Humoral and Cellular Immune Responses Induced by 3a DNA Vaccines against Severe Acute Respiratory Syndrome (SARS) or SARS-Like Coronavirus in Mice[▽]

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Vaccine development for severe acute respiratory syndrome coronavirus (SARS-CoV) has mainly focused on the spike (S) protein. However, the variation of the S gene between viruses may affect the efficacy of a vaccine, particularly for cross-protection against SARS-like CoV (SL-CoV). Recently, a more conserved group-specific open reading frame (ORF), the 3a gene, was found in both SARS-CoV and SL-CoV. Here, we studied the immunogenicity of human SARS-CoV 3a and bat SL-CoV 3a DNA vaccines in mice through electroporation immunization followed by enzyme-linked immunosorbent, enzyme-linked immunospot, and flow cytometry assays. Our results showed that high levels of specific humoral responses were induced by SARS-CoV 3a and SL-CoV 3a DNA vaccines. Furthermore, a strong Th1-based cellular immune response was stimulated by both DNA vaccines. The vaccines stimulated gamma interferon production mainly by CD8$^+$ T cells and interleukin-2 (IL-2) mainly by CD4$^+$ T cells. Of interest, the frequency of IL-2-positive cells elicited by the SARS-CoV 3a DNA vaccine was significantly higher than that elicited by the SL-CoV 3a DNA vaccine. In summary, our study provides a reference for designing cross-protective DNA vaccines based on the group-specific ORFs of CoVs.

Severe acute respiratory syndrome coronavirus (SARS-CoV) is the etiologic agent of SARS (8, 19). On the basis of the full-length genome sequence of SARS-CoV, all predicted open reading frames (ORFs) are divided into two groups: (i) those with clear homologies to other CoVs (including replicase and structural genes) and (ii) the eight group-specific ORFs with no clear homology to any known genes in the database (17, 22). To date, vaccine studies have focused on the roles of viral structural proteins (3, 10); little is known regarding the function of the group-specific proteins. One of these group-specific genes, the 3a gene, is located between the S and E loci of the membrane protein genes and encodes a 31-kDa protein with 274 amino acids (aa). A bioinformatics study suggests that the 3a protein contains three putative transmembrane domains in topology with a 34-aa N-terminal extracellular matrix and a C-terminal intracellular domain containing aa 134 to 274 (23). The 3a protein is localized in the rough endoplasmic reticulum/Golgi compartments and interacts with the S and M glycoproteins. Recently, the 3a protein was shown to form an ion channel and modulate virus release (16). The 3a protein may play important roles in viral replication (1), raising the possibility that the 3a protein could be a potential target for vaccine and drug design.

Indeed, the N-terminal fragment of the 3a protein elicited strong and potentially protective humoral responses in infected patients (27). The amino acids (aa 15 to 28) in the ectodomain of the 3a protein were also shown to induce neutralizing antibodies in a previous study (2). However, whether the full length of the 3a DNA is immunogenic remains fully undetermined. Recently, SARS-like CoV (SL-CoV), which has a close genetic homology to SARS-CoV, was isolated from horseshoe bats (genus *Rhinolophus*) (14, 15). Most of the group-specific ORFs that exist in SARS-CoV, including the 3a gene, are also present in SL-CoV. The 3a protein of SL-CoV is ~83% identical to that of SARS-CoV at the amino acid level. To date, the immunogenicity and the biological function of 3a in SL-CoV remain unknown. To better understand the biological function of 3a, we investigated the immunogenicity of SARS-CoV and SL-CoV 3a as DNA vaccines in BALB/c mice.

MATERIALS AND METHODS

Construction of plasmids. The full-length 3a gene was amplified from SARS-CoV WH20 (GenBank accession no. AY772062) or bat SL-CoV Rm1 (GenBank accession no. NC_009696; kindly provided by Zhengli Shi of the Wuhan Institute of Virology, Chinese Academy of Science) and subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) to construct the pcDNA3.1H3 or pcDNA3.1B3 recombinant plasmid, respectively. The accuracy of the constructs was confirmed by restriction enzyme digestion and sequencing. Plasmids were purified using Qiagen Mega Prep columns (Qiagen, Hilden, Germany), dissolved in endotoxin-free Tris-EDTA (TE) buffer to give a final concentration of 1 mg/ml, and stored at −80°C until use.

