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Vaccine

Priming with SARS CoV S DNA and boosting with SARS CoV S epitopes specific for CD4+ and CD8+ T cells promote cellular immune responses

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Abstract

Cellular immune response plays an important role in antiviral immunity. In our previous study, immunization of mice with severe acute respiratory syndrome coronavirus (SARS CoV) spike (S) DNA vaccine could induce both humoral and cellular immunity in response to a pool of entire overlapping S peptides. Identification of functional dominant epitopes in SARS CoV S protein for T cells is crucial for further understanding of cellular immune responses elicited by SARS CoV S DNA vaccine. In present study, mice were immunized with SARS CoV S DNA vaccine. Subsequently, a pool of 17–19 mers overlapped SARS CoV S peptides, which served as immunogens, were scanned to identify the specific epitopes for T cells. Two H-2^d restricted CD4+ T epitopes, N60 (S435–444) and P152 (S1111–1127), and two H-2^d restricted CD8+ T cell epitopes, N50 (S365–374) and P141 (S1031–1047) were identified by three different methods, enzyme-linked immunosorbent assay (ELISA), enzyme linked immunospot assay (ELISPOT) and fluorescence activated cell sorter (FACS). The dominant CD4+ T cell epitope (N60) and CD8+ T cell epitope (N50) located in the receptor-binding domain (RBD) of SARS CoV S protein, which mediated virus combining and fusing to susceptible cells. Importantly, our novel finding is that mice primed with SARS S DNA vaccine and boosted with T cell epitopes (N50 and N60) could promote antigen specific CD4+ and CD8+ T cell immune responses. Our study provides valuable information for the design of vaccine for SARS study.
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Keywords: SARS CoV; T cells; Epitope; Vaccines

1. Introduction

Severe acute respiratory syndrome (SARS) is a new infectious disease caused by a novel coronavirus named as SARS CoV [1,2], which infected 8096 people and killed 774 in 2003 [3]. SARS CoV consists of four major structural proteins: spike (S), membrane (M), envelope (E) and nucleacapsid (N). S protein is a large type I transmembrane glycoprotein responsible for receptor binding and membrane fusion [4,5]. The angiotension converting enzyme 2 (ACE-2) on the susceptible cell surface is served as a receptor for SARS CoV. And a small 193 amino acid fragment on SARS CoV residue (S318–510) was characterized as a minimal functional receptor-binding domain (RBD) [6,7]. Moreover, CD209L (L-SIGN) is another receptor for SARS CoV [8]. It has been reported that SARS CoV infection induces both humoral and cellular immunity. Neutralizing antibodies specific for SARS CoV were detected on days 5–10 after the onset of syndrome in SARS patients. The levels of antibodies peaked on days 20–30 and sustained for more than 150 days [9]. Sera from convalescent SARS patients could be

transferred to SARS patients for the treatment during the SARS outbreak [10]. At the early stage of SARS, although levels of serum cytokines including IL-2, IL-10 and IL-12 increased significantly, CD3^{+}, CD4^{+} and CD8^{+} T cell counts in peripheral blood decreased compared to normal individuals [11]. In addition, SARS CoV associated memory CD4^{+} and CD8^{+} T cells specific for SARS CoV S, N and E proteins were generated and sustained for more than 2 years [12–14].

Currently, there is no effective drug to prevent or treat SARS. Therefore, developing SARS vaccines will be an effective way to prevent SARS spreading. Several vaccines for SARS CoV have been developed and proved to be effective in animals [15]. Whole SARS CoV particles inactivated by formaldehyde, UV light or β-propiolactone could induce neutralizing antibodies in animals, and these vaccines were being tested in clinic trials in China [16]. Yang et al. reported that a candidate DNA vaccine encoding the full-length S protein induced neutralizing antibodies, which could protect mice from SARS CoV challenge [17]. After immunization with SARS CoV DNA vaccine, cellular immunity was induced by the evidence of high frequencies of IFN-γ producing effector/memory CD4^{+} and CD8^{+} T cells in mice [18]. Additionally, adenovirus, attenuated para-influenza virus and modified attenuated modified vaccinia virus Ankara (MVA) vaccines encoding SARS CoV S protein were used to immunize animals. Results indicated that these vaccines could elicit high titers of neutralizing antibodies [19–21].

