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Studies of SARS virus vaccines

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Summary

This study was designed to develop inactivated, protein-based and recombinant adeno-associated virus (rAAV)-based vaccine candidates for prevention of SARS-CoV infection. We demonstrated that the titer of serum neutralizing antibody was up to 1:640 when immunized mice intranasally with inactivated SARS-CoV either alone or with adjuvant. In mice immunized with adjuvant, specific IgA was detected in tracheal-lung wash fluid by immunofluorescence. We further found that protein-based receptor binding domain in S protein of SARS-CoV (RBD-Fc) is able to induce effective neutralizing antibody, which could provide protection from SARS-CoV infection in animal model. Since we failed to generate low temperature adaptable animal SARS-CoV like virus by cell cultures, we have alternately constructed a rAAV-based live vaccine candidate, which expresses RBD of SARS-CoV S protein (RBD-rAAV), and evaluated its immune responses and protection against SARS-CoV infection in animal models. The results showed that: (1) a single dose of RBD-rAAV vaccination could induce sufficient neutralizing antibody against SARS-CoV infection; (2) two more doses of repeated immunization boosted production of the neutralizing antibody to about 5 fold higher than that of the single dose immunization; (3) more importantly, the RBD-rAAV vaccination provoked a prolonged antibody response with continually increasing levels of neutralizing activity; and (4) intranasal vaccination of RBD-rAAV induced local IgA and systemic IgG neutralizing antibodies and specific T cell responses, which provided protective effects to SARS-CoV infection in the animal model. Taken together, our findings suggest that inactivated vaccine, RBD-Fc and RBD-rAAV can be further developed into vaccines for prevention of SARS and that intranasal vaccination may be the preferred route of administration.

Introduction

Severe acute respiratory syndrome (SARS) is a recently emerged human disease associated with pneumonia [1, 2]. The disease is unusual in its predilection to affect health care workers. A novel SARS-associated coronavirus (SARS-CoV) was identified as the etiological agent of SARS [3, 4] and it was further confirmed by the fact that the virus causes a similar disease in cynomolgous macaques [5]. Although SARS has been under the control after June 2003, epidemiologists and scientists in the world have predicted that the outbreak may be recurrent in the coming years. Vaccine development for the prevention of SARS still remains a high priority due to the possibility of reemergence of the disease. We have started initial exploration on the development of inactivated SARS virus vaccine since April 2003. This study was designed to investigate the hypothesis that inactivated vaccine, protein-based vaccine and rAAV live vaccine may be crucial in prevention of SARS. Our objectives were: (1) evaluation of inactivated vaccine candidate; (2) construction and evaluation of RBD-Fc candidate; and (3) construction and evaluation of RBD-rAAV vaccine candidate. The study was benefit from the expertise of the investigators, who have extensive experience in vaccine development, virology and immunology, in general, and in SARS research, specifically. The P3 facilities for virus cultures have been used in our previous studies since 2003 but the P3 animal facilities was just available in Jan, 2007, so that the SARS-CoV challenge animal experiments were finally done by April, 2007.

Methods

Virus strains and inactivation of SARS-CoV

SARS-CoV strain GZ50 (GenBank accession number AY 304495) was isolated from the nasopharyngeal wash fluid of a female patient who suffered from SARS in Guangzhou. Full-length sequencing and phylogenetic analysis showed that GZ50 lay between the reported Hong Kong strains, the Canadian and US strains [6].

Formaldehyde at 1:2000 concentration at 4°C for 72 hours completely inactivated GZ50. Crude inactivated virus solution was spun at 38,000rpm for 16 hours with 20% sucrose cushion, and the precipitate was resuspended in PBS. Inactivation of the virus was confirmed by using 100 times concentrated formaldehyde treated virus to inoculate Vero cells. When no CPE was detected, cell supernatants were blindly passaged for three passages. Cell cultures were fixed with cold acetone and stained with SARS antibody positive convalescent serum by indirect immunofluorescent assay and no positively stained cells were found.

Vaccination of inactivated vaccine

Balb/c mice were used in all experiments. Eighty µg of inactivated virus was used per mouse for subcutaneous (s.c.) immunization with alum as the adjuvant. For intranasal (i.n.) immunization, 50

µg of inactivated viruses in 30µl of PBS was used per mouse with or without adjuvant. The adjuvant used for i.n. immunization was either phosphorothiate-modified CPG oligonucleotide (1 µg/mouse), or cholera toxin B (10µg/mouse). The polyethylene glycol (PEG mw 6000) was used to precipitate the inactivated virus, and was adjusted to 25 µg in 20 µl of PBS for intranasal immunization.

Mice underwent light ether anesthesia were immunized intranasally with 15 µl of inactivated virus or with 15 µl of inactivated virus containing adjuvant to each nostril. For PEG-precipitated inactivated virus, 10 µl was delivered into each nostril. Groups of mice were immunized totally four times and serum anti-SARS-coV was measured by neutralization tests. Two weeks after the last boosting, mice were sacrificed and tracheal-lung wash fluid was collected by infusion of the tracheal-lung tract with 1 ml of PBS per mouse.

Construction and preparation of RBD protein

A 193-aa RBD domain of SARS-CoV S protein (residues 318–510) and fused with the Fc domain of human IgG1 (RBD-Fc) was cloned and expressed. Briefly, a plasmid encoding RBD-Fc fusion protein was transfected into 293T cells using Fugene 6 transfection reagents according to the manufacturer's protocol. Supernatants were harvested 72 h post-transfection. Recombinant RBD-Fc protein was purified by protein A-Sepharose 4 Fast Flow. A full-length S protein of SARS-CoV expressed in expresSF⁺® insect cells with recombinant baculovirus D3252 was purchased from the Protein Sciences Corporation.

Vaccination of RBD protein vaccine and SARS-CoV challenge

A group of five female BALB/c mice at the age of 4-6 weeks were vaccinated intramuscularly (i.m.) with 10 µg of purified RBD-Fc resuspended in PBS in the presence of Freund's complete adjuvant (FCA), and boosted twice with freshly prepared emulsion of 5 µg immunogen and Freund's incomplete adjuvant (FIA) at 3-week intervals. The mice were boosted for the third time with the same amount of RBD-Fc + FIA 12 months after the first vaccination and were challenged with SARS-CoV five days later. Mice injected with the same amount of PBS were used as the negative control. For each group, mouse sera were collected before immunization and at one-month interval post-vaccination. Sera were kept at –20°C until use.

Construction and titration of RBD-rAAV viral vector

The recombinant AAV encoding a 193-aa RBD domain (residues 318–510) of SARS-CoV S protein (RBD-rAAV) was constructed and titrated. Briefly, RBD-rAAV plasmid was co-transfected with pHelper and pAAV-RC plasmids into 293T cells using a calcium phosphate transfection method according to the manufacturer's protocol. Transfected cells and supernatant were harvested 72 h post-transfection. Recombinant AAV was purified by chloroform-NaCl-PEG8000 method and titrated by real-time quantitative PCR (Q-PCR).

Vaccination of RBD-rAAV via i.m. and i.n. routes and sample collection

Groups of Balb/c mice were vaccinated with RBD-rAAV or blank AAV, respectively, via the i.m. and i.n. routes. For the i.m. vaccination, mice were given with a single-prime dose (i.m.P) or prime-boost doses at 1.5-month interval (i.m.B) of RBD-rAAV (2×10^{11} VP/dose). For the i.n. vaccination, mice were immunized with a single-prime dose (i.n.P) or prime-boost doses at an interval of 0.5 months (i.n.B) of RBD-rAAV (2×10^{10} VP/dose). Two groups of mice i.m. or i.n. vaccinated with prime-boost doses of blank AAV were used as negative controls. Four mice/group were challenged with SARS-CoV 1 month after the booster vaccination (young mice), and five mice/group were boosted at the end of 12 months post-vaccination and challenged with SARS-CoV 15 days later (aged mice).

