

A valid protective immune response elicited in rhesus macaques by an inactivated vaccine is capable of defending against SARS-CoV-2 infection

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32

33 **Abstract**

34 With the relatively serious global epidemic outbreak of SARS-CoV-2 infection, public
 35 concerns focus on not only clinical therapeutic measures and public quarantine for
 36 this disease but also the development of vaccines. The technical design of our
 37 SARS-CoV-2 inactivated vaccine provides a viral antigen that enables the exposure of
 38 more than one structural protein based upon the antibody composition of COVID-19
 39 patients' convalescent serum. This design led to valid immunity with increasing
 40 neutralizing antibody titers and a CTL response detected post-immunization of this
 41 vaccine by two injections in rhesus macaques. Further, this elicited
 42 immunoprotection in macaques enables not only to restrain completely viral
 43 replication in tissues of immunized animals, compared to the adjuvant control and
 44 those immunized by an RBD peptide vaccine, but also to significantly alleviate
 45 inflammatory lesion in lung tissues in histo-pathologic detection, compared to the
 46 adjuvant control with developed interstitial pneumonia. The data obtained from
 47 these macaques immunized with the inactivated vaccine or RBD peptide vaccine
 48 suggest that immunity with a clinically protective effect against SARS-CoV-2 infection
 49 should include not only specific neutralizing antibodies but also specific CTL
 50 responses against at least the S and N antigens.

51 Introduction

52 Since the end of last year, a new species of coronavirus, a contagious agent
53 capable of causing acute and severe respiratory infection and pneumonia through
54 airborne transmission, has rapidly caused global public concern and even popular
55 panic (1-3); the specific coronavirus was named SARS-CoV-2 by the World Health
56 Organization (WHO) (4). Updated data have reported more than 3 million infection
57 cases in the adult age group (5), with a death rate of approximately 2-10%, mostly in
58 the elderly population (6, 7). With its high pathogenicity and infectivity and its spread
59 in 200 countries and areas in a short time (8-10), not only multiple measures for
60 clinical treatment and cure and public isolation but also vaccine development are
61 urgently needed, and vaccine development could play a proactive role in controlling
62 this epidemic (11). However, as SARS-CoV-2 is a new viral agent with an unknown
63 infection mechanism and an unclear interaction with the immune system, vaccine
64 development should first answer some basic questions, including characterization of
65 antigenic component of this virus and its immunogenicity; validation of the
66 protective immunity elicited by the viral antigen via certain immune processes; and
67 whether the process of eliciting immunity to a viral antigen may be associated with
68 immunopathogenesis during viral infection, similar to many concerns related to
69 antibody-dependent enhancement (ADE) (12, 13). Based on these considerations,
70 the work here raised the following inferential idea: the fact that SARS-CoV-2 infects
71 cells through its spike (S) protein undergoing membrane binding with angiotensin
72 converting enzyme 2 (ACE2) molecules in the cell membrane indicates the S protein

73 is a major viral antigen that elicits a neutralizing antibody response (14, 15), while
 74 the nucleocapsid (N) protein, the other major structural component, acts as an
 75 antigenic stimulator of the innate immune response through its recognition as a
 76 pathogen-associated molecule pattern (PAMP) by cellular pattern recognition
 77 receptors (PRRs) in epithelial cells (16, 17). If this is the case, the N antigen should be
 78 significant in the study of viral vaccines, and the antibodies against the N protein may
 79 play a role in the antiviral immunity expected in vaccine development. Logically,
 80 these N-specific antibodies should be considered with the S-specific antibodies to be
 81 related to ADEs, which might exist in SARS-CoV-2-infected individuals (12). Our work
 82 describes a SARS-CoV-2 inactivated vaccine developed based on the above deduction
 83 and suggests a characterized immune response capable of defending against viral
 84 attack in rhesus macaques immunized with this vaccine, while the possible ADEs
 85 induced by existing antibodies against the S and N proteins are negated, as indicated
 86 by pathological observation and immunological detection. Conclusively, the
 87 inactivated vaccine is identified as having application potential.

88

89 **Results**

90 **The antigenicity of the SARS-CoV-2 inactivated vaccine interacting with** 91 **convalescent serum from COVID-19 patients**

92 Previous studies of viral inactivated vaccines have indicated that the capacity of
 93 vaccines to elicit valid antiviral immunity in immunized individuals depends upon the
 94 viral antigenicity that is required to have viral antigenic components and structures

displayed to the immune system of the body during the natural infectious process (18-20). With little knowledge about SARS-CoV-2, our inactivated vaccine created using a specific inactivating process was developed and investigated for its antigenicity by studying its interaction with convalescent serum, which was inferred to reflect, to some extent, the valid immune reactions of recovered individuals against viral infection. The results of a series of experiments were helpful in developing this vaccine. First, dozens of convalescent serum samples showed a neutralizing effect on the seed virus of the vaccine with varied titers of 1:16-256 (Fig. 1a), which suggests a potential quantized responsiveness of immunity leading to recovery in infected individuals. Furthermore, 2D electrophoresis and immune blotting using the convalescent serum suggested that not only the S protein but also the N protein and other proteins in this inactivated vaccine were recognized by the serum, which was more extensive recognition than that observed by blotting using a mAb against the S or N protein (Fig. 1b), and importantly, the anti-N antibody appeared to be an ascendant component. This result suggests that convalescent serum contains more antiviral antibodies than simply neutralizing antibodies and that these additional antibodies may play multiple functions in antiviral immunity. Our work using ELISA plates coated with the purified S or N protein or whole virions of the inactivated vaccine detected the convalescent serum and showed an interesting relationship between the anti-S, anti-N and anti-whole virion antibodies in the serum (Fig. 1c). The design of our inactivated vaccine based on this relationship allowed exposure of the N and S antigens, which was determined by

117 visible electron microscopy observation using convalescent serum, mAb-S or mAb-N;
118 the results showed similar interactions of the virion composing the inactivated
119 vaccine and these antibodies (Fig. 1d).

120

121 **Immunization of rhesus macaques with the SARS-CoV-2 inactivated vaccine elicits**
122 **effective immunity with indexes of humoral and cellular reactions**

123 Based on immunological studies of the SARS-CoV-2 inactivated vaccine in mice, our
124 work basically integrated the GMTs of neutralizing antibodies observed in the
125 convalescent serum from COVID-19 patients and those detected in the serum from
126 mice immunized with the vaccine at various doses (Supplemental Fig. 1). The
127 immune dose of 100 EU was determined to elicit neutralizing antibody titers in the
128 range of 1:16-64 through the intramuscular route with two inoculations on days 0
129 and 14 (Supplemental Fig. 1). Furthermore, 3 doses of 200, 100 or 20 EU were used
130 to immunize groups A (4 macaques), B (3 macaques) and C (3 macaques),
131 respectively, followed by a booster immunization on the 14th day, while 10 macaques
132 were used as adjuvant controls. The immunological evaluation of these immunized
133 macaques on day 7 after the 2nd inoculation indicated increasing neutralizing
134 antibody titers (Fig. 2a), with GMTs of 107.6, 25.4 and 2 found in the 3 dose groups,
135 respectively, and titers lower than 1:4 were observed in 2 macaques in the low-dose
136 group. This result suggests a dose-dependent relationship for vaccine immunization.
137 However, ELISA analysis with plates coated with the S or N protein indicated no
138 obvious difference in trends of the titers of anti-S and anti-N antibodies (Fig. 2b).

