

# Immunogenicity, safety, and protective efficacy of an inactivated SARS-associated coronavirus vaccine in rhesus monkeys

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## Abstract

An inactivated vaccine for severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) was evaluated in rhesus monkeys. The monkeys were inoculated intramuscularly (i.m.) with 0.5, 5, 50, or 5000  $\mu\text{g}$  of vaccine, or PBS as control, and boosted on day 7. After 3 weeks, they were challenged with the NS-1 strain of SARS-CoV. The humoral and mucosal immune responses, clinical signs, chemical indices and viremia were monitored following the immunization and challenge. The control animals who received PBS developed atypical SAR-CoV infection after viral challenge, according to clinical, virological and pathological findings. No systematic side effects were observed in vaccinated animals post-immunization, even in at the high dose of 5000  $\mu\text{g}$ . The 50  $\mu\text{g}$  dosage of vaccine elicited SARS-CoV specific immune responses against viral infection as compared to the partial immunity elicited by 0.5 and 5  $\mu\text{g}$  doses. The results show that this inactivated vaccine can induce effective concomitant humoral and mucosal immunity against SARS-CoV infection, is safe in monkeys, and the vaccine maybe a good candidate for clinical trials.

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**Keywords:** SARS-CoV; Inactivated vaccine; Rhesus monkeys

## 1. Introduction

As the first emerging life-threatening and highly contagious epidemic of the 21st century, severe acute respiratory syndrome (SARS) spread to more than 30 countries across five continents with excess morbidity and mortality. Through the efforts of an international consortium of laboratories, a new type of coronavirus, SARS-associated coronavirus (SARS-CoV), was identified as the causative agent [1].

Conventional coronaviruses cause economically devastating diseases in livestock, poultry, and laboratory rodents. Several coronaviruses can cause fatal systemic diseases in

animals, including feline infectious peritonitis virus (FIPV), hemagglutinating encephalomyelitis virus (HEV) of swine, and some strains of avian infectious bronchitis virus (IBV) and mouse hepatitis virus (MHV) [2,3]. In general, each coronavirus causes disease in only one animal specie. In immunocompetent hosts, infection elicits neutralizing antibodies and cell-mediated immune responses that kill infected cells. In immunocompetent SARS patients, neutralizing antibodies were detected 2–3 weeks after the onset of disease, and 90% of patients recover without hospitalization [4].

Based on what were learned from animal coronavirus and serologic responses in SARS patients [5,6], control of SARS seems most likely to be achieved by vaccination. As lack of understanding of the pathogenesis of SARS and its etiology, some efforts toward developing a SARS vaccine, such

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as expressing viral proteins in vitro, introducing attenuating mutations into virus, or engineering virus genome as vector, require considerable research set-up time [7]. Therefore, the classic approach using inactivated, cell-culture based SARS virus is likely to be the fastest and easiest way for SARS vaccine development, on the ground of experiences of many commercial vaccines including inactivated or oral polio vaccines, and rabies vaccine [8,9].

In the present study, we reported a pre-clinical evaluation of an inactivated vaccine candidate against SARS-CoV for immunogenicity, safety, and protectivity in non-human primate, rhesus monkey.

## 2. Materials and methods

### 2.1. Virus and vaccine

The virus used for candidate inactivated SARS-CoV vaccine was SARS-CoV Z-1 strain, isolated from the blood of the first SARS patient from Zhejiang Province, China, in 2003. The vaccine is a formaldehyde inactivated whole virus, prepared in cultured Vero cells, provided by the Wuhan Institute of Biological Products in Hubei Province.

The virus used for challenge was the Chinese SARS-CoV representative strain NS-1, which was isolated from the urine of an acute-phase SARS patient during the epidemic in China's Ningxia province in 2003. The titer of the preparation is  $1 \times 10^8$  PFU/ml.

### 2.2. Animal immunization and challenge

All animals were obtained from Yunnan Animal Cultivation Center in Yunnan, China. Eighteen 2- to 5-year-old rhesus monkeys (numbered #1–#18; half of them male and half female), with body weights ranging from 3.5 to 4.5 kg, were used in this study. All monkeys had been tested negative for antibodies against SARS-CoV. The animals were housed in individual cages in a biosafety level three (BSL-3) containment facility, maintained at constant room temperature with a 12-h light/12-h dark photoperiod, and fed apples and pelleted diet every day. Before handling, bleeding, immunization, and challenge, monkeys were anesthetized intramuscularly (i.m.) with ketamine hydrochloride (10–40 mg/kg). All procedures

involving the probable infectious samples were conducted in the BSL-3 laboratory.

