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**Development of spike receptor-binding domain nanoparticle as a vaccine candidate
against SARS-CoV-2 infection in ferrets**

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a causative agent of COVID-19 pandemic, enters host cells *via* the interaction of its Receptor-Binding Domain (RBD) of Spike protein with host Angiotensin-Converting Enzyme 2 (ACE2). Therefore, RBD is a promising vaccine target to induce protective immunity against SARS-CoV-2 infection. In this study, we report the development of RBD protein-based vaccine candidate against SARS-CoV-2 using self-assembling *H. pylori*-bullfrog ferritin nanoparticles as an antigen delivery. RBD-ferritin protein purified from mammalian cells efficiently assembled into 24-mer nanoparticles. 16-20 months-old ferrets were vaccinated with RBD-ferritin nanoparticles (RBD-nanoparticles) by intramuscular or intranasal inoculation. All vaccinated ferrets with RBD-nanoparticles produced potent neutralizing antibodies against SARS-CoV-2. Strikingly, vaccinated ferrets demonstrated efficient protection from SARS-CoV-2 challenge, showing no fever, body weight loss and clinical symptoms. Furthermore, vaccinated ferrets showed rapid clearance of infectious viruses in nasal washes and lungs as well as viral RNA in respiratory organs. This study demonstrates the Spike RBD-nanoparticle as an effective protein vaccine candidate against SARS-CoV-2.

43

Introduction

44 SARS-CoV-2, originally named 2019-nCoV upon initial isolation from Wuhan, China in
45 December 2019, has caused a global outbreak of coronavirus disease-19 (COVID-19) with
46 significant socioeconomic impacts (1, 2). From the continuously growing numbers of diagnoses
47 and deaths, COVID-19 was declared a public health emergency of international concern
48 (PHEIC) in January 2020 and soon declared a pandemic by WHO in March 2020 (3, 4). As of
49 Jan 27th 2021, more than 100 million people have been infected with SARS-CoV-2, among
50 which 2 million died (5). Although approximately 80% of the confirmed SARS-CoV-2 infections
51 are asymptomatic or show mild flu-like symptoms, 20% of the infections progress to severe
52 pneumonia and acute respiratory distress syndrome requiring hospitalization and mechanical
53 ventilation (6, 7). The overwhelming number of SARS-CoV-2 patients has rapidly devastated the
54 availability of health-care resources (8). Shortage of medical resources and staff in conjunction
55 with the overwhelming number of patients have exacerbated the quality of medical care and
56 eventually increased mortality rates of COVID-19 (9). Although a significant proportion of the
57 infected patients have recovered, many of them report cardiovascular, pulmonary, and
58 neurologic symptoms lasting after the recovery (10, 11). Thus, strong preventive measures are
59 essential to halt the pandemic and its destructive effects on the global public health, as well as
60 the economy.

61 SARS-CoV-2 is a member of the *Coronaviridae* family, carrying a single positive-
62 stranded RNA genome within the viral envelope (2). Although at least seven coronaviruses are
63 known as etiological agents of mild respiratory illnesses in human infection, the family has not
64 been closely associated with severe illnesses until the relatively recent outbreaks of SARS-CoV,
65 MERS-CoV, and SARS-CoV-2 (1, 12). Emergence of these pathogens and the COVID-19
66 pandemic have called for an urgent global research efforts to investigate the pathogenesis of
67 coronaviruses. The SARS-CoV-2 RNA genome is approximately 30 kilobases and encodes for
68 structural proteins such as –Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N)–

69 and non-structural proteins such as papain-like protease, chymotrypsin-like protease, and RNA-
70 dependent RNA polymerase (13). The heavily glycosylated S protein protruding from the virion
71 surface is the key bridge between the virus and the host cell, playing a crucial role in host cell
72 receptor recognition, virion attachment, and ultimately entry into the host cell. S is a member of
73 the Class I viral fusion protein which undergoes trimerization upon cleavage into S1 and S2
74 domains by a host cellular protease, furin. While S1 confers specificity in cell tropism through its
75 Receptor-Binding Domain (RBD) which directly interacts with the receptor of SARS-CoV-2,
76 Angiotensin-Converting Enzyme 2 (ACE2), S2 mediates membrane fusion via formation of a
77 trimeric hairpin structure from its heptad repeat domains (14). Therefore, S1 RBD has been
78 considered as one of the most promising candidates in vaccine development to protect against
79 Coronaviruses (15-17). Its efficacy has previously been shown to induce potent neutralizing
80 antibodies against MERS-CoV (18). Furthermore, previous studies of neutralizing antibodies
81 from naturally recovered patients of SARS-CoV-2 infections have mapped their epitopes to be
82 S1 and RBD (19, 20), implicating RBD-targeting antibodies in successful immunity against
83 SARS-CoV-2 (21-23). Thus, most of the currently developed vaccines against SARS-CoV-2,–
84 despite of their diversity in vaccine approaches,– include RBD in their immunogens (24-28).

85 One major limitation of small soluble proteins alone as vaccine candidates is that our
86 immune system only reacts efficiently against immunogens of nanometer range in size (29, 30).
87 Therefore, many protein vaccines using viral proteins are developed into virus-like particles
88 (VLPs) which are multiprotein structures that mimic the organization and conformation of native
89 viruses, but lack the viral genome. However, this approach is limited to a few pathogens that are
90 capable of self-assembling into VLPs upon overexpression of the viral protein, such as Hepatitis
91 B virus (HBV) surface antigen (HBsAg) and Human Papillomavirus (HPV) L1 protein (31-33).
92 Fortunately, the latest advances in molecular biology and nanotechnology have overcome this
93 limitation by adopting nanoparticle engineering to serve as platforms for vaccines. The efficacy
94 of these nanoparticle-engineered vaccines outperforms traditional vaccines, such as whole

95 inactivated vaccines of bacterial and viral pathogens (34-38). Moreover, recent studies have
96 shed light on the immunological advantages of nanoparticle-based vaccines in nearly every step
97 of the humoral and cellular immunity: efficient antigen transport to draining lymph nodes,
98 antigen presentation by follicular dendritic and helper T cells, as well as high level of activation
99 of the germinal centers (30, 39, 40). Among the genetically engineered nanoparticles, ferritin is
100 the most well-characterized in the bionanotechnology field. Ferritin, ubiquitous through
101 kingdoms of life, has a conserved role in minimizing damage to cell from reactive oxygen
102 species formed from the Fenton reaction upon excess iron (II). Due to its natural tendency to
103 self-assemble into 24-meric homopolymer and amenability via fusion peptides, ferritin is an
104 ideal candidate for drug delivery and vaccine development (41, 42). Most importantly, its
105 exceptional chemical and thermal stability does not require stringent temperature control,
106 enabling streamlined distribution process, especially in areas with limited resources for cold-
107 chain supplies (41, 43). One of the recently engineered ferritin for vaccine development is the
108 self-assembling *Helicobacter pylori*-bullfrog (*Rana Catesbeiana*) hybrid ferritin which carries
109 NH₂-terminal residues from the lower subunit of bullfrog ferritin on the core of *Helicobacter pylori*
110 ferritin to form radially projecting tails (38). The *H. pylori* ferritin-based nanoparticle has been
111 reported to be an effective platform for vaccines to carry trimeric glycoproteins for presenting
112 viral immunogens on its threefold axis points. Most importantly, it provides stronger protective
113 immunity at a lower dose than soluble immunogens against Influenza and Epstein-Barr viruses,
114 while minimizing the risk of autoimmunity through its genetic diversity from heavy and light
115 chains of human ferritin (38, 44, 45).

116 Despite recent efforts to develop mouse models that fully recapitulates human SARS-
117 CoV-2 infection, the current hACE2-transgenic mouse model fails to mimic pathogenic progress
118 and symptoms of COVID-19 in humans. Ferrets (*Mustela putorius furo*) on the other hand, are
119 naturally susceptible to human respiratory viruses—Respiratory Syncytial virus (48), Influenza
120 virus (49, 50), and SARS-CoV (51, 52)—making ferret models ideal to study respiratory virus

121 infections in humans. In addition, ferrets share anatomy of upper and lower respiratory tracts,
122 architecture of terminal bronchioles, and density of submucosal glands to those of humans (46,
123 47). Recently, we and others have shown that SARS-CoV-2-infected ferrets develop immune
124 responses and pathogenic progress similar to humans', and shed virus through nasal wash,
125 saliva, urine, and fecal samples, which highly recapitulate human SARS-CoV-2 infection (53-
126 56). Furthermore, we have also demonstrated the efficacy of the ferret model in drug discovery
127 for SARS-CoV-2 (57). Thus, ferrets represent an infection and transmission animal model of
128 SARS-CoV-2 that should facilitate the development of SARS-CoV-2 therapeutics and vaccines.

129 Here, we demonstrate the immunogenic efficacy of self-assembling spike RBD-ferritin
130 nanoparticle (RBD-nanoparticle) as an efficient SARS-CoV-2 vaccine antigen. We purified the
131 RBD-nanoparticle from transfected HEK293T cells and immunized ferrets via intramuscular (IM)
132 and intranasal (IN) routes to monitor the induction of neutralizing antibodies. Furthermore, we
133 challenged the vaccinated ferrets with SARS-CoV-2 and observed protective immunity against
134 SARS-CoV-2. Taken together, we propose the self-assembling RBD-nanoparticles as a
135 potential vaccine candidate that effectively protects against SARS-CoV-2 infection.

