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

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## ABSTRACT

COVID-19 vaccines are being developed urgently worldwide, among which single-shot adenovirus vectored vaccines represent a major approach. Here, we constructed two novel adenovirus vectored COVID-19 vaccine candidates on simian adenovirus serotype 23 (Sad23L) and human adenovirus serotype 49 vectors (Ad49L) carrying the full-length gene of SARS-CoV-2 spike protein (S), designated Sad23L-nCoV-S and Ad49L-nCoV-S vaccines, respectively. The immunogenicity elicited by these two vaccine strains was individually evaluated in mice. Specific humoral and cellular immune responses were proportionally observed in a dose-dependent manner, and stronger response was obtained by boosting. Furthermore, five rhesus macaques were intramuscularly injected with a dose of $5\times10^9$ PFU Sad23L-nCoV-S vaccine for prime vaccination, followed by boosting with $5\times10^9$ PFU of Ad49L-nCoV-S vaccine at 4-week interval. Three macaques were injected with Sad23L-GFP and Ad49L-GFP vectorial viruses as negative controls. Both mice and macaques tolerated well the vaccine inoculations without detectable clinical or pathologic changes. In macaques, prime-boost vaccination regimen induced high titers of $10^{3.16}$ S-binding antibody (S-BAb), $10^{2.75}$ cell receptor binding domain (RBD)-BAb and $10^{2.38}$ neutralizing antibody (NAb) to pseudovirus a week after boosting injection, followed by sustained high levels over 10 weeks of observation. Robust IFN-γ secreting T-cell response (712.6 SFCs/$10^6$ cells), IL-2 secreting T-cell response (334 SFCs/$10^6$ cells) and intracellular IFN-γ expressing

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

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

## INTRODUCTION

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

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

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

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

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

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

## RESULTS

### Production and characterization of Sad23L-nCoV-S and Ad49L-nCoV-S vaccines

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

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

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

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

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

C57BL/6 mice (n=5 per group) were intramuscularly injected with individual Sad23L-nCoV-S and Ad49L-nCoV-S vaccines at doses of $10^7$, $10^8$ and $10^9$ PFU or by prime-boost immunization with these two adenovirus vectored vaccines ($10^9$ PFU/each) at 4 week interval and compared with vectors and PBS controls, respectively. Mice tolerated well the various doses of vaccines or vector controls at 28 or 56 days, presenting no obvious change of weight and body temperature when compared with injected PBS (Fig. 2A). In addition, histopathological examination of 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 $5\times10^9$ PFU Sad23L-nCoV-S vaccine or Sad23L-GFP control,
147 and then at 4 week interval, animals were injected with a second dose of $5\times10^9$ 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 $10^9$ PFU Sad23L-nCoV-S on day 0 and a
202 boost dose of $10^9$ 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.

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

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

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

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

Five rhesus macaques were intramuscularly injected first with a dose of $5\times10^9$ PFU Sad23L-nCoV-S vaccine, then with a boost injection of $5\times10^9$ PFU Ad49L-nCoV-S vaccine 4 weeks later, while three sham rhesus macaques controls were injected with equal doses of Sad23L-GFP and Ad49L-GFP viruses, respectively (Fig. 5A). Blood samples were collected weekly from these two groups of animals. Both BAb (S-BAb and RBD-BAb) and NAb (sVNT and pVNT) levels were titrated in the vaccinated group but not in sham group. BAb and NAb reactivity increased at week 2 post prime-immunization, at week 4 (W0 for boost-immunization), at weeks 5 and 6 and then stayed at high levels up to week 10 (Fig. 5, B-E). Titers of $10^{3.16}$ S-BAb (Fig. 5B), $10^{2.75}$ RBD-BAb (Fig. 5C), $10^{2.01}$ sVNT($ID_{50}$) (Fig. 5D) and $10^{2.38}$ pVNT($ID_{50}$) (Fig. 5E) were quantified and found higher than the corresponding antibody titers found in sera from 25 asymptomatic, 14 mild and 9 severe COVID-19 convalescent patients (Fig. 5, B-E). Strong correlation between BAb and NAb titers was observed ($P$<0.0001, R=0.784-0.927; Fig. 5, F and G), suggesting the reliability of these antibody quantitative assays.

PBMCs were isolated from whole blood of pre- and post-immunized monkeys for evaluation of T cell responses to S peptides, S and RBD protein by ELISpot and ICS (Fig. 6). Prime vaccination with a dose of Sad23L-nCoV-S vaccine induced an increase of IFN-γ secreting T cell response to S peptides (406.6-526.3 SFCs/million cells) at weeks 2 and 4 post prime-vaccination, and then boosting with a dose of 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 $TNF\alpha^+$ $CD8^+$ or $IL\text{-}2^+$ $CD4^+$/$CD8^+$ 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;

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

## DISCUSSION

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

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

In this study, we generated two novel adenovirus vectored COVID-19 vaccines encoding the full-length S gene of SARS-CoV-2. The intact S glycoprotein rather than the shorter S or RBD proteins was shown to be the most effective antigen eliciting protective immunity against SARS-CoV-2 infection in DNA vaccines and Ad26 vectored vaccines *(12,13,26)*. The vectors Sad23L and Ad49L originated from simian adenovirus type 23 and human adenovirus type 49, respectively *(22,23)*, are novel adenoviral vector. Comparing Ad5-vectored COVID-19, ChAdox1 nCoV-19 and Ad26.COV2.S vaccines *(8-12,14,21)*, three attractive aspects emphasized in this study are highlighted below.

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

Secondly, the prime-boost vaccination regimen with two heterologous adenoviruses vectored Sad23L-nCoV-S and Ad49L-nCoV-S vaccines examined in mice and rhesus macaques suggested that the boost with Ad49L-nCoV-S vaccine significantly enhanced the levels of neutralizing antibody and IFN-γ expressing $CD4^+$/$CD8^+$ T cell responses following prime-immunization with Sad23L-nCoV-S vaccine (Fig. 4-6). In addition, specific IFN-γ secretion T cell response was prolonged at high level after boosting vaccination. Two recent publications compared prime only with a single-dose of ChAdOx1 nCoV-19 and homologous boost with a second dose of ChAdOx1 nCoV-19 vaccine in mice and pigs, and human clinical trials, showed that boost vaccination significantly increased and prolonged the level of binding or neutralizing antibody response to SARS-CoV-2 *(11,20)*. By boosting with Ad5-S 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 ($<5\times10^{10}$ vp) with prime-boost
336 immunization regimen should theoretically reduce severe adverse reaction induced by
337 a high dose of adenovirus vectored vaccine ($>5\times10^{10}$ 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;

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

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

## MATERIALS AND METHODS

### Rhesus macaques and ethics statement

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

The welfare issues (housing, feeding, environmental enrichment, etc.) were in accordance with the recommendations of the Weatherall report (https://acmedsci.ac.uk/more/news/the-use-of-non-human-primates-in-research). Animals were individually housed in spacious cages and were provided with commercial food pellets supplemented with appropriate treats. Drinking water was provided ad libitum from an automatic watering system. Animals were monitored daily for health and discomfort. Blood samples were obtained using sterilized needle and syringe from the venous vessels of animal legs.

**Cells and mice**

HEK-293A, HEK-293T and HEK293T-hACE2 cells (Sino Biological) were maintained in complete Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gbico) and incubated at 37℃ in 5% $CO_2$.

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 ℃ 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**

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

**Adenovirus neutralizing antibody assay**

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

**Animal immunization**

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

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

Five rhesus macaques aged 11 to 14 years (Table S1) were first injected intramuscularly with a dose of $5\times10^9$ PFU Sad23L-nCoV-S vaccine, and then at 4 week interval received a second dose of $5\times10^9$ PFU Ad49L-nCoV-S vaccine. Three rhesus macaques aged 11 to 13 years (Table S1) were vaccinated by prime-boost regimen with a dose of $5\times10^9$ PFU Sad23L-GFP and a dose of Ad49L-GFP vectorial adenoviruses as sham control.

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

The microtiter plates (Corning, USA) were coated overnight with 2μg/ml of SARS-CoV S or RBD proteins (Sino Biological, China). Serum samples were 2-fold serially diluted and S or RBD binding antibody (S-BAb or RBD-BAb) were detected by ELISA. Secondary antibodies were goat anti-mouse IgG-HRP (Beijing Bersee Science and Technology, Co. LTd, China), rabbit anti-monkey IgG-HRP (Bioss, China) and goat anti-human IgG-HRP conjugates (Sigma, USA), respectively. 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 ℃, 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 ℃, 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

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

**ELISpot**

Monkey (or mouse) IFN-gamma, IL-2 or IL-4 ELISpotPLUS kits (MabTech) were used to determine SARS-CoV-2 S antigen-specific T lymphocyte response. Rhesus macaque's PBMCs ($2\times10^5$ cells/well) or mouse splenocytes ($5\times10^5$ cells/well) were stimulated with S peptides, S or RBD protein (5μg/ml) in triplicates, respectively. Seventy-nine peptides encoding for amino acid sequence of SARS-CoV-2 S protein were predicted (http://www.iedb.org/) and synthesized by Guangzhou IGE Biotechnology LTD (Table. S3). Spots were counted with a CTL Immunospot Reader

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

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

Mouse splenocytes ($2\times10^6$ cells/well) or monkey PBMCs ($1\times10^6$ cells/well) were stimulated with S peptide pools (3μg/ml each peptide), or medium as negative control, in triplicates. After 4h, the cells were incubated with Golgi Plug (BD) for 12h at 37℃. Cells were collected and stained with anti-mouse or anti-monkey CD3, CD4 and CD8 surface marker antibodies (BD). Cells were fixed with IC fixation buffer, permeabilized with permeabilization buffer (BD) and stained with anti-mouse or anti-monkey interferon-γ (IFN-γ), interleukin-2 (IL-2) and tumor necrosis factor α (TNF-α) (BD). All samples were tested with BD FACS Canton flow cytometer (BD).

