonotic SARS-related viruses and SARS-CoV-2  
91 variants <sup>6</sup>. Therefore, multiple vaccine designs and modalities have protected against  
92 heterologous sarbecovirus challenge in animal models. Importantly, humans infected with

93 SARS-CoV 2003 and/or SARS-CoV-2 generate neutralizing monoclonal antibodies capable of  
94 neutralizing SARS-related zoonotic viruses and SARS-CoV-2 variants<sup>11-15</sup>. These human  
95 monoclonal antibodies protected mice and monkeys from sarbecovirus infection<sup>11,16</sup>. These  
96 studies indicated that elicitation of protective neutralizing antibody responses against  
97 sarbecoviruses is achievable.

98         Despite demonstrating proof-of-principle that vaccines can elicit broad immunity against  
99 genetically divergent sarbecoviruses<sup>5-8,17</sup>, no study to date has demonstrated vaccine-mediated  
100 protection against both sarbecovirus and merbecovirus betacoronaviruses. While stem-helix  
101 antibodies isolated from humans can protect against Group 2b SARS-CoV and SARS-CoV-2 as  
102 well as Group 2c MERS-CoV in highly pathogenic mouse models<sup>18</sup>, current vaccination  
103 strategies do not reproducibly induce immunity targeting these conserved S2 epitopes. Therefore,  
104 alternative vaccination strategies that effectively target sarbecoviruses and merbecoviruses are  
105 needed.

106         SARS-CoV-2 spike mRNA vaccines do not protect mice against challenge with  
107 genetically divergent zoonotic SARS-related viruses and SARS-CoV<sup>6</sup>. This suggests that  
108 currently used SARS-CoV-2 mRNA spike vaccines are unlikely to strongly protect against future  
109 SARS-related, SARS-CoV-2-related zoonotic viruses, or highly evolved SARS-CoV-2 variants  
110 of concern that could emerge in the future<sup>19,20</sup>. We therefore developed a trivalent RBD vaccine  
111 composed of sarbecovirus and merbecovirus RBDs from zoonotic pre-emergent, human  
112 epidemic, and pandemic coronaviruses. In this study, we evaluated the immunogenicity and  
113 protective efficacy against SARS-CoV and MERS-CoV in mice. We show that a monovalent  
114 SARS-CoV-2 RBD nanoparticle can protect against heterologous sarbecovirus challenge but  
115 does not protect against merbecovirus challenge. Conversely, the trivalent RBD scNP generates

116 neutralizing antibodies and prevents severe sarbecovirus disease and merbecovirus infections.

117 This study demonstrates proof-of-concept in an *in vivo* challenge setting that a vaccine that

118 protects against merbecoviruses and sarbecoviruses is an achievable goal.

119

## 120 **RESULTS**

### 121 **Generation and validation of trivalent RBD ferritin nanoparticle vaccine.**

122 We previously reported that a ferritin scNP monovalent SARS-CoV-2 RBD vaccine  
123 elicited broadly neutralizing antibodies against bat zoonotic pre-emergent betacoronaviruses,  
124 SARS-CoV, and SARS-CoV-2 variants in non-human primates<sup>7,16</sup>. To broaden the response of  
125 this SARS-CoV-2 RBD vaccine, we sought to generate a vaccine that increased the  
126 immunogenicity against the high risk *Merbecovirus* (also called Group 2c coronavirus) subgenus  
127 of betacoronaviruses, which includes MERS-CoV<sup>2</sup>. We designed a trivalent sortase-A-  
128 conjugated 24-mer ferritin nanoparticle (scNP) vaccine displaying SARS-CoV-2 RBD, SARS-  
129 related bat RsSHC014 RBD, and MERS-CoV EMC RBD (Figure 1A and S1A)<sup>10</sup>. Equimolar  
130 ratios of each RBD were conjugated to 24 acceptor sites on the 24-mer ferritin nanoparticle. In  
131 addition to the sarbecovirus SARS-CoV-2 RBD, RsSHC014 RBD was chosen for inclusion  
132 because it is a pre-emergent ACE2-binding sarbecovirus<sup>19</sup> to which the SARS-CoV-2 RBD  
133 nanoparticle generated only low levels of neutralizing antibodies<sup>7,16</sup>. We used negative stain  
134 electron microscopy (NSEM) to visualize the sortase A conjugated trivalent vaccines and  
135 demonstrated successful RBD conjugation (Figure 1B and S1B). The trivalent RBD scNP  
136 recapitulated the stability of the individual RBDs indicating the conjugation reaction had no  
137 deleterious effects on RBD folding or stability (Figure S1C and D).

138 To validate the efficient conjugation of SARS-CoV-2/RsSHC014/MERS-CoV RBDs as a  
139 trivalent vaccine, we also performed biolayer interferometry (BLI) binding analyses with human  
140 monoclonal antibodies that recognize Group 2b and Group 2c coronavirus spike epitopes and  
141 human ACE2. MERS-CoV RBD-specific mAbs JC57-14 and CDC-C2 only recognized MERS-  
142 CoV spike and the trivalent RBD vaccine (Figure 1C). Similarly, SARS-CoV-2 RBD-specific  
143 mAbs DH1284 and DH1041 bound only to SARS-CoV-2 spike and the trivalent RBD vaccine  
144 (Figure 1C). Group 2b RBD cross-reactive mAbs DH1047, DH1235, CR3022, and S309 bound  
145 to SARS-CoV-2 spike, RsSHC014 spike, and the trivalent RBD vaccine with highest magnitude  
146 but not to MERS-CoV spike or HIV Env. Finally, the negative control stem-helix mAb  
147 DH1057.1 bound to RsSHC014 spike and SARS-CoV-2 spike but not to the trivalent RBD  
148 vaccine, MERS-CoV spike, or HIV Env. Overall, the trivalent RBD nanoparticle bound to all the  
149 various Group 2b and 2c RBD antibodies, whereas no one spike protein recapitulated this  
150 breadth of reactivity. These BLI binding analyses suggest that the trivalent SARS-CoV-  
151 2/RsSHC014/MERS-CoV RBD scNP vaccine was efficiently conjugated and the RBD  
152 immunogens are properly recognized by various Group 2b and Group 2c-reactive monoclonal  
153 antibodies.

