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A chimeric multi-epitope DNA vaccine elicited specific antibody response against severe acute respiratory syndrome-associated coronavirus which attenuated the virulence of SARS-CoV *in vitro*

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ABSTRACT

Epitope-based vaccines designed to induce antibody responses specific for severe acute respiratory syndrome-associated coronavirus (SARS-CoV) are being developed as a means for increasing vaccine potency. In this study, we identified four B cell epitopes from the spike (S) and membrane (M) protein through bioinformatics analysis and constructed a multi-epitope DNA vaccine. Intramuscular immunization of mice with this vaccine was sufficient to induce specific prime as well as a long-term memory humoral immune response to at least two candidate epitopes, $S_{437-459}$ and M_{1-20}. A DNA prime–protein boost strategy greatly enhanced the antibody generation and the immune sera not only reacted with the lysates of SARS-CoV-infected Vero cells but also neutralized the cytopathic effect of SARS by 75% at 1:160 dilution. The novel immunogenic S protein peptide revealed in this study provides new target for SARS vaccine design; and our work indicated multi-epitope DNA vaccine as an effective means for eliciting polyvalent humoral immune response against SARS-CoV.

1. Introduction

Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) is the etiologic agent causing SARS, a severe and highly contagious infectious disease spreading worldwide during the year 2003 [1]. The severe morbidity and mortality associated with SARS make it imperative that effective vaccines for the prophylaxis and therapy of the disease be developed and evaluated.

Although lot of efforts have been made to understand the roles of various immune effectors in protective immunity and identifying protective antigens recognized by these effector cells, no conclusive information is available on the immune correlates of protection to SARS. However, the convalescent sera were reported very efficient to neutralize SARS-CoV infection [2], and spike-mediated infection could be inhibited by sera from SARS patients [3], SARS patients derived antibodies to S and M proteins efficiently neutralized SARS-CoV infectivity [4], indicating neutralizing antibodies as one of the important protective immune effectors. The SARS-CoV encodes four major structural proteins, known as nucleocapsid (N) protein, envelope (E) protein, membrane (M) protein and spike (S) protein [5]. M protein plays a crucial role in assembly and budding of virions [6]; and S protein is critical for the viral binding to its cellular receptor, angiotensin-converting enzyme 2(ACE2) through the receptor binding domain (RBD) [7,8]. Hence, identification of neutralization epitopes within S and M sequence may enable the design of epitope-based SARS vaccines [9]. A couple of immunodominant epitopes from S and M protein of SARS-CoV have been identified through online epitope predication software or epitope mapping technologies and used for potential vaccine targets and therapeutic reagents [10–12].

A multiple B cell epitope DNA vaccine strategy is adopted in the present study since epitope-based approach brings vaccine with increased safety, the possibility of rational epitopes engineering, and accurate immune focusing which would contribute to the promotion of the potency and breadth of the specific immune response [13–15]. Multi-epitope-based vaccines designed to induce antibody responses and CTL responses specific for SARS-CoV and other viruses such as HIV-1 have been successfully developed as a means for addressing vaccine potency and viral heterogeneity [16–18].

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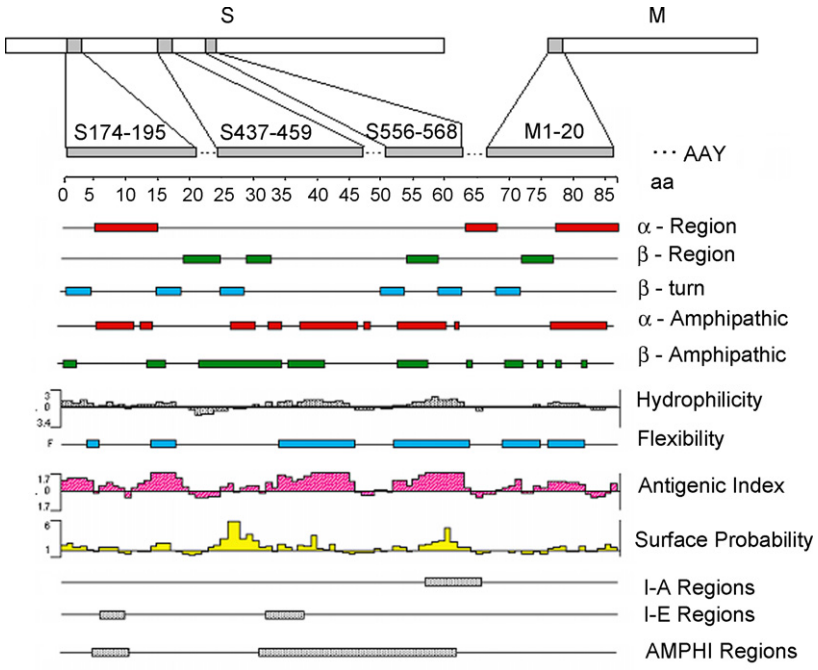

