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Enhanced Induction of SARS-CoV Nucleocapsid Protein-Specific Immune Response Using DNA Vaccination followed by Adenovirus Boosting in BALB/c Mice

Ma Chunling[a] Yao Kun[a] Xu Jian[a] Qin Jian[b] Sun Hua[c] Zhu Minsheng[d]

[a]Department of Microbiology and Immunology, Nanjing Medical University, [b]College of English, Hehai University, [c]Nanjing Center for Disease Prevention and Control, and [d]Model Animal Research Institute, Nanjing University, Nanjing, PR China

Key Words

DNA vaccine · Severe acute respiratory syndrome-associated coronavirus · Nucleocapsid protein · Adenovirus vector

Abstract

Objective: To investigate immunogenicity in the induction of humoral and cellular immune responses to genetic vaccines of the recombinant severe acute respiratory syndrome-associated coronavirus (SARS-CoV)-N gene expressing the same protein plasmid, pcDNA3.1-N, and replication-defective adenoviral vector, rAd-N, in a pcDNA3.1-N prime-rAd-N boost regimen and the reverse sequence in a rAd-N prime-pcDNA3.1-N boost regimen. ***Method:*** After the mice had been immunized intramuscularly and/or intraperitoneally with pcDNA3.1-N and rAd-N in prime-triple boost immunization, humoral and cellular immune responses were detected. ***Results:*** After detection, different levels of anti-N humoral and cellular responses are shown compared to controls. The humoral immune response was induced more effectively by the DNA priming and recombinant adenovirus boosting regimen and the reverse sequence of heterogeneous combinations. There is a significant difference between heterogeneous and homologous vaccinations. However, the cytotoxic T lymphocyte (CTL) response was not significantly altered by the different prime-boost immunizations or the recombinant adenovirus of pcDNA3.1-N prime-rAd-N boost regimen alone, but lymphoproliferation and interferon-γ (IFN-γ) secretion were all enhanced by heterologous combination immunizations compared to homologous combinations. For the reverse sequence immunization regimen, lymphoproliferation, IFN-γ and CTL responses were all significantly weaker compared with pcDNA3.1-N prime-rAd-N boost regimen. ***Conclusion:*** Taken together, of all the combinations, the prime-triple boost immunization of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N can effectively induce SARS-CoV-N-specific and strong humoral and cellular immune responses in mice. The present results suggest that DNA immunization followed by recombinant adenovirus boosting could be used as a potential SARS-CoV vaccine in the induction of an enhanced humoral and cellular immune response.

Introduction

The devastation caused by the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) illustrates that new infectious diseases keep emerging, and the development of SARS-CoV vaccines are urgently required. From November 2002 to June 2003, SARS-CoV spread to many countries on several continents and

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Yao Kun
Department of Microbiology and Immunology
Nanjing Medical University
Nanjing 210029 (PR China)
Tel. +86 25 8686 2901, Fax +86 25 8650 8960, E-Mail yaokun@njmu.edu.cn

brought great suffering to people with regard to health and economy. Through the efforts of an international team it was identified as a new type of coronavirus [1]. Like the other 3-group coronaviruses, the SARS-CoV genome is about 30 kb in length, encodes four major structural proteins, spike (S), envelope (E) and membrane (M) glycoproteins and nucleocapsid (N) protein, and has several small nonstructural open reading frames found between the genes for S and E and between the genes for M and N [2, 3].

The N protein of SARS-CoV is 422 amino acids long, sharing 20–30% homology with the N protein of other coronaviruses. Previous studies indicate that N proteins of other coronaviruses are extensively phosphorylated, highly basic, and associated with viral RNA to form a helical ribonucleoprotein, which comprises the viral core structure. The biological function of coronavirus N protein is thought to participate in the replication and transcription of viral RNA and interfere with the cell cycle processes of the host cells. Importantly, the N protein in many coronaviruses is highly immunogenic and abundantly expressed during infection [2–5]. This suggests that the N gene might be very critical in the prophylaxis and diagnosis of the SARS-CoV infection.

Genetic immunization with DNA vaccines induces both antibody and cytotoxic T lymphocytes (CTLs) because they are expressed through the class-I and class-II antigen-processing pathway [6]. Therefore, DNA vaccine approaches have been applied to generate protective immunity to various pathogens [7–12], but to date the strength of the immune response induced by DNA vaccines has been relatively weak compared with conventional vaccines. A variety of strategies are being developed to increase DNA vaccine efficiency. Some approaches use the co-injection of plasmids coding for immune-enhancing genes such as interleukin-2 or granulocyte-macrophage colony-stimulating factor [13, 14]. Others aim to facilitate DNA uptake, such as the use of liposomes, encapsulation into microparticles, or bacteria [15–17]. However, currently one of the most successful protocols is the recombinant viral vector in combination with DNA vaccine. The advantage of such recombinant viral vaccines is their high efficacy in generating humoral and cellular immune responses. A number of different viruses, such as adenovirus, vaccinia, rabies virus, canarypox virus, simian immunodeficiency virus and murine leukemia virus, have been used to construct recombinant viral vaccines [18–24]. Among these, the replication-defective recombinant adenovirus is viewed to be a favorably choice as a viral vector vaccine, since it appears to be both safe and to induce strong humoral and cellular antigen-specific immune responses [25–28].

Regarding the clinical characteristics of SARS-CoV infection, the development of efficient SARS-CoV genetic vaccines capable of inducing strong humoral and cellular responses is urgent and essential for preventing its re-emergence. In the current study, we constructed a SARS-CoV N gene recombinant plasmid (pcDNA3.1-N) and an adenovirus vector (rAd-N) as vaccines to inoculate BALB/c mice in a prime-triple boost regimen in an attempt to determine which combination is able to induce strong SARS-CoV-N-specific humoral and cellular immune responses.