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### 155 **Immunogenicity of monovalent versus trivalent scNP vaccines in mice.**

156 To compare the immunogenicity of the monovalent versus the trivalent RBD scNP  
157 vaccines, we vaccinated aged BALB/c two times four weeks apart (Figure 2A). The Toll-like  
158 receptor 4 agonist glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) was used as the  
159 adjuvant for both vaccine groups and adjuvant-only controls (Figure 2A)<sup>21</sup>. We immunized mice  
160 with 10 $\mu$ g of the monovalent SARS-CoV-2 RBD scNP vaccine and also 10 $\mu$ g of the trivalent

161 SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP vaccine adjuvanted with 5 $\mu$ g of the GLA-SE  
162 adjuvant. We measured serum binding IgG antibodies against sarbecovirus and merbecovirus  
163 spike ectodomain matching the RBDs present in the vaccine and SARS-CoV spike, which was  
164 not in the vaccine. In mice vaccinated twice with the SARS-CoV-2/RsSHC014/MERS-CoV  
165 trivalent RBD scNP vaccine, high titers of spike binding IgG antibodies against human outbreak  
166 SARS-CoV Tor2 isolate (Figure 2B), bat pre-emergent RsSHC014 (Figure 2C), the SARS-CoV-  
167 2 Wuhan-1 outbreak isolate (Figure 2D), and the MERS-CoV EMC isolate (Figure 2E) were  
168 observed. In agreement with the IgG binding to various Group 2b and Group 2c spikes, we also  
169 observed serum antibody blocking of hACE2 binding to SARS-CoV-2 Spike and hDPP4 binding  
170 to MERS-CoV in trivalent RBD scNP vaccinated mice (Figure 2F and 2G). Only the trivalent  
171 scNP vaccine elicited robust serum antibody responses capable of blocking hDPP4 (Figure 2G).  
172 The monovalent SARS-CoV-2 RBD scNP vaccine also elicited high titers of binding IgG  
173 antibodies two weeks post boost against the three sarbecovirus spikes SARS-CoV Tor2 isolate,  
174 RsSHC014, SARS-CoV-2 Wuhan-1 isolate and human ACE2-blocking antibodies (Figure 2B-D,  
175 2F). However, immunization with SARS-CoV-2 RBD scNP did not elicit binding IgG to MERS-  
176 CoV spike or DPP4-blocking antibodies (Figure 2E 2G). These data indicated that the  
177 monovalent SARS-CoV-2 RBD scNP vaccine elicited cross-reactive binding and serum-  
178 blocking antibodies to Group 2b but not Group 2c coronaviruses. Thus, the trivalent RBD scNP  
179 vaccine improved antibody responses compared to the monovalent SARS-CoV-2 RBD scNP by  
180 eliciting serum antibodies to spikes from all three highly pathogenic human betacoronaviruses  
181 and a pre-emergent bat coronavirus.

182 To gain insights about the antibody binding breadth and depth elicited by the trivalent  
183 RBD scNP vaccine, we assessed serum IgG binding in vaccinated mice to thirteen RBDs of

184 animal and human coronaviruses from Groups 1, 2, and 4<sup>2,3</sup>. The RBD panel included a canine  
185 CoV-huPn, OC43, WIV-1, SARS-CoV GZ02, ZC45, GXP4L, BANAL-236, MERS-CoV,  
186 NL140422, HKU4, HKU5, BtKY06, and porcine deltacoronavirus Haiti (Figure 2H). The  
187 trivalent SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP vaccine elicited high IgG binding  
188 responses against five different Group 2b RBDs and four different Group 2c RBDs (Figure 2H).  
189 IgG binding was not observed against Group 1, Group 2a, 2d, and Group 4 coronaviruses (Figure  
190 2H). Notably, the MERS-CoV RBD in the trivalent vaccine elicited high binding responses  
191 against MERS-CoV and HKU5 and markedly lower binding was observed against NL140422  
192 and HKU4 (Figure 2H). This heterogenous binding across Group 2c RBDs suggests that Group  
193 2c RBDs may share fewer conserved epitopes as compared to Group 2b RBDs (Figure 2H). In  
194 contrast, the monovalent SARS-CoV-2 RBD scNP vaccine only elicited high IgG binding  
195 responses to Group 2b betacoronaviruses (Figure 2H), demonstrating more limited breadth than  
196 the trivalent RBD scNP. Together, these findings indicated that the superior trivalent RBD scNP  
197 vaccine elicited broad IgG responses against Group 2b and 2c viruses, and its IgG response  
198 exhibited depth reacting with multiple human and animal CoV RBDs from Group 2b and 2c  
199 coronaviruses.

200

### 201 **Induction of SARS-CoV-2, RsSHC014, MERS-CoV neutralizing antibodies.**

202 We then measured serum neutralizing antibody responses against Group 2b and Group 2c  
203 coronaviruses using live-virus assays. At baseline, both the monovalent SARS-CoV-2 RBD and  
204 trivalent SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP-vaccinated mice had undetectable  
205 neutralizing antibodies against the highly transmissible SARS-CoV-2 BA.1, SARS-CoV Urbani,  
206 and MERS-CoV. Following two immunizations, monovalent SARS-CoV-2 RBD scNP



207 vaccinated mice elicited serum neutralizing antibodies against SARS-CoV-2 BA.1 with a median  
208 ID<sub>80</sub> of 1832 (Figure 3A). Similarly, monovalent vaccinated mice elicited potent serum  
209 neutralizing antibodies against SARS-CoV Urbani with a median ID<sub>80</sub> of 1157 (Figure 3B).  
210 Undetectable serum neutralizing antibodies were observed against MERS-CoV EMC (Figure  
211 3C). In contrast to the monovalent vaccine, we observed potent serum neutralizing antibodies  
212 against MERS-CoV by the trivalent SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP vaccine  
213 with a median ID<sub>80</sub> of 3424 (Figure 3C). The trivalent SARS-CoV-2/RsSHC014/MERS-CoV  
214 RBD scNP vaccine also elicited serum neutralizing antibodies against SARS-CoV-2 BA.1 and  
215 SARS-CoV-1 Urbani with ID<sub>80</sub> values of 251 and 625, respectively. Importantly, undetectable  
216 serum neutralizing antibodies were measured in the adjuvant-only vaccinated mice. Thus, the  
217 monovalent SARS-CoV-2 RBD scNP vaccine elicited neutralizing antibodies against pandemic  
218 and epidemic sarbecoviruses whereas trivalent SARS-CoV-2/RsSHC014/MERS-CoV RBD  
219 scNP vaccines elicited neutralizing antibodies against pandemic and epidemic sarbecoviruses  
220 and MERS-CoV.