Animals were immunized according to the protocol in Table 1. Eighteen monkeys were randomized into six groups. Monkeys #13 and #14 who received the highest dose of vaccine were used to evaluate safety of the vaccine. The injection region was depilated in advance when local reactions to the vaccine were evaluated.

On day 22 post-immunization, 12 immunized monkeys (#1–#12) and two control monkeys (#17, #18) were challenged with the NS-1 strain of SARS CoV, at a dose of  $10^8$  plaque-forming units (PFU) per animal. Intratracheal (IT) administration of virus was performed by cricothyroid membrane puncture. All animals were sacrificed and necropsied on day 15 post-challenge. Venous blood was collected on days 0, 3, 7, 11, 14, 21 post-immunization and on days 2, 5, 8, 15 post-challenge. Serum was separated and stored at  $-20^\circ\text{C}$  for subsequent tests. In addition, lightly heparinized blood samples (10 IU heparin per ml of blood) were collected for hematological examination before and after immunization and two times after post-challenge. Nasopharyngeal secretions were collected with aseptic swabs on days 0, 7, 14, 21 post-immunization and on days 3, 5, 7, 9 post-challenge, washed in 0.5 ml saline, and centrifuged at 4000 rpm for 5 min. The supernatant was stored at  $-20^\circ\text{C}$  for IgA ELISA and reverse-transcriptase polymerase chain reaction (RT-PCR) assays. The mixture of feces and urine samples were collected pre-, post-immunization, and on days 3, 5, 7, 9 post-challenge.

Necropsies were conducted on day 15 post-challenge, and tissue samples were obtained from the lungs, kidneys, liver, heart, and brain for pathological examination. They were fixed in formalin, embedded, sectioned and stained with hematoxylin and eosin. Tissue samples of lung and kidneys were also frozen for RT-PCR assay and virus isolation in Vero cell culture.

### 2.3. ELISA assays for IgG and IgA antibodies

Humoral and mucosal immune responses were assayed by conventional indirect ELISA as described below. Purified SARS-CoV antigen was provided by the Institute of Microbiology and Epidemiology, Chinese Military Medical Academy (Beijing, China). Serum samples were tested for

Table 1  
The protocol of immunizing rhesus monkeys with inactivated SARS-CoV

No. of rhesus monkeys	Immunization dose ( $\mu\text{g}$ )	Immunization route	Immunization times	Immunization days	Active virus challenge
4 (#1–4)	0.5	i.m.	2	Day 0 and 7	$1 \times 10^8$ PFU
4 (#5–#8)	5	i.m.	2	Day 0 and 7	$1 \times 10^8$ PFU
4 (#9–#12)	50	i.m.	2	Day 0 and 7	$1 \times 10^8$ PFU
2 (#13, #14)	5000	i.m.	2	Day 0 and 7	ND
2 (#15, #16)	PBS <sup>a</sup>	i.m.	2	Day 0 and 7	ND
2 (#17, #18)	PBS <sup>a</sup>	i.m.	2	Day 0 and 7	$1 \times 10^8$ PFU

Vaccines were diluted to equal volumes in PBS before vaccination. Seven days post-immunization monkeys were boosted i.m., with the same dose of inocula.

<sup>a</sup> PBS: phosphate-buffered saline.

IgG in duplicate in serial 2-fold dilutions from 1:100 in PBS containing 5% BSA. The nasopharyngeal IgA was tested directly in the supernatant washings obtained from the swabs.

