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Immune responses against SARS-coronavirus nucleocapsid protein induced by DNA vaccine

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Abstract

The nucleocapsid (N) protein of SARS-coronavirus (SARS-CoV) is the key protein for the formation of the helical nucleocapsid during virion assembly. This protein is believed to be more conserved than other proteins of the virus, such as spike and membrane glycoprotein. In this study, the N protein of SARS-CoV was expressed in *Escherichia coli* DH5 α and identified with pooled sera from patients in the convalescence phase of SARS. A plasmid pCI-N, encoding the full-length N gene of SARS-CoV, was constructed. Expression of the N protein was observed in COS1 cells following transfection with pCI-N. The immune responses induced by intramuscular immunization with pCI-N were evaluated in a murine model. Serum anti-N immunoglobulins and splenocytes proliferative responses against N protein were observed in immunized BALB/c mice. The major immunoglobulin G subclass recognizing N protein was immunoglobulin G2a, and stimulated splenocytes secreted high levels of gamma interferon and IL-2 in response to N protein. More importantly, the immunized mice produced strong delayed-type hypersensitivity (DTH) and CD8⁺ CTL responses to N protein. The study shows that N protein of SARS-CoV not only is an important B cell immunogen, but also can elicit broad-based cellular immune responses. The results indicate that the N protein may be of potential value in vaccine development for specific prophylaxis and treatment against SARS.

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Keywords: Severe acute respiratory syndrome-coronavirus; Nucleocapsid protein; Immune response; Cell-mediated immunity; DNA vaccine

A worldwide outbreak of severe acute respiratory syndrome (SARS) has been associated with a novel coronavirus, termed with SARS-Coronavirus (SARS-CoV), which has strong infectivity and pathogenicity (Drosten et al., 2003; Falsey et al., 2003; Ksiazek et al., 2003). Recently, several new cases have been confirmed in Taiwan and mainland of China, Singapore, and the Philippines. The coronaviruses are large, enveloped positive-stranded RNA viruses. The spike glycoprotein protein (S) forms conspicuous peplomer structures on the surface of the virus particle and is responsible for many of the biological properties of the virus, such as attachment to cell receptors, penetration, and spread by virus-induced cell to cell fusion (Gagneten et

al., 1995; Popova and Zhang, 2002; Sanchez et al., 1999; Taguchi and Shimazaki, 2000). Antibodies to this protein not only neutralize the virus in vitro, but also provide protection against lethal virus challenge (Daniel and Talbot, 1990; Taguchi et al., 1995; Torres et al., 1995). Although the S protein is the immunodominant structural component, coronavirus infection also induces immune responses to other structural proteins, such as the membrane glycoprotein (M) and the nucleocapsid protein (N). The predicted N protein of SARS-CoV is a highly charged, basic protein of 422 amino acids with seven successive hydrophobic residues near the middle of the protein (Marra et al., 2003; Rota et al., 2003). The cellular immune response against N protein of some animal coronaviruses can enhance the recovery from the virus infection (Glansbeek et al., 2002; Wasmoen et al., 1995; Wesseling et al., 1993).

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Immunization with plasmid DNA encoding microbial antigens has provided protective immunity in some animal models and is therefore considered a potentially useful vaccine strategy (Fuller et al., 2002; Kamili et al., 2004; Padua et al., 2003). This technique involves the transfer of a target antigen gene into muscle cells by a plasmid vector with subsequent endogenous production and presentation for the induction of systemic immune responses. In this aspect, DNA immunization appeared to mimic natural viral infection. Hence, DNA vaccines may provide a useful tool to dissect the immunogenicity of a certain viral protein and define its roles in viral immunity.

In the present study, the intact N protein of SARS-CoV was expressed in *Escherichia coli* and its antigenicity was analyzed with the pooled sera from convalescence phase of SARS patients. Furthermore, a candidate DNA vaccine containing the N gene was constructed and its immunogenicity was evaluated in mice.

