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## Induction of SARS-nucleoprotein-specific immune response by use of DNA vaccine

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### Abstract

Induction of effective cytotoxic T lymphocyte (CTL) and/or a specific antibody against conserved viral proteins may be essential to the development of a safe and effective severe acute respiratory syndrome coronavirus (SARS-Cov) vaccine. DNA vaccination represents a new strategy for induction of humoral and cellular immune response. To determine the ability of SARS-Cov nucleoprotein (N protein) to induce antiviral immunity, in this report, we established a stable C2C12 line expressing SARS-Cov N protein, which was used as a target for specific CTL assay. We also expressed recombinant N proteins in *Escherichia coli* and prepared N protein-specific polyclonal antibodies. C3H/He mice were immunized with N protein-expressible pcDN-fn vector by intramuscular injections. We found that the DNA vaccination induced both N protein-specific antibody and specific CTL activity to the target. When C3H/He mice were immunized by three separate injections, high antibody titre (1:3200–1:6400, average titre is 1:4580) and high CTL activity ( $67.4 \pm 8.4\%$  ( $E:T = 25:1$ ),  $69.6 \pm 6.7\%$  ( $E:T = 50:1$ ) and  $71.8 \pm 6.2\%$  ( $E:T = 100:1$ )) were observed. In the case of two vaccine injections, CTL activity was also high ( $56.6 \pm 12.7\%$  ( $E:T = 25:1$ ),  $57.4 \pm 11.7\%$  ( $E:T = 50:1$ ) and  $63.0 \pm 6.3\%$  ( $E:T = 100:1$ )). However, antibody titres were much lower (1:200–1:3200, average titre is 1:980). Our results suggest that SARS-Cov nucleocapsid gene might be a candidate gene for SARS DNA vaccination.

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### 1. Introduction

The causative agent of severe acute respiratory syndrome (SARS) was identified as a new type of coronavirus, the SARS coronavirus (SARS-Cov). Determining immunogenicity of the virus proteins for possible vaccine development is important for SARS prevention.

SARS-Cov genome contains five major open reading frames (ORF) encoding the replicase polyprotein, the spike (S), the envelope (E), membrane (M) glycoproteins and the nucleocapsid protein (N) in the same order and of approximately the same size as those of other coronaviruses [1–3]. Additionally, SARS-Cov also has several small non-structural ORFs that are found between the S and E genes and between the M and N genes.

The structural proteins encoded by the five ORFs function during host cell entry and virion morphogenesis and release. During virion assembly, N protein binds to viral RNA and leads to formation of the helical nucleocapsid. It is predicted that N protein is a highly charged, basic protein of 422 amino acids with a short lysine rich region suggestive of a nuclear localization signal [1].

For vaccine development, it is critical to generate protective immune responses including specific antibody and cytotoxic T lymphocyte (CTL) generation. Some reports demonstrated that protective responses elicited by antigens of some viruses that were not present on the surface of the virion, such as the N protein, were more likely to be due to CTLs. For example, nucleoproteins of Ebola virus [4,5], measles virus [6], lymphocytic choriomeningitis virus [7] and influenza virus [8,9] may induce protective CTLs. In porcine coronavirus, transmissible gastroenteritis virus (TGEV), N protein is a representative antigen for the T-cell response and may induce cellular and humoral immune response [10].

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The immunogenicity of SARS N protein, especially for induction of specific CTL, remains unknown.

Recent clinical data showed that immunological responses of SARS patients were greatly changed after infection [11–14], such as neutralization antibody production, T lymphocytes ratio alternation, etc. Recently, Wang [15] demonstrated that the N protein fragment inhibited binding of SARS-Cov lysate to serum antibody of SARS patients and bound to antibodies in >94% SARS serum. Based on these observations, we hypothesize that N protein expressed in viral infected cells may be a potent mediator of the body's immune system, and thus N protein may be a potential target for SARS-Cov vaccine. To address this issue, we detected N protein-specific immune response by use of DNA vaccine and, as expected, found that DNA vaccination with N protein could induce specific antibody and specific CTLs. Our results implicates that SARS N protein may be a target for DNA vaccination.

## 2. Materials and methods

### 2.1. Mice

Female C3H/He mice, 4–5 weeks, were purchased from Shanghai Experimental Animal Center (Shanghai, China). Female adult New Zealand rabbits, 5–6 kg, were purchased from Nanjing Experimental Animal Center (Nanjing, China).