Purified viral antigen was diluted to 5 µg/ml in 0.1 M sodium carbonate buffer (pH 9.6) just prior to use. Flat-bottomed 96-well microplates (Costar, Corning, Inc., USA) were coated with 100 µl of the antigen solution at 4 °C for 16 h. The plate was blocked with 100 µl/well of 15% FBS in PBS for 2 h at 37 °C. After washing with PBS containing 0.05% Tween 20 (PBS-T), the plate was incubated at 37 °C for 1 h with 100 µl of serum dilution or nasopharyngeal washings. The plate was washed and incubated at 37 °C for 1 h with horseradish peroxidase-conjugated goat anti-monkey affinity purified IgG (Bethyl Laboratories, Inc., USA) diluted 1:40,000 in T-PBS buffer containing 5% BSA, or horseradish peroxidase-conjugated goat anti-monkey affinity purified IgA (α) (KPL, Europe) diluted 1:2000. After washing with PBS-T, wells were developed by TMB for 20 min at room temperature in the dark. The reaction was stopped by the addition of 100 µl 1 M H<sub>2</sub>SO<sub>4</sub>. Optical density (OD) of colored product was measured at 450 nm using a plate reader (RT-2100C, Rayto Corporation). The IgG ELISA titers were expressed as the reciprocal of the highest dilution with an OD value >0.1 and at least 2.1-fold that of the negative control. A significant mucosal IgA antibody response was defined as at least 2.1-fold that of the control. Pre-inoculation samples from the same monkeys were used as controls.

#### 2.4. Neutralization antibody assay

Neutralizing antibody was detected by the inhibition of CPE in cultured Vero-E6 cell monolayers inoculated with the challenge virus strain. The serum samples on day 14 post-immunization were heat-inactivated and serially diluted 2-fold, from 1:5 to 1:1280, and then were mixed with 100 TCID<sub>50</sub> of the virus. After water incubation for 1 h at 37 °C, the mixture was inoculated onto 96-well plates of Vero-E6 cells, using eight wells per dilution. Cultures were held at 37 °C and in 5% CO<sub>2</sub>, with daily microscopic examination for cytopathic effect (CPE). When the virus control (no serum) showed complete CPE, the maximum dilution of each serum that completely prevented CPE in 50% of the test-wells was calculated by the Reed Muench formula when the virus control (no serum) showed complete CPE. [10].

#### 2.5. Serum cytokine analysis

In vitro quantitative determination of monkey IL-4 and IFN-γ in serum were assayed by a quantitative “sandwich” ELISA (Biosource International, Inc., USA). The assay was performed according to the manufacturer’s instruction. The OD value at 450 nm was measured with the ELISA plate reader described above. Standard curves were plotted in light of OD values of standard samples and used to calculate the concentration of cytokines IFN-γ and IL-4, separately.

#### 2.6. Clinical observation

Following immunization and challenge, each animal was monitored daily for clinical signs, such as local reaction at the injection site, changes in activity, rectal temperature, appetite, rash, frequency of defecation and stool consistency, etc.

Hematological examination was conducted on pocH-100i, SYSMEX. Liver and kidney functions were measured by blood level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), gamma glutamyl transferase (GGT), urea and creatinine clearance (CR) on RT-2100C (Rayto Corporation, Shenzhen). The average levels of animals in the same group before immunization were used as the normal levels.

Chest X-ray exams at the posterior–anterior position and lateral position were performed for all animals before sacrifice.

#### 2.7. Virological evaluation

##### 2.7.1. RT-PCR

Evidence of virus in blood, nasopharyngeal swab, and the mixture of feces and urine samples were detected by reverse-transcriptase polymerase chain reaction. Total RNA kit (Promega) and Trizol kit (Invitrogen) were used for RNA extraction of different samples. Random primers were used for the preparation of complementary DNA templates for PCR. The forward and reverse primers used for RT-PCR were 5′-CAGAGTTGTGGTTTCAAGTG-3′ (nucleotides 21431 to 21450) and 5′-CACAGAGTAATCAGCAACAC-3′ (nucleotides 22538 to 22519), respectively. The reverse transcription was conducted at 43 °C for 1 h then at 95 °C for 5 min. Amplification of the cDNAs was performed at the reaction conditions of 94 °C for 2 min followed by 35 cycles of 94 °C for 10 s, 55 °C for 30 s, and 68 °C for 3 min, then 68 °C extending for 7 min.

##### 2.7.2. Virus isolation

One hundred microliters per well of serum, filtered nasopharyngeal washings and 10% tissue homogenates were inoculated onto Vero cells monolayer in 24-well plates (Costar, Corning, Inc., USA). After incubation at 37 °C for 6 ~ 8 h in a 5% CO<sub>2</sub> incubator, the inoculum was replaced with complete MEM (Gibco) containing 2% bovine serum. The culture plates were observed for 7 days and blindly passaged two times if no SARS-CoV specific cytopathogenic effects (CPE) appeared.

