Induction of Specific Immune Responses by Severe Acute Respiratory Syndrome Coronavirus Spike DNA Vaccine with or without Interleukin-2 Immunization Using Different Vaccination Routes in Mice[▽]

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DNA vaccines induce humoral and cellular immune responses in animal models and humans. To analyze the immunogenicity of the severe acute respiratory syndrome (SARS) coronavirus (CoV), SARS-CoV, spike DNA vaccine and the immunoregulatory activity of interleukin-2 (IL-2), DNA vaccine plasmids pcDNA-S and pcDNA–IL-2 were constructed and inoculated into BALB/c mice with or without pcDNA–IL-2 by using three different immunization routes (the intramuscular route, electroporation, or the oral route with live attenuated *Salmonella enterica* serovar Typhimurium). The cellular and humoral immune responses were assessed by enzyme-linked immunosorbent assays, lymphocyte proliferation assays, enzyme-linked immunospot assays, and fluorescence-activated cell sorter analyses. The results showed that specific humoral and cellular immunities could be induced in mice by inoculating them with SARS-CoV spike DNA vaccine alone or by coinoculation with IL-2-expressing plasmids. In addition, the immune response levels in the coinoculation groups were significantly higher than those in groups receiving the spike DNA vaccine alone. The comparison between the three vaccination routes indicated that oral vaccination evoked a vigorous T-cell response and a weak response predominantly with subclass immunoglobulin G2a (IgG2a) antibody. However, intramuscular immunization evoked a vigorous antibody response and a weak T-cell response, and vaccination by electroporation evoked a vigorous response with a predominant subclass IgG1 antibody response and a moderate T-cell response. Our findings show that the spike DNA vaccine has good immunogenicity and can induce specific humoral and cellular immunities in BALB/c mice, while IL-2 plays an immunoadjuvant role and enhances the humoral and cellular immune responses. Different vaccination routes also evoke distinct immune responses. This study provides basic information for the design of DNA vaccines against SARS-CoV.

An international outbreak of severe acute respiratory syndrome (SARS), an atypical form of pneumonia, caused about 8,000 cases and 774 deaths across more than 30 countries, starting from its emergence in mid-November 2002 until 7 August 2003. The etiology of SARS was identified as a new coronavirus (CoV), named SARS-CoV (13, 22, 29, 37). The SARS-CoV genome is approximately 29,000 bp and encodes four main structure proteins, including the spike (S) protein, the membrane (M) protein, the envelope (E) protein, and the nucleocapsid (N) protein (9, 22, 37). The S glycoprotein is responsible for binding to receptors on host cells and plays an important role in membrane fusion (6, 21, 30, 32, 33). Moreover, antibodies to this protein not only neutralize the virus in vitro but also protect against lethal SARS-CoV challenge (21, 38, 46). It has previously been shown that pseudotype lentiviral particles bearing the SARS-CoV S protein could be inhibited by sera from SARS patients (21). In addition, the CD8 T-cell epitopes identified in the SARS-CoV S protein have been shown to elicit a T-cell response in SARS-CoV-infected pa-

tients (39). Hence, the S protein is an attractive target for both therapeutics and vaccine development. Several SARS-CoV S-protein-based vaccines have been shown to generate antibodies and cellular immune responses (25, 42, 44). Therefore, our study focused on the SARS-CoV S protein as a target antigen for the development of a DNA vaccine.

DNA vaccines induce both cellular and humoral immune responses to produce long-lasting immunity against infectious diseases (31). However, the low immunogenicity of DNA-based vaccines could compromise the application of such vaccines (18, 28). In recent years, many efforts have been made to enhance the immune responses elicited by DNA vaccines, including through the coexpression of cytokines, the use of heterologous prime-boost regimens, and the use of the conventional route of delivery of DNA vaccines.

Plasmid cytokine adjuvants can be used to augment DNA vaccine-elicited humoral and cellular immune responses in animal models (2, 3, 7, 8). Interleukin-2 (IL-2) is a potent cytokine that can activate multiple compartments of the immune system. Several studies have reported that the immune responses to DNA vaccines can be dramatically enhanced by coadministration of plasmids encoding the IL-2 gene. Examples of this phenomenon have been reported for DNA vaccines against bovine herpes virus type 1 (26), hepatitis C virus (18),

hepatitis B virus (7, 8), bovine viral diarrhea virus (28), human immunodeficiency virus (27), foot-and-mouth disease virus (41), and measles virus (31). The IL-2 gene has not been used as a cytokine adjuvant in SARS-CoV DNA vaccines, and therefore, its immune-modulating effects on the SARS-CoV S-protein DNA vaccine were investigated in the current study.

