

1 **Prime-boost vaccination of mice and Rhesus macaques with two novel**
2 **adenovirus vectored COVID-19 vaccine candidates**

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29

30 **ABSTRACT**

31 COVID-19 vaccines are being developed urgently worldwide, among which
32 single-shot adenovirus vectored vaccines represent a major approach. Here, we
33 constructed two novel adenovirus vectored COVID-19 vaccine candidates on simian
34 adenovirus serotype 23 (Sad23L) and human adenovirus serotype 49 vectors (Ad49L)
35 carrying the full-length gene of SARS-CoV-2 spike protein (S), designated
36 Sad23L-nCoV-S and Ad49L-nCoV-S vaccines, respectively. The immunogenicity
37 elicited by these two vaccine strains was individually evaluated in mice. Specific
38 humoral and cellular immune responses were proportionally observed in a
39 dose-dependent manner, and stronger response was obtained by boosting.
40 Furthermore, five rhesus macaques were intramuscularly injected with a dose of
41 5×10^9 PFU Sad23L-nCoV-S vaccine for prime vaccination, followed by boosting
42 with 5×10^9 PFU of Ad49L-nCoV-S vaccine at 4-week interval. Three macaques were
43 injected with Sad23L-GFP and Ad49L-GFP vectorial viruses as negative controls.
44 Both mice and macaques tolerated well the vaccine inoculations without detectable
45 clinical or pathologic changes. In macaques, prime-boost vaccination regimen
46 induced high titers of $10^{3.16}$ S-binding antibody (S-BAb), $10^{2.75}$ cell receptor binding
47 domain (RBD)-BAb and $10^{2.38}$ neutralizing antibody (NAb) to pseudovirus a week
48 after boosting injection, followed by sustained high levels over 10 weeks of
49 observation. Robust IFN- γ secreting T-cell response (712.6 SFCs/ 10^6 cells), IL-2
50 secreting T-cell response (334 SFCs/ 10^6 cells) and intracellular IFN- γ expressing

51 CD4⁺/CD8⁺ T cell response (0.39%/0.55%) to S peptides were detected in the
52 vaccinated macaques. It was concluded that prime-boost immunization with
53 Sad23L-nCoV-S and Ad49L-nCoV-S vaccines can safely elicit strong immunity in
54 animals in preparation of clinical phase 1/2 trials.

55

56 **Key word:** COVID-19 vaccines, simian adenovirus 23 vector, human adenovirus 49
57 vector, prime-boost vaccination, mice and non-human primates.

58

59 INTRODUCTION

60 Novel coronavirus disease 2019 (COVID-19) usually presents as severe acute
61 respiratory syndrome triggered by SARS-CoV-2 infection (1, 2), which has become
62 globally pandemic and killed nearly one million people worldwide (WHO
63 Coronavirus Disease [COVID-19] Dashboard) (3). Currently the most urgent need is
64 to develop safe and effective vaccines that prevent SARS-CoV-2 infection.

65 SARS-CoV-2 is a positive-sense single-stranded RNA virus, encoding four
66 structural proteins, including spike (S), envelope (E), membrane (M) and
67 nucleocapsid (N) (4, 5). The S protein is a glycoprotein carrying the cell receptor
68 binding domain (RBD), and is a major protective antigen that may elicit potent
69 neutralizing antibody (NAb) and cellular immunity (4, 5). Therefore, S protein has
70 been the primary target antigen for developing recombinant vaccines.

71 According to the World Health Organization (WHO) report for a draft landscape
72 of COVID-19 candidate vaccines on August 28, 2020, there are 33 vaccine candidates
73 in clinical evaluation, which are mainly distributed across five types of
74 biotechnological platforms, *i.e.*, inactivated virus, DNA, RNA, protein subunit and
75 non-replicating viral vector vaccines (6). Among COVID-19 candidate vaccines in
76 clinical trials, five non-replicating adenovirus vectored vaccines progressed to the
77 frontline, of which one (CanSino Biological Inc./Beijing Institute of Biotechnology)
78 has been approved for emerging use in Chinese military personal, one (Gamaleya
79 Research Institute) has been registered in Russia, and two have been in initial phase

80 III clinical trial (University of Oxford/AstraZeneca; Janssen Pharmaceutical
81 Companies), and one in phase I clinical trial (ReiThera/LEUKOCARE/Univercells),
82 respectively. Recombinant adenovirus vectors originated from various serotype of
83 strains have displayed good safety profiles and induced broad and strong humoral and
84 cellular immune responses, which have been widely used for research and
85 development of vaccines (7). Based on the published data regarding human
86 adenovirus type 5 (Ad5), chimpanzee adenovirus type 25Y (ChAdOx1) and human
87 adenovirus type 26 (Ad26) vectorial COVID-19 vaccines (8-14), results showed that a
88 single-shot vaccine prevented SARS-CoV-2 pneumonia in rhesus macaques and
89 hamsters, and elicited significant immune response in the majority of recipients in
90 phase I/II clinical trials. However, relatively weaker protection in animals and lower
91 immune response in humans were observed with a single dose of vaccine compared
92 with prime-boost immunizations by two or three doses of inactivated virus or mRNA
93 vaccines (15-19). Enhancement of immune response was evidenced by prime and
94 boost vaccination regimens with homologous boosting of ChAdOx1 nCoV-19 in
95 animals and humans (11,20), or with heterologous Ad26 and Ad5 vectored
96 COVID-19 vaccines in humans (21).

97 In this study, two novel simian adenovirus type 23 (SAdV23) and human
98 adenovirus type 49 (HAdV49) derived vectors (Sad23L and Ad49L) were used for
99 developing the COVID-19 vaccines carrying the full-length S gene of SARS-CoV-2
100 (22, 23) designated Sad23L-nCoV-S and Ad49L-nCoV-S vaccines, respectively.

101 These two adenovirus vectored COVID-19 vaccines presented high infectious titers
102 and low frequencies of pre-existing immunity in humans. Immunogenicity was
103 extensively evaluated in mice and rhesus macaques by prime-boost vaccinations with
104 these two novel heterologous adenovirus vectored vaccines.

105

106 **RESULTS**

107

108 **Production and characterization of Sad23L-nCoV-S and Ad49L-nCoV-S** 109 **vaccines**

110 An optimized and synthesized full-length S gene of SARS-CoV-2 was cloned into the
111 deleted E1 region under cytomegalovirus (CMV) promoter regulation within two
112 novel adenovirus vectorial Sad23L and Ad49L plasmids, designated as
113 Sad23L-nCoV-S or Ad49L-nCoV-S, respectively (Fig. 1A). The recombinant
114 adenoviruses were rescued from HEK-293A. A large amount of Sad23L-nCoV-S and
115 Ad49L-nCoV-S candidate vaccines were produced from HEK-293A cell cultures, and
116 further purified and titrated over 10^{11} PFU/ml.

117 Expression of S protein was verified in Sad23L-nCoV-S and Ad49L-nCoV-S
118 vaccine strains infected HEK-293A cells by Western blot with rabbit polyclonal
119 anti-RBD antibodies and COVID-19 patient's serum IgG, respectively, but not in the
120 Sad23L-GFP and Ad49L-GFP vectorial viruses infected cells (Fig. 1B). The
121 expression of S protein in the vaccine strains-infected HEK-293A cells was also

122 observed in red color by an immunofluorescence staining, but not in the
123 adenovirus-GFP infected control cells (Fig. 1C). These results indicated that
124 Sad23L-nCoV-S and Ad49L-nCoV-S vaccines could effectively produce
125 SARS-CoV-2 S protein in the infected cells.

126 To measure pre-existing immunity to these two vectors, 600 healthy blood donor
127 samples were collected from six cities crossing the south, north, east, west and central
128 regions of China and were tested for neutralizing antibodies (NAb) reacting with
129 Sad23L-GFP, Ad49L-GFP and Ad5-GFP viruses, respectively. The seroprevalence of
130 Sad23L, Ad49L and Ad5 was 10.2%, 2.2% or 75.2% respectively (Fig. 1D),
131 indicating that Sad23L and Ad49L vectors had low pre-exposure rate in the Chinese
132 population.

133

134 **Animal tolerance of Sad23L-nCoV-S and Ad49L-nCoV-S vaccine inoculation**

135 C57BL/6 mice (n=5 per group) were intramuscularly injected with individual
136 Sad23L-nCoV-S and Ad49L-nCoV-S vaccines at doses of 10^7 , 10^8 and 10^9 PFU or by
137 prime-boost immunization with these two adenovirus vectored vaccines (10^9
138 PFU/each) at 4 week interval and compared with vectors and PBS controls,
139 respectively. Mice tolerated well the various doses of vaccines or vector controls at 28
140 or 56 days, presenting no obvious change of weight and body temperature when
141 compared with injected PBS (Fig. 2A). In addition, histopathological examination of
142 brain, lung, heart, liver, kidney and muscle tissues (at intramuscular injection site and

143 para-tissues) did not present significant pathological lesions over 4 weeks following
144 the prime only or prime-boost injections (Fig. S1).

145 Vaccination (n=5) and sham control (n=3) groups of rhesus macaques (Table S1)
146 were first injected with 5×10^9 PFU Sad23L-nCoV-S vaccine or Sad23L-GFP control,
147 and then at 4 week interval, animals were injected with a second dose of 5×10^9 PFU
148 Ad49L-nCoV-S vaccine or Ad49L-GFP control, respectively. During the course of
149 immunization, clinical parameters were monitored for eight weeks. All monkeys
150 displayed normal appetite and mental state, and no obvious change of weight and
151 body temperature was observed with these eight animals (Fig. 2B). Hematological
152 and biochemical examination of blood samples showed no notable variation when
153 comparing the vaccinated or vector control animals during 8 weeks pre-vaccination
154 and post-vaccination (Fig. S2; Table S2).

155

156 **A single-shot immunization of Sad23L-nCoV-S or Ad49L-nCoV-S vaccine** 157 **induced specific immune response in mice**

158 To evaluate the immunogenicity of individual Sad23L-nCoV-S and Ad49L-nCoV-S
159 vaccines, a single dose of 10^7 , 10^8 or 10^9 PFU Sad23L-nCoV-S or Ad49L-nCoV-S
160 vaccine was intramuscularly injected to C57BL/6 mice (n=5/group). Vector control
161 groups (n=5/group) received 10^9 PFU Sad23L-GFP or Ad49L-GFP viruses, and naïve
162 control group (n=5) received an equal volume of PBS, respectively. Four weeks
163 post-immunization, titers of S or RBD binding antibody (S-BAb or RBD-BAb) were

164 quantified by ELISA in a dose-dependent manner with both Sad23L-nCoV-S and
165 Ad49L-nCoV-S immunized mice, but not in the control groups ($P<0.001$, Fig. 3,
166 A-D). A dose of 10^9 PFU Sad23L-nCoV-S vaccine induced antibody titers of $10^{4.27}$
167 S-BAb and $10^{3.98}$ RBD-BAb (Fig. 3, A and B), while the same dose of
168 Ad49L-nCoV-S vaccine induced titers of $10^{3.02}$ S-BAb and $10^{2.42}$ RBD-BAb,
169 respectively (Fig. 3, C and D).

170 The neutralizing antibody (NAb) titers to SARS-CoV-2 were titrated in two
171 individual vaccine immunized mice by surrogate virus based NAb test (sVNT) and
172 pseudovirus-based NAb test (pVNT) at 50% inhibitory dilution (ID_{50}), respectively
173 (Fig. 3, E-H). A dose of 10^9 PFU Sad23L-nCoV-S vaccine induced NAb titers of
174 $10^{2.41}$ sVNT(ID_{50}) and $10^{2.79}$ pVNT (ID_{50}) (Fig. 3, E and F), and Ad49L-nCoV-S
175 vaccine induced titers of $10^{1.38}$ sVNT (ID_{50}) and $10^{1.57}$ pVNT(ID_{50}), respectively (Fig.
176 3, G and H).

177 Specific T-cell response of isolated splenocytes was examined in vaccinated and
178 sham mice after stimulation with S peptides, S protein and RBD protein, respectively
179 (Fig. 3, I-N; Fig. S3). A single dose of Sad23L-nCoV-S vaccine elicited strong
180 specific IFN- γ secreting T cell response to S peptides (450.2-898.4 SFCs/million cells,
181 Fig. 3I) and S protein (81.5-246.8 SFCs/million cells, Fig. 3J), while a dose of
182 Ad49L-nCoV-S vaccine induced similar pattern of IFN- γ secreting T cell response
183 (336.8-857.1 or 82.8-232 SFCs/million cells, Fig. 3, K and L). Both vaccines elicited
184 T cell response significantly higher than sham controls ($P<0.001$), but response to

185 RBD protein was not significantly different ($P>0.05$, Fig. S3, A and B). Specific
186 intracellular levels of IFN- γ , TNF- α and IL-2 expressing CD4⁺ or CD8⁺ T cell
187 response to S peptides were measured by ICS, in which significantly higher frequency
188 of IFN- γ and TNF- α but not IL-2 expressing CD4⁺/CD8⁺ T-cells was found in both
189 Sad23L-nCoV-S and Ad49L-nCoV-S vaccinated mice compared to sham mice
190 ($P<0.001$, Fig. 3, M and N; Fig. S3, C-F).

191 Overall, individual Sad23L-nCoV-S or Ad49L-nCoV-S vaccinated mice
192 developed specific BAb and NAb antibodies and T cell responses to S protein, RBD
193 protein or S peptides of SARS-CoV-2 in a dose-dependent fashion (Fig. 3),
194 suggesting strong immunogenicity of the two novel adenovirus vectored COVID-19
195 vaccines.

196

197 **Prime-boost immunization of mice with Sad23L-nCoV-S and Ad49L-nCoV-S** 198 **vaccines**

199 To further improve reactivity and longevity of immune response to vaccines, the
200 prime-boost vaccination regimen was utilized to immunize C57BL/6 and BALB/c
201 mice (n=5/group) with a prime dose of 10⁹ PFU Sad23L-nCoV-S on day 0 and a
202 boost dose of 10⁹ PFU Ad49L-nCoV-S on day 28 (Fig. 4A). In comparison with
203 prime immunization with Sad23L-nCoV-S, a booster with Ad49L-nCoV-S
204 significantly increased BAb and NAb titers in C57BL/6 and BALB/c mice ($P<0.001$;
205 Fig. 4, B-G), and titers were maintained at high level for 10 weeks of monitoring (Fig.

206 **4, D and G**). Profiling of IgG subclasses showed a predominant serum IgG2a to RBD
207 protein associating with Th1 response in prime-boost vaccinated mice (Fig. S4).
208 Regarding specific T cell response, the boosting with Ad49L-sCoV-S on
209 Sad23L-nCoV-S prime immunization enhanced or stabilized specific IFN- γ -secretion
210 T cell responses to S peptides, S protein or RBD protein (Fig. 4H), as well as levels of
211 intracellular cytokines IFN- γ and TNF- α but not of IL-2 response to S peptides
212 compared with single dose of vaccine vaccinated or sham control C57BL/6 and
213 BALB/c mice (Fig. 4I; Fig. S5).

214 Taken together, the results suggest that prime-boost vaccination of two species of
215 mice with Sad23L-nCoV-S followed by Ad49L-nCoV-S enhanced specific immune
216 response to SARS-CoV-2 when compared with prime vaccination only with a
217 single-shot of Sad23L-nCoV-S or Ad49L-nCoV-S vaccine.