Results

Expression of the N protein of SARS-CoV in E. coli and identification of its antigenicity

The plasmid pHT-N encodes a fusion protein about 50 kDa with 6 histidines and other 25 amino acid residues at its N-terminal. The bands of recombinant bacteria-expressed N proteins could be visualized in SDS-PAGE as predicted. There were two other proteins of smaller molecular mass following purification with Ni-NTA affinity resin in addition to the protein band corresponding to the complete fusion protein (Fig. 1). Western blot analysis of the total bacterial protein was carried out to determine the antigenicity of the N protein. As shown in Fig. 1, all of the three proteins could react with the pooled serum of SARS patients.

Expression of N protein in COS1 cells

Intracellular expression of the N protein following transfection with the pCI-N construct was evaluated in COS1 cells by Western blot analysis. As shown in Fig. 2, lysate of COS1 cells transfected with pCI-N could react with the pooled serum of SARS patients, and the molecular mass is consistent with 46 kDa of the whole N protein. The lysate of COS1 cells transfected with control plasmid pCI-neo could not develop the binding reaction.

Antibody response to SARS-CoV N protein in mice

Mouse sera were collected at weeks 0, 2, 4, 6, 8, respectively (i.e., at 6 h prior to each of the three immunizations and at weeks 2 and 4 after the last immunization). The recombinant N protein-coated microtiter plates were used for detection of the total IgG and IgG subclass antibodies by ELISA. For the DNA vaccine pCI-N immunized mice, the specific antibodies were detectable after the first boost, and the antibody positive rates were 33%, 75%, and 75% at weeks 4, 6, and 8, respectively. As shown in Table 1, there is significant difference of ELISA values between pCI-N immunized group and control group after the first boost. The difference increases gradually following the second boost. As shown in Table 2, the increased level of IgG2a against the N protein was more significant than that of IgG1 during the observation period.

Splenocyte proliferation and cytokine secretion assays

The splenocytes from DNA vaccine pCI-N immunized mice responded significantly to the N protein in vitro while they failed to respond to the unrelated antigen transferrin. The splenocytes from control mice showed no response either to N protein or transferrin. When stimulated with N protein, the splenocytes from DNA vaccine immunized

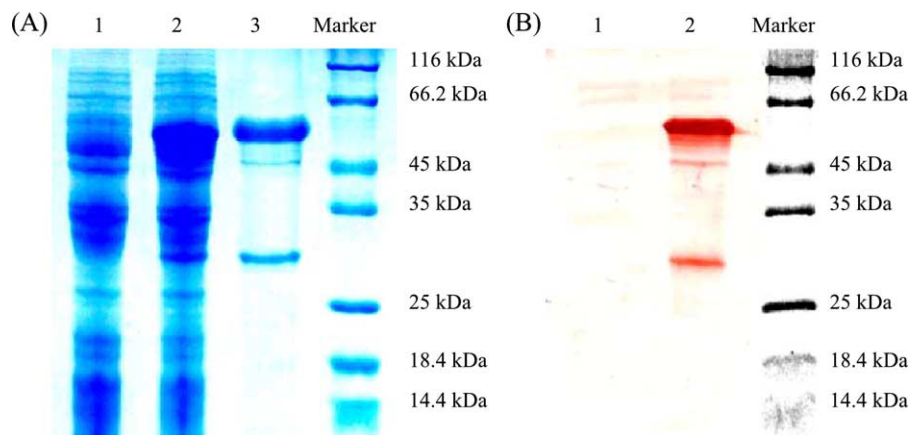


Fig. 1. Expression of SARS-CoV N protein in *E. coli* DH5 α . *E. coli* DH5 α was transformed with the recombinant plasmid pHT-N and cultured with 0.6 mmol/L IPTG for induction of the SARS-CoV N protein expression, and the lysates of the bacteria were prepared for analysis. (A) SDS-PAGE analysis. Lane 1, *E. coli* DH5 α culture without IPTG induction; Lane 2, *E. coli* DH5 α culture with IPTG induction; Lane 3, the purified N protein using Ni-NTA affinity resin. (B) Western blot analysis. Lane 1, *E. coli* DH5 α culture without IPTG induction; Lane 2, *E. coli* DH5 α culture with IPTG induction.