139 Furthermore, ELISPOT analysis of IFN- γ specificity also showed a positive cytotoxic T
140 lymphocyte (CTL) response with no dose difference after stimulation with the S or N
141 antigen (Fig. 2c). These results seem to suggest that the neutralizing antibodies
142 against the S antigen showing a dose-dependent effect related to vaccine
143 immunization are one of the components elicited in the humoral immune reaction.

144

145 **The integrated immune response elicited by the vaccine is capable of restraining**
146 **viral replication in the respiratory and alimentary tracts of challenged macaques**

147 Based on the data obtained from above immunological detection of the SARS-CoV-2
148 inactivated vaccine in rhesus macaques, we designed a viral challenge test involving
149 10 macaques immunized with the vaccine as described above to identify the
150 immunoprotective effect of the vaccine (Fig. 3a). The other 10 macaques were used
151 as adjuvant controls for observation of clinical manifestations and viral shedding, and
152 one challenged macaque was sacrificed under anesthesia at each time point.
153 Another 2 macaques, which were immunized with a ferritin-fused peptide from the
154 RBD region of the S protein expressed in CHO cells twice with inoculations on days 0
155 and 14 via the intramuscular route, and their neutralizing antibody titers reached
156 1:16-32, but no anti-N antibody set-up (Supplemental Fig. 2) was used as a parallel
157 control. All animals were challenged with wild-type virus with dose of 2×10^5
158 CCID₅₀/each animal via the nasal route. Following viral challenge, monitoring of the
159 body temperature; the viral load in pharyngeal secretions, nasal secretions, and anal
160 swabs; and viremia was performed each day. The results suggested that no obvious

161 fluctuation in body temperature was observed in any immunized macaques,
 162 including the two immunized with the peptide vaccine, compared with the 10
 163 adjuvant control animals (Fig. 3b). The detection of viral loads indicated that viral
 164 shedding occurred in the nasal cavity of the macaques immunized with the
 165 inactivated vaccine, which is the site of virus challenge and the viral load decreased
 166 from 36 copies/100 μ l - 3×10^3 copies/100 μ l on day 1 to less than 50 copies/100 μ l
 167 on day 2 and was maintained at that level through day 15 post infection (Fig. 3c),
 168 while the viral loads in pharyngeal and anal swabs were lower than 50 copies/100 μ l
 169 (Fig. 3c). Values of 10^3 - 10^4 copies/100 μ l and higher were found in the pharyngeal,
 170 nasal and anal swabs of the adjuvant control macaques for at least 8-9 days (Fig. 3c).
 171 In the analysis of the peripheral blood, no positive result was found in the inactivated
 172 vaccine group, but a peak value was observed on days 5-7 in the positive control
 173 group (Fig. 3c). Interestingly, the two macaques immunized with the peptide vaccine
 174 had neutralizing antibody titers of 1:16-32 and showed values higher than 10^4 and
 175 10^3 copies/100 μ l in nasal and anal swabs, respectively, on days 3-5 and 6-8 (Fig. 3c).
 176 These results suggest not only that the inactivated vaccine elicits a valid
 177 immunoprotective effect but also that an integrated immune response rather than
 178 neutralizing antibodies alone may be needed to restrain viral replication in the
 179 respiratory and/or alimentary tracts.

180

181 **The immunity induced by the inactivated vaccine can disrupt viral proliferation in**
 182 **organs and largely alleviate pathological damage to tissues**

183 Generally, the experimental index of immunoprotective effects on animal models for
184 the study of inactivated virus vaccines includes viral replication and the virus-induced
185 damage to tissues of vaccine-immunized animals during challenge tests with a
186 wild-type virus (21, 22). Our work here also focused on the detection of the viral
187 loads in tissues and pathological lesions in these tissues in immunized macaques,
188 which were sacrificed under anesthesia on day 3, 5, 7, 9, or 15 post viral challenge.
189 All tissue samples collected from these challenged animals, including adjuvant
190 control monkeys and the monkeys immunized with the RBD peptide vaccine, were
191 used for the detection of viral loads and pathological observation. q-RT-PCR results
192 suggested that almost all samples from various tissues of the animals in the
193 inactivated vaccine immunization group showed values lower than 50 copies/100 mg
194 and only those in spleens were higher than 100 copies/100 mg (Fig. 4a). Macaque No.
195 19177, which was immunized with the RBD peptide vaccine and sacrificed under
196 anesthesia on day 9, was found to have 635, 2200 and 140 copies/100 mg in the
197 intestine, cervical lymph nodes and spleen, respectively (Fig. 4a); and macaque No.
198 19295, which was in the same group and sacrificed under anesthesia on day 15, was
199 found to have 288,670 copies/100 mg in the cerebellum (Fig. 4a). However, the
200 evaluation of adjuvant control animals showed a trend toward increasing viral loads
201 in various organs during the observation period (Fig. 4a). In the pathological
202 detection at 5 time points, the lung tissues of 3 groups of macaques immunized with
203 the inactivated vaccine showed slight and nonspecific inflammatory reactions,
204 including a few local aggregations of inflammatory cells, inflammatory exudation in

205 some alveoli and bronchioles, slight hyperplasia of the epithelial tissue in a few
206 alveolar tissues and slight congestive reactions (Fig. 4b). The two macaques
207 immunized with the RBD peptide vaccine presented similar changes (Fig. 4b), while
208 the macaques in the adjuvant control group presented more severe inflammatory
209 reactions, which were recognized as interstitial pneumonia based upon the
210 histopathologic observation (Fig. 4b). These results suggest that the immunity
211 elicited by the inactivated vaccine can restrain viral proliferation in various tissues in
212 immunized individuals and alleviate pathological lesions in tissues caused by viral
213 replication. Interestingly, elimination of the challenge virus by this immunity in
214 macaques did not seem to be due to the existence of neutralizing antibodies. Two
215 macaques immunized with the inactivated vaccine with a neutralizing antibody titer
216 lower than 1:4 were capable of completely restraining viral replication *in vivo*, but
217 the other two immunized with the RBD peptide vaccine had a titer of 1:16-32 and
218 were not capable of completely eliminating viral replication.