Expression of recombinant proteins in vitro. 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) at 37°C in a 5% CO$_2$ incubator. Transfection was carried out using Lipofectin reagent, following the manufacturer's protocols (Gibco, Gaithersburg, MD). Briefly, cells were grown to 80% confluence in a 35-mm dish and transfected with 3 μg of recombinant plasmid. The cells were harvested a 48 h posttransfection, washed twice with phosphate-buffered saline

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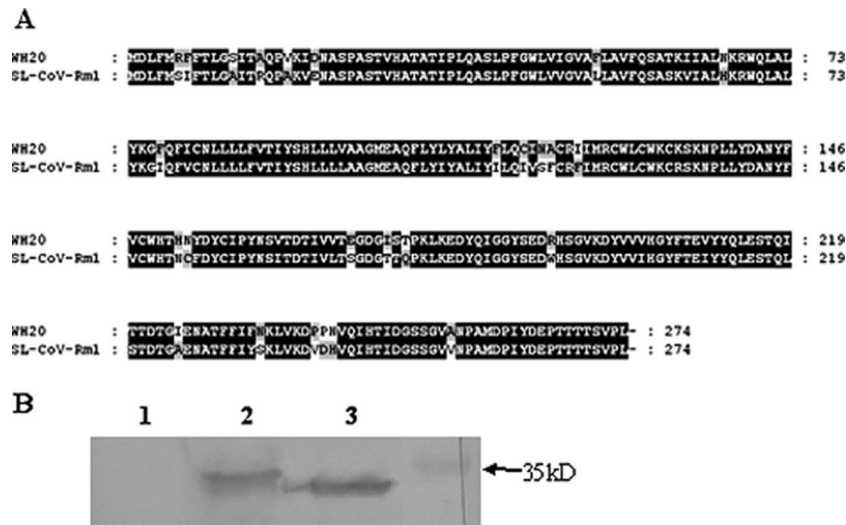
FIG. 1. Amino acid sequence alignment and in vitro expression of 3a proteins. (A) The amino acid sequences of full-length 3a from SARS-CoV and SL-CoV were aligned with ClustalX 1.83 and edited using GenDoc. (B) The full-length 3a gene from SARS-CoV or SL-CoV was cloned into pcDNA3.1(+) to make pcDNA3.1H3 or pcDNA3.1B3, respectively. The expression of 3a protein was analyzed at 48 h posttransfection by Western blotting. Lane 1, pcDNA3.1; lane 2, pcDNA3.1H3; lane 3, pcDNA3.1B3.

(PBS), and then suspended in sample buffer to verify the expression of protein 3a by Western blotting analysis.

Antibodies. Purified anti-CD28, anti-CD4–phycoerythrin (anti-CD4–PE), anti-CD8–PE–Cy5, anti-gamma interferon–fluorescein isothiocyanate (anti-IFN-γ–FITC), anti-interleukin-2–FITC (anti-IL-2–FITC), and isotype-matched control antibodies were purchased from eBioscience (San Diego, CA).

Mice and immunization. Female BALB/c mice, 6 to 8 weeks old, were purchased from the Experimental Animal Center at Hubei Medical College. Animals were maintained under pathogen-free conditions, randomly divided into four groups (five mice each), and immunized with 30 µg DNA per mouse by electroporation every 3 weeks. Mice were boosted in the same manner on days 21 and 42. Three mice of each group were sacrificed by cervical dislocation, and splenocytes were isolated on day 52.

Cell preparations. Single-cell suspensions of spleens were prepared from the immunized mice. Briefly, spleens from freshly killed mice were disrupted in Nycoprep (Axis-Shield, Oslo, Norway) by using monofilament nylon filters, and the cells were collected and centrifuged at $800 \times g$ for 30 min. Cell pellets were suspended in 10 ml RPMI 1640 and then centrifuged at $250 \times g$ for 10 min at room temperature. Cells were suspended in RPMI 1640 supplemented with 10% FBS at a concentration of 1×10^7 cells/ml before the enzyme-linked immunospot (ELISPOT) assay and flow cytometry analysis.