**Nested-PCR amplification**

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

**Statistical analyses**

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

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# SUPPLEMENTARY MATERIALS

667

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## Author contributions

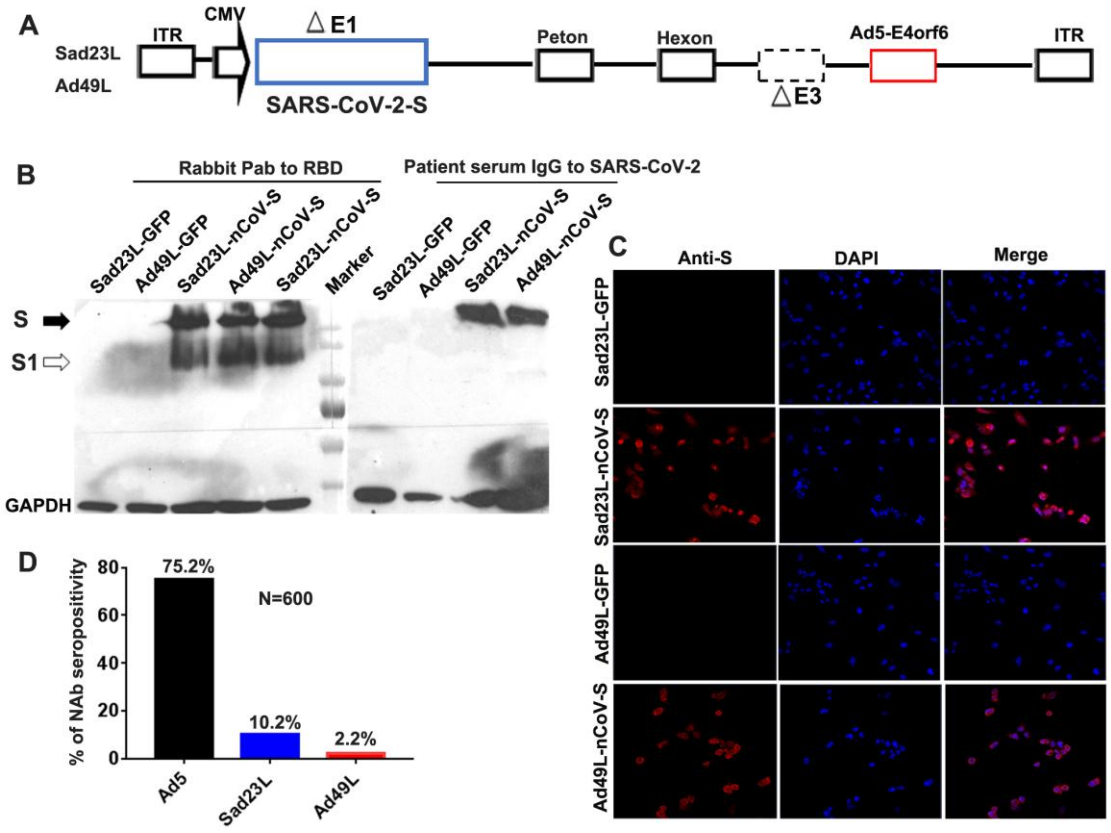
C.L., L.S., T.L. and Y.Z. designed the research. L.S., P.Z., B.L., C.Y., C.L.L., Q.W., L.Z. and T.L. carried out the experiments. X.T., J.L., S.H., J.Z. and Y.F. provided COVID-19 patient or healthy blood donor samples. S.L., C.L., Y.Z. and J.P.A. analyzed data and wrote the paper.

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**

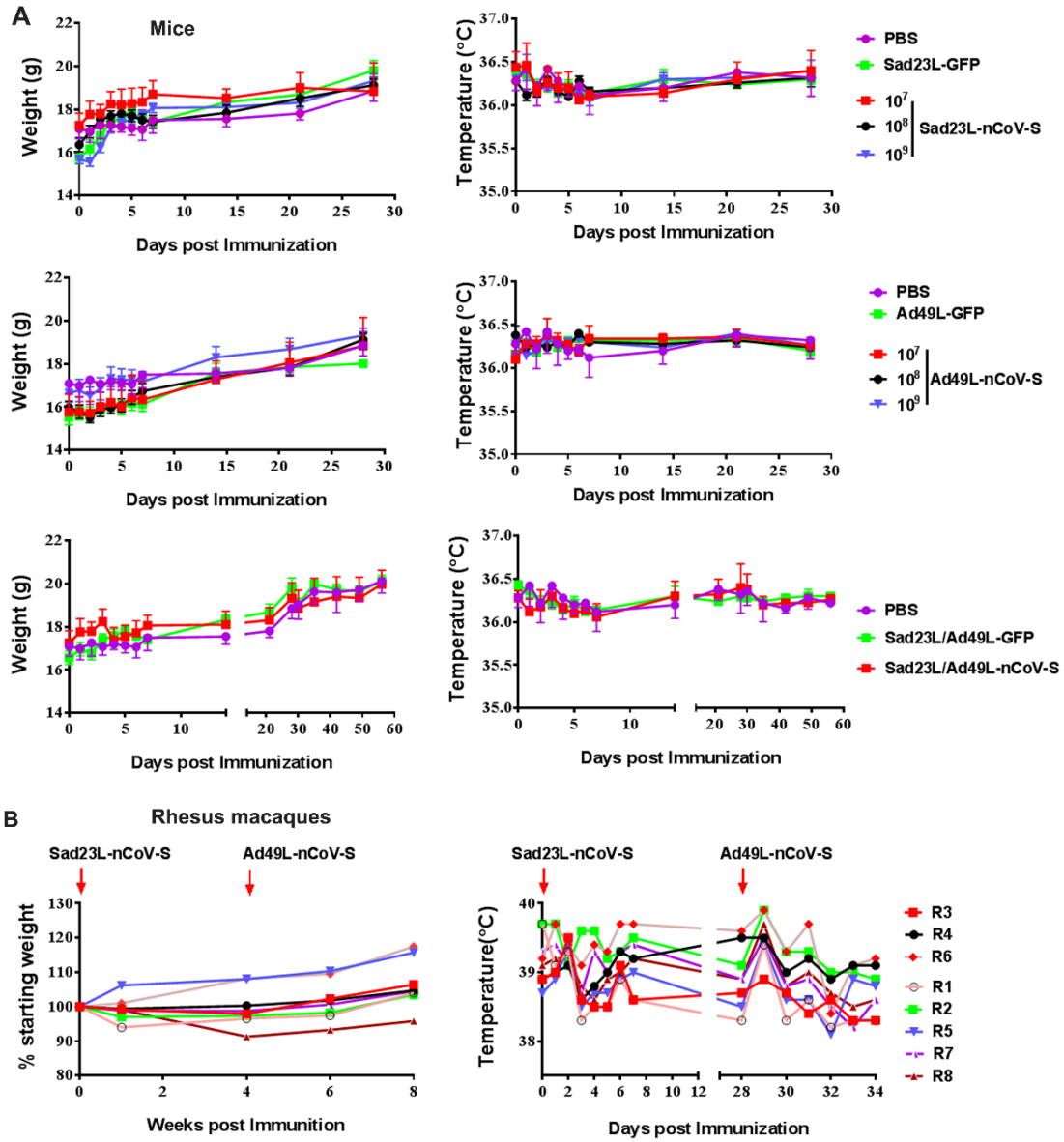
691 All data associated with this paper can be found in the main text or the supplementary
692 materials. Two novel adenoviral vectors (Sad23L and Ad49L) are available under a
693 material transfer agreement with BRK upon request to the corresponding author.