ELISA for systemic IgG and local IgA detection

Specific IgG and IgA against SARS-CoV in mouse sera and lung flush were tested by ELISA as described previously [7, 8].

Neutralization assay

Titers of neutralizing antibodies (NA) in sera and lung flush of mice were detected in FRhK-4 and/or Vero E6 cells as described previously [9].

ELISPOT assay

The assay was performed using an ELISPOT mouse kit (Mabtech, Sweden) according to the manufacturer's protocol. In brief, 96-well ELISPOT plates were coated with anti-IL-2 and -IFN- γ monoclonal antibodies (mAbs) overnight at 4°C, and blocked by sterile RPMI-1640 containing 10% FBS for 2 h at room temperature. Single-cell suspensions prepared from the spleens of vaccinated mice were added to the wells at the concentration of 2×10^5 cells/well. Cells were incubated for 24 h in the presence or absence of an identified MHC-H-2^d restricted SARS-CoV-specific cytotoxic T lymphocyte (CTL) peptide (S365-374, KCYGVSATKL) plus anti-mouse CD28 mAb (1 μ g/ml) at 37°C with 5% CO₂. Plates were washed with PBS, followed by incubation with biotinylated labeled anti-mouse IL-2 and IFN- γ mAbs at 1:1000 for 2 h at room temperature. After additional washes, wells were incubated with streptavidin-conjugated HRP for 1 h at room temperature. Wells were extensively washed again, and developed with TMB substrate solutions included in the kit. Spots of IL-2 and IFN- γ producing T cells were counted by using an automated ELISPOT reader system and ImmunoSpot 3 software. Results were expressed as the number of spot-forming cells (SFC) per 10^6 input cells.

Cell surface markers/intracellular cytokine staining and FACS

Single-cell suspensions (2×10^6) from spleens and lungs of vaccinated mice were stimulated with or without SARS-CoV S-specific CTL peptide (S365-374, KCYGVSATKL, 1 μ g/ml) plus anti-mouse CD28 (1 μ g/ml). Phorbol myristate acetate (PMA, 5 ng/ml; Sigma, USA) and ionomycin (250 ng/ml) were used as positive controls. Cells with stimulatory agents were incubated for 5 h at 37°C with 5%

CO₂ in the presence of GolgiPlugTM containing Brefeldin A (1 µl/ml). The cells were fixed using a Cytotfix/CytopeimTM Plus kit in accordance with the manufacturer's protocol, and stained directly with conjugated mAbs specific for cell surface antigens [anti-mouse-CD3 (PerCP) and anti-mouse-CD8 (APC)] and intracellular cytokines [anti-mouse-IL-2 (PE) and anti-mouse-IFN-γ (FITC)] for 30 min at 4°C. Appropriate isotype-matched controls for cytokines were included in each staining. The stained cells were analyzed using a flow cytometer. Lymphocyte population was gated by forward light scatter versus side light scatter, and 10,000 events for CD3⁺CD8⁺ lymphocyte subpopulation were acquired to determine the percentage of CD8⁺ T cells positive for specific cytokines. FACS data were analyzed by CellQuest software.

SARS-CoV challenge in mice

Mice were anaesthetized with isoflurane and i.n. inoculated with 50 µl of SARS-CoV strain GZ50 (100TCID₅₀) according to the National animal care and use guidelines in an approved animal BSL-3 laboratory. The mice were sacrificed 3 days (for young mice) or 8 days (for aged mice) after virus challenge, and the lungs removed. The lung tissues were stored at -80 °C for virological tests or were fixed immediately with 10% buffered formalin for histopathological analysis.

Quantitative reverse-transcriptase polymerase chain reaction (Q-RT-PCR)

The viral RNA copies in lung tissues of challenged mice were determined by Q-RT-PCR as described previously [7, 10].

Histopathological analysis

The lung tissues of challenged mice were immediately fixed in 10% buffered formalin and embedded in paraffin wax. Sections were made of 4–6 µm thickness and mounted on slides. Histopathological changes caused by SARS-CoV infection were examined by haematoxylin and eosin (H&E) staining and viewed under the light microscope.

Statistical analysis

Values were presented as mean with standard error (SE). Statistical significance among different vaccination groups was calculated by Student's *t* test using Stata statistical software. *P* values less than 0.05 were considered significant.

Results

Antibody responses in inactivated vaccine immunized mice

After two subcutaneous injections of 80µg of inactivated virus, only low titer of ELISA antibody (1:8) was detected in 3 out of 5 mice of the s.c. group, while sera from the i.n. groups were all negative. However, when sera from the s.c. and from the i.n. groups were assayed by neutralization test (NT), all showed positive results (Fig 1). All s.c. mice developed high titers of neutralizing antibodies

(NA). After 4 doses of i.n. immunization of inactivated virus with and without adjuvant, substantial levels of serum NA were detected in all the mice. Due to the viscosity of PEG precipitated inactivated SARS-CoV, although a lower dosage of virus was used for i.n. immunization, the serum neutralizing antibody titer was 1:160 in all mice of this group. When tracheal- lung-wash fluid was tested for anti-SARS IgA by IF, no positive staining was detected in the group immunized only with inactivated virus. However, strong IF staining at 1:5 dilution was shown in all groups of mice immunized with the virus plus the adjuvants, and in mice immunized with PEG-inactivated virus (Fig 2).

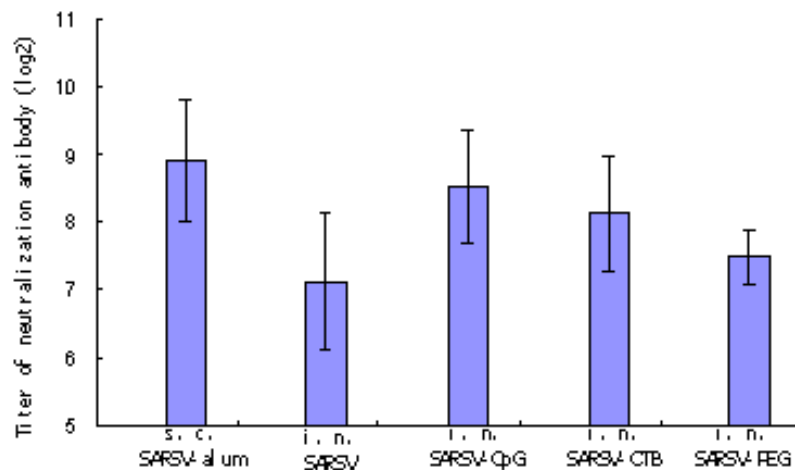


Fig 1. The titer of anti-SARS-CoV neutralization antibody in sera from immunized mice The bars represent the neutralizing titer and standard deviation of each group of immunized mice i.e. s.c., i.n., CPG, CTB.

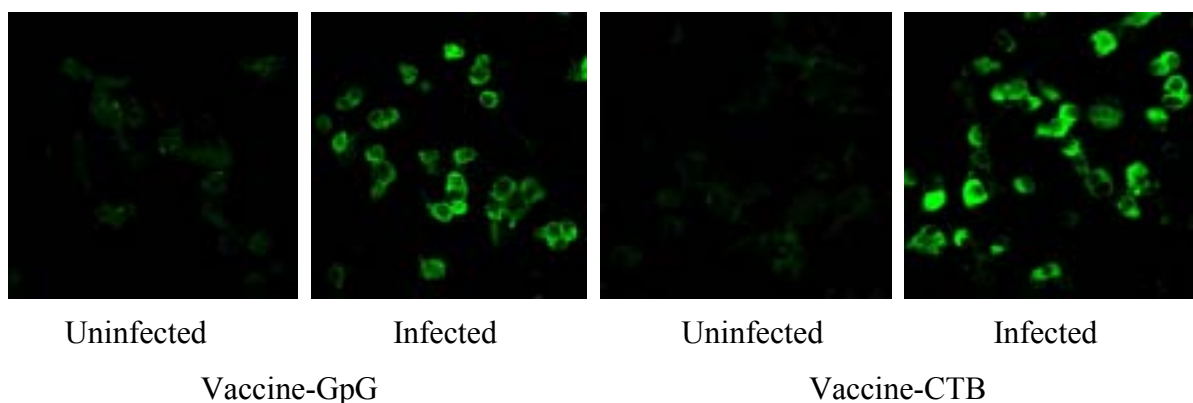


Fig 2 Immunofluorescent study of anti-SARS-CoV IgA in tracheal-lung wash fluid from different groups of intranasal immunized mice SARS-CoV infected cells and non-infected control cells were stained separately with tracheal-lung wash fluid from different groups of immunized mice. FITC-labeled anti-mouse IgA was used as second antibodies for the immunofluorescence (IF).