In addition to DNA adjuvants, the vaccination route is another important factor that influences the efficiency of immunization. A number of methods have been developed to increase the efficiency of plasmid delivery (15). Apart from the conventional intramuscular (i.m.) route of immunization, recent experiments have demonstrated that electroporation can greatly enhance vaccination with plasmids and is associated with increased levels of gene expression. Additionally, electroporation displays an adjuvant quality that increases gene expression (48). Another convenient DNA vaccine delivery system is oral vaccination with live attenuated *Salmonella enterica* serovar Typhimurium (12, 35). The use of attenuated strains of *Salmonella* as vehicles for the delivery plasmid DNA in vivo is an effective method for the induction of strong cell-mediated and humoral immune responses at mucosal sites (1, 4, 10, 16, 19).

Thus, we were particularly interested in immunization by electroporation and immunization by the oral route with live attenuated *Salmonella enterica* serovar Typhimurium in vivo. Few studies have directly compared the immune responses generated by the use of different routes of vaccination for SARS-CoV DNA immunization. Therefore, in this study, the immunogenicities of vaccinations with SARS-CoV S-protein DNA with or without an IL-2-expressing vector delivered by the i.m. route, electroporation, and the oral route with live attenuated *S. enterica* serovar Typhimurium were compared in a mouse model.

MATERIALS AND METHODS

Plasmids and bacterial strains. pcDNA3.1(+) (Invitrogen, Carlsbad, CA) was used as the vector for all DNA vaccines. The full-length S-protein gene of SARS-CoV was amplified from pFastBacDULL-S (constructed by the State Key Laboratory of Virology, Wuhan Institute of Virology). The mouse IL-2 gene was kindly provided by Cui Bo-an (Henan Agricultural University, Zhengzhou, People's Republic of China). The S-protein and IL-2 genes were subcloned into pcDNA3.1 to construct recombinant plasmids pcDNA-S and pcDNA-IL-2, respectively. The accuracies of the constructs were confirmed by restriction digestion and sequencing. DNA plasmids were purified with MegaPrep columns (QIAGEN), dissolved in endotoxin-free phosphate-buffered saline (PBS) to a final concentration of 2 $\mu g/\mu l$, and stored at $-20°C$.

The attenuated *S. enterica* serovar Typhimurium strain CS022 (ATCC 14028; *phopc*) was kindly provided by Guo Ai-zhen (Huazhong Agricultural University, School of Animal Medicine, Wuhan, People's Republic of China) and was used as a carrier for oral genetic immunization.

Animals and immunization. Six- to 8-week-old female BALB/c mice were purchased from the Center of Experimental Animal of Hubei Medical College and were randomly divided into groups (eight animals per group). The animals were provided with pathogen-free water and food. The mice were immunized three times at 2-week intervals by the i.m. route, electroporation, or the oral route with *S. enterica* serovar Typhimurium. The immunization schedule is summarized in Table 1.

The immunization dose was 200 μg plasmid per animal and was injected into the quadriceps muscles. For immunization by electroporation, the animals were anesthetized and injected with 30 μg plasmid pcDNA-S i.m. in the rear thigh. Two-needle-array electrodes were inserted into the muscles immediately after the injection of DNA by electroporation, and the array was inserted longitudinally relative to the direction of the muscle fibers. In vivo electroporation was performed with a model 820 square-wave generator (BTX, San Diego, CA), and the parameters were as follows: 20-V/mm distance between the electrodes, 50-ms pulse length, and six pulses with a reversal of polarity after three pulses. For oral

TABLE 1. Immunization schedule for the different forms of vaccines

Group	Vaccine	Route of administration	Dose per mouse
1	pcDNA-S	i.m.	200 μg
2	pcDNA-S + pcDNA-IL-2	i.m.	200 + 50 μg
3	pcDNA-S	i.e.[a]	30 μg
4	pcDNA-S + pcDNA-IL-2	i.e.	30 + 8 μg
5	pcDNA-S	Oral[b]	5×10^9 CFU
6	pcDNA-S + pcDNA-IL-2	Oral	5×10^9 CFU + 1×10^9 CFU
7	pcDNA3.1	i.m.	200 μg
8	pcDNA-IL-2	i.m.	200 μg
9	PBS	i.m.	100 μl
10	pcDNA3.1	Oral	5×10^9 CFU
11	CS022	Oral	5×10^9 CFU

[a] i.e., electroporation.
[b] Oral immunization with attenuated *S. enterica* serovar Typhimurium CS022.

immunization, attenuated *S. enterica* serovar Typhimurium strain CS022 cells harboring the pcDNA-S or the pcDNA-IL-2 DNA vaccine were cultured and grown until they reached an optical density at 600 nm (OD_{600}) of 1.0. The cells were harvested by centrifugation and resuspended at the highest required density in PBS (Sigma). The inoculum of *S. enterica* serovar Typhimurium was diluted to the appropriate concentration with 0.1 ml 10% sodium bicarbonate buffer. Each mouse was immunized by oral gavage with 5×10^9 CFU *S. enterica* serovar Typhimurium transformed with pcDNA-S.