Materials and Methods

Cells, Plasmids

SP2/0 cells and Vero cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 medium (Gibco, Grand Island, N.Y., USA) supplemented with 100 μg/ml treptomycin, 100 units/ml penicillin and 10% heat-inactivated fetal bovine serum (FBS, Sijiqing, Hangzhou, China). Human embryonal kidney 293A cells purchased from Invitrogen Corporation (San Diego, Calif., USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% FBS. The LacZ gene recombinant adenoviral expression vector was purchased from Invitrogen Corporation. The DNA expression plasmid of pcDNA3.1-N, rabbit antisera (IgG polyclonal antibody anti-SARS-CoV-N protein) and the recombinant N protein were prepared as described before [29].

Construction of Recombinant Adenovirus

The construction of the replication-defective recombinant adenovirus expressing the structural protein N of SARS-CoV (rAd-N) has been described previously [30]. The same adenovirus inserting the LacZ gene was used as a negative control. The virus was amplified in 293A cells and the titer was measured by the End-Point Dilution Assay on 293A cells.

Transfection of the Plasmid, pcDNA3.1-N, into SP2/0 Cells and Infection of the Recombinant Adenovirus, rAd-N, into Vero Cells

The plasmid constructs of pcDNA3.1-N and pcDNA3.1 were transfected into SP2/0 cells derived from BALB/c mouse plasmacytoma using the lipofectamine 2000 reagent (Invitrogen). The cells were cultured in RPMI 1640 medium supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin and 10% heat-inactivated FBS, selected in the medium containing G418 (Promega, Madison, Wisc., USA) for 2 weeks. G418-resistant stable clones were screened by Western blot analysis. Western blot-positive cell clones of SP2/0 were treated with 25 μg/ml of mitomycin C (Promega) for 1 h and washed 3 times before being subjected to CTL assay. Vero cells were infected with rAd-N or rAd-LacZ, at a multiplicity of infections of 100, and incubated for 48 h at 37°C in 5% CO_2, and then lysed and subjected to Western blot analysis.

Table 1. Procedure of the immunization experiment with the pcDNA3.1-N prime-rAd-N boost regimen

Groups	Prime/ 0 week	First boost/ 2 weeks	Second boost/ 4 weeks	Third boost/ 6 weeks
1	pcDNA3.1-N	pcDNA3.1-N	pcDNA3.1-N	pcDNA3.1-N
2	pcDNA3.1-N	pcDNA3.1-N	pcDNA3.1-N	rAd-N
3	pcDNA3.1-N	pcDNA3.1-N	rAd-N	rAd-N
4	pcDNA3.1-N	rAd-N	rAd-N	rAd-N
5	rAd-N	rAd-N	rAd-N	rAd-N
6	pcDNA3.1	pcDNA3.1	pcDNA3.1	pcDNA3.1
7	rAd-LacZ	rAd-LacZ	rAd-LacZ	rAd-LacZ

BALB/c mice were divided into 7 groups with 5 mice each.

Table 2. Procedure of the immunization experiment with the rAd-N prime-pcDNA3.1-N boost regimen

Groups	Prime/ 0 week	First boost/ 2 weeks	Second boost/ 4 weeks	Third boost/ 6 weeks
8	rAd-N	rAd-N	rAd-N	pcDNA3.1-N
9	rAd-N	rAd-N	pcDNA3.1-N	pcDNA3.1-N
10	rAd-N	pcDNA3.1-N	pcDNA3.1-N	pcDNA3.1-N
11	pcDNA3.1	pcDNA3.1	pcDNA3.1	pcDNA3.1
12	rAd-LacZ	rAd-LacZ	rAd-LacZ	rAd-LacZ

BALB/c mice were divided into 5 groups with 10 mice each.

Western Blot Analysis

To detect SARS-CoV N protein expression, the SP2/0 cells were transfected with pcDNA3.1-N and pcDNA3.1, and Vero cells were infected with rAd-N and rAd-LacZ. At 48 h after transfection or infection, the cells were lysed in 1× SDS-PAGE loading buffer and then heated for 5 min at 95°C. Protein samples were separated by 5–12% SDS-PAGE and proteins electroblotted to polyvinylidene difluoride membranes (Hybaid, Heidelberg, Germany). Following blocking of nonspecific protein-binding sites using 5% (w/v) dried milk in Tris-buffered saline containing 0.5% (v/v) Tween 20, the membranes were incubated with a 1:500 dilution of rabbit anti-SARS-CoV-N protein antisera. After washing, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated antibody as the secondary antibody at a dilution of 1:1,000. All antibodies were diluted in 5% milk in Tris-buffered saline containing 0.5% (v/v) Tween 20. Signals of immunoreactive bands were detected using the sensitive substrate of the diaminobenzidine kit (Wuhan Boster, China) for Western blot analysis.

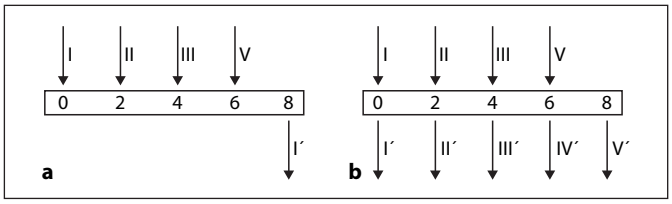

Fig. 1. a I, II, III and IV show that mice were immunized in the week indicated; I′ shows that blood and spleens were sampled, and sera and splenocytes were prepared in the week indicated in order to detect the antibody, lymphoproliferation, IFN-γ and CTL responses specific to SARS-CoV-N protein. **b** I, II, III and IV show that mice were immunized in the week indicated; I′, II′, III′, IV′ and V′ show that blood and spleens were sampled, and sera and splenocytes were prepared in the week indicated in order to detect the antibody, lymphoproliferation, IFN-γ and CTL responses specific to SARS-CoV-N protein.