218

219 **Rhesus macaques' specific immune response to prime-boost vaccination with**
220 **combined Sad23L-nCoV-S and Ad49L-nCoV-S vaccines**

221 Eight rhesus macaques aged 11-14 years were selected and tested for the baseline
222 values of antibody and T-cell response to SARS-CoV-2 S from blood samples in
223 pre-vaccination at week -1 or week 0 (Fig. 5A; Table S1). The pre-existing NAb titer
224 to Sad23L, Ad49L or Ad5 was detected < 1:10 in serum samples from all eight
225 animals (Table. S1).

226 Five rhesus macaques were intramuscularly injected first with a dose of 5×10^9
227 PFU Sad23L-nCoV-S vaccine, then with a boost injection of 5×10^9 PFU
228 Ad49L-nCoV-S vaccine 4 weeks later, while three sham rhesus macaques controls
229 were injected with equal doses of Sad23L-GFP and Ad49L-GFP viruses, respectively
230 (Fig. 5A). Blood samples were collected weekly from these two groups of animals.
231 Both BAb (S-BAb and RBD-BAb) and NAb (sVNT and pVNT) levels were titrated
232 in the vaccinated group but not in sham group. BAb and NAb reactivity increased at
233 week 2 post prime-immunization, at week 4 (W0 for boost-immunization), at weeks 5
234 and 6 and then stayed at high levels up to week 10 (Fig. 5, B-E). Titers of $10^{3.16}$
235 S-BAb (Fig. 5B), $10^{2.75}$ RBD-BAb (Fig. 5C), $10^{2.01}$ sVNT(ID₅₀) (Fig. 5D) and $10^{2.38}$
236 pVNT(ID₅₀) (Fig. 5E) were quantified and found higher than the corresponding
237 antibody titers found in sera from 25 asymptomatic, 14 mild and 9 severe COVID-19
238 convalescent patients (Fig. 5, B-E). Strong correlation between BAb and NAb titers
239 was observed ($P < 0.0001$, $R = 0.784-0.927$; Fig. 5, F and G), suggesting the reliability
240 of these antibody quantitative assays.

241 PBMCs were isolated from whole blood of pre- and post-immunized monkeys
242 for evaluation of T cell responses to S peptides, S and RBD protein by ELISpot and
243 ICS (Fig. 6). Prime vaccination with a dose of Sad23L-nCoV-S vaccine induced an
244 increase of IFN- γ secreting T cell response to S peptides (406.6-526.3 SFCs/million
245 cells) at weeks 2 and 4 post prime-vaccination, and then boosting with a dose of
246 Ad49L-nCoV-S vaccines enhanced the IFN- γ secreting T cell response (583.9-712.6

247 SFCs/million cells) at weeks 5 to 8 (Fig. 6A). IFN- γ secreting T cell reaction to S and
248 RBD proteins stayed at high levels after prime-boost immunizations (Fig. 6, B and C).
249 Relatively high IL-2 secreting T cell response to S peptides, S and RBD proteins was
250 observed (Fig. 6, D-F), but weak IL-4 secreting T cell response by ELISpot (Fig. S6).
251 Frequency of intracellular IFN- γ expressing CD4⁺/CD8⁺ T-cell responses to S
252 peptides was observed in vaccinated macaques at weeks 2 to 8 (Fig. 6, G-J),
253 significantly higher than observed in pre-vaccination and sham controls ($P<0.01$).
254 Frequency of intracellular TNF- α expressing CD4⁺ T cell response was also found
255 significantly different between vaccinated and sham monkeys ($P<0.05$; Fig. S7, A and
256 B), but intracellular TNF α ⁺ CD8⁺ or IL-2⁺ CD4⁺/CD8⁺ T cell response to S peptides
257 was not statistically different between groups ($P>0.05$; Fig. S7, C-F).

258 In summary, prime-boost vaccination with Sad23L-nCoV-S and Ad49L-nCoV-S
259 vaccines at an interval of 4 weeks elicited higher levels of specific antibody and T-cell
260 responses against SARS-CoV-2 in rhesus macaques, which was recommended as
261 COVID-19 vaccine candidates for clinical trials in humans.

262

263 **Biodistribution of Sad23L-nCoV-S and Ad49L-nCoV-S vaccine strains in the** 264 **tissues of inoculated mice**

265 Neutralizing antibody titers to Sad23L and Ad49L vectors were determined in rhesus
266 macaques and mice after prime only and prime-boost vaccinations (Fig. 7, A and B;

267 Fig. S8). A high NAb reactivity to an individual adenoviral vector of either Sad23L or
268 Ad49L in macaques (1:1280 or 1:640) and mice (1:576 or 1:352) was induced post
269 prime immunization, but not enhanced post boost vaccination with a heterologous
270 vector (Fig. 7, A and B), which might limit the homologous boosting of adenovirus
271 vectored vaccines. The lung, spleen, liver and muscle tissues (at intramuscular
272 injection site and para-tissues) from prime only or prime-boost immunized C57BL/6
273 mice were examined 4 weeks after inoculation of vaccines in order to assess vaccine
274 delivery by amplifying specific adenoviral hexon sequences of both Sad23L and
275 Ad49L vectors. The predicted 500bp PCR band was detected in lung, spleen and liver
276 tissues after Sad23L-nCoV-S vaccination, while the PCR band was observed in lung
277 and liver tissues but not found in the spleen after Ad49L-nCoV-S vaccination (Fig.
278 7C). The expression of S antigen was observed in splenocytes and hepatocytes from
279 prime-only Sad23L-nCoV-S or Ad49L-nCoV-S and prime-boost vaccines inoculated
280 C57BL/6 mice by immunofluorescence staining (Fig. 7D), but not found in lung and
281 muscle tissues (Fig. S9).

282

283 **DISCUSSION**

284

285 A safe and effective COVID-19 vaccine is one of the most wanted goods in the world.
286 Among 33 COVID-19 candidate vaccines in clinical trials (WHO report on 28 August
287 2020) (6), 9 vaccines have initiated clinical phase III trial, and experimental data from

288 pre-clinical and/or clinical studies have been published (8-21). In the frontline of
289 COVID-19 vaccine candidates in clinical trial phase III, the range of approaches
290 includes three inactivated virus, two lipid nanoparticle (LNP)-encapsulated mRNA
291 and four non-replicating adenovirus vectored vaccines (6). Beyond general comments
292 on the disadvantages of major types of COVID-19 vaccines (24,25), the limitations of
293 inactivated SARS-CoV-2 vaccines are crucial biosafety concerns regarding large
294 amounts of infectious virus being processed above biological safety level-3 (BSL-3)
295 condition and production capacity; for LNP-mRNA vaccines instability and lack of
296 evidence for efficacy are puzzling; for adenovirus vectored vaccines, pre-existing
297 immunity to the carrier virus and the relative weakness of single-shot immunization
298 appear limiting factors.

299 In this study, we generated two novel adenovirus vectored COVID-19 vaccines
300 encoding the full-length S gene of SARS-CoV-2. The intact S glycoprotein rather
301 than the shorter S or RBD proteins was shown to be the most effective antigen
302 eliciting protective immunity against SARS-CoV-2 infection in DNA vaccines and
303 Ad26 vectored vaccines (12,13,26). The vectors Sad23L and Ad49L originated from
304 simian adenovirus type 23 and human adenovirus type 49, respectively (22,23), are
305 novel adenoviral vector. Comparing Ad5-vectored COVID-19, ChAdox1 nCoV-19
306 and Ad26.COVS vaccines (8-12,14,21), three attractive aspects emphasized in this
307 study are highlighted below.

308 Firstly, there is a low-seroprevalence of pre-existing antibodies to Sad23L and
309 Ad49L vectors in humans. According to the investigation of NAb to three types of
310 adenoviruses in Chinese population, the prevalence of both Sad23L and Ad49L NAb
311 was below 10%, while the prevalence of Ad5 NAb was over 75% (Fig. 1D). However,
312 the pre-existing anti-Ad5 immunity might partly limit vaccine effectiveness,
313 especially for populations aged over 50 (8,9,24,25), while the low seroprevalence of
314 antibodies to novel adenoviral vectors such as ChAdox1 (11), Ad26 (12,14,21,27),
315 and Sad23L and Ad49L used in this study might avoid a negative impact on vaccine
316 efficacy (22-24).

317 Secondly, the prime-boost vaccination regimen with two heterologous
318 adenoviruses vectored Sad23L-nCoV-S and Ad49L-nCoV-S vaccines examined in
319 mice and rhesus macaques suggested that the boost with Ad49L-nCoV-S vaccine
320 significantly enhanced the levels of neutralizing antibody and IFN- γ expressing
321 CD4⁺/CD8⁺ T cell responses following prime-immunization with Sad23L-nCoV-S
322 vaccine (Fig. 4-6). In addition, specific IFN- γ secretion T cell response was prolonged
323 at high level after boosting vaccination. Two recent publications compared prime only
324 with a single-dose of ChAdOx1 nCoV-19 and homologous boost with a second dose
325 of ChAdOx1 nCoV-19 vaccine in mice and pigs, and human clinical trials, showed
326 that boost vaccination significantly increased and prolonged the level of binding or
327 neutralizing antibody response to SARS-CoV-2 (11,20). By boosting with Ad5-S
328 vaccine the Ad26-S vaccine primed immunization, a higher level of immunity was

329 observed in humans as reported in a recent Russian study (21). Compared with
330 homologous boosting of ChAdOx1 nCoV-19 or boosting of Ad5-S vaccines (11,21),
331 heterologous prime-boost vaccinations with Sad23L-nCoV-S and Ad49L-nCoV-S
332 vaccines have the advantage of avoiding vector's immunity interfering with prime
333 vaccination or enhancing pre-existing Ad5 immunity (22-25).

334 Thirdly, in order to achieve an effective vaccine immunity, a low dose of two
335 heterologous adenovirus vectored vaccines ($<5 \times 10^{10}$ vp) with prime-boost
336 immunization regimen should theoretically reduce severe adverse reaction induced by
337 a high dose of adenovirus vectored vaccine ($>5 \times 10^{10}$ vp) with prime only
338 immunization in clinical trials (8,9,11,14). In this study, a relatively low dose of
339 Sad23L-nCoV-S and Ad49L-nCoV-S vaccines elicited a robust immunity in both
340 young mice and older rhesus macaques (aged 11-14 years), but no obvious clinical
341 symptoms or histopathological changes were observed (Fig. 2; Fig. S1; Fig. S2).

342 There are two limitations to this work. Considering biosafety and restriction for
343 SARS-CoV-2 virus manipulation, conventional live virus neutralization test (cVNT)
344 and virus injection challenge experiments have not been performed in this study.
345 However, sVNT and pVNT reported in this study have well demonstrated a
346 significant correlation with cVNT in NAb titers to SARS-CoV-2 ($P < 0.0001$,
347 $R = 0.7678-0.8591$) (28). This was also evidenced by close correlation between ELISA
348 and pVNT or cVNT by other studies using adenovirus vectored COVID-19 vaccines
349 in rhesus macaques ($P < 0.0001$, $R = 0.8314-0.8427$) (12), hamsters ($P < 0.0001$;

350 $R=0.7849$) (13) and human clinical phase II trial ($P<0.0001$, $R=0.72-0.75$) (9). In
351 addition, compared with a panel of convalescent serum samples from COVID-19
352 patients the level of binding or neutralizing antibodies observed in this study was
353 favorable and in line with all other published data from COVID-19 vaccines.
354 Protective efficacy of Sad23L-nCoV-S and Ad49L-nCoV-S vaccines for prime-boost
355 vaccination of animals might be referred to ChAdOx1 nCoV-19 or Ad26.COV2.S
356 vaccine immunized rhesus macaques against SARS-CoV-2 challenge (10,12), because
357 Sad23L and ChAdOx1, Ad49L and Ad26 were classified in the same species E or D
358 of adenoviruses, respectively (7).

359 In conclusion, two novel adenovirus vectored COVID-19 vaccines were
360 produced, which could, when used in succession, safely elicit robust humoral and
361 T-cell immune response to SARS-CoV-2 in mice and Rhesus macaques. Prime-boost
362 vaccination regimen with priming of Sad23L-nCoV-S and boosting of
363 Ad49L-nCoV-S vaccines were recommended to develop the better protective
364 immunity against SARS-CoV-2 in humans. These two vaccines are being planned for
365 clinical phase I/II trials after an extensive safety evaluation has been carried out in
366 pre-clinical animal examination.

367

368 **MATERIALS AND METHODS**

369

370 **Rhesus macaques and ethics statement**

371 Eight healthy outbred male rhesus macaques (*Macaca mulatta*) aged 11-14 years were
372 randomly allocated to this study (Table S1). Experimentation and sample collection
373 were ethically approved and carried out by the Huazheng Laboratory Animal
374 Breeding Centre, Guangzhou, China. All animal care and experimental procedures
375 (NFYYLASOP-037) were in accordance with national and institutional policies for
376 animal health and wellbeing.

377 The welfare issues (housing, feeding, environmental enrichment, etc.) were in
378 accordance with the recommendations of the Weatherall report
379 (<https://acmedsci.ac.uk/more/news/the-use-of-non-human-primates-in-research>).

380 Animals were individually housed in spacious cages and were provided with
381 commercial food pellets supplemented with appropriate treats. Drinking water was
382 provided ad libitum from an automatic watering system. Animals were monitored
383 daily for health and discomfort. Blood samples were obtained using sterilized needle
384 and syringe from the venous vessels of animal legs.

385

386 **Cells and mice**

387 HEK-293A, HEK-293T and HEK293T-hACE2 cells (Sino Biological) were
388 maintained in complete Dulbecco's modified Eagle's medium (DMEM, Gibco)
389 supplemented with 10% fetal bovine serum (FBS, Gbico) and incubated at 37 °C in
390 5% CO₂.

391 Female C57BL/6 and BALB/c mice were obtained from the Animal
392 Experimental Centre of Southern Medical University, Guangdong, China.

393

394 **COVID-19 patients' serum samples**

395 A total of 48 convalescent serum samples were provided by The First People's
396 Hospital of Foshan, Shenzhen or Guangzhou Center for Disease Control and
397 Prevention (CDC), China. The samples were collected from 25 asymptomatic, 14
398 mild and 9 severe COVID-19 infected subjects. All serum samples were inactivated
399 for 40 min by heating at 56 °C in the water bath.

400

401 **Construction of two novel adenovirus vectored COVID-19 vaccine strains**

402 According to the description of Sad23L vector (SAdV23, GenBank: AY530877.1)
403 (22,23), the replication defective adenoviral vector Ad49L was constructed by
404 deleting the E1 and E3 regions of the full-length human adenovirus serotype 49
405 genome (HAdV49, GenBank: DQ393829.1). The E4 region open reading frame 6
406 (orf6) was replaced by the corresponding element of human adenovirus type 5 (Ad5)
407 in order to improve virus propagating efficiency. The full-length S protein gene of
408 SARS-CoV-2 (GenBank: MN908947.3) was optimized and synthesized (Beijing
409 Genomics Institute, China) and was cloned into adenoviral vectors Sad23L and
410 Ad49L, respectively. The recombinant adenoviral constructs Sad23L-nCoV-S and
411 Ad49L-nCoV-S were rescued, and the novel adenovirus vectored COVID-19 vaccine

412 strains were propagated from HEK-293A packaging cells. The vaccine strains were
413 serially passaged for stability by 12 generations when the full cytopathic effect
414 appeared. Virus purification was performed by cesium chloride density gradient
415 centrifugation as previously described (23).