All of the groups containing pcDNA-IL-2 were immunized as follows: 50 μg pcDNA-IL-2 per mouse by the i.m. route, 8 μg by electroporation, and 1×10^9 CFU by oral immunization. The DNA dosage and the numbers of CFU of the attenuated *S. enterica* serovar Typhimurium strain used to treat the mice were optimized by a series of preliminary experiments.

Analysis of humoral immune response. Anti-SARS-CoV antibody levels in serum were assessed by enzyme-linked immunosorbent assay (ELISA). Chemically killed SARS-CoV was purified and used as the detection antigen. Optimized concentrations (5 $\mu g/ml$) of antigens were coated onto 96-well plates (Costar) overnight at 4°C. The plates were washed and blocked with 1% bovine serum albumin buffered solution for 1 h at 37°C prior to a 2-h incubation with mouse serum diluted 1:100 at 37°C. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG; Sigma). The color was developed by adding *para*-nitrophenylphosphate substrate, and the A_{405} was read with a plate reader (Bio-Rad). The values obtained for sera from the mice in the experimental groups were considered positive when they were ≥ 2.1 times the value for the control group. Values of <0.05 were not included.

A similar ELISA protocol was followed to assess S-protein-specific IgG and its subclasses (IgG1 and IgG2a). In this case, recombinant S protein expressed in *Escherichia coli* was purified and was used as the detection antigen. Horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, and IgG2a (Sigma) were used as secondary antibodies. The OD_{490} (A_{490}) was read.

LPA. The antigen-specific lymphocyte proliferation assay (LPA) was performed as described previously (17). In brief, 10 days following the final injection, the mice were killed and single-cell suspensions were prepared from the spleens for each group. Splenocytes (2×10^5 per well) in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum were seeded in 96-well plates in triplicate. The cultures were stimulated under the following various conditions for 60 h at 37°C and 5% CO_2: 5 $\mu g/ml$ concanavalin A (ConA; positive control), 5 $\mu g/ml$ purified S-protein antigen (specific antigen), 5 $\mu g/ml$ bovine serum albumin (irrelevant antigen), or medium alone (negative control). The CellTiter 96 Aqueous One solution reagent (20 μl; Promega) was added to each well, according to the manufacturer's protocols. Following a 4-h incubation at 37°C, the A_{490} was read. Proliferative activity was estimated by using the stimulation index (SI), which was calculated from the mean OD_{490} for the antigen-containing wells divided by the mean OD_{490} for wells without the antigen.

SARS-CoV S-protein-specific ELISPOTs. The cellular immune responses to SARS-CoV were assessed by gamma interferon (IFN-γ) and IL-4 enzyme-linked immunospot assays (ELISPOTs) with mouse splenocytes. The assays were performed according to the instruction manual (U-CyTech, The Netherlands). Ninety-six-well plates were coated with 5 $\mu g/ml$ rat anti-mouse IFN-γ or IL-4 (100 μl/well) in PBS overnight. The plates were then washed three times with PBS containing 0.25% Tween 20 and blocked with PBS containing 5% fetal bovine serum for 2 h at 37°C. After three additional washes with PBS containing 0.25% Tween 20, 1×10^5 splenocytes in 100 μl reaction buffer containing 2 $\mu g/ml$ purified S protein were added to each well. The plates were incubated for 16 h
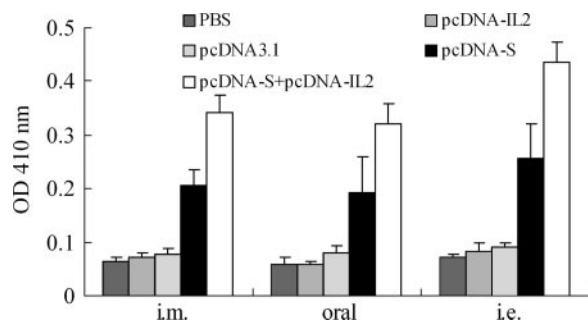
FIG. 1. Antibody responses to SARS-CoV induced by vaccinations with or without pcDNA–IL-2 by different immunization routes in mice. Serum samples (eight per group) were taken 10 days after the final immunization. Data are presented as means ± SDs. Values of ≥2.1 were considered positive by taking into account the absolute ratio of the responses in postimmunization serum/naïve serum.
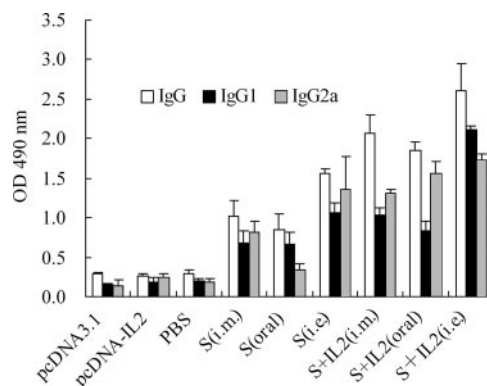