221

## 222 **Protective efficacy of trivalent RBD nanoparticle vaccine against Group 2b and Group 2c** 223 **CoVs.**

224 To evaluate the protective efficacy of the trivalent RBD scNP against sarbecovirus and  
225 merbecovirus infection with highly pathogenic coronaviruses, we challenged mice with either a  
226 heterologous, lethal mouse-adapted SARS-CoV virus (MA15)<sup>22</sup>, or a highly pathogenic mouse-  
227 adapted MERS-CoV virus (m35c4)<sup>23,24</sup>. Aged BALB/c mice immunized with the trivalent  
228 SARS-CoV-2/RsSHC014/MERS-CoV RBD were protected from weight loss (Figure 4A) and  
229 mortality (Figure 4B) after SARS-CoV MA15 challenge. This protection was likely due to

230 conserved RBD epitopes shared among sarbecoviruses<sup>7,10,11,25</sup>. Notably, the monovalent SARS-  
231 CoV-2 RBD scNP vaccine also protected against heterologous SARS-CoV MA15 challenge,  
232 whereas the adjuvant-only-vaccinated controls had 40% mortality by day 4 post infection (Figure  
233 4B). Compared to adjuvant-only controls, both monovalent and trivalent RBD scNP had reduced  
234 lung virus replication at day 2 post infection as measured by infectious virus plaque assays  
235 (Figure 4C). However, only the trivalent scNP- vaccinated mice had lower infectious SARS-CoV  
236 replication in the nasal turbinates at day 2 post infection as measured by plaque assay compared  
237 to the adjuvant-only-vaccinated controls (Figure 4D). Moreover, the trivalent RBD scNP vaccine  
238 also mediated increased protection against upper airway replication of SARS-CoV in mice.

239 As we observed strong protection from heterologous and highly pathogenic SARS-CoV  
240 MA15, we evaluated whether the trivalent vaccine also protected against challenge in a highly  
241 pathogenic mouse-adapted MERS-CoV model<sup>23,24</sup>. Like adjuvant-only controls, DPP4  
242 transgenic mice vaccinated twice with a monovalent SARS-CoV-2 RBD scNP vaccine  
243 experienced severe MERS-CoV disease including weight loss (Figure S2A), high levels of  
244 infectious virus replication in the lung and nasal turbinates (Figure S2B and C). Similarly, by day  
245 4 post infection SARS-CoV-2 RBD scNP-vaccinated mice exhibited significant weight loss and  
246 high amounts of virus replication in the lung (Figure S2D). In contrast, mice vaccinated twice  
247 with the trivalent SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP vaccine were protected from  
248 weight loss (Figure S2A). Unlike adjuvant-only controls and SARS-CoV-2 RBD monovalent  
249 vaccinated mice, we observed complete protection from lung virus replication at day 2 post  
250 infection in the trivalent SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP 2X vaccinated group  
251 (Figure S2B). However, we did not observe complete suppression of nasal turbinate MERS-CoV  
252 replication at day 2 post infection and lung virus replication at day 4 post infection in the

253 trivalent SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP 2X vaccinated group (Figure S2C-  
254 D).

255 To evaluate if additional boosting could increase the protective efficacy in the upper  
256 airways of the trivalent SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP vaccine, we repeated  
257 the vaccination study in the DPP4-modified mice that are susceptible to MERS-CoV infection  
258 and disease. We vaccinated mice three times four weeks apart with either the trivalent RBD  
259 scNP or SARS-CoV-2 RBD scNP (Figure S3A). Notably, mice immunized three times (3X)  
260 showed an increase in serum binding IgG against Group 2b and 2c coronavirus RBDs indicating  
261 the additional boost augmented antibody responses (Figure S3B). Three immunizations with the  
262 trivalent SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP vaccine completely protected mice  
263 from weight loss (Figure 5A), and lung virus replication at days 3 and 5 following MERS-CoV  
264 challenge (Figure 5B, 5D). Importantly, mice vaccinated 3x with the trivalent vaccine were fully  
265 protected from MERS-CoV replication in the nasal turbinates (Figure 5C). In contrast, the  
266 adjuvant-only control and the SARS-CoV-2 RBD monovalent scNP vaccine group exhibited  
267 marked weight loss following MERS-CoV challenge (Figure 5A) and had high levels of virus  
268 replication in the lungs and nasal turbinates at day 3 (Figure 5B and C). At day 5 post infection,  
269 virus replication remained high in these two groups of mice (Figure 5D). Therefore, a three-dose  
270 vaccination strategy achieved a high degree of protection in both the lower and upper airway  
271 after challenge with MERS-CoV.

272

## 273 **DISCUSSION**

274 Given the more than 6.8 million deaths attributed to the SARS-CoV-2 pandemic,  
275 vaccines that protect against the known highly pathogenic human coronaviruses are needed<sup>26,27</sup>.

276 This current study demonstrated a trivalent receptor binding domain sortase-conjugated  
277 nanoparticle vaccine induced neutralizing antibodies against all three highly pathogenic human  
278 betacoronaviruses and protected against both heterologous Group 2b (*Sarbecovirus* subgenus)  
279 and homologous Group 2c (*Merbecovirus* subgenus) coronavirus infections. This vaccine is an  
280 advance over current SARS-CoV-2 mRNA vaccines, which lack protection against other human  
281 pathogenic betacoronaviruses such as SARS-CoV and MERS-CoV<sup>6</sup>. The trivalent vaccine is  
282 also an advance beyond current Group 2b-focused RBD nanoparticle vaccines. The monovalent  
283 SARS-CoV-2 RBD scNP vaccine used in this study elicited high concentrations of IgG  
284 antibodies against Group 2b RBDs, and in previous studies was shown to neutralize recent  
285 known SARS-CoV-2 variants including highly mutated BA.4/BA.5 omicron sub-strains<sup>10</sup>.  
286 Moreover, the monovalent SARS-CoV-2 RBD scNP vaccine protects against sarbecoviruses  
287 SARS-CoV, SARS-CoV-2, and RsSHC014<sup>10</sup>. However, this SARS-CoV-2 RBD nanoparticle  
288 did not generate cross-reactive antibodies against Group 2c spike<sup>7</sup>. Notably, monovalent scNP  
289 SARS-CoV-2 vaccines that protect mice and monkeys against SARS-CoV-2 and sarbecovirus  
290 challenge<sup>7,10</sup> did not protect against MERS-CoV challenge. The lack of broadly reactive Group  
291 2b and 2c antibodies is expected given that MERS-CoV and SARS-CoV-2 RBDs differ in  
292 overall structure<sup>28,29</sup>. Therefore, “universal” vaccine approaches targeting SARS-CoV-2 variants  
293 may be distinct from those approaches needed for vaccines against antigenically and genetically  
294 distant coronaviruses.

