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Immunization of Mice with a DNA Vaccine Based on Severe Acute Respiratory Syndrome Coronavirus Spike Protein Fragment 1

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ABSTRACT

According to data in GenBank, a gene encoding SARS spike protein fragment 1 (S1) was synthesized. After recombination with an immunostimulatory sequence (ISS), the gene was cloned into the plasmid pIRES to produce pIRES-ISS-S1. On confirmation of the expression of S1 protein by indirect immunofluorescence assay (IFA), after the transfection of pIRES-ISS-S1 into BHK-21 cells, the DNA vaccine was repeatedly administrated to BALB/c mice. $CD4^+$ and $CD8^+$ spleen T lymphocytes were analyzed by flow cytometry (FCM) to evaluate T cell-mediated immune responses, the antigen-specific responses of T cells were evaluated by cytotoxic T lymphocyte (CTL) assay, and the level of IgG in antisera from immunized mice was determined by enzyme-linked immunosorbent assay. Results showed that the counts of spleen $CD4^+$ and $CD8^+$ T lymphocytes were increased, that the T cell-mediated immune responses showed antigen specificity, and that IgG was significantly induced with DNA vaccines pIRES-ISS-S1 and pIRES-S1 at titers of 1:320 and 1:160, respectively. These results are promising for the protective immunization of humans.

INTRODUCTION

Severe acute respiratory syndrome (SARS) was first reported in late 2002 in China's Guangdong Province. Since then, it has become a worldwide threat to people's health, and the disease has spread to more than 30 countries and regions. The SARS virus, a novel Coronavirus (SARS-CoV), is composed of a single-stranded, positive-sense RNA of approximately 29 kb. Its genome contains five major open reading frames encoding the replicase polyprotein and the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, as in other coronaviruses (15,16). The S protein, as a type I membrane glycoprotein, is the largest structural protein and the main component of the characteristic spikes on the exterior surface of coronaviruses. The S protein of SARS-CoV is composed of a knob unit (S1) and a stem unit (S2), referring to the N-terminal and C-terminal glycopolypeptide fragments, respectively, from the cleavage of precursor S protein by special proteases (5,7,12). The S1 unit of the spike protein is located on the outer surface of the virion and is believed to function by binding to the exterior receptor on host cells. It has been identified as a host protective antigen and used as a candidate vaccine against coronaviruses (4).

Although the spread of the virus was eventually controlled in 2003, it may still be in circulation in the animal reservoir and there is a possibility that it may return to human beings at any time (17). Thus, it is essential for us to develop a safe and effective SARS-CoV vaccine to

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prevent the possible reemergence of the SARS epidemic in humans. A DNA vaccine is considered a prospective vaccine candidate for SARS-CoV because it may induce neutralization antibodies and cellular immune responses to SARS-CoV (20,22). In this study, a DNA vaccine encoding SARS-CoV S protein fragment 1 (S1) was constructed by inserting a recombinant S1 gene and an immunostimulatory sequence (ISS). The addition of an ISS may enhance immune responses. Mice were immunized with the recombinant DNA vaccine, and the immune effects were then analyzed.

MATERIALS AND METHODS

Plasmid construction

According to the SARS-CoV genomic sequence reported in GenBank (accession number NC_004718), the gene encoding S1 protein (2260 bp) and the designed ISS (195 bp), which included an extended Kozak sequence, a PADRE helper T (Th) cell epitope sequence, and a tetanus toxin (TT) Th epitope sequence, were synthesized by chemical methods, and the fusion gene, by incorporating the ISS into the 5′ terminus of the S1 protein-encoding gene, was constructed and cloned into the pKS(−) plasmid (Biotechs, Dalian, China). The fusion gene was cut with *Bam*HI and *Xba*I from the plasmid pKS(−) and inserted into the eukaryotic expression plasmid pIRES (Clontech, Palo Alto, CA) to generate pIRES-ISS-S1.

