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Receptor-binding domain of SARS-CoV spike protein induces long-term protective immunity in an animal model

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Abstract

Development of effective vaccines against severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) is still a priority in prevention of re-emergence of SARS. Our previous studies have shown that the receptor-binding domain (RBD) of SARS-CoV spike (S) protein elicits highly potent neutralizing antibody responses in the immunized animals. But it is unknown whether RBD can also induce protective immunity in an animal model, a key aspect for vaccine development. In this study, BALB/c mice were vaccinated intramuscularly (i.m.) with 10 μg of RBD-Fc (RBD fused with human IgG1 Fc) and boosted twice at 3-week intervals and one more time at 12th month. Humoral immune responses of vaccinated mice were investigated for up to 12 months at a 1-month interval and the neutralizing titers of produced antibodies were reported at months 0, 3, 6 and 12 post-vaccination. Mice were challenged with the homologous strain of SARS-CoV 5 days after the last boost, and sacrificed 5 days after the challenge. Mouse lung tissues were collected for detection of viral load, virus replication and histopathological effects. Our results showed that RBD-Fc vaccination induced high titer of S-specific antibodies with long-term and potent SARS-CoV neutralizing activity. Four of five vaccinated mice were protected from subsequent SARS-CoV challenge because no significant virus replication, and no obvious histopathological changes were found in the lung tissues of the vaccinated mice challenged with SARS-CoV. Only one vaccinated mouse had mild alveolar damage in the lung tissues. In contrast, high copies of SARS-CoV RNA and virus replication were detected, and pathological changes were observed in the lung tissues of the control mice. In conclusion, our findings suggest that RBD, which can induce protective antibodies to SARS-CoV, may be further developed as a safe and effective SARS subunit vaccine.

Keywords: SARS-CoV; Receptor-binding domain; Protective immunity; Subunit vaccine

1. Introduction

Severe acute respiratory syndrome (SARS) is a novel infectious disease caused by SARS coronavirus (SARS-CoV), which led to several hundred deaths among thousands of cases. Although SARS has been successfully contained, re-emergence of SARS-CoV from animal reservoirs is still a potential risk for future epidemic, which is supported by continual reports of finding SARS-CoV-like coronavirus in small animals, such as civets, raccoon dogs [1–3] and bats [4,5]. SARS outbreak may also recur in the future by the virus escaping from laboratory accidents [6,7]. Therefore, development of safe and effective SARS vaccines for prevention of SARS-CoV infection is an important issue on current SARS research.

SARS-CoV, the causative agent of SARS, contains the genome encoding the nonstructural replicase polyprotein (rep) and structural proteins spike (S), envelope (E), membrane (M) and nucleocapsid (N) [8,9]. Its S protein is responsible for virus binding to the receptor, angiotensin-converting

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enzyme 2 (ACE2), and subsequent virus entry into the host cells [10,11]. The S protein of SARS-CoV has also been demonstrated to be the main antigen in inducing high titer of neutralizing antibodies [12–14], and in eliciting protective immunity against infection in challenged animals [15–17]. Thus, it is implied to be the main target in development of SARS vaccines.

A number of vaccine candidates based on SARS-CoV S have been reported in terms of their abilities in inducing neutralizing antibodies and protective immunity [15,18–20]. These candidates can be grouped into inactivated viruses-based, protein-based, DNA-based and virus-based vaccines [18,20–23]. Many viruses, including VSV, rhabdovirus, adenovirus, adeno-associated virus (AAV), modified vaccinia virus Ankara (MVA) and attenuated parainfluenza virus, have been used for expressing SARS-CoV S protein as SARS vaccine candidates [12,15,24–27]. Bukreyev et al. [24] reported that vaccination of African green monkeys with an attenuated parainfluenza virus encoding SARS-CoV S resulted in production of SARS-CoV-specific neutralizing antibodies and protection of animals from virus challenge. These findings suggest that S protein of SARS-CoV is a good target for development of SARS-CoV vaccines.

Since most DNA-based and virus-based vaccine candidates have still caused some safety concerns, protein-based vaccine may be an alternative candidate. Indeed, the full-length S protein or its S1 subunit could induce potent neutralizing antibody responses in the immunized animals [13,25,28]. However, it may also elicit antibodies that enhance virus infection [25,29] or cause liver damage in animals challenged with SARS-CoV [23]. In this regard, the receptor-binding domain (RBD), a fragment of the S protein, which has been demonstrated to be a major neutralization determinant, has implied to be an ideal candidate for development of subunit vaccines against SARS [30–34]. In this study, we further evaluated its long-term immune responses and protective immunity against SARS-CoV infection in a mouse model.