Fig. 1. Schematic diagram of the design of a multi-epitope DNA vaccine. The four selected B cell epitopes from S and M protein ($S_{174-195}$, $S_{437-459}$, $S_{556-568}$ and M_{1-20}) were screened from the SARS-CoV spike protein and M protein and engineered into a DNA vaccine separated one from another with alanine–alanine–tyrosine (AAY) spacers. Evaluation of the hydrophilicity, surface probability, hydrophobicity, antigenic value, flexibility and secondary structure of the multi-epitope chimera protein was performed by DNASTAR software. The synthetic nucleotide sequence was then incorporated into a pVAX1 vector. Codon optimization was employed in constructing this plasmid.

In the present study, we identified four B cell epitopes from the S and M protein of the SARS-CoV and a DNA plasmid vaccine was designed to express the four B cell epitopes as a single Ag and tested for immunogenicity in BALB/c mice. Intramuscular injection of this DNA vaccine induced antibody response against SARS-CoV. The reactivity of the serum antibodies to each of the four epitopes was evaluated. DNA prime–protein boost strategy was utilized to improve the vaccine efficiency. The protective activity of the immune sera was evaluated by *in vitro* neutralization assay.

2. Materials and methods

2.1. Identification of SARS-CoV vaccine candidate B cell epitopes

Since the two identified human coronaviruses (HCoV-OC43 and HCoV-229E) only triggered mild upper respiratory infection [19], the low-homologous motifs of their relative SARS-CoV, designated as S_{1-217}, $S_{425-513}$ and M_{1-20} were first screened by comparing sequence between 40 different Chinese SARS-CoV isolates and 36 various coronaviruses. The SARS-CoV protein sequences in the NCBI GenBank database (Genbank AY274119) representing a Canadian Tor2 isolate were analyzed to identify potential B cell epitopes according to the algorithms concerning the hydrophilicity, surface probability, hydrophobicity, antigenic value, flexibility and secondary structure using DNASTAR software. Four segments designated as $S_{174-195}$, $S_{437-459}$, $S_{556-568}$ and M_{1-20} were selected for construction of multi-epitope DNA vaccine.

2.2. Design and construction of the multi-epitope DNA vaccine

The four selected epitopes from S and M protein were engineered into a DNA vaccine and separated one from another with AAY spacers to enhance appropriate epitope processing (Fig. 1). The multi-epitope gene was constructed using overlapping oligonucleotides in a PCR-based synthesis with the sequence as "*AGATCT***GCC ACC**ATG GAGAAGTCCGGCAACTTTAAGCACTTACGCG-AGTTTGTGTTTAAGAACAAGGACGGCTTTCTGTACGCCGCCTACAACTA-CAAGTACAGGTACCTGAGACACGGCAAGCTGAGGCCCTTTGAGAGAG-ACATCTCCAACGTGCCCGCCGCCTACTCCGACTTCACTGACTCCGTTCG-CGACCCCAAGACCTCCGCCGCCTACATGGCCGACAACGGCACCATCAC-CGTGGAGGAGCTGAAGCAGCTGCTGGAGCAGTGGAACTAATAG *GAATTC*". The construct included a Kozak sequence (bold) at the N-terminus and the gene was optimized for mammalian codon usage according to the previous study [20]. The synthetic nucleotide sequence was then incorporated into expression vector pVAX1 (Invitrogen, USA), pcDNA4-his/myc (Invitrogen, USA) eukaryotic plasmid vector as well as pET-32a (Novagen, Germany) prokaryotic vector. The final plasmids identified as pVAX-epis, pcDNA4-his/myc-epis and pET-32a-epis were purified using Qiagen MegaPrep columns (Valencia, CA) according to manufacturer's instructions and were adjusted to 1 mg/ml in PBS buffer, pH 7.0.