Vaccination with RBD-Fc induced long-term and potent SARS-CoV S-specific antibodies with strong neutralizing activity

As shown in Fig 3A, RBD-Fc vaccination induced a prolonged and potent humoral immune response with IgG specific to SARS-CoV S protein as tested by ELISA, reaching the highest titer at the 3rd and 4th month post-vaccination. Although the titer decreased slightly afterwards, high titer of IgG antibody maintained for at least 6 months. Specific IgG antibody increased rapidly after the 3rd boost (12 months after the first vaccination) Fig 3B further demonstrated that the antibodies induced by vaccination of RBD-Fc exhibited strong neutralizing activity with a similar pattern of the ELISA antibodies to RBD. However, neither ELISA S-specific antibodies nor neutralizing antibodies (NA) were detected in the mouse sera from the control group.

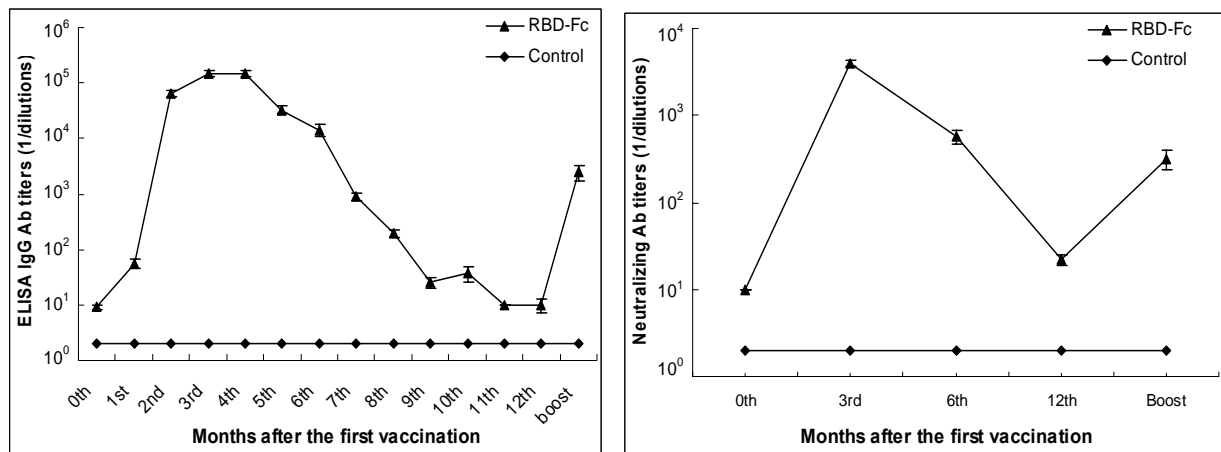


Fig 3. Detection of anti-S antibodies and virus neutralizing antibodies in mouse sera. (A) Titers of anti-S antibodies measured by ELISA for 12 months at monthly basis and five days after the last boost. Data are presented as the geometric means \pm SE of five mouse sera collected at different time points. (B) Titers of the SARS-CoV neutralizing antibody in sera collected at 0, 3, 6, and 12 months post-vaccination and sera collected five days after the last boost. The titers of neutralizing antibodies were determined as the highest dilutions of sera that could completely prevent CPE in at least 50% of the wells and are presented as the geometric means \pm SE of five mouse sera in each group.

Neutralizing antibodies elicited by RBD-Fc vaccination played an important role in inhibiting SARS-CoV infection in challenged mice

Five days after challenged with SARS-CoV through the intranasal route, mice were sacrificed and lung tissues were collected. Virus RNA copies in the lung tissues were determined by Q-RT-PCR. As shown in table 1, viral RNA copies for both the RBD-Fc group and the control group were reversely correlated with the titer of the NA. These results suggested that the NT induced by RBD-Fc vaccination play an important role in prevention of SARS-CoV infection in virus challenged mice.

Table 1 SARS-CoV RNA copies in the lung tissues measured by Q-RT-PCR

Group	Mouse ID	Neutralizing antibody titers	RNA copies/g of lung tissues
RBD-Fc vaccination	M1	57	3.13×10^3
	M2	189	Undetectable
	M3	505	Undetectable
	M4	452	Undetectable
	M5	404	Undetectable
Control	C1	<4	1.39×10^7
	C2	<4	3.77×10^7
	C3	<4	1.39×10^5
	C4	<4	1.98×10^6
	C5	<4	1.45×10^6

Vaccination with RBD-Fc suppressed SARS-CoV replication in the virus challenged mice

SARS-CoV replication was detected by titration of the inoculated virus in lung tissues to evaluate the efficacy of RBD-Fc vaccination in protecting SARS-CoV infection (Fig 4). All of five mice in the control group vaccinated with PBS had high titers of virus replication in the lungs after the SARS-CoV challenge. However, the mice vaccinated with RBD-Fc were either completely (4 of 5 mice) or partially protected (1 of 5 mice) from SARS-CoV challenge. These data indicate that vaccination with RBD-Fc is able to establish protective immunity to prevent mice from subsequent SARS-CoV challenge.

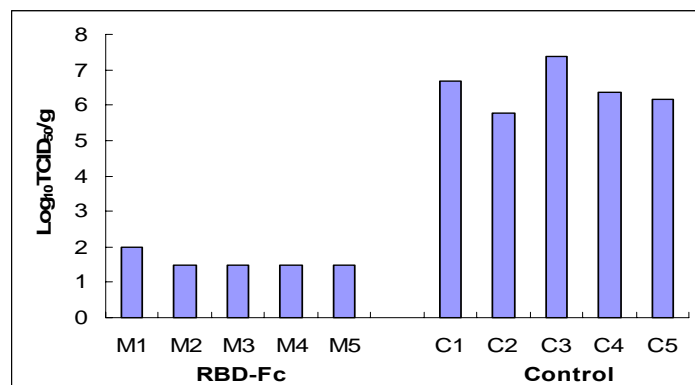


Fig 4 RBD-Fc protected vaccinated mice from subsequent challenge with SARS-CoV. Groups of five BALB/c mice vaccinated with RBD-Fc or PBS were intranasally challenged with 10^6 TCID₅₀ of SARS-CoV five days after the last dose of vaccination. The titers of SARS-CoV replicated in the lung tissues of the mice were detected five days after the challenge.

Mice vaccinated with RBD-Fc did not develop histopathological changes in the lung tissues

Histopathological changes in lungs from the RBD-Fc vaccinated and control mice were observed on the H&E stained lung tissue sections. Lung tissues from the control mice revealed significant histopathological changes, including diffuse alveolar damages characterized by disruption of

alveolar walls and flooding of alveolar lumina with serosanguineous exudates admixed with neutrophils and alveolar macrophages, thickened alveolar walls lined by type 2 pneumocytes, and alveolar macrophages in alveolar lumina (Fig 5A). However, except which from the M1 mouse, lung tissues from other four RBD-Fc vaccinated mice exhibited no significant histopathological changes, but showed similar histological structures as the normal mouse lung tissues (Fig 5B). These results further confirm that RBD-Fc vaccine is able to prevent mice from subsequent SARS-CoV infection.