416

417 **Histopathological examination.**

418 Mice tissues were stained with hematoxylin and eosin (H&E) and examined
419 microscopically for histopathological changes by Guangzhou Huayin Medical Science
420 Company Limited (Guangzhou, China).

421

422 **Western blotting**

423 HEK-293A cells were infected with Sad23L-nCoV-S and Ad49L-nCoV-S strains,
424 respectively, and Sad23L-GFP and Ad49L-GFP vectorial viruses were used as mock
425 control. The expression of SARS-CoV-2 S protein was analyzed by Western blotting
426 with rabbit polyclonal antibody to SARS-CoV-2 RBD (Sino Biological, China) and
427 heat-inactivated human serum samples from Chinese COVID-19 infected patients.
428 Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was included as a loading
429 control. The membranes were washed five times and developed by Supersignal West
430 Pico Plus chemiluminescent substrate (Thermo Scientific, USA).

431

432 **Immunofluorescence staining**

433 Cells infected with vaccine strains or vector control viruses were fixed in cell culture
434 plates, while tissues were collected from vaccinated and control mice. Cell layers or
435 tissue frozen sections were incubated with human monoclonal antibody to
436 SARS-CoV-2 RBD (OkayBio, China), and then washed with PBST. Anti-human
437 IgG-Alexa Fluor 594 antibody (Thermo Scientific, USA) in 1% BSA-PBST was
438 added to the cells for 30 min at 37 °C. DAPI was added to stain cell nuclei.

439

440 **Adenovirus neutralizing antibody assay**

441 Human plasma samples were collected from 600 healthy blood donors at six blood
442 centers (100 per center) across China, including Shenzhen (south), Guangzhou (south),
443 Yichang (central), Harbin (northeast), Chengdu (southwest) and Xian (west) blood
444 centers. Plasmas were tested on HEK-293A cells for neutralizing Sad23L-GFP, or
445 Ad49L-GFP vectorial viruses by green fluorescent activity assay as previously
446 described (22,29).

447

448 **Animal immunization**

449 Female C57BL/6 mice (5-6 weeks, n=5 each group) were individually inoculated
450 intramuscularly (i.m.) with a dose of 10^7 , 10^8 and 10^9 PFU Sad23L-nCoV-S or
451 Ad49L-nCoV-S vaccine, respectively. A dose of 10^9 PFU Sad23L-GFP or
452 Ad49L-GFP vectorial virus and an equivalent volume of PBS were used as sham
453 control.

454 Female C57BL/6 and BALB/c mice (5-6 weeks, n=5 each group) were prime
455 inoculated intramuscularly with a dose of 10^9 PFU Sad23L-nCoV-S vaccine, and then
456 at 4 week interval were boosted with a dose of 10^9 PFU Ad49L-nCoV-S vaccine. A
457 dose of 10^9 PFU Sad23L-GFP and a dose of 10^9 PFU Ad49L-GFP were used as sham
458 control.

459 Five rhesus macaques aged 11 to 14 years (Table S1) were first injected
460 intramuscularly with a dose of 5×10^9 PFU Sad23L-nCoV-S vaccine, and then at 4
461 week interval received a second dose of 5×10^9 PFU Ad49L-nCoV-S vaccine. Three
462 rhesus macaques aged 11 to 13 years (Table S1) were vaccinated by prime-boost
463 regimen with a dose of 5×10^9 PFU Sad23L-GFP and a dose of Ad49L-GFP vectorial
464 adenoviruses as sham control.

465

466 **Enzyme-linked immunosorbent assay (ELISA)**

467 The microtiter plates (Corning, USA) were coated overnight with $2 \mu\text{g/ml}$ of
468 SARS-CoV S or RBD proteins (Sino Biological, China). Serum samples were 2-fold
469 serially diluted and S or RBD binding antibody (S-BAb or RBD-BAb) were detected
470 by ELISA. Secondary antibodies were goat anti-mouse IgG-HRP (Beijing Bersee
471 Science and Technology, Co. LTd, China), rabbit anti-monkey IgG-HRP (Bioss,
472 China) and goat anti-human IgG-HRP conjugates (Sigma, USA), respectively.
473 Endpoint titers were defined as the highest reciprocal serum dilution that yielded an

474 absorbance > 0.2 and a ratio of signal than cutoff (S/CO) > 1. Log10 end point titers
475 were reported (26).

476 Goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 heavy chain-HRP conjugates were
477 used for IgG sub-classification according to manufacturer's instruction (Abcam, UK).
478

479 **Surrogate virus neutralization test (sVNT)**

480 The surrogate virus based neutralization test (sVNT) was used for measuring
481 neutralizing antibody (NAb) to SARS-CoV-2 as previously described (28). Briefly,
482 microtiter plates (Corning) were coated overnight with 2µg/ml hACE2 protein (Sino
483 Biological, Beijing, China) at 4 °C, followed by blocking with OptEIA assay diluent
484 (BD). The HRP-RBD conjugate (3 ng) was pre-incubated with 100 µl of diluted
485 serum samples for 1 h at 37 °C, then added into hACE2 pre-coated plate for 1 h at
486 room temperature. Plates were washed five times by PBST. A colorimetric signal was
487 developed with TMB, and equal volume of stop solution was added to terminate the
488 reaction. The absorbance reading was performed at 450 nm and 570 nm. Inhibition
489 rate (%) = (1 - sample optical density value/negative control optical density value) ×
490 100.

491

492 **Pseudovirus neutralization test (pVNT)**

493 Pseudoviruses expressing a luciferase reporter gene were generated for measuring of
494 NAb to SARS-CoV-2 as previously described (26). Briefly, the packaging construct

495 psPAX2 (Addgene), plasmid pLenti-CMV Puro-Luc (Addgene), and
496 pcDNA3.1-SARS-CoV-2 SΔCT (deletion of the cytoplasmic tail) were co-transfected
497 into HEK-293T cells. The supernatants were collected 48 hours post-transfection and
498 the pseudoviruses were purified by filtration through 0.45 μm filter. The pVNT titers
499 were measured with HEK293T-hACE2 cells in 96-well tissue culture plates.
500 Two-fold serial dilutions of heat-inactivated serum samples were prepared and mixed
501 with 50 μl of pseudovirus. After incubation at 37 °C for 1h, the serum-virus mixture
502 was added to HEK293T-hACE2 cells. Forty-eight hours after infection, cells were
503 lysed in Steady-Glo Luciferase Assay (Promega) according to the manufacturer's
504 instructions. The pVNT titer of SARS-CoV-2 antibody was defined as the sample
505 dilution at which a 50% inhibition rate. Inhibition rate (%) = $(1 - \text{sample RLU}/\text{virus}$
506 $\text{control RLU}) \times 100$. Log₁₀ pVNT(ID₅₀) titer was reported.

507

508 **ELISpot**

509 Monkey (or mouse) IFN-gamma, IL-2 or IL-4 ELISpotPLUS kits (MabTech) were
510 used to determine SARS-CoV-2 S antigen-specific T lymphocyte response. Rhesus
511 macaque's PBMCs (2×10^5 cells/well) or mouse splenocytes (5×10^5 cells/well) were
512 stimulated with S peptides, S or RBD protein (5μg/ml) in triplicates, respectively.
513 Seventy-nine peptides encoding for amino acid sequence of SARS-CoV-2 S protein
514 were predicted (<http://www.iedb.org/>) and synthesized by Guangzhou IGE
515 Biotechnology LTD (Table. S3). Spots were counted with a CTL Immunospot Reader

516 (Cellular Technology Ltd). The results were expressed as spot forming cells (SFCs)
517 per million cells.

518

519 **Intracellular cytokine staining (ICS) and flow cytometry**

520 Mouse splenocytes (2×10^6 cells/well) or monkey PBMCs (1×10^6 cells/well) were
521 stimulated with S peptide pools ($3 \mu\text{g/ml}$ each peptide), or medium as negative control,
522 in triplicates. After 4h, the cells were incubated with Golgi Plug (BD) for 12h at 37°C .
523 Cells were collected and stained with anti-mouse or anti-monkey CD3, CD4 and CD8
524 surface marker antibodies (BD). Cells were fixed with IC fixation buffer,
525 permeabilized with permeabilization buffer (BD) and stained with anti-mouse or
526 anti-monkey interferon- γ (IFN- γ), interleukin-2 (IL-2) and tumor necrosis factor α
527 (TNF- α) (BD). All samples were tested with BD FACS Canton flow cytometer (BD).

528

529 **Nested-PCR amplification**

530 Genomic DNA was extracted from homogenized tissue of adenovirus vectored
531 vaccine inoculated mice by High Pure Viral Nucleic Acid Kit (Roche). DNA
532 fragments of Sad23L and Ad49L vectors were amplified by nested PCR with primers
533 specific to adenoviral hexon sequences (Table S4) (23).

534

535 **Statistical analyses**

536 Data are analyzed with unpaired two-tailed t test, one-way ANOVA. Neutralizing
537 antibody titer data were log transformed before analysis. Neutralizing antibody titer
538 data generated by the RBD-BAb and sVNT or S-BAb and pVNT assays were
539 compared using Spearman nonparametric correlation. Statistically significant
540 differences are indicated with asterisks (* $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$). All
541 graphs are generated with GraphPad Prism 7 software.

542

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636

637

638 **SUPPLEMENTARY MATERIALS**

639 Fig. S1. Histopathological examination from Sad23L-nCoV-S and Ad49L-nCoV-S

640 vaccines inoculated C57BL/6 mice

641 Fig. S2. Kinetic change of hematological and clinical biochemistry indexes during the

642 course of vaccinated or sham control rhesus macaques

643 Fig. S3. Specific T cell response of splenocytes from C57BL/6 mice (n=5/group)

644 immunized with a single dose of Sad23L-nCoV-S or Ad49L-nCoV-S vaccine

645 Fig. S4. IgG subclass antibodies against RBD protein in sera of C57BL/6 and

646 BALB/c mice immunized by prime only or prime-boost with Sad23L-nCoV-S and

647 Ad49L-nCoV-S vaccines

648 Fig. S5. Frequency of IL-2 expressing CD4⁺/CD8⁺ T cell responses of splenocytes

649 from prime-boost immunized C57BL/6 and BALB/c mice with Sad23L-nCoV-S and

650 Ad49L-nCoV-S vaccines

651 Fig. S6. IL-4 secreting T cell response in prime-boost immunized rhesus macaques

652 with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines

653 Fig. S7. Frequency of intracellular TNF α and IL-2 expressing T cell response in

654 PBMCs from rhesus macaques immunized with Sad23L-nCoV-S and Ad49L-nCoV-S

655 vaccines or sham controls

656 Fig. S8. Measurement of serum NAb titers to Ad49L and Sad23L vectors in

657 vaccinated macaques

658 Fig. S9. Examination of SARS-CoV-2 S protein in the tissues of Sad23L-nCoV-S and
659 Ad49L-nCoV-S immunized mice

660 Table S1. Basic information for rhesus macaques pre-vaccination

661 Table S2: Measuring of hematological and biochemistry indexes of rhesus macaques
662 in the course of pre- and post-vaccination with Sad23L-nCoV-S and Ad49L-nCoV-S
663 vaccines

664 Table S3. Peptides derived from amino acid sequences of SARS-CoV-2 S protein
665 used in ELISpot and ICS

666 Table S4. Nested-PCR primers specific for hexon of Sad23L or Ad49L vector

667

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683 **Author contributions**

684 C.L., L.S., T.L. and Y.Z. designed the research. L.S., P.Z., B.L., C.Y., C.L.L., Q.W.,
685 L.Z. and T.L. carried out the experiments. X.T., J.L., S.H., J.Z. and Y.F. provided
686 COVID-19 patient or healthy blood donor samples. S.L., C.L., Y.Z. and J.P.A.
687 analyzed data and wrote the paper.

688 **Competing interests:** S.L., P.Z., B.L., and C.L.L. were partly sponsored by BRK

689 company. All other authors declare that they have no competing interests.

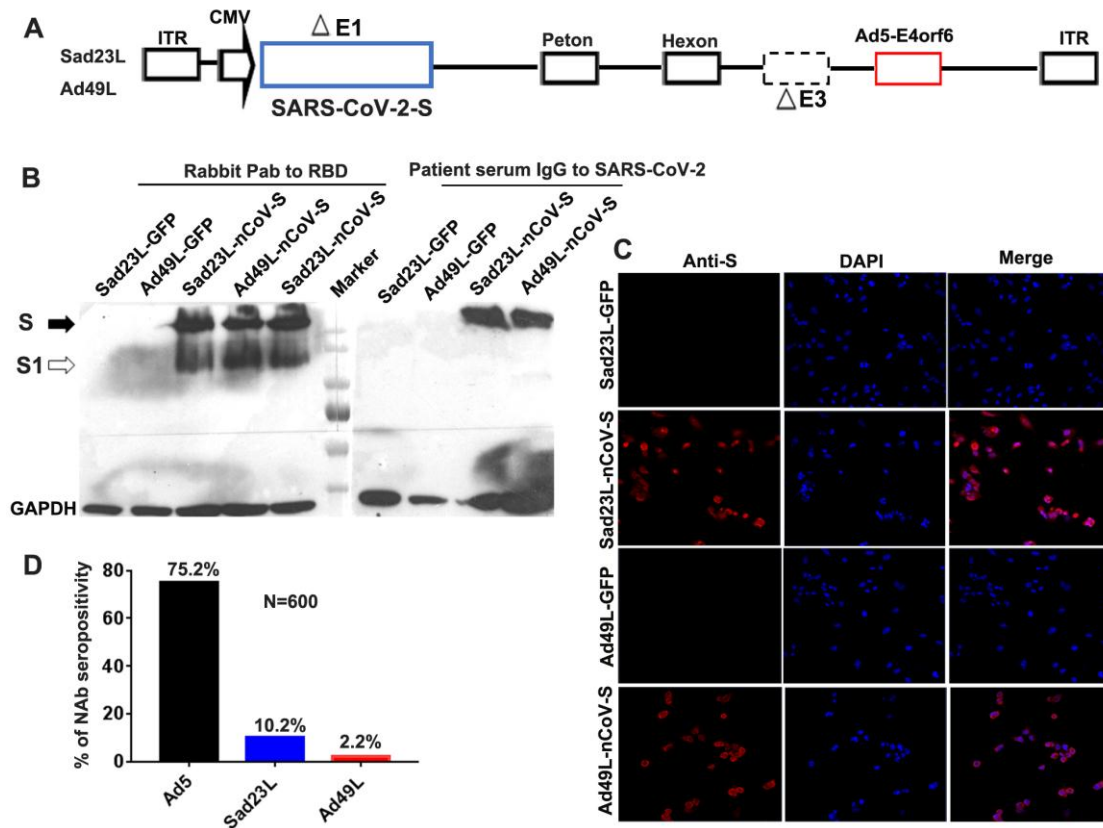
690 **Data and materials availability**

691 All data associated with this paper can be found in the main text or the supplementary

692 materials. Two novel adenoviral vectors (Sad23L and Ad49L) are available under a

693 material transfer agreement with BRK upon request to the corresponding author.