2. Materials and methods

2.1. Virus, cells and recombinant proteins

The BJ01 strain of SARS-CoV (GenBank accession no. AY278488) was propagated in Vero E6 cells under the conditions of the biosafety level 3 laboratories (BSL-3) [35,36]. Titer of this virus strain was determined by the 50% tissue-culture infectious dose ($TCID_{50}$) in Vero E6 cells. A 193-aa RBD domain of SARS-CoV S protein (residues 318–510) fused with the Fc domain of human IgG1 (RBD-Fc) was produced as described previously [30,31]. Briefly, a plasmid encoding RBD-Fc fusion protein was transfected into HEK293T cells using Fugene 6 transfection reagents (Boehringer Mannheim) according to the manufacturer's protocol. Supernatants were harvested 72 h post-transfection.

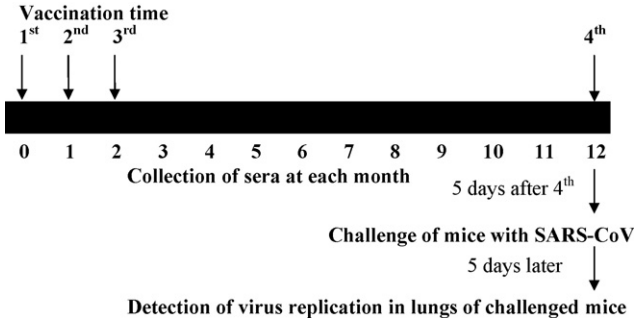

Fig. 1. Schematics for RBD-Fc vaccination and SARS-CoV challenge. BALB/c mice were i.m. immunized with RBD-Fc fusion protein suspended in PBS plus adjuvant (vaccination group) or PBS only (control group) four times in total. Serum was collected from each mouse at a 1-month interval. The mice in both groups were intranasally challenged with SARS-CoV 5 days after the last vaccination, followed by detection of virus replication and histopathological changes in the mouse lung tissues 5 days after the challenge.

Recombinant RBD-Fc protein was purified by protein A-Sepharose 4 Fast Flow (Amersham Biosciences, USA). A full-length S protein of SARS-CoV Urbani (accession no. AY278741) expressed in expresSF$^{+®}$ insect cells with recombinant baculovirus D3252 was purchased from the Protein Sciences Corporation (Bridgeport, CT) [13,14].

2.2. Animal vaccination with SARS-CoV RBD-Fc

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 phosphate-buffered solution (PBS, pH 7.2) 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 5 days later (Fig. 1). 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 1-month interval post-vaccination. Sera were kept at −20 °C until use.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Mouse sera were tested against the recombinant SARS-CoV S protein by ELISA. Briefly, 1 μg/ml of the recombinant S 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 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 (Zymed, South San Francisco, CA) at

37 °C for 1 h. After three washes, the reaction was visualized by addition of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) and measured for the absorbance at 450 nm (A450) by an ELISA plate reader (Victor 1420 Multilabel Counter, Perkin-Elmer, USA).

2.4. Neutralization assay for SARS-CoV infection

Titers of the neutralizing antibody in mouse sera vaccinated with the recombinant RBD-Fc protein were detected in Vero E6 cells as described by Qu et al. [37]. Briefly, Vero E6 cells were seeded at 2×10^4 cells/well in 96-well culture plates and cultured at 37 °C for 24 h to form a monolayer. Serial two-fold dilutions of serum samples were incubated with 100 $TCID_{50}$ of SARS-CoV BJ01 strain at 37 °C for 1 h and subsequently added to the monolayer of Vero E6 cells at tetrad. Cells infected with 100 $TCID_{50}$ SARS-CoV and without the virus were applied as positive and negative controls, respectively. Cytopathic effect (CPE) in each well was observed daily and recorded on day 3 post-infection. The neutralizing titers of mouse antisera that completely prevented CPE in 50% of the wells were calculated by Reed-Muench method.

2.5. Challenge of vaccinated mice with SARS-CoV

Five days after the last boost with RBD-Fc, mice were anaesthetized with isoflurane and intranasally (i.n.) inoculated with 50 μl of diluted SARS-CoV BJ01 strain (10^6 $TCID_{50}$) according to the national animal care and use guidelines in a BSL-3 laboratory. Vaccinated mice were sacrificed 5 days after the challenge and lungs of the mice were removed. The lung tissues were stored at −80 °C for virological tests or were fixed with 4% paraformaldehyde and stored at 4 °C for histopathological analysis.