### 2.2. Cell line and transfection

C2C12 is a myoblast cell line derived from C3H mice. The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 µg/ml streptomycin and 100 u/ml penicillin and 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL). Electroporation transfection was performed exactly as described by the manufacturer's manual for Cell Porator II (Gibco BRL).

### 2.3. Construction of expression vectors

SARS-Cov nucleocapsid protein (also nucleoprotein) gene (*n* gene was mentioned in following text) was synthesized chemically based on sequence reported (AY304495, GeneBank). At the 5'(ATG) and 3'(TAA) ends of full coding region, *EcoRI* and *BamHI* restriction sites were introduced respectively for subcloning into pGEM3Z. The resultant plasmid (pGEM-fn) was digested with *EcoRI* and *BamHI* and a 1.2 kb released fragment was then ligated to pcDNA3.1 vector digested with the same enzymes. The recombinant plasmid (pcDN-fn) could express full length N protein in mammalian cells and was used as a DNA vaccine. pcDNA3.1 vector was used for vaccination control. To express recombinant SARS N protein in *Es-*

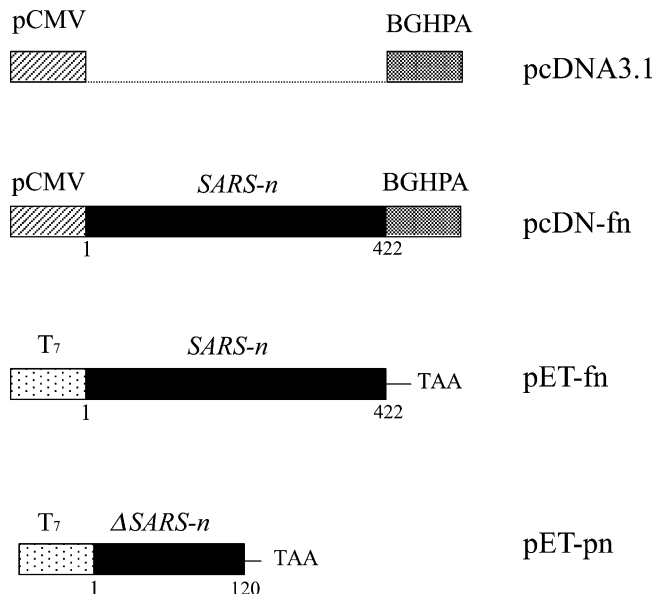


Fig. 1. Schematic diagram of plasmid constructs expressing SARS-Cov N protein in mammalian expression vector and prokaryotic expression vector. The vehicle vector for DNA vaccination, pcDNA3.1 contains a CMV promoter and BGH polyadenylation sequence. Full length of *n* gene (labeled as SARS-*n* in this figure) released from pGEM-fn by *EcoRI/BamHI* digestion was inserted into pcDNA3.1, the resultant plasmid was indicated as pcDN-fn. For expressing protein in *E. coli*, *EcoRI* site in 5' end of *n* gene was mutated to *NdeI* site and thereafter inserted *NdeI/BamHI* fragment into pET24a vector. The resultant plasmid is indicated as pET-fn. pET-pn indicates a truncated mutant of pET-fn in which only 1–120 aa region ( $\Delta$ SARS-*n*) is remained as described in Section 2. TAA indicates stop codon. The number is the amino acid position of N protein sequence.

*cherichia coli* for polyclonal antibody preparation, we used pfu DNA polymerase chain reaction with primer pair P1 (5'-GAATTCATATGTCTGATAATGGACCCC-3') and P2 (5'-AAGGATCCTTATGCCTGAGTTGAATCAG-3'), and modified the SARS *n* gene in pGEM-fn by introducing *NdeI* and *BamHI* site to the 5' end and 3' end, respectively. pET-fn expression plasmid was made by ligating the *NdeI/BamHI* *n* gene fragment into pET24a vector digested with *NdeI* and *BamHI*. To make an expression plasmid of truncated N protein, pET-fn was cut with *HindIII* and *BamHI* to remove the region from 121 to 422 aa and then self ligated after blunting the ends by Klenow large fragment. The resultant plasmid (pET-pn) was able to express truncated N protein in *E. coli*. All constructs are summarized in Fig. 1.