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 $10^7$,
714 $10^8$ or $10^9$ PFU Sad23L-nCoV-S vaccine, $10^9$ PFU Sad23L-GFP and an equal volume
715 of PBS controls (top panel); a dose of $10^7$, $10^8$ or $10^9$ PFU Ad49L-nCoV-S vaccine,
716 $10^9$ 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 $5\times10^9$
720 PFU Sad23L-nCoV-S and a dose of $5\times10^9$ PFU Ad49L-nCoV-S vaccines (R1, R2, R5,
721 R7 and R8), or $5\times10^9$ PFU Sad23L-GFP and a dose of $5\times10^9$ PFU Ad49L-GFP
722 controls (R3, R4 and R6).
723

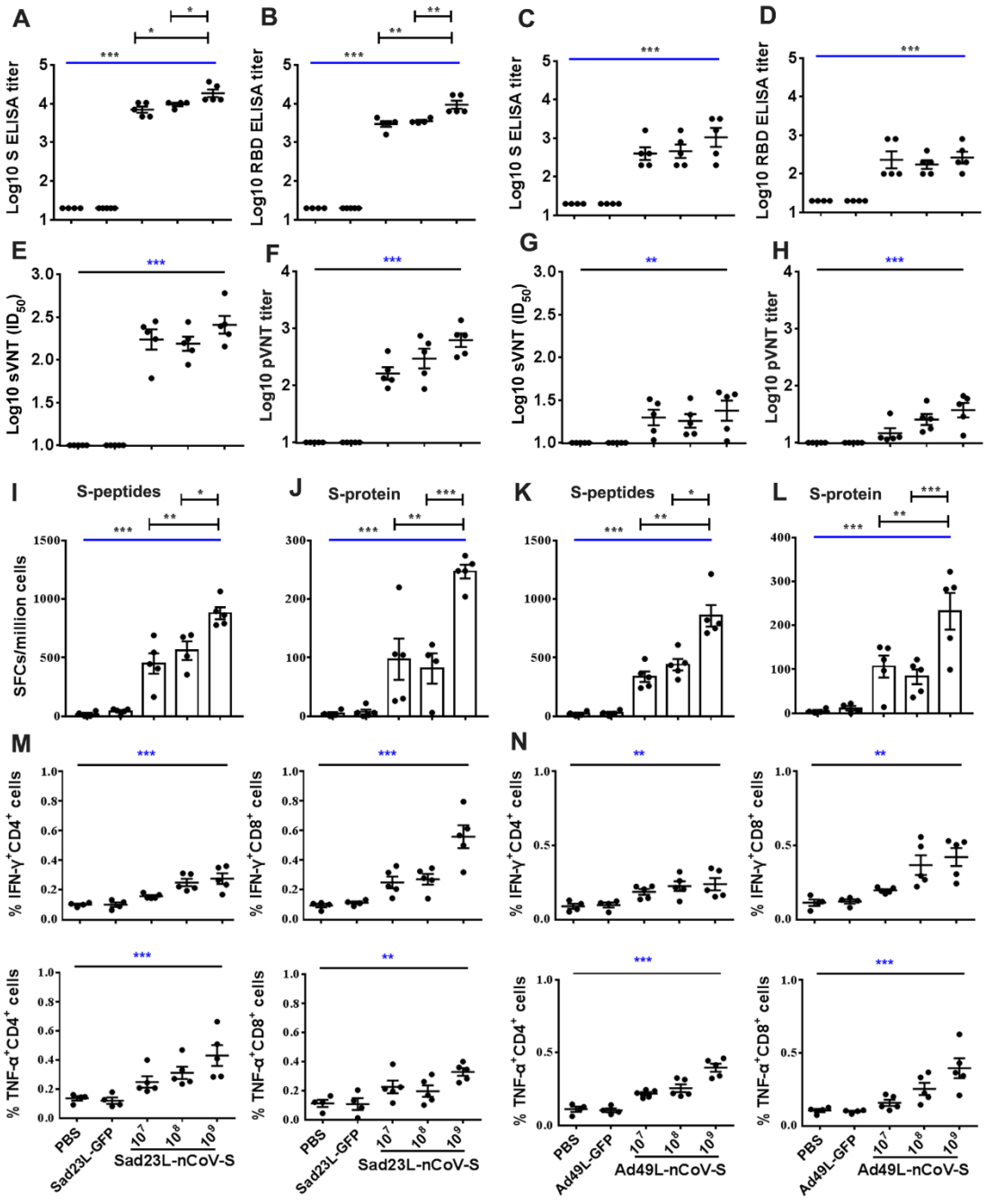



**Fig 3. Specific antibody and T cell response of C57BL/6 mice inoculated with a single shot of Sad23L-nCoV-S or Ad49L-nCoV-S vaccine at three different doses.** C57BL/6 mice (n=5/group) were immunized by a single dose of $10^7$, $10^8$ or $10^9$ PFU Sad23L-nCoV-S or Ad49L-nCoV-S vaccine. Mice sera and splenocytes were collected for measurement of antibody level and T cell response 4 weeks post-immunization. (**A-D**) S or RBD binding antibody (S-BAb or RBD-BAb) titers were obtained by ELISA. (**E-H**) Neutralizing antibody (NAb) titers were obtained by 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 ± 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

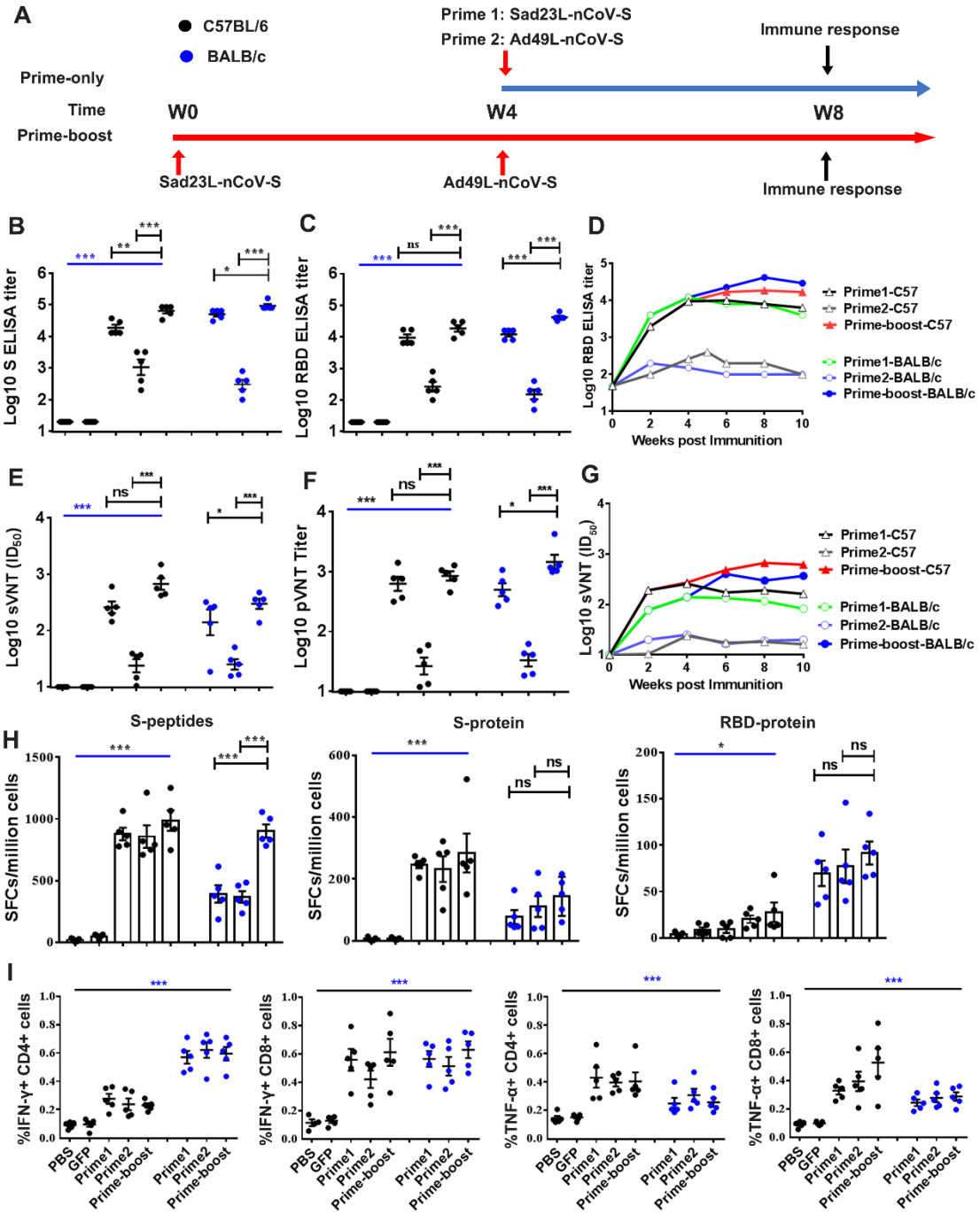



**Fig. 4. Specific humoral and cellular immune response of C57BL/6 and BALB/c mice to prime-boost immunization with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines.** (**A**) C57BL/6 and BALB/c mice (n=5/group) were prime immunized with a dose of $10^9$ PFU Sad23L-nCoV-S vaccine and boosted with a dose of $10^9$ PFU Ad49L-nCoV-S vaccine at 4 week interval. Sera and splenocytes were collected from vaccinated or control mice for measurement of antibody and T cell responses 4 weeks after both prime only and boosting immunizations. (**B-D**) Anti-S-BAb and RBD-BAb

