

# Augmentation of immune responses to SARS coronavirus by a combination of DNA and whole killed virus vaccines

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## Abstract

We studied the immunogenicity of a DNA SARS-vaccine, a whole killed virus, or a whole killed and DNA vaccine combination. The DNA vaccine contained a plasmid encoding the SARS coronavirus (SARS-CoV) S protein under the control of the human CMV promoter and intron A. The whole killed virus vaccine was comprised of SARS-CoV, propagated in Vero-E6 cells, with subsequent  $\beta$ -propylactone inactivation and formulated with aluminum hydroxide adjuvant. Mice immunized twice with the DNA vaccine and once with the whole killed virus elicited higher antibody responses than mice immunized three times with the DNA vaccine or once with the whole killed virus vaccine. Mice immunized twice with the whole killed virus vaccine elicited higher antibody responses than mice immunized three times with the DNA vaccine or once with the whole killed virus vaccine. However, a combination of the vaccines induced T-helper type 1 (Th1) immune responses while the whole killed virus vaccine induced T helper type 2 (Th2) immune response. These results demonstrate that combination of the DNA vaccine and the whole killed virus vaccine can be used to enhance the magnitude and change the bias of the immune responses to SARS-CoV. © 2005 Elsevier Ltd. All rights reserved.

**Keywords:** SARS coronavirus; DNA vaccine

## 1. Introduction

Severe acute respiratory syndrome (SARS) is the latest in a series of emerging infectious diseases. This acute and often severe respiratory illness emerged in Southern China in late 2002 and subsequently spread to other countries early the following year. The SARS epidemic was contained at 8098 cases with 774 deaths. In addition to the human misery, there was enormous economic damage caused by this agent [1].

The causative agent of SARS was identified as a new type of coronavirus, the SARS coronavirus (SARS-CoV). The SARS-CoV is an enveloped virus with a positive single-stranded RNA genome 29,727 kb in length. Consistent with other coronaviruses, the genome encodes an RNA-dependent

RNA polymerase and other replication associated proteins at its 5' end and viral structural proteins (the S, E, M, N proteins) and several putative uncharacterized proteins at its 3' end [2,3].

Recent studies indicate that the SARS-CoV spike protein (S) is expressed as a non-cleaved glycoprotein with an apparent mass of ~180 kDa [4]. Based on sequence comparison, the SARS-CoV S is predicted to be a class I fusion protein [2,3]. The angiotensin-converting enzyme 2 (ACE2) has been reported to function as a receptor for SARS-CoV [5], and amino acids 270–510 of S protein are required for interaction with the receptor [6] suggesting that this protein would be an ideal target for a vaccine.

Prior experience in infectious disease control suggests that vaccination will be one of the most effective measures to prevent future SARS outbreaks. To develop a vaccine, the SARS-CoV S gene has been expressed in different vector systems [7–10] and the findings to date have identified S

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as the only significant protective antigen among SARS-CoV structural proteins [7].

Previously, Yang and colleagues [9] have described that a DNA plasmid, expressing S, induced SARS-CoV neutralization and protective immunity in mice. Here, we describe a prime-boost combination of a DNA vaccine with a whole killed SARS-CoV vaccine. This vaccine combination was found to be more immunogenic in mice as compared to DNA vaccine or whole killed virus vaccine alone.

## 2. Materials and methods

### 2.1. DNA vaccine

The S gene cDNA of the SARS-Tor-2 strain was kindly provided by Dr. Rachel Roper, University of Victoria. The C-terminus of the spike gene was fused with a V5 14 amino acid sequence tag to facilitate subsequent detection of gene expression. The spike gene was first cloned into vector pBKSII(+) with restriction enzymes *NheI* and *XbaI*. This step of cloning allowed us to insert the spike gene into the DNA vaccine vector pMASIA using the *BamHI* site and the resultant construct was designated pLL70 (Fig. 1A). Plasmid constructs were characterized by restriction digestions and purified with EndoFree Plasmid Maxi kit (Qiagen).

### 2.2. Whole killed virus vaccine

Stock SARS-CoV Tor-2 was obtained from Dr. Booth, National Microbiology Laboratory, Winnipeg, Manitoba. The virus was passaged three times on Vero-E6 cells, obtained from the same source and used as stock for all experiments.

The preparation was titrated by plaque assay and found to contain  $4 \times 10^7$  pfu/mL.