694 **Figures and legends**



695

696 **Fig 1. Characterization of Sad23L-nCoV-S and Ad49L-nCoV-S vaccines.** (A)

697 Recombinant adenovirus constructs Sad23L-nCoV-S and Ad49L-nCoV-S carrying

698 the full-length S gene of SARS-CoV-2 under CMV promotor regulation within the

699 deleted E1 region of Sad23L or Ad49L vector. (B) Western blot analysis for the

700 expression of S protein from Sad23L-nCoV-S or Ad49L-nCoV-S infected HEK-293A

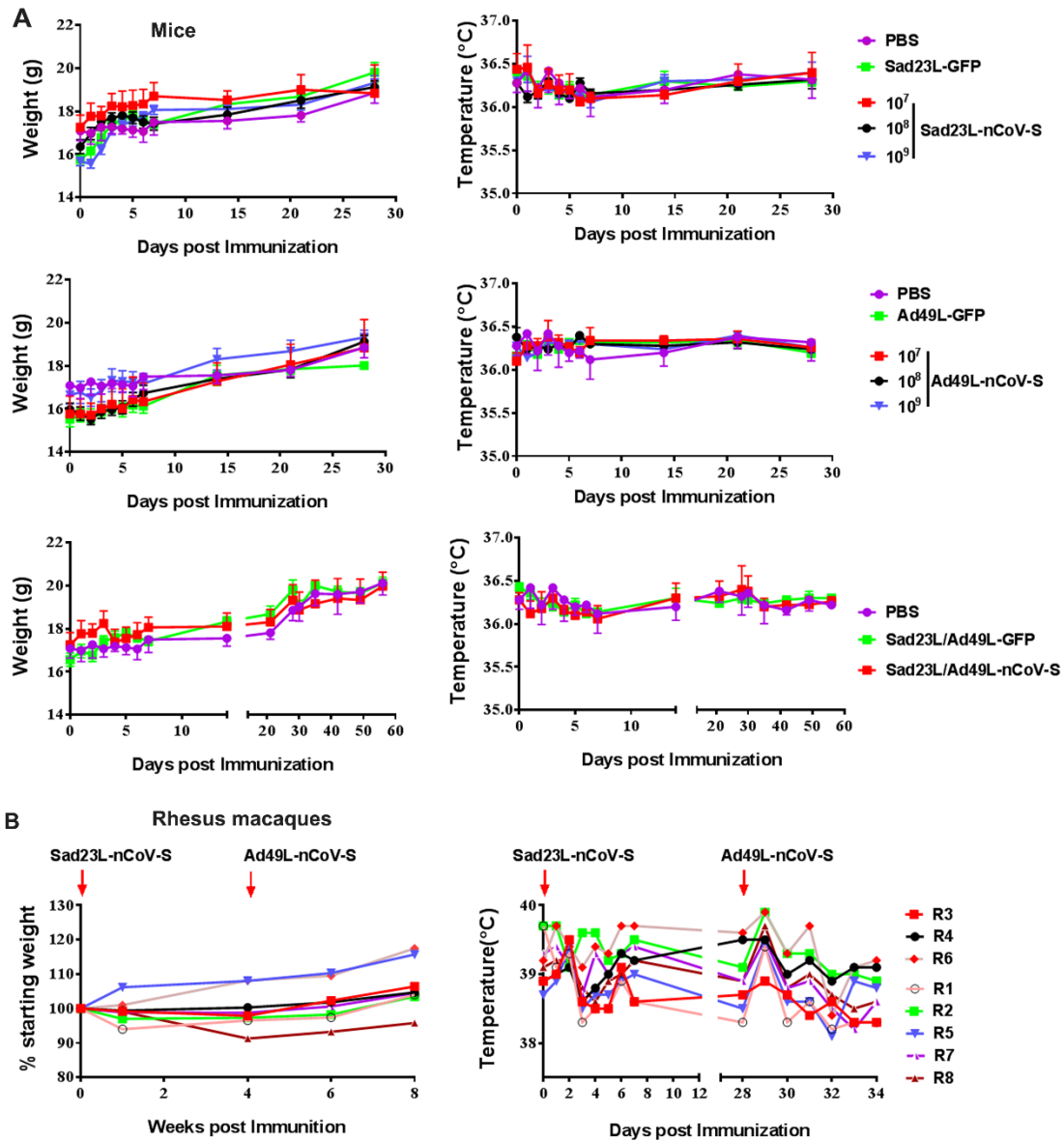
701 cell lysates by rabbit polyclonal antibody to RBD and heat-inactivated COVID-19

702 patent's serum IgG. Sad23L-GFP or Ad49L-GFP virus infected cells were used as

703 mock controls. (C) Expression of S protein in HEK-293A cells detected by

704 immunofluorescence staining. (D) Seroprevalence of neutralizing antibody to Ad5,

705 Ad49L or Sad23L vector in 600 healthy blood donors.

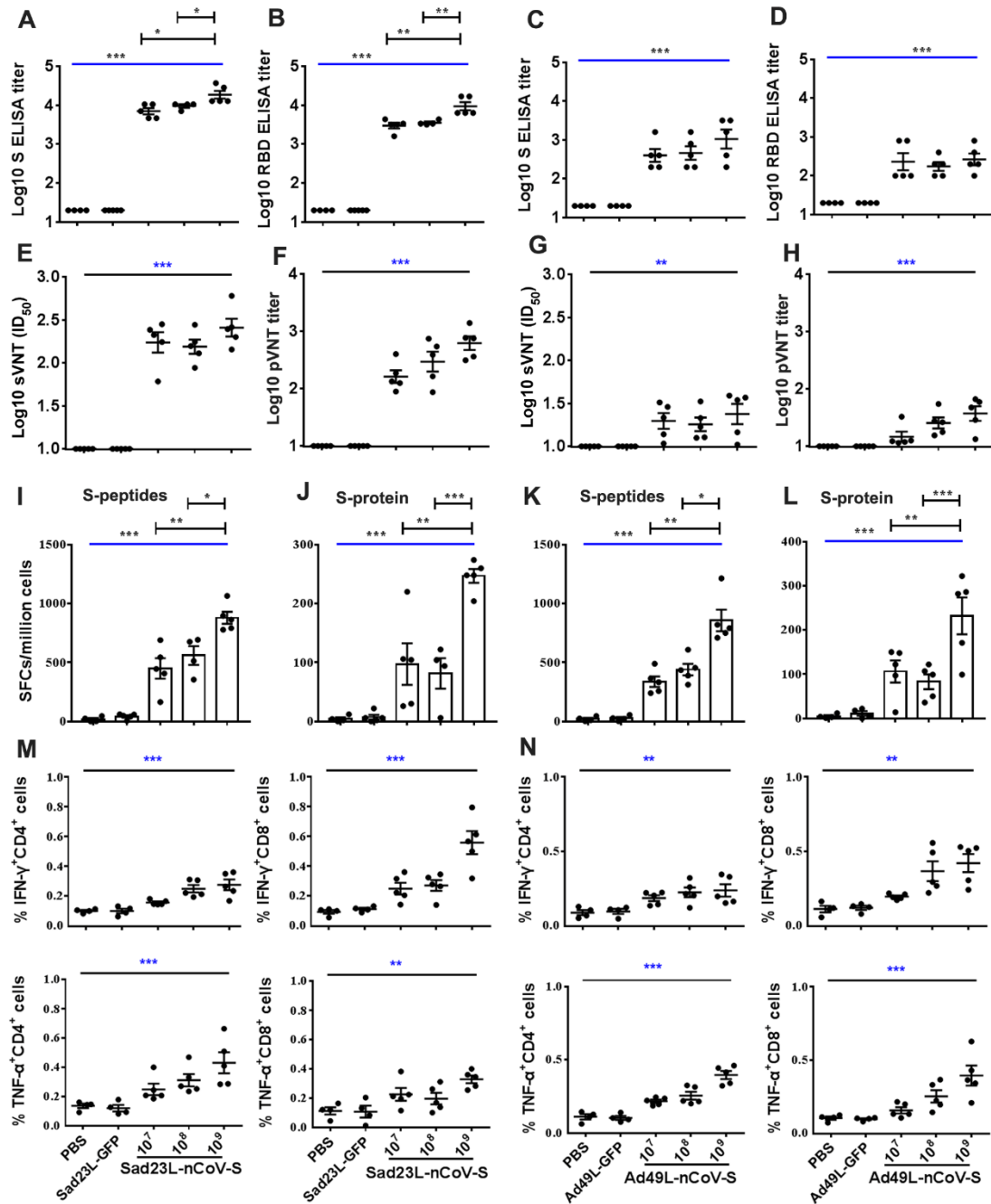


706

707 **Fig. 2. Examination of weight and body temperature of mice and rhesus**
 708 **macaques inoculated with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines.**

709 Animals were intramuscularly immunized by prime only with either Sad23L-nCoV-S
 710 or Ad49L-nCoV-S vaccine at three different doses, or by prime-boost with first
 711 Sad23L-nCoV-S and boost with Ad49L-nCoV-S vaccines at 4 week interval. Body
 712 weight and temperature were monitored during the course up to 60 days for mice or 8
 713 weeks for monkeys. (A) C57BL/6 mice (n=5/group) immunized with a dose of 10⁷,
 714 10⁸ or 10⁹ PFU Sad23L-nCoV-S vaccine, 10⁹ PFU Sad23L-GFP and an equal volume
 715 of PBS controls (top panel); a dose of 10⁷, 10⁸ or 10⁹ PFU Ad49L-nCoV-S vaccine,
 716 10⁹ PFU Ad49L-GFP and an equal volume of PBS controls (middle panel); a dose of

717 10^9 PFU Sad23L-nCoV-S followed by a dose of 10^9 PFU Ad49L-nCoV-S, or 10^9 PFU
718 Sad23L-GFP and 10^9 PFU Ad49L-GFP vectorial viruses and an equal volume of PBS
719 controls (low panel). **(B)** Rhesus monkeys (11-14y) inoculated with a dose of 5×10^9
720 PFU Sad23L-nCoV-S and a dose of 5×10^9 PFU Ad49L-nCoV-S vaccines (R1, R2, R5,
721 R7 and R8), or 5×10^9 PFU Sad23L-GFP and a dose of 5×10^9 PFU Ad49L-GFP
722 controls (R3, R4 and R6).
723



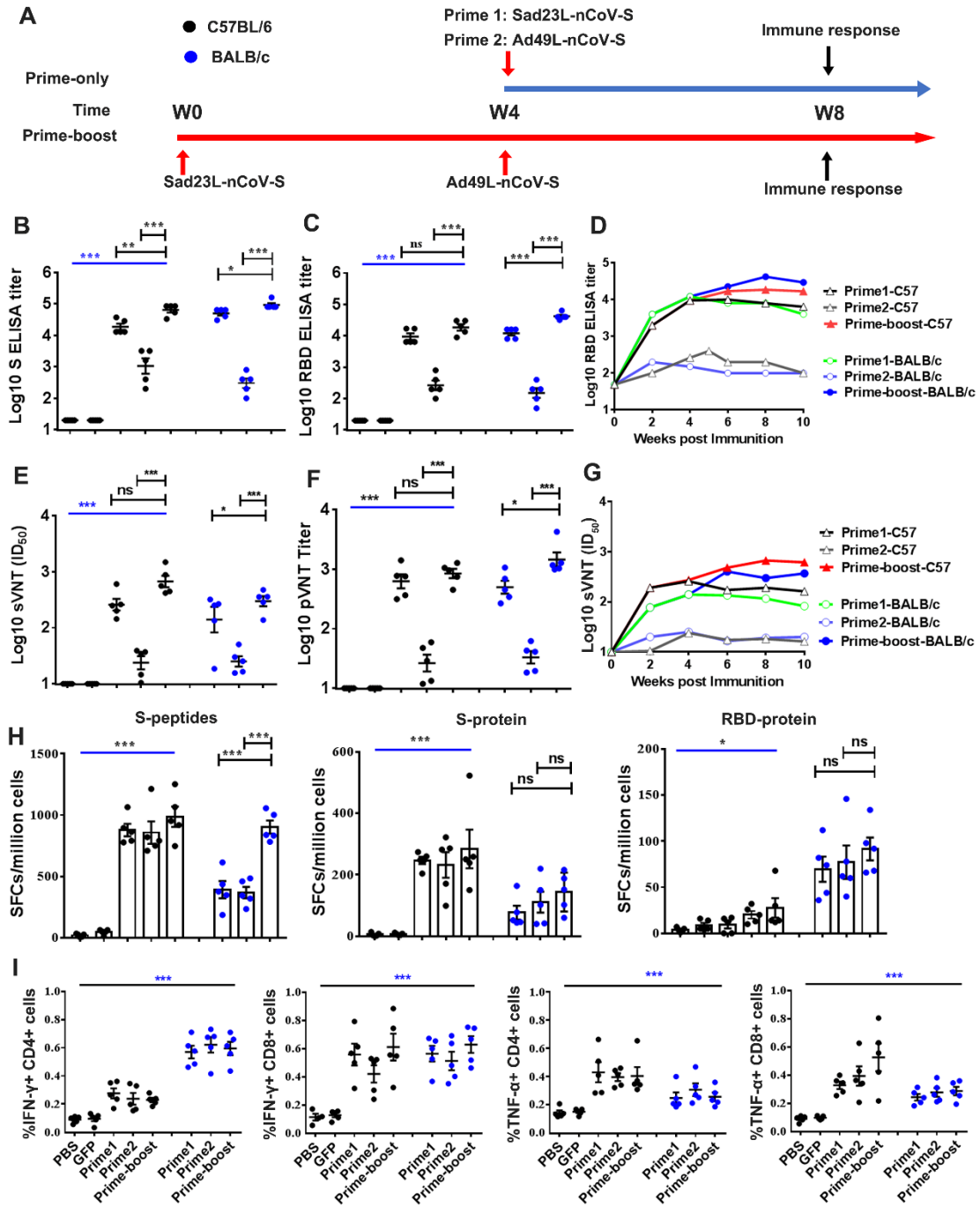
724

725 **Fig 3. Specific antibody and T cell response of C57BL/6 mice inoculated with a**
 726 **single shot of Sad23L-nCoV-S or Ad49L-nCoV-S vaccine at three different doses.**

727 C57BL/6 mice (n=5/group) were immunized by a single dose of 10⁷, 10⁸ or 10⁹ PFU
 728 Sad23L-nCoV-S or Ad49L-nCoV-S vaccine. Mice sera and splenocytes were
 729 collected for measurement of antibody level and T cell response 4 weeks
 730 post-immunization. (A-D) S or RBD binding antibody (S-BAb or RBD-BAb) titers
 731 were obtained by ELISA. (E-H) Neutralizing antibody (NAb) titers were obtained by
 732 surrogate virus-based neutralization test (sVNT) or pseudovirus-based neutralization

733 test (pVNT). **(I-L)** IFN- γ secreting T cell response (spot forming cells [SFCs]/million
734 cells) of splenocytes to S peptides and S protein from Sad23L-nCoV-S or
735 Ad49L-nCoV-S immunized mice was measured by ELISpot, respectively. **(M and N)**
736 Frequency of IFN- γ or TNF- α expressing CD4⁺ and CD8⁺ T cell response of
737 splenocytes to S peptides from Sad23L-nCoV-S or Ad49L-nCoV-S immunized mice
738 was obtained by ICS. Data is shown as mean \pm SEM (standard errors of means). *P*
739 values are analyzed by one-way ANOVA with 2-fold Bonferroni adjustment.
740 Statistically significant differences are shown with asterisks (*, *P*<0.05; **, *P*< 0.01
741 and ***, *P*< 0.001).