Transfection

Five hundred thousand BHK-21 cells were seeded onto coverslips in six-well plates and incubated at 37°C in a CO_2 incubator until the cells were 50–80% confluent. The next day, 10 μg of plasmid DNA in 100 μl of reduced-serum minimal essential medium (MEM) was mixed with 6 μl of Lipofectamine reagent (Invitrogen, Carlsbad, CA) in 100 μl of reduced-serum MEM. The mixture was incubated at room temperature for at least 30 min before it was added to 800 μL of reduced-serum MEM, and then the total mixture was added to the cells. After the cells had been incubated for 5 h at 37°C in a humidified incubator, 1 mL of medium containing 5% fetal calf serum was added to each well.

Confirmation of the expression of S1 protein in BHK-21 cells by indirect immunofluorescence assay

Expression of the S1 protein in BHK-21 cells was confirmed by indirect immunofluorescence assay (IFA). Two days after transfection the medium was aspirated, and the transfected cells were washed three times with 1× phosphate-buffered saline (PBS) and then fixed with a mixture of methanol and acetone. The cells were immersed in 1% bovine serum albumin (BSA) blocking buffer for 2 h to block nonspecific binding, and anti-SARS positive sera from recovered SARS patients were incubated with the cells for 1 h at room temperature. After washing the cells with 1× PBS three times, each for 5 min, 100 μl of fluorescein-conjugated anti-human IgG (Invitrogen) diluted in 0.025% Evans blue in PBS was added per coverslip and incubated in the dark at room temperature for 2 h. After washing the cells with 1× PBS three times, each for 5 min again, the cells were examined with a fluorescence microscope (Olympus, Tokyo, Japan).

Screening of target cells

BALB/c mouse mastocytoma cells (P815) were screened with ampicillin (Amp, 500 μg/mL) after transfection with recombinant plasmid pIRES-S1. When most of the control group cells were dead, the cells transfected with plasmid pIRES-S1 were incubated in RPMI 1640 medium including Amp (200 μg/mL) for 2 weeks. Expression of S1 protein on the surface of P815 cells was also confirmed by IFA. The cells expressing S1 protein were used as target cells in the cytotoxic T lymphocyte (CTL) assay.

Immunization of mice with DNA vaccine

Six-week-old female BALB/c mice were randomly divided into three groups of 10 mice. Each mouse was immunized with 200 μg of plasmid pIRES-ISS-S1, pIRES-S1, or pIRES via the quadriceps. The mice were boosted in the same way at week 3 and week 5 after the first immunization. Serum samples were collected by tail bleeding on days 10, 21, 35, 45, and 55 after the primary immunization. Preimmune serum and antiserum were prepared from blood samples and stored at −20°C. Ten days after the last immunization, five mice from each group were killed, blood samples were collected from the ophthalmic venous plexus, and spleen cells were cultured in 10% fetal calf serum (FCS)–RPMI 1640.

Flow cytometry

$CD4^+$ and $CD8^+$ spleen T lymphocytes were analyzed by flow cytometry. The cells were treated with fluorescence-activated cell-sorting (FACS) lysing solution and FACS permeabilizing solution (BD Biosciences, San Jose, CA). The permeabilized cells were subsequently stained with various combinations of phycoerythrin (PE)-labeled goat anti-mouse antibodies to CD4 and CD8. Aliquots of 1×10^6 cells were resuspended in 50 μL of PBS and phenotyped according to standard protocols. Flow cytometric analysis was conducted with a FACSCalibur (BD Biosciences), acquiring 10,000 events.

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1   GAA TTC GGA TCC GCC GCC ACC ATG GGT ATG CAG GTG CAG ATT CAG AGC CTG   51
1    EcoRI   BamH1      Korak      M   G   M   Q   V   Q   I   Q   S   L   10

52  TTT CTG TTG TTG CTG TGG GTT CCA GGT TCT CGT GGT GCT AAA TTT GTT GCT  102
11   F   L   L   L   L   W   V   P   G   S   R   G   A   K   F   V   A   27
103 GCC TGG ACC CTG AAA GCT GCC GCT GGT GGC GGT CAG TAT ATT AAA GCT AAT  153
28   A   T   T   L   K   A   A   A   G   G   G   Q   Y   I   K   A   N   44
154 AGC AAA TTT ATT GGT ATT ACC GAA CTG GCT GCC GCT GAT ATC              195
45   S   K   F   I   G   I   T   E   L   A   A   A   D   I              58
                                                   EcoRV
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FIG. 1. Immunostimulatory sequence (ISS).