2.3. Prokaryotic expression of the chimeric multi-epitope protein

The pET-32a-epis plasmid was transformed into *Escherichia coli* strain BL21. The expressed chimeric protein with a 6xHis tag was purified by a Ni^{2+} affinity chromatography column according to the manufacturer's instructions (Novagen, Germany) and was eluted with PBS for future analysis.

2.4. Western blot analysis of the Eukaryotic expressing multi-epitope protein

293T cell was transfected with pcDNA4-his/myc-epis using Lipofectamine 2000 reagent. Cell lysates and supernatants were collected 48 h post-transfection. After separation by 10% SDS-PAGE, samples were transferred onto a nitrocellulose membrane by electroblotting. The membrane was incubated with monoclonal mouse

anti-myc antibody (Santa Cruz, USA) at 4 °C overnight then with HRP-conjugated goat anti-mouse IgG (Santa Cruz, USA) at 37 °C for 1 h. After washing the membrane was developed with enhanced chemiluminescence Kit (Piece Corp., USA).

2.5. Immunohistochemical analysis of chimeric multi-epitope protein in vivo

BALB/c mice were intramuscularly injected with 100 μg pcDNA4-his/myc-epis or mock plasmid, 36 h later; quadriceps were surgically removed and made frozen sections. The section was dried at 37 °C for 45 min, fixed with acetone then treated with 0.3% H_2O_2 in methanol to quench endogenous peroxidase. After blocking by normal serum, it was incubated with monoclonal mouse anti-myc tag antibody; biotinylated secondary antibody and ABC reagent were used following the protocol of ABC Kit (R.T.U. VECTASTAIN® UNIVERSAL, Vector Lab) before DAB staining.

2.6. Animals, immunization, and serum collection

BALB/c mice at 6–8-week-age were obtained from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. All animals were housed in the pathogen-free mouse colony and all animal experiments were performed according to the guidelines for the Care and Use of Medical Laboratory animals (Ministry of Health PR China, 1998) and the guidelines of the Laboratory Animal Ethical Commission of Fudan University. Mice were injected with 100 μg pVAX-epis or mock plasmid in 100 μl PBS into the tibialis anterior muscle by two legs on week 0, 3 and 6. Then 20 μg chimeric proteins in 50 μl PBS was administered subcutaneously (s.c.) on week 10 or 18 as a boosting immunization. To optimize the prime–boost strategy, 100 μg pVAX-epis DNA i.m. immunization was followed by three 20 μg chimeric proteins boosting on week 3, 5 and 7. Blood samples were collected by retro-orbital bleeding.

2.7. ELISA measurement of anti-SARS serum IgG

ELISA plates were coated with 10 μg/ml individual synthesized epitope peptide, mixed synthesized peptides or purified prokaryotic expressed SARS-CoV S protein fragment (125–683aa) (Shukang Biotechnology, Shanghai) at 4 °C overnight and blocked with 10% goat serum in PBST (PBS with 0.05% Tween 20) for 1 h. After washing serum sample were added in duplicate (1:100) and incubated at 37 °C for 1 h. After another three times of washing, HRP-coupled goat anti-mouse IgG (Sigma–Aldrich, USA) was added before color development with OPD. The reaction was stopped with 2 M H_2SO_4 and absorption at 490 nm was measured by a microplated reader (BioLab, USA).