Confluent monolayers of Vero-E6 cells, grown in 5% fetal bovine serum (FBS), were infected with stock SARS-CoV at a multiplicity of 0.1 pfu/cell. The infected cell lysates were harvested at 48 h post infection at which time the yield of virus was shown to be maximal. The cell fraction was collected by centrifugation, resuspended in Tris-buffered saline pH 7.8, subjected to three freeze–thaw cycles, re-centrifuged and the supernatant fraction pooled with the medium fraction and loaded on a 20% solution of sorbitol in Tris buffered saline and ultra-centrifuged in a SW 32 rotor at 28,000 rpm for 2 h at 4 °C. The pellets were resuspended in Tris-buffered saline and pooled to a final volume of 1.5 mL from each 150 mL of the original culture supernatant.

The purified virus preparations were inactivated by the addition of a 10  $\mu$ L of a 10% beta-propiolactone solution in 0.1 M Tris pH 7.8 to each 0.9 mL of virus to which 0.1 mL of MEM had been added as a pH indicator. The preparations were kept at 4 °C and mixed on an hourly basis for the first 8 h, with 0.1 M NaOH being added if the pH was becoming too acidic. After 24 h, the preparation was placed in a 37 °C water bath, the indicator color was monitored and pH adjusted every 5 min.

### 2.3. Mouse immunizations

Two independently performed immunizations studies (experiments 1 and 2) were conducted in 6- to 8-week-old female BALB/c mice. The immunization groups (five mice per group) and experimental design for each study are summarized in Table 1. All immunizations were given subcutaneously and the experiments were finished on day 63 (experiment 1) and on day 40 (experiment 2).

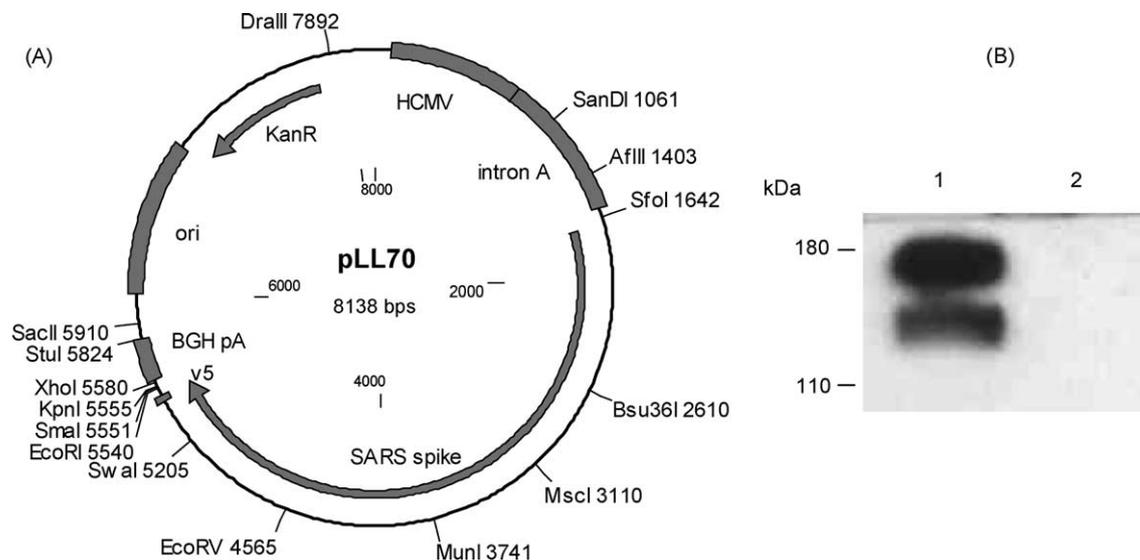


Fig. 1. (A) Schematic diagram of the plasmid pLL70. (B) Western blot analysis of SARS-CoV S protein. Proteins from pLL70 transfected 293 cells (lane 1) and transfected with empty vector DNA (lane 2) were separated by SDS-PAGE (7.5% gel) under reducing conditions and transferred to a membrane. Since the S protein was linked to a V5 tag, the separated proteins were probed using anti-V5 monoclonal antibodies. Sizes of molecular weight marker bands are shown on the left.