Tissue histologic and immunohistochemical assays were performed as described previously. [11]

#### 2.8. Statistical analysis

Statistical comparisons of the WBC counts pre-, post-immunization and post challenge in each group, and IgA OD values, cytokines profiles of different vaccine dosages were

performed with one-way ANOVA in SPSS 11.5 software. Significance was set at  $P < 0.05$ .

### 3. Results

#### 3.1. Safety and protective efficacy assessment

##### 3.1.1. Post-vaccination clinical signs

All monkeys survived the intramuscular inoculation of the candidate vaccine in doses of up to 5000  $\mu\text{g}$  and the IT challenge of active SARS-CoV NS-1 strain at  $1 \times 10^8$  PFU up to the time of necropsy 2 weeks later. Following immunization, all animals appeared normal in activity and appetite, and had normal weight gain. No systematic complications were observed. Specifically, none of the monkeys showed skin rash at the vaccine injection site, while very high dose (5000  $\mu\text{g}$ ) caused mild local reactions (induration, less than 1.6 cm in diameter). Hematological assay showed no significant differences pre- and post-immunization in 6 groups (Fig. 1), and no elevation of serum concentrations of ALT, AST, TP, ALB, GGT, RUEA and CR greater than twice the mean pre-immunization level (data not shown). It must be noted, however, that these characteristics varied among the monkeys.

##### 3.1.2. Post-challenge clinical signs

Following live virus challenge, no remarkable changes were observed in rectal temperature in all vaccine-immunized animals. Other clinical symptoms such as coughing, lethargy, and rash were not shown in the vaccinated animals. Likewise, normal body weight gain was observed in all the vaccinated animals. However, in the PBS-inoculated control animals, although no overt clinical respiratory symptoms appeared, minor lethargy and loose stools were observed in both animals after 3–5 days post-challenge. Monkey #18 had temporary skin rash, but no significant change in body temperature. Monkey #17 developed fever ( $0.5^\circ\text{C}$  above the level before challenge) on day 10 post-challenge. The body weight of monkey #17 decreased slightly after the fever developed, while no obvious change observed in monkey #18.

After live virus challenge, hematological assays showed reduction in white blood cell (WBC) counts in animals receiving 0.5  $\mu\text{g}$  of vaccine ( $P < 0.01$ ) but no changes were measured in other immunized groups. In control monkeys,

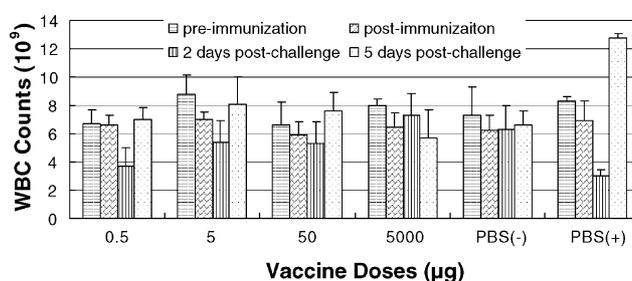


Fig. 1. WBC counts of rhesus monkeys pre-, post-immunization, 2 and 5 days post-challenge. Each column represents the mean  $\pm$  S.E. of four animals in each group.

transient drops in WBC counts were detected on day 2 post-challenge, but rapid increases with increases of lymphocyte classification were observed on day 5 (Fig. 1), compared to the average level of all animals pre-immunization. Serum biochemical indices did not show any significant increase up to the day of sacrifice (data not shown).

#### 3.2. X-ray and pathological findings

Chest X-ray found no radiological or pathological abnormalities were found in the immunized animals before or after challenge (Fig. 2A). Focally thickened and deranged streaks were identified in the lungs of two PBS-control monkeys after challenge. Tissue samples obtained at necropsy from the affected regions as demonstrated on X-ray showed the walls of the pulmonary alveoli were thickened obviously due to edema of alveolar epithelia, proliferation of interstitial cells, interstitial lymphocytes and macrophage inflammatory infiltration, and hyaline-membrane formation on alveolar walls (Fig. 2B). Immunohistochemical analysis showed SARS-CoV antigen in the alveolar epithelia (Fig. 2C). No remarkable lesions were shown in other tissues.