FACS data were analyzed with SPSS software (version 10.0 for Windows; SPSS, Chicago, IL).

Cytotoxic T lymphocyte assay

Splenocytes from the immunized mice were suspended in complete culture medium (RPMI 1640 supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 10 mM HEPES, 2 mM L-glutamine, penicillin [100 U/mL], and streptomycin [100 μg/mL]) with concanavalin A (2 μg/mL) and interleukin (IL)-2 (10 U/mL) and cultured for 1–2 days. P815 target cells were treated with mitomycin C (25 μg/mL) for a further 4 h to become stimulator cells. Stimulator cells were pelleted and washed four or five times with RPMI 1640. Splenocytes (4×10^7 cells) were incubated with stimulator cells at a splenocyte-to-stimulator ratio of 10:1 for 6 days at 37°C in 5% CO_2 to become effector cells.

To measure the specific lysis of these target cells, we used the lactate dehydrogenase (LDH) release assay. This assay provides results similar to those obtained with the standard chromium release assay and does not require the use of radioisotopes. In a 96-well round-bottom plate, effector cells were incubated with P815 target cells at effector:target (*E*:*T*) ratios of 25:1, 50:1, and 100:1 for 4 h in phenol red-free RPMI 1640 containing 3% FCS, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). Supernatant samples, 100 μl from each well, was transferred by centrifugation to another 96-well plate, and lysis was determined by measuring LDH release, using a nonradioactive cytotoxicity assay kit (Promega, Madison, WI). Released LDH converts the added substrate tetrazolium salt into a red formazan product, and the strength of the color of the product is proportional to the number of lysed cells. Absorbance values from supernatants were measured at 490 nm with an enzyme-linked immunosorbent assay (ELISA) microplate reader. The percentage of specifically lysed P815 target cells for a given effector cell sample was calculated according to the following formula: specific lysis (%) = (OD of experimental LDH release − OD of effector cell spontaneous LDH release − OD of target cell spontaneous LDH release)/(OD of maximum target LDH release − OD of target spontaneous LDH release) × 100, where OD is optical density. All determinations were made in triplicate.

Detection of antibody against S1 protein in sera

The IgG level in antiserum samples was determined by ELISA. Polystyrene plates coated with standard antigens from the detection kit for analyzing anti-SARS IgG antibody (Beijing BGI-GBI Biotech, Beijing, China) were incubated with 10% BSA in PBS for 1 h at 37°C, and then washed with PBS containing 0.1% Tween 20. Antisera (100 μL, in 5% BSA) were added to the wells and the plates were incubated for 1 h at room temperature. After washing all wells, 100 μl of goat anti-mouse

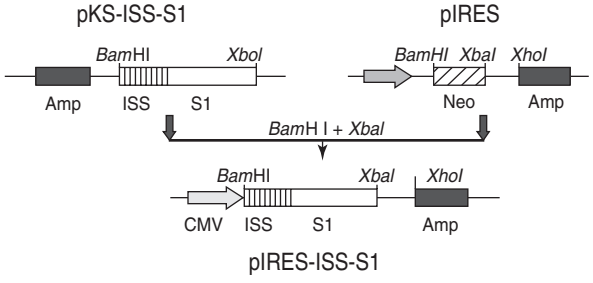

FIG. 2. Strategy for construction of plasmid pIRES-ISS-S1. A fragment from *Bam*HI- and *Xba*I-digested pKS-ISS-S1 was inserted into the *Bam*HI–*Xba*I site of eukaryotic expression plasmid pIRES by enzyme digestion and ligation.