Table 1  
Immunizations schedule

Group	Priming immunization and schedule	Boost immunization and schedule
Experiment 1		
DNA	pLL70 DNA, 50 µg, days 0 and 21	pLL70 DNA, 50 µg, day 50
DNA + WKV	pLL70 DNA, 50 µg, days 0 and 21	Whole killed SARS-CoV/Alum, 15 µg, day 50
WKV (1)	Whole killed SARS-CoV/Alum, 15 µg, day 35	
Control-1	pMASIA DNA, 50 µg, days 0 and 21	pMASIA DNA, 50 µg, day 50
Experiment 2		
WKV (2)	Whole killed SARS-CoV/Alum, 15 µg, day 0	Whole killed SARS-CoV/Alum, 15 µg, day 28
Control-2	PBS, day 0	PBS, day 28

#### 2.4. Western blot

To confirm the expression of the spike gene *in vitro*, 293 cells were transfected with 1 µg of plasmid pLL70 expressing the spike gene or empty vector pMASIA together with FuGene 6 reagent (Roche). Cells were lysed with 0.5% NP-40, 50 mM HEPES buffer pH7.8, and protease inhibitor cocktail (Roche). Cell lysates were cleared by centrifugation before the protein concentration was determined by a Bradford assay. For Western blot, 20 µg of protein were subjected to electrophoresis on SDS-7.5% polyacrylamide gel and transferred onto PVDF membrane (Amersham). The membrane was probed with anti-V5 monoclonal antibody (Invitrogen) and HRP-conjugated secondary antibody. Specific proteins on the membrane were visualized by ECL-plus reagent (Amersham).

#### 2.5. SARS-CoV S-specific ELISA

Antibodies titers in serum from immunized mice were measured by ELISA. Ninety-six-well plates were coated overnight with 0.1 mL/well of 1 µg/mL purified recombinant spike protein. The plates were washed five times in phosphate buffer saline (PBS) containing 0.05% Tween-20. Sera serially diluted with PBS containing 0.5% gelatin and 0.05% Tween-20 were added to respective wells. After a 2-h incubation, the plates were washed, and 1/10000 diluted biotinylated goat anti-mouse antibodies (Caltag Laboratories) were added. After a 1-h incubation, the plates were washed, and a 1/5000 diluted alkaline phosphatase conjugated streptavidin (Jackson ImmunoResearch) was added. After a 1-h incubation, the plates were washed eight times, developed with 4-nitrophenylphosphate (Sigma), stopped with 1% HCl, and analyzed at 405 nm using an ELISA plate reader.

#### 2.6. Neutralization test

Sera were tested for antibody to SARS-CoV in a standard virus neutralization test. Each serum was heated at 56 °C for 30 min and duplicate serial 2-fold dilutions from 1:10 to 1:2560 were each incubated with 100 pfu of SARS-CoV Tor-2 for 2 h, then added to monolayers of Vero-E6 cells. Cultures were examined after 72 h for characteristic CPE. The dilution below the one at which CPE was first noted was

deemed the antibody titre. For each assay, reference serum from a designated SARS convalescent patient was used as a positive control and serum from a subject not infected with SARS as negative control.

#### 2.7. SARS-CoS-specific ELISPOT

Cellular immune responses to SARS-CoV were assessed by IFN-γ and IL-4 ELISPOT assay using murine splenocytes. Unifilter 96-well plates coated overnight with 0.1 mL/well of 1.25 µg/mL rat anti-mouse IFN-γ (or IL-4) (BD PharMingen) were washed once with RPMI 1640 (Life Technologies) containing 10% FBS, and incubated in triplicate with 10<sup>5</sup> splenocytes/well in a 0.1 mL RPMI 1640 media with 10% FBS containing 30 µg/mL synthetic peptides. Sequences of the four unique synthetic peptides were derived from SARS-CoV S protein (AZ1, APNYTQHTSSMRGVYYPDE-IFRSDT; AZ2, TGNVNYKYRYLRHGKLRPFER; AZ3, LTPSSKRFQPFQFGRDVSDFDTSVRDPK; AZ4, LQP-ELDSFKEELDKYFKNHT).

After 48 h of incubation at 37 °C in a CO<sub>2</sub> incubator, the plates were washed five times in PBS containing 0.05% Tween-20, and incubated overnight at 4 °C with biotinylated rat anti-mouse INF-γ (or IL-4) antibody (BD PharMingen, 0.1 mL/well, 1.25 µg/mL). After washing with PBS containing 0.05% Tween-20, the plates were incubated for 1.5 h with a 1/500 dilution of streptavidin-alkaline phosphatase (Jackson ImmunoResearch). After eight washes with water, the plates were developed with SIGMA FAST 5-brom-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Development was stopped by washing with tap water, and plates were air-dried and read with the aid of a microscope.

