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## Inactivated SARS-CoV vaccine elicits high titers of spike protein-specific antibodies that block receptor binding and virus entry

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### Abstract

The only severe acute respiratory syndrome (SARS) vaccine currently being tested in clinical trial consists of inactivated severe acute respiratory syndrome-associated coronavirus (SARS-CoV). However, limited information is available about host immune responses induced by the inactivated SARS vaccine. In this study, we demonstrated that SARS-CoV inactivated by  $\beta$ -propiolactone elicited high titers of antibodies in the immunized mice and rabbits that recognize the spike (S) protein, especially the receptor-binding domain (RBD) in the S1 region. The antisera from the immunized animals efficiently bound to the RBD and blocked binding of RBD to angiotensin-converting enzyme 2, the functional receptor on the susceptible cells for SARS-CoV. With a sensitive and quantitative single-cycle infection assay using pseudovirus bearing the SARS-CoV S protein, we demonstrated that mouse and rabbit antisera significantly inhibited S protein-mediated virus entry with mean 50% inhibitory titers of 1:7393 and 1:2060, respectively. These data suggest that the RBD of S protein is a major neutralization determinant in the inactivated SARS vaccine which can induce potent neutralizing antibodies to block SARS-CoV entry. However, caution should be taken in using the inactivated SARS-CoV as a vaccine since it may also cause harmful immune and/or inflammatory responses.

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The global outbreak of severe acute respiratory syndrome (SARS) in 2002/2003, caused by a novel coronavirus (SARS-CoV) [1–5], resulted in a cumulative total of more than 8000 cases and about 900 deaths in 29 countries ([www.who.int/csr/sars/en/](http://www.who.int/csr/sars/en/)). New SARS cases were reported in South China in early 2004 (WHO), suggesting that this deadly virus may recur in the future. Therefore, it is highly imperative to develop effective and safe vaccines to prevent and control new SARS epidemic.

Currently, a number of SARS vaccine candidates, including inactivated SARS-CoV vaccines, DNA vaccines, and attenuated virus vaccines expressing SARS-

CoV specific protein, are being developed [6–9]. Among them, the inactivated SARS-CoV vaccine is the only one tested in clinical trial in China [8,10]. Although several reports have showed that SARS-CoV inactivated with formaldehyde, UV light, or  $\beta$ -propiolactone can induce virus neutralizing antibodies in the immunized animals [11–13], host immune responses induced by the inactivated virus have not been well characterized.

Similar to other coronaviruses, SARS-CoV is an enveloped positive-strand RNA virus, featuring a large viral genome encoding the replicase polyproteins, the spike (S), envelope (E), and matrix (M) glycoproteins, the nucleocapsid protein (N), and other small proteins with unknown functions [3,5]. It is expected that vaccination with the inactivated SARS-CoV vaccine can elicit antibody responses against a number of viral proteins.

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Therefore, it is necessary to determine which virus protein is the major antigen responsible for eliciting neutralizing antibodies.

The S protein of SARS-CoV is a large transmembrane glycoprotein that contains S1 domain (residues 15–680) and S2 domain (residues 681–1255), responsible for receptor binding and membrane fusion, respectively. Entry of SARS-CoV into target cells is initiated by binding of the S1 domain to angiotensin-converting enzyme 2 (ACE2), the functional receptor for SARS-CoV on susceptible cells [14–17]. The S2 domain changes conformation through association of heptad repeat 1 and 2 (HR1 and HR2) regions to form a six-helix bundle, resulting in fusion of the viral envelope with the target cell membrane [18,19]. It has been shown that a small fragment (residues 318–510) in the S1 region is the minimal receptor-binding domain (RBD) [20–22].

Recent studies have demonstrated the S protein of SARS-CoV to be a major inducer of neutralizing antibodies among structural proteins [23] and the S proteins expressed by the DNA vaccine, the attenuated vaccinia virus or the parainfluenza virus can elicit protective immunity against virus challenge [6,7,9,23]. We have recently shown that the S protein of SARS-CoV contains several immunodominant domains that do not induce neutralizing antibodies [24], but the RBD in the S1 region is a highly potent inducer of neutralizing antibodies in immunized animals [25].