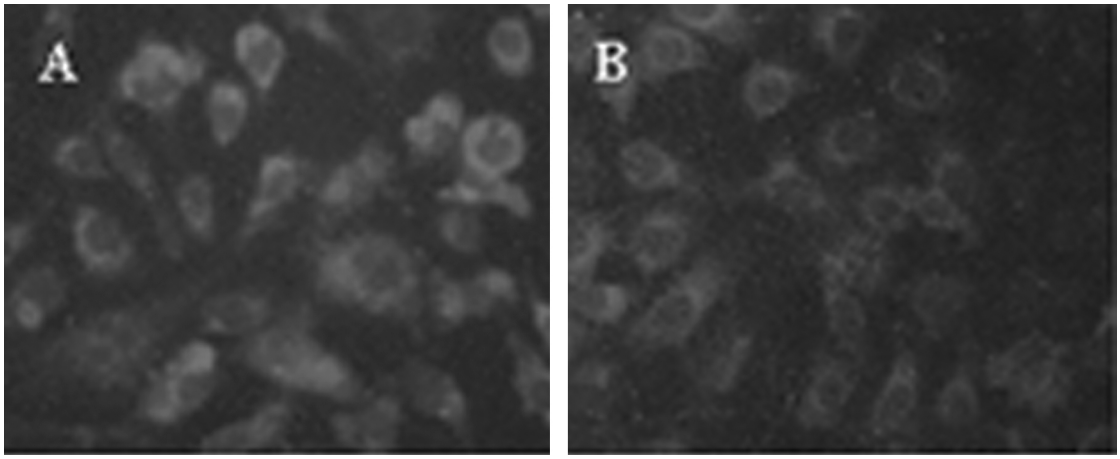

FIG. 3. Detection of expressed S1 protein by immunofluorescence microscopy. BHK-21 cells were transfected with pIRES (**A**) or the recombinant pIRES-ISS-S1 (**B**). Expression of SARS-CoV S1 protein was recognized by anti-SARS-CoV antibody [immunofluorescence shown as intracytoplasmic staining (yellow–green signal) in (**B**) positive, no yellow-green signal in control (**A**). Cells were counterstained with Evans blue and photographs were taken at an original magnification of ×1000.

immunoglobulin–horseradish peroxidase conjugate (SABC, diluted 1:5000) was added to each well and the plates were incubated for 1 h at room temperature. The reaction was developed with a solution containing phenylenediamine (2 mg/mL), 0.03% H_2O_2 in 0.1 M citrate phosphate buffer (pH 5.0) and was stopped with 50 μL of 4 N H_2SO_4. The OD was measured at 490 nm with an ELISA microplate reader (Tecan Group, Maennedorf, Austria). Sera from immunized mice were considered positive if the mean OD was 2-fold greater than that of the preimmune sera. The end point of the dilution for antibody was defined as that giving an optical density greater than 0.10 and that was at least 2-fold higher than that of control sera at the same dilution.

RESULTS

Gene synthesis and plasmid construction

The gene sequence encoding SARS-CoV S1 protein (2260 bp) was synthesized by chemical methods according to the SARS-CoV genomic sequence reported in GenBank (accession number NC_004718). Similarly, an ISS composed of a Kozak sequence, a PADRE (pan-DR epitope) Th cell epitope, and a tetanus toxin (TT) Th epitope was synthesized as a genetic adjuvant and was incorporated into the 5′ terminus of the S1 gene. The ISS is represented in Fig. 1. The recombinant consisting of S1 and ISS was cloned into pIRES to make the DNA vaccine pIRES-ISS-S1 as shown in Fig. 2.

The construction of plasmid pIRES-ISS-S1 was confirmed to be as shown in Fig. 2 by sequencing (data not shown).

Identification of expression of S1 protein in BHK-21 cells by IFA

To confirm the expression of S1 protein in mammalian cells, pIRES-ISS-S1 plasmid was transfected into BHK-21 cells. On immunofluorescence staining with sera from SARS-CoV-positive patients, cells transfected with pIRES-ISS-S1 clearly exhibited positive staining (yellow–green fluorescence) in the cytoplasmic region, indicating the expression of S1 protein and recognition by the SARS-CoV antisera, in contrast to those cells trans-

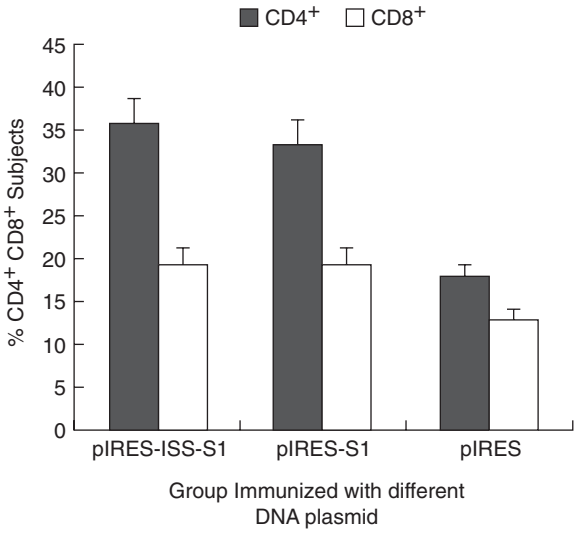

FIG. 4. Percentages of $CD4^+$ and $CD8^+$ T cells in spleens of mice immunized with pIRES-ISS-S1, pIRES-S1 plasmid, and pIRES control plasmid.