ELISA analysis. An enzyme-linked immunosorbent assay (ELISA) was used to determine the titers of mouse sera as previously described (3). Briefly, 10 µg/ml of purified SARS-CoV or SL-CoV 3a protein (aa residues 126 to 274) was used to coat 96-well microtiter plates (Corning Costar, Acton, MA) at 4°C overnight. After being blocked with 1% bovine serum albumin, 1:250-diluted mouse sera were added and incubated at 37°C for 1 h, followed by three washes with PBS containing 0.05% Tween 20. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (1:3,000; Sigma, St. Louis, MO) at 37°C for 1 h. The reaction was visualized by addition of the substrate *para*-nitrophenyl phosphate, and absorbance at 405 nm was measured by an ELISA plate reader (Bio-Rad, Hercules, CA).

ELISPOT assay. Nitrocellulose membranes of 96-well ELISPOT plates (Millipore, Molsheim, France) were prewet with 15 µl 70% ethanol and then coated overnight at 4°C with 100 µl anti-mouse IFN-γ or IL-4 monoclonal antibody (MAb) at 15 µg/ml (Mabtech, Stockholm, Sweden). The antibody-coated plates were blocked with RPMI 1640 containing 10% FBS for at least 2 h at room temperature, and then, 1×10^6 splenocytes in 100 µl medium (RPMI 1640, 10% FBS, 10 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin) containing 10 µg/ml purified SARS 3a protein were incubated for 20 h at 37°C. All stimulation conditions were tested in triplicate, and cell viability was confirmed by adding 4 µg/ml of concanavalin A (Sigma). The plates were washed five times with PBS-0.05% Tween and then incubated with 100 µl of biotinylated anti-mouse IFN-γ or IL-4 MAb (1 µg/ml in PBS-0.5% FBS; Mebtech) for 2 h at room

temperature. After five washes, 100 µl of streptavidin-horseradish peroxidase reagent was added. Following a 1-h incubation at room temperature and five subsequent washes, 100 µl of TMB substrate was added for 15 min. The reaction was terminated by discarding the substrate solution and washing the plates under running tap water. After drying, the spots were scanned and countered by ELISPOT image analysis (Biosys, Karben, Germany). Responses were considered positive if a minimum of 10 spot-forming cells per 10^6 splenocytes were detected above the background level.

Cell surface marker and intracellular cytokine staining. Ten days after the final boost, splenocytes were suspended in RPMI 1640 supplemented with 10% FBS and seeded in triplicate in 96-well plates containing corresponding purified proteins (10 µg/ml) and anti-CD28 MAb (1 µg/ml). Six hours later, monensin (15 µl/ml; eBioscience) was added to each well and blocked at 37°C for 3 h. Then, splenocytes were suspended in PBS and stained with PE-conjugated anti-CD4 and PE–Cy5-conjugated anti-CD8 for 20 min in the dark. After one wash with PBS, the cells were fixed, permeabilized, and stained with anti-IFN-γ or anti-IL-2–FITC for 30 min at room temperature. The corresponding isotype controls were included for each staining. Cells (5,000 to 10,000) were acquired and analyzed with a flow cytometer (EPICS ALTRA II; Beckman, Fullerton, CA). Data were analyzed using EXOPO analysis software.

Statistical analysis. All data are presented as means ± standard deviations (SD). SPSS 13.0 for Windows was used for statistical analysis. Differences in humoral or cellular immune responses between groups were analyzed by one-way analysis of variance methods. The least-significant-difference t test was used for between-group comparison. P values of <0.05 were considered statistically significant.