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

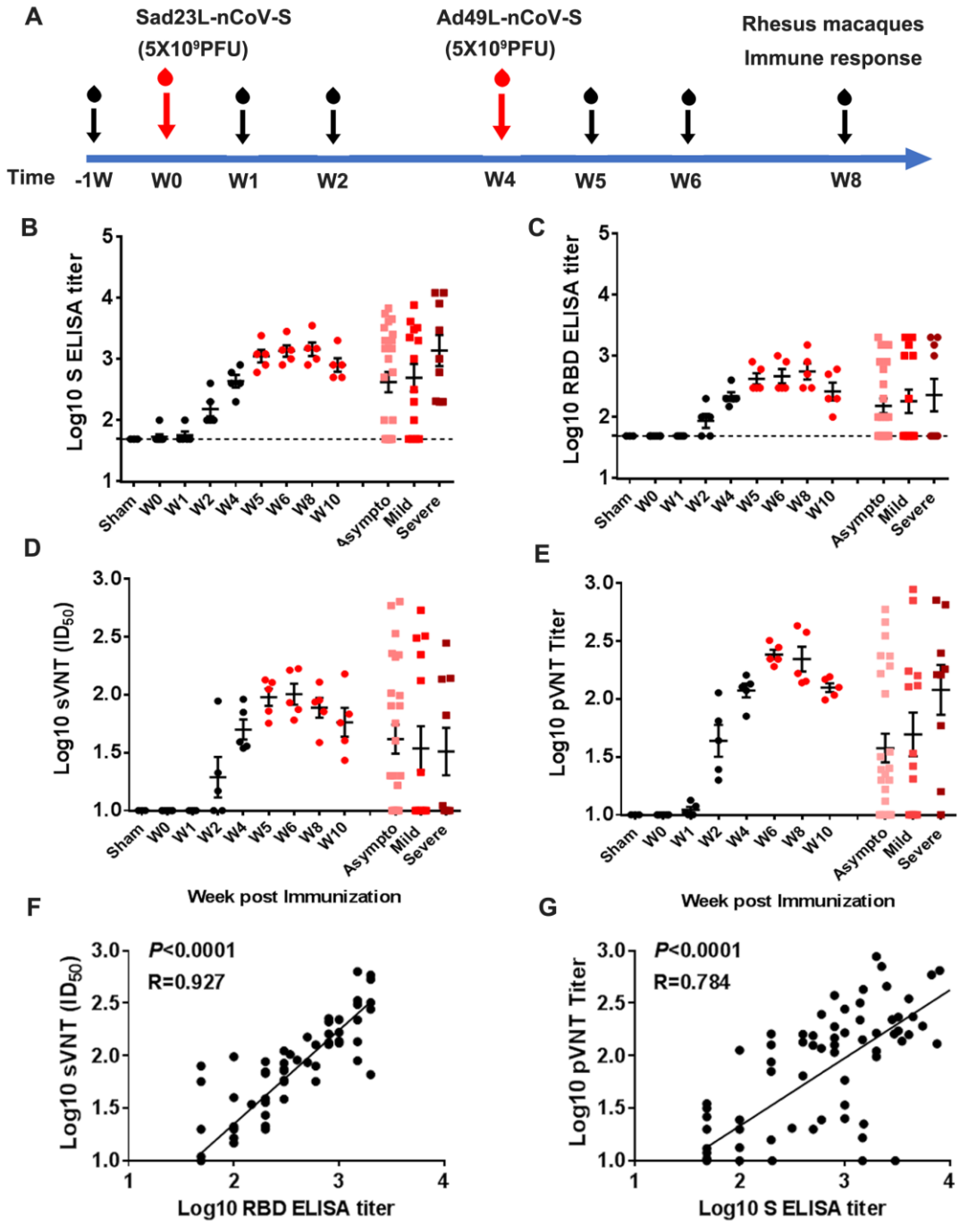



**Fig. 5. Antibody reactivity of Rhesus macaques to prime-boost vaccination with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines.** (**A**) Five rhesus macaques were prime immunized with $5\times10^9$ PFU of Sad23L-nCoV-S vaccine and boosted with $5\times10^9$ PFU of Ad49L-nCoV-S vaccine at 4 week interval. Blood samples were collected weekly from immunized or sham control macaques. Three macaques first immunized with $5\times10^9$ PFU of Sad23L-GFP viruses and boosted with $5\times10^9$ PFU of 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

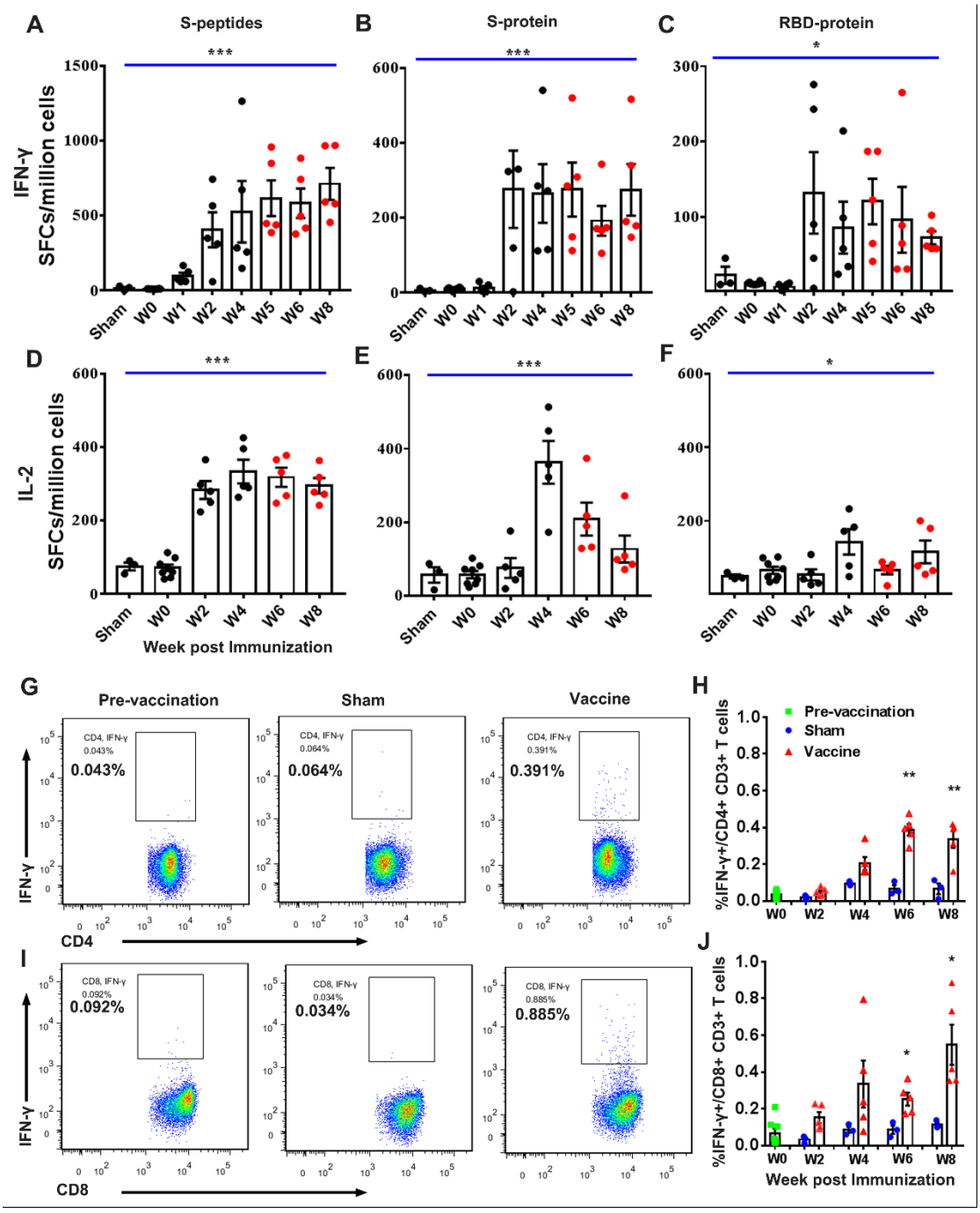
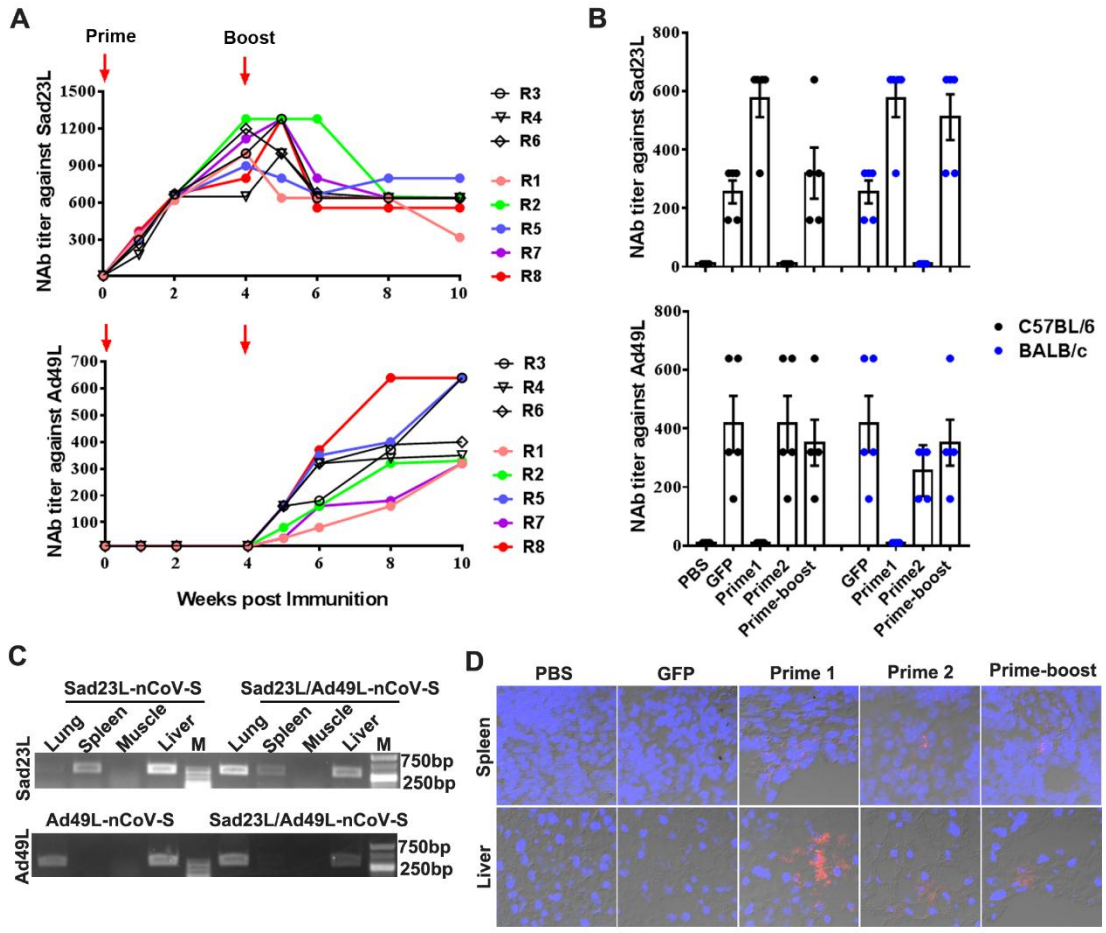