742



743

744 **Fig. 4. Specific humoral and cellular immune response of C57BL/6 and BALB/c**

745 **mice to prime-boost immunization with Sad23L-nCoV-S and Ad49L-nCoV-S**

746 **vaccines. (A)** C57BL/6 and BALB/c mice (n=5/group) were prime immunized with a

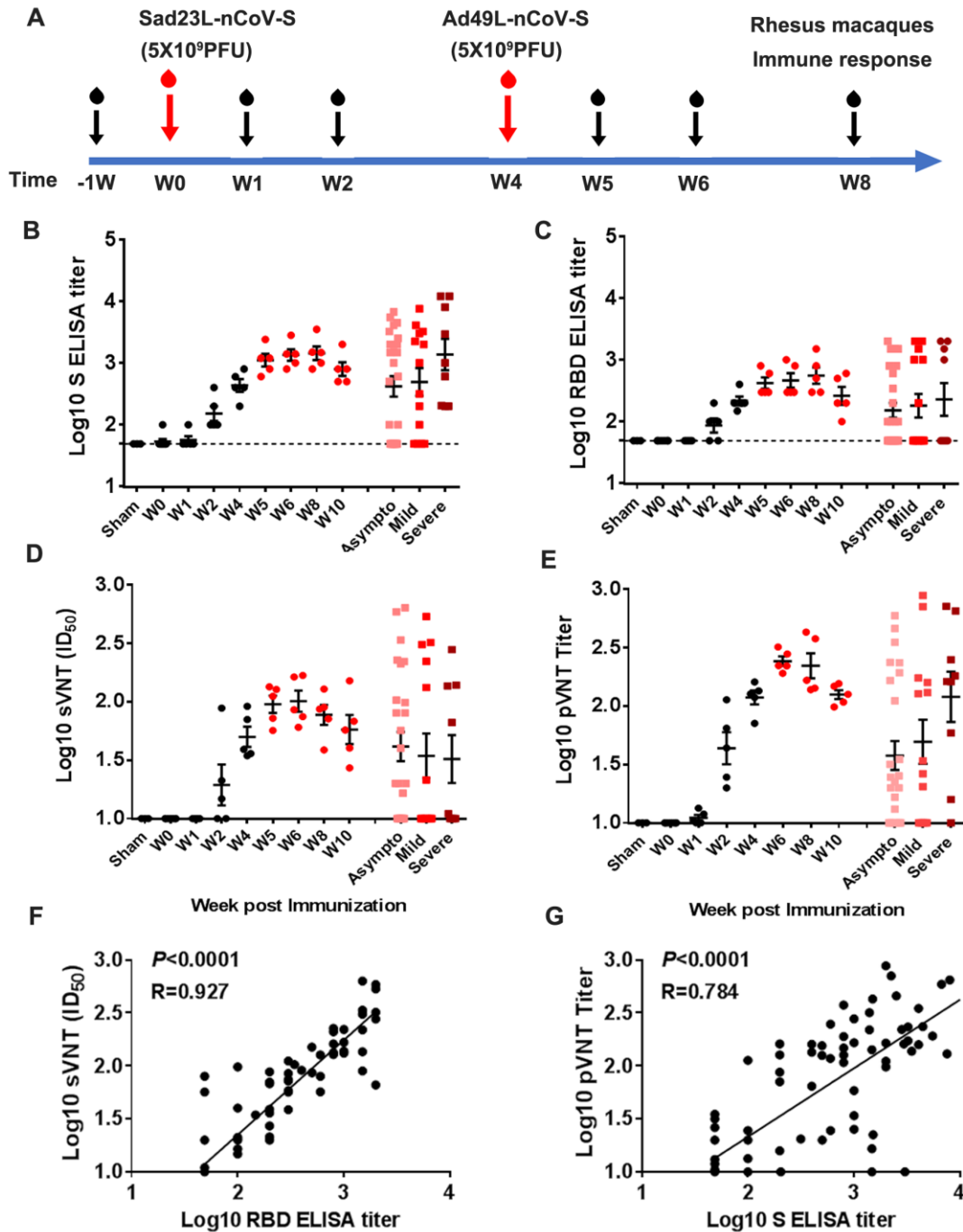
747 dose of 10⁹ PFU Sad23L-nCoV-S vaccine and boosted with a dose of 10⁹ PFU

748 Ad49L-nCoV-S vaccine at 4 week interval. Sera and splenocytes were collected from

749 vaccinated or control mice for measurement of antibody and T cell responses 4 weeks

750 after both prime only and boosting immunizations. **(B-D)** Anti-S-BAb and RBD-BAb

751 titers determined by ELISA. **(E-G)** NAb titers measured by sVNT and pVNT. **(H)**
752 IFN- γ secreting T cell response (SFCs/million cells) to S peptides, S or RBD protein
753 measured by ELISpot. **(I)** Frequency of IFN- γ or TNF- α expressing CD4⁺ and CD8⁺
754 T cell response to S peptides determined by ICS. Data are shown as a mean \pm SEM. *P*
755 values are analyzed with two-tailed t test. Statistically significant differences are
756 shown with asterisks (*, *P*<0.05; **, *P*< 0.01 and ***, *P*< 0.001).
757



758

759 **Fig. 5. Antibody reactivity of Rhesus macaques to prime-boost vaccination with**

760 **Sad23L-nCoV-S and Ad49L-nCoV-S vaccines. (A) Five rhesus macaques were**

761 **prime immunized with 5×10⁹ PFU of Sad23L-nCoV-S vaccine and boosted with**

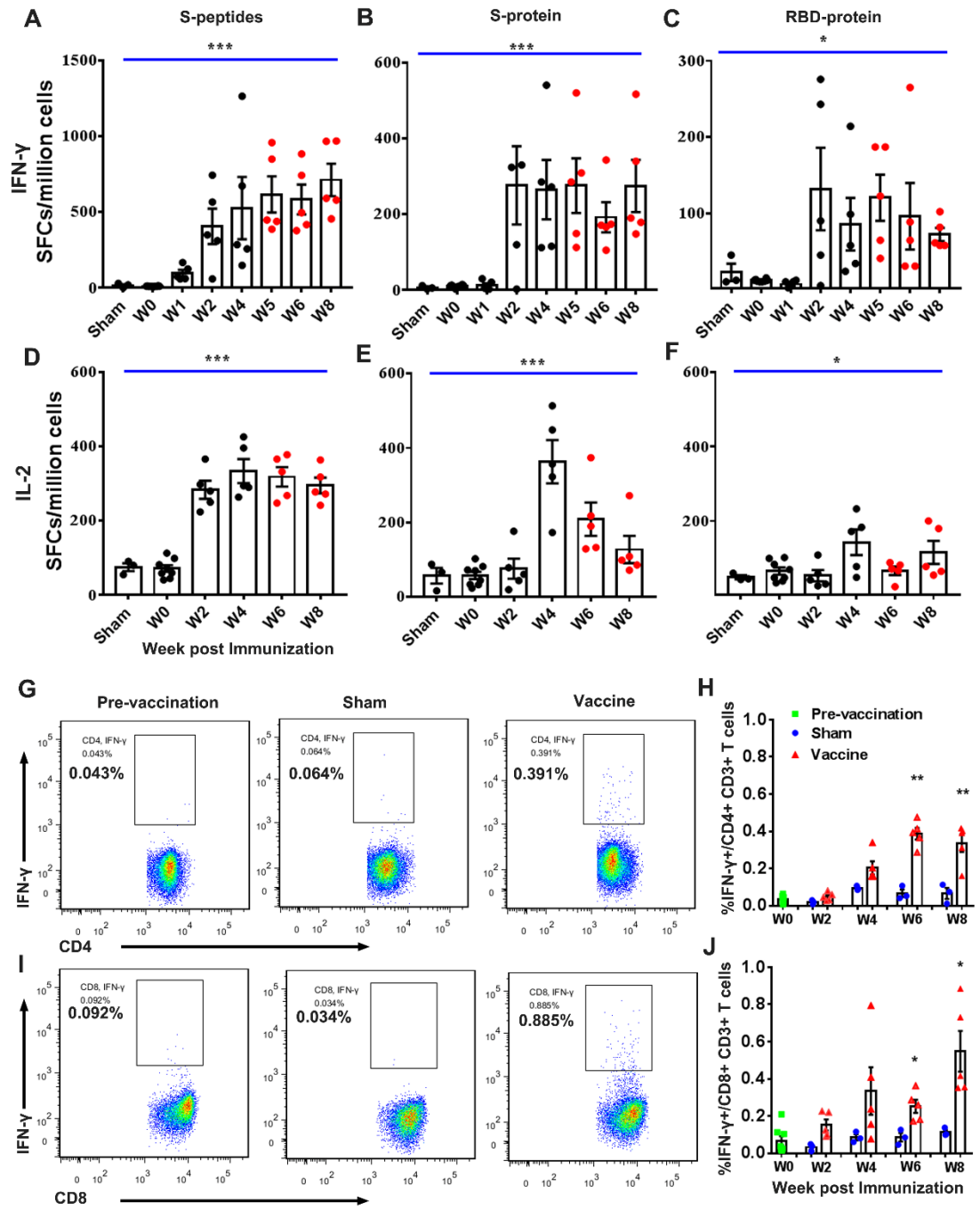
762 **5×10⁹ PFU of Ad49L-nCoV-S vaccine at 4 week interval. Blood samples were**

763 **collected weekly from immunized or sham control macaques. Three macaques first**

764 **immunized with 5×10⁹ PFU of Sad23L-GFP viruses and boosted with 5×10⁹ PFU of**

765 **Ad49L-GFP viruses were used as sham controls. Convalescent serum samples from**

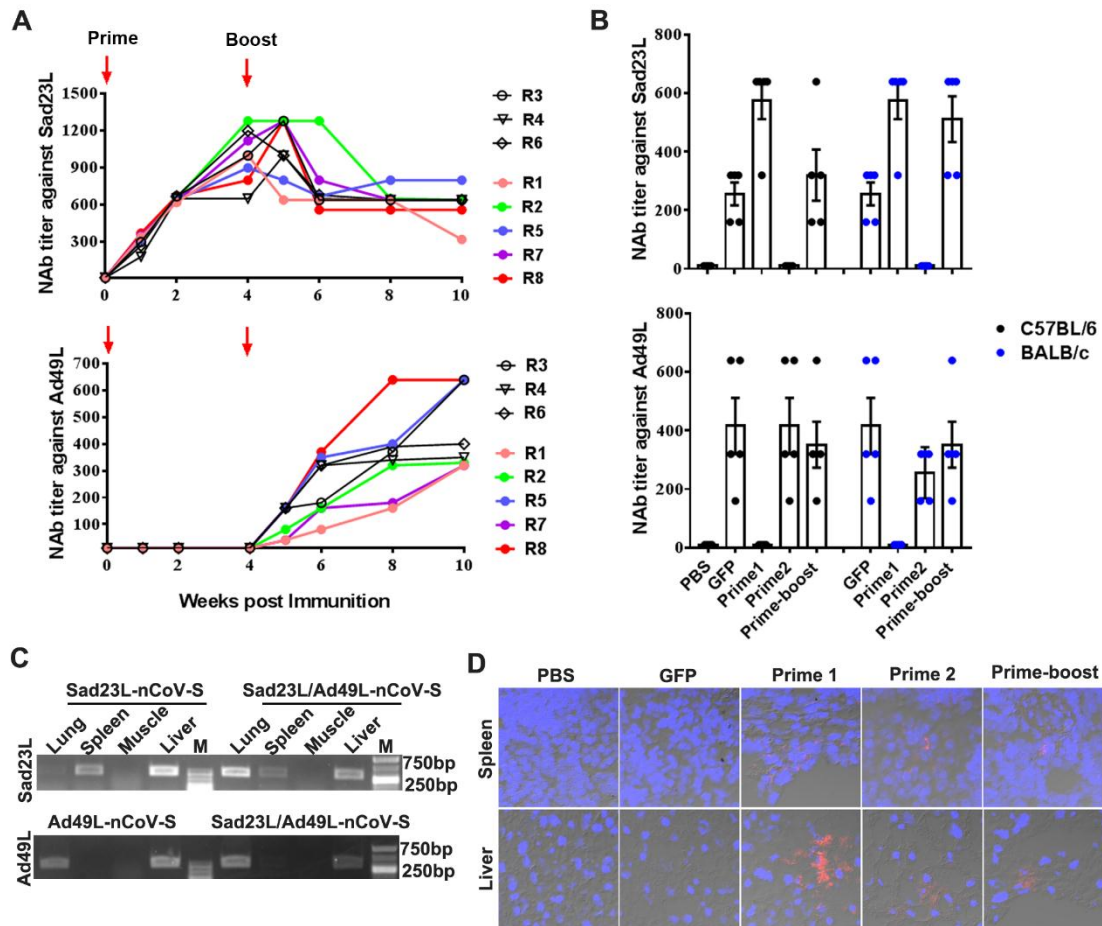
766 25 asymptomatic, 14 mild and 9 severe COVID-19 infected patients were taken as
767 positive controls. **(B and C)** S-BAb and RBD-BAb titers were tested by ELISA. **(D**
768 **and E)** NAb titers were measured by sVNT and pVNT. **(F and G)** Correlation
769 between RBD-BAb and sVNT or S-BAb and pVNT assay were compared using
770 Spearman nonparametric correlation, respectively.
771



772

773 **Fig. 6. Specific T cell response of PBMCs from Rhesus macaques immunized**
 774 **with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines or sham controls by**
 775 **prime-boost vaccination regimen. (A-C) IFN- γ or (D-F) IL-2 secreting T cell**
 776 **response (SFCs/million cells) to S peptides, S or RBD protein was measured by**
 777 **ELISpot. (G-J) Frequency of intracellular IFN- γ expressing CD4⁺/CD8⁺ T cell**
 778 **responses to S peptides was determined by ICS, respectively. Data are shown as mean**

779 \pm SEM. P values are calculated with two-tailed t test. Statistically significant
780 differences are shown with asterisks (*, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$).



781

782 **Fig 7. Biodistribution of Sad23L-nCoV-S and Ad49L-nCoV-S vaccines in**

783 **inoculated animals. (A)** Serum NAb titers to Sad23L and Ad49L vectors were

784 measured in macaques immunized by prime-boost inoculation with two vaccines at 4

785 week interval, or **(B)** in C57BL/6 and BALB/c mice 4 weeks post prime only or

786 prime-boost vaccination with two vaccines or vectorial controls. **(C)** Nested-PCR

787 amplification of Sad23L or Ad49L-hexon gene (500bp) in tissues of C57BL/6 mice 4

788 weeks after inoculation by prime only or prime-boost immunization with

789 Sad23L-nCoV-S and Ad49L-nCoV-S vaccines. **(D)** Expression of S protein in

790 splenocytes and hepatocytes of tissue frozen sections from vaccine immunized or

791 control C57BL/6 mice by immunofluorescence staining with a human monoclonal

792 antibody to SARS-CoV-2 S and DAPI.