RESULTS

In vitro expression of 3a proteins. The 3a gene of SARS-CoV or SL-CoV was subcloned into the pcDNA3.1(+) vector to make the DNA vaccine plasmid pcDNA3.1H3 or pcDNA3.1B3, respectively. The plasmids were transfected into 293T cells, and the expression of 3a proteins was evaluated by Western blotting. At 48 h posttransfection, a strong specific band of 3a protein was detected in pcDNA3.1H3- or pcDNA3.1B3-transfected cells (Fig. 1B, lanes 2 and 3), but no such band was detected in the pcDNA3.1-transfected cells (Fig. 1B, lane 1). Though 3a of SL-CoV is ~83% identical to that of SARS-CoV at the amino acid level (Fig. 1A), we see a
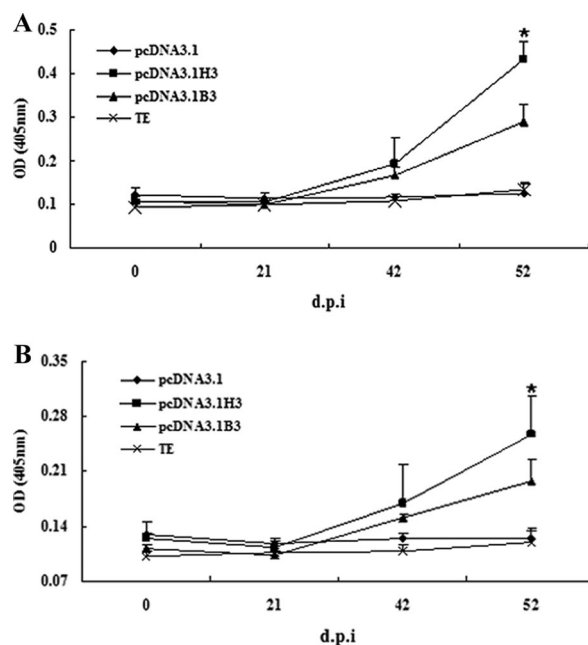
FIG. 2. 3a-specific antibody responses in immunized mice. Sera from each group were collected before each immunization. Ten days after the final boost, the mice were sacrificed and the specific antibody responses were examined by ELISA. SARS-CoV 3a protein (A) and SL-CoV 3a protein (B) were used as a coating antigen. Data shown are the means ± SD of results from two independent experiments using five animals from each group, with each condition tested in triplicate (∗, P values of <0.05 for comparison with pcDNA3.1B3). OD, optical density; d.p.i., days postimmunization.
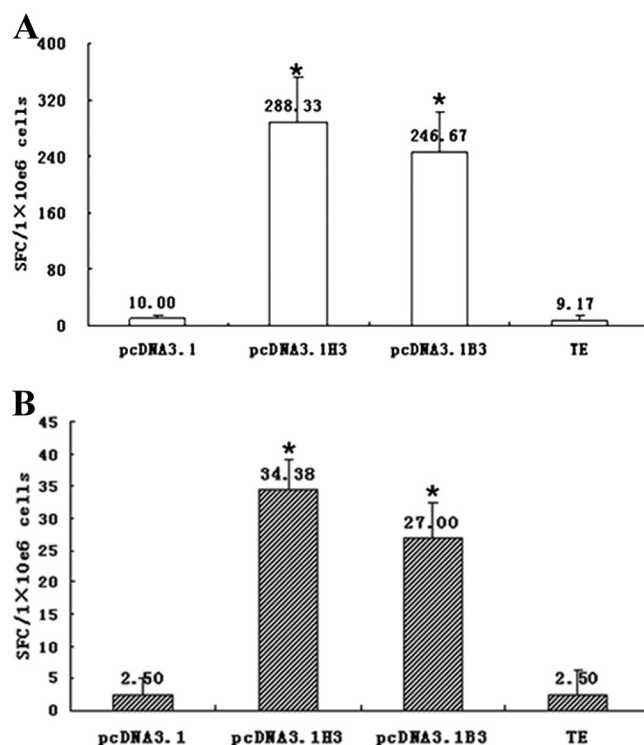

FIG. 3. Specific IFN-γ and IL-4 production in immunized mice. Mice were sacrificed 10 days after the final boost, and the frequency of IFN-γ (A)- or IL-4 (B)-producing cells at the single-cell level was determined by an ELISPOT assay. Data shown are the means ± SD of results from two independent experiments using three animals from each group, with each condition tested in triplicate (∗, P values of <0.05 for comparison with control groups).

lower molecular weight upon expression in vitro (Fig. 1B), suggesting a posttranslational modification difference.

Humoral immune responses to 3a DNA vaccines. To investigate the humoral immune responses, mice were immunized with pcDNA3.1, pcDNA3.1H3, pcDNA3.1B3, or TE buffer by electroporation. Sera were collected 10 days after the final boost. Specific antibody responses were assessed by analyzing mouse sera with ELISA, while SARS or SL-CoV 3a protein was expressed and purified as a coating antigen, as previously described (11). As shown in Fig. 2, all vaccinated groups induced specific antibody responses compared to the control groups immunized with pcDNA3.1 or TE ($P < 0.01$). After the first boost, there was no significant difference in humoral response between SARS and SL 3a DNA vaccines ($P > 0.05$). Following the final boost, the humoral response in SARS 3a DNA-vaccinated mice was significantly increased compared to that in SL 3a DNA-vaccinated mice ($P < 0.05$).