2.8. Western blotting analysis of the binding capacity of anti-sera

Anti-sera (week 10) derived from mice receiving DNA priming 1× (week 0) and protein boosting 3× (week 3, 5 and 7) was used for Western blotting analysis and neutralization assay. Inactivated SARS-CoV(strain Z-1 (6) IV, China) lysates were provided by the biosafety level 3 laboratory, Wuhan institute of Biological Products, China and mixed with equal volume of loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). After separation by 12% SDS-polyacrylamide gel electrophoresis (PAGE), the protein band was then transferred onto a nitrocellulose membrane. After blocking with 5% skimmed milk in PBS for 2 h, the membrane was incubated with 1:100 diluted serum overnight at 4 °C and then with an HRP-conjugated goat anti-mouse IgG for 2 h at 37 °C before color development with enhanced chemiluminescence kit (Piece Corp., USA).

2.9. Virus neutralization assay

Virus neutralization assay was performed in the biosafety level 3 laboratory. Vero E6 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum, 100 U/ml of penicillin G per ml, and 100 μg/ml of streptomycin. Viral infection was performed by adding 100 μl medium containing 50 × $TCID_{50}$ of SARS-CoV (strain Z-1 (6) IV, China) or 50 μl serial twofold diluted serum premixed with 50 μl 50 × $TCID_{50}$ of SARS-CoV for 1 h to plate containing 4×10^4 cells per well. Cytopathic effects (CPE) were observed 96 h afterwards by microscopy and confirmed by crystal violet staining. CPE of cells was blindly evaluated by two pathologists on a scale of 0 to 4, in which − represents 100% cell viability; + represents 0–25% cell death or apoptosis; ++ represents 25–50% cell death or apoptosis; +++ represents 50–75% cell death or apoptosis; ++++ represents 75–100% cell death or apoptosis.

2.10. Statistical analysis

Data were presented as means and standard deviation. One-way ANOVA test was used to compare difference of antibody titers between all the groups of mice. Difference between two groups of mice was compared using wilcoxon-matched pairs test. A probability of less than 0.05 was taken as significant. Statistical calculation was performed using the GraphPad Prism (version 4.0) statistical program.

3. Results

3.1. Identification of potential B cell epitopes from S and M protein of SARS-CoV and construction of chimeric multi-epitope DNA vaccine

S and M protein were screened for potential B cell epitopes using epitope prediction software online and four candidate B cell epitopes designated as $S_{174–195}$ (EKSGNFKHLREFVFKNKDGFLY), $S_{437–459}$ (NYKYRYLRHGKLRPFERDISNVP), $S_{556–568}$ (SDFTDSVRDPKTS) and $M_{1–20}$ (MADNGTITVEELKQLLEQWN) were selected for construction of a multi-epitope DNA vaccine (Fig. 1).

3.2. The prokaryotically expressed multi-epitope chimera was immunogenic

In vitro expression of the multi-epitope gene was first demonstrated using pET-32a prokaryotic expression system. The corresponding multi-epitope protein fused with a trxA fragment and a 6xHis tag expressed in BL21 cells was purified by a Ni^{2+} affinity chromatography. It was shown in Fig. 2A that the multi-epitope construct was well expressed *in vitro* with a molecular weight about 28 kDa. The immunogenicity of this prokaryotic expressing protein was proved by s.c. injection of 20 μg chimera protein into mice emulsified with complete Freund's adjuvant (CFA) which resulted in induction of peptide-specific serum IgG 3 weeks post-immunization (Fig. 2B).

3.3. Eukaryotic expression of the multi-epitope chimera gene in vitro and in vivo

The multi-epitope chimera gene was also cloned into a pcDNA4-his/myc eukaryotic expression vector. The *in vitro* transfection