BALB/c mice immunized with SARS CoV S peptides specific for B cells (S91–102, S424–435, S458–469 and S1065–1076) with Freund adjuvant could trigger a rapid and highly effective immune response in vivo [22]. Meanwhile, four human HLA-0201 restricted CD8^{+} T cell epitopes S411–420, S978, S1203 and SSp-1 were demonstrated in recovered SARS patients [23–25].

In order to identify the epitopes of SARS CoV S antigen for T cells, mice were immunized with SARS CoV DNA vaccines. One hundred and sixty nine SARS CoV S peptides were mapped for specific stimulation of T cells to produce IFN-γ. Our data confirmed previous studies [19] and demonstrated that two dominant H-2[d] restricted epitopes N50 and N60 were specific for CD8^{+} and CD4^{+} T cells, respectively. Importantly, our novel finding is that mice boosted with T cell epitopes could specifically and significantly increase the cellular immune responses of both CD4^{+} and CD8^{+} T cells, suggesting that epitopes for T cells could be used for vaccination in SARS study.

2. Materials and methods

2.1. Mice

Female BALB/c and C57BL/6 mice, 6–8 weeks old, were purchased from Zhongshan University Animal Center (Guangzhou, China) and maintained in animal care facility under pathogen-free conditions.

2.2. SARS CoV S DNA vaccine, S peptides and CpG ODN sequence

Plasmids encoding severe acute respiratory syndrome coronavirus (SARS CoV) spike (S) protein was constructed as described [17] and kindly provided by Dr. Gary J. Nabel from the Vaccine Research Center (VRC), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD, USA. Plasmid DNA was purified by plasmid purified kit (QIAGEN). The 260/280 ratios ranged from 1.8 to 2.0. The endotoxin content from purified plasmid DNA was below 10 EU/ml in which the level had no effect on the immune response. Seventeen to nineteen-mer peptides with 10 amino acids (aa) overlap spanning the entire SARS CoV S protein were synthesized and kindly provided by Dr. Richard A. Koup from VRC, NIAID, NIH, Bethesda, MD, USA. In total, there are 169 peptides. Pools (1–8) of 20 consecutive peptides were prepared, and pool 9 contained 9 peptides (P161–169). Peptides were dissolved in DMSO at 25 mg/ml and used at the final concentration of 1 μg/ml (for each) in all experiments, except for the dose response studies. Peptides N50 (S365–374, KCYGVSATKL) and N60 (S435–444, NYNYKYRYLR) were synthesized and kindly provided by School of Pharmaceutical Sciences, Sun Yat-sen University, Guangdong, China. The CpG oligodeoxynucleotide (ODN) 1826 (TCC ATG ACG TTC CTG ACG TT) was synthesized by Sangon Corp. (Shanghai, China).

2.3. Antibodies

Purified anti-CD28 and anti-CD16/CD32, PerCP conjugated anti-CD4, FITC conjugated anti-CD62L, PE conjugated anti-CD8, APC-Cy7 conjugated anti-CD8, PE or APC conjugated anti-IFN-γ, FITC conjugated anti-IL-2 and isotype-matched control monoclonal antibodies (mAbs) were purchased from BD/PharMingen (San Diego, CA). FITC conjugated anti-IL-7R mAb was obtained from eBioscience (San Diego, CA).

2.4. Immunization

BALB/c and C57BL/6 mice were injected intramuscularly (i.m.) with 50 μg truncated SARS CoV S plasmid DNA in 100 μl PBS in the left thigh. Mice were boosted twice with 3-week intervals. In DNA prime-peptides boost experiments, BALB/c mice were immunized twice with 50 μg SARS CoV S plasmid DNA with 3-week intervals in the left thigh. Three weeks later, mice were boosted twice with 3-week intervals by subcutaneous (s.c.) injection of peptides (N50 and N60, 50 μg of each) with or without 25 μg CpG ODN at the base of tail. PBS and CpG ODN were administrated alone as controls. Each experiment was repeated 2–3 times with consistent results and 3–5 mice were used in each experiment.