Mice and Immunization Experiment

Six- to 8-week-old female BALB/C mice were purchased from Shanghai Experiment Animal Center (Shanghai, China) and divided into 12 groups. Groups 1–7 contained 5 mice each, and groups 8–12 contained 10 mice each. For DNA immunization, mice were injected intramuscularly (i.m.) via quadricep muscles with 100 μg of pcDNA3.1-N or pcDNA3.1 plasmid DNA dissolved in 100 μl sterile phosphate-buffered saline (PBS, 100 μg/100 μl). For recombinant adenovirus immunization, mice were injected intraperitoneally (i.p.) with 1.55×10^9 or 2.4×10^8 plaque-forming units of rAd-N or rAd-LacZ diluted in 0.5 ml sterile PBS. Each immunization was at 2-week intervals. The detailed immunization procedure and the time of collecting samples are shown in table 1 and 2 and figure 1.

Detection for SARS-CoV-N-Specific IgG Antibody

The BALB/C mice of groups 1–7 were sacrificed by cervical dislocation after their blood had been collected from the retro-orbital plexus using a capillary tube at week 8. Blood was collected and incubated for 4 h at 37°C, centrifuged for 10 min at 8,000 *g*, and the supernatants were transferred into new sterile tubes. For inactivation, sera were incubated for 30 min at 55°C and stored in –20°C. Blood from the mice of groups 8–12 were collected from 2 mice of each group at weeks 0, 2, 4, 6 and 8, and prepared as described above. The antibody activity of these sera were determined by enzyme-linked immunosorbent assay (ELISA). The ELISA kit for the diagnosis of SARS-CoV infection was purchased from Beijing Huada Aijier Corp. (Beijing, China). In this kit, 96-well plates were coated with inactivated SARS-CoV and the kit was used according to the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm with 600 nm for a reference wavelength. The antibody titers were expressed relatively as the values of $OD_{450\ nm}$.

Lymphoproliferation and IFN-γ Assay

Spleens were removed from mice that had been sacrificed by cervical dislocation. Splenocytes separated from individual mice of each group (5 mice from groups 1–7 at week 8, and 2 mice from groups 8–12 at weeks 0, 2, 4, 6 and 8) were used for proliferation and interferon-γ (IFN-γ) detections. After washing three times with PBS (pH 7.4), the splenocytes were resuspended at a final concentration of 2×10^6 cells/ml cultured in 12-well plates, and of 5×10^5 cells/well cultured in flat-bottom 96-well plates in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mmol/ml L-glutamine, 50 μmol/ml mercaptoethanol and 20 IU/ml of interleukin-2 (Promega) for detection. The suspensions were restimulated with recombinant N protein (5 μg/ml) for 72 h in 5% CO_2 at 37°C as experimental groups; splenocytes stimulated with phytohemagglutinin (PHA, Guangzhou, China) at a concentration of 10 μg/ml were used as positive controls, and those without stimulation were used as negative controls. Each culture condition was assessed in triplicate. 72 h after incubation, the 96-well plate was stained with 10 μl 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Roche, Germany) per well. One hundred microliters of dimethyl sulfoxide were added to each well to stop reactions after 4 h of incubation. The OD values were measured by a microplate reader at a test wavelength of 550 nm and a reference wavelength of 690 nm.

For the IFN-γ measurement, culture supernatants were harvested after 72 h of culture and stored at –70°C until assayed. IFN-γ levels were measured in culture supernatants using a standard ELISA assay. A commercially available mouse IFN-γ ELISA kit (Jingmei, China) was used according to the manufacturer's instructions.

Cytotoxic T Lymphocyte Assay

Splenocytes were harvested, prepared and maintained as described above, and a total of 1×10^7 splenocytes from each mouse were restimulated with 4×10^5 SP2/0 target cells, which express N protein stably, and treated with mitomycin C for 6 days at 37°C in 5% CO_2 in a 12-well plate before CTL assay.

Target cells (SP2/0 cells) were added at 1×10^4 cells/well to 96-well plates containing 2.5×10^5, 5×10^5 or 1×10^6 of restimulated effector cells (splenocytes) from each mouse and each concentration in triplicate. The effector:target (E:T) cell ratios were adjusted to 25:1, 50:1 and 100:1, respectively. After incubation at 37°C in 5% CO_2 for 4 h, lactate dehydrogenase (LDH) activity released into the supernatants was measured followed the manufacturer's instructions. The LDH detection kit was purchased from Roche Corporation (Germany). Maximal LDH release was determined by target cells cultured in medium added to 1% Triton X-100, spontaneous LDH release was determined by target cells cultured in medium alone. The percentage of specific cytotoxic activity was calculated as (experiment LDH release – spontaneous LDH release)/(maximal LDH release – spontaneous LDH release) × 100%.

Statistical Analysis

The data of this study are presented as mean ± SD. Statistical analyses were performed with SPSS version 10.0. Comparisons of mean humoral and cellular immune response were performed by two-tailed t tests for 2 groups of mice or by ANOVA for more than 2 groups. In all cases, $p < 0.05$ was considered statistically significant.

Results

Expression of the SARS-CoV N Protein in pcDNA3.1-N-Transfected Cells and rAd-N-Infected Cells

SP2/0 cells transfected with pcDNA3.1-N and pcDNA3.1 were selected in medium containing G418 for 2 weeks. G418-resistant stable clones were screened by Western blot assay. A 47-kDa band corresponding to the SARS-CoV-N protein was observed in the lane of SP2/0 cells transfected with pcDNA3.1-N, but not in the lane of pcDNA3.1-transfected cells (fig. 2a). Whether the recombinant adenovirus, rAd-N, could express the SARS-CoV-N protein in Vero cells was examined. Vero cells infected with rAd-N or rAd-LacZ were lysed and subjected to Western blot analysis. As demonstrated in figure 2a. A 47-kDa band corresponding to the SARS-CoV-N protein was clearly detected in rAd-N-infected cells, but not observed in rAd-LacZ-infected Vero cells.