295       Importantly, the SARS-CoV-2 RBD was sufficient in the monovalent vaccine for  
296 eliciting cross-reactive IgG antibodies against all tested sarbecoviruses. To bolster immunity  
297 against sarbecoviruses, the trivalent RBD nanoparticle includes SHC014 RBD. Conversely, the  
298 MERS-CoV RBD in the trivalent RBD vaccine elicited a range of high and low binding IgG

299 titers to the four Group 2c RBDs tested. The inability of a single Group 2c RBD to elicit high  
300 titers of cross-reactive IgG to all Group 2c RBDs tested indicates that Group 2c RBDs may share  
301 less epitope conservation compared to Group 2b RBDs.

302         The development of antibody-based MERS vaccines has been of concern given reports  
303 that antibody dependent enhancement of infection can occur *in vitro* with MERS-CoV-reactive  
304 antibodies<sup>30</sup>. Increased virus replication that is mediated by IgG antibodies is a classical  
305 surrogate of antibody-dependent enhancement that is observed for flaviviruses like dengue virus  
306<sup>31</sup>. In our study, we observed potent serum antibody neutralization of MERS-CoV *in vitro* and no  
307 evidence of increased virus replication upon challenge of mice immunized with MERS-CoV  
308 RBD. It is also important to note that we did not observe increased lung or nasal turbinate  
309 MERS-CoV replication relative to adjuvant only controls in mice vaccinated with the  
310 monovalent SARS-CoV-2 RBD scNP vaccine even though this vaccine did not protect against  
311 MERS-CoV challenge. This is an important observation as it suggests that individuals that have  
312 SARS-CoV-2 immunity to the RBD are unlikely to experience more severe disease when  
313 exposed to MERS-CoV or to a distinct Group 2c coronavirus that is antigenically like MERS-  
314 CoV.

315         A limitation to our study is that mucosal antibody responses were not measured. Thus,  
316 the durability of vaccine-elicited neutralizing antibodies in the upper airway is not known.  
317 Similarly, tissue-resident memory B and T cells responses were not profiled in the nasal airways  
318 or lungs<sup>17</sup>. Another limitation to our study is the lack of a heterologous Group 2c challenge in  
319 the trivalent scNP vaccine expressing MERS-CoV RBD. However, this is currently a limitation  
320 of the broad coronavirus pathogenesis field as MERS-CoV is the only known Group 2c human

321 respiratory coronavirus that can replicate and cause disease in mice expressing humanized DPP4  
322 receptors.

323 Finally, our study shows the utility of the sortase conjugate nanoparticle platform for  
324 rapidly and easily generating broadly protective vaccines. The trivalent RBD scNP vaccine is a  
325 viable strategy for vaccine-mediated protection against the three highly pathogenic Group 2b and  
326 2c betacoronaviruses - SARS-CoV, SARS-CoV-2 and its variants, and MERS-CoV. Moving  
327 forward it will be critical to assess if this trivalent RBD scNP vaccine also protect against Group  
328 2b and Group 2c coronaviruses in additional mouse models that express human ACE2 in the  
329 upper and lower airway epithelium as is observed in humans <sup>32</sup> and in other MERS-CoV mouse  
330 challenge models <sup>33</sup>. Our results suggest that universal vaccine approaches targeting Group 2b  
331 and Group 2c coronaviruses is achievable via multivalent delivery of RBDs via adjuvanted  
332 nanoparticle vaccines. The protective Group 2c immunity generated by the trivalent RBD  
333 nanoparticle is important since previous MERS-CoV outbreaks have had case fatality rates as  
334 high as 40% <sup>34</sup>, far exceeding the 1-10% rate reported for SARS-CoV-2 and SARS-CoV <sup>35</sup>. The  
335 next generation of coronavirus vaccines will need to broaden protection to include both Group 2b  
336 and 2c coronaviruses. Additionally, these findings have important implications for slowing down  
337 or preventing the spread of pre-emergent, zoonotic coronaviruses poised for human emergence  
338 <sup>19,20</sup>.

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342

343 **STAR METHODS**

### 344 **RBD sortase A conjugated nanoparticle vaccine production**

345 The receptor-binding domains (RBDs) from SARS-CoV-2 Wuhan-Hu1 isolate, MERS-CoV  
346 EMC isolate, and BatCoV RsSHC014 were expressed with a Sortase A donor sequence  
347 (LPETGG) encoded at the C terminus. An HRV-3C cleave site, an 8x His-tag, and a twin  
348 StrepTagII (IBA) were added C-terminal to the Sortase A donor sequence. The RBDs were each  
349 expressed by transient transfection using 293Fectin in Freestyle 293 cells and purified by  
350 StrepTactin affinity chromatography (IBA) followed by Superdex200 size-exclusion  
351 chromatography as described previously<sup>7,36</sup>. *Helicobacter pylori* ferritin particles were expressed  
352 with an N-terminal pentaglycine Sortase A acceptor sequence at the end of each subunit. 6x His-  
353 tags were included C-terminal to an HRV-3C cleavage site to enable affinity purification of the  
354 Ferritin particles. Prior to conjugation, RBDs, ferritin subunits, and pentamutant Sortase A<sup>37</sup>  
355 were buffer exchanged into 50mM Tris, 150mM NaCl, 5mM CaCl<sub>2</sub> at pH 7.4. The components  
356 were combined at a ratio of 360 μM total RBD (360 μM SARS-CoV-2 RBD for monovalent  
357 RBD scNP, or 120μM each of SARS-CoV-2, RsSHC014, and MERS-CoV RBD for the trivalent  
358 RBD scNP), plus 120uM Ferritin, plus 100uM Sortase A, and incubated at room temperature for  
359 4 hours. After incubation, the conjugated RBD-bearing nanoparticles were separated from free  
360 unconjugated reactants by size-exclusion chromatography using a Superose6 16/600 column.  
361 Conjugate nanoparticle assembly was confirmed by NSEM and by Western blot under both  
362 reducing and non-reducing conditions.

363

### 364 **Biolayer interferometry (BLI)**

365 Antibody binding was determined using a FortéBio Bio-Layer Interferometry instrument  
366 (Sartorius Octet Red96e) at 25°C with a shake speed of 1000 rpm. Antibodies were diluted to 20

367  $\mu\text{g/mL}$  in a flat bottom 96-well plate (Greiner) with .22 $\mu\text{m}$  filtered phosphate buffered saline pH  
368 7.4 and 0.05% Tween 20 (PBS-T). The antigens were diluted to a concentration of 50 $\mu\text{g/mL}$   
369 using PBS-T. Hydrated Anti-hIgG Fc Capture (AHC) biosensors (Sartorius #18-5060) were  
370 equilibrated for 60 second and then antibodies were loaded to biosensors for 300 seconds. After a  
371 60-second wash and a 180-second baseline step, biosensors were then dipped into the diluted  
372 antigens for a 200-second association. Next, antibody and antigens allowed to dissociate for 300  
373 seconds. Data was analyzed using Data Analysis HT 12.0 software. The negative control  
374 antibody, CH65, was indicated as a reference sensor and subtracted from the remaining ligand  
375 sensor measurements. Data was then aligned to the average of the baseline step and plotted using  
376 GraphPad Prism 9 software.