FIG. 2. Detection of SARS-CoV S-protein-specific IgG and other subclasses in vaccinated mice. Mouse sera (eight per group) were collected 10 days after the final immunization and were assayed for IgG1 and IgG2a antibodies against the S protein of SARS-CoV. Data are presented as means ± SDs.

at 37°C in 5% CO_2 and then washed 10 times with PBS. Biotinylated anti-mouse IFN-γ or IL-4 monoclonal antibody at a 1:500 dilution was subsequently added, and plates were incubated for 2 h at room temperature. After the plates were washed, streptavidin-biotinylated horseradish peroxidase was added and the plates were incubated for an additional hour at room temperature. Following five washes with PBS, individual plates containing IFN-γ or IL-4 were developed to obtain dark spots after a 10-min reaction with the peroxidase substrate 3-amino-9-ethylcarbazole. The reactions were stopped by rinsing the plates with demineralized water. The plates were air dried at room temperature, and the absorbance was read by using an ELISPOT reader (Hitech Instruments). The number of spot-forming cells per 10^6 splenocytes was calculated. The medium backgrounds consistently had <10 spot-forming cells per 10^6 splenocytes.

Determinations of $CD4^+$ and $CD8^+$ cells in peripheral blood mononuclear cells. Flow cytometry was used to monitor the expression of T-cell surface markers. Direct immunofluorescence was performed by standard techniques, as described elsewhere (5). $CD4^+$ and $CD8^+$ cells were assessed 10 days after the final boost with each vaccine. Lymphocytes were isolated from peripheral blood mononuclear cells and stained with the following monoclonal antibodies: fluorescein isothiocyanate-labeled anti-mouse CD3 antibody, phycoerythrin–Cy-5-labeled anti-mouse CD4 antibody, phycoerythrin-labeled anti-mouse CD8 antibody (BD PharMingen), or the corresponding isotype controls. One hundred thousand cells were acquired on a FACSCalibur flow cytometer, and the data were analyzed with WinMDI software (Becton Dickinson, Lincoln Park, NJ).

Statistical analysis. All data are presented as means for the immunized mice in each group ± standard deviations (SDs). SPSS 13.0 software for Windows was used for statistical analysis. Differences in the humoral and the cellular immune responses between groups were assessed by using single-factor analysis of variance. The least-significant-difference *t* test was used for between-group comparisons. *P* values of <0.05 were considered statistically significant.

RESULTS

Antibody responses to SARS-CoV. In the present study, plasmid pcDNA-S or pcDNA-S plus pcDNA–IL-2 was used to immunize mice by the i.m. route, electroporation, or the oral route with attenuated *S. enterica* serovar Typhimurium. To examine the humoral responses elicited by these vaccines, the levels of anti-SARS-CoV antibodies in the immunized mice were determined by ELISA. Sera collected 10 days after the final boost with each vaccine were assayed, and the results are shown in Fig. 1. In all groups, immunization induced significantly higher levels of anti-SARS-CoV IgG compared to those in the control groups immunized with pcDNA3.1, pcDNA–IL-2, or PBS (*P* < 0.01). Immunization with pcDNA-S by electroporation induced the highest level of antibody among those achieved by the three routes of vaccination, while immunization by the oral route evoked the lowest level of antibody

among those achieved by the three routes of vaccination. The antibody levels in the groups to which pcDNA and IL-2 were coadministered were significantly higher than those in the groups that received pcDNA-S alone (*P* < 0.05). For the groups immunized with pcDNA-S plus pcDNA–IL-2, the antibody responses induced by i.m. injection were comparable to those induced by immunization by the oral route (*P* > 0.05) but were significantly lower than those induced by immunization by electroporation (*P* < 0.01).

S-protein-specific antibodies and antibody subclasses. Ten days after the final immunization, the levels of S-protein-specific antibodies in mouse sera were determined by ELISA (Fig. 2). All vaccinated mice developed substantial antibody responses, whereas the animals in the control groups did not show any detectable S-protein-specific antibody response. The tendency for the mice in the immunized groups to achieve an S-protein-specific IgG antibody response was consistent with that for the mice to achieve an anti-SARS-CoV antibody response (Fig. 1).