#### 3.3. Virological assays

Specific amplification product was obtained by RT-PCR in most of the samples from the 2 PBS-control monkeys challenged by the virus (Table 2). Furthermore, virus was isolated from lung tissue of these animals by Vero cell culture. CPE in the Vero cells were first noted between day 3 and day 5 after subculture of tissue specimens from animals #17 and

Table 2  
Excretion of SARS-CoV by experimentally inoculated rhesus monkey (RT-PCR)

Time after challenge (days)	Monkey #17			Monkey #18		
	Nasopharyngeal swab	Faeces	Blood	Nasopharyngeal swab	Faeces	Blood
0	–	–	–	–	–	–
3	–	–	+	–	–	+
5	–	–	+	–	–	+
7	+	–	ND <sup>a</sup>	+	+	ND <sup>a</sup>
9	+	+	ND <sup>a</sup>	ND <sup>a</sup>	+	ND <sup>a</sup>

The presence of SARS-CoV RNA in samples by PCR is indicated with “+”, absence with “–”.

<sup>a</sup> ND: not done.

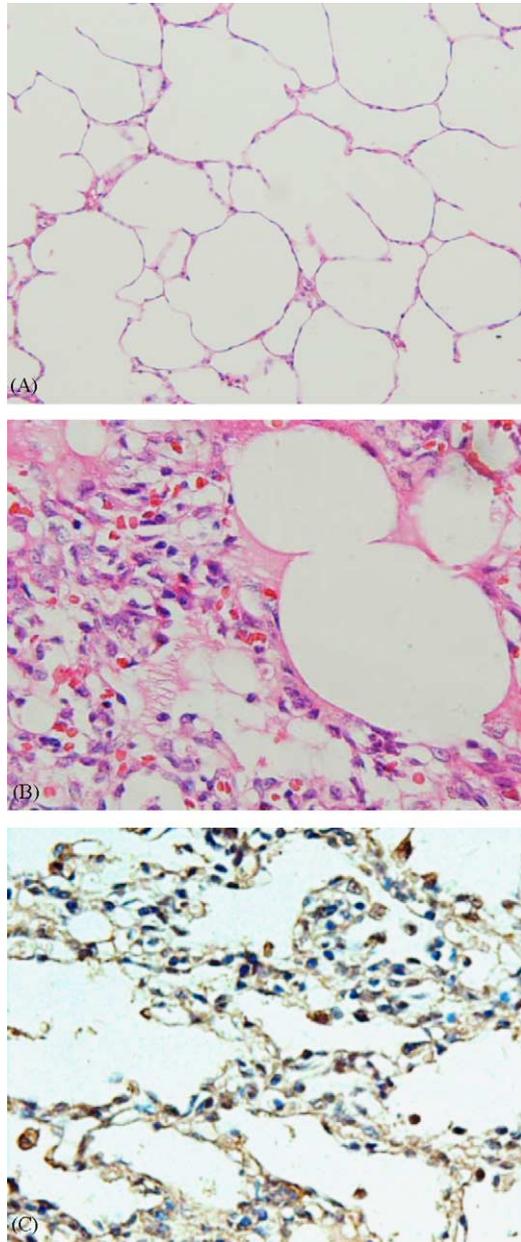


Fig. 2. (A) Post-immunization necropsy lung tissue from monkey #12. Normal pulmonary alveoli and epithelia were shown. Hematoxylin-eosin stain, 400 $\times$ ; (B) Post-challenge necropsy lung tissue from monkey #17, showing the walls of the pulmonary alveoli were thickened obviously due to edema of alveolar epithelia, proliferation of interstitial cells, interstitial lymphocytes and macrophage inflammatory infiltration, and hyaline-membrane formation on alveolar walls. Hematoxylin-eosin stain, 400 $\times$ ; and (C) Immunohistochemical staining of lung section from monkey #17. Serum samples from convalescent SARS patients were used as the primary antibody. SARS-CoV antigen was shown in pneumocytes. 400 $\times$ .

#18. RT-PCR was negative in all pre-challenged samples and in most of the challenged samples of the vaccinated monkeys. Blood samples of the 2 animals receiving 0.5  $\mu\text{g}$  of vaccine (#1 and #2) and one animal receiving 5  $\mu\text{g}$  (#6) were positive by RT-PCR. However, no virus was recovered from these samples by culture. At the end of the observation pe-

riod, pathogen excretor (RT-PCR or virus isolation positive) amounted to 0 in 50  $\mu\text{g}$  of vaccine group, one of four animals in 5  $\mu\text{g}$  of vaccine, and two of four in 0.5  $\mu\text{g}$  of vaccine, two of two in PBS immunized group.