219

220 **The immune response elicited in macaques by the inactivated vaccine presents an** 221 **immunologically dynamic process**

222 Previous reports on COVID-19 suggest it to be an acute and severe inflammatory
223 disease of the respiratory system (1, 23) and lead to the deduction that the
224 pathological mechanism may be due to not only viral infection but also a process of
225 excessive immune reaction, especially ADE, induced by the virus (12). In this case,
226 the study of the SARS-CoV-2 inactivated vaccine in an animal model should be

227 focused on the immunologically dynamic process associated with the elicitation of
228 specific antiviral immunity through monitoring various indexes of the immune cell
229 population, which should be maintained in a stable dynamic state (24). Our work
230 detected altered proportions of different immune cells in the total population of
231 PBMCs after viral challenge in macaques immunized with the inactivated vaccine.
232 The results suggested that compared to that of the adjuvant control animals (Fig. 5a),
233 the dynamic alteration in the PBMC population in all animals immunized with
234 inactivated vaccine included increases in the proportions of T cells, NK cells, T cells
235 with IFN- γ specificity and Treg cells (Fig. 5a, b), in which upregulation of the
236 percentages of IFN- γ -specific T cells and Treg cells was observed on days 9 and 5 post
237 viral challenge, respectively (Fig. 5b). Furthermore, the detection of some
238 inflammatory factors, including IL-2, IL-4, IL-5, IL-6 and TNF- α , in the serum of all
239 animals indicated that viral challenge was capable of slightly upregulating IL-2 and
240 IL-5 levels in the inactivated vaccine group (Fig. 5c). These results suggest not only
241 the dynamic activation of the immune system induced in macaques immunized with
242 the inactivated vaccine.

243

244 Discussion

245 The global public health crisis induced by SARS-CoV-2, which leads to acute and
246 severe infectious respiratory disease and/or pneumonia, is driving the rapid
247 development of a suitable vaccine (3, 25). Facing this emergency requirement, a
248 basic scientific question that first needs to be addressed is the antigen composition

capable of eliciting effective immunity in a vaccine; however, no clear antigenic analysis of different viral structural proteins is available. We investigated the interaction of our inactivated vaccine and convalescent serum from COVID-19 patients based on the deduction that the recovery of a patient means his/her immune system was capable of controlling and/or eliminating the virus, and convalescent serum may contain antibodies effective in this process. The data from this study suggest that anti-S and anti-N antibodies may play similar roles in convalescent serum and that both types of antibodies, but especially anti-N antibodies, show a strong relationship with the general antibody response to the virus. This result led to our technical design of an inactivated vaccine capable of exposing the S protein and N protein upon immunization into animals. In the viral challenge test with macaques, this vaccine elicited a specific immune response with a neutralizing antibody titer that increased depending on the dose and specific CTL response against the S-antigen, N-antigen and virion. The ELISA confirmed that both anti-S and anti-N antibodies were elicited in these animals, which is different from the serum of animals immunized with the RBD peptide vaccine containing only anti-S antibodies that showed neutralizing activity of 1:16-32. Usually, neutralizing antibodies are capable of blocking the binding of a virus to receptors and are an index indicative of effective antiviral immunity (26, 27). In our study of the inactivated vaccine, an immunoprotective effect against viral challenge was observed in macaques immunized with high and medium doses of the inactivated vaccine, which were associated with increasing neutralizing antibody titers, while similar

271 protective effects were observed in low-dose immunized animals with a neutralizing
272 antibody GMT of 2 and a CTL response similar to those of the high- and
273 medium-dose groups. Interestingly, the immunity elicited in macaques immunized
274 with the RBD peptide vaccine was unable to completely restrain viral proliferation in
275 some tissues, even though the animals possessed neutralizing antibodies with titer of
276 1:16-32. Two animals were found to have high viral shedding in the nasopharynx
277 and/or alimentary tract during later times of viral infection with slightly more serious
278 pathological inflammatory reactions in the lungs than animals in the high- and
279 medium-dose groups treated with inactivated vaccine immunization. These results
280 suggest that the immunity elicited by our inactivated vaccine can provide systematic
281 immune protection against viral infection not only by producing upregulation of
282 neutralizing antibody titers but also by functioning through the CTL response
283 associated with increased anti-N antibody levels. This conclusion that integrated
284 immunity involving antibodies against various viral proteins elicited by an inactivated
285 vaccine is needed for the effective prevention of SARS-CoV-2 infection, at least in
286 macaques, is supported by observation of macaques immunized with the RBD
287 peptide vaccine. All data obtained here may lead to a logical analysis of the
288 interaction of SARS-CoV-2 with the immune system, which indicates that the N
289 protein may play an important role in the activation of innate immunity in epithelial
290 cells to further the specific antiviral immune response via the N protein being a
291 major viral antigen that interacts with PRRs in cells, while the S protein greatly
292 impacts the elicitation of neutralizing antibodies and interacts with PRRs to a lesser

293 degree than the N protein. In this case, a valid systematic immunoprotective
294 response against SARS-CoV-2 infection should include at least anti-S and anti-N
295 antibodies, and innate immunity may contribute largely to this response and should
296 be considered in vaccine development. The technical design of our inactivated
297 vaccine enables N and S antigen exposure and elicits antibodies against both viral
298 proteins, which indicates that this vaccine is of practicable significance. However, the
299 observation of immunized macaques with a low titer of neutralizing antibodies due
300 to the very low antigen amount does not suggest that ADE exists. Based on these
301 data, this vaccine was permitted to enter clinical trial by CFDA with approval number
302 of 2020L00020.

303

304 **Methods**

305 ***Virus and cells***

306 The SARS-CoV-2 virus used was isolated from the respiratory secretions of an adult
307 male patient at Yunnan Hospital of Infectious Diseases in Kunming in January 2020.
308 The virus proliferated in Vero cells (ATCC, Manassas, USA) and was purified by plaque
309 cloning. The cloned virus was identified via genomic sequencing and named KMS-1
310 (GenBank No: MT226610.1). Vero cells were cultured in DMEM (Corning, NY, USA)
311 containing 5% fetal bovine serum (FCS; HyClone, Logan, USA).

312

313 ***Viral titration***

314 All experimental procedures were performed under BSL-3 laboratory conditions.

315 Virus samples were serially diluted 10-fold with serum-free DMEM (Corning, NY, USA).
316 Different dilutions of the virus were added to a 96-well plate. Each dilution (100 µl
317 per well) was added to 8 parallel wells. Then, 100 µl of Vero cell suspension was
318 added to each well at a concentration of 2.5×10^5 cells/ml. After the plate was
319 incubated at 37°C in 5% CO₂ for 7 days, the cytopathic effect (CPE) was observed and
320 assessed with an inverted microscope (Nikon, Tokyo, Japan).