### 3. Results

#### 3.1. *In vitro* expression of SARS-CoV S protein by plasmid vector

We evaluate the protein expression by the DNA vaccine vector in 293 cells at 24 h post-transfection by Western blot analysis using antibody against the V5 epitope. The S protein was detected in cell lysates as a doublet with an estimated upper band molecular weight 180 kDa

when the lysate was boiled and analyzed under reducing SDS-PAGE conditions (Fig. 1B, lane 1). This experiment indicated that full-length SARS-CoV S protein was expressed in mammalian cells in two differently glycosylated forms.

### 3.2. Characterization of the whole killed virus vaccine

After centrifugation, aliquots of the resuspended pellets were examined by electron microscopy for evidence of spike protein on the virus, subjected to SDS-PAGE and Western blot with a SARS patient convalescent serum and tested for infectivity by TCID<sub>50</sub> on Vero-E6 cell monolayers in 96-well microtitre plates. Acceptable lots of purified virus had titres of  $>10^9$  TCID<sub>50</sub>/mL, displayed prominent bands corresponding to 180 and 50 kDa on SDS-PAGE and Western blot and had an abundance of virus with well defined spike proteins evident by EM (Fig. 2). After inactivation procedure, aliquots were tested for infectivity on Vero-E6 cell cultures, SDS-PAGE and Western blot. Acceptable preparations displayed prominent bands corresponding to 180 and 50 kDa on SDS-PAGE and Western blot and had no evidence of infectivity (data not shown).

### 3.3. SARS-CoV S-specific antibody immune responses in vaccinated mice

In the first experiment, two groups of BALB/c mice were primed with a DNA vaccine and boosted with DNA or a whole killed SARS-CoV vaccine; the third group of mice was immunized with the whole killed SARS-CoV vaccine without the DNA priming. In the second experiment, BALB/c mice

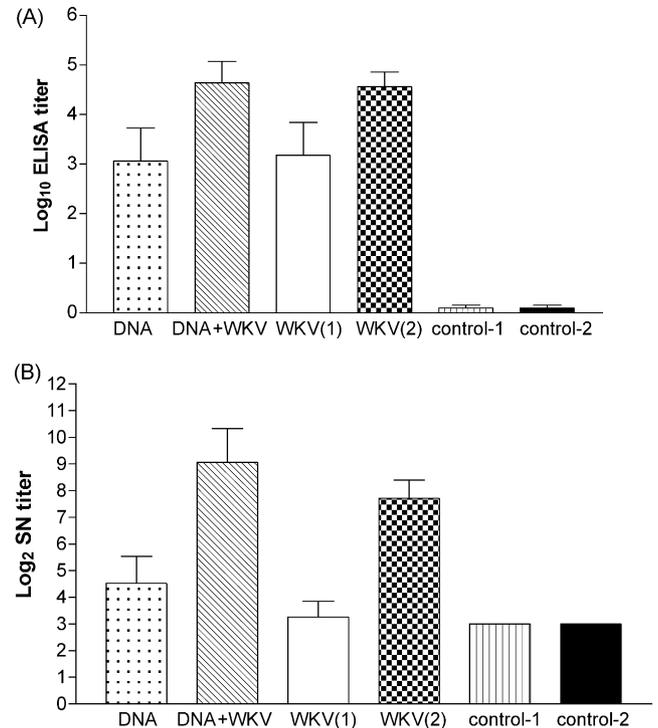


Fig. 3. Humoral immune responses in the immunized BALB/c mice. (A) SARS-CoV S-specific total IgG titers in sera. (B) SARS-CoV neutralization antibody titers in sera. Error bars represent the standard deviation of the mean of five mice per group.

were immunized twice at a four-week interval with a whole killed SARS-CoV vaccine. Following immunizations, the humoral immune responses were assessed in sera by ELISA and in vitro virus neutralization.