ELISA Assay and Western Blot Analysis of the Anti-SARS-CoV-N IgG Antibody

SARS-CoV-N-specific antibody response was assessed using the standard ELISA kit for diagnosis of SARS-CoV infection. When the sera from immunized mice were separated at week 8 (groups 1–7) or at weeks 0, 2, 4, 6 and 8 (groups 8–12). The mean antibody levels of the different immunized groups in the induction of an N-protein-specific antibody response were detected and the results are shown in figure 3, and among those 7 groups, the maximum 1.258 ± 0.231 was induced by a combination of pcDNA3.1-N/pcDNA3.1-N/

pcDNA3.1-N/rAd-N at week 8. The heterogeneous combinations of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N, pcDNA3.1-N/pcDNA3.1-N/rAd-N/rAd-N, and pcDNA3.1-N/rAd-N/rAd-N/rAd-N were more efficient in the induction of SARS-CoV-N-specific antibody response than the two homologous combinations of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N and rAd-N/rAd-N/rAd-N/rAd-N at week 8 (fig. 3a; $p < 0.05$). After the mice of groups 8–12 had been sacrificed at 2-week intervals from week 0 to week 8, we observed that titers (fig. 3b) of anti-N antibody increased to the maximum 1.209 ± 0.043 in week 6 after the mice had been immunized with rAd-N/pcDNA3.1-N /pcDNA3.1-N (group 10), but at week 8 these three heterogeneous combinations of rAd-N/rAd-N/rAd-N/pcDNA3.1-N, rAd-N/rAd-N/pcDNA3.1-N/pcDNA3.1-N, and rAd-N/pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N were not found to be significantly increased ($p > 0.05$). They were equivalent or less when compared with the antibody levels at week 6. Among all humoral immune response experiments, the combination of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N induced the highest SARS-CoV-N-specific antibody response at week 8 in BALB/c mice and rAd-N/pcDNA3.1-N/pcDNA3.1-N did so at week 6 ($p > 0.05$).

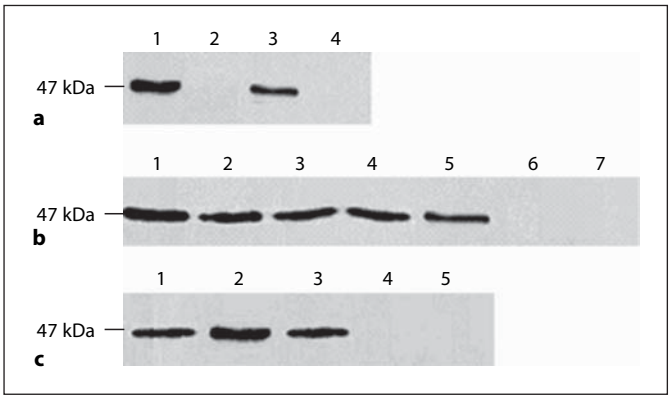

Fig. 2. Western blot analysis for N protein expression and SARS-CoV-N-specific IgG antibodies. **a** Western blot analysis for N protein expression. Lane 1: Lysates of SP2/0 cells transfected with pcDNA3.1-N. Lane 2: Lysates of SP2/0 cells transfected with pcDNA3.1 as negative control. Lane 3: Lysates of Vero cells infected with rAd-N. Lane 4: Lysates of Vero cells infected with rAd-LacZ as negative control. **b** Seven sera pools from mice of groups 1–7 were used as primary antibodies and purified N protein as antigens to perform the Western blot analysis. To lanes 1–5 were added sera from experimental groups, and to lanes 6 and 7 were added sera from the 2 groups of negative controls. **c** Five sera pools from mice of groups 8–12 were used as primary antibodies and purified N protein as antigens to perform the Western blot analysis. To lanes 1–3 were added sera from experimental groups, and to lanes 4 and 5 were added sera from the 2 groups of negative controls.