fected with the parental plasmid pIRES, which showed no reactivity with the antisera (no yellow–green fluorescence) (Fig. 3).

Determination of CD4$^+$ and CD8$^+$ spleen T lymphocyte counts

To evaluate T cell-mediated immune responses in mice immunized with the DNA vaccine, CD4$^+$ and CD8$^+$ spleen T lymphocyte counts were done by flow cytometry. The results are shown in Fig. 4. Both CD4$^+$ and CD8$^+$ T lymphocyte counts in mice immunized with pIRES-ISS-S1 and pIRES-S1 were significantly higher than those in mice immunized with pIRES; on the other hand, those in mice immunized with pIRES-ISS-S1 were slightly higher than those in mice immunized with pIRES-S1. These results demonstrated that the DNA vaccine pIRES-ISS-S1 and pIRES-ISS-S1 could greatly induce cellular immune responses in BALB/C mice, and the ISS actually enhanced the cellular immune responses to a certain extent *in vivo*.

Induction of CTL responses in immune mice

Cytotoxic activity was measured in a nonradioactive lactate dehydrogenase (LDH) release assay. Specific lysis rates are shown in Fig. 5. Both plasmids pIRES-ISS-S1 and pIRES-S1 could elicit highly specific CTL activities compared with the empty vector pIRES ($p < 0.01$). The specific lysis rate of the pIRES-ISS-S1 group was slightly higher than that of the pIRES-S1 group, which showed that the splenocytes had antigen specificity and that the designed T cell epitopes, acting as an immunoadjuvant, could enhance cellular immunity, although not prominently.

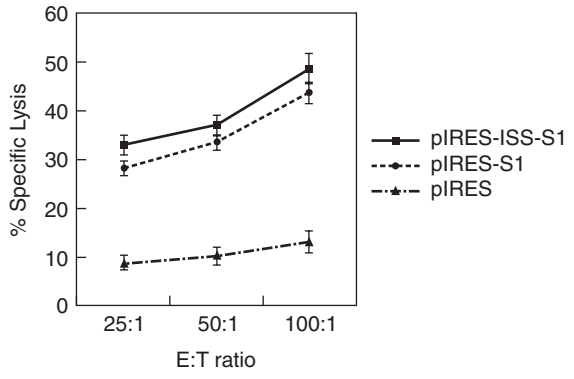

FIG. 5. Specific lysis of antigen-sensitized target cells by restimulated effector cells from immunized mice. *E*:*T* ratio, effector cell:target cell ratio.

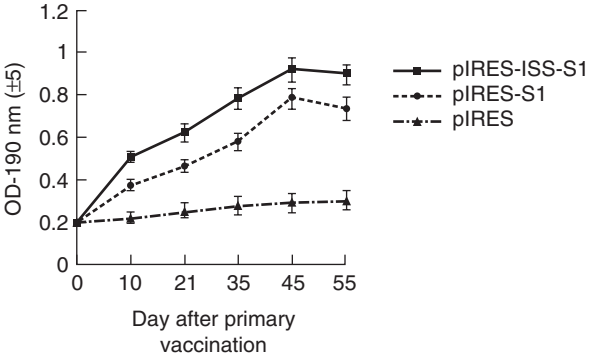

FIG. 6. Antibody levels in immunized mice. Antibody titer was measured by ELISA on days 0, 10, 21, 35, 45, and 55. Antibody titer of antisera was expressed as mean ± standard deviation of five preimmunization serum samples.