### 3.4. Immunogenicity assessment

#### 3.4.1. Serum IgG antibody response

As listed in Table 3, the PBS-control monkeys remained negative for SARS-CoV IgG antibody during the 21 days pre-challenge period. Most of the vaccinated monkeys (11/12, 92%) developed detectable IgG titers (>100) by 7 days post first immunization, but two animals receiving 5  $\mu\text{g}$  of vaccine (#2 and #6) became IgG negative after second immunization, and remained negative by 21 days post first immunization. This result suggests that there is difference in the levels of immune responses among monkeys. All monkeys receiving 50  $\mu\text{g}$  of vaccine demonstrated specific IgG responses by 7 days post first immunization, and the antibody titers elevated gradually after second immunization. All of the animals receiving 50  $\mu\text{g}$  of vaccine exhibited high level antibody titers ( $\geq 1600$ ) by 21 days post first immunization, compared with three of four animals receiving 5  $\mu\text{g}$  of vaccine and two of four animals receiving 0.5  $\mu\text{g}$  of vaccine. The increase of doses appeared to enhance immune responses. The antibody titers at day 21 indicated that 50  $\mu\text{g}$  of inactivated vaccine was more immunogenic than 0.5 and 5  $\mu\text{g}$  of vaccine. We also detected the IgG Ab level on days 8 and 15 following inoculation, the Ab levels increased obviously in most of animals in the 0.5 and 5  $\mu\text{g}$ -dose groups and all of the animals in the 50  $\mu\text{g}$ -dose group.

#### 3.4.2. Mucosal immunology

Nasopharyngeal antibody responses to intramuscular vaccinations are listed in Fig. 3. Five (41.7%) of the 12 immunized monkeys had SARS-CoV specific secretory IgA (sIgA) antibody (Fig. 3).

#### 3.4.3. Serum neutralization activity

After immunization of the inactivated SARS-CoV vaccine, different levels of the neutralization antibody were detected on day 14 in all three immunized groups (Fig. 4). We found that all antisera showed various abilities to inhibit

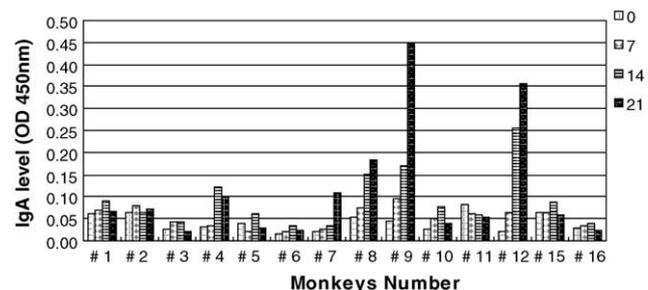


Fig. 3. The SARS-CoV specific sIgA response in nasopharyngeal washings after the initial immunization.

Table 3  
IgG antibody titers of monkeys immunized with inactive SARS CoV candidate

Dose(μg)	Animal	Days post first immunization							
		0	3	7	11	14	21	28 (day 8 p.i.)	35 (day 15 p.i.)
0.5	#1	<100	<100	<100	<100	<100	200	800	3200
	#2	<100	<100	100	<100	<100	<100	100	100
	#3	<100	<100	200	<100	400	800	1600	3200
	#4	<100	<100	100	<100	800	1600	3200	1600
5	#5	<100	100	200	400	800	3200	>12800	>12800
	#6	<100	<100	100	<100	<100	<100	<100	200
	#7	<100	<100	100	100	400	1600	1600	3200
	#8	<100	100	400	800	1600	3200	6400	6400
50	#9	<100	100	400	800	1600	>6400	>12800	>12800
	#10	<100	100	200	400	400	1600	6400	6400
	#11	<100	<100	100	200	800	>3200	12800	12800
	#12	<100	<100	200	400	400	1600	3200	>12800
PBS	#15	<100	<100	<100	<100	<100	<100	NT <sup>a</sup>	NT <sup>a</sup>
	#16	<100	<100	<100	<100	<100	<100	NT <sup>a</sup>	NT <sup>a</sup>