**Fig. 6. Specific T cell response of PBMCs from Rhesus macaques immunized with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines or sham controls by prime-boost vaccination regimen.** (**A-C**) IFN-γ or (**D-F**) IL-2 secreting T cell response (SFCs/million cells) to S peptides, S or RBD protein was measured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

779 ± 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**

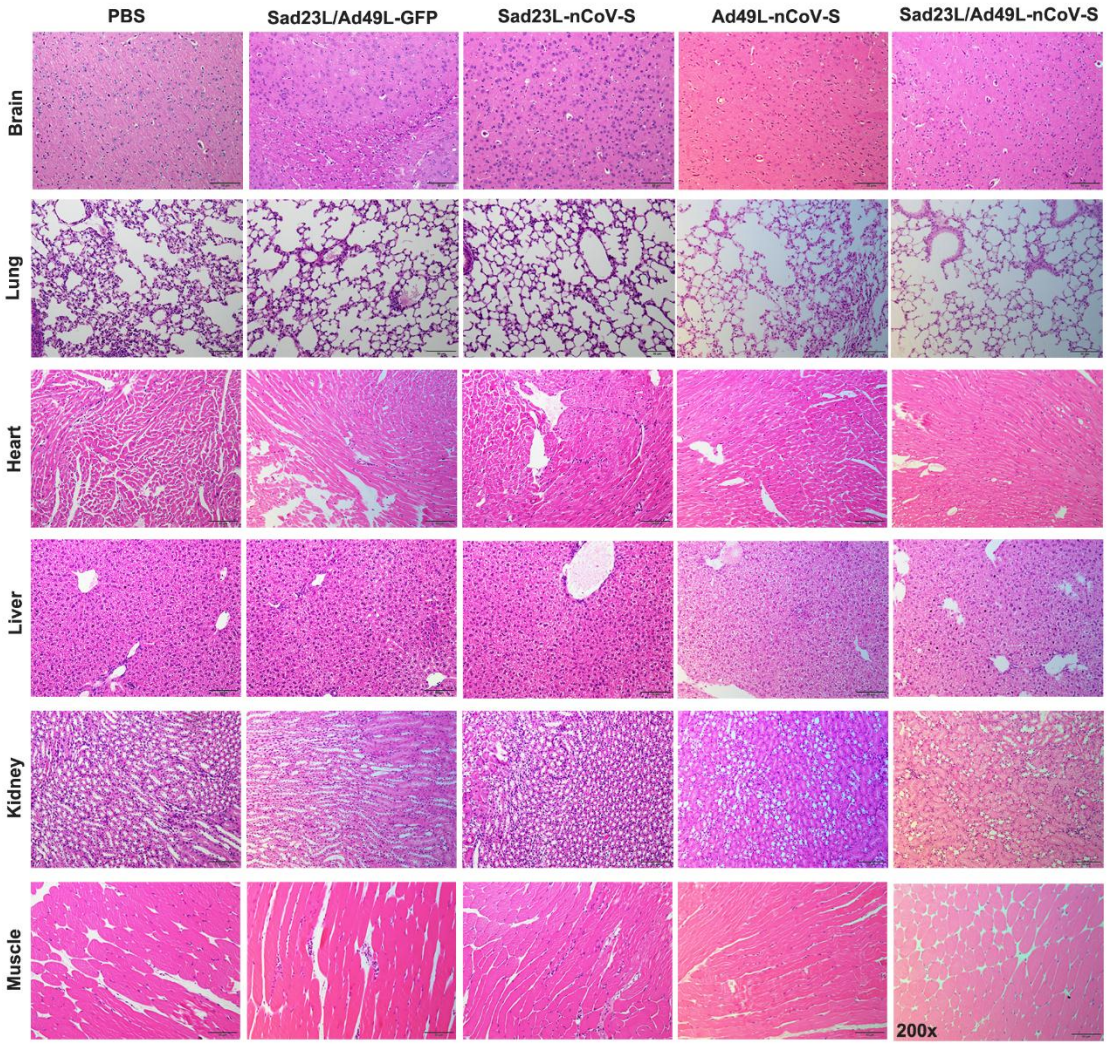



**Fig. S1. Histopathological examination from Sad23L-nCoV-S and Ad49L-nCoV-S vaccines inoculated C57BL/6 mice.** Histopathological examination was carried out for brain, lung, heart, liver, kidney and muscle tissues (at intramuscular injection site and para-tissues) of mice in 4 weeks post prime only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

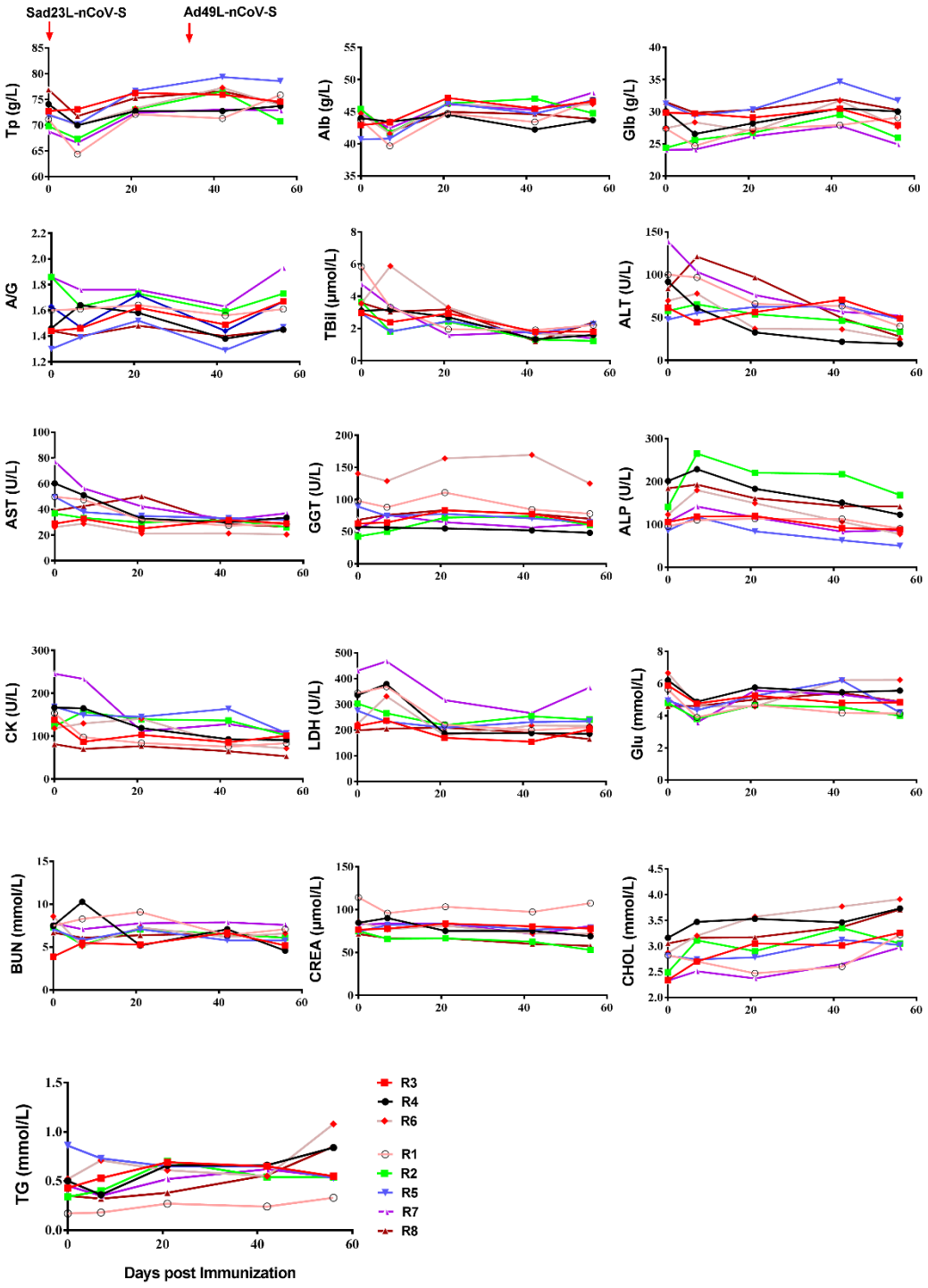



**Fig. S2. Kinetic change of hematological and clinical biochemistry indexes during the course of vaccinated or sham control rhesus macaques.** Rhesus macaques were intramuscularly inoculated by prime-boost immunization with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines at an interval of 4 weeks. A panel of

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.