2.5. Cell culture and IFN-γ ELISA

Mice were sacrificed. Lymph node, spleen and lung from individual mouse were harvested one to two weeks after the final boost vaccination. Single-cell suspensions were prepared and plated in a 96-well micro-titer plate at 4×10^5 cells/200 μl per well. Pooled SARS CoV S peptides (1 μg/ml for each) or single peptide (1 μg/ml) with anti-mouse CD28 mAb (1 μg/ml) were added to cultures. Supernatants of cell cultures were collected 72 h later, and levels of IFN-γ were assessed by specific ELISA kit (BD PharMingen) according to the manufacturer's protocol. The detection limit of the IFN-γ assay kit was 3.13 pg/ml.

2.6. ELISPOT assay for IFN-γ

Assay was performed using mouse ELISPOT set (BD PharMingen) according to the instruction provided by the manufacture. Briefly, single-cell suspensions were prepared from spleens of mice after vaccination, and plated in 96 well microplates (Millipore) pre-coated with anti-IFN-γ antibody overnight at 4 °C and blocked by RPMI-1640 containing 10% FBS. Cells were incubated for 14–20 h in the presence or absence of selected SARS CoV S peptides plus anti-CD28 (1 μg/ml) at 37 °C and 5% CO_2. Wells were washed and incubated with biotinylated labeled anti-mouse IFN-γ antibody for 2 h at room temperature. After washing, wells were incubated with streptavidin conjugated horseradish peroxidase for 1 h at room temperature. Wells were extensively washed again, and developed with 3-amino-9-ethyl-carbazole (AEC) substrate solutions. After drying, wells were counted by ChampSpot II ELISPOT reader (Sage Creation, China).

2.7. Cell surface and intracellular cytokine staining

Single-cell suspensions from lymph node, spleen and lung of mice after vaccination were stimulated with or without SARS CoV S peptides plus anti-CD28 (1 μg/ml) for 5 h at 37 °C and 5% CO_2. Brefeldin A (10 μg/ml, Sigma) was added in the last 4 h incubation. Cells were washed, fixed with 4% paraformaldehyde, and permeabilized in PBS buffer containing 0.1% saponin (Sigma), 0.1% BSA and 0.05% NaN_3 overnight at 4 °C. Cells were then stained with conjugated mAbs specific for cell surface antigens CD4, CD8, CD62L and IL-7Rα (CD127), and intracellular cytokines IFN-γ and IL-2 for 20–30 min at 4 °C in dark. Cells (300,000–600,000) were acquired on flow cytometer (BD Calibur) and data were analyzed with the program FlowJo version 6.0 (Tree Star, Inc., USA). Isotype-matched controls for cytokines were included in each staining.

2.8. IgG ELISA assay

The titers of peptide (N50, N60) specific antibodies in sera from SARS CoV S DNA immunized mice were mea-sured by ELISA. Briefly, sera were obtained from mice 1–2 weeks after boost vaccination. The 96-well plates were pre-coated overnight with 5 μg/ml SARS peptides N50 and N60, respectively. Unrelated peptide K9I (KYGVSVQVI, *Lister innocua*) was included as negative control, and truncated SARS CoV S protein was used as positive control. Plates were washed by PBST (0.05% Tween 20 in PBS) and blocked for 2 h in 10% FBS containing PBS at room temperature. Wells were washed again and serial-diluted serum samples were added to the wells in triplicate for 2 h at room temperature. After washing, horseradish per-oxidase conjugated anti-mouse IgG (Jackson, USA) was added at 1:2000 dilution for 1 h at room temperature. Wells were extensively washed and substrates tetramethylbenzidine (TMB) and hydrogen peroxide (BD PharMingen) were added to wells. Twenty minutes later, 10% H_2SO_4 were added to stop reaction. Plates were read at 450 nm by using Elx800 universal microplate reader (BIO-TEK, USA).