In this study, we demonstrated that mice and rabbits immunized with the inactivated SARS-CoV vaccine developed significant antibody responses against the S protein, especially against the RBD in the S1 region and that their antisera were able to efficiently block receptor binding and inhibit virus entry into target cells.

## Materials and methods

**Recombinant SARS-CoV spike proteins.** Plasmid encoding S protein S1 domain (residues 12–672) tagged with C9 at the C-terminus (designated as S1-C9) and plasmid encoding a 193-amino acid fragment of RBD (residues 318–510) linked to the Fc domain of human IgG1 (designated as RBD-Fc) were kindly provided by Dr. M. Farzan at the Harvard Medical School [15,21]. RBD-Fc and S1-C9 proteins were expressed in 293T cells transfected with the plasmids using Fugene 6 reagents (Boehringer–Mannheim, Indianapolis, IN) according to the manufacturer's protocol. Supernatants were harvested 72 h post-transfection. S1-C9 was purified by affinity chromatography with anti-C9 mAb 1D4 (National Cell Culture Center, Minneapolis, MN), and RBD-Fc was purified by protein A-Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ). A full-length S protein expressed in expresSF<sup>+</sup> insect cells infected with recombinant baculovirus D3252 was purchased from the Protein Sciences (Bridgeport, CT).

**Preparation of inactivated SARS-CoV.** SARS-CoV strain BJ01 (GenBank Accession No. AY278488) was propagated in Vero E6 cells as described previously [24,26]. The infected cells were harvested and completely lysed by three cycles of freeze–thaw.  $\beta$ -Propiolactone (Sigma–Aldrich, St. Louis, MO) was then added to the lysates at 1:2000 ratio and incubated at 37 °C for 2 h. The inactivated virus was

centrifuged at 10,000 rpm for 20 min. After removal of cell debris, the supernatants were desalted with Sephadex G-50, concentrated with PEG-8000, and filtered with Sepharose-CL 2B, sequentially. The inactivated SARS-CoV in the final preparation, with >95% purity as analyzed by HPLC, was confirmed by observing the coronavirus-like particles under an electron microscope and by determining the reactivity with convalescent sera from SARS patients in Western blots.

**Immunizations of mice and rabbits.** Four Balb/C mice and four NZW rabbits were primarily immunized intradermally with 10 and 30  $\mu$ g of purified viral particles inactivated by  $\beta$ -propiolactone, respectively, as immunogen in the presence of Freund's complete adjuvant (FCA), and boosted twice with the same amount freshly prepared emulsion of immunogen and Freund's incomplete adjuvant (FIA) at 2-week intervals. Pre-immune sera (preimmune) were collected before starting immunization and antisera were collected 5 days after the second boost.

**Enzyme-linked immunosorbent assay.** Mouse and rabbit sera were tested against the recombinant S proteins (S protein, S1-C9 or RBD-Fc) by enzyme-linked immunosorbent assay (ELISA). Briefly, 1  $\mu$ g/ml of each recombinant protein was coated to 96-well microtiter plates (Corning Costar, Acton, MA) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with 2% non-fat milk, serially diluted mouse or rabbit sera were added and incubated at 37 °C for 1 h, followed by three washes with PBS containing 0.1% Tween 20. Bound antibodies were detected with HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Zymed, South San Francisco, CA) at 37 °C for 1 h, followed by washes. The reaction was visualized by addition of the substrate 3,3',5,5'-tetramethylbenzidine (TMB) and absorbance at 450 nm (A450) was measured by an ELISA plate reader (Tecan US, Research Triangle Park, NC). Mouse and rabbit sera reactivities with SARS-CoV were tested by ELISA with commercially available diagnostic kits using the mixture of proteins purified from viral lysates as coating antigens (Beijing Genomics Institute, Beijing, China).

**Receptor-binding assay.** Inhibitory effects of the mouse or rabbit antisera on RBD-Fc binding to receptor ACE2 were tested using an ELISA-based assay. Briefly, recombinant soluble ACE2 (R&D systems, Minneapolis, MN) was coated at 2  $\mu$ g/ml to 96-well ELISA plates (Corning Costar) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with 2% non-fat milk, 0.5  $\mu$ g/ml of RBD-Fc was added to the wells in the presence or absence of serially diluted mouse or rabbit sera. After incubation at 37 °C for 1 h, the HRP-conjugated goat anti-human IgG (Zymed) and the substrate TMB were used for detection.