### 2.4. Polyclonal antibody preparation

After transformation of BL21(DE3) with pET-fn and pET-pn, the recombinants were induced by 0.2 mM IPTG for 5 h. Cell pellets were collected by centrifugation and lysed by 8M urea and ultrasonic. The lysates were loaded onto DEAE-A50 column pre-equilibrated by Tris buffer (20 mM Tris-HCl pH 8.3, 5 mM 2-ME, 2 mM EDTA), and the fraction passing through was collected for further purification. Proteins in this fraction were resolved by

SDS-PAGE gel electrophoresis and recovered from the gel by KCl staining method [16].

For making anti-N protein polyclonal antibodies, purified N proteins were mixed with Freund's adjuvant and immunized sequentially in rabbits three to four times. The sera were collected a week after final immunization and the antibody titres were detected with enzyme-linked immunosorbent assay (ELISA).

### 2.5. Determination of serum IgG by ELISA

The mice were bled and their sera were assayed for antibody activity by standard quantitative ELISA. Briefly, 10 µg purified protein was coated in each well of ELISA plate by which captured reactive antibody from serum. The binding IgG was detected by HRP-labeled secondary antibody. A serum pool from several non-immune mice was used as normal serum control.

### 2.6. DNA vaccination of mice

Plasmid DNA for vaccination was prepared with a Qiagen plasmid purification kit. The purified DNA was dissolved in 20% sucrose at a concentration of 1 mg/ml. Female C3H/He mice were immunized by intramuscular injections with 0.05 ml of plasmid DNA in a separate site on each side of the quadriceps muscle, followed by one or two booster vaccination at 2 week intervals [17]. In all cases, a total volume of 50 µl was injected per muscle, using an insulin syringe with 5 G needle.

### 2.7. Splenocyte re-stimulation *in vitro*

For cytotoxicity assays, mice splenocytes were harvested 2 weeks after both two and three injection protocols. *In vitro* re-stimulation of primed splenocytes was achieved by C2C12-N, a C2C12 cell line expressing N protein made by transfection of pcDN-fn plasmid into C2C12 cells as described in the Section 2.2. The single-cell suspension of splenocytes was prepared from individual mouse spleen in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 µM 2-mercaptoethanol and 20 iu/ml of interleukin 2. A total of  $3 \times 10^7$  splenocytes were then re-stimulated with  $6 \times 10^5$  Mitomycin C-treated C2C12-N target cells for 5 days at 37 °C in 5% CO<sub>2</sub> prior to CTL assay. The target cells were been treated with 10 µg/ml of Mitomycin C for 5 h and washed 3 times.

### 2.8. Cytotoxic T-cell assay

Target cells were washed three times and added in triplicate at the rate of  $1 \times 10^4$  cells per well to 96-well round-bottom plates containing different amounts of re-stimulated effector cells. The effector:target ratios were adjusted from 100:1 to 25:1. After incubation at 37 °C in

5% CO<sub>2</sub> for 4 h, LDH activity released into the supernatants was determined according to the method published previously [18]. Maximal LDH release was made by treating cells with PBS containing 1% Triton-X100; spontaneous LDH release was determined from the wells containing the target cells and medium only. The percent specific lysis was calculated as follows:  $100 \times (\text{experimental LDH release} - \text{spontaneous LDH release}) / (\text{maximum LDH release} - \text{spontaneous LDH release})$ .

## 3. Results

### 3.1. Expression and purification of SARS-N protein in *E. coli*

To detect SARS-n gene expression in C2C12-N target cells and measure N protein-specific antibody produced in vaccinated mice, recombinant N protein and polyclonal antibody were prepared first. Full length and truncated N genes were inserted into pET24a expression vector, respectively. After induction with IPTG, as we expected, both pET-fn and pET-pn cell lysates showed obvious extra expression bands of ~ 48 and 15 kDa, respectively, as shown in Fig. 2A and B. It was also found that both proteins were expressed in soluble form other than in inclusion body form. The results of Western blot assay showed that these two proteins could also react with the serum antibody of mice immunized with pcDN-fn DNA vaccine as shown in Fig. 2C. We purified the recombinant proteins by DEAE-A50 absorption and SDS-PAGE gel extraction method. The resultant pure proteins were injected into New Zealand rabbits for polyclonal antibody preparation and two kinds of antibodies (FN-Ab, anti-full length N protein, and PN-Ab, anti-partial N protein) were obtained. The titration of both antibodies was over 1:30,000 averagely.