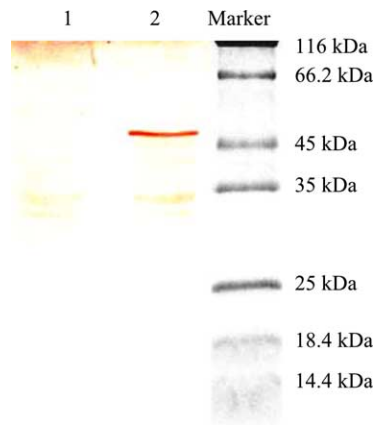


Fig. 2. Expression of SARS-CoV N protein in transiently transfected COS1 cells. COS1 cells were transfected with recombinant plasmid pCI-N or pCI-neo. After 48 h, the cells were lysed for Western blot analysis. Lane 1, lysate of COS1 cells transfected with pCI-neo; Lane 2, lysate of COS1 cells transfected with pCI-N.

group secreted significant levels of IFN- γ , IL-2, IL-4, and IL-10 compared with the control group. IFN- γ and IL-2 levels were increased more than 10- to 30-fold, respectively. IL-4 and IL-10 by stimulated splenocytes were also increased 5- to 6-fold in animals inoculated with N protein expression plasmid (Table 3).

DTH responses of mice

Four mice per group were subjected to detection of the SARS-CoV N protein-specific DTH reaction using the footpad swelling response at week 4 after the second boost. The DNA vaccine pCI-N immunized mice developed significant swelling, in which the difference of thickness between the left and right footpads at 24 h after N protein inoculation was $14.4 \pm 3.5 \times 10^{-2}$ mm, while that of the control plasmid immunized mice was only $3.3 \pm 1.4 \times 10^{-2}$ mm ($P < 0.01$). In fact, we found that the antibody activity was not paralleled with the DTH response because two antibody-negative mice also developed significant footpad swelling.

Cytotoxic T lymphocyte response

To assess CTL activity in vitro, syngeneic BALB/c-derived P815 mastocytoma cells were transfected with a plasmid construct pcDNA3.1/Hygro-N. Western blot analysis showed that the selected clonal P815 cells stably expressed the N protein of the SARS-CoV (data not presented). The results illustrate the CTL activity exhibited by splenocytes derived from pCI-N or mock plasmid immunized mice at effector/target ratios of 25:1, 50:1, and 100:1 against the P815N04 cells expressing N protein compared with native P815 target cells. As shown in Fig. 3, the splenocytes from the mock plasmid immunized mice showed no significant CTL killing activity against N protein expressing P815 cells or parental P815 cells. It is noteworthy that splenocytes from pCI-N immunized mice specifically kill only the N protein expressing P815 cells.

Discussion

The N protein of coronavirus binds to a defined packaging signal on viral RNA, leading to the formation of the helical nucleocapsid during virion assembly (Nguyen and Hogue, 1997). In the present study, we found three kinds of SARS-CoV N proteins with different molecular mass expressed in *E. coli* and all of them could react with the pooled sera from convalescence phase of SARS patients. Two of the proteins with lower molecular mass may be degraded from the amino terminus of the intact N fusion protein since both could be purified by Ni-NTA affinity resin (the six histidine residues are located at the amino terminal of the fusion protein). In our observation, the N protein is more sensitive to react with sera of SARS patients relative to the S protein of SARS-CoV expressed in *E. coli* (Zhao et al., 2004). This is consistent with those found in some animal coronaviruses, indicating that the N protein may be a sensitive antigen for SARS-CoV infection (Wesseling et al., 1993). But it is still necessary to observe its time course to evaluate the meaning for early diagnosis of SARS-CoV infection.