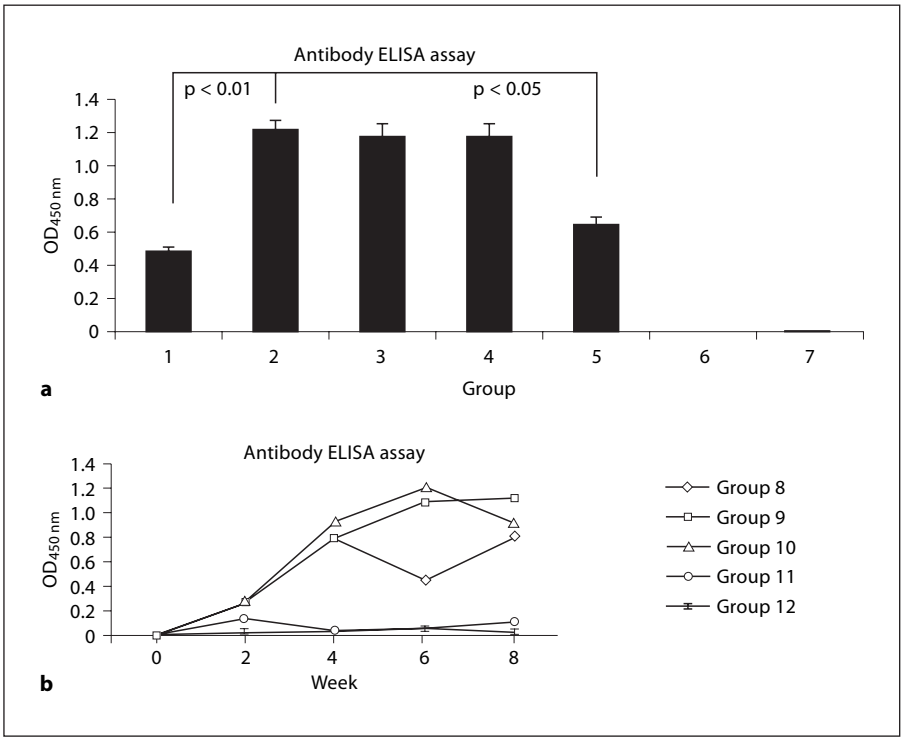

Fig. 3. ELISA assay of SARS-CoV N-protein-specific IgG antibodies. Mice (groups 1–7 (**a**) and groups 8–12 **b**)) were immunized with twelve combinations (shown in tables 1 and 2) in prime-triple boost immunization at 100 μg/mouse of pcDNA3.1-N or pcDNA3.1 and at 1.55×10^9 or 2.4×10^8 pfu/mouse of rAd-N or rAd-LacZ at 2-week intervals. ELISA was used to measure IgG antibody titers in sera at a dilution of 1:100 of individual mice and data are expressed as mean ± SD based on the $OD_{450\ nm}$ values of 5 mice in each group.

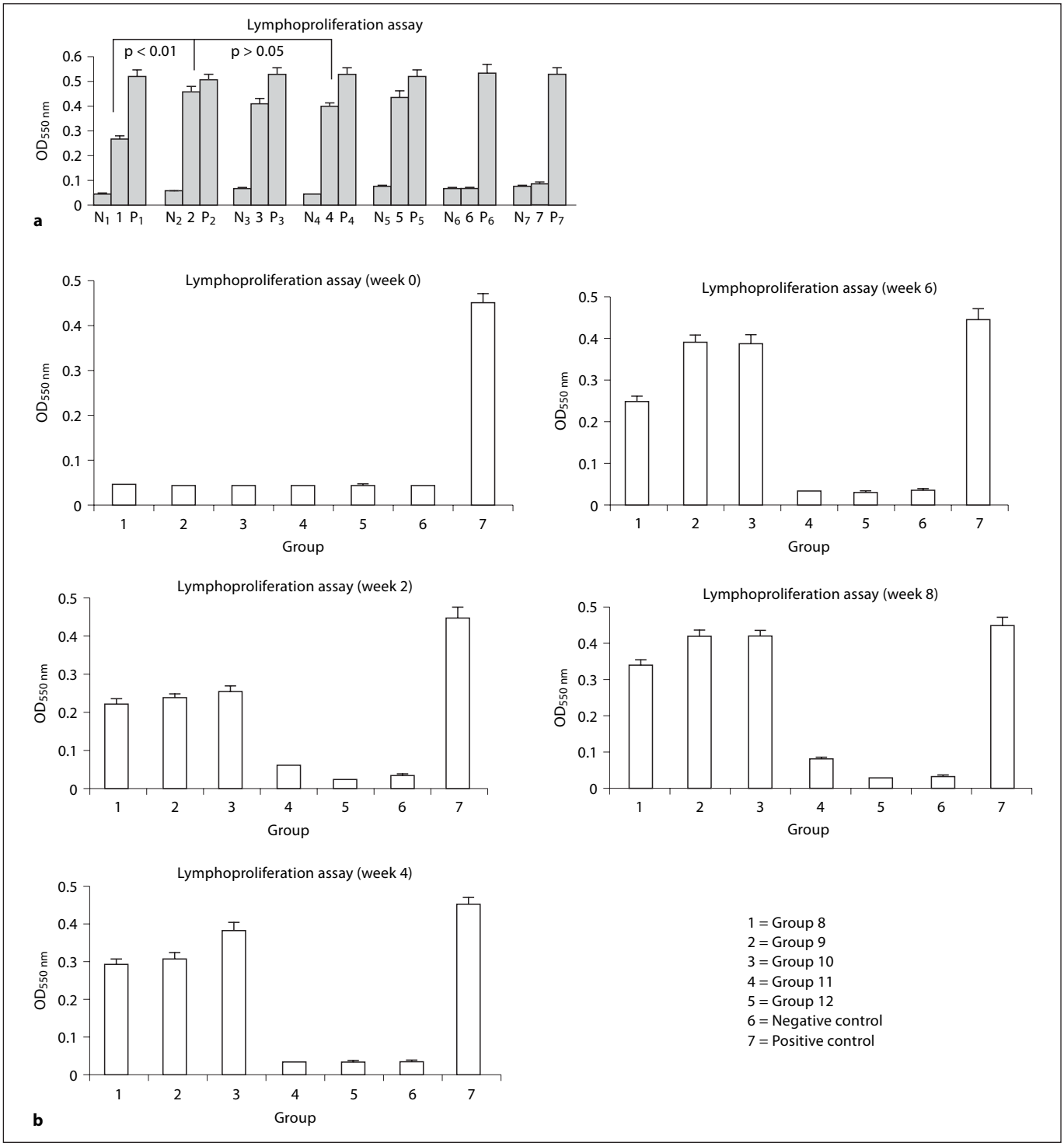

Fig. 4. Lymphoproliferative response assay. **a** Splenocytes from a single mouse each of groups 1–7 were stimulated in vitro: with 5 μg/ml of purified recombinant N protein in a well of a 96-well plate as experimental groups, labeled 1–7; with 10 μg/ml of PHA as positive control, labeled P_1–P_7, and without stimulation as negative controls, labeled N_1–N_7. **b** Splenocytes from a single mouse each of groups 8–12 were stimulated in vitro: with 5 μg/ml of purified recombinant N protein in a well of a 96-well plate as experimental groups, labeled 1–5; with 10 μg/ml of PHA as positive controls, labeled 7, and without stimulation as negative controls, labeled 6. Proliferation responses were detected by the MTT method and calculated as means of triplicate wells. Data are reported as the mean value ± SD of $OD_{550\,nm}$ of 5 or 2 mice in each group.