### 3.2. Stable expression of N protein in C2C12 cells and as target cells for measuring CTL response

To determine expression of pcDN-fn in myoblast cells, C2C12 cells were transfected with pcDN-fn plasmid by electroporation. G418 resistant stable lines were screened by Western blot assay and an N protein-expressible stable clone was amplified for target cell identification. Initially, we used PN-Ab as the first antibody and detected a unique band of size 48 kDa as shown in Fig. 3. This suggests no post-translational modification of SARS-Cov nucleocapsid gene in C2C12 cells.

### 3.3. Induction of SARS-N protein-specific antibody

C3H/He mice were injected intramuscularly with 100 µg of plasmid DNA for two times at 0 and 2nd week or for three times at 0, 2nd and 4th week. Anti-N protein antibody was measured at the 6th week after the first injec-

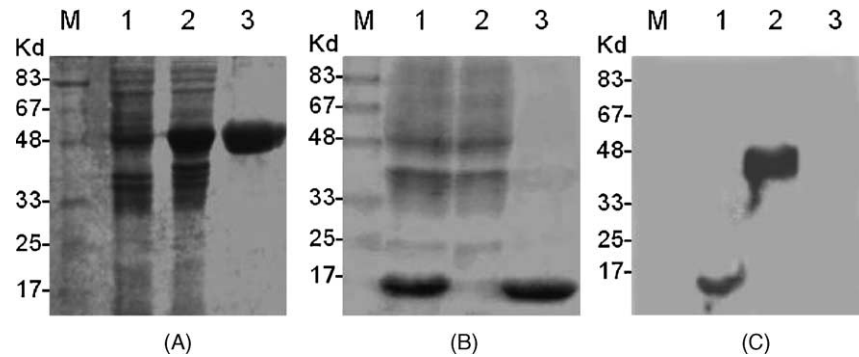


Fig. 2. Expression and purification of SARS-Cov N protein in *E. coli*. Full length of *n* gene (1–422 aa) (pET-fn) and partial *n* gene (1–120 aa) (pET-pn) were inserted into pET24a vector and transformed into BL21 (DE3) *E. coli*. After induction with IPTG, cells were harvested and lysed by 8 M urea and ultrasonic. Cell lysate was centrifuged. Recombinant proteins in supernatants were purified by DEAE-A50 absorption and gel extraction method [16]. A: expression of full length N protein in *E. coli*. Lane M: marker; lane 1: without IPTG induction; lane 2: with IPTG induction; lane 3: purified protein. B: expression of partial N protein in *E. coli*. Lane M: marker; lane 1: with IPTG induction; lane 2: without IPTG induction; lane 3: purified protein. C: Western blot assay. Cell lysates were resolved on SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was then incubated with 1:1000 serum of C3H/He mice which had received pcDN-fn vaccination and HRP labeled secondary antibody, sequentially. Positive bands were visualized by ECL reagent. Lane M: marker, lane 1: cell lysate of pET-pny lane 2: cell lysate of pET-fn; lane 3: BL21 (DE3) control cell lysate.

tion. Sera were collected from the mice injected with the pcDN-fn construct or pcDNA3.1 control and the titers were quantified by ELISA with full length or truncated recombinant N proteins. As shown in Fig. 4, anti-N protein antibodies were detected in mice vaccinated with pcDN-fn DNA. When the mice received two injections, specific antibody level was much lower than three injections. Antibody titre was also calculated and defined as dilution folds at which OD490 nm reading value was three times over control serum level ( $P/N > 3$ ). The result showed that the antibody titres of the two injections group were 1:200–1:3200, and averaged 1:985. For groups receiving three injections, titres were

1:3200–1:6400, and the average was 1:4580. The anti-N protein antibody raised by DNA vaccination also reacted specifically with recombinant N protein expressed in *E. coli* as shown in Fig. 2C.