136

137

Results

138 **Purification and characterization of RBD-ferritin nanoparticles**

139 Kanekiyo et al. have discovered the use of engineered ferritin in vaccine developments
140 by fusion with viral immunogens (38, 44). Briefly, the NH₂-terminal tail from the lower subunit of
141 bullfrog ferritin was fused to *H. pylori* ferritin so that the bullfrog-originated tail and viral
142 immunogen were fused by the linker and presented on the threefold axis points of the *H. pylori*
143 ferritin core. The human codon-optimized RBD of SARS-CoV-2 Wuhan-Hu-1 strain
144 (NC_045512) was fused to the IL-2 signal peptide at the amino terminus and the *H. pylori*-
145 bullfrog ferritin at the carboxyl terminus to generate the RBD-ferritin fusion. A computer-assisted
146 modeling predicts the 3D structure of RBD-ferritin nanoparticles with RBD forming radial
147 projections on the threefold axis point of fully assembled nanoparticles (Fig. 1A). Ferritin and
148 RBD-ferritin fusion proteins were readily purified from the supernatants of transfected HEK293T
149 cells (Fig. 1B). To demonstrate the 24-mer self-assembly of ferritin nanoparticles, purified ferritin
150 and RBD-ferritin proteins were subjected to size exclusion chromatography with columns
151 designed to have a maximum resolution for proteins with kilodalton and megadalton ranges of
152 molecular weight. As a result, the purified ferritin nanoparticles and RBD-nanoparticles showed
153 peaks at approximately 408 kDa and 1350 kDa, respectively, corresponding to 24-mers of each
154 protein (Fig. 1C). These results indicate that RBD-ferritin protein is readily purified from
155 mammalian cells to homogeneity and efficiently assembles into 24-mer nanoparticles.

156

157 **Immunization with RBD-nanoparticle induces neutralizing antibody in ferrets**

158 To test the vaccine efficacy of purified RBD-nanoparticles, we immunized 16-20 months-
159 old ferrets (n=10/immunization route), which is equivalent to 30 years of age in humans. While
160 intramuscular (IM) immunization is the most widely used route for vaccine delivery, intranasal
161 (IN) immunization closely resembles infection with respiratory pathogens and efficiently
162 stimulates mucosal immunity (58). Ferrets were injected with 15µg RBD-nanoparticle via IM

163 route only or both IM and IN routes over 31 days with boosting immunizations at days 14 and 28
164 (Fig. 2A). Blood was drawn from each ferret prior to primary and boosting immunizations on
165 days 14 and 28. All ferrets vaccinated with RBD-nanoparticles produced strong neutralizing
166 antibodies after the second boosting immunization performed at day 28. Neutralization titer did
167 not show statistically significant difference between the routes of immunization (Fig. 2B). These
168 data indicate that RBD-nanoparticle immunization induces strong neutralizing antibody
169 regardless of the route of immunization.

170

171 **Immunization with RBD-nanoparticle promotes rapid viral clearance and protects ferrets** 172 **from SARS-CoV-2 challenge**

173 Immunized ferrets were challenged with $10^{5.0}$ TCID₅₀/mL of NMC2019-nCoV02 strain
174 SARS-CoV-2 three days after the last immunization at day 31 and monitored for clinical
175 symptoms resembling COVID-19. Ferrets with adjuvant only-immunization was included as
176 control group. Over a total of 10 days from the day of challenge infection, ferrets with adjuvant
177 only-immunization showed increase in body temperature and decrease in body weight (Fig. 3A).
178 On the contrary, ferrets immunized with RBD-nanoparticle did not show any change in either
179 body temperature or body weight (Fig. 3A and B). Minor body weight changes in ferrets
180 immunized by IM route showed a statistically insignificant difference compared to the adjuvant
181 only-immunized ferrets (Fig. 3B). On the other hand, ferrets immunized by IM and IN routes
182 provided stronger protection with high statistical significance against body weight loss as shown
183 by the minimal reduction of body weight followed by a constant increase thereafter (Fig. 3B).
184 Nasal wash samples were collected every other day for 10 days after the virus challenge, and 3
185 ferrets were sacrificed at 3 and 6 days post-infection (dpi) to harvest the lungs. Consistent with
186 the trend shown in body temperature and weight, immunized ferrets showed rapid viral
187 clearance in nasal washes (Fig. 3C) and lungs (Fig. 3D) of both groups of vaccinated ferrets. It

188 should be noted that IM and IN immunization showed slightly more effective viral clearance in
189 nasal washes at 4 dpi than IM immunization (Fig. 3C).

190 To further investigate the potency of protective immunity by RBD-nanoparticle, we
191 challenged the immunized ferrets with a higher titer ($10^{6.0}$ TCID₅₀/mL) of SARS-CoV-2 following
192 the same immunization protocol (Fig. 2A). Consistently, RBD-nanoparticles-immunized ferrets
193 showed no increase of body temperature compared to adjuvant only-immunized ferrets (Fig.
194 S1). While adjuvant only-immunized ferrets suffered from cough, runny nose, and reduction in
195 movement, RBD-nanoparticles-immunized ferrets showed only mild reduction in movement on
196 the 2nd and 3rd days after the high virus titer challenge (Table 1). On the other hand, IN and IM
197 immunization showed more potent protective immunity upon challenge with a high virus titer
198 than IN immunization only (Fig. S2). IN and IM immunization led to faster clearance of infectious
199 virus in nasal washes at 4 and 8 dpi than IM immunization alone (Fig. S2A). Infectious virus
200 titers of lungs were also lower in IN and IM-immunized ferrets than in IM-immunized ferrets (Fig.
201 S2B). These data demonstrate that RBD-nanoparticle induces strong protective immunity to
202 suppress SARS-CoV-2-induced clinical symptoms and promote viral clearance. Moreover, a
203 combination of IN and IM immunization induces stronger anti-viral immunity against challenge of
204 high titer SARS-CoV-2 than IM immunization alone.

205

206 **RBD-ferritin vaccination blocks lung damage from SARS-CoV-2 challenge**

207 COVID-19 has most commonly been shown to be associated with a spectrum of lung
208 damage. To compare lung histopathologies among immunized ferrets, RNAscope *in situ*
209 hybridization and histopathological examination were conducted (Fig. 4). Lung tissues
210 harvested from naïve ferrets were included as negative controls (Fig. 4D). RNAscope *in situ*
211 hybridization results showed that the adjuvant only-immunized ferrets had a number of SARS-
212 CoV-2 RNA-positive cells at 3 and 6 dpi with infiltration of numerous inflammatory immune cells
213 (Fig. 4A-E). At 3 dpi, IM- or IM and IN-immunized ferrets showed considerable reduction of viral

214 RNAs in the lungs compared to adjuvant only-immunized ferrets (Fig. 4). At 6 dpi, lung tissues
215 of IM- or IM and IN-immunized ferrets showed complete clearance of viral RNAs (Fig. 4F-G),
216 while adjuvant only-immunized ferrets still showed high viral RNAs (Fig. 4E). Finally, IM- or IM
217 and IN-immunized ferrets showed little or no infiltration of inflammatory immune cells in infected
218 lung (Fig. 4B-G). These data show that RBD-nanoparticle immunization accelerates viral
219 clearance in the lung and suppresses the infiltration of inflammatory immune cells.

220

Discussion

221 Since the first discovery in Wuhan, China in late 2019, SARS-CoV-2 has rapidly spread
222 around the world and was declared a pandemic in 3 months. Confirmed infection and death
223 counts have skyrocketed to over 88 million infections and 2 million deaths, and the statistics are
224 still on a continuous rise. Although 80% of the infections do not progress to severe COVID-19,
225 the recent surge in infections and severe patients have led to subsequent increase in mortality
226 rates (8, 9). While several vaccines were approved at accelerated rates (59, 60), additional
227 indepth study of mRNA-based vaccines regarding safety concerns and long-term effects still
228 need to be addressed as they are the first approved mRNA human vaccine of its kind.
229 Moreover, taking the growing evidence of reinfections into consideration, recovered patients
230 cannot be completely excluded from the population requiring vaccination (61-64). Therefore,
231 there still is a constant need for alternative vaccine approaches against SARS-CoV-2 using
232 relatively well-characterized approaches. Recent advances in nanotechnology has favorably
233 allowed the application of nanoparticles in the field of vaccinology to develop safer yet potent
234 vaccines. One of the most promising candidates is *H. pylori*-bullfrog ferritin that has been
235 genetically engineered to carry a protruding tail from the bullfrog on the self-assembling ferritin
236 core of *H. pylori*, and serves as a platform to build nanoparticles of immunogen. This approach
237 has proven higher efficacy in lower dose than traditional protein subunit vaccines. This
238 approach also highlights lower risk of vaccine-related adverse effects and potentially greater
239 accessibility to the public with reduced production cost (38, 44, 45). Importantly, inherent
240 stability of ferritin nanoparticles from heat and chemicals may shed light to remove the necessity
241 of strict cold-chain supply required for the currently distributing mRNA-based vaccines (41).