321

322 ***Inactivated vaccine***

323 The SARS-CoV-2 inactivated vaccine was developed by the Institute of Medical
324 Biology (IMB), Chinese Academy of Medical Sciences (CAMS). Briefly, the virus seed
325 strain for the vaccine was inoculated into Vero cells, which was provided by WHO
326 with batch number of UCC91-02 of main seed for vaccine production, and obtained
327 the verification approval number of 201501 from National Institute for Food and
328 Drug Control (NIFDC) of China, in BSL-3 environment. The viral harvest was
329 inactivated by formaldehyde in rate of 1:4000 for 48 hours, which was found enable
330 to break viral membrane. After this inactivation, the chromatograph process using
331 Core-700 gel medium was performed for the first purification. Further, viral antigenic
332 component collected was concentrated for the second inactivated with
333 beta-propiolactone in rate of 1:2000 destructing viral genomic structure. After the
334 second purification by the same medium, the vaccine stock was evaluated with
335 various quality indexes including antigen content, immunogenicity, sterility and
336 residues test etc. The viral antigen content was measured via ELISA assay, in which,

the antibodies against S, N protein and whole virion were used to coat plate for the detections of each antigenic component. A solution of the inactivated virus vaccine stock was then emulsified in 0.5 mg/ml Al(OH)₃ adjuvant, constituting the final vaccine.

Ethics

Human --- Convalescent serum samples were collected from patients diagnosed with new coronavirus pneumonia at Yunnan Hospital of Infectious Diseases, Kunming Third People's Hospital and CDC of Xianyang city, Hubei province, with the patients providing informed consent. The protocols were reviewed and approved by the Experimental Management Association of the IMB, CAMS (approval number: DWSP 202003 004).

Animal --- The animal experiment was designed and performed according to the principles in the "Guide for the Care and Use of Laboratory Animals" and in "Guidance for Experimental Animal Welfare and Ethical Treatment". The protocols were reviewed and approved by the Experimental Animal Management Association of the IMB, CAMS (approval number: DWSP 202003 005). All animals were fully under the care of veterinarians at the IMB, CAMS.

Animals

Mouse --- Four-week-old female BALB/c mice (Beijing Vital River Laboratory Animal Technologies Co. Ltd, Beijing, China) were housed in a laboratory (ABSL-3) within the

specific pathogen-free facility at the IMB, CAMS. Mice were anesthetized with inhaled 2% isoflurane for all procedures, with every effort made to minimize suffering.

Monkey --- Rhesus monkeys (age 1.5-2 years) were bred and fed pellets (IMB, CAMS, China) and fresh fruits in a laboratory (ABSL-3) at the IMB, CAMS.

Immunization

Mouse --- Mice were randomly divided into four groups, intramuscularly immunized with the vaccine at 200 ELISA units (EU; viral antigen concentration determined by ELISA), 100 EU or 20 EU or with Al(OH)₃ adjuvant alone, which was used as a control, on days 0 and 14. Blood samples were collected for neutralization assays on day 7 after the booster injection.

Monkey --- Rhesus monkeys were randomly divided into four groups intramuscularly immunized with the vaccine at 200 EU (N=4), 100 EU (N=3) or 20 EU (N=3) or with Al(OH)₃ adjuvant alone (N=10) as a control on days 0 and 14. The monkeys were tested for neutralizing antibodies and IFN-γ-secreting cells in blood samples taken on day 7 after the booster injection.

Viral challenge

SARS-CoV-2 infection (2×10^5 CCID₅₀/monkey) via nasal spray was performed under ABSL-3 laboratory conditions for the immunized macaques by inactivated vaccine and adjuvant control group. As a parallel control, two monkeys immunized with a

381 peptide vaccine (Supplemental Fig. 2) were also infected with the patient-derived live
382 SARS-CoV-2 strain. All animals were monitored daily for clinical signs. Pharyngeal
383 secretion, nasal secretion, anal swab and blood samples were obtained every day
384 after infection. Blood was collected under appropriate anesthesia to alleviate pain
385 and minimize suffering.

386 In the 200 EU-dose vaccine group, a monkey was euthanized on days 3, 5, 7 and 9
387 postinfection (dpi). In the 100 EU-dose vaccine group, a monkey was euthanized on
388 days 3, 7 and 15 dpi. In the 20 EU-dose vaccine group, a monkey was euthanized on
389 days 5, 9 and 15 dpi. In the adjuvant control group, a monkey was euthanized at each
390 time point (days 3, 5, 7, 9 and 15 dpi). In the RBD peptide vaccine group, a monkey
391 was euthanized on days 9 and 15 dpi. The blood and organs of the euthanized
392 monkeys were obtained for various experiments. The remaining 5 macaques in the
393 adjuvant control groups were used for observation of clinical manifestations and viral
394 shedding.

395

396 ***2D protein electrophoresis-Western blot analysis***

397 Purified virus samples were resuspended in 2D lysis buffer (8 M urea, 2 M thiourea, 4%
398 CHAPS, 100 mM DTT, and 2% IPG buffer). Total protein was quantified using the
399 PlusOne 2D Quant Kit (GE Healthcare Europe GmbH, Freiburg, Germany) according to
400 the manufacturer's instructions. Approximately 200 µg of protein were first
401 separated based on their pI using immobilized linear gradient strips (Immobiline™
402 DryStrip, Amersham Biosciences Europe GmbH, Freiburg, Germany) covering the pH

range 4-7 and then separated by 12% SDS-PAGE. Proteins were transferred to a polyvinylidene fluoride membrane. The transferred membrane was blocked in 5% bovine serum albumin (BSA)-Tris-buffered saline/Tween 20 (Tris-HCl, 100 mM, pH 7.5; NaCl, 0.9%; and Tween-20, 0.2%) and treated with convalescent serum, anti-S (Snio Biological, Beijing, China), anti-N antibody (Snio Biological) and horseradish peroxidase (HRP)-labeled secondary antibody (Abcam, MA, USA) to visualize the proteins according to the standard protocol of the enhanced chemiluminescence (ECL) reagent.

411

412 ***Neutralizing antibody test***

Heat-inactivated serum was diluted and coincubated with live virus (100 lgCCID₅₀/well) for 2 h at 37°C, followed by addition of Vero cells (10⁵/mL) of 100 µl to the mixture. Then, the plates were incubated at 37°C in 5% CO₂ for 7 days. The CPEs were observed and assessed with an inverted microscope (Nikon) to determine the neutralizing antibody titer of the serum. The geometric mean titers (GMTs) of neutralizing antibodies were measured.

419 .