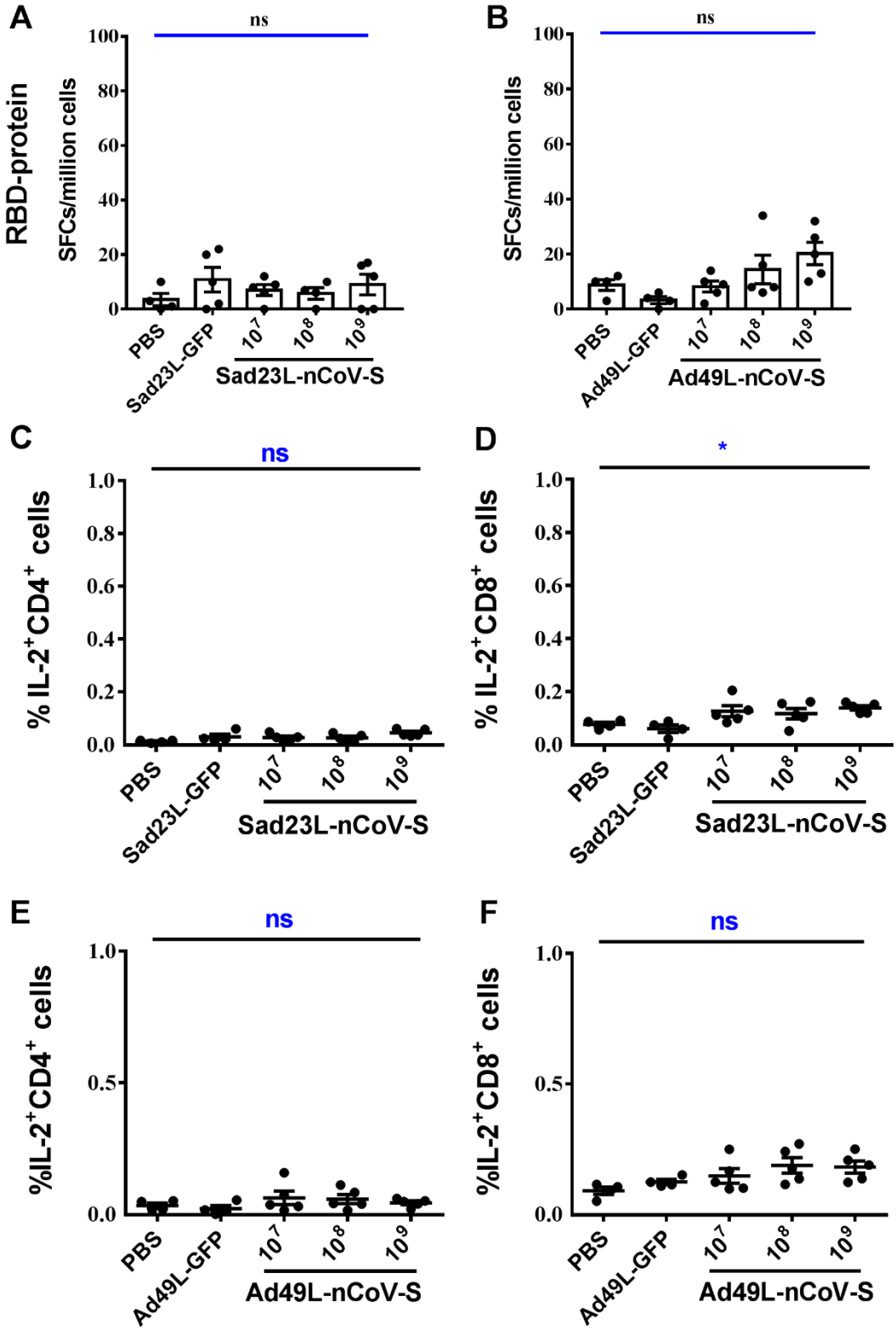



**Fig. S3. Specific T cell response of splenocytes from C57BL/6 mice (n=5/group) immunized with a single dose of Sad23L-nCoV-S or Ad49L-nCoV-S vaccine.** (**A**

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 ± 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.

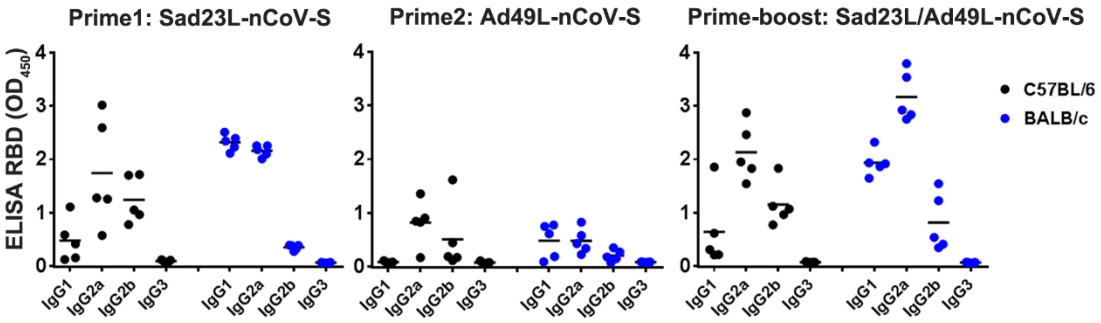



**Fig. S4. IgG subclass antibodies against RBD protein in sera of C57BL/6 and BALB/c mice immunized by prime only or prime-boost with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines.** The sera were collected 4 weeks post prime only or prime-boost immunized C57BL/6 and BALB/c mice. IgG subclass antibodies in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

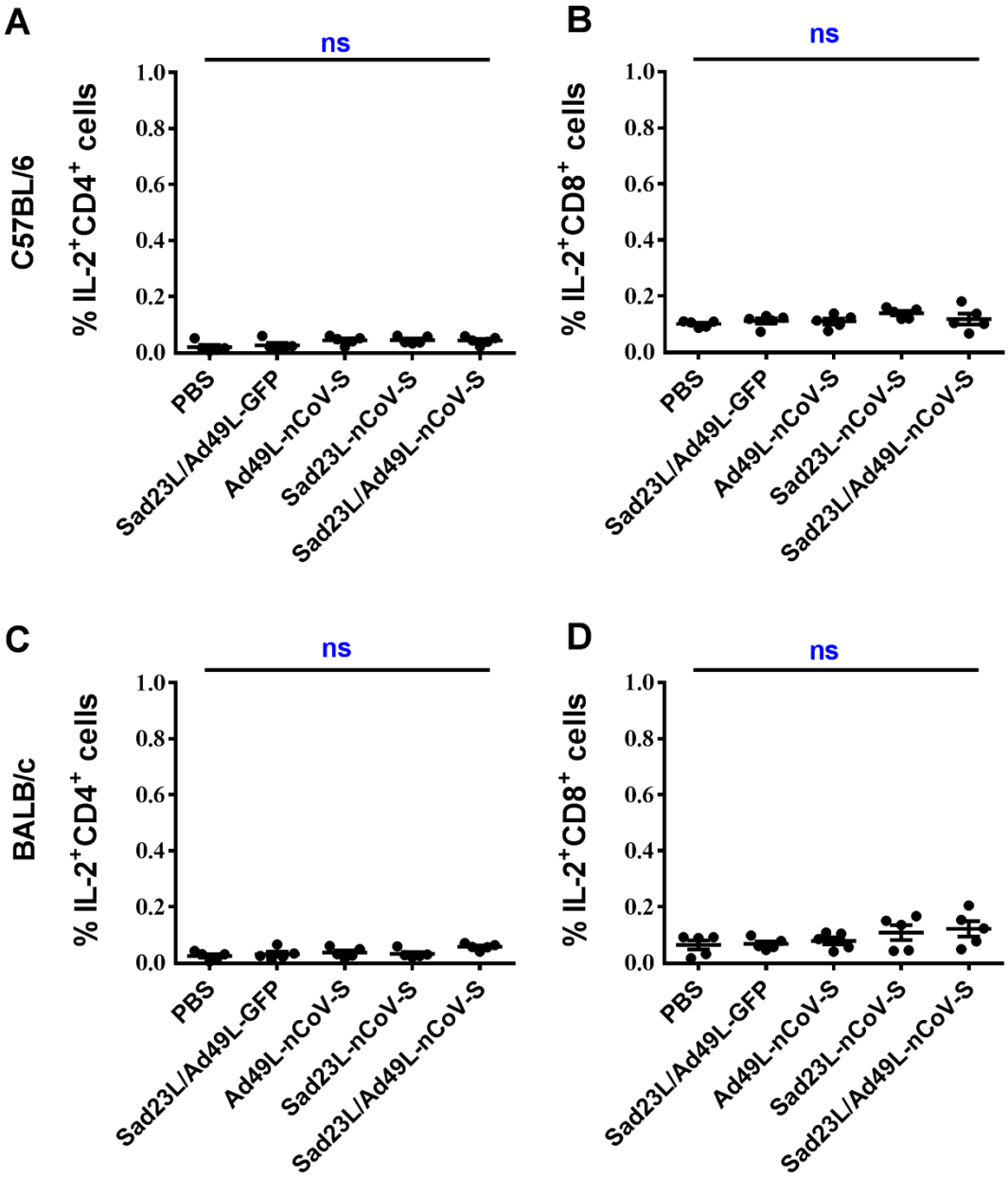



**Fig. S5. Frequency of IL-2 expressing CD4+/CD8+ T cell responses of splenocytes from prime-boost immunized C57BL/6 and BALB/c mice with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines.** (**A** and **B**) Frequency of IL-$2^+$ $CD4^+$ or $CD8^+$ T cell responses of splenocytes to S peptides from C57BL/6, or (**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.