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

The viral RNA copies in lung tissues were determined by Q-RT-PCR according to the protocol described by Zhai et al [38]. The limit of detection for live virus in lung samples was 1×10^3 copies/g. Briefly, total RNA was extracted from 20 mg of the lung tissues using RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. SARS-CoV RNA was quantified in a 30 μl mixture containing 10 μl RNA, 15 μl 2 × TaqMan® one-step RT-PCR Master Mix (ABI, 4309169), 0.75 μl 40 × multiscribe, 0.25 μM each forward primer Af (Taq-772F 5′-AAG CCT CGC CAA AAA CGT AC-3′), reverse primer Ar (Taq-1000R 5′-AAG TCA GCC ATG TTC CCG AA-3′) and probe (Taq-955T 5′-FAM-TCA CGC ATT GGC ATG GAA GTC ACA CT-TAMRA) (TIB Molbiol, Berlin, Germany) using a fluorometric PCR instrument (ABI 7300).

2.7. Determination of viral titers and total virus

The virus replication was determined by titration of the inoculated virus in lung tissues collected from sacrificed mice. Briefly, the lung tissues were homogenized to a final concentration of 10% (w/v) suspension in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA). Tissue homogenates were centrifuged, filtrated and inoculated into the monolayer of Vero E6 cells seeded in 96-well tissue-culture plates. Results were evaluated after 3 days of the culture under phase-contrast microscopy, and viral titers using a CPE-based $TCID_{50}$ test were calculated by Reed-Muench method. Viral titers were expressed as $Log_{10}TCID_{50}$/g of tissues, with the lowest detection limit of 1.5 $Log_{10}TCID_{50}$/g. The total amount of virus was calculated by multiplying the weight of the lung tissues and the viral titers measured in 10% of tissue homogenates.

2.8. Histopathological assay

The lung tissues were immediately fixed in 4% buffered paraformaldehyde and embedded in paraffin wax. Sections were made at 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 light microscopy.

3. Results

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

As shown in Fig. 2A, RBD-Fc vaccination induced a prolonged and potent humoral immune response with IgG specific to SARS-CoV S protein in the animals as tested by ELISA, reaching the highest titer ($1{:}1.5 \times 10^5 \pm 1.6 \times 10^4$, Mean ± SE) at the 3rd and 4th month post-vaccination. Although the titer decreased slightly afterwards, high titer of S-specific IgG antibody maintained for at least 6 months until it dropped to $1{:}9.0 \times 10^2 \pm 1.6 \times 10^2$ at the end of the 7th month. Specific IgG antibody increased rapidly after the 3rd boost (12 months after the first vaccination) and reached the titer of $1{:}2.5 \times 10^3 \pm 7.7 \times 10^2$ before SARS-CoV challenge. Fig. 2B 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. The neutralizing antibodies reached the highest level at the 3rd month ($1{:}4.0 \times 10^3 \pm 3.5 \times 10^2$) after the vaccination with RBD-Fc. The neutralizing antibody level slightly decreased to $1{:}5.8 \times 10^2 \pm 1.0 \times 10^2$ by the end of the 6th month but dropped down to less than 1:40 at the end of the 12th month. The titer of the neutralizing antibody to SARS-CoV increased again after the third boost, reaching the titer of