IgG1 and IgG2a levels were measured to determine the humoral immune response profiles. As shown in Fig. 2, the anti-SARS-CoV IgG subtype profile revealed that both IgG1 and IgG2a were induced by all immunization regimens. The antibody levels in the group vaccinated with the combination vaccine showed greater increases than those in the groups vaccinated with pcDNA-S, although the antibody subclass profiles were similar in both groups. The antibodies produced in the animals vaccinated i.m. were mainly of the IgG2a subclass. The level of the IgG1 subclass that was induced in the group vaccinated by the mucosal (oral) route was appreciably higher than the level of the IgG2a subclass that was induced. Of interest, in the groups immunized by electroporation, IgG2a was predominant when the mice were immunized with pcDNA-S alone, but IgG1 was the major subclass in the animals vaccinated with pcDNA–IL-2 plus pcDNA-S. In most cases, coadministration of pcDNA–IL-2 and pcDNA-S mainly enhanced the antibody response, but it did not change the antibody subclass types.

S-protein-specific T-cell proliferation. The activation and the proliferation of lymphocytes play a critical role in both the
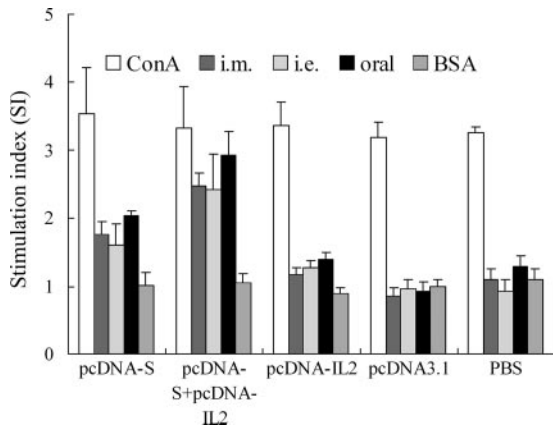
FIG. 3. S-protein-specific LPA. Pooled splenocytes were obtained from mice (three mice per group) immunized with the DNA vaccine on day 10 postimmunization with pcDNA–IL-2. Splenocytes were stimulated in vitro with S protein (test groups), ConA (positive controls), and bovine serum albumin (irrelevant antigen controls). Splenocytes from the control groups (immunized with pcDNA–IL-2, pcDNA3.1, or PBS) were stimulated with the S protein and served as negative controls and sham controls. The SI was calculated by use of the following formula: (mean OD of ConA- or antigen-stimulated proliferation)/(mean OD of nonstimulated proliferation). Each bar represents the mean SI $\pm$ SD for three mice.

humoral and the cellular immune responses induced by vaccination. Therefore, we next evaluated whether vaccination with pcDNA-S in the presence or the absence of immunization with pcDNA–IL-2 by the different routes could influence antigen-specific T-cell proliferation. As shown in Fig. 3, higher levels of lymphocytes stimulated by the S protein were observed in mice immunized with pcDNA-S alone or with pcDNA-S plus pcDNA–IL-2 than in the controls ($P < 0.01$). The level of cell proliferation in animals coimmunized with pcDNA–IL-2 was appreciably higher than that in animals immunized with pcDNA-S alone ($P < 0.05$). The level of splenocyte proliferation in response to oral immunization with pcDNA-S or pcDNA-S plus pcDNA–IL-2 was consistently and significantly higher than that in response to immunization by the other two routes ($P < 0.05$). Immunization by the i.m. route induced a stronger T-cell response than immunization by electroporation. These results indicate that immunization with pcDNA-S and pcDNA-S plus pcDNA–IL-2 elicits recognizable levels of

antigen-specific T-cell responses and that immunization by the oral routes evokes the strongest immune response.

S-protein-specific Th1- and Th2-type responses. ELISPOT was used to assess the magnitudes of the S-protein-specific IFN-γ (Th1) and IL-4 (Th2) T-cell responses after the mice were vaccinated with pcDNA-S DNA or pcDNA-S plus pcDNA–IL-2. The protein dosage used for stimulation and the number of splenocytes were optimized to induce IFN-γ and IL-4 T-cell responses (data not shown). Splenocytes from vaccinated mice were harvested 10 days after the final vaccination, and S-protein-specific IFN-γ and IL-4 levels were enumerated by ELISPOTs. As shown in Fig. 4A and B, only small numbers of nonspecific IFN-γ- and IL-4-secreting cells were detected in the control groups by ELISPOTs (≤ 10 spots/10^6 cells). By ELISPOT, the background counts in wells containing splenocytes in the absence of mitogens or nominal antigens were about the same as those for the control groups. Significant numbers of S-protein-specific IFN-γ and IL-4 spots were detected for all the immunized groups by ELISPOTs ($P < 0.01$). Compared to immunization with pcDNA-S alone, a two- or threefold increase in antigen-specific IFN-γ-secreting cell numbers was detected in mice coimmunized with pcDNA and IL-2 by all three immunization routes ($P < 0.05$). However, the IL-4-secreting cell number was not as high as the IFN-γ-secreting cell number in the groups to which pcDNA and IL-2 were coadministered and was appreciably higher only than that in the group immunized with pcDNA-S alone.