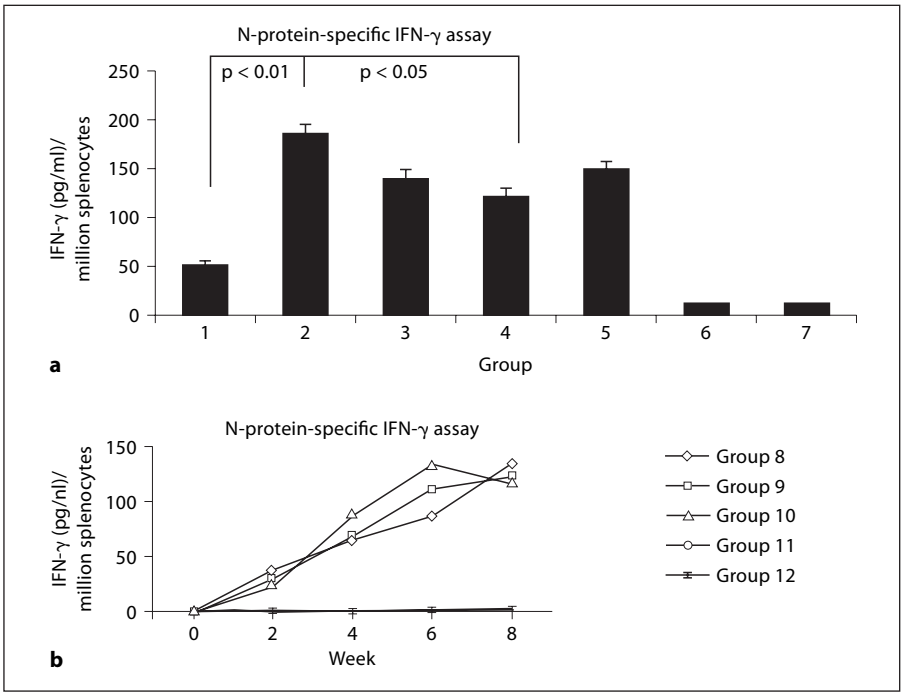

Fig. 5. ELISA assay of IFN-γ secretion. IFN-γ levels were measured in splenocyte culture supernatants from a single mouse of each immunized group (groups 1–7 (**a**), groups 8– 12 (**b**)) using a standard ELISA assay. A commercially available mouse IFN-γ ELISA kit was used according to the manufacturer's instructions. IFN-γ amounts are reported as the mean value ± SD of 5 or 2 mice in each group.

In order to detect whether the antisera removed from the immunized mice of each group were specific to the N protein of SARS-CoV, 12 serum pools from these immunized mice were collected at week 8 as the primary antibody and the purified recombinant N protein as antigen were subjected to Western blot analysis as described above. Five 47-kDa bands of groups 1–5 and three of groups 8–10 corresponding to the SARS-CoV-N protein were clearly detected but not observed in the lanes of the 4-negative control groups (fig. 2b, c).

Lymphoproliferation and IFN-γ Secretion Assay

To analyze the cellular immune response, splenocytes restimulated with recombinant N protein, PHA, and without stimulation were used as positive and negative experimental control groups, respectively, for the lymphoproliferative assay. The lymphoproliferative response levels of groups 1–7 are shown in figure 4a and those of groups 8–12 are shown in figure 4b.

In this experiment, the lymphoproliferation response was 0.478 ± 0.034 after immunization with the combination of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N at week 8 and this was the maximum among groups 1–7, and the slightly weak lymphoproliferation response of 0.432 ± 0.056 was induced by the rAd-N/rAd-N/rAd-N/rAd-N combination. They were all significantly higher than the other combinations ($p < 0.01$).

Among the mice of groups 8–12, the lymphoproliferation response increased to the highest level (0.405 ± 0.078) at week 8, induced by combinations of rAd-N/rAd-N/pcDNA3.1-N/pcDNA3.1-N and rAd-N/pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N. In addition, during immunization with the rAd-N/pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N combination, we observed that the lymphoproliferation response was sustaining at a higher level from week 4 to week 8. During immunization with the rAd-N/rAd-N/pcDNA3.1-N/pcDNA3.1-N combination, the lymphoproliferation response was sustained at a higher level from week 6 to week 8, but they were all much less ($p < 0.01$) than immunizations with the pcDNA3.1-N-prime-rAd-N boost regimen as described above. Among all mice of groups 1–12, the pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N combination induced the maximum lymphoproliferation response at week 8.

In the same culture, the secretion of IFN-γ was detected using a standard ELISA, and the results show that the mice of groups 1–7 immunized with the pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N combination induced the maximum (186.35 pg/ml) SARS-CoV-N-specific secretion of IFN-γ. The other combinations induced relatively less amounts (45.68, 145.90, 123.44,146.87, <17, and <17 pg/ml) of IFN-γ specific to SARS-CoV-N protein (fig. 5a).

From the results (fig. 5b) of combinations in groups 8–12, we found that the rAd-N/pcDNA3.1-N/pcDNA3.1-

N/pcDNA3.1-N combination stimulated the highest IFN-γ secretion (142 μg/ml) at week 6, and the rAd-N/rAd-N/rAd-N/pcDNA3.1-N combination did so at week 8, but they were all much less than the amounts of IFN-γ stimulated by the pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N combination ($p < 0.01$).