377

### 378 **Negative stain electron microscopy of RBD nanoparticles**

379 Negative stain electron microscopy was performed as previously described<sup>7</sup>. The RBD  
380 nanoparticle protein was thawed in an aluminum block at room temperature for 5 min. The RBD  
381 scNP was diluted to a final concentration of 0.2 mg/mL into room-temperature buffer containing  
382 150 mM NaCl, 20 mM HEPES pH 7.4, 5g/dL glycerol and 8 mM glutaraldehyde. After 5 min,  
383 the cross-linking was quenched by the addition of 1 M Tris pH 7.4 to a final concentration of 75  
384 mM Tris and incubated for 5 min. Carbon-coated grids (EMS, CF300-cu-UL) were glow-  
385 discharged for 20 s at 15 mA, and subsequently a 5- $\mu\text{l}$  drop of quenched sample was incubated  
386 on the grid for 10–15 s. The grid was blotted and then stained with 2g/dL uranyl formate. After  
387 air drying, grids were imaged with a Philips EM420 electron microscope operated at 120 kV, at  
388 49,000 $\times$  magnification and images captured with a 76 megapixel CCD camera at a pixel size of  
389 2.4  $\text{\AA}$ .



390

### 391 **Processing of negative-stain images**

392 The RELION 3.0 program was used for all negative-stain image processing following previously  
393 published procedures <sup>7</sup>. Images were CTF-corrected with CTFFIND and particles were picked  
394 using a nanoparticle template. Extracted particle stacks were underwent 2 or 3 rounds of 2D class  
395 averaging and selection to discard irrelevant particles and background picks.

396

### 397 **Mouse vaccinations and virus challenge experiments**

398 Aged BALB/c (#047) retired breeder female mice were purchased from Envigo and were used  
399 for SARS-CoV-1 MA15 challenge studies. B6 male and female mice modified at the DPP4 locus  
400 <sup>23</sup> to allow pathogenesis by mouse-adapted MERS-CoV m35c4 <sup>24</sup> were bred in house and used at  
401 ~20-25 weeks of age. The Toll-like receptor 4 agonist glucopyranosyl lipid adjuvant–stable  
402 emulsion (GLA-SE) was used as the adjuvant for the vaccine immunogens. Mouse vaccination  
403 studies were performed intramuscularly with GLA-SE-adjuvanted SARS-CoV-2 RBD scNP,  
404 GLA-SE-adjuvanted SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP, or GLA-SE-adjuvant  
405 only for the control group. Vaccine immunogens were administered at 10 µg of the RBD scNP  
406 vaccines formulated with 5 µg of adjuvant. Mice were immunized at week 0 and week 4 for the  
407 2X prime-boost vaccine regimen, and at week 0, week 4, and week 8 for the 3X prime-boost-  
408 boost vaccine regimen. Mice were then moved into the BSL3 and acclimated for a few days.  
409 Prior to challenge, mice were anesthetized with an intraperitoneal delivery of xylazine and  
410 ketamine and given a lethal dose of SARS-CoV-1 MA15 <sup>22</sup>:  $1 \times 10^4$  PFU/ml. For the MERS-  
411 CoV challenge studies, mice were challenged with mouse-adapted MERS-CoV m35c4 <sup>24</sup>.

412

### 413 **Binding ELISA against coronavirus antigen panel**

414 For coronavirus antigen-binding assays, 384-well ELISA plates (Costar #3700) were coated with  
415 2 µg/ml antigens in 0.1M sodium bicarbonate overnight at 4°C. Plates were then washed 1X and  
416 blocked for 2 h at room temperature with SuperBlock (1X phosphate buffered saline (PBS)  
417 containing 4% (w/v) whey protein 15% normal goat serum/0.5% Tween-20/0.05% sodium  
418 azide). Mouse serum samples were collected at baseline before prime, two weeks post prime,  
419 four weeks post prime, two weeks post boost, and two weeks post the second boost. Mouse  
420 serum samples were added at 1:30 dilution in SuperBlock and diluted 3-fold through 12 dilution  
421 spots to generate binding curves. Diluted serum samples were bound to coated plates in  
422 SuperBlock for 1h at room temperature. Plates were then washed 2X and a horseradish  
423 peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (SouthernBiotech 1030-  
424 05) was added in SuperBlock at a 1:16,000 dilution. Secondary antibody was bound for 1h and  
425 then washed 4X and detected with 20µL SureBlue Reserve (KPL 53-00-03) for 15 min.  
426 Colorimetric reactions were stopped by adding 20µL of 1% HCL stop solution. Plates were read  
427 at 450nm and area under the curve (AUC) was calculated from the serially diluted mouse serum  
428 samples.

429

### 430 **Live virus neutralization assays**

431 All live virus assays were performed in a BSL-3 laboratory. Full length SARS-CoV Urbani,  
432 SARS-CoV-2 Wuhan-1 expressing the BA.1 spike, and MERS-CoV were designed to express  
433 nanoluciferase (nLuc) as described previously<sup>38,39</sup>. SARS-CoV Urbani and SARS-CoV-2 BA.1  
434 stocks were generated and titrated in Vero E6 (C1008) cells and MERS-CoV stocks were titrated  
435 in Vero 81 (CCL-81) cells. For the live virus neutralization assays, cells were plated at 20,000

436 cells per well in clear bottom, black-walled 96-well plates the day prior to the assay. On the day  
437 of the assay, mouse serum samples diluted 1:40 and serially diluted 3-fold to eight dilutions.  
438 Serially diluted mouse serum was added at a 1:1 volume with diluted virus and incubated for 1 h.  
439 Antibody-virus dilutions were then added to cells at 800 PFU per well and incubated at 37°C  
440 with 5% CO<sub>2</sub>. Following a 24hr incubation, plates were read by adding 25µL of Nano-Glo  
441 Luciferase Assay System (Promega). Luminescence was measured by a Spectramax M3 plate  
442 reader (Molecular Devices). Fifty percent virus neutralization titers were calculated using  
443 GraphPad Prism via four-parameter dose-response curves.