#### 3.4. Induction of CTL in vivo by pcDN-fn DNA

To examine CTL level in DNA-immunized mice, splenocytes were collected from individual mice 2 weeks after two or three injections with pcDN-fn and pcDNA3.1. Splenocytes cultures were re-stimulated in vitro for 5 days with Mitomycin C-treated C2C12-N cells. Cytolysis activity of the splenocytes was measured in a LDH release assay. When C3H/He mice were immunized by three injections, as expected, the group of vaccinated mice that received pcDN-fn developed a much higher level of target lytic activities:  $67.4 \pm 8.4\%$  ( $E:T = 25:1$ ),  $69.6 \pm 6.7\%$  ( $E:T = 50:1$ ) and  $71.8 \pm 6.2\%$  ( $E:T = 100:1$ ). In the case of two injections, CTL activities were also at a high level:  $56.6 \pm 12.7\%$  ( $E:T = 25:1$ ),  $57.4 \pm 11.7\%$  ( $E:T = 50:1$ ) and  $63.0 \pm 6.3\%$  ( $E:T = 100:1$ ) as shown in Fig. 5 (left part and right upper part). There was no significant difference between groups the received two or three injections. CTL activities of both groups were much higher than that of pcDNA3.1 control as shown in Fig. 5 (right lower part).

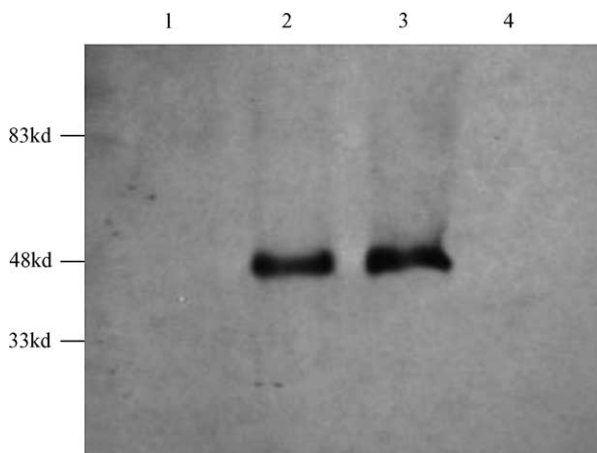


Fig. 3. Expression of SARS-Cov nucleocapsid protein in C2C12 cells.  $2 \times 10^6$  C2C12 cells were harvested and washed twice with cold HBS buffer (21 mM HEPES pH7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM  $\text{Na}_2\text{HPO}_4$ , 6 mM glucose) and re-suspended with 0.5 ml HBS buffer mixed with 10  $\mu\text{g}$  pcDN-fn DNA. The cell suspension was electroporated (60  $\mu\text{F}$ , 200 V). The transfected cells were selected with G418 (250  $\mu\text{g}/\text{ml}$ ) and stable N protein-expressing clones were screened by Western blot assay. Lane 1: C2C12 cell control; lane 2: N protein-expressible line 1; lane 3: N protein-expressible line 2; lane 4: no N protein expression line.

## 4. Discussion

SARS-Cov vaccine may be a very effective method for preventing SARS. A successful vaccine should be able to induce proper humoral and/or cellular immune response. DNA vaccination has been used to express antigens in vivo for the generation of both humoral and cellular immune responses, and is a promising therapy, especially for life-threatening diseases. In this report, we detected