**Single-cycle SARS pseudovirus infection assay.** SARS pseudovirus bearing SARS-CoV S protein and a defective HIV-1 genome expressing luciferase as reporter was prepared as previously described [21,27,28]. In brief, 293T cells were co-transfected with a plasmid encoding codon-optimized SARS-CoV S protein and a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.RE) using Fugene 6 reagents (Boehringer–Mannheim). Supernatants containing SARS pseudovirus were harvested 48 h post-transfection and used for single-cycle infection. Briefly, ACE2-transfected 293T (293T/ACE2) cells were plated at 10<sup>4</sup> cells/well in 96-well tissue-culture plates and grown overnight. The supernatants containing pseudovirus were preincubated with 2-fold serially diluted mouse or rabbit sera at 37 °C for 1 h before addition to cells. The culture was re-fed with fresh medium 24 h later and incubated for an additional 48 h. Cells were washed with PBS and lysed using lysis reagent included in a luciferase kit (Promega, Madison, WI). Aliquots of cell lysates were transferred to 96-well Costar flat-bottomed luminometer plates (Corning Costar, Corning, NY), followed by addition of luciferase substrate (Promega). Relative light units (RLU) were determined immediately in the Ultra 384 luminometer (Tecan US).

**Results**

*Inactivated SARS-CoV induced high titer of S protein-specific antibodies in mice and rabbits*

SARS-CoV particles inactivated with  $\beta$ -propiolactone were used for immunizing mice and rabbits. After three immunizations, all the immunized mice and rabbits developed high levels of antibody responses against SARS-CoV as detected by ELISA with commercially available diagnostic kits that use the mixture of proteins purified from viral lysates as coating antigen (mean end-point titers of mouse and rabbit antisera were 1:102,400 and 1:83,200, respectively), while the preimmune sera from the mice and rabbits did not react with the viral antigens (Fig. 1).

To determine antibody responses specific for the SARS-CoV S protein in the immunized mice and rab-

bits, a recombinant full-length S protein was used as an antigen in ELISA. The data in Figs. 2A and 3A indicated that the mice and rabbits immunized with the inactivated viruses developed high titers of antibodies against the S protein. The mean end-point titers of mouse and rabbit antisera were 1:51,200 and 1:25,600, respectively. It is expected that antibodies reacting with the S protein may contain an antibody population spe-