Supplementary Materials

Prime-boost vaccination of mice and Rhesus macaques with two novel adenovirus vectored COVID-19 vaccine candidates

The PDF file includes

Fig. S1. Histopathological examination from Sad23L-nCoV-S and Ad49L-nCoV-S vaccines inoculated C57BL/6 mice

Fig. S2. Kinetic change of hematological and clinical biochemistry indexes during the course of vaccinated or sham control rhesus macaques

Fig. S3. Specific T cell response of splenocytes from C57BL/6 mice (n=5/group) immunized with a single dose of Sad23L-nCoV-S or Ad49L-nCoV-S vaccine

Fig. S4. IgG subclass antibodies against RBD protein in sera of C57BL/6 and BALB/c mice immunized by prime only or prime-boost with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines

Fig. S5. Frequency of IL-2 expressing CD4⁺/CD8⁺ T cell responses of splenocytes from prime-boost immunized C57BL/6 and BALB/c mice with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines

Fig. S6. IL-4 secreting T cell response in prime-boost immunized rhesus macaques with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines

Fig. S7. Frequency of intracellular TNF α and IL-2 expressing T cell response in PBMCs from rhesus macaques immunized with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines or sham controls

Fig. S8. Measurement of serum NAb titers to Ad49L and Sad23L vectors in vaccinated macaques

Fig. S9. Examination of SARS-CoV-2 S protein in the tissues of Sad23L-nCoV-S and Ad49L-nCoV-S immunized mice

Table S1. Basic information for rhesus macaques pre-vaccination

Table S2: Measuring of hematological and biochemistry indexes of rhesus macaques in the course of pre- and post-vaccination with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines

Table S3. Peptides derived from amino acid sequences of SARS-CoV-2 S protein used in ELISpot and ICS

Table S4. Nested-PCR primers specific for hexon of Sad23L or Ad49L vector

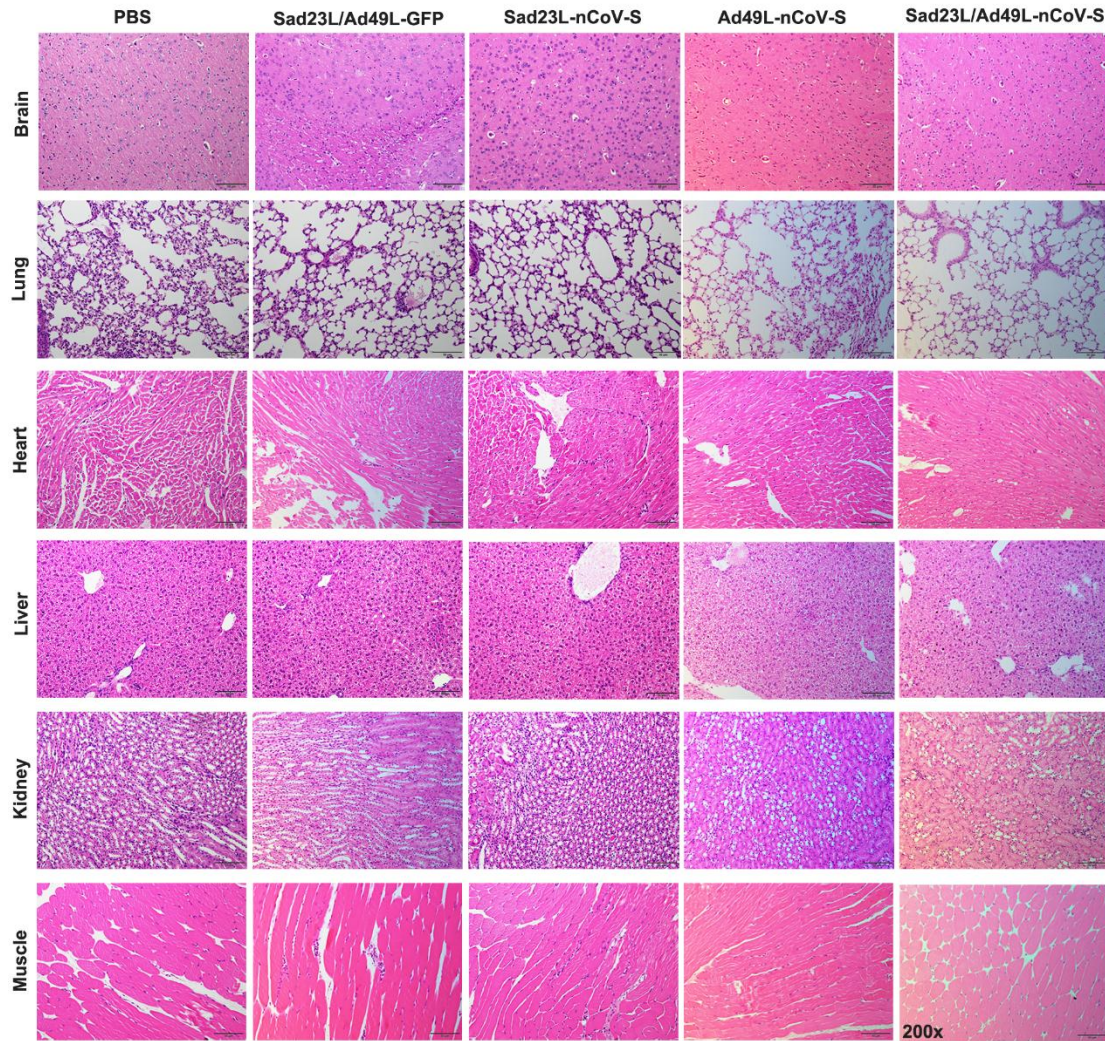


Fig. S1. Histopathological examination from Sad23L-nCoV-S and Ad49L-nCoV-S vaccines inoculated C57BL/6 mice. Histopathological examination was carried out for brain, lung, heart, liver, kidney and muscle tissues (at intramuscular injection site and para-tissues) of mice in 4 weeks post prime only or prime-boost immunization with these two vaccines. Tissues were stained with hematoxylin and eosin.

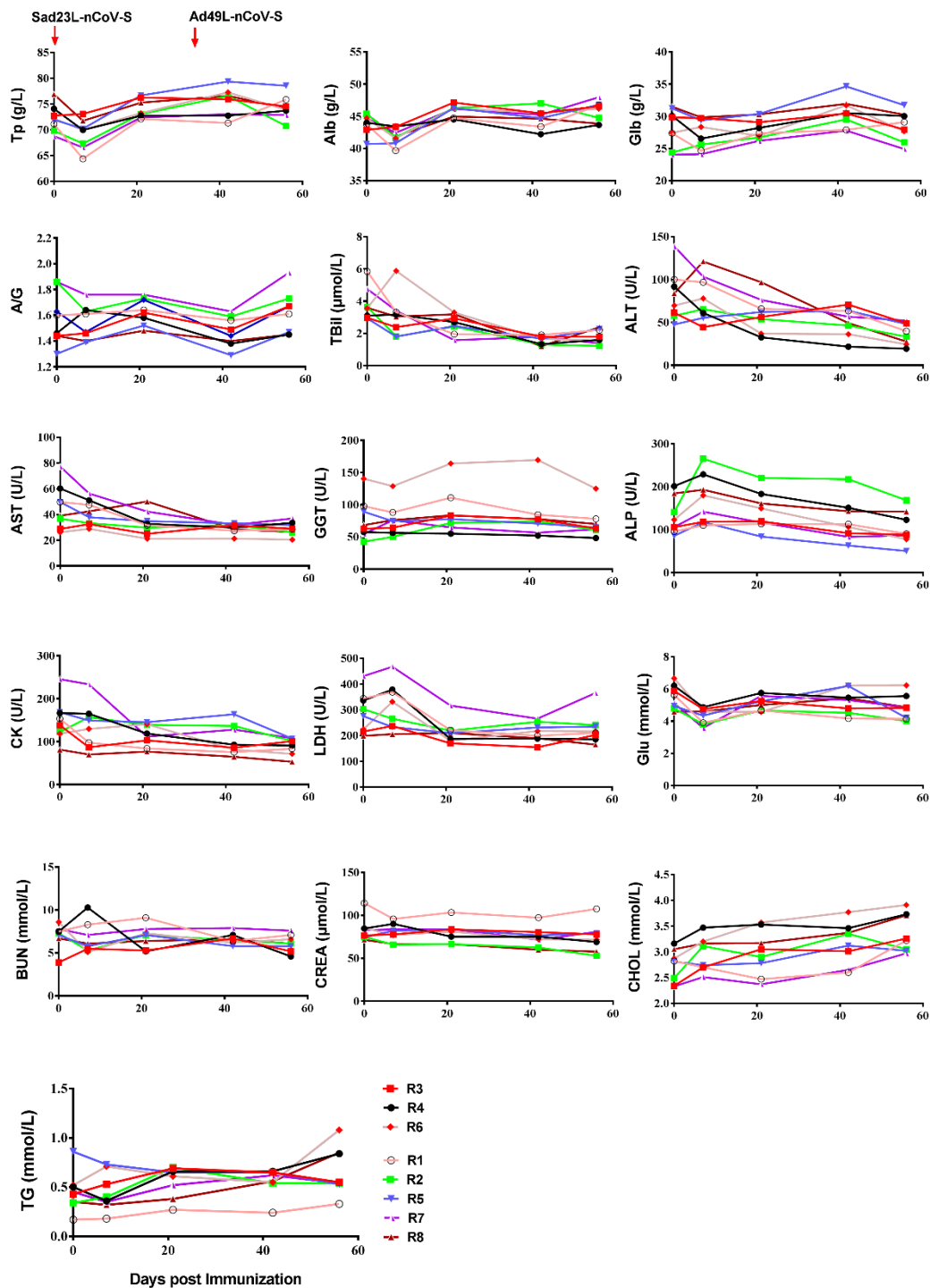


Fig. S2. Kinetic change of hematological and clinical biochemistry indexes during the course of vaccinated or sham control rhesus macaques. Rhesus macaques were intramuscularly inoculated by prime-boost immunization with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines at an interval of 4 weeks. A panel of

hematological and biochemical indexes were measured from blood samples at different time points. TP, Total protein; Alb, Albumin; Glb, Globulin; A/G, Albumin/globulin ratio; TBil, Total bilirubin; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, γ -glutamyltranspeptidase; ALP, Alkaline phosphatase; CK, Creatine kinase; LDH, Lactate dehydrogenase; Glu, Glucose; BUN, Blood urea nitrogen; CREA, Creatinine; CHOL, Total cholesterol; TG, Triglycerides. R1-R8 indicate rhesus macaques.

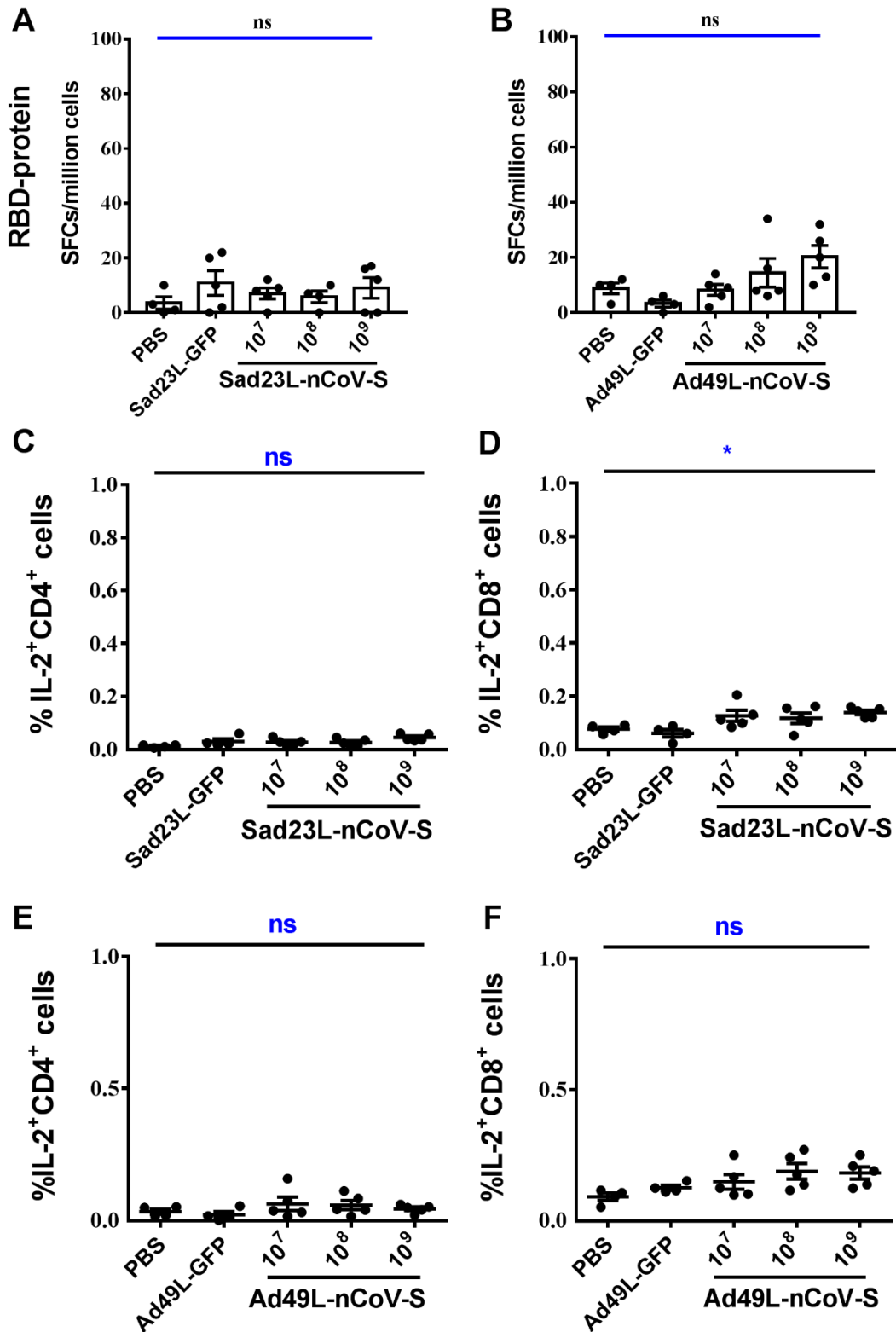


Fig. S3. Specific T cell response of splenocytes from C57BL/6 mice (n=5/group) immunized with a single dose of Sad23L-nCoV-S or Ad49L-nCoV-S vaccine. (A

and **B**) Specific IFN- γ secreting T cell response (spot forming cells [SFCs] per million cells) to RBD protein was measured by ELISpot, respectively. (**C** and **D**) Frequency of IL-2⁺ CD4⁺ or CD8⁺ T cell response to S peptides from mice elicited by Sad23L-nCoV-S vaccines was measured by ICS, respectively. (**E** and **F**) Frequency of IL-2⁺ CD4⁺ or CD8⁺ T cell response to S peptides from mice immunized by Ad49L-nCoV-S vaccines was measured by ICS, respectively. Data were shown as means \pm SEM (standard errors of means). *P* values were analyzed by one-way ANOVA with 2-fold Bonferroni's test. Statically significant differences were showed with asterisks (*, *P*<0.05; **, *P*< 0.01 and ***, *P*< 0.001). ns, *P*>0.05 and no significant difference.

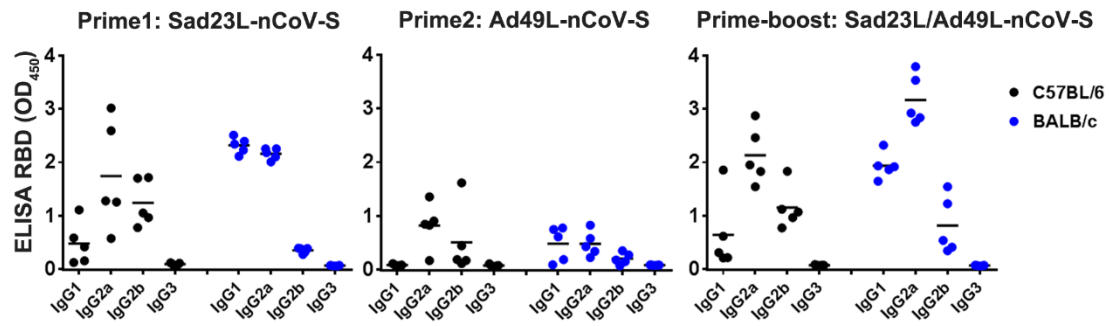


Fig. S4. IgG subclass antibodies against RBD protein in sera of C57BL/6 and BALB/c mice immunized by prime only or prime-boost with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines. The sera were collected 4 weeks post prime only or prime-boost immunized C57BL/6 and BALB/c mice. IgG subclass antibodies in sera were detected against RBD protein by ELISA with secondary antibody-HRP conjugate specific to mouse IgG subclass.