444

#### 445 **Biocontainment and biosafety**

446 All experiments handling live viruses, including mouse-adapted coronaviruses, were performed  
447 in an animal biosafety level 3 (BSL-3) laboratory. Laboratory workers performing BSL-3  
448 experiments wore powered air purifying respirators (PAPR), Tyvek coverall suits, double booties  
449 covering footwear, and double gloves. All recombinant coronavirus work was approved by the  
450 UNC Institutional Biosafety Committee (IBC). All animal work was approved by the UNC  
451 Institutional Animal Care and Use Committee (IACUC). All BSL-3 work was performed in a  
452 facility conforming to requirements recommended in the Microbiological and Biomedical  
453 Laboratories, by the U.S. Department of Health and Human Services, the U.S. Public Health  
454 Service, and the U.S. Center for Disease Control and Prevention (CDC), and the National  
455 Institutes of Health (NIH).

456

#### 457 **Statistical analysis**

458 Non-parametric Kruskal-Wallis tests were used to compare lung and nasal turbinate infectious  
459 virus replication in challenged mice and neutralizing antibody assays. A Dunn's test was used to  
460 correct for multiple comparisons. A Chi square log-rank test was used for the survival analysis.  
461 Statistical analyses were performed in GraphPad Prism 9.

462

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468

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470 A.S., R.S.B., B.F.H., K.O.S., designed experiments. D.R.M., T.D.G., A.S., M.L.M, N.J.C., K.G.,  
471 T.S., R.J.E., K.M., performed laboratory experiments. D.R.M., T.D.G., A.S., M.L.M, N.J.C.,  
472 K.G., T.S., R.S.B., B.F.H., K.O.S. analyzed the data and provided critical insight. D.R.M. wrote  
473 the first draft of the paper. D.R.M., T.D.G., A.S., M.L.M, N.J.C., K.G., T.S., R.S.B., B.F.H.,  
474 K.O.S. edited the paper. All authors read and approved the final version of the paper.

475

476 **Competing interests:** B.F.H. and K.O.S. have filed US patents regarding the nanoparticle  
477 vaccine. R.S.B. is on the scientific advisory boards of VaxArt, Invivyd, and Takeda.

478

479 **Data and materials availability:** All data associated with this paper is in the paper and  
480 Supplementary Materials. Reagents developed in this study, including vaccine immunogens and

481 viruses will be made available by contacting R.S.B., B.F.H. and K.O.S. following the completion  
482 of a material transfer agreement.

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683 **Figure legends:**

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685 **Figure 1. Design and characterization of trivalent RBD scNP vaccines.** (A) Ferritin

686 nanoparticles were conjugated with sortase A tagged Group 2b SARS-CoV-2 RBD, Group 2b

687 RsSHC014 RBD, and Group 2c MERS-CoV RBD. (B) Visualization of trivalent scNP was

688 performed via negative stain electron microscopy. (C) Validation of trivalent scNP vaccine by

689 Biolayer Interferometry. Trivalent RBD scNP antigenicity was done by assessing binding of the

690 trivalent vaccine and various Group 2b and Group 2c spikes to human ACE2, MERS-CoV RBD

691 mAbs, SARS-CoV-2 RBD mAbs, Group 2b cross-reactive RBD mAbs, and an S2 mAb. HIV-1

692 envelope was included as a negative control antigen.

693

694 **Fig. 2. IgG binding responses in mice immunized with monovalent SARS-CoV-2 RBD scNP**

695 **vaccine, trivalent SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP, and adjuvant alone.**

696 Mouse sera was measured at pre-prime, pre-boost, and two-week post boost against the

697 following spike antigens (A) SARS-CoV Tor2, (B) RsSHC014, (C) SARS-CoV-2, and (D)

698 MERS-CoV. (E) Vaccine-elicited hACE2-blocking serum responses in monovalent, trivalent,

699 and adjuvant-only vaccinated mice. (F) Vaccine-elicited hDPP4-blocking serum responses in

700 monovalent, trivalent, and adjuvant-only vaccinated mice. (G) Cross-reactivity of monovalent,

701 trivalent, vs adjuvant-only IgG responses against Group 1 (Canine CoV-HuPn), Group 2a

702 (OC43), 2b (WIV-1, SARS-CoV GZ02, ZC45, GXP4L, and BANAL-236), 2c (MERS-CoV,

703 NL140422, HKU4, and HKU5), 2d (BtKY06), and Group 4 (Porcine deltaCoV Haiti)

704 coronavirus RBDs.

705

706 **Figure 3. Neutralizing antibodies elicited against Group 2b and Group 2c**

707 **betacoronaviruses.** Live virus neutralizing activity against SARS-CoV-2 BA.1, SARS-CoV  
708 Urbani, and MERS-CoV EMC. Mouse sera at baseline and post boost are shown in 2X  
709 vaccinated mice are shown. Blue circles denote monovalent SARS-CoV-2 RBD scNP vaccinated  
710 mice. Red squares denote trivalent SARS-CoV-2/RsSHC014/MERS-CoV RBD scNP vaccinated  
711 mice. Gray triangles denote adjuvant-only control mice. Numerical values in the graphs denote  
712 the median ID<sub>80</sub> values (n = 16; \*P < 0.05, \*\*P < 0.005, \*\*\*P < 0.0005, and \*\*\*\*P < 0.0001).

713

714 **Figure 4. Protective efficacy of monovalent vs trivalent RBD scNP vaccines against SARS-**

715 **CoV challenge in mice.** (A) Weight loss in monovalent SARS-CoV-2 RBD, trivalent SARS-  
716 CoV-2/RsSHC014/MERS-CoV RBD vaccinated scNP, and adjuvant-only vaccinated mice. (B)  
717 Percent survival in vaccinated mice vs control following lethal SARS-CoV Urbani MA15  
718 challenge. Statistical significance of the survival curves is from a Chi square log-rank test. (C)  
719 Infectious virus replication in the lung of vaccinated mice at day 2 following infection. Statistical  
720 significance is from a Kruskal-Wallis test following a Dunn's multiple comparison correction  
721 test. (D) Infectious virus replication in nasal turbinates at day 2 post infection. Statistical  
722 significance is from a Kruskal-Wallis test following a Dunn's multiple comparison correction  
723 test. Blue circles represent the monovalent vaccinated mice. Red squares represent the trivalent  
724 vaccinated mice. Grey triangles denote the adjuvant-only vaccinated mice. \*P < 0.05, \*\*P <  
725 0.005, \*\*\*P < 0.0005, and \*\*\*\*P < 0.0001

726

727 **Figure 5. Protective efficacy of monovalent vs trivalent RBD scNP vaccines against MERS-**

728 **CoV challenge in mice.** (A) Weight loss in SARS-CoV-2 RBD monovalent, SARS-CoV-