242 SARS-CoV-2 carries Spike protein to attach to the host receptor ACE2, which triggers
243 membrane fusion for entry into host cells. The RBD of Spike protein confers the specificity to
244 bind ACE2 and therefore is a promising target for vaccine development throughout the
245 *Coronaviridae* family. As also shown from previously developed vaccine candidates against

246 Coronaviruses (17, 65, 66), we selected the RBD as a vaccine antigen. However, soluble
247 antigen is weakly immunogenic and therefore require higher dose of antigen along with an
248 adjuvant, which correlates with higher risk of vaccine-related adverse effects (29). In this study,
249 we engineered the fusion of SARS-CoV-2 Spike RBD with *H. pylori*-bullfrog ferritin to develop
250 RBD-nanoparticle vaccine. Ferrets immunized with RBD-nanoparticles carried efficient
251 neutralizing antibodies against SARS-CoV-2 and were protected from fever and body weight
252 loss upon SARS-CoV-2 challenge. These clinical symptoms corresponded to the accelerated
253 viral clearance in nasal washes and lungs following SARS-CoV-2 challenge. We further
254 investigated the vaccine potential of RBD-nanoparticles by challenging the immunized ferrets
255 with a high virus titer ($10^{6.0}$ TCID₅₀/mL). Immunized ferrets showed considerably reduced clinical
256 symptoms, such as body weight loss, cough, runny nose, and movement activity, upon
257 challenge of high titer SARS-CoV-2. Moreover, RNAscope analyses showed rapid viral
258 clearance in the lungs of immunized ferrets compared to those of adjuvant only-immunized
259 ferrets. Histological analysis also showed little or no lung tissue damage and inflammatory
260 immune cell infiltration in immunized ferrets. As seen from other protein vaccines such as HPV
261 VLP that requires prime-boost regimens (67), the first immunization alone was not sufficient to
262 induce neutralizing antibodies. IN + IM immunization elicited more potent protective immunity
263 upon challenge of high titer SARS-CoV-2 than IM immunization alone, which is consistent with
264 previous reports showing stronger induction of mucosal immunity upon IN than IM immunization
265 to protect against respiratory pathogens such as MERS-CoV (65, 66, 68), Influenza virus (69),
266 and *Mycoplasma pneumoniae* (70). To differentiate vaccine efficacy between IN immunization
267 and IN and IM immunization, we repeated the viral challenge with high titer ($10^{6.0}$ TCID₅₀/mL)
268 and observed the improvement of viral clearance in lung and nasal washes from IN and IM-
269 immunized ferrets. However, as IN and IM immunization was employed together in this study,
270 further investigation is required to directly compare vaccine efficacy between IN immunization
271 and IM immunization against SARS-CoV-2 infection. Also, ferrets challenged with $10^{6.0}$

272 TCID₅₀/mL virus titer showed delayed viral clearance compared to ferrets challenged with 10^{5.0}
273 TCID₅₀/mL virus titer. However, 10^{5.0} TCID₅₀/mL is already excessive and not physiologically
274 relevant to real clinical setting.

275 In this study, we integrated SARS-CoV-2-derived immunogen into self-assembling
276 nanoparticle to develop an effective vaccine candidate against COVID-19. IM-immunized
277 animals showed strong induction of neutralizing antibody, rapid clearance of respiratory track
278 virus, and clear suppression of clinical symptoms, which is further enhanced in combination with
279 intranasal immunization. However, additional comprehensive studies are needed to understand
280 the humoral and cellular immunity elicited by RBD-nanoparticle administration and differential
281 activation of IgA-mediated mucosal immunity upon different immunization routes. Taken
282 together, our study indicated that immunization with self-assembling SARS-CoV-2 RBD-
283 nanoparticle elicits protective immunity against SARS-CoV-2 infection, showing its potential as a
284 vaccine candidate in the midst of the COVID-19 pandemic.

285

Material and Methods

286 Material and Reagents

REAGENT or RESOURCE	VENDOR	CATALOG NO.
Recombinant DNA		
pFUSEN-hIgG1Fc	Invivogen	pfcn-hg1
<i>H. pylori</i> -bullfrog recombinant ferritin	Jefferey Cohen and Gary Nabel at NIAID	
SARS-CoV-2 Spike codon-optimized to human codon usage	Genscript	MC_0101081
Chemicals		
Polyethylene imine	Polysciences	23966
Valproic acid	Sigma	P4543
AddaVax adjuvant	Invivogen	Vac-adx-10
RNAscope reagent	ACD	322360
RNAscope probe	ACD	848561
Gill's Hematoxylin #1	Polysciences	24242
Purification		
Labscale TFF system	Sigma	C1975
TFF filter – 100 kDa MWCO	Sigma	PXB100C50
TFF filter – 500 kDa MWCO	Sigma	PXB500C50
NGC Medium-pressure liquid chromatography system	Bio-rad	
BioFracFraction collector	Bio-rad	7410002
Superdex 200 Increase 10/300 GL	Cytiva	45-002-570
HiPrep 16/60 Sephacryl S-500 HR	Cytiva	28935606

287

288 Expression vector construction

289 The gene encoding the recombinant ferritin engineered from *Helicobacter pylori* non-
290 heme ferritin and 2nd to 9th residues of bullfrog (*Rana catesbeiana*) ferritin lower subunit was a
291 gift from Gary Nabel (44). Gene encoding Spike of SARS-CoV-2 (GenBank NC_0101080)
292 codon-optimized for human codon usage (GenBank MC_0101081) was purchased from
293 Genscript (pUC57-2019-nCoV-S). RBD was used to generate a fragment encoding RBD-
294 SSGGASVLA linker-recombinant ferritin. For expression plasmid, a commercially available
295 pFUSE vector (Invivogen) was engineered to replace human ferritin light chain gene promoter

296 with SV40 promoter. Genes encoding the recombinant ferritin and the RBD-linker-ferritin
297 fragment were cloned into the plasmid vector.

298

299 **Computer-assisted three-dimensional model of nanoparticles**

300 Previously solved structures of *H. pylori* ferritin nanoparticle (PDB: 3EGM) and SARS-
301 CoV-2 RBD (PDB: 7JMP) were processed with PyMol (Schrodinger) and Autodesk Meshmixer
302 (Autodesk). The model was generated to reflect the linker connecting the end of RBD to the
303 start of *H. pylori* ferritin monomer.

304

305 **Expression and purification of nanoparticles**

306 HEK293T cells were directly purchased from American Type Culture Collection (ATCC)
307 and maintained in DMEM medium (Gibco) supplemented with 10% FBS (Gibco) and 1%
308 penicillin/streptomycin (Gibco). The cells were transiently transfected with polyethylenimine
309 (Polysciences) and respective vector plasmids in Opti-MEM and FreeStyle 293 medium (Gibco)
310 supplemented with 3mM valproic acid. Supernatants containing the nanoparticle were harvested
311 72 h after transfection and concentrated with Labscale TFF system equipped with 100 kDa and
312 500 kDa MWCO filters (Millipore Sigma). The concentrates were purified by size exclusion
313 chromatography (NGC Medium-Pressure Liquid Chromatography, Bio-Rad) using Superdex
314 200 10/300 GL and HiPrep 16/60 Sephacryl S-500 HR (Cytiva) running degassed PBS at
315 0.4ml/min. Standard curves were plotted using Gel filtration LMW/HMW calibration Kit (Cytiva)
316 running at same conditions. Collected fractions were verified for their yield and purity via SDS-
317 PAGE and stored at -80°C in 10% glycerol (Invitrogen).

318

319 **Virus propagation**

320 NMC2019-nCoV02 strain of SARS-CoV-2 was isolated from a patient diagnosed with
321 COVID-19 and tested positive for SARS-CoV-2 in February, 2020 in South Korea. Vero cells

322 were used to propagate the virus in DMEM medium (Gibco) supplemented with 1%
323 penicillin/streptomycin (Gibco) at 37°C. The viruses were harvested 72 h later and stored at -
324 80°C until use.

325

326 **Animal Care**

327 Male and female ferrets of 16-20 months old and tested seronegative for Influenza A,
328 MERS-CoV, and SARS-CoV were purchased from ID Bio Corporation (Cheongju, Korea). The
329 ferrets were housed in ABSL3 facility within Chungbuk National University (Cheongju, Korea)
330 with 12 h light/dark cycle with access to water and diet. All animal cares were performed strictly
331 following the animal care guideline and experiment protocols approved by the Institutional
332 Animal Care and Use Committee (IACUC) in Chungbuk National University.

333

334 **Ferret immunizations and viral challenge**

335 RBD-ferritin nanoparticles (volume: 300ul) and AddaVax adjuvant (volume: 300ul) were
336 administered into the legs through intramuscular injection and/or intranasal route. Subsequently,
337 ferrets were intranasally infected with $10^{5.0}$ or $10^{6.0}$ TCID₅₀/mL SARS-CoV-2. Body weight and
338 temperature were measured, and veterinary clinical symptoms were observed every day. Blood
339 and nasal washes were collected every other day for 10 days. Three animals per group were
340 sacrificed at days 3 and 6 to collect lung tissues with individual scissors. Infectious viruses from
341 the nasal washes and lung tissues were quantified by inoculation onto Vero cells. Veterinary
342 symptoms were scored accordingly to our previous publication (57).

343

344 **Titration of neutralizing antibody in serum**

345 The neutralizing antibody assay against SARS-CoV-2 was carried out using a micro-
346 neutralization assay in Vero cells. Collected ferret serum specimens were inactivated at 56°C
347 for 30 min. Initial 1:2 serum dilutions were made with the medium, and two-fold serial dilutions of

348 all samples were made to a final serum dilution of 1:2 to 1:256. For each well, 50 μ L of serially
349 diluted serum was mixed with 50 μ L (equal volume) of 100 TCID₅₀ of SARS-CoV-2 and
350 incubated at 37°C for 1 h to neutralize the infectious virus. The mixtures were then transferred
351 to Vero cell monolayers. Vero cells were incubated at 37°C in 5% CO₂ for 4 days and monitored
352 for 50% reduction in cytopathic effect (CPE).

353

354 **RNAscope**

355 SARS-CoV-2 RNA (Spike gene) was detected using the Spike-specific probe (Advanced
356 Cell Diagnostics, Cat. # 848561) and visualized using RNAscope 2.5 HD Reagent Kit RED
357 (Advanced Cell Diagnostics, Cat. # 322360). Lung tissue sections were fixed in 4% neutral-
358 buffered formalin and embedded in paraffin, according to the manufacturer's instructions,
359 followed by counterstaining with 50% Gill's hematoxylin #1 (Polysciences, cat # 24242-1000).
360 Slides were viewed using Olympus IX 71 (Olympus, Tokyo, Japan) microscope with DP
361 controller software to capture images.