Cellular immune responses to 3a DNA vaccines. In order to assess the production of cytokines elicited by 3a DNA vaccines, the frequency of IFN-γ- or IL-4-producing cells at the single-cell level was determined by an ELISPOT assay. As shown in Fig. 3A and B, both SARS-CoV and SL-CoV 3a DNA vaccines induced specific IFN-γ and IL-4 responses in mice, compared with the levels for the control groups ($P < 0.01$), but there was no significant difference between the IFN-γ and IL-4 responses induced by different 3a DNA vaccines. Only a low number of nonspecific spots were detected in the control groups (≤10 spots/10^6 cells). It was noted that the levels of IFN-γ secretion

were much higher than those of IL-4 in both 3a DNA vaccine-immunized groups, indicating a strong Th1 bias in the cellular immune response. Because Th1 cells can elicit a phagocyte-mediated defense against infections, Th1-dominated immune responses stimulated by 3a may play an important role in virus clearance.

Subsets of specific memory T-cell responses. To determine the immune responses of T-cell subsets generated after immunization, Th1 cytokine IFN-γ- and IL-2-positive cells in CD4$^+$ or CD8$^+$ cell populations were analyzed using flow cytometry. As shown in Fig. 4A, 3a-specific CD4$^+$ or CD8$^+$ IFN-γ-producing T cells were detected in splenocytes. Both 3a DNA vaccines enhanced specific T-cell responses, compared with the levels for the control groups. The frequency of IFN-γ in the CD8$^+$ T-cell populations was higher than that in the CD4$^+$ T cells. The frequency of IL-2-producing cells in CD4$^+$ or CD8$^+$ T cells was also analyzed. As shown in Fig. 4B, IL-2-positive cells were the predominant population in CD4$^+$ T cells and to a lesser extent in CD8$^+$ T cells. Surprisingly, there was a significant difference in IL-2-producing T-cell response between the two 3a genes in both CD4$^+$ and CD8$^+$ T cells ($P < 0.01$).

DISCUSSION

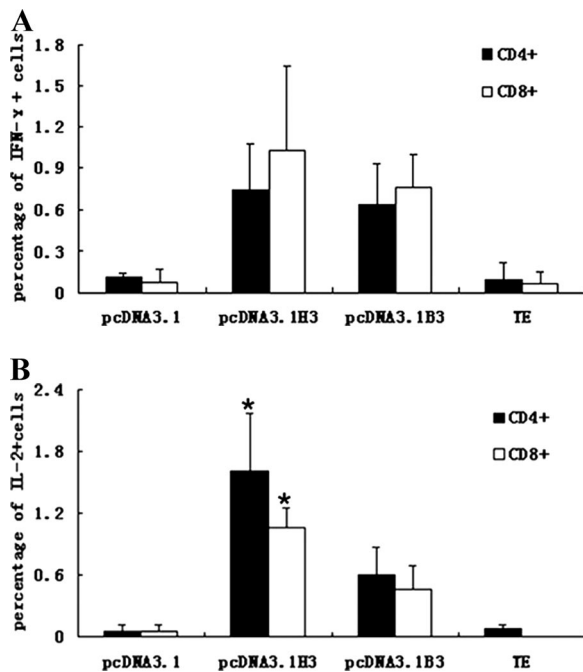The outbreak of SARS in 2003 caused thousands of infections and hundreds of deaths around the world. To prevent

FIG. 4. Frequencies of IFN-γ- and IL-2-positive cells in CD4$^+$ or CD8$^+$ splenocytes. Splenocytes were isolated from vaccinated mice 10 days after the final immunization. IFN-γ (A)- and IL-2 (B)-positive cells in CD4$^+$ or CD8$^+$ cell populations were analyzed by flow cytometry. Data shown are the means ± SD of results from two independent experiments using three animals from each group, with each condition tested in triplicate (*, P values of <0.05 for comparison with pcDNA3.1B3).