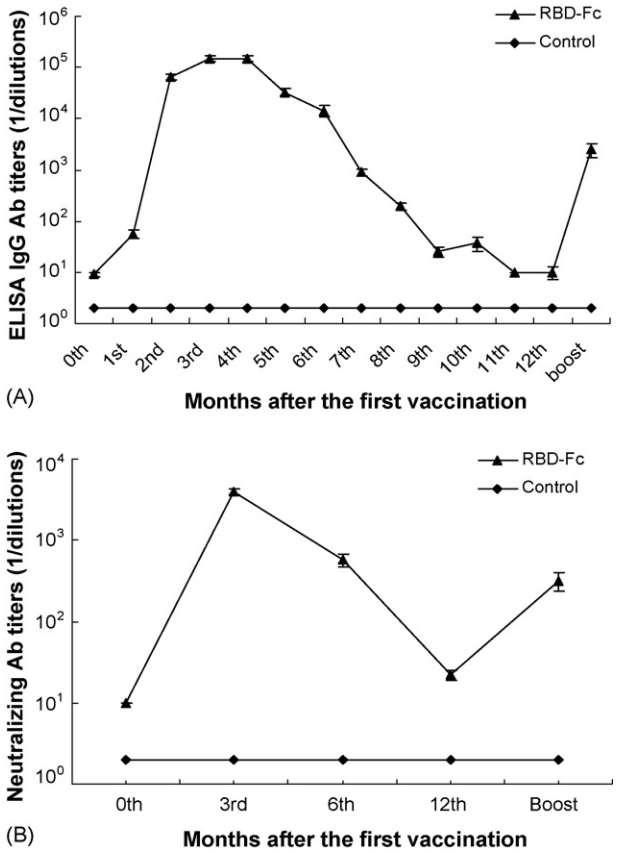

Fig. 2. Detection of anti-S antibodies and virus neutralizing antibodies in mouse sera. (A) Titers of anti-S antibodies measured by ELISA for 12 months at monthly basis and 5 days after the last boost. Data are presented as the geometric means ± SE of five mouse sera collected at different time points. (B) Titers of the SARS-CoV neutralizing antibody in sera collected at 0, 3, 6 and 12 months post-vaccination and sera collected 5 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 ± SE of five mouse sera in each group.

$1{:}3.2 \times 10^2 \pm 1.9 \times 10^2$ before SARS-CoV challenge. However, neither ELISA S-specific antibodies nor neutralizing antibodies were detected in the mouse sera from the control group.

3.2. 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 and described as RNA copies/g of tissues. As shown in Table 1, viral RNA copies for both the RBD-Fc group and the control group were negatively correlated with the titer of the neutralizing antibodies against SARS-CoV. The higher titer of the neutralizing antibodies was detected in the sera, the lower copy number of viral RNA was found in the lung tissues. M1 mouse in the RBD-Fc vaccination group developed the lowest titer of neutralizing antibody (1:57) and showed detectable viral RNA copies (3.13×10^3), while no viral RNA was detectable in lung tissues of the other four mice in this group as they presented higher titers of the neutralizing antibody (>1:189). Similarly, no neutralizing antibody response in the PBS control group was correlated to the higher levels of RNA copies in the lung tissues (1.39×10^5 to 3.77×10^7). These results suggested that the neutralizing antibodies induced by RBD-Fc vaccination play an important role in prevention of SARS-CoV infection in virus challenged mice.

3.3. 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. 3). 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 (four of five mice) or partially protected (one of five mice, M1 which showed lower level of neutralizing antibody in its serum) from SARS-CoV challenge. These data indicate that vaccination with RBD-Fc

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

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

[a] Titers of neutralizing antibodies in sera collected from each mouse 5 days after the last vaccination and before the virus inoculation were determined. RNA copies in the lung tissues of mice challenged with SARS-CoV were measured by Q-RT-PCR and expressed as RNA copies/g of lung tissues. M1–M5 and C1–C5 are RBD-Fc-vaccinated mice and control mice, respectively. The limit of detection for live virus in the lung was 1×10^3 copies/g.

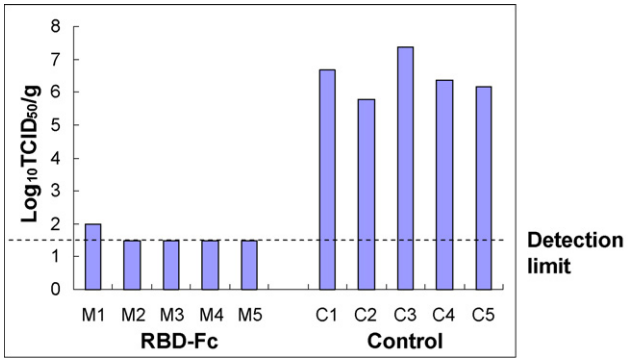

Fig. 3. RBD-Fc protected vaccinated mice from subsequent challenge with SARS-CoV. Groups of five BALB/c mice vaccinated with RBD-Fc or PBS were intranasally challenged with 10^6 $TCID_{50}$ of SARS-CoV 5 days after the last dose of vaccination. The titers of SARS-CoV replicated in the lung tissues of the vaccinated mice and control were detected 5 days after the challenge, and expressed as $Log_{10}TCID_{50}$/g of tissues. M1–M5 are mice vaccinated with RBD-Fc, and C1–C5 indicate the control mice. The limit of detection was 1.5 $Log_{10}TCID_{50}$/g.

is able to establish protective immunity to prevent mice from subsequent SARS-CoV challenge.