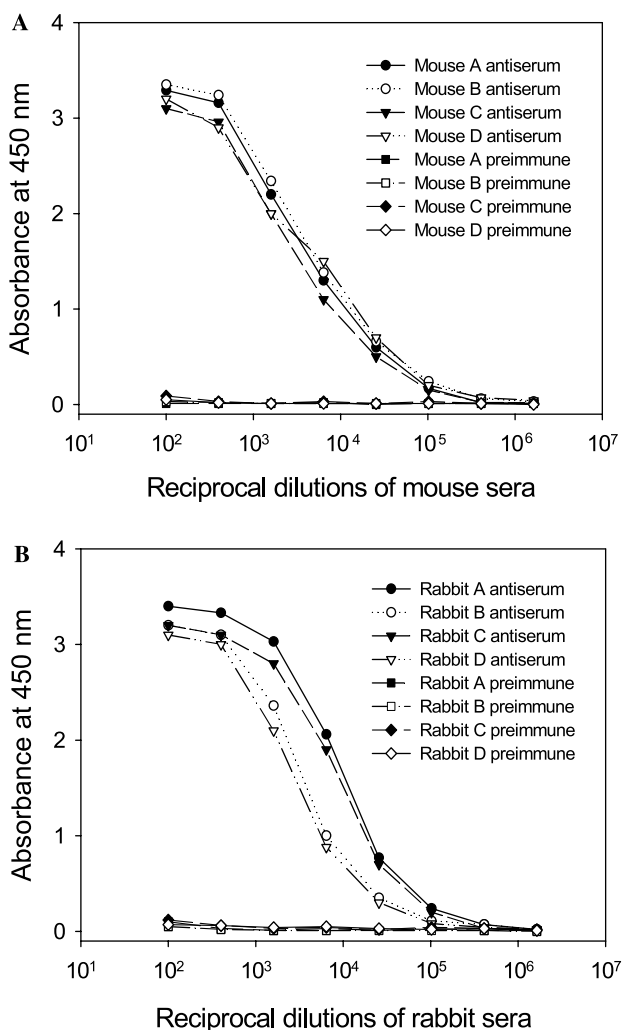


Fig. 1. Antibody responses against SARS-CoV in the immunized mice (A) and rabbits (B). Mouse and rabbit sera were tested at a series of 4-fold dilutions by commercial diagnostic ELISA kit using a mixture of proteins purified from viral lysates as coating antigens.

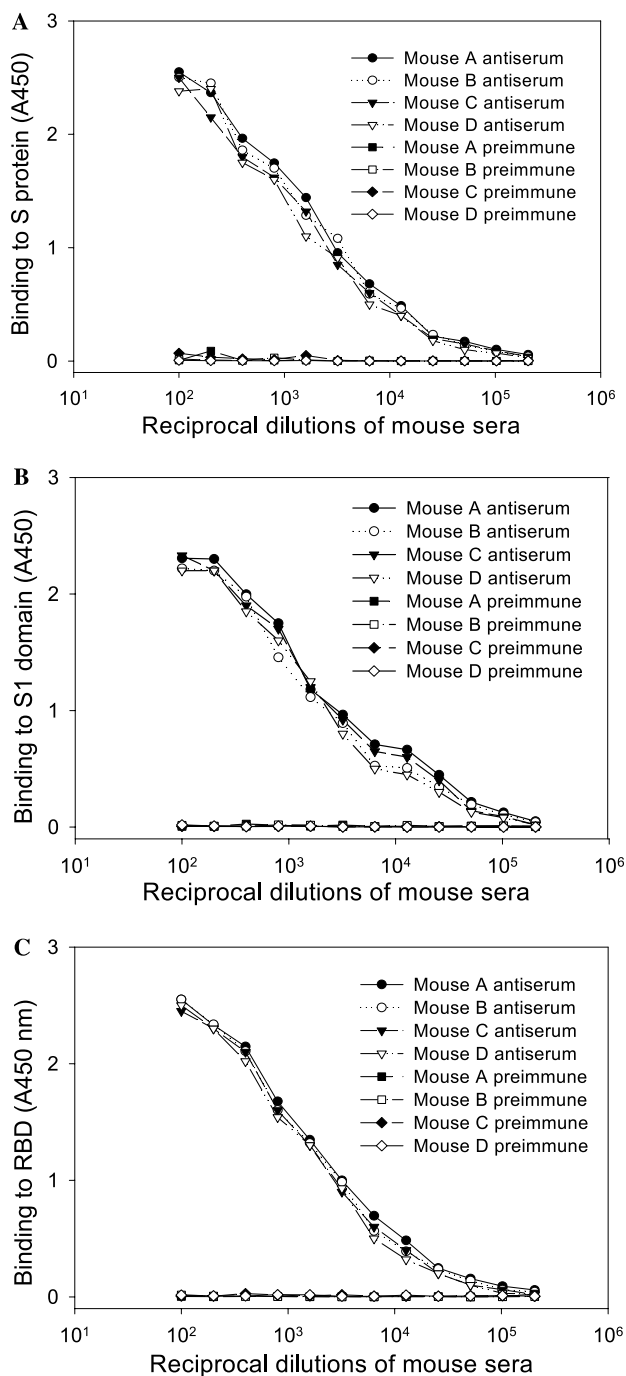


Fig. 2. Inactivated SARS-CoV induced high titers of antibodies in mice against full-length S protein (A), S1-C9 (B), and RBD-Fc (C). Mouse sera were tested at a series of 2-fold dilutions by ELISA.