Table 1
Detection of IgG antibody against SARS-CoV N protein in sera of DNA immunized mice

DNA construct	Serum samples obtained at week				
	0	2	4	6	8
pCI-neo	0.08 \pm 0.03	0.10 \pm 0.02	0.12 \pm 0.02	0.13 \pm 0.02	0.14 \pm 0.03
pCI-N	0.09 \pm 0.03 ^a	0.13 \pm 0.02 ^b	0.22 \pm 0.08 ^c	0.37 \pm 0.16 ^d	0.43 \pm 0.17 ^e

BALB/c mice were immunized with 200 μ g of DNA vaccine pCI-N or control plasmid pCI-neo three times at 2-week intervals. The mouse serum samples were collected at weeks 0, 2, 4, 6, 8 post-immunization and detected for IgG against N protein of SARS-CoV. The data are presented as mean value of ELISA of 1:100 diluted serum samples \pm SD at various time courses. $n = 12$ mice.

^a $P > 0.5$, compared with that of pCI-neo immunized group.

^b $P > 0.05$, compared with that of pCI-neo immunized group.

^c $P < 0.01$, compared with that of pCI-neo immunized group.

^d $P < 0.01$, compared with that of pCI-neo immunized group.

^e $P < 0.001$, compared with that of pCI-neo immunized group.

Table 2
IgG subclasses against SARS-CoV N protein in DNA immunized mice

IgG subclasses	Serum samples obtained at week				
	0	2	4	6	8
IgG2a	0.09 ± 0.03	0.15 ± 0.03	0.30 ± 0.08	0.39 ± 0.11	0.44 ± 0.13
IgG1	0.09 ± 0.02	0.11 ± 0.02	0.16 ± 0.04	0.20 ± 0.05	0.21 ± 0.07

BALB/c mice were immunized as described previously. The serum samples were assayed for IgG2a and IgG1 against N protein of SARS-CoV. The data are presented as mean values of ELISA of 1:50 diluted serum samples ± SD at various time courses; $n = 12$ mice.

The DNA construct pCI-N, which was proved to be able to express the N protein in eukaryotic cells, was used to immunize BALB/c mice. The mouse serum anti-N antibody response was detectable in the pCI-N immunized group after the second immunization and continued to increase after boosts. Seventy five percent of the mice developed anti-N IgG antibody after three times of immunization. It is unlikely that the antibodies against N protein play a neutralizing role due to its internal position in the virion. Some researchers reported that antibodies to N protein of murine hepatitis virus (MHV, an animal coronavirus) had no neutralizing activity in vitro, but a monoclonal antibody (MAb) to the protein prevented the cytopathic effect of MHV on L cells, and passive transfer of this antibody protected mice from acute MHV infection (Lecomte et al., 1987; Nakanaga et al., 1986). That whether the antibodies to N protein of SARS-CoV have the similar protective effect in vivo needs to be further investigated.

Cell-mediated immune response represents an important defense mechanism against virus infection, which involved cytotoxic T lymphocyte activity or T cell-derived antiviral cytokines and so on (Altfeld et al., 2001; Eckels et al., 2000). There is evident malfunction of cell immunity in SARS patients, which may be one of the important causative factors for this disease (Li et al., 2003). Therefore, to enhance specific cellular immune response by vaccine administration is likely to accelerate the clearance of the virus and represents a potential alternative therapeutic method against SARS. As an internal protein of SARS-CoV, N protein is more conserved than surface protein such as S or M proteins, which makes it an ideal candidate for inclusion in a broadly protective vaccine for induction of cellular immune response. It was reported that vaccination with adenovirus vector expressing N protein of MHV could protect a significant fraction of mice against a lethal infection, which indicates that the N protein can generate

a protective immune response (Wasmoen et al., 1995; Wesseling et al., 1993). Splenic lymphocytes derived from plasmid pCI-N inoculated mice stimulated with recombinant N protein in vitro demonstrated significant proliferative responses, whereas the mice failed to respond to a control antigen transferrin.