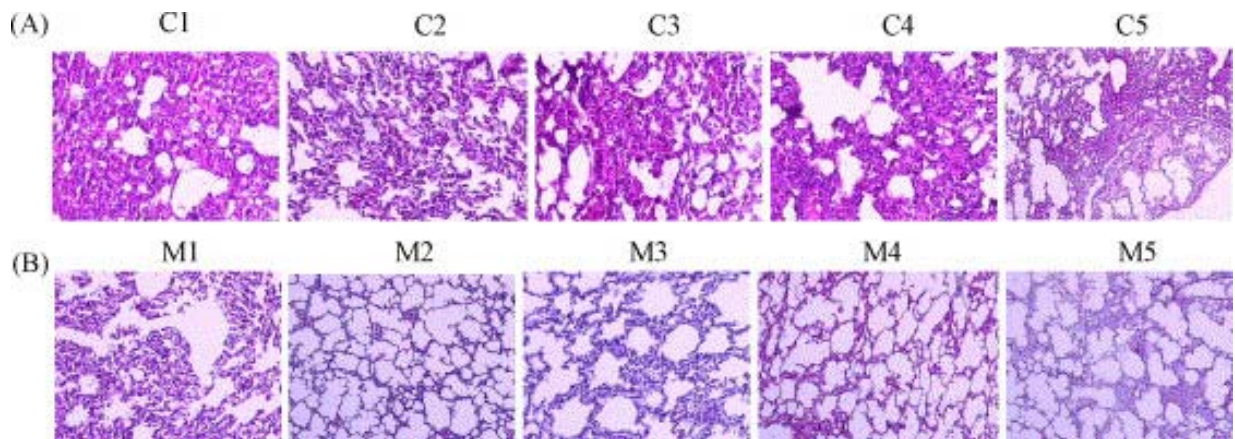


Fig 5 Histopathological examinations of mouse lung tissues. Mouse lung tissue sections were stained with H&E and examined under microscopy (200 \times). (A) Histopathological changes of lung tissues from control mice (C1-C5). (B) Histological characterization of lung tissues from RBD-Fc vaccinated mice (M1-M5).

Single dose of RBD-rAAV vaccination induced sufficient SARS-CoV specific antibody responses with neutralizing activity in mice

Groups of 5 to 10 BALB/c mice were vaccinated with single dose of RBD-rAAV, blank AAV (negative control), or with inactivated SARS-CoV suspended in PBS or in adjuvant Alum (positive control). As shown in Fig 6A, the titers of SARS-CoV specific antibodies in the sera from single dose of RBD-rAAV immunized mice reached 1:880 at 4 months post-vaccination, while inactivated virus vaccination peaked earlier and at a slightly low level (1:686 with Alum, $P > 0.05$) or at a significantly low level (1:384 without Alum, $P < 0.05$) at 2 months post-immunization. Fig 6B shows that the NA level rose continuously from 1: 48 at 1 month to 1: 108 at 4 months post-vaccination, but NA level of animals given the inactivated virus vaccination peaked earlier, at 2 months after immunization, reaching a similar level as that of vaccinated with the inactivated virus suspended in Alum (1:109, $P > 0.05$), and 3 folds higher than that was given the inactivated virus suspended in PBS (1:42, $P < 0.05$). The results indicated that although the RBD-rAAV vaccination induced a lower level of NA than vaccination with inactivated SARS-CoV plus Alum at 1 – 2 months after immunization, it can deliver a prolonged effect with an increasing neutralizing antibody titer at 4 months post-vaccination, which was similar or higher than inactivated virus vaccination with or without Alum ($P > 0.05$).

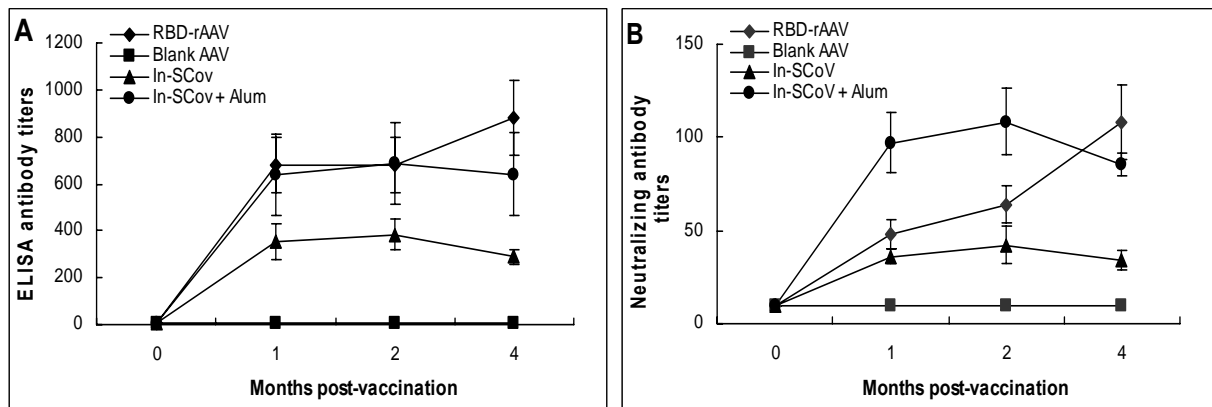


Fig 6 Evaluation of specific antibody responses in single dose of RBD-rAAV vaccinated mice.

(A) Serum samples were collected from the mice at pre-vaccination (0 month), 1, 2, and 4 months post-immunization. SARS-CoV specific antibodies in the serum samples were detected by ELISA.

(B) The neutralizing antibody titers in the sera were measured by a neutralization assay. The data were presented as the mean values \pm SE.

Repeated vaccinations of RBD-rAAV effectively induced high levels of SARS-CoV specific antibodies with neutralizing activity

In a further experiment, groups of 5 mice were given 2 repeated doses and the animals were bled at different times over a period of 5.5 months following the first dose. In the animals given the RBD-rAAV vaccine, the level of the SARS-CoV specific antibody determined by ELISA increased continuously throughout the experiment to reach a titer of 1:5120 at 5.5 months post-vaccination (Fig. 7A). In animals given the inactivated virus, the antibody levels reached the peak (1:3584 without Alum and 1:4754 with Alum) at 4 months post-vaccination. The antibody level determined at the end of the experiment 5.5 months after the first dose, was slightly higher for animals, which were given the RBD-rAAV vaccine than those which were given the inactivated virus, but the difference was not statistically significant ($P > 0.05$).

The NA level in RBD-rAAV group of animals also rose continuously throughout the duration of the experiment of 5.5 months, as did the levels of ELISA antibody, whereas the antibody level of the animals receiving the inactivated virus vaccines peaked on the 4th month. At the end of the experiment, 5.5 months after the first dose, the NA level of RBD-rAAV group was 512, which is similar as the peak antibody levels of animals given the inactivated virus vaccine, (1:512 without Alum and 1:640 with Alum), and it is 5 times higher than the antibody level induced by a single dose of this vaccine (Fig 7B).

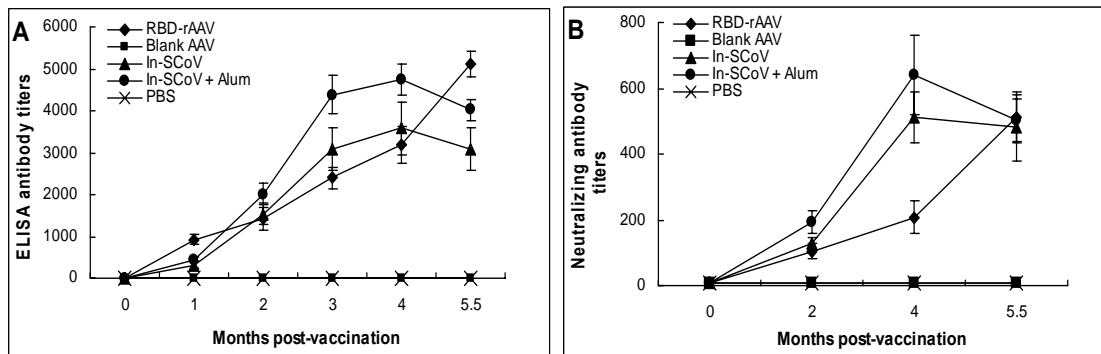


Fig 7 Detection of specific antibody responses in three doses of RBD-rAAV vaccinated mice. SARS-CoV specific antibody responses in mice vaccinated with three doses of RBD-rAAV or controls were tested by ELISA (A) and neutralization assay (B). The experiments were repeated two times and the data were presented as the mean values \pm SE.