729 2/RsSHC014/MERS-CoV RBD vaccinated scNP, and adjuvant-only vaccinated mice following  
730 MERS-CoV intranasal challenge. Lung virus replication in monovalent, trivalent, and adjuvant-  
731 only controls at day 3 post infection. (C) Infectious virus replication in nasal turbinates at day 3  
732 post infection. (D) Lung infectious virus replication at day 5 post infection. P values shown in all  
733 panels are from a Kruskal-Wallis test following a Dunn's multiple comparisons test. \*P < 0.05,  
734 \*\*P < 0.005, \*\*\*P < 0.0005, and \*\*\*\*P < 0.0001

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747 **Supplemental Figure legends:**

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750 **Figure S1. Structural and biophysical characterization of the trivalent SARS-CoV-**

751 **2/SHC014/MERS RBD scNP. (A)** Analytical size exclusion chromatography with a Superose6

752 column showing a homogenous protein nanoparticle at the expected elution volume. **(B)**

753 Negative stain electron microscopy of trivalent RBD scNP. 2D class average is shown on the

754 bottom left and a raw image of a single nanoparticle is shown on the top left. The raw image of  
755 the carbon grid is shown on the right. **(C and D)** Differential scanning fluorimetry of the  
756 individual components and assembled trivalent RBD scNP. Melting temperature is defined as the  
757 inflection temperature ( $T_i$ ) on the 350 nm/330 nm ratio curve.

758

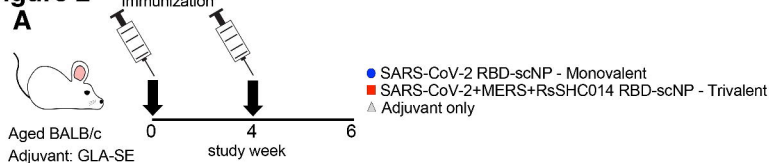
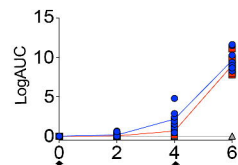
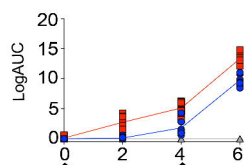
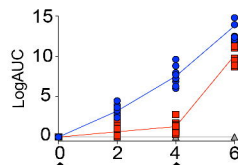
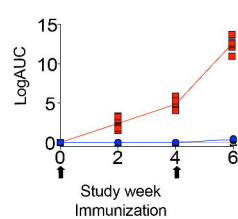
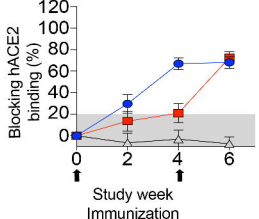
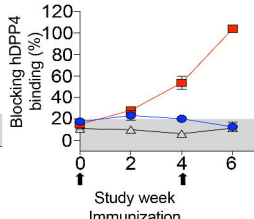
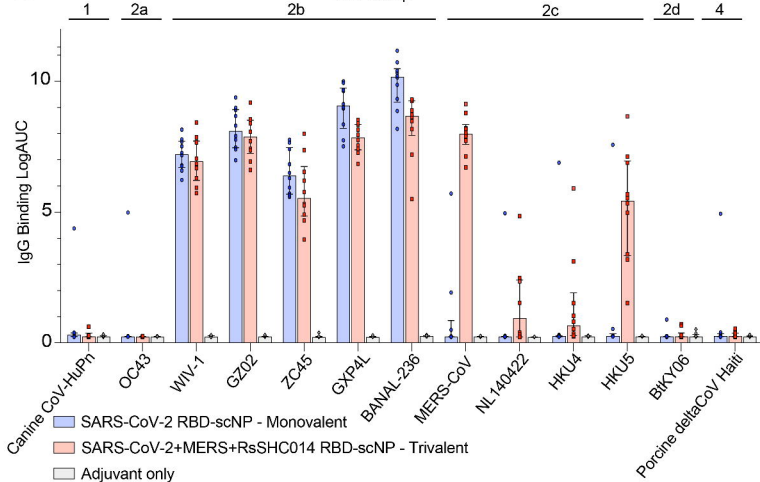
759 **Figure S2. Protective efficacy of monovalent vs trivalent RBD scNP vaccines against**  
760 **MERS-CoV challenge in 2X vaccinated mice.** **(A)** Weight loss following MERS-CoV  
761 intranasal challenge in monovalent, trivalent, and adjuvant-only vaccinated mice. **(B)** Infectious  
762 virus replication in the lung at day 2 post infection. **(C)** Infectious virus replication at day 2 post  
763 infection in nasal turbinates. **(D)** Infectious virus replication at day 4 post infection. A Kruskal-  
764 Wallis test with a Dunn's multiple comparison correction test was used for calculating statistical  
765 significance in all panels. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ , and \*\*\*\* $P < 0.0001$

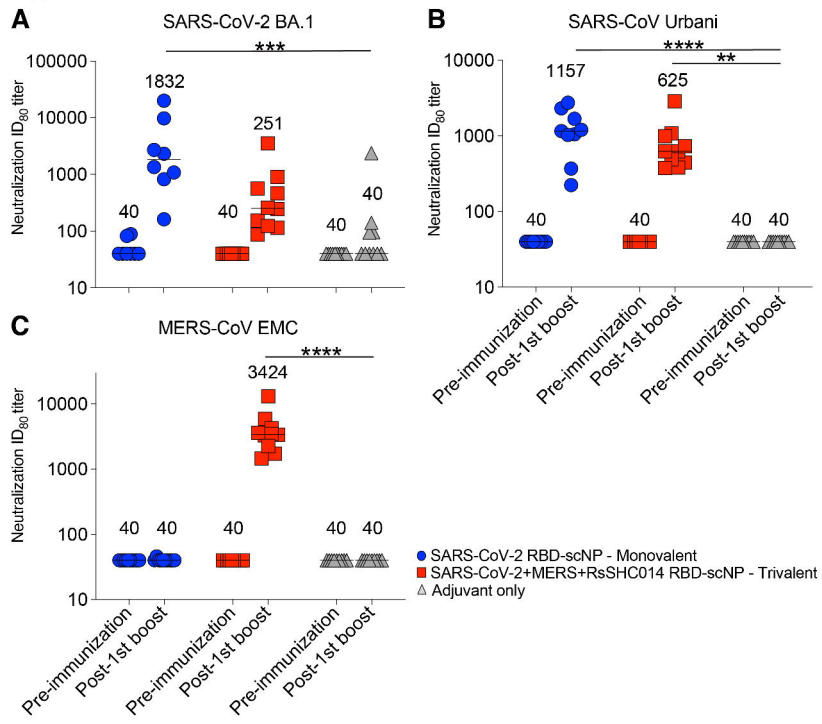
766

767 **Figure S3. Immunogenicity of monovalent vs trivalent RBD scNP vaccines in 2X vs 3X**  
768 **vaccinated mice.** **(A)** vaccination schema with monovalent scNP, trivalent scNP, and adjuvant  
769 only (GLA-SE). **(B)** LogAUC IgG binding comparison of 2X (prime-boost) vs 3X (prime-boost-  
770 boost) vaccinated mice with monovalent, trivalent, and adjuvant-only against genetically  
771 divergent RBDs from Group 1 (canineCoV-HuPn). Group 2a (OC43), Group 2b (WIV-1, GZ02,  
772 ZC45, GXP4L, and BANAL-236), Group 2c (MERS-CoV, NL140422, HKU4, and HKU5),  
773 Group 2d (BtKY06), and Group 4 (Porcine deltacoronavirus Haiti).