Immunization of the mice by the oral route, which induced the lowest IgG level and appreciably more of the IgG1 isotype, elicited significantly higher levels of IFN-γ and IL-4 than immunization by the i.m. route ($P < 0.01$) or electroporation ($P < 0.05$). Moreover, in all groups, IFN-γ was induced to a much higher level than IL-4. These results suggest that this vaccine formulation is more immunogenic and likely induces a stronger Th1 bias.

CD8$^+$- and CD4$^+$-lymphocyte responses. Since activated CD4$^+$ and CD8$^+$ T lymphocytes are among the most crucial components of antiviral effectors, the responses to these lymphocytes were assessed in the vaccinated mice (Fig. 5). Flow cytometric analysis of unstimulated cells was used to standardize the background responses, and there was little variation in the responses among nonimmunized mice. The vaccinations with pcDNA-S and pcDNA-S plus pcDNA–IL-2 significantly increased the percentages of activated CD4$^+$ and CD8$^+$ cells
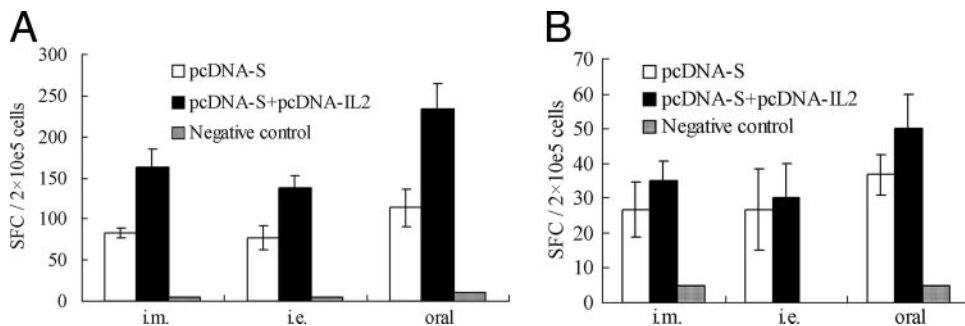

FIG. 4. SARS-CoV S protein-specific IFN-γ (A) and IL-4 (B) ELISPOT results. The numbers of IFN-γ-secreting cells in the spleens of mice harvested 10 days after the final immunization and stimulated in vitro with the S protein are shown. The results represent the averages for triplicate wells for three mice and are expressed as means $\pm$ SDs.
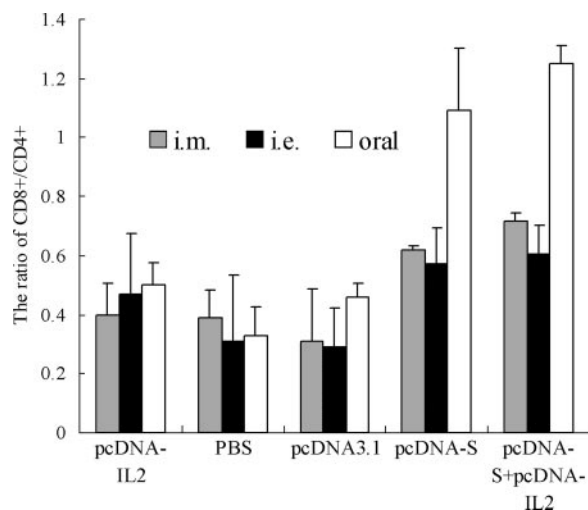
FIG. 5. Analysis of CD8$^+$ and CD4$^+$ lymphocytes by flow cytometry. Peripheral blood mononuclear cells were isolated from vaccinated mice ($n = 5$) 10 days after the final immunization. CD4$^+$ and CD8$^+$ T cells from healthy and immunized BALB/c mice were counted. Values are expressed as means of the ratio of CD8$^+$/CD4$^+$ $\pm$ SDs.

compared to the percentages for the control groups ($P < 0.01$). The numbers of activated CD4$^+$ and CD8$^+$ cells increased in all immunized groups. The CD8$^+$/CD4$^+$ ratio in the groups immunized with pcDNA-S plus pcDNA–IL-2 was higher than that in the groups immunized with pcDNA-S for all three immunization routes, but the difference was not statistically significant ($P > 0.05$). Moreover, immunization by the oral route induced a substantial CD8$^+$ response, while immunization by electroporation induced a negligible CD8$^+$ response. This result paralleled the Ig response of the group immunized by electroporation, which induced the highest level of CD4$^+$ and the lowest level of CD8$^+$. These results further reinforce the fact that the S-protein DNA vaccine can elicit a T-cell response in mice by use of the three immunization routes and that IL-2 expression can enhance the T-lymphocyte activity induced by the S-protein DNA vaccine.