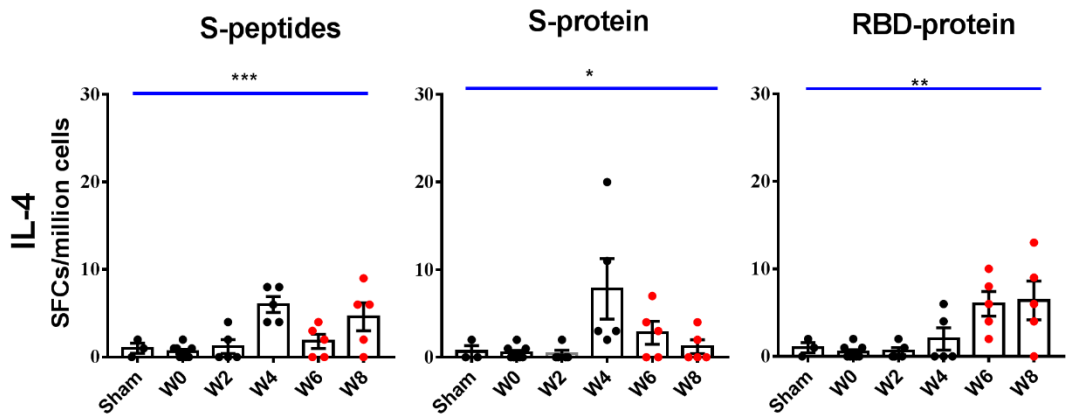



**Fig. S6. IL-4 secreting T cell response in prime-boost immunized rhesus macaques with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines.** The number of specific IL-4 secreting T cells (SFCs/million cells) to S peptides, S or RBD protein in PBMCs of monkeys was measured by ELISpot, respectively. Data were shown as means ± 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.

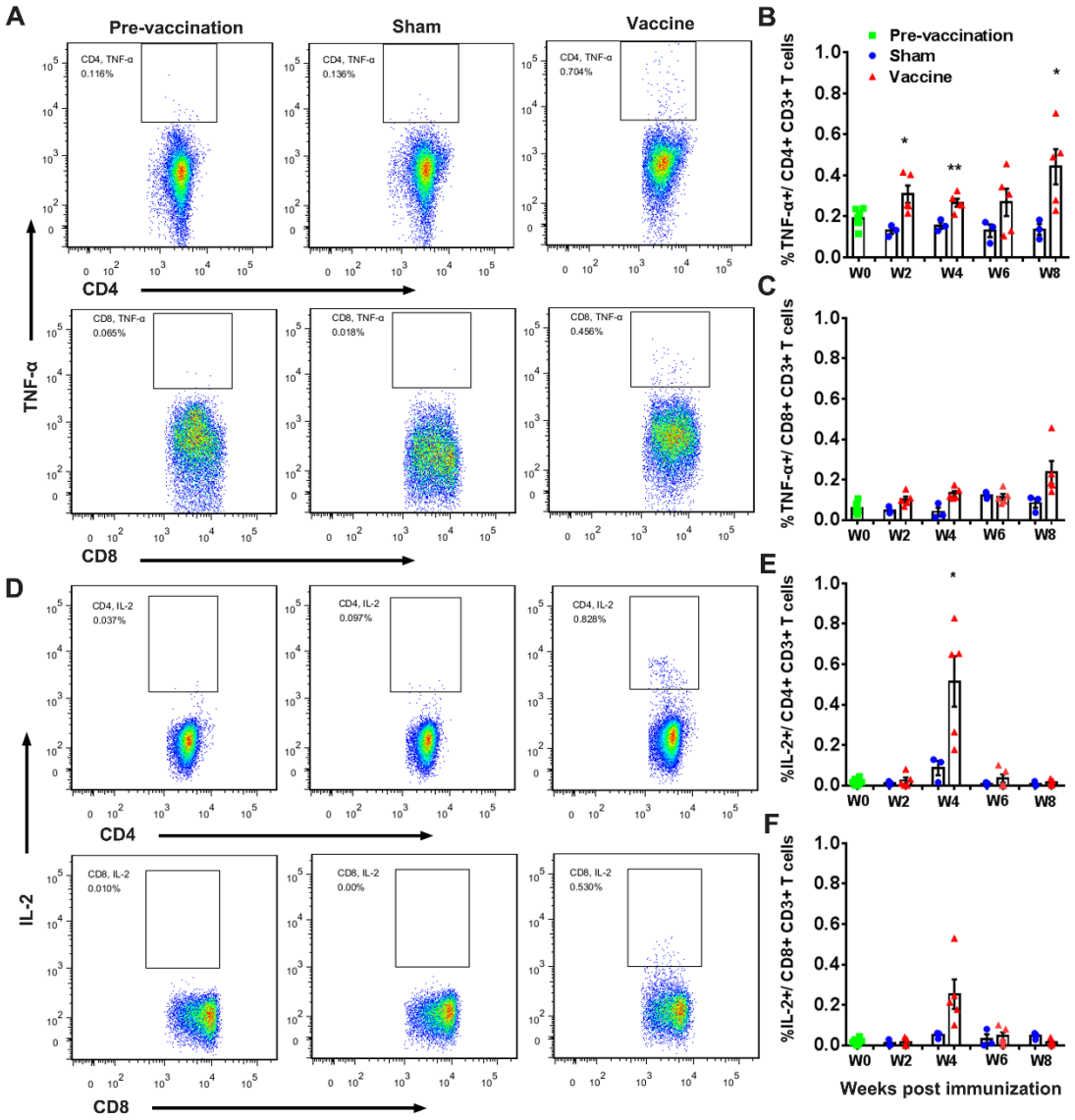



**Fig. S7. Frequency of intracellular TNFα and IL-2 expressing T cell response in PBMCs from rhesus macaques immunized with Sad23L-nCoV-S and Ad49L-nCoV-S vaccines or sham controls.** (A-C) Frequency of TNF-$\alpha^+$ $CD4^+$/$CD8^+$ T cell response to S peptides. (D-F) Frequency of TNF-$\alpha^+$ $CD4^+$/$CD8^+$ T cell response to S peptides. Data were shown as means ± 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.