420 ***ELISA***

The S protein (Sanyou Biopharmaceuticals Co., Ltd., Shanghai, China), the N protein (Sanyou Biopharmaceuticals Co., Ltd.) and purified viral antigen were used to coat separate wells of 96-well ELISA plates (Corning, NY, USA) at a concentration of 5 µg/well and incubated at 4°C overnight. Then, the plates were blocked with 5%

BSA-phosphate-buffered saline (PBS), incubated with serum samples, and visualized with an HRP-conjugated antibody (Abcam, MA, USA) and TMB substrate (Solarbio, Beijing, China) according to previously described methods (28). The absorbance of each well at 450 nm was measured using an ELISA plate reader (Gene Company, Beijing, China), and the following equation was used: resulting OD = (experimental well OD) – (mock well OD). The standards used were commercial antibodies at known concentrations (Snio Biological). Antibody concentrations were calculated according to a standard curve.

433

434 ***ELISPOT***

An ELISPOT assay was performed with the Monkey IFN- γ ELISPOT Kit (Mabtech, Cincinnati, OH, USA) according to the manufacturer's protocol. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from the blood by lymphocyte isolation (Ficoll-Paque PREMIUM; GE Healthcare, Piscataway, NJ, USA) and plated in duplicate wells. Three different stimulators, purified SARS-CoV-2 antigen, recombinant S-protein (Sanyou Biopharmaceuticals Co., Ltd.) and recombinant N-protein (Sanyou Biopharmaceuticals Co., Ltd.), were added to separate wells. The positive control was phytohemagglutinin (PHA). The plate was incubated at 37°C for 24 h, after which time the cells were removed, and the spots were developed. The colored spots were counted with an ELISPOT reader (CTL, Shaker Heights, OH, USA).

445

446 ***Electron microscopy***

Purified inactivated SARS-CoV-2 preparations were coincubated with convalescent serum, a monoclonal antibody (mAb) against S (mAb-S) or N (mAb-N) (Solarbio, Beijing, China) at 37°C for 24 h, stained with 1% phosphotungstic acid and observed by transmission electron microscopy (Hitachi, Kyoto, Japan).

451

Quantitation of the viral load by q-RT-PCR

Total RNA was extracted from blood and tissue samples from experimental monkeys with TRIzol reagent (Tiangeng, Beijing, China). According to the protocol, q-RT-PCR was performed using the One Step PrimeScript™ RT-PCR Kit (Perfect Real Time; TaKaRa). The primers used for q-RT-PCR were selected to be specific for the E and ORF1ab sequences in the SARS-CoV-2 genome (Table 1). Viral copies were quantified according to vitro-synthesized RNA, and the quantity was expressed as a relative copy number, determined by the equation $[(\mu\text{g of RNA}/\mu\text{l}) / (\text{molecular weight})] \times \text{Avogadro's number} = \text{viral copy number}/\mu\text{l}$ (29).

461

Histopathological examinations

The organs of experimental monkeys were fixed in 10% formalin, embedded in paraffin, sliced into 4-μm sections and stained with hematoxylin and eosin (H&E). Morphology was assessed with an inverted microscope (Nikon).

466

Immune cell populations

PBMCs were isolated from monkeys by lymphocyte isolation (Ficoll-Paque PREMIUM;

469 GE Healthcare). Anti-CD3, anti-CD20 and anti-CD16 antibodies were added to the
470 PBMCs. The mixtures were incubated at room temperature (RT) for 30 min in the
471 dark. Reagents for red blood cell lysis (BD) and membrane permeabilization (BD)
472 were added in sequence. After washing with PBS twice, the cells were resuspended
473 in PBS and detected using a flow cytometer (BD, USA). T cells (CD3⁺), B cells (CD20⁺)
474 and NK cells (CD16⁺) were evaluated. Furthermore, the T cells were typed into T
475 helper (Th) 1, Th2, regulatory T (Treg) and Th17 cells. The PBMCs were coincubated
476 with an anti-CD4 antibody (BD) at RT for 30 min. Red blood cell lysis (BD) and
477 membrane permeabilization (BD) reagents were added. After incubating with the red
478 blood cell lysis and membrane permeabilization reagents and washing with PBS twice,
479 the cells were stained with anti-FOXP3, anti-IL-4, anti-IFN- γ and anti-IL-17A
480 antibodies for 30 min at RT and then washed once. The percentages of immune cells
481 were detected using a flow cytometer (BD). Th1 cells (CD4⁺/IFN- γ ⁺), Th2 cells
482 (CD4⁺/IL-4⁺), Treg cells (CD4⁺/FOXP3⁺) and Th17 cells (CD4⁺/IL-17A⁺) were assessed.

483

484 ***Cytokine assay***

485 The levels of IL-2, IL-4, IL-5, IL-6, TNF- α and IFN- γ in the serum of experimental
486 monkeys were detected with the Non-Human Primate (NHP) Th1/Th2 Cytokine Kit
487 (BD) according to the manufacturer's protocol. Briefly, serum was added into a tube
488 containing detection beads. Then, the PE detection reagent was added, and the
489 mixtures were incubated at RT for 2 h in the dark. After washing, the beads were
490 resuspended in wash buffer and detected using a flow cytometer (BD). The levels of

491 these cytokines were calculated according to a standard curve.

492

493 ***Statistical analysis***

494 Data are shown as the mean and standard deviation. GraphPad Prism software (San
495 Diego, CA, USA) and STATA (Version 15.0; STATA Corp., College Station, TX, USA) were
496 used for statistical analyses. The association of antibodies against S, N and virus
497 (WVN) was Spearman rank correlation analysis.

498

499 **Author contributions**

500 QHL, LDL and CGL conceived and designed the experiments; HBC, ZPX, RXL, STF, HL,
501 ZLH, KWX, YLiao, LCW, XQL, TWM, YYY, LG, JBY, HWZ, XLX, JL, YLiang, DDL, HZ, GRJ,
502 FMY, YGH, XW, CC, XQD, YLi, HLZ, JZ, HJY, JFY and WLZ performed the experiments;
503 QHL, LDL, YZ and ZMZ analyzed the data; XQD, XFZ, CH, YCC, JP, KLM, LW, WGD, DS,
504 HLZ, RJJ, LY, MJX, LYi, ZXZ, ND, HYang and WY contributed reagents/materials/analysis
505 tools; and QHL wrote the paper.

506

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510 Province.

511

512 **Conflicts of Interest**

513 All authors have completed the Unified Competing Interest form and declare that
514 they have no financial and non-financial competing interests. The corresponding
515 authors had full access to all the data generated in the present study and assume full
516 responsibility for the final submission of this manuscript for publication.