It is known that subsets of Th cells can be distinguished by the pattern of cytokines that they produce. Th1 cells produce IFN- γ , IL-2, and lymphotoxin and play a critical role in directing cell-mediated immune responses, which are important for clearance of intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which are important for humoral responses (Gor et al., 2003; Maldonado-Lopez and Moser, 2001; Mosmann, 1992). To distinguish the activation between Th1 and Th2 subsets, profiles of cytokines released by N-stimulated splenocytes were examined. Immunization of mice with plasmids pCI-N resulted in a significant increase in Th1 cytokine production. IFN- γ and IL-2 levels were increased more than 10- to 30-fold, respectively. The production of the Th2 cytokine IL-4 and IL-10 by stimulated splenocytes was also increased 5- to 6-fold in animals inoculated with N protein expression plasmid. These results indicated that the expression of N protein by DNA vaccine activated both Th1 and Th2 subsets, although the activation level of Th1 subset was much more significant. The results were agreeable with the tendency of elevated IgG2a antibody against N protein in murine sera.

An important natural mechanism for elimination of virus in vivo is an antigen-specific cell-mediated immunity (CMI). CD8⁺ T cells recognize viral antigens in the form of short peptides presented by MHC class I molecules on the surface of virus-infected cells. The recognition of these viral peptides in the context of MHC molecules by CD8⁺ T cells can trigger the specific lysis of virally infected cells (Kagi et al., 1995) or noncytopathic intracellular inactiva-

Table 3
Splenocyte responses of mice immunized with DNA vaccine

DNA construct N	Splenocyte stimulation index		Amount of cytokine produced (pg/ml)			
	N Protein	Transferrin	IFN- γ	IL-2	IL-4	IL-10
pCI-neo	1.09 ± 0.05	1.05 ± 0.05	30 ± 3	11 ± 3	24 ± 3	10 ± 3
pCI-N	4.11 ± 0.10	1.02 ± 0.03	368 ± 15	349 ± 11	152 ± 9	47 ± 6

Splenocytes (2×10^6 /well) obtained from different groups of immunized mice were cultured in presence of N protein (3 μ g) or transferrin (20 μ g) in 96-well flat-bottomed microtiter plates in a final volume of 200 μ l. Cell-free supernatants were harvested after 72 h and cytokine levels were measured by standard ELISA kits and the splenocyte stimulation index was detected. Data represent means ± SD of triplicate cultures. One representative experiment of two is shown.

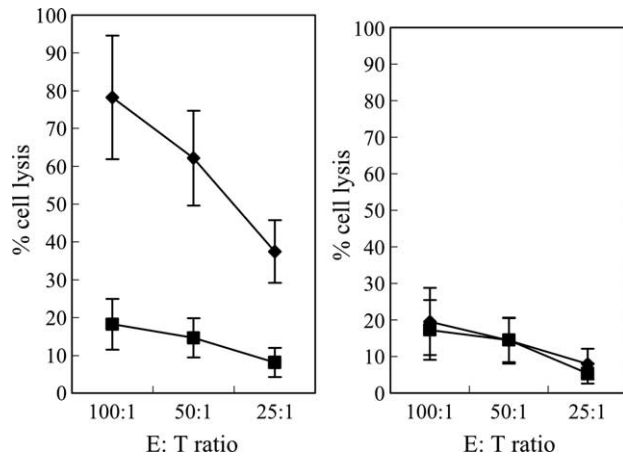


Fig. 3. CTL responses of BALB/c mice immunized with the DNA vaccine. Mice were injected with DNA vaccine for three times. Single spleen cell suspensions were assayed after *in vitro* stimulation with SARS-CoV N protein-expressing P815 cells (P815N04) for 6 days. The effector cells were then tested against target P815N04 (◆) and parental P815 cells (■) in a ^{51}Cr -release assay at the E/T ratios indicated. Values are means of triplicate determinations. The effector cells were from pCI-N immunized mice (left panel) or control plasmid pCI-neo immunized mice (right panel).

tion of virus mediated by cytokines such as gamma interferon and tumor necrosis factor α (Guidotti and Chisari, 2000; Mosmann et al., 1997). CD8^+ CTL has thus been implicated as playing a role in recovery or clearance of virus during animal coronavirus and SARS-CoV infection (Castro and Perlman, 1996; Glass and Lane, 2003; Wang and Chen, 2004).