Cytotoxic T Lymphocyte Assay

We tested various combinations of pcDNA3.1-N prime-rAd-N boost immunizations and the reverse sequence immunization of rAd-N prime-pcDNA3.1-N boosts in the induction of SARS-CoV-N-specific CTLs in mice. At E:T cell ratios of 25:1, 50:1 and 100:1, there were no significant differences among the four combinations of pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N, pcDNA3.1-N/pcDNA3.1-N/rAd-N/rAd-N, pcDNA3.1-N/rAd-N/rAd-N/rAd-N, and rAd-N/rAd-N/rAd-N/rAd-N in the induction of the SARS-CoV-N-specific CTL response. Each of them was more efficient than the pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N combination using the pcDNA3.1-N prime-rAd-N boost regimen as well as the negative control. However, the pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N combination induced the maximum (about 75%) cell lysis activity at an E:T cell ratio of 100:1 ($p < 0.05$; fig. 6a).

Splenocytes from 2 mice in each group immunized with reverse sequence combinations in the induction of a SARS-CoV-N-specific CTL response were detected at weeks 0, 2, 4, 6 and 8 (fig. 6b). The results show that immunization with rAd-N/rAd-N/pcDNA3.1-N/pcDNA3.1-N induced the highest level of CTL response of 40% at an E:T cell ratio of 100:1 at week 8, and the other two heterogeneous combinations were slightly weak ($p > 0.05$). However, compared to the immunization with the pcDNA3.1-N-prime-rAd-N boost regimen, the reverse sequence immunization induced a relatively weaker CTL response ($p < 0.05$). Among all combinations in the induction of a CTL response, immunization with pcDNA3.1-N/pcDNA3.1-N/pcDNA3.1-N/rAd-N induced about 75% cell lysis activity (at an E:T cell ratio of 100:1) specific to SARS-CoV-N protein, and it is the highest level of CTL response in this experiment.

Discussion

Genetic immunization is an encouraging approach to the development of more effective vaccines in the control of some severe viral infections [31]. To prevent and control the re-emerging and transmission of SARS-CoV, except for inactivated vaccine experiments [32–34], several different research reports on DNA vaccines of SARS-CoV have been recommended, such as S gene recombinant DNA vaccine [35–38], N gene recombinant DNA vaccine [39, 40], and a combination of S, M and N gene recombinant DNA vaccines [41, 42]. These genes are delivered and expressed by different plasmids or viral vectors and elicit different levels of humoral and cellular immune responses. DNA vaccine has been shown to possess several important advantages, including the ability to induce CTLs and antibodies through the class-I and class-II antigen-processing pathways, but its efficacy does not meet our expectations [43, 44]. Different methods have been tried to increase the effectiveness of genetic immunization. Priming with a DNA vaccine can augment the efficacy of vaccines based on recombinant viral vectors. The rationale behind this strategy is that DNA priming elicits low-level but persistent immunity followed by strong boosting with virus encoding the same recombinant antigen as the DNA encodes [45]. The ability of the prime-boost regimen to induce a higher response than the DNA vaccine or virus vaccine alone may relate to the ability of recombinant adenovirus to interfere with the maturation of infected dendritic cells, decreasing their ability to present Ag to CD4 T cells, while preserving efficient Ag presentation to CD8 T cells [46, 47]. So far, the most successful DNA immunization is likely to be a consecutive immunization involving priming with plasmid DNA and boosting with recombinant virus [48–50].

Several groups have concentrated on prime-boost immunization, first priming with DNA and then boosting with the live virus vector, and the reverse sequence regimen. For instance, Schneider et al. [51] demonstrated that heterologous priming immunization with plasmid DNA followed by a single boost with a recombinant modified vaccinia virus Ankara provided complete protection against *Plasmodium berghei* sporozoite challenge and induction of high levels of epitope-specific CTLs in mice, whereas other combinations, including virus followed by DNA, DNA followed by DNA, and virus followed by virus, did not induce significant protection at all. Thus, DNA priming followed by virus boosting seems to be the best regimen in prime-boost immunization. However, Matsui et al. [45]. reported that DNA prime-virus boost immunization is not absolutely superior to the reverse sequence in the induction hepatitis C virus core-specific CTLs in mice. They found that the prime-double boost immunization with pCEP4-core (DNA vaccine) priming followed by pCEP4-core and AdexlSR3ST (virus) boosts (pC/pC/aC) has a similar efficacy compared with the