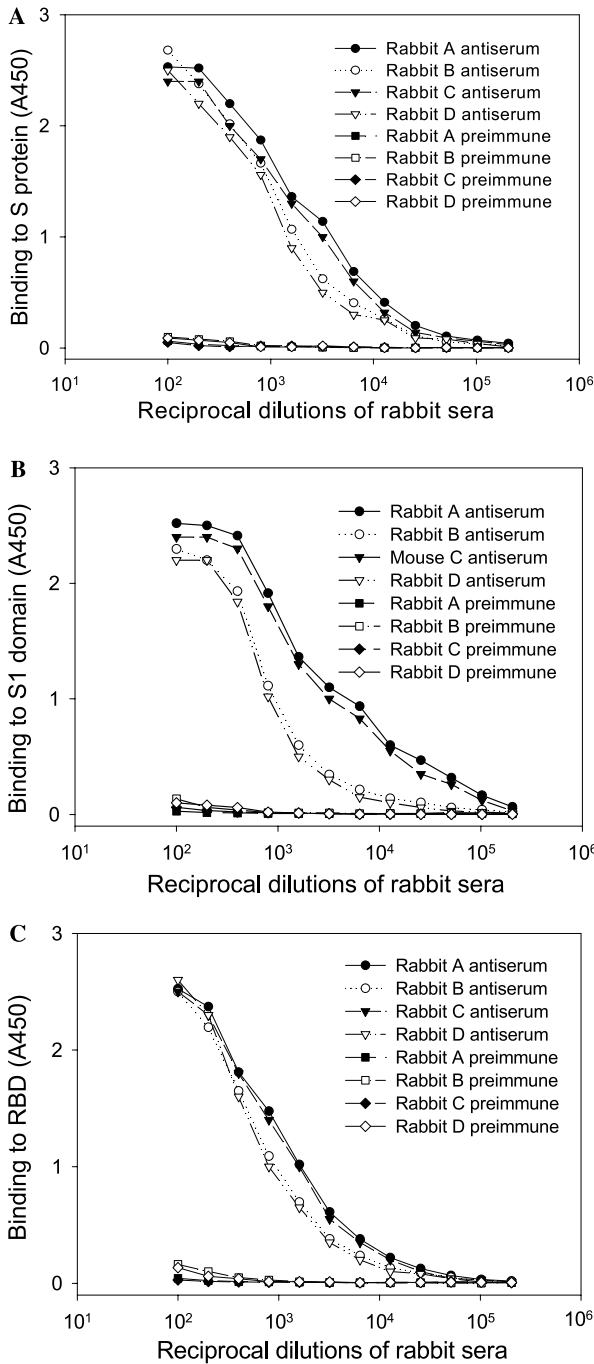


Fig. 3. Inactivated SARS-CoV induced high titers of antibodies in rabbits against full-length S protein (A), S1-C9 (B), and RBD-Fc (C). Rabbit sera were tested at a series of 2-fold dilutions by ELISA.

cific for the S1 region since S1 is a major antigenic determinant in the S protein [24]. We therefore used recombinant S1-C9 as an antigen in ELISA and found that the antibody titers against S1 were comparable to those against the full-length S protein (Figs. 2B and 3B). We then determined whether the antisera reacted with RBD within S1 region since it is a functional domain in S protein responsible for mediating virus binding to

the receptor ACE2 [14–17] and contains neutralizing epitopes [25]. RBD-Fc fusion protein was used as a coating antigen in the ELISA for testing the reactivities of mouse and rabbit antisera. Strikingly, both mouse and rabbit antisera were highly reactive with the RBD-Fc with mean end-point titers of 1:51,200 and 1:25,600, respectively (Figs. 2C and 3C), at similar levels of responses against the full-length S protein. This suggests