Titers are reported as the inverse of the last dilution of serum for which the OD value is greater than 0.1 absorbance units and at least 2.1-fold that of the control.  
<sup>a</sup> NT: not test.

cytopathic effects mediated by SARS-CoV in Vero-E6 cells and higher dosages of vaccine induced higher levels of neutralizing antibody titer (Fig. 4). 50 μg of vaccine appeared the largest neutralizing activity with 1:160 of antibody level on day 21, while the 5 μg of vaccine indicated half of that level on the same day. On day 21, the neutralization titer of 0.5 μg dose group were 1:20, significantly lower than the other two higher dose groups ( $P < 0.01$ ).

3.4.4. Cytokine profiles

To further determine the T helper type of response, cytokines IFN-γ and IL-4 released in serum were quantified with sandwich ELISA. After initial immunization, serum from monkeys receiving 5 μg and 50 μg of vaccine showed significantly increased productions of IFN-γ, which was significantly higher than those of the pre-immunization levels ( $P < 0.05$  and 0.01, respectively), and continued to increase after second immunization. Serum from monkeys receiving 0.5 μg of vaccine demonstrated significantly increased IFN-

γ only after second immunization ( $P < 0.05$ ). No obvious changes were observed in animals of PBS control group. On the other hand, the levels of IL-4 produced pre- or post-vaccination in three immunized groups and control group did not exhibit significant changes (Fig. 5).

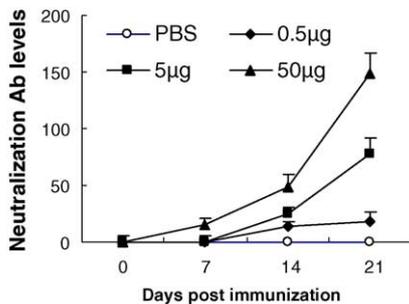


Fig. 4. Neutralizing Ab in rhesus monkey's serum after immunization. Titers were expressed as the reciprocal of the highest dilution at which the CPE was completely inhibited in 50% of the test-wells. Data are presented as the mean ± S.E. for each group.

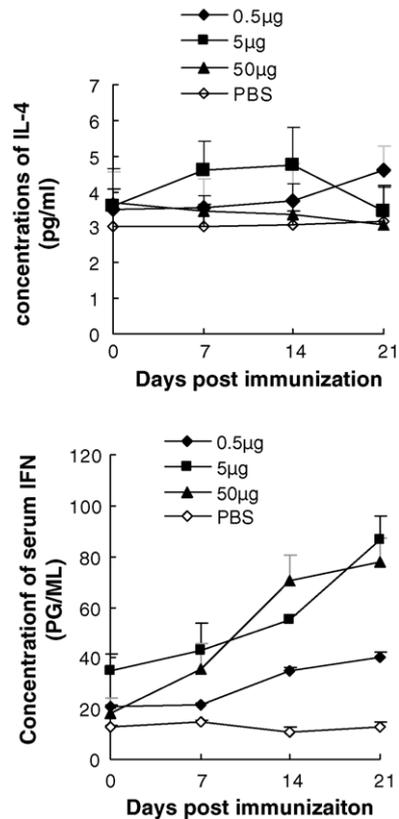


Fig. 5. Serum cytokine concentrations pre- and post-immunization. The OD values were presented as mean ± S.E. of the four animals in each group.

#### 4. Discussion

Although in 2004 there were no significant SARS outbreaks, and none of the few isolated cases resulted in death or secondary transmission, the resurgence of SARS leading to an major epidemic remains a possibility. Development of an effective vaccine against SARS-CoV is crucial in preventing future epidemics.

Early studies with rodents were not successful in reproducing SARS clinical spectrum and/or pathology, and non-human primates showed great value as a model of SARS infection and pathology [12,13]. The suitability of monkey model for SARS-CoV vaccine testing also has been described [14]. According to the summary of the technical meeting on “SARS ANIMAL MODELS”, which was held on 5–6 February 2004, SARS models for infection (mice, effects and monkeys) are available. However, the development of clinical disease models needs to be further addressed. Some factors might affect the outcome of SARS-CoV infection of the different models in different laboratories, such as time of necropsy post-challenge, strain of virus used, genetic background, specific pathogen free (SPF) status and age of the animals, SCV challenge dose and stress of the animals.