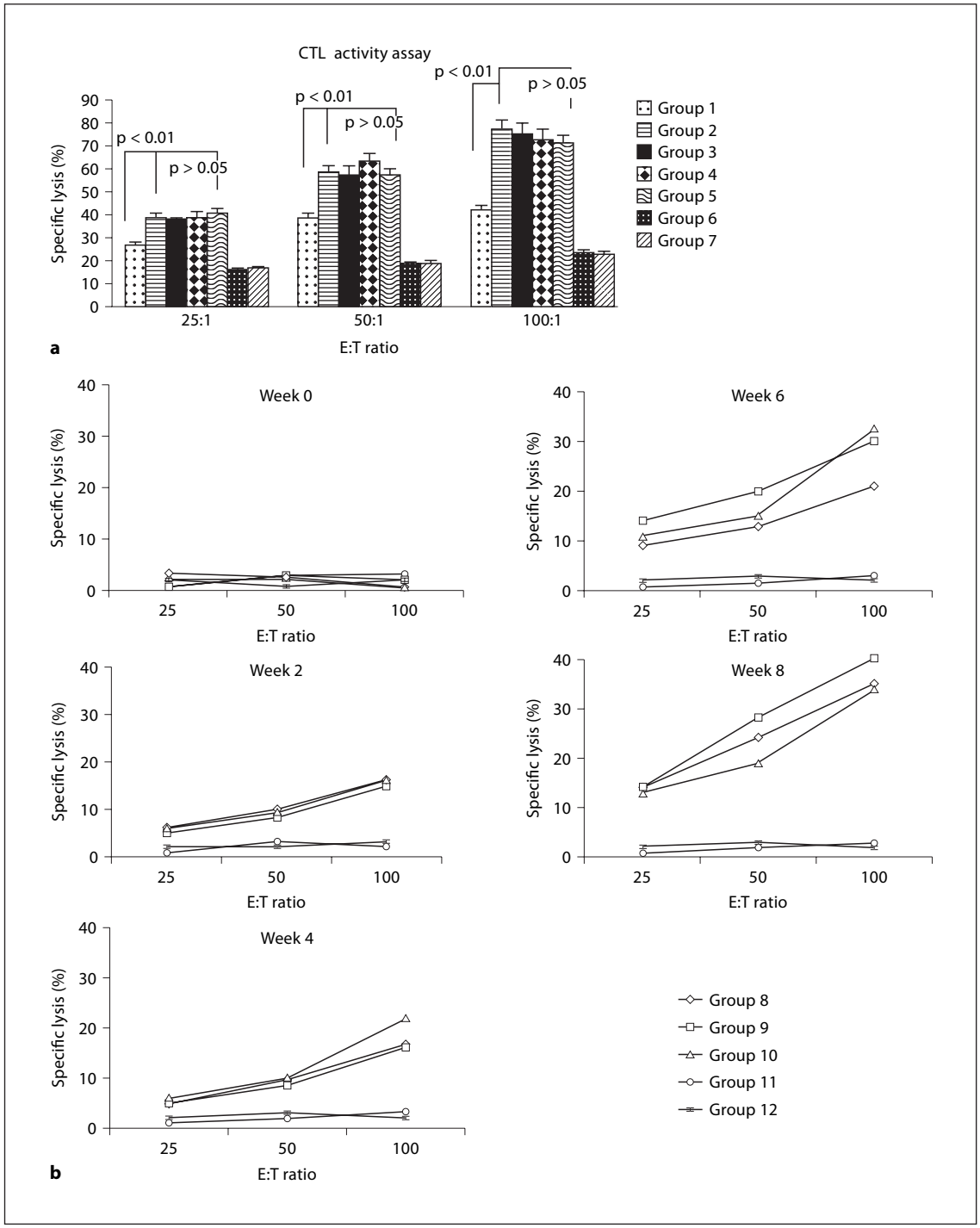

Fig. 6. LDH-release assay for CTLs. Splenocytes from mice of groups 1–7 (**a**) were prepared in week 8, and groups 8–12 (**b**) were prepared in weeks 0, 2, 4, 6 and 8, respectively. Splenocytes from a single mouse of each immunized group were re-stimulated in vitro with N protein-pressible SP2/0 cells for 6 days and tested for cytolytic activity by the LDH-release assay. Cytolytic activity is defined as the specific lysis percentage and is detected at 25:1, 50:1 and 100:1 of E:T cell ratios. Data on the specific lysis percentage of each group were calculated as the mean of 5 or 2 mice in each group and each experimental point represented the mean of triplicate wells.

combination aC/pC/pC. Furthermore, compared to homologous vectors, heterogeneous vectors induce a strong antigen-specific immune response, and the mechanism of this may be that priming with a DNA plasmid vector seems to decrease the development of immune responses to the viral vector itself [45, 52, 53].

In this study, we investigated which combination induced enhanced humoral and cellular immune responses after mice had been immunized with pcDNA3.1-N prime-rAd-N boosts and rAd-N prime-pcDNA3.1-N boost regimen. We compared the homologous prime boosting regimens and those of heterogeneous regimens, and also investigated how many boosts of pcDNA3.1-N are best after mice had been immunized with three combinations of rAd-N prime-pcDNA3.1-N boost regimen. The results of our experiments demonstrate that the DNA vaccine (pcDNA3.1-N) prime-adenovirus vector (rAd-N) boost regimen greatly enhanced the induction of a humoral and cellular immune response. In addition, in our study the rAd-N priming strategy resulted in a relatively weak induction of an immune response specific to SARS-CoV N protein, especially in the induction of CD8 T-cell response when these immunized mice were studied for their CTL activity. Regarding the ability to induce a humoral response, immunization with rAd-N/pcDNA3.1-N/pcDNA3.1-N at week 6 and immunization with rAd-N/rAd-N/pcDNA3.1-N/pcDNA3.1-N at week 8 produced similar levels of SARS-CoV-N-specific antibody response to the immunization with pcDNA3.1-N/ pcDNA3.1-N/ pcDNA3.1-N/rAd-N.

It is likely that the repeated immunizations with recombinant adenovirus vectors may induce sufficient vector immunity to interfere with the presentation of the transgene upon subsequent boosts [54, 55]. In fact, in our study, we observed that both antibody and CTL responses were not enhanced followed the third immunization with rAd-N in the repeated rAd-N prime-pcDAN3.1-N boost regimen. Unfortunately, we were not able to detect the activities of pcDNA3.1-N prime-rAd-N boosts in the induction of humoral and cellular immune responses at weeks 0, 2, 4 and 6. With regard to the times of boost immunization, in this study immunizations with pcDNA3.1 following the rAd-N prime gave higher levels of humoral and cellular immune responses 6 weeks after the third immunization, and the fourth immunization did not enhance this significantly. Therefore, the principle of priming with rAd-N followed by pcDNA3.1-N boosts was not the more the higher, and in this study no more than three times was the better regimen against SARS-CoV infection.

Among all combinations of pcDNA3.1-N prime-rAd-N boosts and rAd-N prime-pcDNA3.1-N boost regimen, pcDAN3.1-N/pcDAN3.1-N/pcDAN3.1-N/rAd-N induced the strongest humoral and cellular immune response, such as antibody, lymphoproliferation, IFN-γ production and cell lysis activity (75% at E:T cell ratio of 100:1). In addition, because the severe restrictions regarding the acquisition of the SARS virus for study purposes, it remains to be ascertained whether these responses are of sufficient extent and durability to afford protection from SARS-CoV infection.

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