362

363 **Statistical Analysis**

364 All figure asterisks indicate statistical significance compared with adjuvant-only group as
365 evaluated by the two-way ANOVA Dunnett's multiple comparisons tests (* indicates $p < 0.05$, **
366 indicates $p < 0.01$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$) and were drawn using
367 GraphPad Prism 8 (GraphPad).

368

369

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376

377

Author Contributions

378 Y.I.K, D.K., Y.K.C. and J.U.J. conceived the study and designed the experiments. Y.I.K.
379 and D.K. performed the experiments. K.M.Y., H.S., S.A.L., M.A.C., S.G.J., S.K., W.J. and C.J.L.
380 helped with the experimental designs and data interpretation/analysis. Y.I.K. and D.K. took the
381 lead to prepare the manuscript with Y.K.C. and J.U.J.

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Competing Financial Interests

384 Dr. Jae U Jung is a scientific adviser of the Vaccine Stabilization Institute, a California
385 corporation.

386

References

- 387 1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P,
388 Zhan F, Ma X, Wang D, Xu W, Wu G, Gao GF, Tan W, China Novel Coronavirus I,
389 Research T. 2020. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N*
390 *Engl J Med* 382:727-733.
- 391 2. Coronaviridae Study Group of the International Committee on Taxonomy of V. 2020. The
392 species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV
393 and naming it SARS-CoV-2. *Nat Microbiol* 5:536-544.
- 394 3. WHO. 2020. Statement on the second meeting of the International Health Regulations
395 (2005) Emergency Committee regarding the outbreak of novel coronavirus (2019-nCoV).
396 [https://www.who.int/news/item/30-01-2020-statement-on-the-second-meeting-of-the-](https://www.who.int/news/item/30-01-2020-statement-on-the-second-meeting-of-the-international-health-regulations-(2005)-emergency-committee-regarding-the-outbreak-of-novel-coronavirus-(2019-ncov))
397 [international-health-regulations-\(2005\)-emergency-committee-regarding-the-outbreak-of-](https://www.who.int/news/item/30-01-2020-statement-on-the-second-meeting-of-the-international-health-regulations-(2005)-emergency-committee-regarding-the-outbreak-of-novel-coronavirus-(2019-ncov))
398 [novel-coronavirus-\(2019-ncov\)](https://www.who.int/news/item/30-01-2020-statement-on-the-second-meeting-of-the-international-health-regulations-(2005)-emergency-committee-regarding-the-outbreak-of-novel-coronavirus-(2019-ncov)). Accessed Jan 11, 2021.
- 399 4. WHO. 2020. Coronavirus disease 2019 (COVID-19) situation report-63.
400 [https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200323-sitrep-](https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200323-sitrep-63-covid-19.pdf?sfvrsn=b617302d_4)
401 [63-covid-19.pdf?sfvrsn=b617302d_4](https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200323-sitrep-63-covid-19.pdf?sfvrsn=b617302d_4). Accessed Jan 11, 2021.
- 402 5. GISAIID. Coronavirus COVID-19 Global Cases by Johns Hopkins CSSE.
403 <https://www.gisaid.org/epiflu-applications/global-cases-covid-19>. Accessed Jan 11, 2021.
- 404 6. Wu Z, McGoogan JM. 2020. Characteristics of and Important Lessons From the
405 Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of
406 72314 Cases From the Chinese Center for Disease Control and Prevention. *JAMA*
407 323:1239-1242.
- 408 7. NIH. 2020. COVID-19 Treatment Guidelines.
409 <https://www.covid19treatmentguidelines.nih.gov/>. Accessed Jan 11, 2021.
- 410 8. Neil A. Harpen KST. 2020. United States Resource Availability for COVID-19.
- 411 9. Ji Y, Ma Z, Peppelenbosch MP, Pan Q. 2020. Potential association between COVID-19
412 mortality and health-care resource availability. *The Lancet Global Health* 8.
- 413 10. Yelin D, Wirtheim E, Vetter P, Kalil AC, Bruchfeld J, Runold M, Guaraldi G, Mussini C,
414 Gudiol C, Pujol M, Bandera A, Scudeller L, Paul M, Kaiser L, Leibovici L. 2020. Long-
415 term consequences of COVID-19: research needs. *Lancet Infect Dis* 20:1115-1117.
- 416 11. Del Rio C, Collins LF, Malani P. 2020. Long-term Health Consequences of COVID-19.
417 *JAMA* doi:10.1001/jama.2020.19719.
- 418 12. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, Hu Y, Tao ZW, Tian JH, Pei YY,
419 Yuan ML, Zhang YL, Dai FH, Liu Y, Wang QM, Zheng JJ, Xu L, Holmes EC, Zhang YZ.
420 2020. A new coronavirus associated with human respiratory disease in China. *Nature*
421 579:265-269.
- 422 13. Chan JF, Kok KH, Zhu Z, Chu H, To KK, Yuan S, Yuen KY. 2020. Genomic
423 characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient
424 with atypical pneumonia after visiting Wuhan. *Emerg Microbes Infect* 9:221-236.
- 425 14. Cai Y, Zhang J, Xiao T, Peng H, Sterling SM, Walsh RM, Jr., Rawson S, Rits-Volloch S,
426 Chen B. 2020. Distinct conformational states of SARS-CoV-2 spike protein. *Science*
427 369:1586-1592.
- 428 15. Kim YS, Son A, Kim J, Kwon SB, Kim MH, Kim P, Kim J, Byun YH, Sung J, Lee J, Yu JE,
429 Park C, Kim YS, Cho NH, Chang J, Seong BL. 2018. Chaperna-Mediated Assembly of
430 Ferritin-Based Middle East Respiratory Syndrome-Coronavirus Nanoparticles. *Front*
431 *Immunol* 9:1093.
- 432 16. He Y, Zhou Y, Liu S, Kou Z, Li W, Farzan M, Jiang S. 2004. Receptor-binding domain of
433 SARS-CoV spike protein induces highly potent neutralizing antibodies: implication for
434 developing subunit vaccine. *Biochem Biophys Res Commun* 324:773-81.

- 435 17. Jiang S, Lu L, Liu Q, Xu W, Du L. 2012. Receptor-binding domains of spike proteins of
436 emerging or re-emerging viruses as targets for development of antiviral vaccines. *Emerg*
437 *Microbes Infect* 1:e13.
- 438 18. Pallesen J, Wang N, Corbett KS, Wrapp D, Kirchdoerfer RN, Turner HL, Cottrell CA,
439 Becker MM, Wang L, Shi W, Kong WP, Andres EL, Kettenbach AN, Denison MR,
440 Chappell JD, Graham BS, Ward AB, McLellan JS. 2017. Immunogenicity and structures
441 of a rationally designed prefusion MERS-CoV spike antigen. *Proc Natl Acad Sci U S A*
442 114:E7348-E7357.
- 443 19. Okba NMA, Muller MA, Li W, Wang C, GeurtsvanKessel CH, Corman VM, Lamers MM,
444 Sikkema RS, de Bruin E, Chandler FD, Yazdanpanah Y, Le Hingrat Q, Descamps D,
445 Houhou-Fidouh N, Reusken C, Bosch BJ, Drosten C, Koopmans MPG, Haagmans BL.
446 2020. Severe Acute Respiratory Syndrome Coronavirus 2-Specific Antibody Responses
447 in Coronavirus Disease Patients. *Emerg Infect Dis* 26:1478-1488.
- 448 20. Liu L, Wang P, Nair MS, Yu J, Rapp M, Wang Q, Luo Y, Chan JF, Sahi V, Figueroa A,
449 Guo XV, Cerutti G, Bimela J, Gorman J, Zhou T, Chen Z, Yuen KY, Kwong PD, Sodroski
450 JG, Yin MT, Sheng Z, Huang Y, Shapiro L, Ho DD. 2020. Potent neutralizing antibodies
451 against multiple epitopes on SARS-CoV-2 spike. *Nature* 584:450-456.
- 452 21. Liu B, Shi Y, Zhang W, Li R, He Z, Yang X, Pan Y, Deng X, Tan M, Zhao L, Zou F,
453 Zhang Y, Pan T, Zhang J, Zhang X, Xiao F, Li F, Deng K, Zhang H. 2020. Recovered
454 COVID-19 patients with recurrent viral RNA exhibit lower levels of anti-RBD antibodies.
455 *Cell Mol Immunol* 17:1098-1100.
- 456 22. Piccoli L, Park YJ, Tortorici MA, Czudnochowski N, Walls AC, Beltramello M, Silacci-
457 Fregni C, Pinto D, Rosen LE, Bowen JE, Acton OJ, Jaconi S, Guarino B, Minola A, Zatta
458 F, Sprugasci N, Bassi J, Peter A, De Marco A, Nix JC, Mele F, Jovic S, Rodriguez BF,
459 Gupta SV, Jin F, Piumatti G, Lo Presti G, Pellanda AF, Biggiogero M, Tarkowski M,
460 Pizzuto MS, Cameroni E, Havenar-Daughton C, Smithy M, Hong D, Lepori V, Albanese
461 E, Ceschi A, Bernasconi E, Elzi L, Ferrari P, Garzoni C, Riva A, Snell G, Sallusto F, Fink
462 K, Virgin HW, Lanzavecchia A, Corti D, Veesler D. 2020. Mapping Neutralizing and
463 Immunodominant Sites on the SARS-CoV-2 Spike Receptor-Binding Domain by
464 Structure-Guided High-Resolution Serology. *Cell* 183:1024-1042 e21.
- 465 23. Wu NC, Yuan M, Liu H, Lee CD, Zhu X, Bangaru S, Torres JL, Caniels TG, Brouwer
466 PJM, van Gils MJ, Sanders RW, Ward AB, Wilson IA. 2020. An Alternative Binding
467 Mode of IGHV3-53 Antibodies to the SARS-CoV-2 Receptor Binding Domain. *Cell Rep*
468 33:108274.
- 469 24. Walsh EE, Frenck RW, Jr., Falsey AR, Kitchin N, Absalon J, Gurtman A, Lockhart S,
470 Neuzil K, Mulligan MJ, Bailey R, Swanson KA, Li P, Koury K, Kalina W, Cooper D,
471 Fontes-Garfias C, Shi PY, Tureci O, Tompkins KR, Lyke KE, Raabe V, Dormitzer PR,
472 Jansen KU, Sahin U, Gruber WC. 2020. Safety and Immunogenicity of Two RNA-Based
473 Covid-19 Vaccine Candidates. *N Engl J Med* 383:2439-2450.
- 474 25. Baden LR, El Sahly HM, Essink B, Kotloff K, Frey S, Novak R, Diemert D, Spector SA,
475 Roupael N, Creech CB, McGettigan J, Kehtan S, Segall N, Solis J, Brosz A, Fierro C,
476 Schwartz H, Neuzil K, Corey L, Gilbert P, Janes H, Follmann D, Marovich M, Mascola J,
477 Polakowski L, Ledgerwood J, Graham BS, Bennett H, Pajon R, Knightly C, Leav B,
478 Deng W, Zhou H, Han S, Ivarsson M, Miller J, Zaks T, Group CS. 2020. Efficacy and
479 Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N Engl J Med*
480 doi:10.1056/NEJMoa2035389.
- 481 26. Sadoff J, De Paepe E, Haazen W, Omoruyi E, Bastian AR, Comeaux C, Heijnen E,
482 Strout C, Schuitemaker H, Callendret B. 2020. Safety and Immunogenicity of the
483 Ad26.RSV.preF Investigational Vaccine Coadministered With an Influenza Vaccine in
484 Older Adults. *J Infect Dis* doi:10.1093/infdis/jiaa409.