2.9. Statistics

Statistical evaluation of differences between means of experimental groups was performed by analysis of variance and a non-parametric two-tailed t test. A value of <0.05 was considered to be significant.

3. Results

3.1. Scanning immunodominant peptides of the SARS CoV S protein in BALB/c mice

To determine the antigenic site on SARS CoV S protein, a set of 169 peptides contained library spanning the entire sequence of SARS CoV S protein was synthesized. Each peptide contains 17–19 mers with 10aa overlapped by adjacent peptides. One hundred and sixty nine peptides were divided into nine pools as described in Section 2.

One to two weeks after the final boost, splenocytes were prepared from SARS CoV S DNA vaccine-inoculated mice and stimulated with each peptide pool. Three days later, IFN-γ levels were detected by ELISA. As shown in Fig. 1A, IFN-γ induced by peptides in pool 3 and pool 8 accounted about 60% and 30% of that induced by the entire S peptides pool, respectively.

Furthermore, peptides in pool 3 (Fig. 1B) and pool 8 (Fig. 1C) were screened as described above. P50 (S358–374, STFFSTFKCYGVSATKL), P51 (S365–382, KCYGVSATKLNDLCFSNV), P59 (S427–444, NIDAT-STGNYNYKYRYLR), P60 (S435–451, NYNYKYRYL-RHGKLRPF) in pool 3 and P141 (S1031–1047, LMSF-PQAAPHGVVFLHV), P144 (S1054–1071, ERNFTTA-PAICHEGKAYF), P151 (S1103–1120, FVSGNCDVVIGI-INNTVY), P152 (S1111–1127, VIGIINNTVYDPLQPEL) in pool 8 seemed to be potential T cell epitopes.
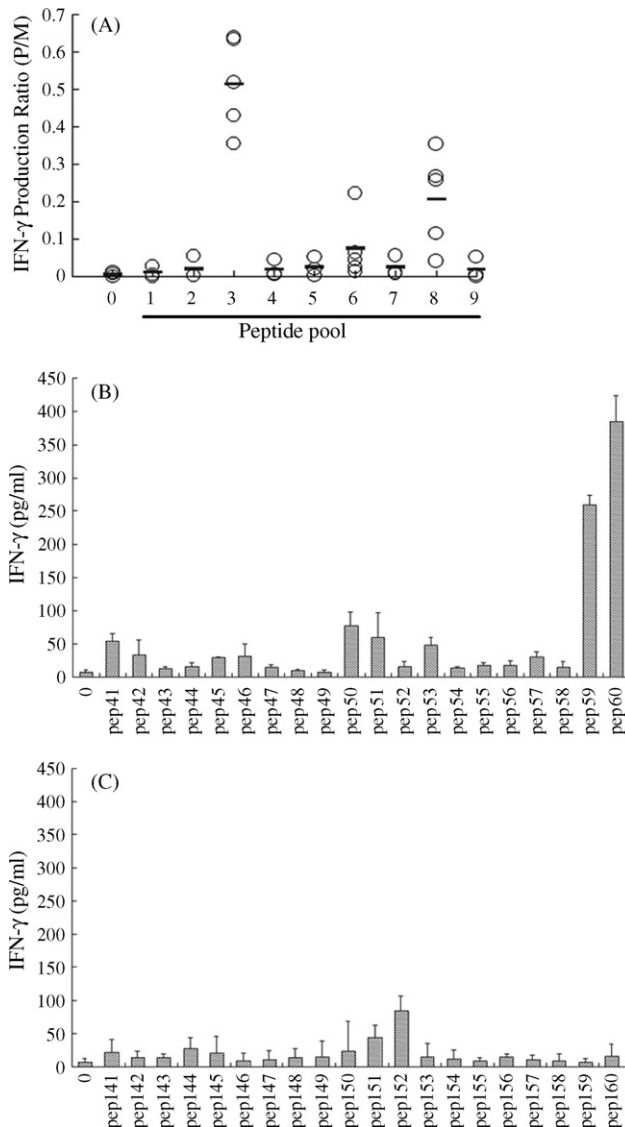
Fig. 1. Determination of epitopes by IFN-γ production. (A) BALB/c mice were immunized i.m. by SARS CoV S DNA. One to two weeks after the final boost immunization, splenocytes were prepared and stimulated with or without peptide pools. Seventy-two hours later, supernatants were collected and levels of IFN-γ were detected by ELISA. Results represented the ratio of IFN-γ levels induced by a single pool (1–9) compared to the mixed S peptide pool in the same experiment. Each open circle represents mean value of an independent experiment. Cross bar represents the average results of three to five times experiments.