The mice immunized with plasmid pCI-N developed significant footpad swelling responses compared with the control plasmid immunized mice. More importantly, generation of DTH activity occurred independent of anti-N antibodies, which suggests that CD8^+ T cell activation may be induced by N protein without a coexisting humoral immune response. The four test mice all developed significant DTH response although two of them failed to produce obvious anti-N antibody. Using the syngeneic BALB/c-derived P815 mastocytoma cells that stably express SARS-CoV N protein as target cells, we observed that the splenocytes from pCI-N immunized mice showed significant CTL activity, which could kill the N protein-expressing target efficiently, but could not kill the native P815 target cells. Our data on the DTH reaction assessed using footpad swelling and results of the CTL assay clearly demonstrated that SARS-CoV-specific CMI was elicited by the DNA construct pCI-N. Recently, the cytotoxic T lymphocyte response targeted to SARS-CoV N protein by DNA immunization was also reported by Zhu et al. (2004) and Kim et al. (2004). Together with our findings, we believe that the N protein of SARS-CoV is an important immunogen for T lymphocytes and may play a concerned role in immunity-mediated virus elimination.

The N protein of transmissible gastroenteritis coronavirus (TGEV) could accelerate the synthesis of neutralizing

antibodies when porcine TGEV-immune cells were stimulated with the combinations of S and N protein *in vitro*, and the reason was referred to the helper T lymphocyte response to the N protein (Anton et al., 1996). Gao et al. (2003) reported that adenoviral delivery of SARS-CoV spike protein S1 fragment, membrane protein, and N protein could induce virus-specific broad immunity in rhesus macaques, and all animals receiving intramuscular injections of recombinant adenovirus vaccine showed N-specific T-cell response and strong neutralizing antibody responses to SARS-CoV infection *in vitro*. From these data, we think that the cellular immune response to the SARS-CoV N protein may contribute to the induction of the neutralizing antibodies, such as the antibodies against the spike protein of the SARS-CoV.

In summary, our results demonstrate that the N protein of SARS-CoV is of strong antigenicity to humans, and DNA vaccination of N protein can induce both humoral and cellular immune responses in mouse. These findings suggest that the N protein should be a useful antigen candidate in the development of SARS vaccines for the specific prophylaxis of SARS-CoV infection.

Materials and methods

Construction of prokaryotic expression plasmid for N gene of SARS-CoV

The full-length N gene (1266 bp in length) of SARS-CoV was synthesized according to the published sequence (GenBank accession number: AY278554) and inserted into the plasmid pUC18. The plasmid containing the N gene was used as the template to amplify the DNA fragment for construction of the prokaryotic expression plasmid using the following primers: NF: 5'-GCTAGCGGATCCACCATGTCGATAATGGACCCCAATC-3'; and NR: 5'-gaattcTTA-TGCCTGAGTTGAATCAGCAG-3'. The amplified DNA was transferred into pMD-18T vector (TaKaRa) using TA-cloning and the correct sequence was verified. The recombinant plasmid pHT-N was constructed by subcloning the N gene into the prokaryotic expression plasmid pProEX HTa (Life Technologies) at its *Bam*HI–*Eco*RI site.

Expression and identification of the N protein of SARS-CoV

The recombinant plasmid pHT-N was transformed into *E. coli* DH5 α for expression of the N protein. A fresh transformant was incubated overnight in liquid LB medium containing 100 mg/L ampicillin. The culture was diluted with 2 \times YT medium at 1% of volume, and incubated at 37 °C until A_{600} reached 0.6 absorbance units. Then IPTG was added at a final concentration of 0.6 mmol/L for the induction of N protein expression. The cultured bacteria were collected after 3 h and subjected to SDS-PAGE and Western blot analyses. Serum antibodies

against SARS-CoV were collected from convalescent SARS patients in Beijing Xiaotangshan Hospital, and inactivated by incubation at 56 °C for 30 min. The patient sera were pooled, and used as the primary antibody in Western blot, while the horseradish peroxidase (HRP) conjugated goat-anti-human IgG (Sigma) was used as the secondary antibody.