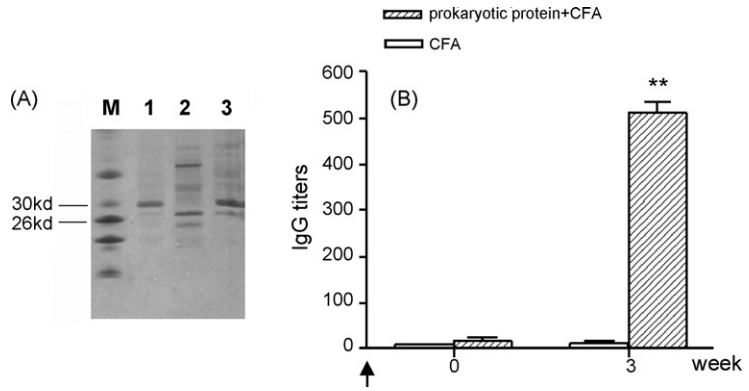

Fig. 2. Prokaryotic expression and the immunogenicity of the chimeric multi-epitope protein. (A) Expression of the chimeric multi-epitope protein with pET-32a system. Purified protein by Ni^{2+} affinity chromatography (lane 1), BL21 lysates without IPTG induction (lane 2) and with 4 h induction (lane 3) were separated by 12% Gel and stained overnight with Coomassie Brilliant Blue G-250. (B) BALB/c mice were s.c. immunized with 20 μg chimera protein emulsified with complete Freund's adjuvant (CFA). 1:50 diluted sera samples were analyzed their reactivity to the 4-epitope mixtures by ELISA assay (*$p < 0.01$ compared with control group).

efficiency was evaluated in 293T cell using Lipofectamine 2000 reagent. Multi-epitope chimera protein expressed in the cell lysates was confirmed by Western blotting (Fig. 3A). To assess the expression and distribution of the chimera gene *in vivo*, tibialis anterior muscles of mice were stained by anti-myc Ab 36 h after 100 μg DNA administration. As shown in Fig. 3B, the eukaryotic encoding multi-epitope gene was well expressed *in vivo*.

3.4. The multi-epitope SARS-CoV DNA vaccine induced epitope-specific prime and memory humoral immune response

To determine the immunogenicity of the multi-epitope SARS-CoV DNA vaccine, BALB/c mice received 3 injections of 100 μg pVAX-epis or mock DNA by 3 weeks intervals. As shown in Fig. 4A, significant increase of IgG level was only observed in multi-epitope DNA immunized mice. The specific serum IgG titer increased with time and reached its peak at week 8 amounting to 512 then declined. A single 20 μg prokaryotic chimera protein boosting on week 10 or 18 quickly restored the humoral response to a higher extent (IgG titer more than 4000) indicating that the multi-epitope DNA vaccine elicited both a prime and memory immune response which lasted at least 3 months. However, priming with a mock DNA followed by protein boosting could only achieve a relatively weak immune response. Reactivity of the anti-sera to the individual epitope peptide showed that $S_{437–459}$ and $M_{1–20}$ peptides were highly immunogenic for the serum antibodies induction while $S_{174–195}$ and $S_{556–568}$ epitopes were relatively weak immunogenic (Fig. 4B). The immune sera could also recognize prokaryotically expressed S and M protein (data not shown).

3.5. DNA prime–protein boost strategy greatly enhanced the antibody response

Various DNA prime–protein boost strategies were investigated to promote the vaccine efficiency. Mice were primed with one DNA/protein injection (week 0) and boosted with three prokaryotic chimera protein s.c. immunization (week 3, 5 and 7). After the last protein boosting, extremely higher level of serum IgG (titer amounted to near 10^6) was only seen in DNA priming/protein boosting immunized mice (Fig. 5, $p < 0.01$) indicating the multi-epitope SARS-CoV DNA vaccine elicited a potent SARS-specific priming antibody response which could be synergistically amplified by the following protein antigen immunization.

3.6. The anti-sera could bind to SARS-CoV S protein and partially neutralize the SARS-CoV infectivity

The week 10 immune sera from mice treated with DNA prime–protein boost 3× (Fig. 5) were first investigated their binding capacity to SARS-CoV proteins by Western blotting assay. The 1:100 diluted serum was shown to bind to the protein in the lysates of viral infected Vero E6 cells with MW of 180 kDa, equivalent to MW of reported S protein (Fig. 6) and to prokaryotic expressed S protein (data not shown), indicating that the anti-sera could recognize the native S proteins expressed by SARS-CoV.