Each individual peptide in pool 3 (B) and pool 8 (C) was screened as described previously. Experiments were done in triplicate. The solid bars represent the mean values ± standard deviation. "0" represents non-stimulated control.

3.2. Identification of potential SARS CoV S epitopes

The potential SARS CoV S epitopes were tested repeatedly by splenocytes from DNA vaccine immunized BALB/c mice. ELISA results demonstrated that P59 and P60 could dramatically induce high levels of IFN-γ after cultured with cells (Fig. 2A). However, ELISPOT results indicated that the

highest frequencies of antigen specific IFN-γ producing cells were induced by P50 and P51 (Fig. 2B).

Furthermore, intracellular IFN-γ and IL-2 were assessed on both CD4+ and CD8+ T cells (Fig. 2C). CD8+ IFN-γ+ cells and low frequency of CD8+IL-2+ T cells could be detected in splenocytes after stimulation with peptides P50, P51 and P141. The results demonstrated these peptides were major histocompatibility complex (MHC) class I molecule-restricted peptides.

In contrast, IFN-γ+ cell populations induced by P59 and P60 were restricted to CD4+ T cells, accompanied by apparent IL-2+ cell populations. It demonstrated that these peptides were MHC class II molecule-restricted peptides. Although peptide 152 could not induce apparent IFN-γ expression, a small population of CD4+ IL-2+ T cells was induced, suggesting that this peptide was MHC-II restricted. The expression of IFN-γ and IL-2 induced by P144 and P151 were too low to be detected by FACS. Therefore, it is difficult to determine whether P144 and P151 induced cytokine production from CD4+ or CD8+ T cells.

3.3. Identification of 10 amino acid peptides: N50 and N60

P50 and P51 as well as P59 and P60 were adjacent peptides, and exhibited similar roles as showed above. Hence, we wondered whether each two peptides contained a same epitope. To test this hypothesis, overlapping sequence between P50 and P51 (N50, KCYGVSATKL) and between P59 and P60 (N60, NYNYKYRYLR) were synthesized.

ELISA (Fig. 3A) and ELISPOT (Fig. 3B) results indicated that peptide N50 and N60 possessed the same abilities to induce IFN-γ production as their original 17aa peptides P50 and P60, respectively. The FACS results also showed that shorting in length had no effects on the MHC restriction of these two peptides (Fig. 4C).

Furthermore, dose-dependent responses of new synthetic peptides N50 and N60 were assessed by ELISPOT. The results showed that frequencies of IFN-γ producing cells induced by N50 were no significantly different when the concentration of N50 was tested from 0.08 to 10 μg/ml. However, the frequency of IFN-γ producing cells decreased quickly from 200/10^6 cells to zero (Fig. 3C) when the concentration of N60 dropped from 10 to 0.08 μg/ml.