517

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584
585
586

Table 1 The primers of qRT-PCR to detect SARS-CoV-2

Name	Sequence (5'-3')
COVID-19 Ef	ACAGGTACGTTAATAGTTAATAGCGT
COVID-19 Er	ATATTGCAGCAGTACGCACACA
E-probe	FAM-ACACTAGCCATCCTTACTGCGCTTCG-TAMR3
COVID-19 orf1f	CCCTGTGGGTTTTACTTAA
COVID-19 orf1r	ACGATTGTGCATCAGCTGA
ORF-probe	FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-TAMR3

Figure legends

Figure 1. The SARS-CoV-2 inactivated vaccine showed more than one antigenic component recognized by convalescent serum derived from COVID-19 patients

- a. The convalescent serum from patients (N=71) could identify and neutralize the virus strain prepared for the SARS-CoV-2 inactivated vaccine. Geometric mean \pm SD.
- b. 2D electrophoresis (upper left) and immune blotting showed the convalescent serum (upper right), mAb-S (lower left) and mAb-N (lower right) could identify the viral proteins in the SARS-CoV-2 inactivated vaccine .
- c. Correlation analysis of the convalescent serum from patients (N=71) identified the S and N proteins and whole virion (WVN) by Spearman rank correlation analysis. The antibody concentrations were tested and calculated according to a standard curve determined by ELISA.
- d. The convalescent serum (left), mAb-S (middle) and mAb-N (right) could identify and enrich the virion, as determined by visible electron microscopy.

Figure 2. The SARS-CoV-2 inactivated vaccine elicited humoral and CTL immune responses in rhesus monkeys

- a. Different doses of the inactivated vaccine induced neutralizing antibodies in rhesus monkeys (N=10). The GMT values for all monkeys in the Al(OH)₃ adjuvant control group (N=10) were <2 (GMT=1 in the picture). Geometric mean \pm SD.
- b. Different doses of the inactivated vaccine elicited antibodies against the S and N

proteins and whole virion (WVN) in rhesus monkeys. The antibody concentrations were tested and calculated according to a standard curve determined by ELISA. Geometric mean \pm SD.

- c. Different doses of the inactivated vaccine elicited IFN- γ -specific immune responses against the S and N proteins and whole virion (WVN) in rhesus monkeys. PBMCs were incubated for 24 h in the presence of a stimulus. Mean \pm SD.

Samples were obtained on day 7 post 2nd inoculation.

Figure 3. The integrated immune response elicited by the inactivated vaccine can restrain viral replication in the respiratory and/or alimentary tracts

- a. The design of the immunity and viral challenge protocol.
- b. Monitoring of the body temperature of rhesus monkeys infected by SARS-CoV-2.

The normal range from 37.5 to 39.5°C (refer to the normal monkeys (N=10) monitored during the same period) is indicated with dotted lines. Inactivated vaccine (200EU, 100EU and 20EU; N=10), RBD peptide vaccine (N=2) or Al(OH)₃ adjuvant (control; N=10). Mean \pm SD.
- c. The viral loads of pharyngeal secretions, nasal secretions, anal swabs and blood from monkeys immunized with the inactivated vaccine (200EU, 100EU and 20EU; N=10), RBD peptide vaccine (N=2) or Al(OH)₃ adjuvant (control; N=10) after infection with live virus. Negative is the copies less than 10 (dotted lines). Mean \pm SD.

Figure 4. The integrated immune response elicited by the inactivated vaccine can eliminate viruses and alleviate the pathological damage induced by the challenge virus

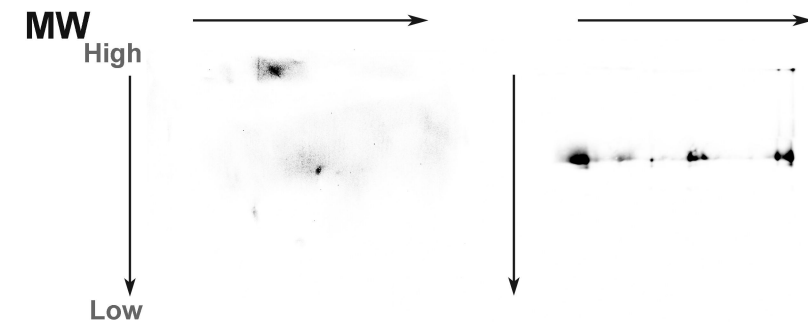
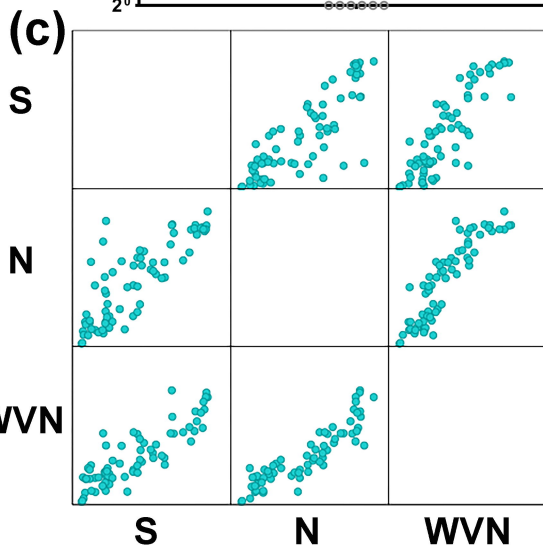
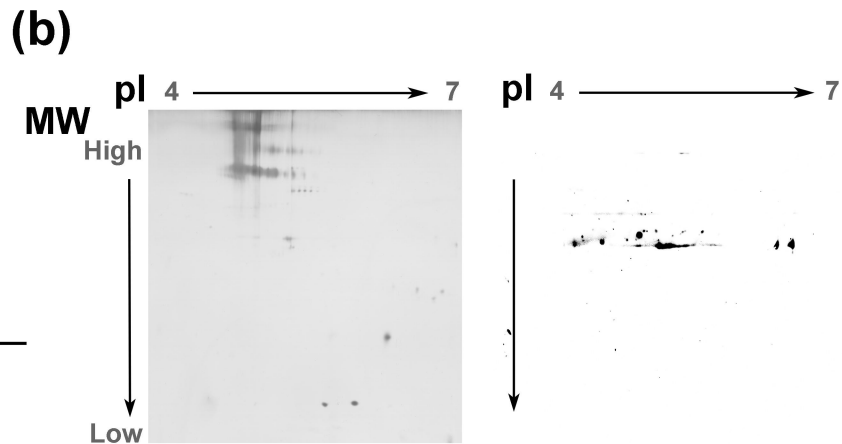
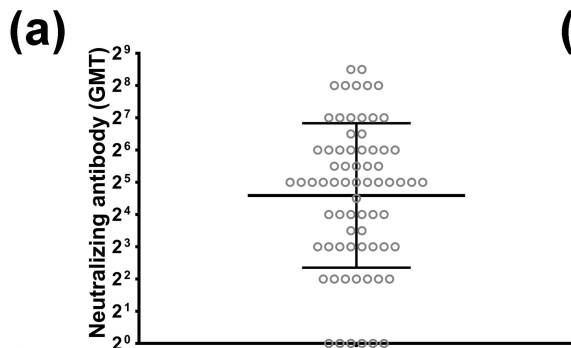
- a. The viral loads of various organs from monkeys immunized with the inactivated vaccine (200EU, 100EU and 20EU; N=10), RBD peptide vaccine (N=2) or Al(OH)₃ adjuvant (control; N=5) after infection with live virus. Negative is the copies less than 10 (dotted lines). Mean ± SD. There are no data on day 15 of the 200EU group, on days 5 and 9 of the 100EU group, on days 3 and 7 of the 20EU group, and on days 3, 5, and 7 of the RBD group.
- b. Typical pathological changes in the lungs of rhesus monkeys immunized with the inactivated vaccine (200EU, 100EU and 20EU; N=10), RBD peptide vaccine (N=2) or Al(OH)₃ adjuvant (control; N=5) and infected with live virus. Congestion (black arrow), infiltration of inflammatory cells (green arrow) and edema (blue arrow). Samples were obtained at 7 dpi (200EU), 7 dpi (100EU), 9 dpi (20EU), 9 dpi (RBD vaccine) and 7 dpi (control).