3.4. 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 (injected with PBS) 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. 4A). However, except M1 mouse with a mild level of the alveolar damage in lung tissues, other four RBD-Fc vaccinated mice exhibited no significant histopathological changes, but showed similar histological structures as the normal mouse in lung tissues (Fig. 4B). These results further confirm that RBD-Fc vaccine is able to prevent mice from subsequent SARS-CoV infection.

4. Discussion

Development of effective and safe SARS vaccines is crucial in prevention of re-occurrence of SARS epidemic. Among four structural proteins of SARS-CoV, S serves as the most important antigen for development of SARS vaccines since it plays essential roles in the host cell tropism and virus entry, and its ability to induce neutralizing antibodies and protective immunity [13,17,20,39,40]. Reports have shown that neutralizing antibodies against S protein protect vaccinated animals from SARS-CoV challenge, thus vaccines encoding the S protein and targeting the humoral immune responses/neutralizing antibodies are indispensable in preventing SARS-CoV infection [16,17,20,28,39,40].

Most of the currently reported SARS vaccine candidates are based on the full-length S protein of SARS-CoV. Although the full-length S protein-based vaccines can elicit neutralizing antibodies and/or protective immunity [13,17,20], it has been reported that this kind of vaccines may also induce harmful inflammatory and immune responses [23,29]. 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 [31,32,41]. Our previous studies have shown that the recombinant RBD can induce high potent neutralizing antibodies against SARS-CoV and its variants

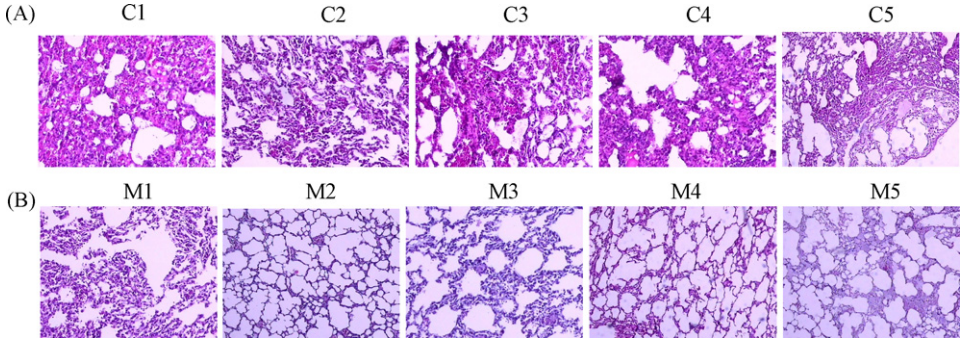

Fig. 4. Histopathological examinations of mouse lung tissues. All sections of mouse lung tissues were stained with H&E and examined under microscopy (original magnification 200×). C1–C5 and M1–M5 are control mice and RBD-Fc vaccinated mice, respectively. (A) Histopathological changes of lung tissues from control mice administered with PBS. Obvious histopathological changes were observed in lung tissues from control mice, 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. (B) Histological characterization of lung tissues from RBD-Fc vaccinated mice. No significant histopathological changes of lung tissues were observed in four of five RBD-Fc-vaccinated mice (M2–M5). Only M1 mouse exhibited mild histopathological damages in lung tissues. But compared to the control mice, M1 mouse revealed a little damage such as thickened alveolar walls with interstitial mononuclear cells and lymphocytes infiltrates, and with congestion but no markedly hemorrhage or effusion in the alveolar spaces of lung tissues.

[31,34], but we do not know whether the RBD-based vaccine can protect animals from SARS-CoV infection.

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. Among five mice vaccinated with RBD-Fc, four mice possessing high titer of neutralizing antibodies exhibited no significant virus replication and histopathological changes in their lungs while only one mouse with low sera neutralizing antibody titer showed marginal level of virus RNA copies, and mild histopathological changes. However, all of the five mice with undetectable neutralizing antibody in the control group presented high level of RNA copies/virus replication, and obvious histopathological changes in their lungs. 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.

In summary, the vaccines containing the RBD of SARS-CoV S protein may induce sufficient neutralizing antibodies and long-term protective immunity against SARS-CoV challenge in the established mouse model. This vaccine candidate may overcome the disadvantages of other vaccine candidates mentioned above, but protect the vaccinated mice from subsequent SARS-CoV challenge. Our results suggest that RBD-Fc vaccine can be further developed as an ideal subunit vaccine for prevention of SARS epidemic.

Acknowledgements

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