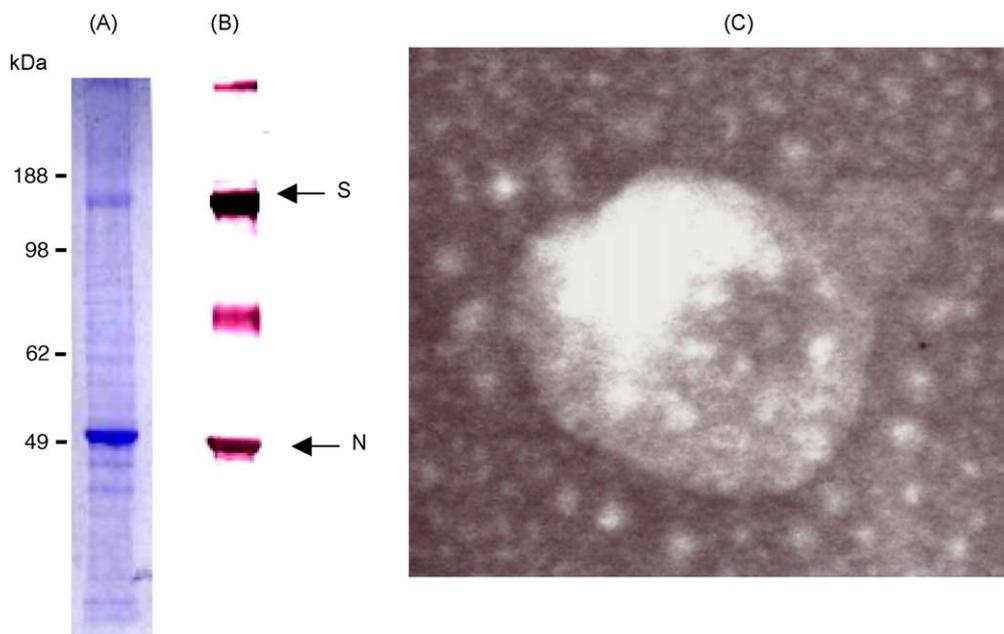


Fig. 2. Analysis of whole killed SARS-CoV virus vaccine. (A) Gel electrophoresis of the proteins from whole killed SARS-CoV vaccine. The proteins were separated by SDS-PAGE (7.5% gel) under reducing conditions and stained by Coomassie blue. (B) Western blot analysis using a SARS patient convalescent serum. (C) EM of the negatively stained SARS-CoV purified from the infected cell lysates.

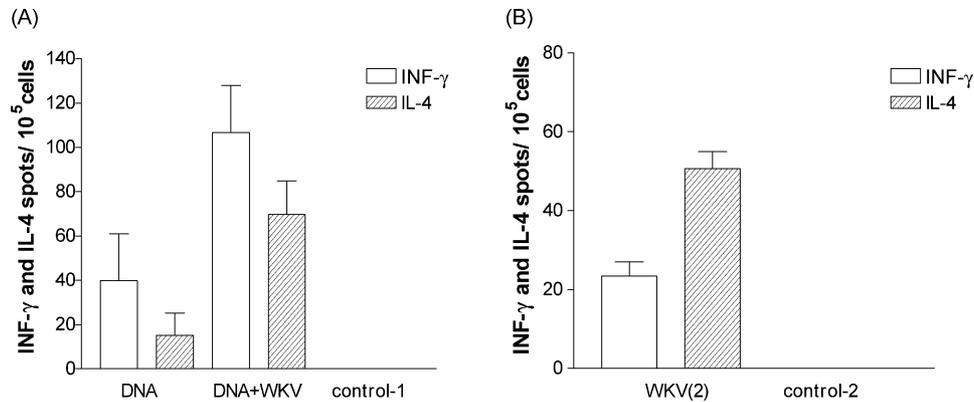


Fig. 4. SARS-CoV S protein-specific INF- $\gamma$  and IL-4 ELISPOT. (A) Data of the experiment 1. (B) Data of the experiment 2. Experimental design for each study is presented in Table 1. The results represent the average of triplicate wells and are expressed as the means and standard errors.

A 10 to 100-fold increase in antibody titer to SARS-CoV S protein was found in mice having received a combination of the vaccines (Fig. 3A). Statistical analysis using these individual titers indicated that the mean titers obtained by a combination of the vaccines were significantly higher than that obtained by immunization only with DNA vaccine or one immunization with whole killed virus vaccine ( $P < 0.05$ , by the Student's  $t$ -test).

The mice immunized with the vaccine combination elicited significantly ( $P < 0.05$ ) higher neutralizing antibody titers (Fig. 3B) than the mice immunized only with DNA vaccine or once with whole killed virus vaccine. These results indicate that immunization with the vaccines combination is more powerful in generating humoral immune responses as compared to responses induced by the DNA vaccine. As for comparison with the whole killed virus vaccine, the vaccine combination is superior to one injection of the whole killed virus vaccine but it elicited the same responses when compared with two injections of the whole killed virus vaccine (Fig. 3).