Similar to other studies, rhesus monkeys in this study experimentally infected with the Chinese NS-1 strain of SARS-CoV did not replicate the severe illness seen in human SARS patients. PBS-control monkeys developed low-grade fevers and mild clinical signs, and showed evidence of viremia and virus replication in the lungs after infection, but no rash appeared. Radiological and pathological findings were consistent with the results from RT-PCR and virus isolation. An enhanced challenge dose may be successful in establishing a more faithful clinical disease model.

The findings in our study showed that the doses of 0.5 and 5  $\mu\text{g}$  protected most of the monkeys against the challenge of  $10^8$  PFU of SARS-CoV strain NS-1, while the dose of 50  $\mu\text{g}$  conferred complete protection in the 4 immunized animals. Although three out of eight immunized animals in two lower dosage groups showed virus excretion in blood after challenge, there was no evidence presented in other samples of the same animals, as well as no significant clinical symptoms and signs. No evidence of infection was found in animals receiving the higher dose of vaccine. Three animals induced lower titers of serum IgG antibody or negative nasopharyngeal sIgA antibody responses. This partial protectivity is likely the result of an inadequate or absent humoral response. These results indicate that the inactivated vaccine used in appropriate doses can elicit safe and protective responses. The findings also imply cross-protection was obtained in the two different virus strains used in this study.

Generally, vaccination by injection route elicits mostly humoral antibodies. Interestingly however, our results showed a high incidence (42%) of mucosal IgA response in rhesus monkeys compared to those induced by other inactivated virus vaccine in humans [15]. This increase in specific antibody production at the mucosa of the upper respiratory tract

may be of importance for the prevention of SARS-CoV virus infection at its early stages, as the mucosal surfaces are the primary route of SARS transmission.

The specific IgG antibody exhibited in most of the immunized animal approximately 7 days post-immunization of inactivated SARS-CoV vaccine with continuous increase, and appeared enhanced by the increase of vaccine doses. The antibody titers induced in high dose group were higher than those of middle and low dose groups. The immunization profile induced in monkeys of present study is similar to those in mice, as well as comparable to those elicited by natural infection in man [16]. Furthermore, the antibody titers continued to increase in most monkeys according to the 2 weeks observation following inoculation. It showed the boost effect of natural infection after the inactivated vaccine immunization. In this study, we also demonstrated that the SARS inactivated vaccine can stimulate rhesus monkeys to produce specific antibodies with neutralization activity. The NABs levels are similar to those induced by the inactivated and DNA vaccine in mice in other studies [17,18], and higher than those induced by adenoviral-based vaccine in monkeys at the same time point [14].

On the other hand, the results of serum cytokine production in the vaccinated monkeys suggest that the vaccination preferentially induces a Th1-driven response. The findings also imply that the inactivated vaccine candidate may enhance the cellular immune response, including the increased production of IFN- $\gamma$  in monkeys. The production of IFN- $\gamma$  can increase the activity of natural killer (NK) cells and inhibits replication of the virus. As observed in a previous study, in SARS patients, the appearance of chest radiographs improved but the viral loads decreased while interleukins and other proinflammatory cytokines increased [19]. Whether or not a specific cellular immune response can be also induced by this inactivated vaccine need further investigation.

The toxicity of this vaccine in monkeys is extremely low; doses of up to 5000  $\mu\text{g}$  caused no clinical sign or biochemical evidence after two injections. Local reactions in immunized monkeys were mild in this high dosage group, and absent in the 3 other vaccinated groups. It can be concluded that the vaccine is well tolerated by experimental animals and can be injected intramuscularly without any appreciable risk of adverse effects.

It has to be noted that antibody mediated enhancement (ADE) discovered in other coronavirus, such as ADE of FIPV infection in some human cell lines, where administration of an inactivated vaccine sensitized cats to subsequent antibody dependent enhancement of infection, leading to more severe peritonitis and death [20,21]. Therefore, further research concerning whether the ADE occurs in the SARS-CoV inactivated vaccine should be considered.

At the same time, it is not known whether the animal model faithfully replicates the complex pathology and symptomatology of the human disease, and it will be necessary to test potential SARS vaccine candidates for their immunogenicity, safety and efficacy in human in the event of future outbreaks.

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