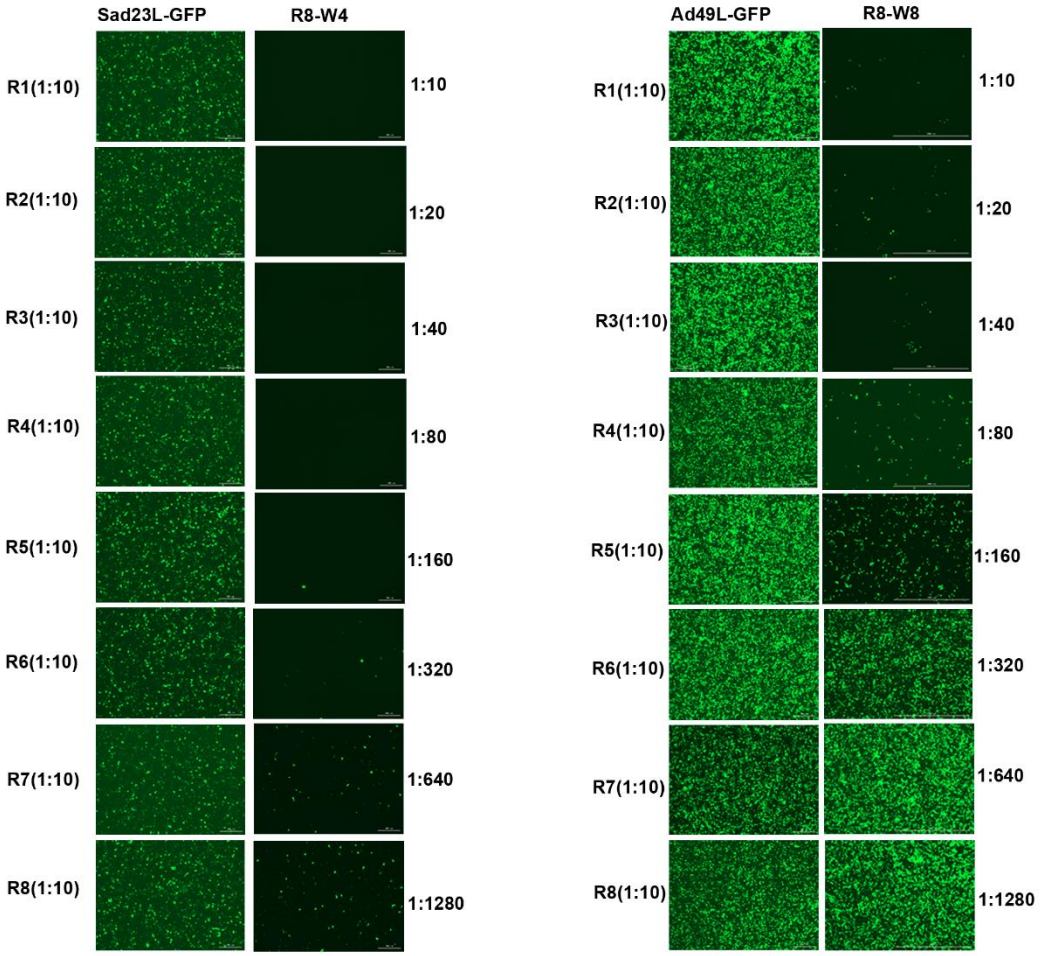



**Fig. S8. Measurement of serum NAb titers to Ad49L and Sad23L vectors in vaccinated macaques.** Two representative plasma samples of vaccinated macaque (R8 at weeks 4 and 8) were tested on HEK-293A cells for neutralizing Sad23L-GFP and Ad49L-GFP vectorial viruses by green fluorescent activity assay.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

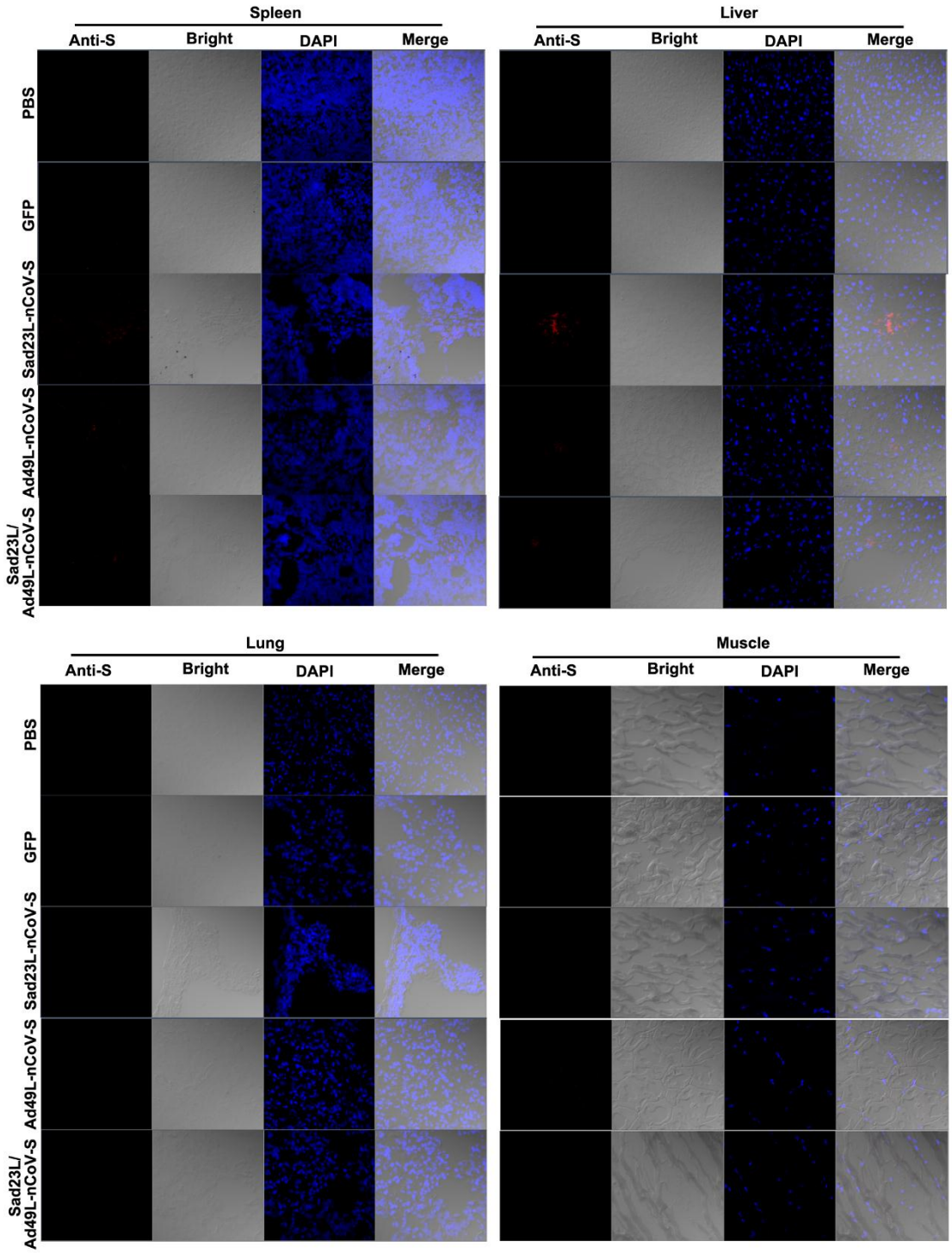



**Fig. S9. Examination of SARS-CoV-2 S protein in the tissues of Sad23L-nCoV-S and Ad49L-nCoV-S immunized mice.** Spleen, liver, Lung and muscle tissues of immunized C57BL/6 mice were examined by immunofluorescence staining with a human monoclonal antibody to SARS-CoV-2 S and DAPI.

**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 g/L | 0 | 71.12 | 69.8 | 72.78 | 74.1 | 71.96 | 72.16 | 68.78 | 76.89 |
| | 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 g/L | 0 | 43.74 | 45.41 | 42.93 | 44.01 | 40.7 | 44.72 | 44.71 | 45.38 |
| | 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 g/L | 0 | 27.38 | 24.39 | 29.85 | 30.09 | 31.26 | 27.44 | 24.07 | 31.51 |
| | 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 µmol/L | 0 | 5.85 | 3.65 | 2.97 | 3.1 | 2.93 | 3.56 | 4.76 | 3.59 |
| | 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 U/L | 0 | 100 | 57.7 | 61.3 | 91.8 | 47.7 | 69.7 | 138.6 | 83.8 |
| | 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 U/L | 0 | 49.7 | 36.7 | 28.8 | 60.2 | 49.7 | 26.2 | 77.3 | 39.1 |
| | 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 U/L** | 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 |
| | 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 U/L** | 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 |
| | 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 U/L** | 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 |
| | 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 U/L** | 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 |
| | 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 mmol/L** | 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 |
| | 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 mmol/L** | 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 |
| | 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 µmol/L** | 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 |
| | 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 mmol/L** | 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 |
| | 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 | TTAPAICHDGKAHF |
| 4 | QGFSALEPLVDLPI | 44 | KTPPIKDFGGFNFS |
| 5 | KTQSLLIVNNATNV | 45 | TTDAVRDPQTLEIL |
| 6 | REFVFKNIDGYFKI | 46 | MSFPQSAPHGVVFL |
| 7 | AAYYVGYLQPRTFL | 47 | LTPTWRVYSTGSNV |
| 8 | FQFCNDPFLGVYYH | 48 | LTDEMIAQYTSALL |
| 9 | VSSQCVNLTTRTQL | 49 | YSNNSIAIPTNFTI |
| 10 | RVYSSANNCTFEYV | 50 | TITSGWTFGAGAAL |
| 11 | VSGTNGTKRFDNPV | 51 | LTESNKKFLPFQQF |
| 12 | VVIGIVNNTVYDPL | 52 | SALLAGTITSGWTF |
| 13 | QVAVLYQDVNCTEV | 53 | ILPDPSKPSKRSFI |
| 14 | SSVLNDILSRLDKV | 54 | FTRGVYYPDKVFRS |
| 15 | FIAGLIAIVMVTIM | 55 | STPCNGVEGFNCYF |
| 16 | YQTSNFRVQPTESI | 56 | CVADYSVLYNSASF |
| 17 | AENSVAYSNNSIAI | 57 | DLCFTNVYADSFVI |
| 18 | RSFIEDLLFNKVTL | 58 | YQPYRVVVLSFELL |
| 19 | AIPTNFTISVTTEI | 59 | FPNITNLCPFGEVF |
| 20 | SIVRFPNITNLCPF | 60 | NNLDSKVGGNYNYL |
| 21 | SAPHGVVFLHVTYV | 61 | YLYRLFRKSNLKPF |
| 22 | VAYSNNSIAIPTNF | 62 | DSKVGGNYNYLYRL |
| 23 | FAMQMAYRFNGIGV | 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 | AEHVNNSYECDIPI | 75 | IAGLIAIVMVTIML |
| 36 | TFGAGAALQIPFAM | 76 | AKNLNESLIDLQEL |
| 37 | AISSVLNDILSRLD | 77 | QYIKWPWYIWLGFI |
| 38 | AEIRASANLAATKM | 78 | VMVTIMLCCMTSCC |
| 39 | IRGWIFGTTLDSKTQSLL | 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 |