Intranasal vaccination induced a shorter-duration systemic humoral immune response but a stronger and prolonged mucosal IgA response than intramuscular vaccination

To evaluate the long-term systemic humoral immune response to RBD-rAAV vaccination, and to compare the differences between immune responses to vaccination via i.m. and i.n. routes, serum samples collected from vaccinated mice at different time points were detected by ELISA for specific IgG antibody to SARS-CoV. As shown in Fig 8A, i.m. prime-boost immunization of RBD-rAAV (RBD.im.B) induced a high level of IgG antibody response, which reached the peak within 3 months, maintained the plateau level for 3 more months, and gradually decreased to a moderate level at 12 months post-immunization. A single-prime dose i.n. vaccination of RBD-rAAV did not induce significant antibody response (data not shown). After booster (RBD.in.B), the vaccination quickly elicited a high level of IgG antibody response, reaching the highest titer 1 month post-vaccination, which was almost the same level as that of RBD.im.B. between the months 3 and 6. However, the IgG antibody level also dropped down to a low level a month later and was maintained at a similar level thereafter. NA levels in these serum samples were further detected by neutralization assay, which showed a similar pattern as that of the IgG antibody responses (Fig 8B). These results indicated that i.n. vaccination induced a similar NA level but shorter-duration systemic humoral immune response than i.m. immunization.

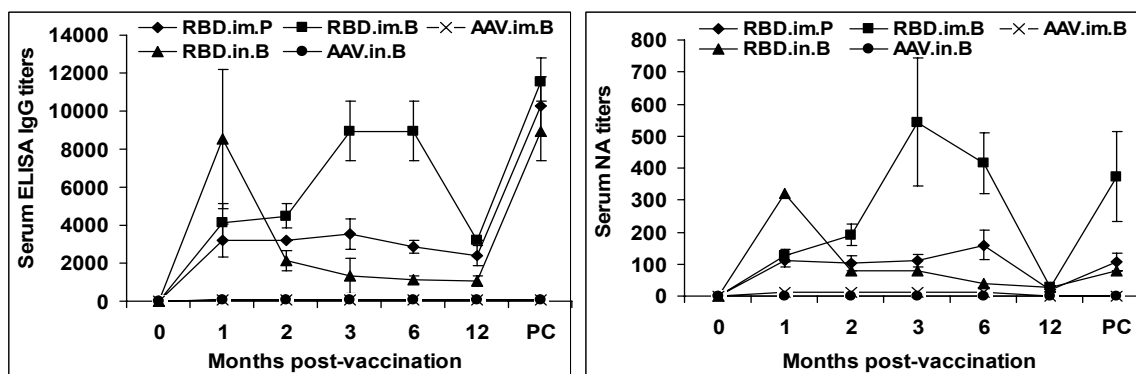


Fig 8 Systemic humoral immune responses to RBD-rAAV vaccinations. (A) SARS-CoV-specific IgG in serum samples was detected by ELISA and reported at 0, 1, 2, 3, 6, 12 months post-vaccination and pre-challenge (PC). (B) Serum NA titers measured by neutralization assay. The data are presented as mean \pm SE of 5 mice per group.

To assess the ability of i.n. vaccination to induce local immune response, mucosal IgA SARS-CoV-specific antibody was further detected by ELISA in the lung flush of vaccinated mice. As shown in Fig 9A, RBD-rAAV i.n. prime-boost (RBD.in.B) induced strong IgA antibody response, which was significantly higher than that elicited by RBD-rAAV i.m. with a single-prime dose (RBD.im.P) and prime-boost doses (RBD.im.B), respectively ($P = 0.004$). Blank AAV (AAV.im.P, AAV.im.B) did not elicit detectable IgA antibody in lung flush ($OD_{450} < 0.05$). These data indicated that the i.n. rather than i.m. vaccination route could induce strong mucosal immune response. Titers of IgA antibody and NA induced by RBD-rAAV i.n. prime-boost in mouse lung flush were further analyzed by ELISA and neutralization assay at 0.5-month intervals. It was shown that the mucosal IgA antibody level reached its peak at 1 month post-vaccination, and gradually decreased to a low level in the following five months (Fig 9B). Strikingly, the lung flush from RBD-rAAV i.n. prime-boost vaccinated mice (RBD.in.B) contained high-level and long-lasting NA against SARS-CoV, which was highly detectable during the detection period of 6 months (Fig. 9C). The above data indicated that i.n. vaccination of RBD-rAAV induced a long-term mucosal immune response with neutralizing activity, implying that mucosal vaccination with RBD-rAAV should provide effective protective immune response against SARS-CoV.

For all vaccination groups, although IgG antibodies had dropped down to low levels at 12 months post-vaccination, it rebounded quickly when the mice were re-boostered (Fig. 8). These results suggested that RBD-rAAV may induce long-term memory immune responses, especially after booster immunization, by both i.m. and i.n. routes.

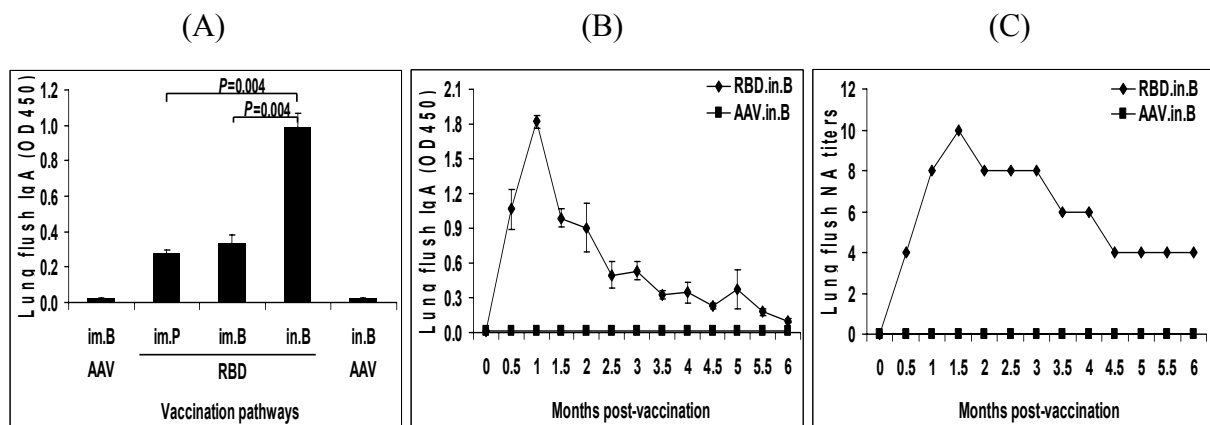


Fig 9 Local humoral immune response to RBD-rAAV vaccinations. (A) Comparison of mucosal IgA in i.m. and i.n. vaccinated mice. (B) Monitoring of mucosal IgA antibody levels in mice

i.n. vaccinated with RBD-rAAV. (C) Detection of NA titers in lung flush of i.n. vaccinated mice.