Determination of anti-SARS antibody level

Significant anti-SARS-CoV IgG was found in mice immunized with pIRES-ISS-S1 and pIRES-S1 ($p < 0.01$). The level of anti-SARS-CoV IgG increased markedly 10 days after primary immunization with pIRES-ISS-S1 and pIRES-S1 and remained at a high level from 35 days. The highest antibody titers reached were 1:320 (group immunized with pIRES-ISS-S1) and 1:160 (group immunized with pIRES-S1) ($p < 0.01$) on day 45. Control mice immunized with pIRES did not develop detectable antibody responses, whereas mice immunized with pIRES-ISS-S1 presented stronger antibody responses than did mice immunized with pIRES-S1 (Fig. 6). These results showed that the ISS could enhance antibody responses markedly.

DISCUSSION

The most effective strategy for preventing SARS infection is vaccine inoculation. Usually, traditional vaccines, such as inactivated vaccine and attenuated vaccine, are too restricted to be used effectively because of some disadvantages. Inactivated vaccine has such deficiencies as T cell independence, major histocompatibility complex restriction, and serotype conversion caused by vaccination, which may affect the immunological analysis of epidemic monitoring (23). Furthermore, inactivated vaccine is more expensive for vaccination of people in developing countries because the vaccine requires large amounts of viral antigen. Attenuated live vaccine, on the other hand, is able to efficiently elicit protective immune responses with a smaller amount of virus because the vaccine virus can propagate and synthesize viral antigen in the inoculated body (6,13), and attenuated vaccine virus has residual virulence or pathogenic mutation during vi-

ral propagation in the body (13). A DNA vaccine may be a better vaccine candidate for SARS-CoV. DNA vaccine, in the broad sense, relies on a ring of nucleic acid to encode an antigen that elicits further protective responses to protect against infectious disease when taken up by the body's immune system. DNA vaccination also efficiently elicits cellular immune responses, including cytotoxic T lymphocyte (CTL) responses, in addition to humoral immunity, DNA vaccine-mediated cellular immunity provided protection (18).

Although the S protein is believed to be variable, it had been identified as the host protective antigen and often used as a vaccine candidate for coronaviruses (4,9,10) because this protein is believed to be incorporated into the viral envelope before the mature virion is released (11), and the receptor-binding domain (RBD) in the S1 subunit of S protein contains multiple conserved neutralizing epitopes. So it has been suggested that recombinant proteins containing the RBD may be used to develop a safe and effective SARS vaccine (8). In this article, a gene encoding the S1 protein of SRAS-CoV and an ISS were synthesized and the fusion gene was cloned into plasmid pIRES to develop DNA vaccine pIRES-ISS-S1. This DNA vaccine was injected into mice and induced cell-mediated immune responses and humoral responses against SARS-CoV.

At present, there are several research articles about DNA vaccines encoding the S protein of SARS-CoV (18,20,21); these data have suggested that the S protein can induce neutralizing antibodies and protective responses in immunized mice. To increase immunogenicity and vaccine efficacy, we added an ISS as an adjuvant to the 5′ terminus of the S1 gene to enhance $CD4^+$ Th cells and the durability of immune responses (1). Although the pan-DR epitope (PADRE) is specifically engineered to be immunogenic in humans (3,14), it has the ability to bind to murine $I\text{-}A^b$ molecules, so it can be used in mice as a helper T epitope (2). In our study, we fused PADRE and the tetanus toxin (TT) Th epitope sequence to the 5′ terminus of the S1 protein gene to enhance antibody responses. We found that addition of these epitopes to the pIRES-S1 vaccine markedly enhanced the antibody titer (Fig. 6) and elicited cellular immune responses slightly more than did the pIRES-S1 vaccine. In this article, both $CD4^+$ and $CD8^+$ spleen T lymphocyte counts increased significantly in mice immunized with pIRES-ISS-S1 and pIRES-S1, compared with mice immunized with pIRES, and the T cell proliferating responses had antigen specificity. After immunization, B cells from immunized mice were elicited to produce higher antibody titer (1:320 and 1:160). Although we do not know whether humoral immune responses mediated by the DNA vaccine can induce resistance to SARS-CoV challenge, it has been reported that neutralizing antibody at 1:10 titer is commonly considered protective (19). These results show that the DNA vaccine pIRES-ISS-S1 can elicit more efficient immune responses than DNA vaccine encoding only the S1 gene. This offers promise for the protective immunization of humans.

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