- 485 27. Keech C, Albert G, Cho I, Robertson A, Reed P, Neal S, Plested JS, Zhu M, Cloney-
486 Clark S, Zhou H, Smith G, Patel N, Frieman MB, Haupt RE, Logue J, McGrath M,
487 Weston S, Piedra PA, Desai C, Callahan K, Lewis M, Price-Abbott P, Formica N, Shinde
488 V, Fries L, Lickliter JD, Griffin P, Wilkinson B, Glenn GM. 2020. Phase 1-2 Trial of a
489 SARS-CoV-2 Recombinant Spike Protein Nanoparticle Vaccine. *N Engl J Med*
490 383:2320-2332.
- 491 28. Cohen AA, Gnanapragasam PNP, Lee YE, Hoffman PR, Ou S, Kakutani LM, Keeffe JR,
492 Wu HJ, Howarth M, West AP, Barnes CO, Nussenzweig MC, Bjorkman PJ. 2021.
493 Mosaic nanoparticles elicit cross-reactive immune responses to zoonotic coronaviruses
494 in mice. *Science* doi:10.1126/science.abf6840.
- 495 29. Gause KT, Wheatley AK, Cui J, Yan Y, Kent SJ, Caruso F. 2017. Immunological
496 Principles Guiding the Rational Design of Particles for Vaccine Delivery. *ACS Nano*
497 11:54-68.
- 498 30. Link A, Zabel F, Schnetzler Y, Titz A, Brombacher F, Bachmann MF. 2012. Innate
499 immunity mediates follicular transport of particulate but not soluble protein antigen. *J*
500 *Immunol* 188:3724-33.
- 501 31. António Roldão MCMM, Leda R Castilho, Manuel J T Carronado, Paula M Alves. 2014.
502 Virus-like particles in vaccine development. *Expert Rev Vaccines* 9:1149-76.
- 503 32. Qian C, Liu X, Xu Q, Wang Z, Chen J, Li T, Zheng Q, Yu H, Gu Y, Li S, Xia N. 2020.
504 Recent Progress on the Versatility of Virus-Like Particles. *Vaccines (Basel)* 8.
- 505 33. Braedon Donaldson ZL, Greg F Walker, Sarah L young, Vernon K Ward. 2018. Virus-like
506 particle vaccines: immunology and formulation for clinical translation. *Expert Rev*
507 *Vaccines* 17:833-849.
- 508 34. Ingale J, Stano A, Guenaga J, Sharma SK, Nemazee D, Zwick MB, Wyatt RT. 2016.
509 High-Density Array of Well-Ordered HIV-1 Spikes on Synthetic Liposomal Nanoparticles
510 Efficiently Activate B Cells. *Cell Rep* 15:1986-99.
- 511 35. Thompson EA, Ols S, Miura K, Rausch K, Narum DL, Spangberg M, Juraska M, Wille-
512 Reece U, Weiner A, Howard RF, Long CA, Duffy PE, Johnston L, O'Neil CP, Lore K.
513 2018. TLR-adjuvanted nanoparticle vaccines differentially influence the quality and
514 longevity of responses to malaria antigen Pfs25. *JCI Insight* 3.
- 515 36. Marcandalli J, Fiala B, Ols S, Perotti M, de van der Schueren W, Snijder J, Hodge E,
516 Benhaim M, Ravichandran R, Carter L, Sheffler W, Brunner L, Lawrenz M, Dubois P,
517 Lanzavecchia A, Sallusto F, Lee KK, Velesler D, Correnti CE, Stewart LJ, Baker D, Lore
518 K, Perez L, King NP. 2019. Induction of Potent Neutralizing Antibody Responses by a
519 Designed Protein Nanoparticle Vaccine for Respiratory Syncytial Virus. *Cell* 176:1420-
520 1431 e17.
- 521 37. Yu F, Wang J, Dou J, Yang H, He X, Xu W, Zhang Y, Hu K, Gu N. 2012. Nanoparticle-
522 based adjuvant for enhanced protective efficacy of DNA vaccine Ag85A-ESAT-6-IL-21
523 against *Mycobacterium tuberculosis* infection. *Nanomedicine* 8:1337-44.
- 524 38. Kanekiyo M, Wei CJ, Yassine HM, McTamney PM, Boyington JC, Whittle JR, Rao SS,
525 Kong WP, Wang L, Nabel GJ. 2013. Self-assembling influenza nanoparticle vaccines
526 elicit broadly neutralizing H1N1 antibodies. *Nature* 499:102-6.
- 527 39. Hu X, Deng Y, Chen X, Zhou Y, Zhang H, Wu H, Yang S, Chen F, Zhou Z, Wang M, Qiu
528 Z, Liao Y. 2017. Immune Response of A Novel ATR-AP205-001 Conjugate Anti-
529 hypertensive Vaccine. *Sci Rep* 7:12580.
- 530 40. Pardi N, Hogan MJ, Naradikian MS, Parkhouse K, Cain DW, Jones L, Moody MA,
531 Verkerke HP, Myles A, Willis E, LaBranche CC, Montefiori DC, Lobby JL, Saunders KO,
532 Liao HX, Korber BT, Sutherland LL, Scearce RM, Hraber PT, Tombacz I, Muramatsu H,
533 Ni H, Balikov DA, Li C, Mui BL, Tam YK, Krammer F, Kariko K, Polacino P, Eisenlohr LC,
534 Madden TD, Hope MJ, Lewis MG, Lee KK, Hu SL, Hensley SE, Cancro MP, Haynes BF,