Intranasal vaccination induced strong CTL responses in spleen and lungs

To examine CTL responses induced by RBD-rAAV vaccination, splenocytes and lung lymphocytes were measured by ELISPOT and FACS. As shown in Fig. 10, i.n. vaccination of RBD-rAAV (RBD.in.B) induced a markedly higher level of antigen specific IL-2⁺ T cells but a slightly lower level of IFN- γ ⁺ T cells in the spleen. In contrast, single dose i.m. or i.n. vaccination with RBD-rAAV did not induce significant IL-2⁺ and IFN- γ ⁺ T cell response (data not shown), suggesting that booster immunization is necessary for inducing antigen specific CTL response. The above data showed that i.n. vaccination could induce much stronger systemic IL-2⁺ CTL response than i.m. vaccination, while IFN- γ ⁺ CTL response elicited by i.m and i.n. routes was of comparable strength. Specific CTL responses induced by RBD-rAAV vaccinations were further evaluated in the mouse splenocytes and lung lymphocytes by cell surface marker and intracellular cytokine staining followed by FACS. As shown in Fig 11A and 11B, RBD-rAAV i.n. vaccination (RBD.in.B) induced a markedly higher frequency of IL-2⁺ cells in the CD3⁺/CD8⁺ T cell population in both splenocytes and lung cells. In addition, IFN- γ -producing CD3⁺/CD8⁺ T cells were significantly higher in splenocytes of RBD-rAAV i.n. vaccinated versus i.m. vaccinated mice, but were similar or slightly lower in lung lymphocytes of i.n. vaccinated versus i.m. immunized mice. These results demonstrated that both i.m. and i.n. vaccination with RBD-rAAV could induce SARS-CoV specific CTL responses, and the i.n. route elicited a higher systemic (in splenocytes) and local (in lungs) CTL responses than the i.m. route.

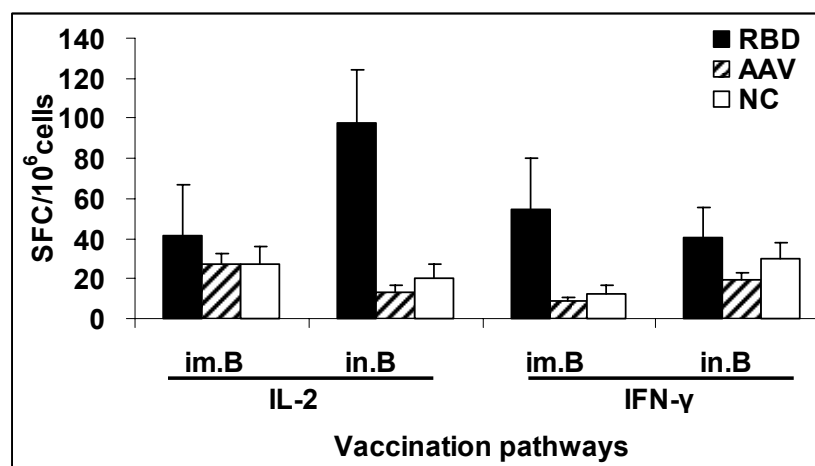


Fig 10 Detection of SARS-CoV specific IL-2 and IFN- γ production. IL-2 and IFN- γ producing T cells stimulated by SARS-CoV S-specific CTL peptide in the spleen were analyzed by ELISPOT. Splenocytes from vaccinated mice were stimulated with SARS-CoV S-specific CTL peptide and anti-CD28 mAb for 24 h. Anti-CD28 alone was applied as the negative control (NC). Frequencies of cytokine-producing cells are expressed as mean + SE of cytokine spot-forming cells (SFC)/10⁶ cells of 5 mice per group.

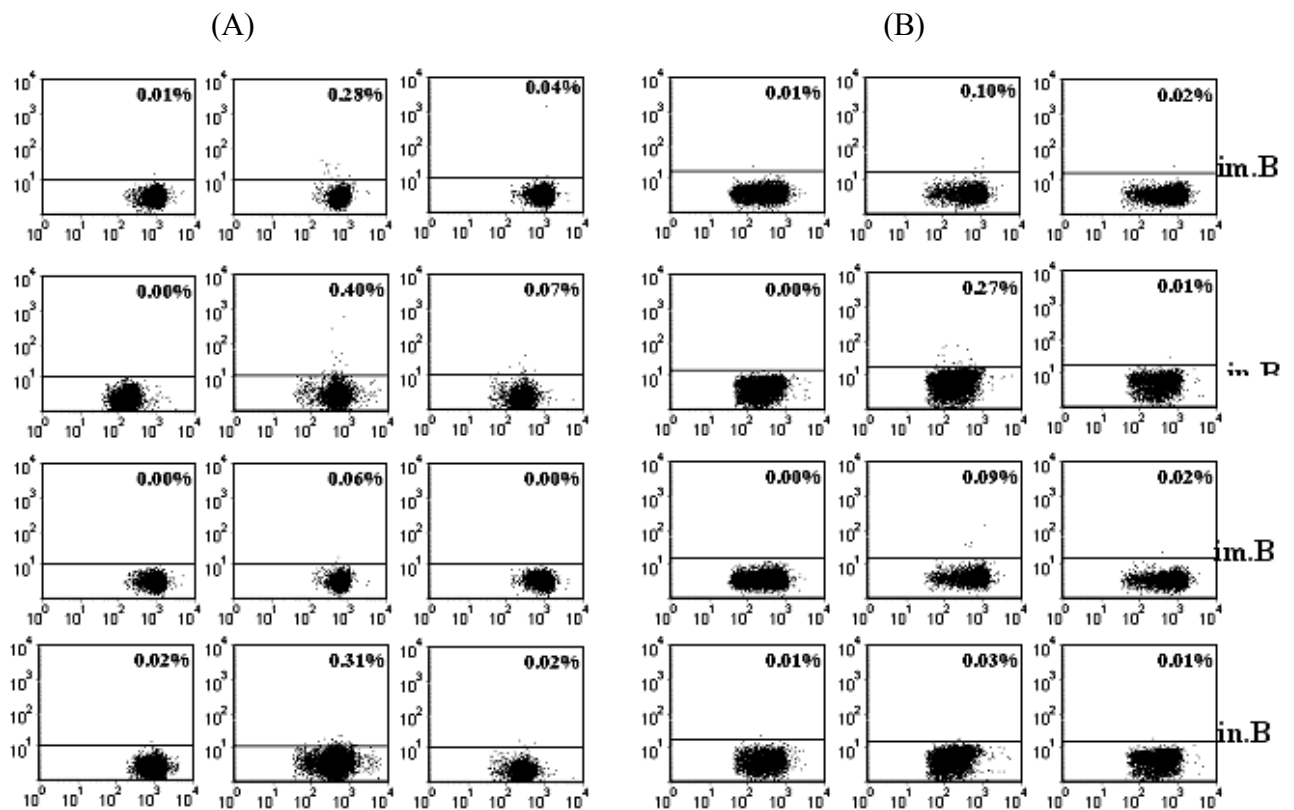


Fig 11 Detection of SARS-CoV specific CTL responses. IL-2 and IFN- γ producing CD8⁺ T lymphocytes stimulated by SARS-CoV S-specific CTL peptide in the spleen (A) and lung (B) were further confirmed by cell surface marker and intracellular cytokine staining using FACS.

RBD-rAAV vaccination suppressed SARS-CoV replication in mouse lungs

The protective efficacies of the vaccinations were further investigated in the mice challenged with 10^5 TCID₅₀ of SARS-CoV strain GZ50. Mice were sacrificed 3 days post-challenge, and virus replication was assessed by viral load in challenged mouse lung tissue by Q-RT-PCR. Fig 12 shows that viral loads (RNA copies/ μ g of lung tissues) in all mice immunized with RBD-rAAV were significantly lower than that of the corresponding control group immunized with blank AAV via i.m. and i.n. routes ($P < 0.05$), indicating that SARS-CoV replication was suppressed in vaccinated mice.