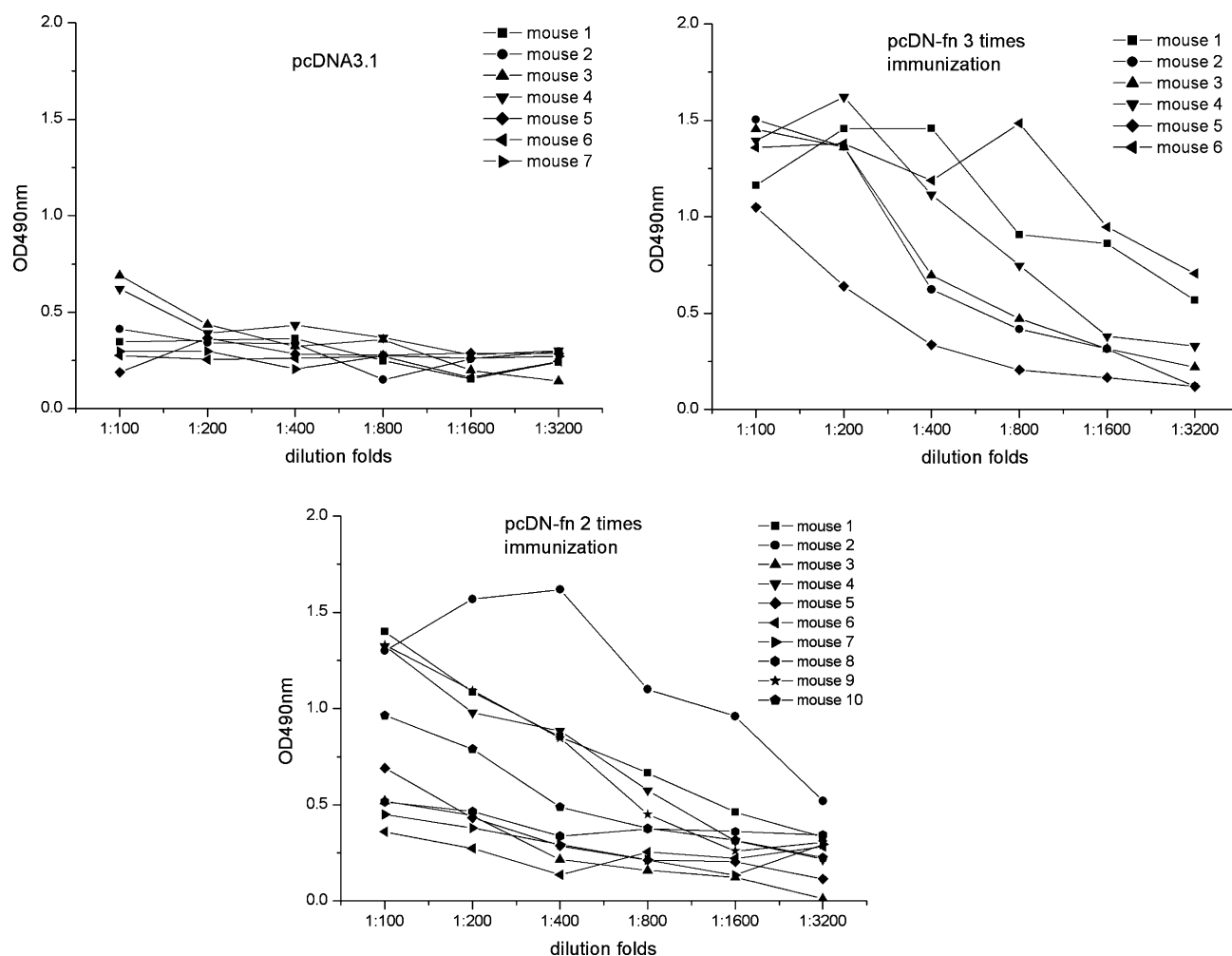


Fig. 4. Induction of SARS-Cov N protein-specific IgG antibody in mice immunized with pcDNA3.1 and pcDN-fn DNA. C3H/He mice given two or three intramuscular injections of the different DNA vaccines at 100  $\mu$ g per mouse were bled at 5th to 6th week post-immunization, and ELISA was used to measure IgG antibody titers in individual mice.

SARS-Cov N protein-specific immune response induced by DNA vaccination and found that specific antibody titres were as high as 1:4580 and specific CTL activities were also high. These results indicate that N protein which, naturally exists in virus particles in after binding of viral RNA, was able to induce strong humoral and cellular immune responses when induced by DNA vaccine, and it might be a prospective candidate gene for development of SARS-Cov vaccine.

During preparation of this paper, Gao et al. [19] published their results concerning SARS-associated coronavirus vaccine in monkeys. They used an adenoviral-based vector that simultaneously expressed spike protein S1 fragment, membrane protein and nucleocapsid protein as a vaccine, and found that it induced a SARS-Cov-specific T-cell and virus neutralizing antibody response in vitro (the specific CTL activity was not detected in this paper). They suggest the requirement of nucleocapsid protein for virus-specific antibody generation. This point was also supported by the data of TGEV [10]. Together with our results, therefore, we be-

lieved that SARS-Cov N protein was of importance not only for enhancement of antibody production but also for specific CTL generation, and N protein was an important target for SARS vaccine.

After infection with SARS-Cov, specific antibody against total virus and N protein could be generated [11–14,20]. Anti-N protein antibody in SARS patients is not detectable in the 1st week after onset of symptom, but it can be detected in almost all SARS patients in the 3rd week after onset of disease. It is believed that antibody responses induced by N protein of SARS is immunodominant and may play an important role in the viral response [13]. Our results in this study showed that level of antibody generation (1:985) after immunization with two injections was much lower than that of three injections (1:4580), but the CTL activity already reached a high level. Thus, we speculate that the cellular immune response might also play an important role in immune response to SARS viral infection.