- 535 Weissman D. 2018. Nucleoside-modified mRNA vaccines induce potent T follicular
536 helper and germinal center B cell responses. *J Exp Med* 215:1571-1588.
- 537 41. He D, Marles-Wright J. 2015. Ferritin family proteins and their use in bionanotechnology.
538 *N Biotechnol* 32:651-7.
- 539 42. Cho KJ, Shin HJ, Lee JH, Kim KJ, Park SS, Lee Y, Lee C, Park SS, Kim KH. 2009. The
540 crystal structure of ferritin from *Helicobacter pylori* reveals unusual conformational
541 changes for iron uptake. *J Mol Biol* 390:83-98.
- 542 43. Brito C, Matias C, Gonzalez-Nilo FD, Watt RK, Yevenes A. 2014. The C-terminal regions
543 have an important role in the activity of the ferroxidase center and the stability of
544 *Chlorobium tepidum* ferritin. *Protein J* 33:211-20.
- 545 44. Kanekiyo M, Bu W, Joyce MG, Meng G, Whittle JR, Baxa U, Yamamoto T, Narpala S,
546 Todd JP, Rao SS, McDermott AB, Koup RA, Rossmann MG, Mascola JR, Graham BS,
547 Cohen JI, Nabel GJ. 2015. Rational Design of an Epstein-Barr Virus Vaccine Targeting
548 the Receptor-Binding Site. *Cell* 162:1090-100.
- 549 45. Kelly HG, Tan HX, Juno JA, Esterbauer R, Ju Y, Jiang W, Wimmer VC, Duckworth BC,
550 Groom JR, Caruso F, Kanekiyo M, Kent SJ, Wheatley AK. 2020. Self-assembling
551 influenza nanoparticle vaccines drive extended germinal center activity and memory B
552 cell maturation. *JCI Insight* 5.
- 553 46. Enkirch T, von Messling V. 2015. Ferret models of viral pathogenesis. *Virology* 479-
554 480:259-70.
- 555 47. Johnson-Delaney CA, Orosz SE. 2011. Ferret respiratory system: clinical anatomy,
556 physiology, and disease. *Vet Clin North Am Exot Anim Pract* 14:357-67, vii.
- 557 48. Chan KF, Carolan LA, Druce J, Chappell K, Watterson D, Young P, Korenkov D,
558 Subbarao K, Barr IG, Laurie KL, Reading PC. 2018. Pathogenesis, Humoral Immune
559 Responses, and Transmission between Cohoused Animals in a Ferret Model of Human
560 Respiratory Syncytial Virus Infection. *J Virol* 92.
- 561 49. Oh DY, Lowther S, McCaw JM, Sullivan SG, Leang SK, Haining J, Arkinstall R, Kelso A,
562 McVernon J, Barr IG, Middleton D, Hurt AC. 2014. Evaluation of oseltamivir prophylaxis
563 regimens for reducing influenza virus infection, transmission and disease severity in a
564 ferret model of household contact. *J Antimicrob Chemother* 69:2458-69.
- 565 50. Chan KF, Carolan LA, Korenkov D, Druce J, McCaw J, Reading PC, Barr IG, Laurie KL.
566 2018. Investigating Viral Interference Between Influenza A Virus and Human Respiratory
567 Syncytial Virus in a Ferret Model of Infection. *J Infect Dis* 218:406-417.
- 568 51. Cameron MJ, Kelvin AA, Leon AJ, Cameron CM, Ran L, Xu L, Chu YK, Danesh A, Fang
569 Y, Li Q, Anderson A, Couch RC, Paquette SG, Fomukong NG, Kistner O, Lauchart M,
570 Rowe T, Harrod KS, Jonsson CB, Kelvin DJ. 2012. Lack of innate interferon responses
571 during SARS coronavirus infection in a vaccination and reinfection ferret model. *PLoS*
572 *One* 7:e45842.
- 573 52. Chu YK, Ali GD, Jia F, Li Q, Kelvin D, Couch RC, Harrod KS, Hutt JA, Cameron C,
574 Weiss SR, Jonsson CB. 2008. The SARS-CoV ferret model in an infection-challenge
575 study. *Virology* 374:151-63.
- 576 53. Schlottau K, Rissmann M, Graaf A, Schon J, Sehl J, Wylezich C, Hoper D, Mettenleiter
577 TC, Balkema-Buschmann A, Harder T, Grund C, Hoffmann D, Breithaupt A, Beer M.
578 2020. SARS-CoV-2 in fruit bats, ferrets, pigs, and chickens: an experimental
579 transmission study. *Lancet Microbe* 1:e218-e225.
- 580 54. Shi J, Wen Z, Zhong G, Yang H, Wang C, Huang B, Liu R, He X, Shuai L, Sun Z, Zhao
581 Y, Liu P, Liang L, Cui P, Wang J, Zhang X, Guan Y, Tan W, Wu G, Chen H, Bu Z. 2020.
582 Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-
583 coronavirus 2. *Science* 368:1016-1020.
- 584 55. Kim YI, Kim SG, Kim SM, Kim EH, Park SJ, Yu KM, Chang JH, Kim EJ, Lee S, Casel
585 MAB, Um J, Song MS, Jeong HW, Lai VD, Kim Y, Chin BS, Park JS, Chung KH, Foo SS,

- 586 Poo H, Mo IP, Lee OJ, Webby RJ, Jung JU, Choi YK. 2020. Infection and Rapid
587 Transmission of SARS-CoV-2 in Ferrets. *Cell Host Microbe* 27:704-709 e2.
- 588 56. Ryan KA, Bewley KR, Fotheringham SA, Slack GS, Brown P, Hall Y, Wand NI, Marriott
589 AC, Cavell BE, Tree JA, Allen L, Aram MJ, Bean TJ, Brunt E, Buttigieg KR, Carter DP,
590 Cobb R, Coombes NS, Findlay-Wilson SJ, Godwin KJ, Gooch KE, Gouriet J, Halkerston
591 R, Harris DJ, Hender TH, Humphries HE, Hunter L, Ho CMK, Kennard CL, Leung S,
592 Longet S, Ngabo D, Osman KL, Paterson J, Penn EJ, Pullan ST, Rayner E, Skinner O,
593 Steeds K, Taylor I, Tipton T, Thomas S, Turner C, Watson RJ, Wiblin NR, Charlton S,
594 Hallis B, Hiscox JA, Funnell S, Dennis MJ, et al. 2021. Dose-dependent response to
595 infection with SARS-CoV-2 in the ferret model and evidence of protective immunity. *Nat*
596 *Commun* 12:81.
- 597 57. Park SJ, Yu KM, Kim YI, Kim SM, Kim EH, Kim SG, Kim EJ, Casel MAB, Rollon R, Jang
598 SG, Lee MH, Chang JH, Song MS, Jeong HW, Choi Y, Chen W, Shin WJ, Jung JU, Choi
599 YK. 2020. Antiviral Efficacies of FDA-Approved Drugs against SARS-CoV-2 Infection in
600 Ferrets. *mBio* 11.
- 601 58. Holmgren J, Czerkinsky C. 2005. Mucosal immunity and vaccines. *Nat Med* 11:S45-53.
- 602 59. FDA. 2020. Pfizer-BioNTech COVID-19 vaccine (BNT162, PF-07302048) Vaccines and
603 related biological products advisory committee briefing document. FDA,
- 604 60. FDA. 2020. MRNA-1273 Vaccines and related biological products advisory committee.
605 FDA,
- 606 61. Tillett RL, Sevinsky JR, Hartley PD, Kerwin H, Crawford N, Gorzalski A, Laverdure C,
607 Verma SC, Rossetto CC, Jackson D, Farrell MJ, Van Hooser S, Pandori M. 2021.
608 Genomic evidence for reinfection with SARS-CoV-2: a case study. *Lancet Infect Dis*
609 21:52-58.
- 610 62. To KK, Hung IF, Ip JD, Chu AW, Chan WM, Tam AR, Fong CH, Yuan S, Tsoi HW, Ng
611 AC, Lee LL, Wan P, Tso E, To WK, Tsang D, Chan KH, Huang JD, Kok KH, Cheng VC,
612 Yuen KY. 2020. COVID-19 re-infection by a phylogenetically distinct SARS-coronavirus-
613 2 strain confirmed by whole genome sequencing. *Clin Infect Dis*
614 doi:10.1093/cid/ciaa1275.
- 615 63. Gupta V, Bhoyar RC, Jain A, Srivastava S, Upadhyay R, Imran M, Jolly B, Divakar MK,
616 Sharma D, Sehgal P, Ranjan G, Gupta R, Scaria V, Sivasubbu S. 2020. Asymptomatic
617 reinfection in two healthcare workers from India with genetically distinct SARS-CoV-2.
618 *Clin Infect Dis* doi:10.1093/cid/ciaa1451.
- 619 64. Volz E, Hill V, McCrone JT, Price A, Jorgensen D, O'Toole A, Southgate J, Johnson R,
620 Jackson B, Nascimento FF, Rey SM, Nicholls SM, Colquhoun RM, da Silva Filipe A,
621 Shepherd J, Pascall DJ, Shah R, Jesudason N, Li K, Jarrett R, Pacchiarini N, Bull M,
622 Geidelberg L, Siveroni I, Consortium C-U, Goodfellow I, Loman NJ, Pybus OG,
623 Robertson DL, Thomson EC, Rambaut A, Connor TR. 2021. Evaluating the Effects of
624 SARS-CoV-2 Spike Mutation D614G on Transmissibility and Pathogenicity. *Cell* 184:64-
625 75 e11.
- 626 65. Kim MH, Kim HJ, Chang J. 2019. Superior immune responses induced by intranasal
627 immunization with recombinant adenovirus-based vaccine expressing full-length Spike
628 protein of Middle East respiratory syndrome coronavirus. *PLoS One* 14:e0220196.
- 629 66. Ma C, Li Y, Wang L, Zhao G, Tao X, Tseng CT, Zhou Y, Du L, Jiang S. 2014. Intranasal
630 vaccination with recombinant receptor-binding domain of MERS-CoV spike protein
631 induces much stronger local mucosal immune responses than subcutaneous
632 immunization: Implication for designing novel mucosal MERS vaccines. *Vaccine*
633 32:2100-8.
- 634 67. Sankaranarayanan R, Prabhu PR, Pawlita M, Gheit T, Bhatla N, Muwonge R, Nene BM,
635 Esmy PO, Joshi S, Poli UR, Jivarajani P, Verma Y, Zomawia E, Siddiqi M, Shastri SS,
636 Jayant K, Malvi SG, Lucas E, Michel A, Butt J, Vijayamma JM, Sankaran S, Kannan TP,

- 637 Varghese R, Divate U, Thomas S, Joshi G, Willhauck-Fleckenstein M, Waterboer T,
638 Muller M, Sehr P, Hingmire S, Kriplani A, Mishra G, Pimple S, Jadhav R, Sauvaget C,
639 Tommasino M, Pillai MR, Indian HPV VSG. 2016. Immunogenicity and HPV infection
640 after one, two, and three doses of quadrivalent HPV vaccine in girls in India: a
641 multicentre prospective cohort study. *Lancet Oncol* 17:67-77.
- 642 68. Baz M, Samant M, Zekki H, Tribout-Jover P, Plante M, Lanteigne AM, Hamelin ME,
643 Mallett C, Papadopoulou B, Boivin G. 2012. Effects of different adjuvants in the context
644 of intramuscular and intranasal routes on humoral and cellular immune responses
645 induced by detergent-split A/H3N2 influenza vaccines in mice. *Clin Vaccine Immunol*
646 19:209-18.
- 647 69. Muszkat M, Greenbaum E, Ben-Yehuda A, Oster M, Yeu'l E, Heimann S, Levy R,
648 Friedman G, Zakay-Rones Z. 2003. Local and systemic immune response in nursing-
649 home elderly following intranasal or intramuscular immunization with inactivated
650 influenza vaccine. *Vaccine* 21:1180-6.
- 651 70. Zhu C, Wu Y, Chen S, Yu M, Zeng Y, You X, Xiao J, Wang S. 2012. Protective immune
652 responses in mice induced by intramuscular and intranasal immunization with a
653 *Mycoplasma pneumoniae* P1C DNA vaccine. *Can J Microbiol* 58:644-52.
- 654