DISCUSSION

In the present study, a new DNA vaccination approach was investigated by coadministering plasmid pcDNA-S with a pcDNA–IL-2 plasmid encoding murine IL-2 cDNA by the use of three immunization routes. We clearly showed that vaccination with SARS-CoV S-protein DNA elicited SARS-CoV S-protein-specific humoral and cellular immune responses and that these responses were significantly enhanced by the coadministration of an IL-2-expressing vector. The animal trial also showed that the administration of these DNA vaccine candidates by different immunization routes could induce a qualitatively different immune response profile. Our findings provide basic information for the design of DNA vaccines targeting other antigenic proteins of SARS-CoV.

From our results on the anti-SARS-CoV and anti-S-protein-specific antibodies and T-cell proliferation, activated CD4$^+$ and CD8$^+$ cells were shown to be successfully evoked after S-protein DNA vaccination. We also found that antigen-spe-

cific T cells were capable of secreting high levels of the Th1 cytokine IFN-γ and moderate levels of the Th2 cytokine IL-4 upon in vitro stimulation with the SARS-CoV S protein. These results indicate that the S-protein DNA vaccine activates both the Th1 and the Th2 subsets, and the level of activation of the Th1 subset was much higher, which correlated with the tendency for the IgG2a antibody levels to be elevated. These findings suggest that the S-protein DNA vaccine is effective in activating both B and T cells to generate anti-S-protein antibodies and cellular immune (mainly Th1) responses in mice. Our results are consistent with those observed in other SARS-CoV DNA vaccine studies (22, 25, 42, 44, 45).

Several studies have indicated that the codelivery of vectors encoding cytokines, such as IL-2, IL-12, IFN-γ, or granulocyte-macrophage colony-stimulating factor, is able to direct the nature of the resulting immune response by augmenting the efficacy of DNA vaccines (18, 24, 28, 36). IL-2 is a cytokine that can potently activate multiple compartments of the immune system, including T-helper cells, cytotoxic T cells, B cells, macrophages, and NK cells (14, 23, 40). Some evidence has suggested that the coadministration of plasmids encoding IL-2 and a given antigen results in the enhancement of both humoral and cell-mediated immune responses to that antigen and mostly favors Th1 cell differentiation (14, 34, 40, 41). In the current study, the immune responses in mice immunized with the SARS-CoV S-protein DNA vaccine alone were compared to those in mice coimmunized with a plasmid encoding IL-2 by analyzing antibodies, T-cell proliferation, T-helper-cell responses, and CD8$^+$ T-cell responses. The mice which received pcDNA–IL-2 adjuvant rapidly generated IgG antibodies (data not shown), and the antibody levels were much higher than those in mice given the antigen-encoding plasmid alone. Both IgG1 and IgG2a antibody levels increased in all groups immunized with the combination, and the dominant isotype did not change by coinjection of the IL-2-expressing vector. A similar pattern was observed in the T-cell immune responses measured by LPA, ELISPOT, and fluorescence-activated cell sorter analysis. Our results showed that immunization with pcDNA-S plus pcDNA–IL-2 elicited recognizably higher levels of T-cell responses compared to those in the groups immunized with pcDNA-S alone. Taken together, our results provide evidence that strategies that include IL-2 as an adjuvant can be used to enhance the protective immunity of candidate SARS-CoV vaccines. These results are consistent with previous findings obtained with animal models of chronic viral infection, which showed that the administration of IL-2 enhances viral antigen-specific Th1 immune responses and improves clinical outcomes (2, 8, 23, 41).

It has been documented that the route and the method of immunization are important modulators of vaccination with DNA vaccines. A DNA vaccine can be delivered by the i.m. route through a needle, by the intradermal route, by the subcutaneous route with a gene gun, by electroporation, or by the oral route with live attenuated bacteria. In general, immunization by injection induces both humoral and cellular immune responses. Recently, it has been found that the application of an electric field to tissues in vivo significantly increases the levels of DNA uptake and gene expression (48). However, oral immunization by the use of *Salmonella* vaccines which bear foreign antigens can induce strong protective immune re-

sponses against a wide variety of infectious diseases in animal models (10, 11, 16, 43), and such oral vaccines can influence the immune profiles by augmenting the mucosal and cellular immunities compared to those achieved by administration by injection. For the SARS-CoV vaccine, it was previously described that immunization with *Salmonella*-carrying S-protein peptides induced protective antibodies (42). Therefore, we investigated whether different vaccination routes could change the immune types, especially when an IL-2 gene adjuvant is combined with the SARS-CoV S DNA vaccine.