To determine the virus-neutralizing activity of the above immune sera, 50 × $CCID_{50}$ dose of SARS-CoV was incubated with serial diluted serum (from 1:20 to 1:1280) then used to infect VeroE6 cell monolayers. Although the serum could not totally block the viral infection, it was found that 1:160 diluted sera reduced

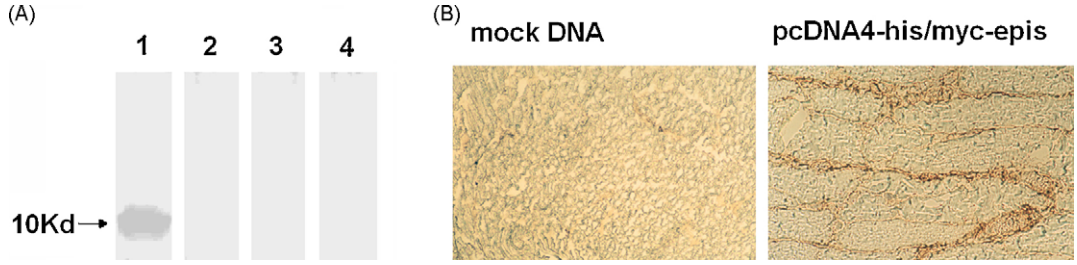

Fig. 3. Eukaryotic expression of the chimeric multi-epitope protein *in vitro* and *in vivo*. (A) Western blotting analysis of the chimeric multi-epitope protein expressed in 293T cells. Presence of the chimeric protein in the cell lysates of 293T cells transfected with pcDNA4-his/myc-epis (1 and 2) or mock plasmid (3 and 4) was determined by an anti-myc monoclonal antibody (1 and 3) or an isoform antibody (2 and 4). (B) Immunohistochemistry analysis of the chimeric multi-epitope protein *in vivo*. Frozen sections prepared from the quadriceps of mice injected with mock plasmid (a) or pcDNA4-his/myc-epis (b) were reacted with an anti-myc monoclonal antibody and then stained with DAB. Magnification: 100×.

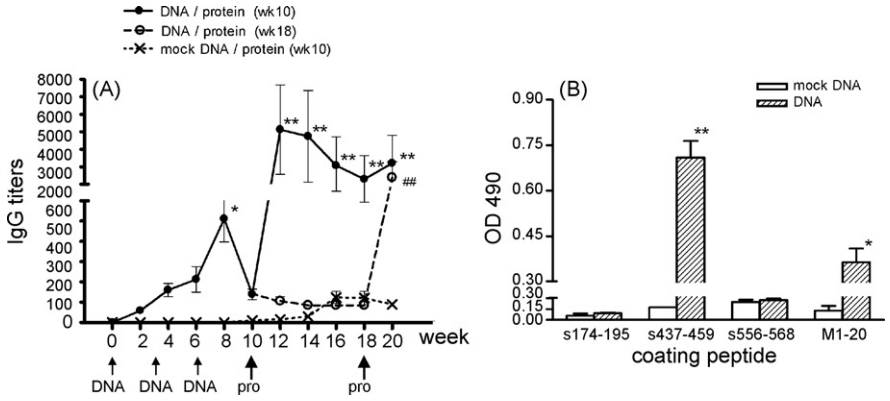

Fig. 4. Humoral immune response induced by the chimeric multi-epitope DNA vaccine. (A) Mice ($n = 12$) were injected with 100 μg pVAX-epis plasmid into the tibialis anterior muscle on week 0, 3 and 6. 20 μg prokaryotic expressed chimeric multi-epitope protein was s.c. injected on week 10 ($n = 6$) or week 18 ($n = 6$) as a boost immunization. Specific IgG level in the 1:100 diluted sera reactive to the mixed epitopes was measured by ELISA assay. Arrows indicate the time points of the 3 DNA injections ($^{**}p < 0.01$ compared with other 2 groups; $^{*}p < 0.05$, $^{\#\#}p < 0.01$ compared with mock DNA priming group). (B) Specific reactivity of the week 8 serum to the four individual predicted epitope peptides was investigated by ELISA assay ($^{*}p < 0.01$).