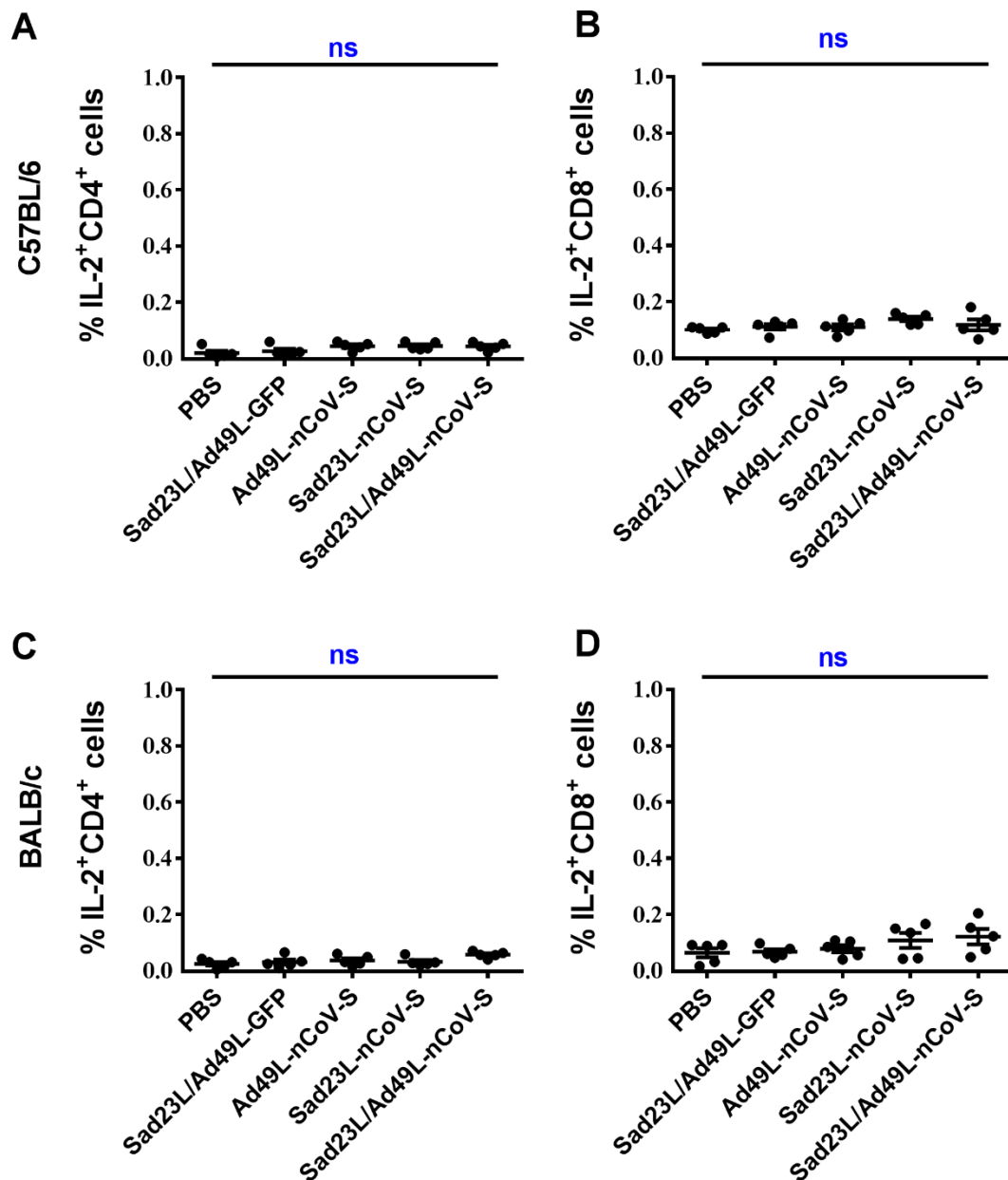


Fig. S5. Frequency of IL-2 expressing CD4⁺/CD8⁺ T cell responses of splenocytes from prime-boost immunized C57BL/6 and BALB/c mice with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines. (A and B) Frequency of IL-2⁺ CD4⁺ or CD8⁺ T cell responses of splenocytes to S peptides from C57BL/6, or (C and D) from BALB/c mice. Data were shown as means ± SEM (standard errors of means). *P* values were analyzed by one-way ANOVA. ns, *P*>0.05 and no significant difference.

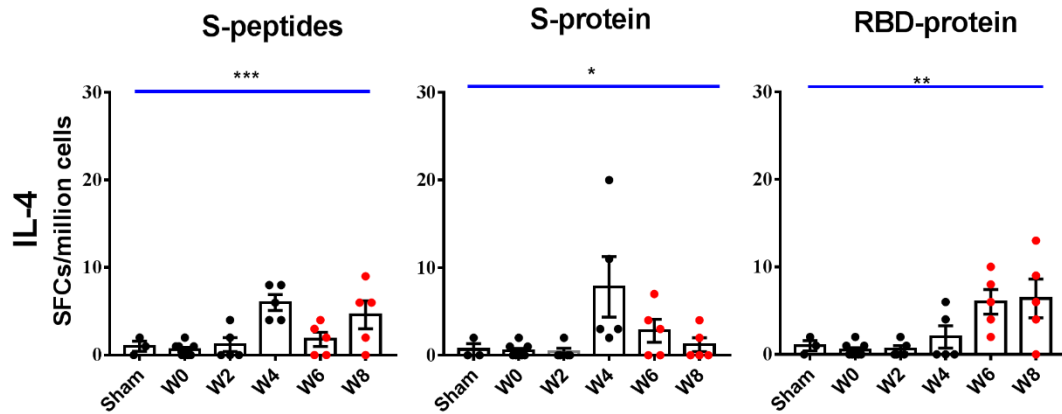


Fig. S6. IL-4 secreting T cell response in prime-boost immunized rhesus macaques with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines. The number of specific IL-4 secreting T cells (SFCs/million cells) to S peptides, S or RBD protein in PBMCs of monkeys was measured by ELISpot, respectively. Data were shown as means \pm SEM (standard errors of means). *P* values were analyzed by one-way ANOVA with 2-fold Bonferroni's test. Statically significant differences were showed with asterisks (*, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$). ns, $P > 0.05$ and no significant difference.

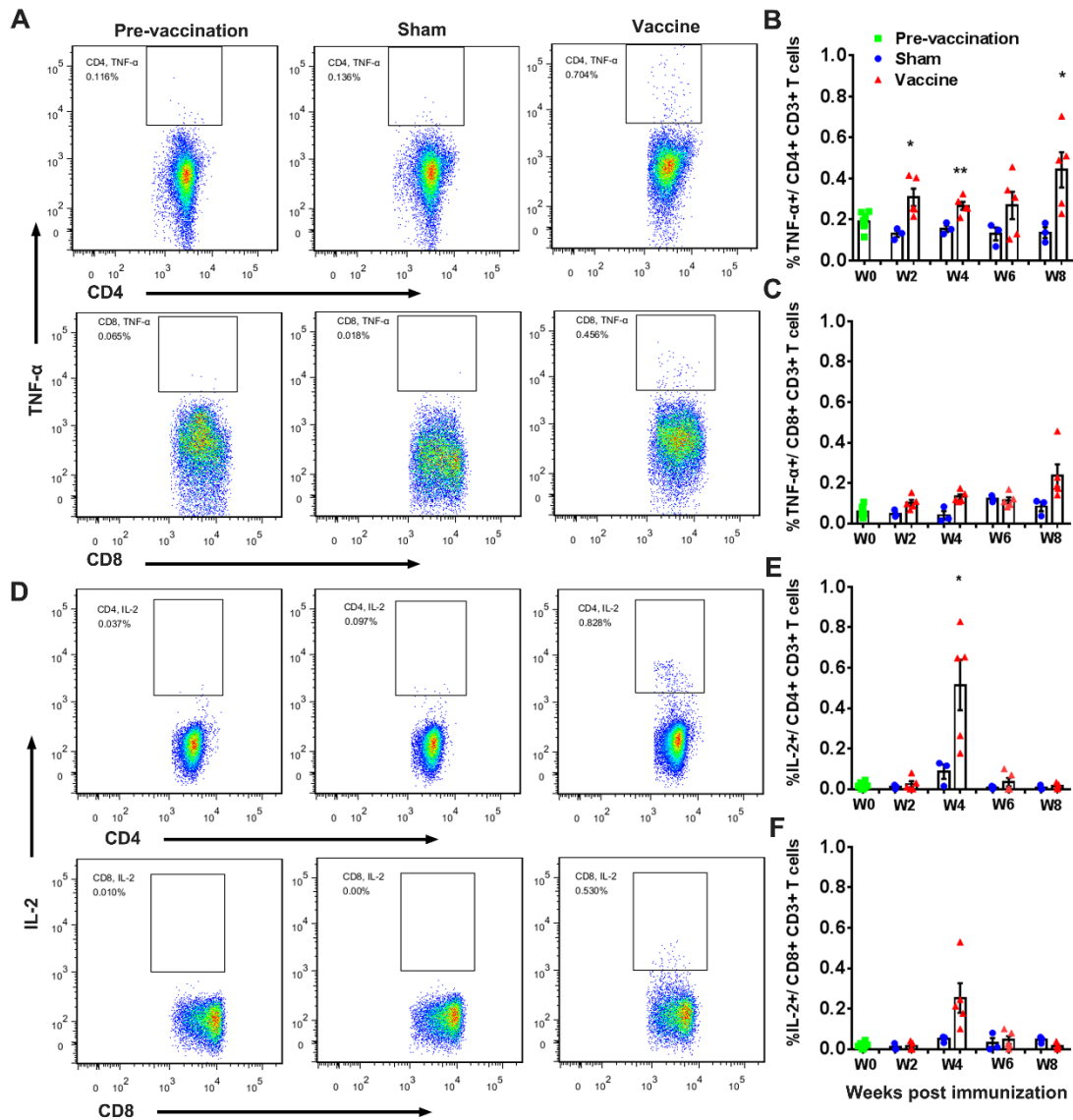


Fig. S7. Frequency of intracellular TNF α and IL-2 expressing T cell response in PBMCs from rhesus macaques immunized with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines or sham controls. (A-C) Frequency of TNF- α ⁺ CD4⁺/CD8⁺ T cell response to S peptides. (D-F) Frequency of TNF- α ⁺ CD4⁺/CD8⁺ T cell response to S peptides. Data were shown as means \pm SEM (standard errors of means). *P* values were analyzed by Student's *t* test. Statically significant differences were showed with asterisks (*, *P* < 0.05; **, *P* < 0.01 and *, *P* < 0.001). ns, *P* > 0.05 and no significant difference.**

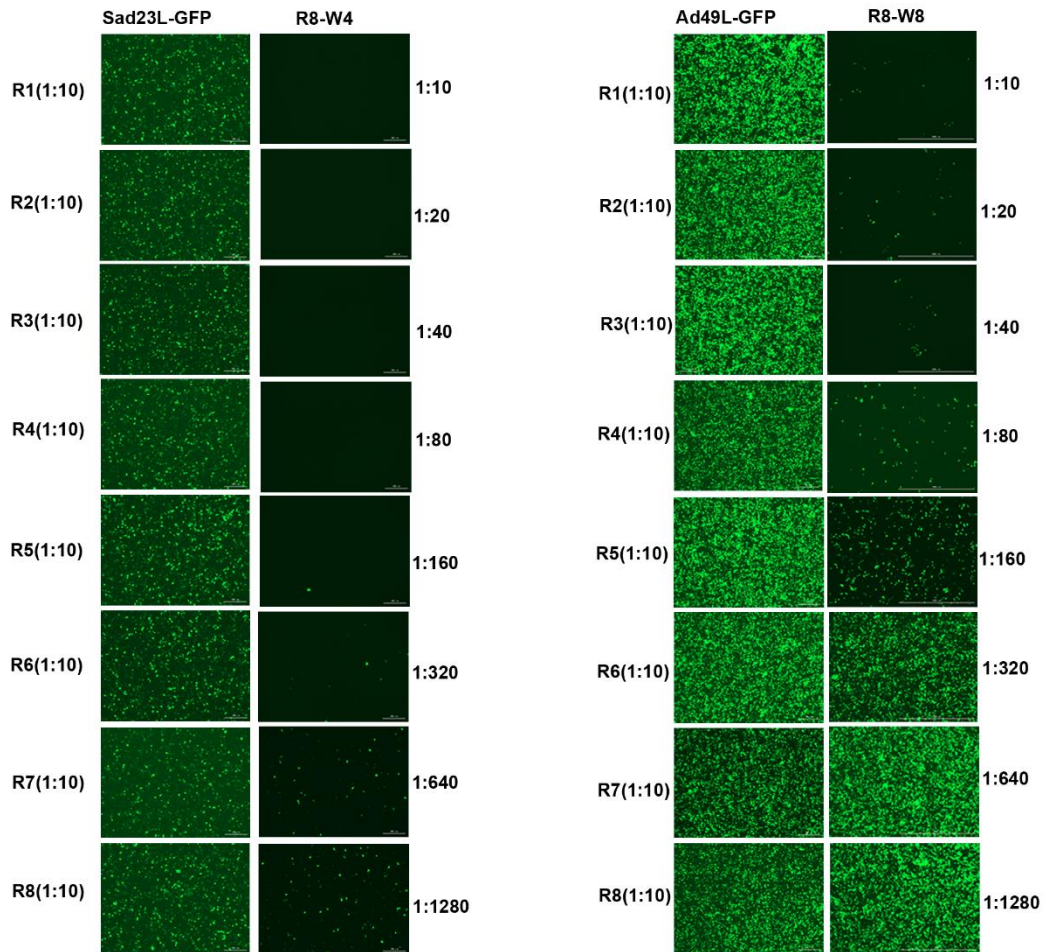


Fig. S8. Measurement of serum NAb titers to Ad49L and Sad23L vectors in vaccinated macaques. Two representative plasma samples of vaccinated macaque (R8 at weeks 4 and 8) were tested on HEK-293A cells for neutralizing Sad23L-GFP and Ad49L-GFP vectorial viruses by green fluorescent activity assay. Neutralization titer was defined as the maximum serum dilution that neutralized 50% of Green fluorescent activity.