One weakness of this study is that we do not have a SARS animal model for determining the protective effects against

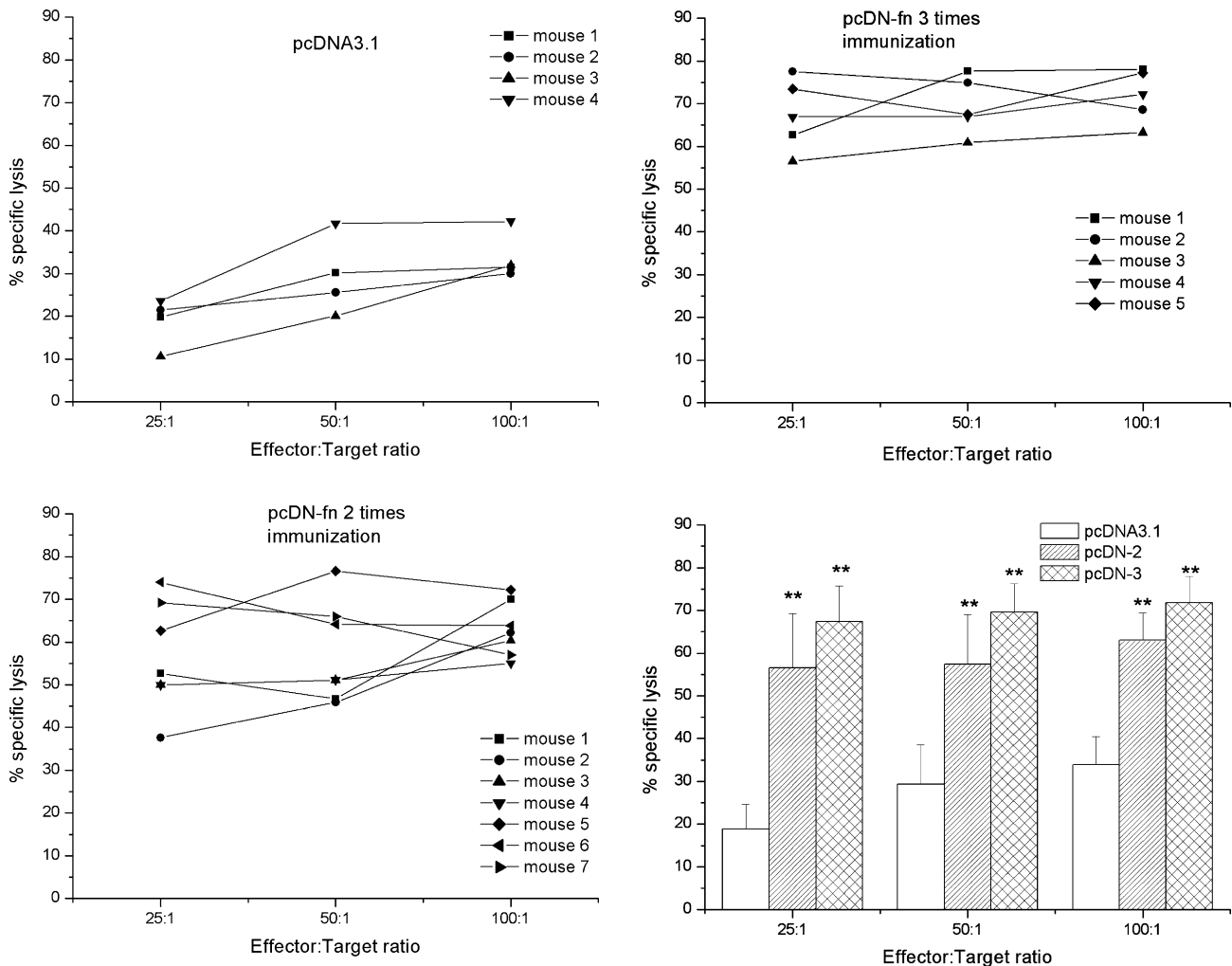


Fig. 5. Induction of SARS-Cov N protein-specific CTLs in mice immunized with pcDN-fn DNA. Splenocytes of the mice receiving pcDN-fn or pcDNA3.1 vaccination were re-stimulated in vitro with N protein-expressible C2C12 cells and tested for cytolytic activity by LDH-release assay. Cytolytic activity was defined as specific lysis percentage and detected at various *E:T* ratio. The values of each experimental point represent the mean of triplicates. \*\* Represents  $P < 0.01$ .

SARS virus due to severe restrictions against acquiring the SARS virus for study purposes.

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