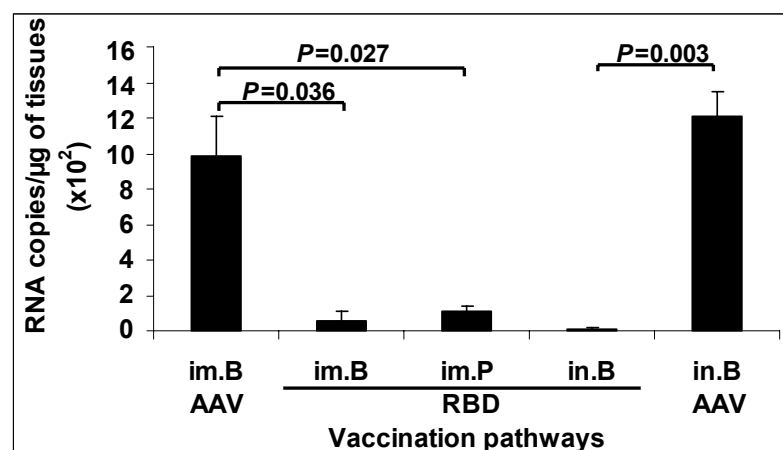


Fig 12 Detection of viral load in lung tissues of challenged mice. Viral load of SARS-CoV in lung tissue from mice i.m. or i.n. vaccinated with a single-prime dose (im.P), or prime-boost doses (im.B, in.B) of RBD-rAAV were determined by Q-RT-PCR. Mice i.m. and i.n. vaccinated with blank AAV were used as negative controls. The data are expressed as mean + SE of RNA copies/ μ g of lung tissue from 4 mice for each group.

Correlation of serological data with virus protection

Mouse sera and lung flush were collected before virus challenge to detect levels of serum specific IgG and IgA antibodies and neutralizing activities. It was shown in Table 2 that there were clear correlation among the level of SARS-CoV-specific serum IgG antibody, lung flush IgA antibody, NA, and the protection against the virus challenge. These data indicated that both mucosal (local) and serum (systemic) specific antibodies, especially NAs, could provide some protection for vaccinated mice from subsequent virus challenge, while mucosal immune response was indispensable for controlling SARS-CoV infection.

Table 2 Correlation of serum IgG, serum NA, mucosal IgA, and virus protection

Group	Vaccinations	Serum IgG titer ($\times 10^3$)	Serum NA titer ($\times 10^2$)	Lung flush IgA titer	Viral RNA copies ($\times 10^2$)/ μ g of tissues
RBD	im.P	3.2	1.2 \pm 0.4	0.20 \pm 0.03	1.1 \pm 0.2
	im.B	8.0 \pm 1.6	3.7 \pm 1.4	0.31 \pm 0.05	0.6 \pm 0.6
	in.B	4.8 \pm 0.9	1.9 \pm 0.7	1.00 \pm 0.07	0.5 \pm 0.2
AAV	im.B	<0.1	<0.05	<0.05	4.8 \pm 1.4
	in.B	<0.1	<0.05	<0.05	12.0 \pm 1.0

RBD-rAAV vaccination provided long-term protection against SARS-CoV challenge

Mice (5 mice/group) were boosted with RBD-rAAV 12 months after the first RBD-rAAV immunization, and challenged with 10^5 TCID₅₀ of SARS-CoV. Challenged mice were sacrificed 8 days post-challenge for examination of histopathological changes. Serious pulmonary interstitial pneumonias were observed in the lung tissues of all control mice vaccinated with blank AAV after SARS-CoV challenge (Fig. 13A). In contrast, mice that had received RBD-rAAV vaccination showed no significant pulmonary effect after virus challenge (Fig. 13B). The above results demonstrated that RBD-rAAV vaccinations lessened the alveolar damage of challenged mouse lungs, and provided long-term protective immunity to prevent vaccinated mice from SARS-CoV infection.

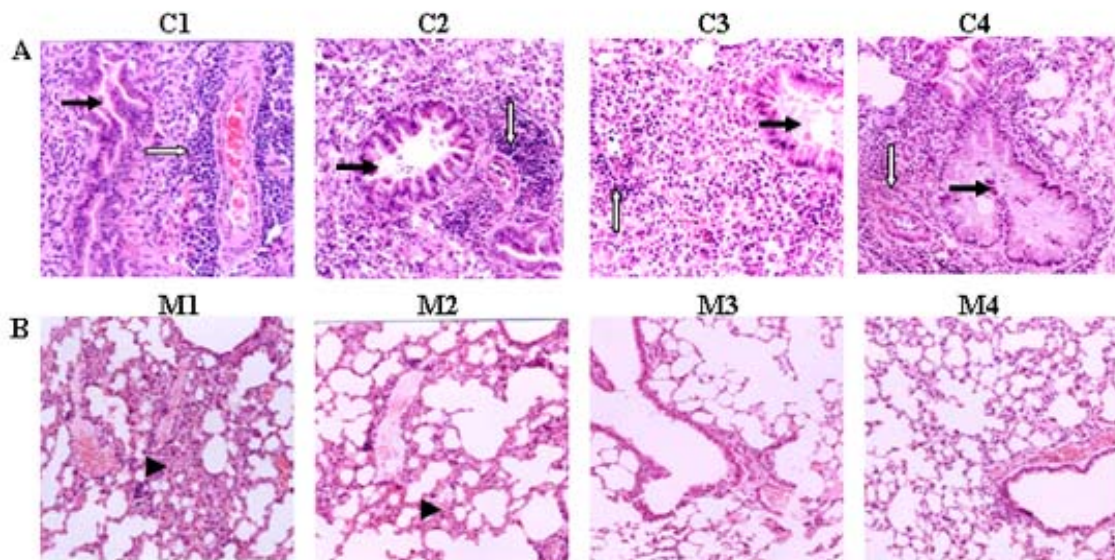


Fig 13 Histopathology of mouse lungs infected with SARS-CoV 8 days post-infection. All sections of mouse lung tissue were stained with H&E and examined under the microscope (original magnification 100 ×). (A) Representative images of histopathological damage of lung tissue from the control mice administered with blank AAV in either i.m. (C1 – C2) or i.n. (C3 – C4) boost vaccinations. These control mice developed interstitial pneumonia. Predominantly infiltrating lymphocytes and mononuclear cells were identified around small blood vessels (open arrow). Pulmonary vascular peripheral lymphocyte infiltration was also shown, with bronchial epithelial cell degeneration, necrosis, desquamation (solid arrow), broadening interstitial spaces, and exudation. (B) Representative images of histopathological changes of lung tissue from mice i.m. vaccinated with a single-prime dose (M1) or prime-boost doses (M2), and i.n. vaccinated with prime-boost doses (M3 – M4) of RBD-rAAV. The lung tissue from a single-prime dose i.m. vaccinated mice showed mild interstitial pneumonia change with focal broadening interstitial spaces and lymphocytic infiltration (arrow head) (M1). Mice with i.m. prime-boost vaccinations developed slightly interstitial pneumonia with normal alveolar, slightly widened pulmonary interval and small lymphocytic infiltration (M2). Mice with i.n. prime-boost vaccinations showed almost normal vascular structure, bronchiole, alveolar, and alveolar lung spacing (M3 – M4).

Discussion

To date, several approaches for developing SARS vaccines have been described, including inactivated virus-based vaccines [11], DNA-based vaccines [12], recombinant subunit vaccines [13], and viral vector-based vaccines [14]. In our studies, we prepared and evaluated three vaccine candidates, inactivated SARS-CoV, RBD protein and RBD-rAAV.

Inactivated SARS-CoV vaccine candidate

Intranasal immunization with inactivated viruses has not succeeded in inducing effective antibodies in other studies, while several approaches to increase the efficacy of intranasal or other mucosal immunization by inactivated viruses, inactivated bacteria or constructs containing viral or bacterial proteins have been presented [15-17]. We used CPG-ODN and CTB as adjuvants and PEG-precipitated inactivated virus was used to potentiate the uptake of the inactivated viruses by antigen presenting cells, and to maintain inactivated viruses at the site of administration. After four intranasal doses of the inactivated virus, serum anti-SARS-CoV neutralizing antibodies were detected, but no anti-SARS-IgA was found in the tracheal-lung wash fluid. In contrast, when the inactivated virus was co-administered intranasally with adjuvant (either CPG or CTB), both serum anti-SARS-CoV neutralizing antibodies and specific IgA in tracheal-lung wash fluid were detected (Fig 1). These results indicate that local IgA antibodies could only be induced by combining the inactivated virus and adjuvant. On the other hand, in mice immunized subcutaneously with inactivated virus, aside from high titer of serum neutralizing antibodies, specific anti-SARS-IgG also could be detected in tracheal-lung wash fluid. However, no anti-SARS-IgA was detected, which indicated that the antibodies detected in the tracheal-lung fluid was not produced locally, but derived from serum antibodies.