The expressed N protein was purified using Ni-NTA affinity resin (Qiagen) according to the manufacturer's directions. The purified N protein was further treated with Detoxi-Gel Endotoxin Removing Gel (PIERCE, Rockford) and tested by the *Limulus* ameocyte lysate assay (Sigma) to ensure that it was free of endotoxin contamination (below detection level <0.1 EU/mL).

Construction of DNA vaccine containing SARS-CoV N gene

The recombinant plasmid pMD18-T containing the full-length N gene was digested with *NheI* and *EcoRI*. Then, the N gene fragment was isolated and inserted into the eukaryotic expression plasmid pCI-neo (Promega) at *NheI*–*EcoRI* site. The recombinant plasmid pCI-N was served as DNA vaccine candidate for the further use.

Expression of SARS-CoV N protein in COS1 cells

COS1 cells were grown in DMEM medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL streptomycin sulfate at 5% CO₂. The recombinant plasmid pCI-N and control vector pCI-neo were transfected into COS1 cells using Lfectamine reagent (Gibco-BRL) according to the manufacturer's instructions, respectively. Logarithmically growing cells in 35 mm dishes were transfected with 1 µg of plasmid and 10 µl of Lfectamine reagent. At 48 h after transfection, the cells were washed twice with ice-cold PBS and lysed in 1×SDS lysis buffer (50 mM Tris (pH 6.8), 50 mM DTT, 2% SDS, 10% glycerol, 100 µg/mL PMSF, 10 µg/mL leupeptin and 0.1% bromophenol blue) for 10 min on ice. Cell lysates were boiled and centrifugated at 12 000 × *g* at 4 °C for 10 min. The supernatants were collected and subjected to Western blot analysis.

DNA immunization of mice

The vaccine candidate pCI-N and control vector pCI-neo were prepared using Qiagen MegaPrep columns (Qiagen). The DNA plasmid was mixed with 3.4% polyvinyl pyrrolidone (PVP, MW 40,000, Sigma) in 0.01 M PBS to a final concentration of 2 g/L for immunization of mice. When used in DNA vaccine formulations, PVP binds to DNA, which facilitates uptake by skin and muscle cells (Alila et al., 1997; Anwer et al., 1998). Six-week-old female BALB/c mice (Xiper-Bikai Experimental Animal Co., LTD, Shanghai) were randomly divided into two groups (12 mice per group). The mice in experimental group were injected

with 200 µg of pCI-N in both tibialis anterior muscles three times at 2-week intervals. The mice in control group received the same amount of the vector pCI-neo in identical route and frequency.

Antibody measurement

Microtiter plates were coated with purified N protein at concentration of 50 mg/L in carbonate buffer. After 4 °C overnight, the coated plates were blocked with 5% nonfat dry milk in PBS. The plates were then used for the detection of the antibody against N protein of SARS-CoV in mice. Mouse sera were collected by retroorbital bleeding and kept in –70 °C. The serum samples were serially diluted with 0.01 M PBS containing 12% goat sera and 0.5% Tween-20 prior to antibody detection. The serum antibody was captured by the coated N protein, and detected by 1:10000 diluted HRP conjugated goat-anti-mouse IgG (Sigma). Color was developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate, and the absorbance at 450 nm (*A*₄₅₀) was measured. It was considered positive when the *A*₄₅₀ value of the mouse serum from experimental group is over or equal to 2.1-time of mean value of the mouse serum from the control group.

A similar protocol was followed for ELISA assessing IgG subclass. Sera from individual mice were 1:50 diluted for detection of IgG2a and IgG1. The HRP-conjugated goat-anti-mouse IgG2a and IgG1 were used as secondary antibodies (SouthernBiotech).