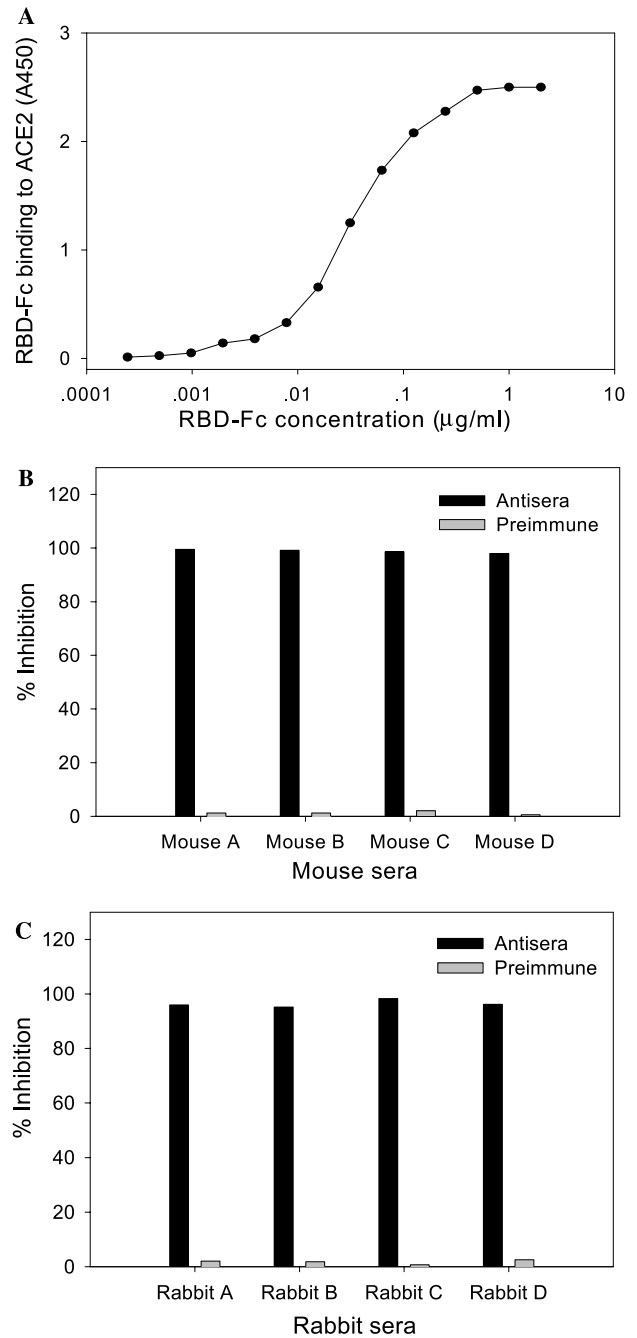


Fig. 4. Inhibition of RBD-Fc binding to ACE2 by antibodies in the mouse and rabbit antisera. (A) RBD-Fc bound to soluble ACE2 in a dose-dependant manner; mouse (B) and rabbit (C) antisera at 1:50 dilution inhibited binding of RBD-Fc to ACE2.

that the RBD on the S protein of the inactivated SARS-CoV vaccine preparation is a major antigenic determinant capable of inducing potent antibody responses in the immunized animals.

*Mouse and rabbit antisera effectively blocked binding of the RBD to ACE2*

SARS-CoV infection is initiated by attachment of its S protein via RBD in the S1 region to the cellular receptor, ACE2. It is interesting to investigate whether the mouse and rabbit antisera that contain RBD-specific antibodies can block receptor binding activity as determined by ELISA using soluble ACE2 as previously described [25]. As shown in Fig. 4A, the RBD-Fc bound to ACE2 in a dose-dependent manner. Both mouse and rabbit antisera at 1:50 dilution could effectively block binding of the RBD-Fc to ACE2, whereas preimmune sera at the same dilution had no inhibitory effect on the receptor binding (Figs. 4B and C). This result

indicates that the inactivated SARS-CoV vaccine induces antibodies specific for RBD, which are capable of blocking receptor binding.

*Mouse and rabbit antisera efficiently inhibited S protein-mediated virus entry into targeted cells*

To investigate whether the antibodies induced by inactivated SARS-CoV in the immunized mice and rabbits inhibit S protein-mediated virus entry, we developed a sensitive, quantitative, and safe single-cycle infection assay using SARS pseudovirus bearing SARS-CoV S protein [25]. As shown in Figs. 5A and 6A, the mouse and rabbit antisera at 1:100 dilution significantly inhibited SARS pseudovirus infection whereas preimmune sera at the same dilution had no inhibitory effect on S protein-mediated virus entry. Both mouse and rabbit antisera inhibited virus entry in a dose-dependent manner with mean 50% inhibitory titers of 1:7393 and 1:2060, respectively. This result further confirms that the inactivated