655 **Table 1. RBD-nanoparticle immunization suppresses clinical symptoms induced by**
656 **challenge with high SARS-CoV-2 titer.**

Group (n = 4/group)	Clinical	0 dpi	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	10 dpi
Adjuvant only	Cough	0	0	0.5	1	1	0	0	0	0	0
	Runny nose	0	0	1.0	1	1	1	1	0.75	0	0
	Movement, activity	0	0	1.25	2	2	1.25	0.75	0.5	0	0
	Total	0	0	2.75	4	4	2.25	1.75	1.25	0	0
RBD- nanoparticle	Cough	0	0	0	0	0	0	0	0	0	0
	Runny nose	0	0	0	0	0	0	0	0	0	0
	Movement, activity	0	0	0.75	0.5	0	0	0	0	0	0
	Total	0	0	0.75	0.5	0	0	0	0	0	0

657

658

659 **A group of adjuvant-immunized or RBD-nanoparticle IM immunized ferrets were**
660 **challenged with $10^{6.0}$ TCID₅₀/mL of SARS-CoV-2 and observed for their clinical symptoms**
661 **– cough, runny nose, movement, and activity. The symptoms were quantified as counts**
662 **per 30 minutes.**

663

664

Figure Legends

665 **Fig 1. Design and purification of RBD-nanoparticle**

666 A. Computer-assisted modeling of RBD-nanoparticle based on previously solved structures of
667 *H. pylori* ferritin (PDB: 3EGM) and SARS-CoV-2 RBD (PDB: 7JMP). RBD forms radial
668 projections on threefold axis point of fully assembled nanoparticle.

669 B. Coomassie staining of purified ferritin-nanoparticle and RBD-nanoparticle following SDS-
670 PAGE.

671 C. Size exclusion chromatography peaks of the concentrated supernatants from HEK293T
672 transfected with plasmids encoding secreted ferritin-nanoparticle or RBD-nanoparticles. The
673 supernatants were concentrated with 100 kDa MWCO and 500 kDa MWCO filters on TFF
674 system and loaded to Superdex 200 Increase 10/300 GL and HiPrep 16/60 Sephacryl S-500 HR
675 gel filtration columns on Bio-rad NGC chromatography system, respectively.

676

677 **Fig 2. Immunization with RBD-nanoparticle elicits neutralizing antibody formation**

678 A. Immunization schedule of ferrets. At day 31, ferrets were challenged with $10^{5.0}$ TCID₅₀/mL of
679 SARS-CoV-2 and observed for clinical symptoms for the following 10 days. One group was
680 immunized with only PBS and adjuvant (only adjuvant-immunized), and two other groups were
681 immunized with 15µg RBD-nanoparticle in adjuvant with 1:1 ratio for total volume of 600µl.

682 B. Serum neutralization titer of adjuvant-immunized, RBD-nanoparticle IM immunized, or RBD-
683 nanoparticle IM and IN immunized ferrets. Neutralizing antibody titers against SARS-CoV-2
684 NMC2019-nCoV02 (100 TCID₅₀) of ferritin-nanoparticle immunized groups were measured in
685 Vero cells with serially diluted ferret sera collected before immunizations at days 0, 14 and 28.

686

687 **Fig 3. Immunization with RBD-nanoparticle promotes rapid viral clearance and** 688 **protects ferrets from SARS-CoV-2 challenge**

689 **A. Body temperature change of adjuvant-immunized, RBD-nanoparticle IM immunized, or**
690 **RBD-nanoparticle IM and IN immunized ferrets upon SARS-CoV-2 challenge.**

691 **B. Body weight change of adjuvant-immunized, RBD-nanoparticle IM immunized, or RBD-**
692 **nanoparticle IM and IN immunized ferrets upon SARS-CoV-2 challenge.**

693 **C. Viral titer in the nasal washes of adjuvant-immunized, RBD-nanoparticle IM immunized,**
694 **or RBD-nanoparticle IM and IN immunized ferrets upon SARS-CoV-2 challenge.**

695 D. Viral titer in the lung tissue homogenates of adjuvant-immunized, RBD-nanoparticle IM
696 immunized, or RBD-nanoparticle IM and IN immunized ferrets upon SARS-CoV-2 challenge.

697

698 **Fig 4. Lung histology and RNAscope of immunized ferrets upon SARS-CoV-2 challenge**

699 Adjuvant-immunized, RBD-nanoparticle IM immunized, or RBD-nanoparticle IM and IN
700 immunized ferrets were intranasally inoculated with $10^{5.0}$ TCID₅₀/mL of SARS-CoV-2. Tissues
701 were harvested on 3 and 6 dpi. RNAscope detected SARS-CoV-2 Spike RNA-positive cells in
702 lung tissues of adjuvant-immunized (A and E), RBD-nanoparticle IM immunized (B and F),
703 and RBD-nanoparticle IM and IN immunized ferrets (C and G). Mock infected ferret lung (D)
704 was included as control. Magnification is x100 and scale bars represents 100 μ m. Insert
705 indicates the magnification (x400) of SARS-CoV-2-positive image and scale bar represents 20
706 μ m. Black arrow indicates SARS-CoV-2 RNA-positive cells.

707

708

709 **Fig S1. Body temperature of RBD-nanoparticle immunized ferrets against challenge with**
710 **high titer SARS-CoV-2**

711 **Body temperature change of adjuvant-immunized, RBD-nanoparticle IM immunized, or**
712 **RBD-nanoparticle IM and IN immunized ferrets upon high titer SARS-CoV-2 challenge.**

713

714 **Fig S2. Respiratory virus titer of RBD-nanoparticle immunized ferrets against challenge**
715 **with high titer SARS-CoV-2**

716 A. Viral titer in nasal washes of adjuvant-immunized, RBD-nanoparticle IM immunized, or RBD-
717 nanoparticle IM and IN immunized ferrets upon high titer SARS-CoV-2 challenge.

718 B. Viral titer in lungs of adjuvant-immunized, RBD-nanoparticle IM immunized, or RBD-
719 nanoparticle IM and IN immunized ferrets upon high titer SARS-CoV-2 challenge. Infectious

720 virus titers were measured and shown as mean \pm SEM.

Figure 1. Design and purification of RBD-nanoparticle

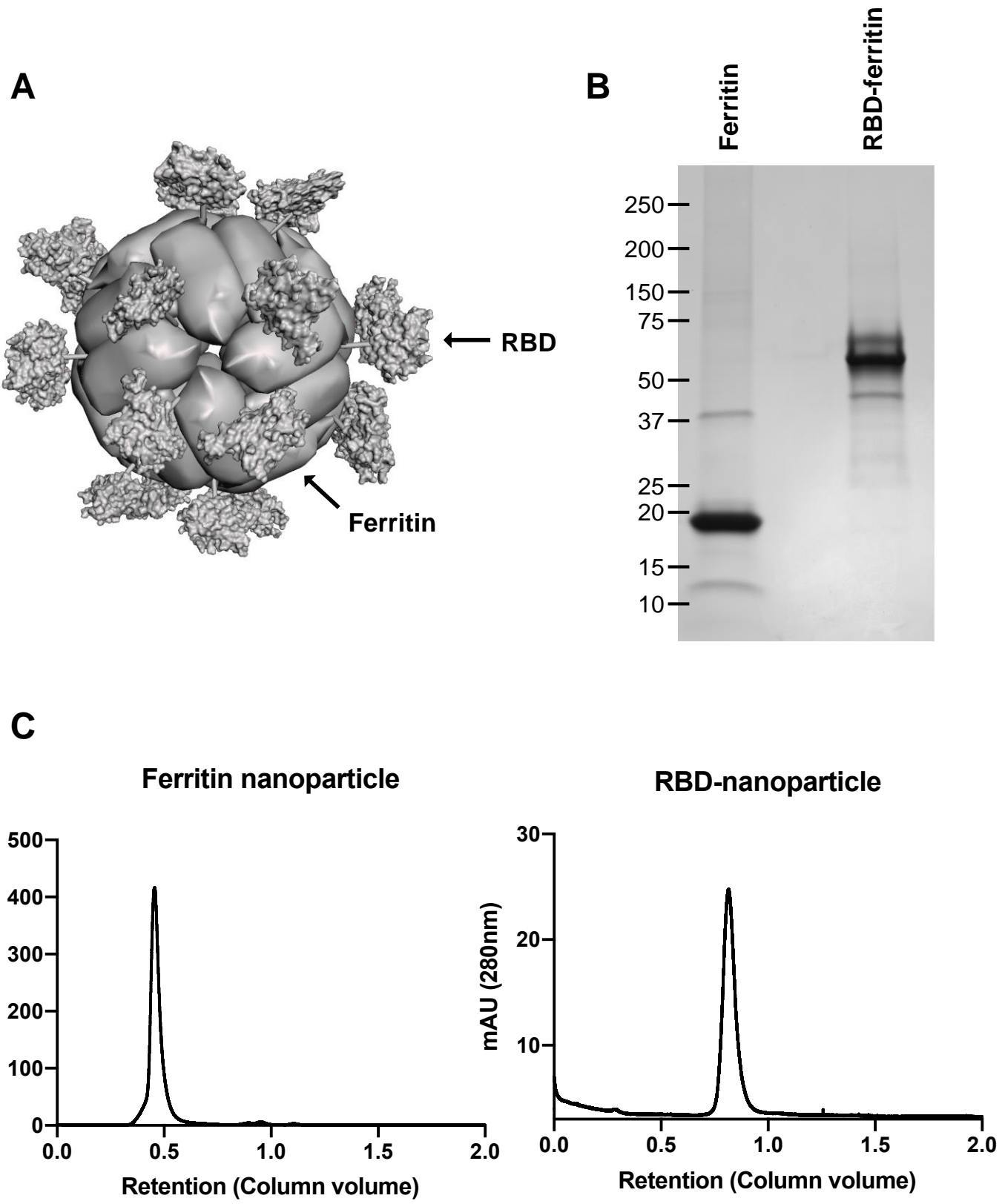


Figure 2. Immunization with RBD-nanoparticle elicits neutralizing antibody formation