Figure 5. The dynamic immune reaction process is maintained in macaques immunized with the inactivated vaccine during viral challenge

- a. The percentages of T, NK and B cells in PBMCs or lung from rhesus monkeys immunized with the inactivated vaccine (N=10) or Al(OH)₃ adjuvant (control; N=5) during viral challenge. Mean ± SD.

- b. The percentages of Th1, Th2, Th17 and Treg cells in PBMCs or lung from rhesus monkeys immunized with the inactivated vaccine (N=10) or Al(OH)₃ adjuvant (control; N=5) during viral challenge. Mean ± SD.
- c. IL-2, IL-4, IL-5, IL-6 and TNF-α levels in the serum of rhesus monkeys immunized with the inactivated vaccine (N=10) or Al(OH)₃ adjuvant (control; N=5) during viral challenge. Mean ± SD.

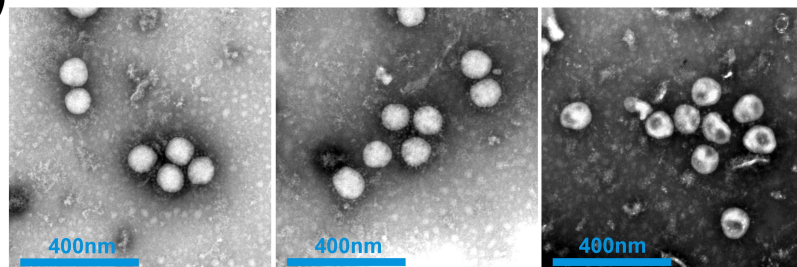
The normal range (refer to the normal monkeys (N=10) monitored during the same period) is indicated with dotted lines.

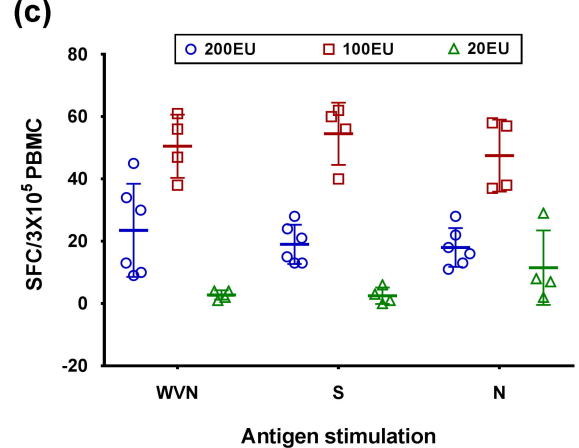
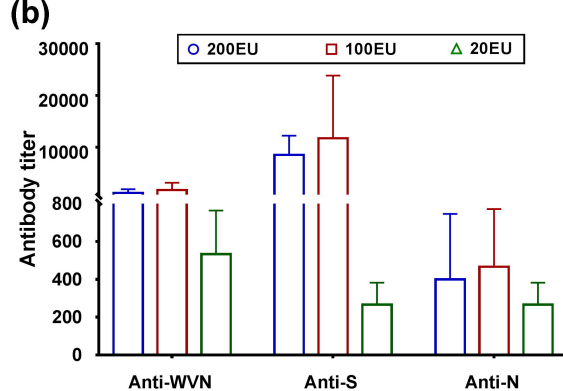
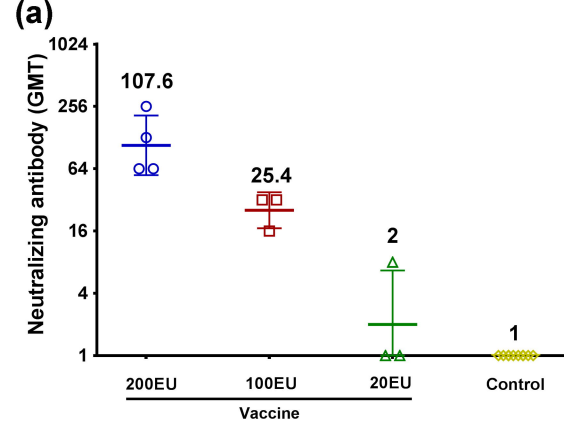