#### 3.4. SARS-CoV S-specific cellular immune responses

To determine the presence of S-specific cellular immune responses, splenocytes were isolated from vaccinated and control mice and antigen-specific responses were measured by INF- $\gamma$  and IL-4 ELISPOT. As shown on Fig. 4A, the mice immunized with a combination of the vaccines developed significantly higher number of S-specific INF- $\gamma$  and IL-4 spots as compared to the mice having received the DNA vaccine alone, while the control mice did not develop any ELISPOT response. We found more INF- $\gamma$  than IL-4 producing cells in splenocytes of the immunized mice. This result suggests that both vaccine regimens generated Th1-type immune response, since INF- $\gamma$  is secreted by Th1-type CD4<sup>+</sup> and CD8<sup>+</sup> cells. In contrast, two injections of the whole killed vaccine induced more IL-4 producing cells than INF- $\gamma$  producing cells (Fig. 4B) which is an indication of Th2-type immune response.

#### 4. Discussion

The first global SARS outbreak has been controlled. However, the origin of the virus remains obscure. Therefore, vigilance must be maintained and appropriate control measures must be available for implementation in the event that it reoccurs. Previous experience in controlling viral diseases suggests that a vaccine may be one of the most effective measures to prevent SARS. Recent studies identified the S protein as the only one which induced significant SARS-CoV neutralization [7]. The SARS-CoV S gene has been expressed in different vector systems [8,10,11] with an ultimate goal to develop a SARS-CoV vaccine. In two reports [9,12] plasmid DNA expressing SARS-CoV S gene fragments was used to develop a DNA vaccine. These authors demonstrated neutralizing antibodies production [12] and protective anti-SARS-CoV immunity [9] in mice. In another study, Tang and colleagues [13] developed an inactivated SARS-CoV vaccine prepared from the whole virus, and demonstrated high level of neutralizing antibodies in mice after immunization with the vaccine.

In the present study, we compare DNA and whole killed SARS-CoV vaccines alone and in combination. Our DNA vaccine vector, pLL70, contained the full-length SARS-CoV S gene under the control of a powerful human CMV promoter and intron A. As we showed previously, the human CMV promoter and intron A improves in vitro gene expression [14]. The sequence of the S protein suggests that it contains an N-terminal signal sequence and C-terminal hydrophobic membrane-anchoring domain. We used full-length S protein because it had been shown that the S protein, anchored on the cell surface, generated a better immune response than secreted versions of the protein [9]. Our findings suggest that the S protein is expressed in 293 transfected cells as a single, uncleaved polypeptide, but in two differentially glycosylated forms, which is in agreement with recently published data [15].

Our results clearly demonstrate that a combination of the vaccines is more immunogenic in mice than the DNA

vaccine alone. Higher antibody responses (as compared to DNA vaccine and the whole killed virus vaccine alone) as well as higher cell-mediated responses (as compared to DNA vaccine alone) were elicited. Although the vaccination strategy consisting of priming with a DNA vaccine followed by boosting with a protein vaccine has been described before for other viruses [16–20], our studies suggest that this strategy is useful for vaccination against SARS-CoV as well.

A combination of the vaccines and the DNA vaccine induced Th1-dominated immune response, while two injections of the whole killed vaccine induced Th2-biased response (Fig. 4). It has been shown previously, that aluminum adjuvants skewed the immune response towards a Th2 response and a DNA vaccine enhanced T-cell immune responses [17,21]. Immunity associated with a Th1-type immune response is thought to be essential for the control intracellular pathogens; therefore, changing the bias of the immune response may be an attractive feature of a vaccine combination strategy.

Understanding the immunity to SARS-CoV is vital to the development of an effective vaccine. Taking into consideration the clinical evidence that the incubation period of SARS is short (5 days to 2 weeks) and that most patients appear to recover within a short time with no persistent or latent infection, it is reasonable to conclude that neutralizing antibody may play an important role in preventing SARS-CoV infection. It has also been shown in laboratory animals that neutralizing antibodies play the most important role in prevention of the viral replication [9,22], and protection can also be achieved by the administration of S-specific monoclonal antibodies [23]. However, the contribution of T-cell immunity cannot be excluded. It was reported that an apparent depletion of T cells occurred in the early SARS-CoV infection of patients, and a gradual increase to normal level was observed as the patients recovered [24]. Moreover, T-cell epitopes in the SARS-CoV S protein elicited specific T-cell immune responses in patients recovered from SARS [25]. Therefore, generation of both humoral and cellular immune responses would be beneficial for vaccine against SARS.

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