To facilitate DNA vaccine delivery in a mouse model, we chose three different immunization routes, the i.m. route, electroporation, and the oral route by the use of live attenuated *S. enterica* serovar Typhimurium. The vaccine with or without pcDNA–IL-2 described here was administered by these immunization routes. After the evaluation and a comparison study of the efficiencies of these immunization schedules, our results showed that immunization by the i.m. route induced a moderate T-cell response and an antibody response, predominantly comprising an IgG2a response, and that these responses were better than those obtained by oral immunization. Vaccination by electroporation resulted in the highest antibody response among the three routes of immunization and a midlevel cellular response. In contrast, when the same DNA vaccine was delivered orally with live attenuated *Salmonella*, vigorous T-cell responses but weak antibody production dominated by the IgG2a subclass was evoked. IgG1 subclass antibody responses were evoked by immunization by all three routes, further suggesting that the processed antigens were recognized by Th2 cells in association with IL-4. In addition, for all three vaccination routes enhanced immune responses were observed in groups coimmunized with IL-2 DNA, including increased antigen-specific proliferative responses, higher levels of IFN-γ production, and increased CD8$^+$ T-cell numbers. These results suggest that those distinct types of immune responses generated were due to the different immunization routes but not to coimmunization with IL-2 DNA. On the basis of our observation that vaccination with DNA by the oral route induced a strong T-cell-mediated immune response, whereas immunization by the i.m. route and electroporation induced moderate T-cell responses but vigorous antibody responses, further studies should be carried out by using a combination of both oral and injection immunizations to stimulate higher cellular and humoral immune responses. In addition, our results also show that the delivery of DNA vaccines by electroporation and orally by using live attenuated *Salmonella* in vivo is an effective method for increasing the level of antigen expression in muscle tissues, leading to marked improvements in immune responses.

It should be noted that delivery of the same DNA by different routes induced different immune response profiles. This might be due to the different ways of presentation of the S-protein antigen by professional antigen-presenting cells when it is delivered by the i.m. route, electroporation, or the oral route. Live attenuated *S. enterica* serovar Typhimurium could selectively infect M cells. If the DNA encoding the S protein was selectively carried into mucosa-associated lymphoid tissue cells, termed M cells, most of the S peptides within the M cells might be cleaved and presented through the major histocompatibility complex (MHC) class I pathway, giving rise to a strong cellular response. On the other hand, only a very small amount of the S-protein peptides generated in the M cells might be secreted and presented by B cells through the MHC class II pathway, resulting in a poor antibody response. In our study, the MHC class I and class II pathways were simultaneously evoked to some extent by both the i.m. and the electroporation immunization routes. This is supported by the findings from a study of the hepatitis B virus DNA vaccine presented by live attenuated *S. enterica* serovar Typhimurium (43). Similar results were also reported by Zheng et al. (47), who showed that live oral vaccination of mice with *S. enterica* serovar Typhimurium delivering DNA-HBsAg (oral DNA vaccine) evoked a vigorous T-cell response and a weak antibody response predominantly of the IgG2a subclass. Thus, different formulations of the same plasmid DNA vaccine can induce distinct immune responses.

Apart from the different ways of antigen presentation by antigen-presenting cells, the reason why the different routes of vaccine administration evoked distinct immune responses to the SARS-CoV S protein may be the different amount of DNA used for the different routes, although the dose of DNA required to stimulate immunity was optimized for the different immunization routes. Further refinements of the immunization conditions, especially the DNA immune dose, are required to ensure the maximal expansion of SARS-CoV S-protein DNA vaccine effectors.

DNA immunization has been well modeled in mice for the assessment of the optimal parameters for immunization and the types of immune responses produced. DNA vaccines also hold promise for use in humans. However, the effects in mice may be more dramatic than those in humans, and the current technologies have significant limitations that prevent the full effectiveness of DNA vaccines in larger animals. Many aspects still remain to be considered prior to the development of a DNA vaccine against SARS-CoV in humans. In the present report, we have demonstrated that the SARS-CoV S-protein DNA vaccine coadministered with an IL-2-expressing plasmid induces specific immune responses in mice. However, we did not run tests with any other animal models; so it is still unknown whether this approach could be applied to other animal models, and its immunogenicity in humans remains to be established. Therefore, it is very important to evaluate the efficacy of this SARS-CoV DNA vaccine in some highly relevant translational models to demonstrate the responsiveness of humans, and further studies should be conducted to validate whether this type of vaccination can be extended to humans.

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