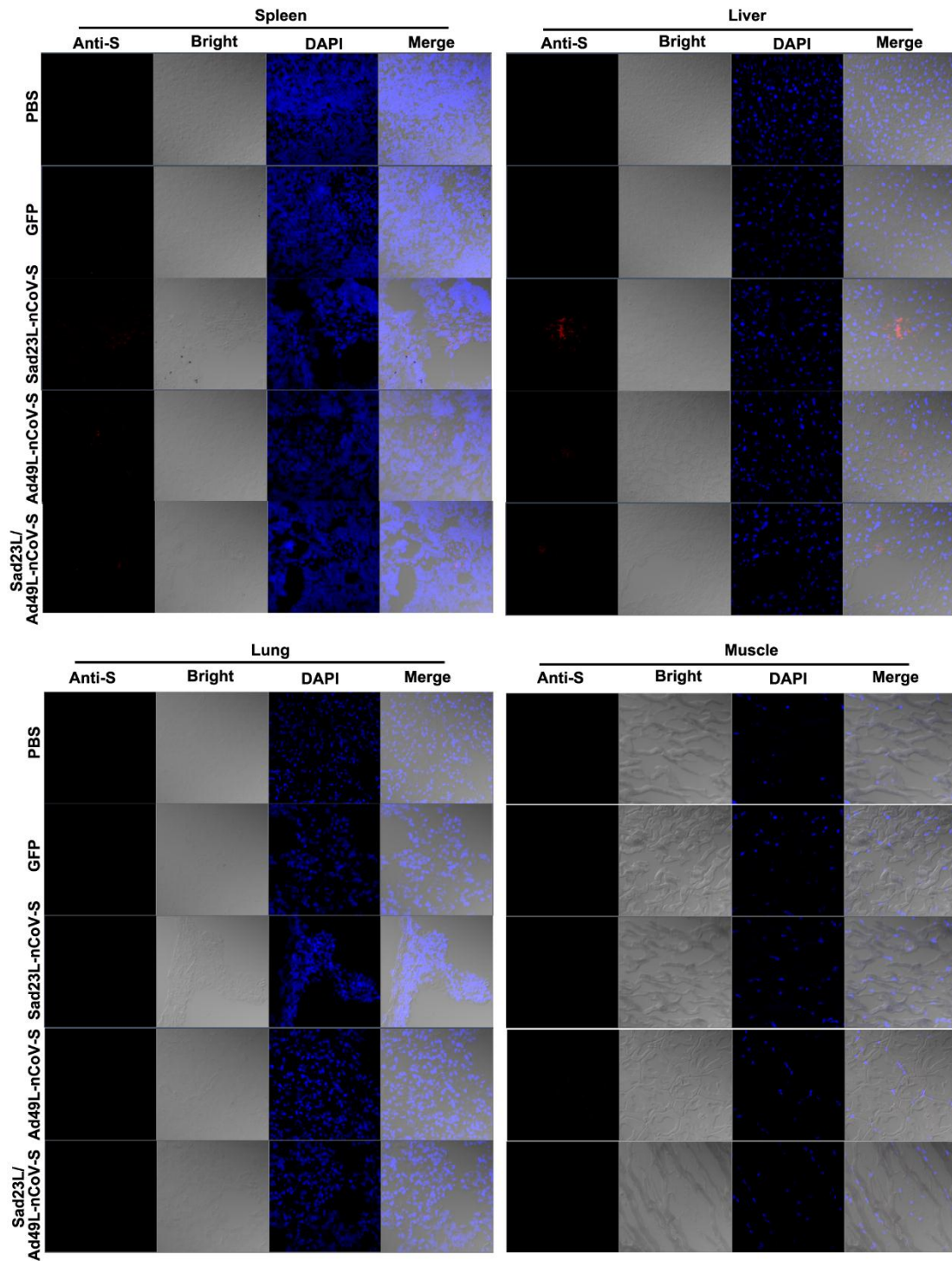


Fig. S9. Examination of SARS-CoV-2 S protein in the tissues of Sad23L-nCoV-S and Ad49L-nCoV-S immunized mice. Spleen, liver, Lung and muscle tissues of immunized C57BL/6 mice were examined by immunofluorescence staining with a human monoclonal antibody to SARS-CoV-2 S and DAPI.

Table S1. Basic information of rhesus macaques pre-vaccination

Group	ID	Macaques (gender)	Age (year)	Weight (kg)	NAb titer of pre-exposure to		
					Ad5	Sad23L	Ad49L
Sham	00159	R3(M)	13	11.79	<1:10	<1:10	<1:10
	06120011	R4(M)	13	6.76	<1:10	<1:10	<1:10
	08050591	R6(M)	12	7.99	<1:10	<1:10	<1:10
Vaccination	01191	R1 (M)	14	11.56	<1:10	<1:10	<1:10
	01399	R2(M)	13	7.51	<1:10	<1:10	<1:10
	00943	R5(M)	13	9.32	<1:10	<1:10	<1:10
	00807	R7(M)	12	10.81	<1:10	<1:10	<1:10
	08040661	R8 (M)	11	8.72	<1:10	<1:10	<1:10

Table S2. Measuring of hematological and biochemistry indexes of rhesus macaques in the course of pre- and post-vaccination with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines

Animal Days		R1	R2	R3	R4	R5	R6	R7	R8
		TP	0	71.12	69.8	72.78	74.1	71.96	72.16
g/L	7	64.37	67.35	73.1	69.97	70.21	69.94	66.56	71.74
	21	72.1	73.03	76.25	72.76	76.67	73.23	72.38	75.28
	42	71.32	76.57	75.93	72.75	79.37	77.27	73.05	76.59
	56	75.88	70.74	74.55	73.72	78.55	73.88	72.9	74.13
Alb	0	43.74	45.41	42.93	44.01	40.7	44.72	44.71	45.38
g/L	7	39.68	41.73	43.36	43.43	40.82	41.59	42.44	41.89
	21	44.74	46.31	47.16	44.55	46.3	46.27	46.18	44.97
	42	43.41	47.02	45.47	42.23	44.72	45.54	45.25	44.64
	56	46.77	44.79	46.59	43.68	46.81	46.19	47.98	43.87
Glb	0	27.38	24.39	29.85	30.09	31.26	27.44	24.07	31.51
g/L	7	24.69	25.62	29.74	26.54	29.39	28.35	24.12	29.85
	21	27.36	26.72	29.09	28.21	30.37	26.96	26.2	30.31
	42	27.91	29.55	30.46	30.52	34.65	31.73	27.8	31.95
	56	29.11	25.95	27.96	30.04	31.74	27.69	24.92	30.26
A/G	0	1.6	1.86	1.44	1.46	1.3	1.63	1.86	1.44
	7	1.61	1.63	1.46	1.64	1.39	1.47	1.76	1.4
	21	1.64	1.73	1.62	1.58	1.52	1.72	1.76	1.48
	42	1.56	1.59	1.49	1.38	1.29	1.44	1.63	1.4
	56	1.61	1.73	1.67	1.45	1.47	1.67	1.93	1.45
TBil	0	5.85	3.65	2.97	3.1	2.93	3.56	4.76	3.59
μmol/L	7	3.32	1.81	2.39	3.17	1.8	5.88	3.41	3.04
	21	1.95	2.42	2.93	2.7	2.46	3.3	1.58	3.19
	42	1.91	1.31	1.8	1.33	1.67	1.66	1.78	1.2
	56	2.21	1.22	1.8	1.6	2.28	1.55	1.38	2.38
ALT	0	100	57.7	61.3	91.8	47.7	69.7	138.6	83.8
U/L	7	96.8	65.5	44.5	61.1	55.4	78	103.3	121
	21	65.9	53.9	56.4	32.6	62.2	36.9	76.3	96.8
	42	63.4	46.4	70.9	21.8	63.7	36.4	56.7	49.3
	56	39.9	33.3	49	19.4	48.1	24.5	52.2	27.7
AST	0	49.7	36.7	28.8	60.2	49.7	26.2	77.3	39.1
U/L	7	47.5	33.3	32.5	51	37.9	28.9	56.3	42.4
	21	31.7	29.8	25	32.7	34.8	21.2	42.3	50.1
	42	27.4	31.6	31.6	29.8	33.1	21.3	31.9	28.4
	56	28.5	26.2	29	33.5	31.7	20.5	36.9	26.5

GGT	0	97.9	42.7	62.7	57.6	89.2	140.5	54.5	68.3
	7	88	50.1	64.3	56.3	74.5	128.8	75.9	76
U/L	21	110.9	71.8	83.2	55	77.7	164	64.9	83.5
	42	84.6	74.2	77.6	52.2	70.8	169.4	56.6	77.8
	56	78.2	60.6	63.3	48.3	64.7	125	61.8	70.1
ALP	0	95.85	141.27	105.9	201.57	85.65	123.09	106.01	184.39
	7	110.17	265.49	118.37	229.04	117.7	179.86	142.02	193.16
U/L	21	113.51	220.7	119.59	183.41	83.99	149.54	116.65	161.14
	42	112.95	217.72	91.87	151.17	63.11	106.35	83.32	142.87
	56	90.59	168.74	88.32	122.93	50.26	77.8	86.85	142.28
CK	0	153.4	122.7	138.3	166.7	168.6	118.5	245.5	81.4
	7	97.8	156.8	87	165.1	149.2	129.6	233.5	70.1
U/L	21	84.1	139	103.1	118.9	145.3	140.5	111.7	76.7
	42	75.6	136.6	86	93	163.9	81.3	128	64.9
	56	83.6	101.9	101.1	90.9	107.1	71.5	108.2	53
LDH	0	344.2	302.9	215.7	336.3	275.3	224.2	430.9	198.9
	7	368.7	265.6	237.2	378.8	233.4	331.6	467.2	205.6
U/L	21	220.9	219.2	170.1	187.3	209.2	180.1	316.6	209.6
	42	199.4	253.9	155	188.5	231.2	217.7	266	189.9
	56	210.4	241.1	202.5	185.8	235	216.5	365.4	164.7
GLU	0	5.63	4.79	5.88	6.2	4.94	6.66	5.11	4.59
	7	3.9	3.81	4.75	4.87	4.34	4.59	3.58	4.6
mmol/L	21	4.67	4.66	5.25	5.75	5.24	4.58	5.57	5
	42	4.17	4.52	4.79	5.46	6.19	6.22	5.31	5.42
	56	4.14	4	4.83	5.56	4.2	6.23	4.85	4.87
BUN	0	7.5	7.3	3.9	7.5	7.1	8.6	7.8	6.7
	7	8.3	5.3	5.5	10.3	5.8	5.1	7.1	6.1
mmol/L	21	9.1	7.1	5.3	5.2	7.1	7.3	7.8	6.4
	42	6.4	6.5	6.7	7.1	5.8	6.2	7.9	6.6
	56	7.1	6.1	5.2	4.6	5.8	6.6	7.6	6.1
CREA	0	114.3	74.9	76.6	84.7	74.2	85.4	82.9	71.4
	7	95.9	65.9	77.8	90.1	82.1	77	83.6	66.8
µmol/L	21	103.3	66.5	83.7	75.2	83.6	81.1	84	66.5
	42	97.3	62.6	80.5	75.2	76.6	72.1	71.4	59.9
	56	107.5	53	78	68.9	78.9	71.6	80.8	57.7
CHOL	0	2.82	2.49	2.34	3.16	2.81	2.87	2.33	3.05
	7	2.7	3.11	2.7	3.47	2.74	3.2	2.51	3.16
mmol/L	21	2.47	2.9	3.05	3.53	2.78	3.57	2.37	3.17
	42	2.6	3.35	3.01	3.46	3.12	3.77	2.65	3.37
	56	3.22	3.05	3.26	3.73	3.02	3.91	2.97	3.71
TG	0	0.17	0.34	0.43	0.5	0.86	0.52	0.45	0.35
	7	0.18	0.4	0.53	0.36	0.73	0.71	0.35	0.32

mmol/L	21	0.27	0.7	0.69	0.66	0.65	0.61	0.52	0.38
	42	0.24	0.54	0.65	0.66	0.65	0.55	0.62	0.56
	56	0.33	0.54	0.55	0.84	0.53	1.08	0.54	0.85

Table S3. Peptides derived from amino acid sequences of SARS-CoV-2 S protein used in ELISpot and ICS

Peptides	Sequence	Peptides	Sequence
1	SSVLHSTQDLFLPF	41	TTRTQLPPAYTNSF
2	FLGVYYHKNNKSWM	42	HTPINLVRDLPQGF
3	FLPFFSNVTWFHAI	43	TTAPAICHGDKAHF
4	QGFSALEPLVDLPI	44	KTPPIKDFGGFNFS
5	KTQSLIVNNTATNV	45	TTDAVRDPQTLEIL
6	REFVFKNIDGYFKI	46	MSFPQSAPHGVVFL
7	AAYYVGYLQPRFTL	47	LTPTWRVYSTGSNV
8	FQFCNDPFLGVYYH	48	LTDEMIAQYTSALL
9	VSSQCVNLTRRTQL	49	YSNNSIAIPTNFTI
10	RVYSSANNCTFEYV	50	TITSGWTFGAGAAL
11	VSGTNGTKRFDNPV	51	LTESNKKFLPFQGF
12	VVIGIVNNTVYDPL	52	SALLAGTITSGWTF
13	QVAVLYQDVNCTEV	53	ILPDPSKPSKRSFI
14	SSVLNDILSRDKV	54	FTRGVYYPDKVFRS
15	FIAGLIAIVMVTIM	55	STPCNGVEGFNCYF
16	YQTSNFRVQPTESI	56	CVADYSVLYNSASF
17	AENSVAYSNNNSIAI	57	DLCFTNVYADSFVI
18	RSFIEDLLFNKVTL	58	YQPYRVVLSFELL
19	AIPTNFTISVTTEI	59	FPNITNLCPFGEVF
20	SIVRFPNITNLCPF	60	NNLDSKVGGNLYL
21	SAPHGVVFLHVTYV	61	YLRLFRKSNLKPF
22	VAYSNNNSIAIPTNF	62	DSKVGGNLYLYRL
23	FAMQMA YRFNGIGV	63	DYSVLYNSASFSTF
24	LIAIVMVTIMLCCM	64	CYGVSPTKLNDLCF
25	TGIAVEQDKNTQEV	65	YGFQPTNGVGYQPY
26	VFAQVKQIYKTPPI	66	RKRISNCVADYSVL
27	VTYVPAQEKNFTTA	67	TRFASVYAWNRKRI
28	NTLVKQLSSNFGAI	68	DFTGCVIAWNSNNL
29	FQPTNGVGYQPYRV	69	SVLYNSASFSTFKC
30	QDVVNQNAQALNTL	70	VSPTKLNDLCFTNV
31	ARSVASQSIIAYTM	71	IAPGQTGKIADYNY
32	CAQKFNGLTVLPPL	72	FKCYGVSPTKLNDL
33	GVTQNVLYENQKLI	73	YSVLYNSASFSTFK
34	ENQKLIANQFNSAI	74	SGINASVVNIQKEI
35	AEHVNSYECDIPI	75	IAGLIAIVMVTIML
36	TFGAGAALQIPFAM	76	AKNLNESLIDLQEL
37	AISSVLNDILSRDL	77	QYIKWPWYIWLGF
38	AEIRASANLAATKM	78	VMVTIMLCCMTSCC
39	IRGWIFGTTLDSTQSSL	79	LGKYEQYIKWPWYI
40	ITPGTNTSNQVAVL		

Table S4. Nested-PCR primers specific for hexon of Sad23L or Ad49L vector

Primers	Sequence (5'-3')
Outer 23-hexon-F1	GATACTCCCGGTGGCACC
Outer 23-hexon-R1	CGAAAATGTTACCTTTACAT
Inner 23-hexon-F2	TGAACGGTCAAGACGAGT
Inner 23-hexon-R2	CCATCTTGACCATCTTTTAC
Outer 49-hexon-F1	GATCTCAGACAAAATGAC
Outer 49-hexon-R1	CGAACAGGTTGCCCTTGGCG
Inner 49-hexon-F2	CTCAGACAAAATGACACT
Inner 49hexon-R2	CCAGCAACAGTTGTATCAGG