Splenocyte proliferation assay

Four mice per group were sacrificed at week 4 after the last immunization. Spleens were isolated and ground between the frosted ends of two glass microscope slides. The cell suspension was then passed through a fine nylon mesh to obtain a single cell suspension. Erythrocytes were lysed by incubation in 0.84% NH₄Cl solution. Splenocytes were cultured in triplicate in 96-well flat-bottomed plates at 2 × 10⁶ cells per well in 200 µl RPMI 1640 containing 10% heat-inactivated fetal bovine serum. Stimulated cells received purified N protein at a final concentration of 15 µg/mL or transferrin (Sigma) at 100 µg/mL, while the control cells received no additional protein stimulation. The plates were incubated at 37 °C in 5% CO₂ for 72 h. Following stimulation, 100 µl of culture supernatant was collected from each well for subsequent cytokine analyses. The splenocyte proliferation of each well was measured by the tetrazolium compound, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), and phenazine ethosulfate (PES) according to the manufacturer's suggestions (Promega) (Mahon et al., 2003). The stimulation index was defined as the ratio of the mean absorbance at 490 nm per well of the stimulated cells and control cells.

Cytokine production assay

Splenocyte proliferation assays were carried out as described above. The supernatants of splenocyte culture were harvested and subjected to measurement for IFN- γ , IL-2, IL-4, and IL-10 with ELISA detection systems (Pharmin-gen) (Pertmer et al., 1996). In brief, the microtiter plates coated with rat anti-mouse IFN- γ , IL-2, IL-4, or IL-10 were incubated with duplicates of serially diluted samples and standards overnight at 4 °C. Plates were then washed and biotinylated secondary antibodies specific for murine IFN- γ , IL-2, IL-4, or IL-10 at 2 μ g/mL were added to each well. After 45 min at 37 °C, plates were washed and avidin-phosphatase (1/500 dilution; Pharmingen) was added at 100 μ l per well. After 30 min at room temperature, the plates were washed again. The color was developed with *p*-nitrophenyl phosphate (Sigma) as the substrate, and the absorbance at 405 nm was measured. The concentrations of cytokines in the supernatants were determined from the standard curve.

Detection of delayed-type hypersensitivity(DTH)response

The DTH response was assessed by a footpad swelling method (Eaton et al., 2001; Shibata et al., 2001). At week 4 after the last immunization, a total of 25 μ l PBS containing 10 μ g of recombinant N protein was injected into the left footpad of the mice in both experimental and control groups. The right footpad received sterile PBS as control. After 24 h, the mice were euthanized and footpad swelling was measured with a spring-loaded metric caliper. Left footpad thickness minus right footpad thickness was calculated.

Cytotoxic cell assay

The SARS-CoV N gene sequence was removed from the plasmid pCI-N by *Nhe*I-*Xba*I digestion, and inserted into an expression vector pcDNA3.1/Hygro(+) with hygromycin as selectable marker (Invitrogen). The resulting plasmid pcDNA3.1/Hygro-N was transfected into P815 cells and the stable transfectants were selected by hygromycin. A hygromycin-resistant cell clone P815N04, which was proved to be able to express endogenously SARS-CoV N protein by Western blot analysis (data not shown), was used for the CTL assays.

The CTL activity was determined by a standard 51 Cr-release assay (Uger and Barber, 1997). Spleen cells from immunized mice were suspended in complete DMEM containing 10% FCS and 50 μ M 2-mercaptoethanol and analyzed for cytotoxic activity 5 days after in vitro stimulation. Recombinant murine IL-2 was added once at day 2 at a concentration of 10 U/mL, and responded cells (5×10^7) were cocultured with 1×10^7 irradiated syngeneic cells P815 N04 (8000 rad) stably expressing the N protein. Cytotoxic effector lymphocyte populations were harvested after 6 days of incubation. A 5-h 51 Cr-release assay was

performed in a 96-well round-bottomed plate using as target cell lines 51 Cr-labeled P815N04 or parental P815 cells. CTL assays were performed at lymphocyte effector/target (E/T) ratios of 100:1, 50:1, 25:1. The amount of 51 Cr released was determined by gamma counting and the specific lysis was calculated according to the following formula: percentage specific lysis = (experiment release – spontaneous release) / (maximum release – spontaneous release). Experimental release represents the mean counts per minute released by target cells in the presence of effector cells. Total release represents the radioactivity released after total lysis of target cells with 5% Triton X-100. Spontaneous release represents the radioactivity present in medium derived from target cells only.

Statistical analysis

Data obtained were analyzed for statistical significance by Student's *t* test. *P* < 0.05 was considered significant.

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