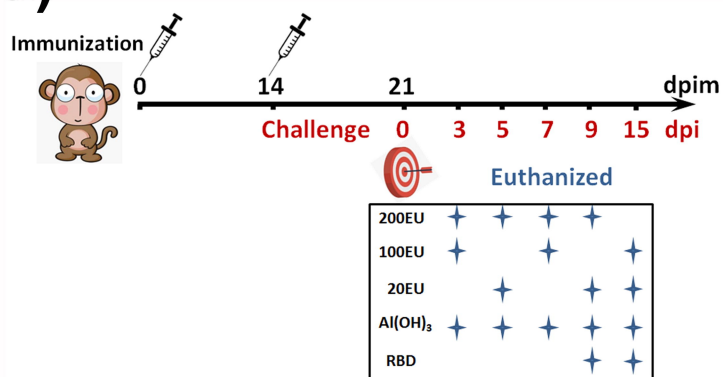
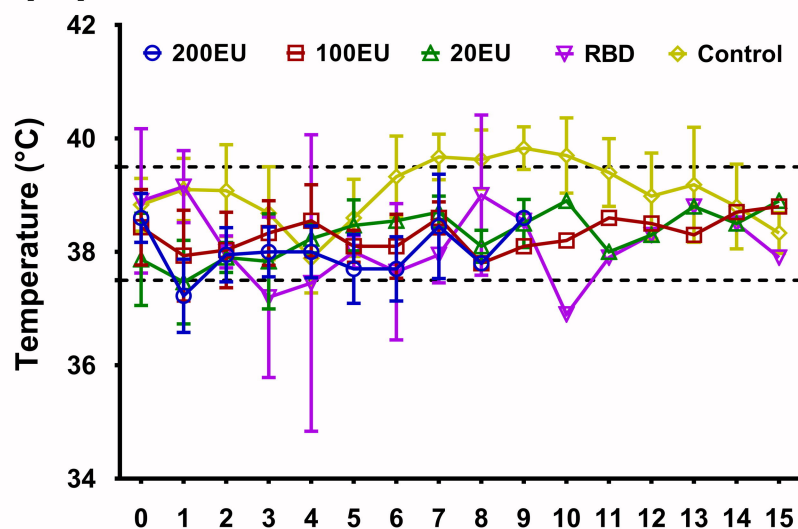
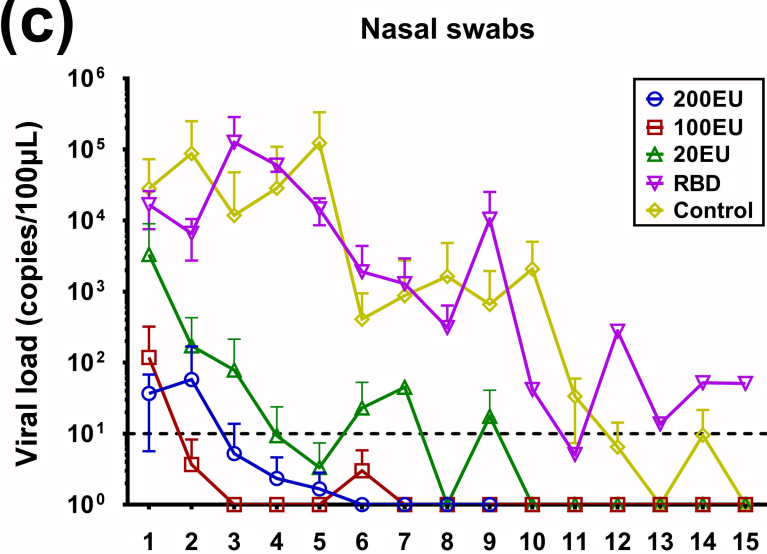
Spearman rank correlation analysis of S antibody, N antibody and total antibody in human convalescent serum.

	Anti-S		Anti-N		Total	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Anti-S	1.00	-				
Anti-N	0.810	<0.001	1.00	-		
Total	0.848	<0.001	0.912	<0.001	1.00	-

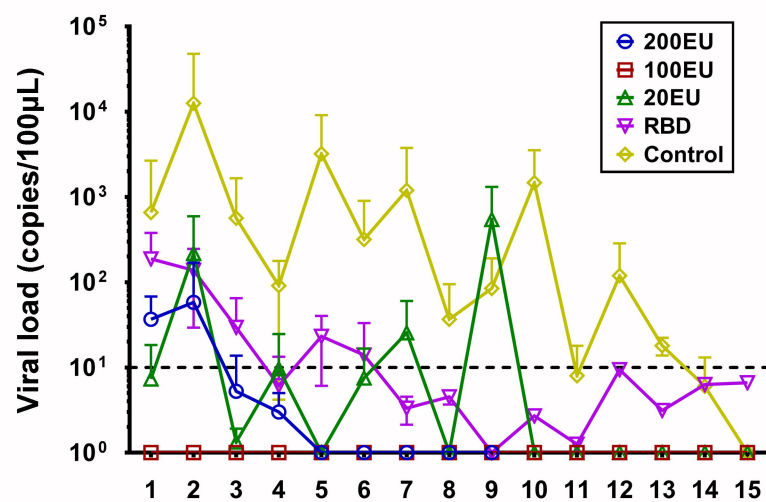
Note: The closer the absolute value of *r* is to 1, the higher the degree of correlation.



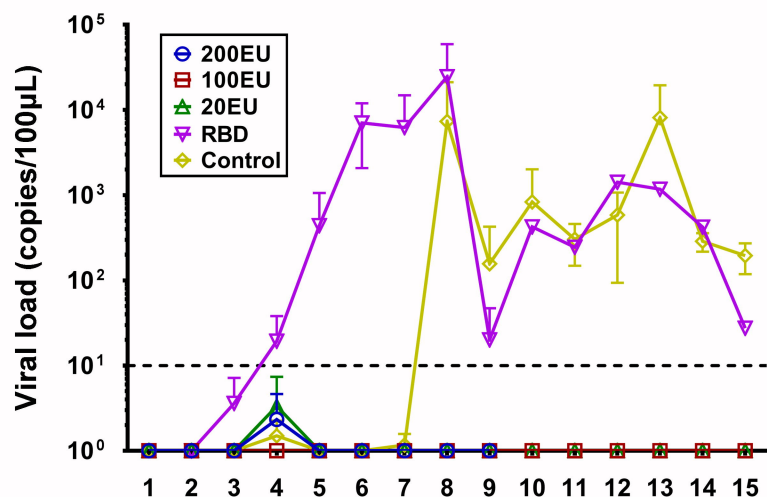


(a)**(b)****(c)**

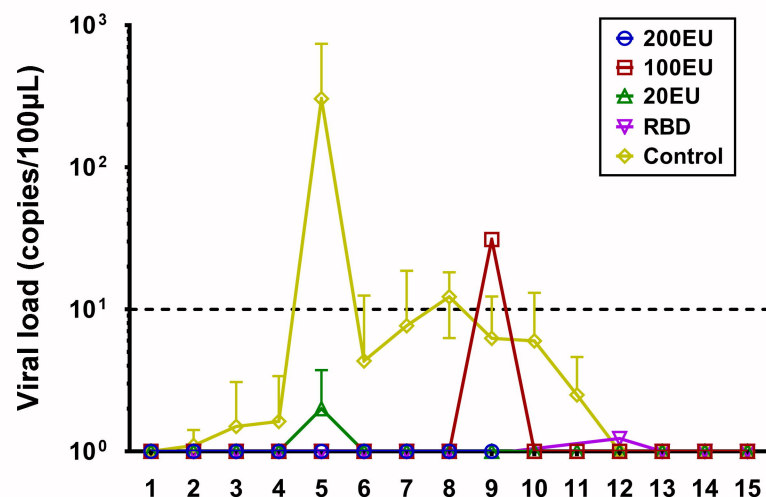
Pharyngeal swabs



Anal swabs



Blood



Days post-infection

Days post-infection