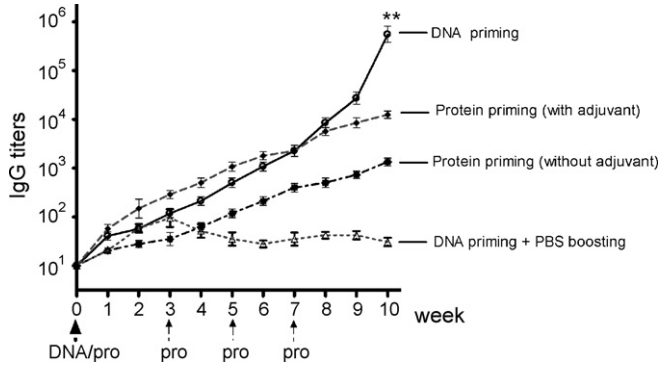

Fig. 5. SARS-CoV–specific humoral immune response was greatly augmented using modified DNA prime–protein boost strategies. Four groups of mice were primed with 100 μg pVAX-epis DNA i.m. immunization or 20 μg chimeric proteins s.c. injection followed by three chimeric protein (20 μg) boosting on week 3, 5 and 7. The specific serum IgG titer was determined by ELISA assay ($^{**}p < 0.01$ compared with protein priming (with adjuvant)).

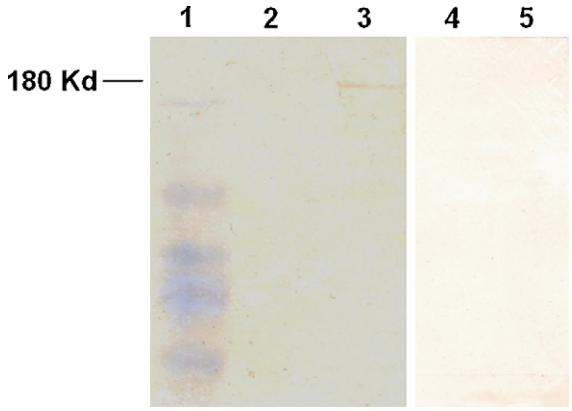

Fig. 6. Western blot analysis of the reactivity of the immune-sera to SARS-CoV lysates. Cell lysates of Vero E6 cells without (lanes 2 and 4) or with SARS-CoV infection (lane 3 and 5) were separated on SDS-PAGE, lane 1 represents the pre-stained marker. Then lane 2 and 3 were hybridized with the anti-sera (week 10) derived from mice receiving DNA priming–protein boosting 3× immunization; while serum from mock DNA-immunized mice were reacted with lane 4 and 5 as negative control.

the CPE percentage of VeroE6 cell from 100 to 25% (Table 1) indicating serum antibodies targeting the predicted spike $_{437–459}$ and $M_{1–20}$ epitope could partially neutralize the infectivity of the virus or attenuate the virulence of the SARS-CoV strain we used.

4. Discussion

Although SARS was eventually contained by the stringent application of infection control measures, high infection rate and inexact animal reservoir of SARS-CoV raise the continuous concern about the viral recurrence. There is an urgent need that an effective and safe vaccine be developed to prevent reemergence and epidemics in the future [21]. Multi-epitope-based vaccine, designed to increase vaccine potency and viral heterogeneity, is an ideal candidate vaccine to control SARS epidemics. Using bioinformatics tools or phage-display peptide library techniques panels of antigenic epitopes could be identified which are easily delivered in the context of DNA or viral vectors for immunization.

The S protein of SARS-CoV comprises major antigenic determinants that induce neutralizing Abs which makes it a major target for vaccine design and immune therapy. Immune response targeted to S as well as M protein was able to neutralize the SARS-CoV infectivity [22]. Taking this into account, three B epitopes from the S protein and one B epitope from the M protein of SARS-CoV were selected and constructed as a multi-epitope chimeric DNA vaccine. Intramuscular injection of DNA vaccine provoked a potent

Table 1
Cytopathic effects (CPE) grade of Vero E6 cells after infection of 50 × $TCID_{50}$ of SARS-CoV (strain Z-1 (6) IV, China) with or without previous neutralization by anti-sera derived from chimeric multi-epitope DNA/protein immunized mice