3.4. Effects of peptide N50 and N60 on different gene background mice

To explore effects of peptide N50 and N60 on different gene background mice, both BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were inoculated with SARS S DNA vaccine. Splenocytes were prepared and stimulated with N50 and N60 alone or both. ELISA (Fig. 4A) and ELISPOT (Fig. 4B) results indicated that N50 could induce a notable IFN-γ response in DNA vaccine-immunized BALB/c mice, but have no effect on the cells from DNA vaccine-immunized
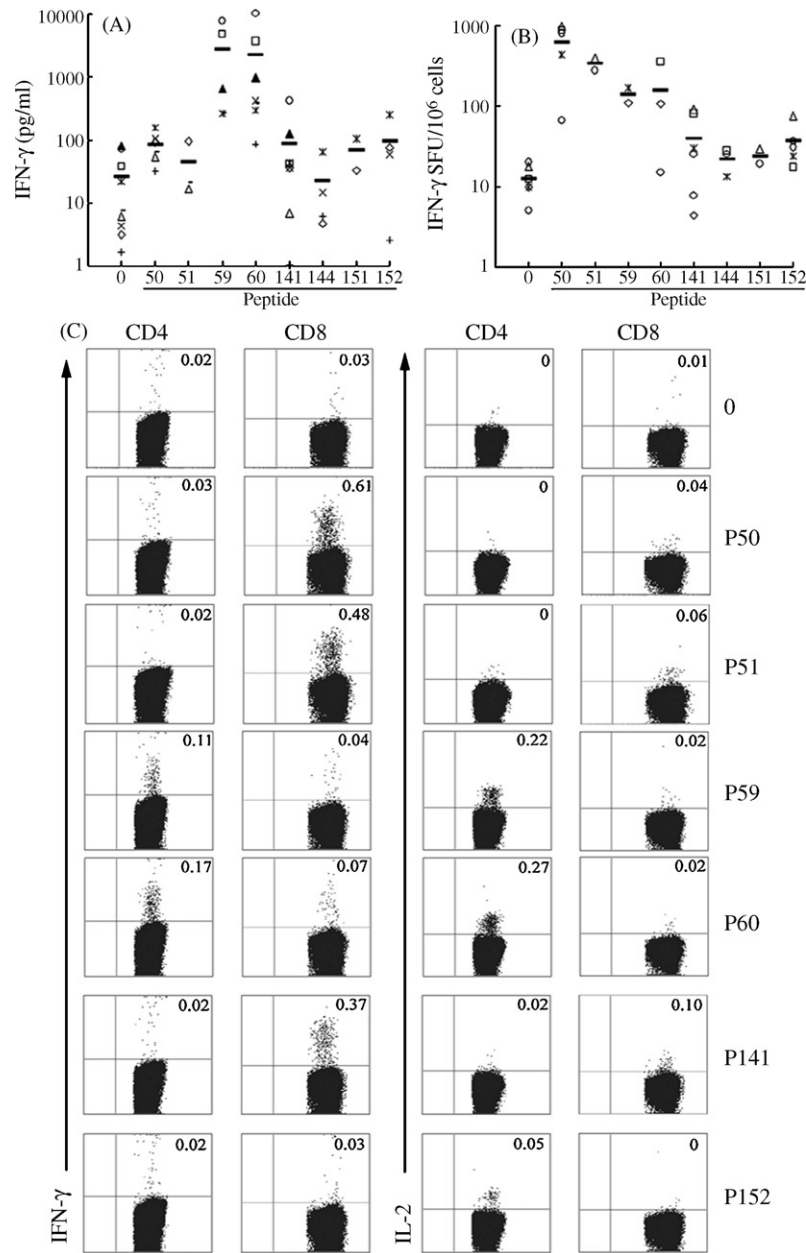
Fig. 2. Verification of potential SARS CoV S epitopes. Potential SARS CoV S epitopes P50, P51, P59 and P60 in pool 3, and P141, P144, P151 and P152 in pool 8 were used to stimulate splenocytes from SARS CoV S DNA immunized BALB/c mice. IFN-γ production was detected by ELISA (A) and ELISPOT (B), respectively. Each symbol represents the results of an individual experiment ($n = 3–7$). In addition, intracellular cytokine staining (C) was performed to determine CD4$^+$ or CD8$^+$ T cell population. "0" represents the non-peptide stimulated control. Numbers at the corner in each sample represent the percentage of positive cells. Representative results of three independent experiments were shown.

C57BL/6 mice. Although N60 could induce considerable IFN-γ producing cells in DNA vaccine-immunized C57BL/6 mice, the level of IFN-γ in supernatants induced by N60 in C57BL/6 mice was about 1/50 of that in BALB/c mice. Interestingly, FACS results demonstrated that N60 as a MHC class II molecule restricted CD4$^+$ T cell epitope in BALB/c mice could apparently elicit an population of CD8$^+$IFN-γ^+ T cells, but not CD4$^+$IFN-γ^+ T cells in C57BL/6 mice (Fig. 4C and D). Moreover, synergistic effects could be observed when

splenocytes from immunized BALB/c mice were stimulated with both N50 and N60 peptides.