Given that CTB was reported more or less toxic in hosts [18], CpG-ODN is non-toxic and induces effective humoral and cellular immune responses in hosts [19, 20]. It is thus a promising adjuvant to be used with SARS-CoV for intranasal immunization. On the other hand, PEG has been used for the purification of inactivated HAV vaccines [21] and PEG-interferon has already been used in clinical trials for treatment of viral hepatitis B and C [22]. So far no serious ill-effects have been described. We therefore used PEG precipitated SARS-CoV inactivated virus for intranasal immunization of mice. Compared to the dosage being used with CPG as the adjuvant, only half the dosage of inactivated virus was necessary to induce both local and serum specific antibodies by using PEG precipitate as the adjuvant. This precipitated inactivated virus is therefore also a good candidate for development of intranasal SARS-CoV inactivated vaccine.

RBD protein vaccine candidate

Although the full-length S protein-based vaccines can elicit neutralizing antibodies and/or protective immunity [23-25], it has been reported that this kind of vaccines may also induce harmful inflammatory and immune responses [26, 27]. This has raised concerns about the efficacy and safety of the vaccines containing or encoding the full-length S protein. We thus proposed to use RBD, a fragment of S protein, rather than the full-length S protein, for development of a safe and effective SARS subunit vaccine since it contains the major neutralizing epitopes [28, 29].

In this study, we compared the antibody responses and protective immunity in mice vaccinated with RBD-Fc and the control mice. We found that RBD-Fc elicited potent and long-term humoral immune responses in vaccinated mice. High titers of SARS-CoV S-specific ELISA antibodies and virus neutralizing antibodies were maintained for at least 6 months. Although the antibodies dropped to a low level at the end of the 12th month, their titers rebound rapidly after the mice were re-boosted with RBD-Fc. These vaccinated mice were protected from SARS-CoV challenge since no significant virus replication was detected in their lungs. Notably, presence of high titers of the neutralizing antibody was a prerequisite for protective immunity. Higher titer of neutralizing antibodies was associated with lower level of RNA copies/virus replication and stronger protective activity against SARS-CoV challenge. Our results indicated that the neutralizing antibody produced in RBD-Fc vaccinated mice plays a significant role in protection of mice from the virus challenge without causing immunopathological damages.

RBD-rAAV vaccine candidate

Above results demonstrated that the RBD region is the main target in eliciting neutralizing antibodies. Therefore, RBD of SARS-CoV S protein was selected as the target immunogen in our study for development of SARS-CoV vaccines. The reason why AAV was used to deliver and express the RBD gene in our study was that it has been demonstrated to be a promising tool for delivery of foreign genes due to its advantages in high virus production and broad host range without causing any known human diseases [30, 31]. Our results showed that as high as 5×10^{12} viral particles/ml of RBD-rAAV could be produced by transfection of 293T cells. It was also confirmed that the recombinant RBD-rAAV was able to efficiently infect different cell lines and express the RBD protein in transduced cell lines, which was recognized by a SARS-CoV neutralizing McAb.

The immune responses and protective immunity of RBD-rAAV were evaluated in animal model. Our results showed that: (1) the intramuscular (*i.m.*) RBD-rAAV vaccination indeed elicited long-term systemic antibody responses with neutralizing activities and T-cell responses, which provided protection against SARS-CoV challenge in mice; (2) the intranasal (*i.n.*) vaccination of RBD-rAAV induced a systemic humoral immune response of comparable strength and shorter duration than the *i.m.* vaccination, but local humoral immune response was much stronger; (3) the *i.n.* vaccination elicited stronger systemic and local SARS-CoV-specific cytotoxic T-cell responses than the *i.m.* vaccination, as evidenced by the higher prevalence of IL-2 and/or IFN- γ producing CD8⁺ T-cells in both lungs and spleen; (4) the *i.n.* vaccination provided better protection than that of the *i.m.* vaccination against SARS-CoV challenge in mice; (5) despite repeated vaccinations, the primary vaccination did not affect the efficacy of subsequent vaccinations, showing a rapid increase in antibody titers after a booster vaccination; (6) higher titers of IgG, IgA and neutralizing antibodies

were associated with lower viral load and less pulmonary pathological damage, and no antibody-mediated disease enhancement effect was observed.

Although the level of humoral immune response induced by the RBD-rAAV vaccination might be lower than that of the RBD-Fc vaccination, RBD-rAAV induced a long-term mucosal immune response, which plays an important role in suppressing virus replication and in minimizing histopathological changes in the lungs of vaccinated mice. Furthermore, RBD-rAAV was able to induce strong specific T-cell responses, which may also play an important role in controlling the virus infection.

Conclusions

This study has proven that the inactivated, RBD-Fc and RBD-rAAV vaccines candidates may be further developed into safe and effective vaccines for prevention of SARS-CoV infection and that intranasal vaccination may be the preferred route of administration.

Implications

The new knowledge gained from this study has provided the theoretical and practical basis for development of effective means and strategies for prevention of SARS, as well as a platform for emergency preparation against other respiratory tract transmitted infectious agents, such as avian flu H5N1, or against sudden attack of aerosol bioterrorism.

Dissemination

The study findings have been published or will be published in international journals and conferences (please see below for details).

Publications

Publications in journals

1. Du L*, Zhao G, Lin Y, Chan C, He Y, Jiang S, Yuen KY, Zhou Y, Zheng BJ. Priming with adeno-associated virus encoding RBD of SARS-CoV S protein and boost with RBD-specific peptides increase immunogenicity in an animal model. Submitted to *Euro J Immunol*.
2. Du L*, Zhao G, Lin Y, Sui H, Chan C, Ma S, He Y, Jiang S, Wu CY, Yuen KY, Jin DY, Zhou Y, **Zheng BJ**. Intranasal vaccination of recombinant adeno-associated virus encoding RBD of SARS-CoV S protein induces strong mucosal immune responses and provides long-term protection against SARS-CoV infection. Submitted to *J. Immunol*.

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Publications in conferences

1. Du L*, Zhou Y, He Y, Ma S, Yuen KY, Jiang S, **Zheng BJ**. Intranasal vaccination with adeno-associated virus encoding the receptor-binding domain of severe acute respiratory syndrome coronavirus S protein induces strong and prolonged systemic and local antibodies with neutralizing activity. The American Society for Virology 25th Annual Meeting (25th ASV). Madison, Wisconsin, USA. Jul. 2006. Abstract number P16-3.
2. Du L*, Zhou Y, He Y, Ma S, Yuen KY, Jiang S, **Zheng BJ**. Vaccination with adeno-associated virus vector encoding severe acute respiratory syndrome coronavirus S protein induces potent and prolonged antibodies. 16th European Congress of Clinical Microbiology and Infectious Diseases (16th ECCMID). Nice, France. Apr. 2006. Abstract number P573.

* Du L is Dr. BJ Zheng's Ph.D student.

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List of Research Workers

Zheng BJ, Du L, Jin DY, Yuen KY, Guan Y, Kao RY, Huang JD, Zhao G, Lin Y, Wu SH, Ng F, Sui H, Zhang H, Chan C, Ma S, He Y, Zhou Y, Jiang S, Wong C, Wang Y, Guo Y, Shen C, Qu D, Yao X, Yuan ZH, Zhong NS, Lu LW, Xie JP, Wen YM.

Financial Statement

Appendices

The publications are attached.