	Serum dilution fold						
	20	40	80	160	320	640	1280
Medium control	++++	++++	++++	++++	++++	++++	++++
Serum from pVAX-treated mice	++++	++++	++++	++++	++++	++++	++++
Non-specific IgG control	++++	++++	++++	++++	++++	++++	++++
Sera from DNA/protein-treated mice	+	+	+	+	++	+++	++++

priming and a relatively long-term (4.5 month) memory antibody response against SARS protein which would have potential protective effect to prevent SARS-CoV epidemics. However, only 2 out of the 4 predicted epitopes ($S_{437-459}$ and M_{1-20}) were immunologically recognized by immune sera (Fig. 4B). This may due to the phenomenon of B cell immunodominance tending to focus the immune response on relatively few dominant epitopes in an antigen. First, antigen availability regulates the hierarchy of peptide epitopes. The folding of the serially connected poly-epitope protein may have caused steric hindrance of certain epitopes while exposing others to the BCR. Second, competition for cytokines may also influence the level of B cell priming and expansion. It was interesting that although the M_{1-20} epitope was immunogenic and could be recognized by the sera (Fig. 4B); Western blotting failed to detect the predicted band corresponding to M protein in the viral lysates. One possible reason was that the first 20 amino acids of M protein might serve as some signal peptide which may be removed from the viral protein.

Our results demonstrated that the $S_{437-459}$ specific immune sera elicited by DNA prime–protein boost immunization were protective by neutralizing the cytopathic effect of SARS. Consistently, $S_{318-510}$ [23] and $S_{324-503}$ [24] fragments of the S protein were reported critical for binding ACE2 indicating the epitope-rich S1 is an important immunodominant domain of SARS-CoV. A nearby epitope, $S_{447-458}$, was also predicted as a neutralizing B cell epitope [25]. Therefore $S_{437-459}$ epitope identified in this study represents another immunogenic and potentially protective B cell epitope in S protein.

A DNA prime–protein boost immunization strategy was employed in this study to enhance the effect of multi-epitope DNA vaccine. Prime–boost vaccination strategies synergistically amplify specific immunity; meanwhile selecting T or B cells having greater avidity and increasing the numbers of memory cells specific for a shared antigen in the prime and boost vaccines [26]. As seen in Fig. 5, the IgG titer induced by DNA prime–protein boost strategy mounted to 10^6 at 8–10 weeks post immunization, much higher than that induced by protein/protein vaccination. The contributing mechanism might be the more efficient stimulation of antigen-specific memory B cells by DNA priming immunization. Maintenance of memory B cell population might be independent of antigen persistence, but memory B cell must be stimulated by antigen to differentiate into short-lived antibody-secreting plasma cells. Therefore, for vaccines to induce long-term protective antibody titers, repeatedly provide or continuously maintenance with antigen in minimal quantities over a prolonged time period is desperately needed for sufficient numbers of memory B cells to mature to plasma cells [27]. DNA transgene expression persists much longer than the cognate protein due to its intrinsic expression property. Exogenous protein degradates *in vivo* quickly with time; while the plasmid DNA persists and stably expresses the antigen at least for 3 months at both RNA and protein level [28]; and the long-term stability of plasmid DNA in muscle even lasts for 19 months [29]. In this study, the expression of DNA-encoding gene could be detected at least for 6 weeks in the muscle (data not shown) which explained the best memory antibody response induced by DNA priming after 8 weeks. $CD4^+$T cells help and cytokines are also required for stimulation of memory B cells. The potent adjuvant properties of CpG nucleotide sequences in DNA vectors is very efficient to stimulate APCs to upregulate the expression of cytokines and co-stimulatory molecules needed for expansion of $CD4^+$T, B and memory B cells. Therefore, DNA priming strategy was more effective than the protein priming to stimulate functional long-term memory B cell response. This is consistent with lot of recent reports demonstrating the efficacy of DNA prime–protein boost protocols to augment antibody levels [30,31].

Taken together, a multi-epitope DNA vaccine capable of stimulating long-term humoral immune response for controlling SARS-CoV infection was designed, showing two epitopes, $S_{437-459}$ and $M_{1-20,}$ are critically important for the generation of immunity with potential protective effect. A heterologous DNA prime–protein boost immunization was demonstrated its effectiveness to elevate the antibody responses.

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