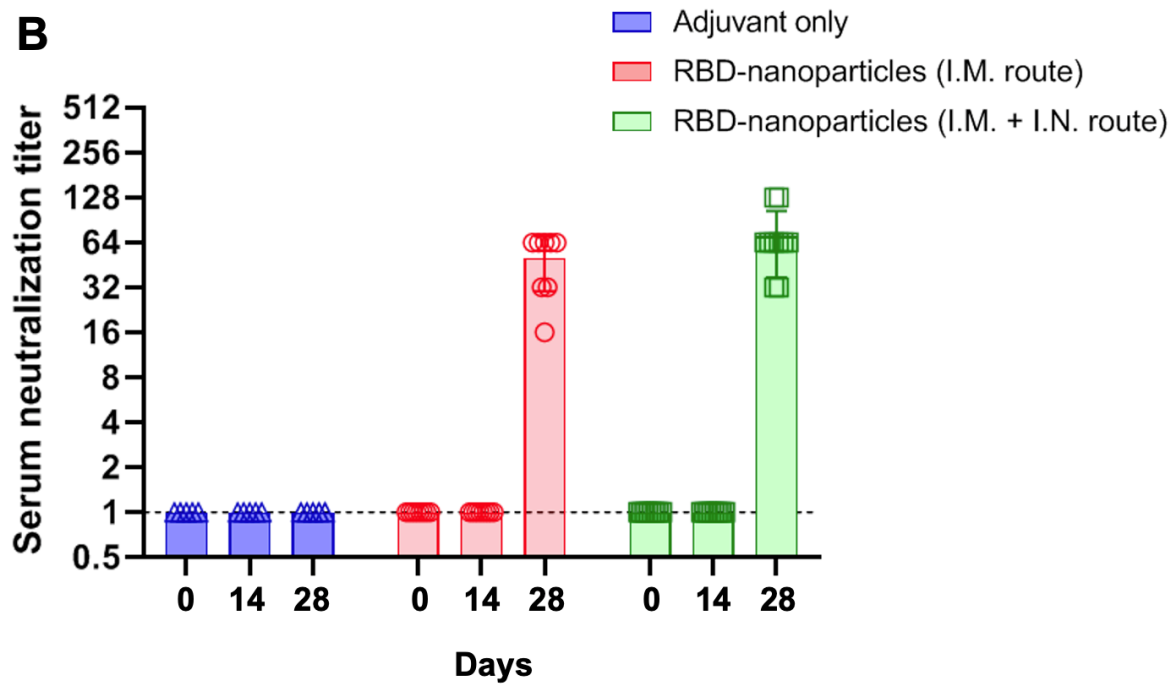
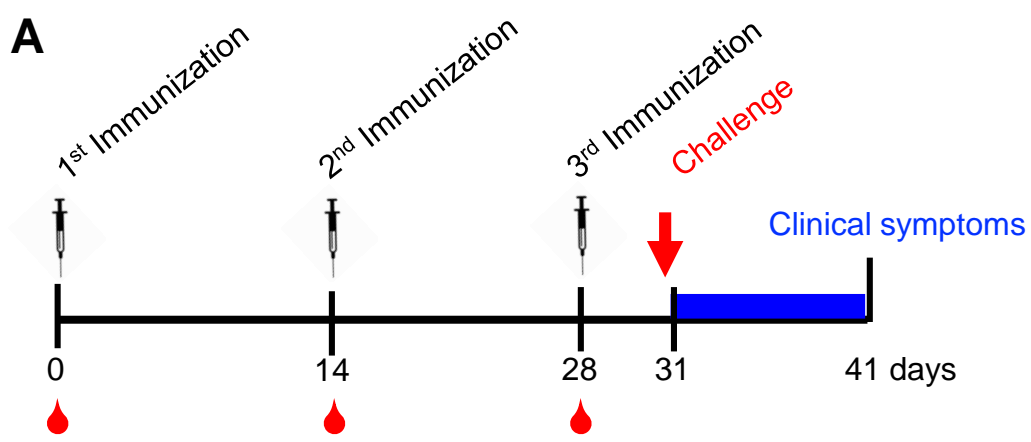
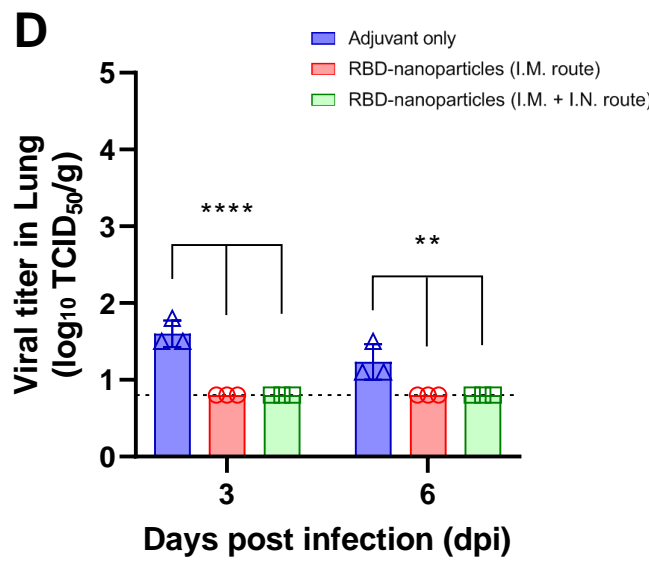
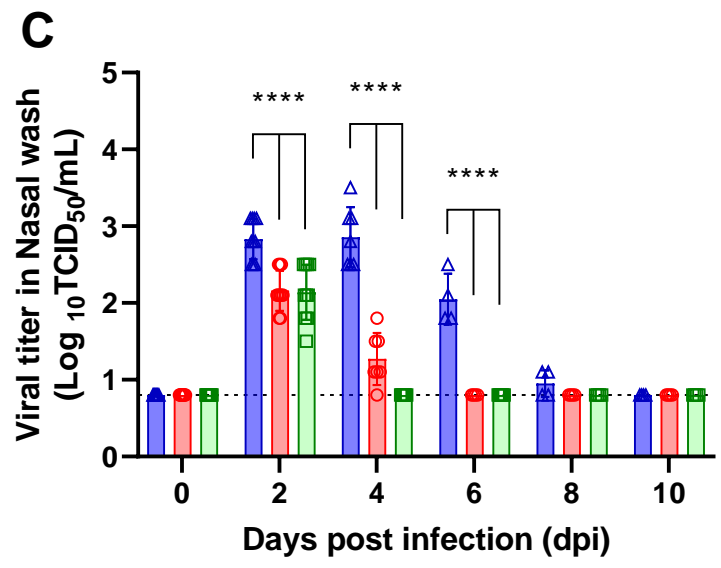
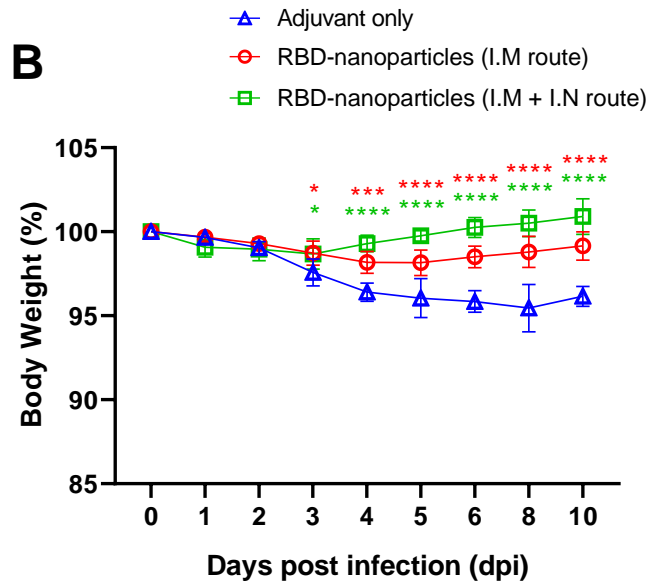
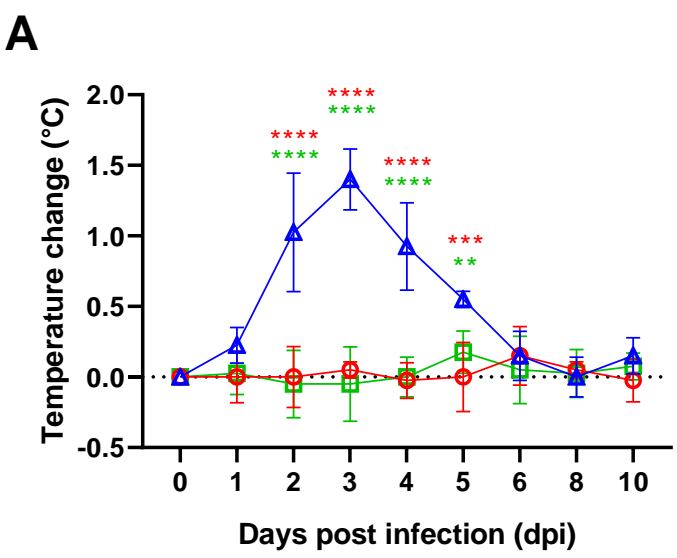


Figure 3. Immunization with RBD-nanoparticle promotes rapid viral clearance and protects ferrets from SARS-CoV-2 challenge



* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$

Figure 4. Lung histology and RNAscope of immunized ferrets upon SARS-CoV-2 challenge