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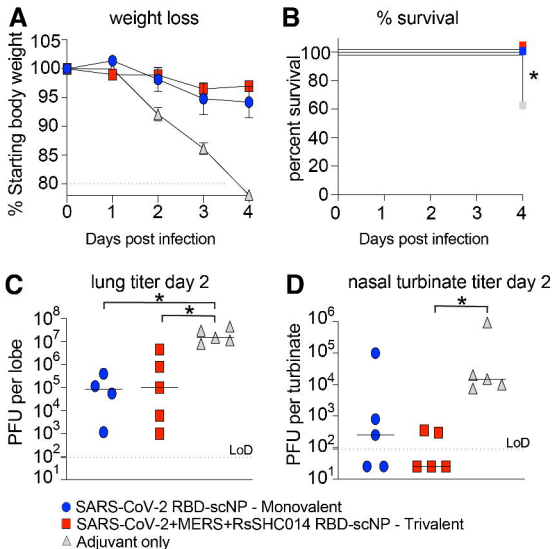
**Figure 2****B** SARS-CoV Tor2**C** RsSHC014**D** SARS-CoV-2**E** MERS-CoV**F** hACE2 serum blocking**G** hDPP4 serum blocking**H** CoV Group

**Figure 3**



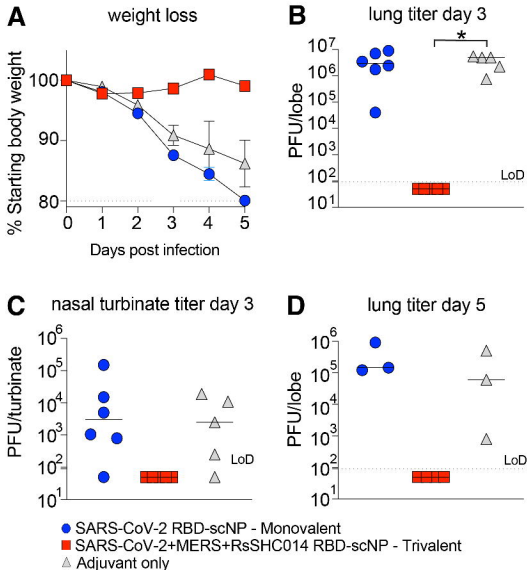
# Figure 4

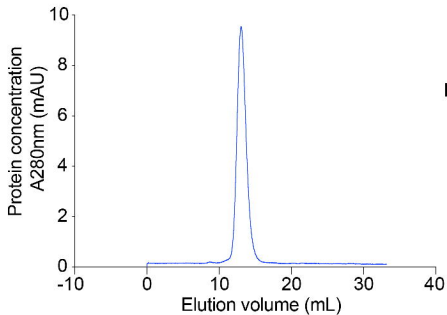
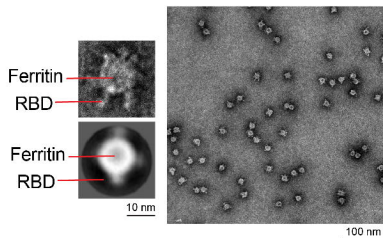
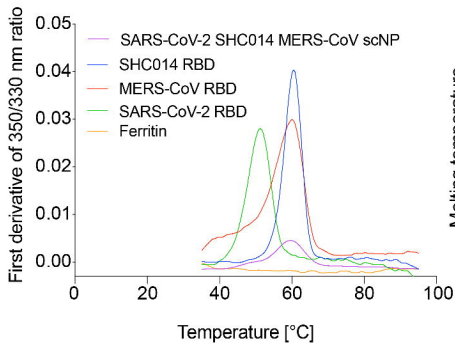
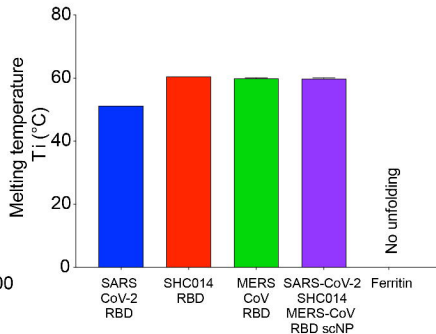
## SARS-CoV challenge



# Figure 5

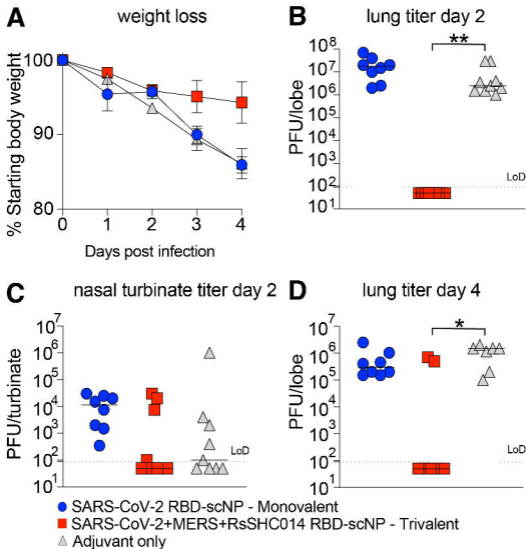
## MERS-CoV challenge

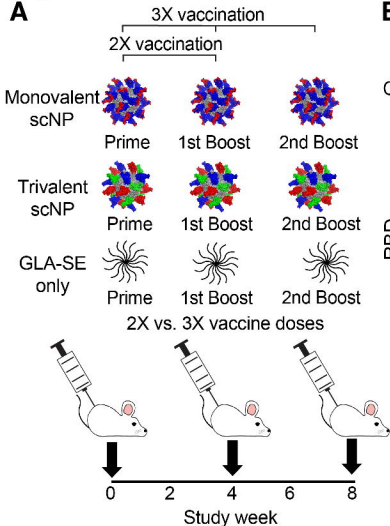


**Figure S1****A****B****C****D**

# Figure S2

## MERS-CoV challenge



**Figure S3****A****B**