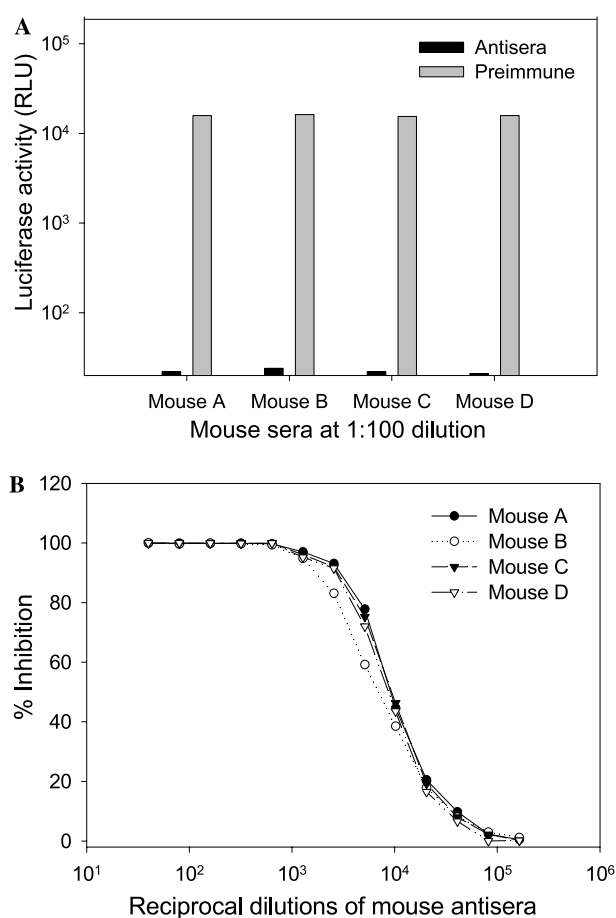


Fig. 5. Inhibition of S protein-mediated virus entry by antibodies in the mouse antisera. (A) Infection of 293T/ACE2 cells by SARS pseudovirus was determined in the presence of preimmune and antisera at 1:100 dilution. (B) Inhibition of SARS pseudovirus infection by mouse antisera at a series of 2-fold dilutions and percentage inhibition was calculated for each sample.

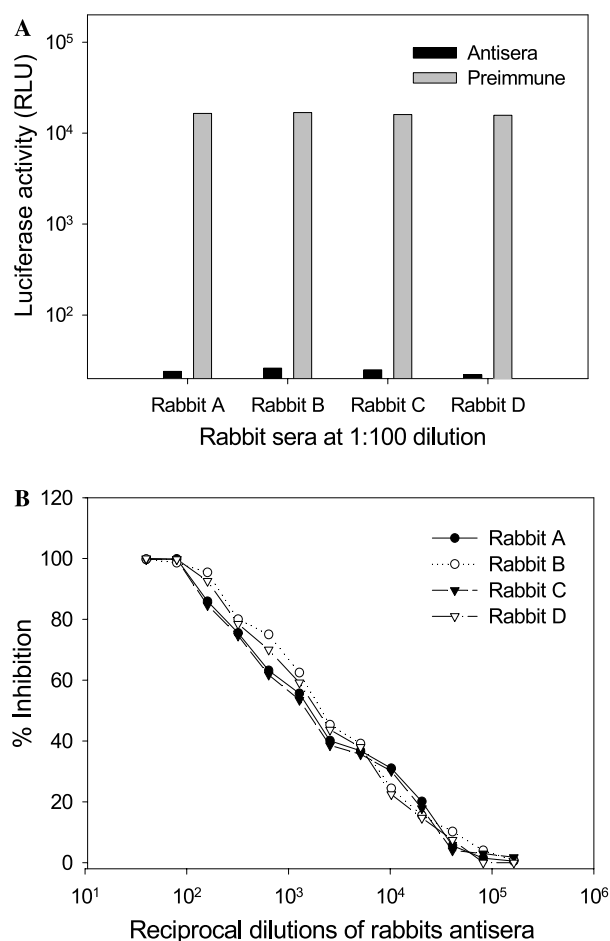


Fig. 6. Inhibition of S protein-mediated virus entry by antibodies in the rabbit antisera. (A) Infection of 293T/ACE2 cells by SARS pseudovirus was determined in the presence of preimmune and antisera at 1:100 dilution. (B) Inhibition of SARS pseudovirus infection by rabbit antisera at a series of 2-fold dilutions and percentage inhibition was calculated for each sample.