another SARS epidemic, continuous efforts have been made toward the development of a prophylactic vaccine. It is generally accepted that an effective SARS vaccine should act not only to prevent the reemergence of SARS-CoV but also to provide cross-protection against SL-CoV that is isolated from horseshoe bats, the possible animal reservoirs of SARS-CoV. To date, a variety of candidate vaccines, such as DNA (26), adenovirus-mediated gene transfer (7), a combination of whole killed virus and DNA (13), inactive virus (21), and recombined proteins or their fragments, are under preclinical or clinical studies. Among these, a DNA vaccine is the most promising strategy because it is safe and can induce both neutralizing antibodies and cellular immune responses. Current SARS vaccine development has mainly been focused on the spike protein. Although spike protein-based vaccines elicit strong cellular immune responses and protective humoral responses, the relatively high variation of the S gene between viruses could compromise its usage, particularly for cross-protection (25). Therefore, other SARS vaccine candidates are urgently needed.

The 3a gene is a group-specific ORF found in both SARS-CoV and SL-CoV. In the current study, we investigated the immunogenicity of SARS-CoV and SL-CoV 3a DNA vaccines in BALB/c mice. From our results, both SARS-CoV and SL-CoV 3a DNA vaccines were found to induce specific humoral and cellular immune responses in mice. The level of 3a-specific antibody was greatly enhanced following two boosts. Our finding is supported by a previous study showing that the amino

acids (aa 15 to 28) in the ectodomain of the 3a protein induced neutralizing antibodies in rabbits (2). Of interest, there was a significant difference ($P < 0.05$) in terms of antibody responses at 52 days postimmunization between the SARS-CoV and SL-CoV 3a DNA vaccines, although the two genes are well conserved (83% at the amino acid level). This difference could be due to the presence of different B- or T-cell epitopes. Further studies are required to address the precise amino acid difference between SARS-CoV and SL-CoV 3a proteins that leads to the change of antigen determinants.

In addition to humoral responses, T-cell immune responses were also demonstrated in our study. T lymphocytes are generally divided into helper (CD4) and CTL (CD8) cells. CD4 T cells can increase the number of memory cells which respond rapidly when reexposed to pathogens and thus play a vital role in protection against virus challenge (5, 6). After activation, naive antigen-specific CD8 T cells are able to proliferate quickly and differentiate into potent effector cells capable of rapid cytokine production and cytolytic killing of target cells (9). CD4 lymphocytes are further subdivided into Th1 and Th2 on the basis of the type of released cytokines (20). Th1 cells secrete IL-2 and IFN-γ. Th2 cells, on the other hand, secrete IL-4 and IL-5. Th1 cells predominantly confer protective immunity and play a crucial role in long-term protection and virus eradication (4, 18), while Th2 cells provide help for antibody production. In the present study, a strong Th1-type response was detected by the ELISPOT assay, which was consistent with the ELISA results; this response may play a vital role in protection against virus challenge. In order to further address the Th1-type response, we examined the frequencies of IFN-γ- and IL-2-positive cells in CD4$^+$ or CD8$^+$ splenocytes by flow cytometric analysis. Of the two Th1 cytokines, IFN-γ is an effector cytokine able to activate macrophage cells and to inhibit viral replication (24); IL-2 is a growth factor which plays important roles in maintaining memory cells and mediating the expansion of both CD4$^+$ and CD8$^+$ T cells (12). Our results revealed that IFN-γ was mainly produced by CD8$^+$ T cells and that IL-2 was predominantly produced by CD4$^+$ T cells. Of interest, the frequency of IL-2-positive cells in CD4$^+$ or CD8$^+$ splenocytes elicited by the SL-CoV 3a DNA vaccine was significantly lower than that elicited by the SARS-CoV 3a DNA vaccine. These data suggest that the gene products of SARS-CoV and SL-CoV 3a DNA may be different in structure or conformation, which could lead the difference in immunogenicity.

Taken together, these data indicate that the 3a DNAs of both SARC-CoV and SL-CoV can be expressed and secreted in the vaccinated mice and activate both B and T cells to induce specific humoral and cellular immune responses. We were unable to do a challenge experiment with mice because Chinese regulations forbid the use of live SARS virus for laboratory studies, but our study demonstrates the immunogenicity of SARS-CoV and SL-CoV 3a DNA vaccines, providing basic information for the design of vaccines based on the group-specific ORFs in the CoV family.

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