SARS-CoV vaccine can elicit S protein-specific neutralizing antibodies in the immunized animals.

## Discussion

Although the outbreak of SARS has been successfully contained with aggressive quarantine measures, SARS may re-emerge in the future. Prior experiences in infectious disease control suggest that vaccination will be one of the most effective measures to prevent SARS epidemic. Generally, the inactivated virus has been considered as a first generation vaccine to prevent viral infectious disease. Therefore, inactivated SARS-CoV vaccine has been developed as one of the major vaccine candidates [8]. Most recently, a vaccine prepared from whole SARS-CoV inactivated with  $\beta$ -propiolactone is being tested in a clinical trial in China [10]. However, there is serious concern over its efficacy and safety in humans since the antigenic properties of inactivated SARS-CoV have not been clearly defined [8].

In this study, we demonstrated that the inactivated SARS-CoV was able to induce high titers of antibodies specific for S protein, especially for RBD in the S1 region, in the immunized mice and rabbits. This suggests that the S protein in the inactivated SARS vaccine preparation maintains its high immunogenicity in the mice and rabbits. This is crucial for development of the inactivated SARS-CoV vaccine since it has been shown that the S protein of SARS-CoV is a major antigen capable of inducing neutralizing antibodies and protective immunity [6,7,9,23]. We have recently shown that the RBD is a major antigenic determinant in the SARS-CoV S protein and that the RBD can induce high titers of SARS-CoV neutralizing antibodies [25]. Here we have demonstrated that the inactivated SARS-CoV vaccine is able to induce high titers of antibodies to RBD, suggesting that this domain is correctly folded in the vaccine preparation and retains appropriate epitopic conformation for eliciting neutralizing antibodies. The antibodies in the antisera from mice and rabbits immunized with the inactivated SARS-CoV could effectively block RBD binding to the receptor ACE2 and potently inhibit S protein-mediated virus entry into susceptible cells, indicating that the antibodies in the immunized animals are specific for the RBD of the S protein and responsible for inhibition of SARS-CoV entry.

Although the RBD in S protein of SARS-CoV can induce potent neutralizing activity, the safety of the inactivated SARS-CoV vaccine should be fully evaluated before it is tested in human clinical trials. The S protein also contains other immunodominant domains which elicit non-neutralizing antibodies [24]. The S protein of feline infectious peritonitis virus (FIPV), a coronavirus, induces antibodies in the immunized animals that do not neutralize, but rather enhance infection after virus chal-

lenge [29–31]. In addition to the S protein, coronaviruses also contain other proteins, which may induce humoral or cellular immune responses that exacerbate the pathology in some coronavirus infections [32]. Some proteins in SARS-CoV may induce harmful immune and/or inflammatory responses, a potential cause of SARS pathogenesis [33,34]. It was reported that SARS-CoV infection of ferrets caused mild liver inflammation and that the liver damage became much more serious if the ferrets were immunized with vaccinia virus-based SARS vaccines before virus challenge [35]. Most recently, Wang and Lu [36] reported that an inactivated SARS-CoV vaccine triggered an autoimmune response against the carbohydrate moieties in an abundant human serum glycoprotein asialo-orosomucoid. Therefore, caution should be taken in application of inactivated SARS-CoV vaccine for prevention of SARS. The antigens that induce harmful immune and/or inflammatory responses should be identified and removed from vaccines. Preparations containing the RBD of S protein may serve as one of the most effective and safest vaccines for the prevention of SARS [25].

In summary, this is the first description that the inactivated SARS-CoV vaccine can induce high titers of antibodies against RBD in the S1 region of the S protein and the RBD-specific antibodies in the antisera can efficiently block receptor binding and virus entry. These data provide important information for understanding the antigenicity and immunogenicity of the SARS-CoV and for developing anti-SARS vaccines.

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