3.5. Subsets of specific memory T cells in response to N50 and N60

To evaluate the capability of peptides N50 and N60 in inducing memory T cell responses, two months after the final boost, cells from lymph nodes, spleens and lungs of BALB/c
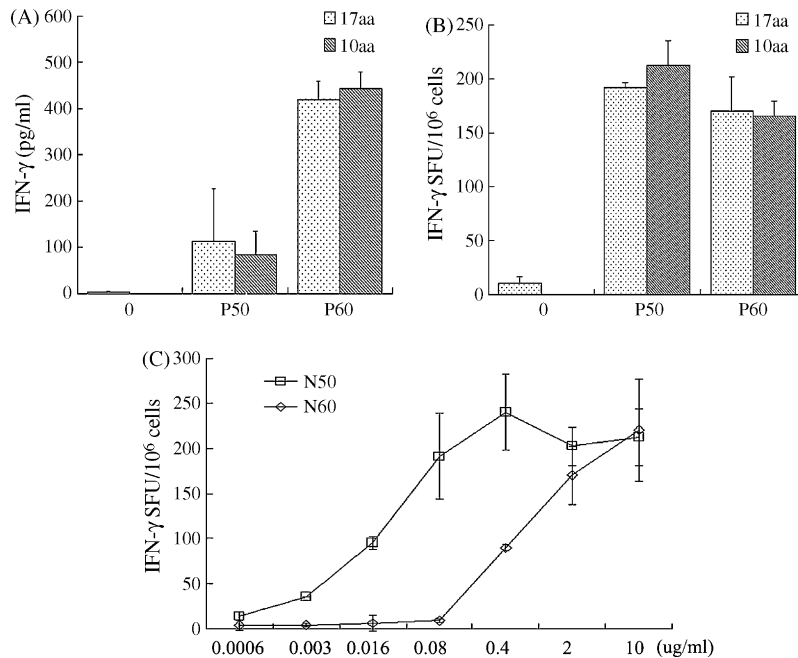
Fig. 3. Identification of new synthetic 10aa CD4 and CD8 epitopes. The overlapped amino acids between P50 and P51 (N50), and between P59 and P60 (N60) were synthesized. Peptides N50 and N60 were used to stimulate splenocytes from SARS CoV S DNA vaccine-immunized mice. P50 and P60 were used as positive controls, respectively. ELISA (A) and ELISPOT (B) were performed as described above. Furthermore, N50 and 60 were serial diluted to stimulate splenocytes from the DNA immunized mice, ELISPOT (C) was performed to detect numbers of antigen specific IFN-γ producing cells. Experiments were carried out in duplicate and representative results were shown. "0" represents non-peptide-cultured negative control.

mice were analyzed. As shown in Fig. 5, after stimulation with peptides, IFN-γ$^+$ T cells could be detected in both CD4$^+$ and CD8$^+$ T cells from spleen and lung. Moreover, different subsets of memory T cells expressing IL-7Rα and CD62L were further analyzed on IFN-γ$^+$ cells. The results showed that the majority of antigen specific IFN-γ$^+$ producing T cells were IL-7Rα$^+$ and CD62L$^-$ in both CD4$^+$ and CD8$^+$ T cells. The frequency of antigen specific IFN-γ$^+$ memory T cells was higher in spleens than that in lungs. Moreover, the memory CD8$^+$ T cell responses were higher than that of CD4$^+$ T cell.

3.6. Enhancement of T cell responses in vivo after boost with peptides N50 and N60

To explore the effects of N50 and N60 on T cell response in vivo, after DNA vaccine priming, BALB/c mice were boosted twice with peptides N50 and N60 in the presence or absence of adjuvant CpG ODN. One to two weeks after the last boosting, cells from lymph nodes and spleens were isolated and stimulated with peptides N50 and N60 in vitro. The IFN-γ ELISPOT results showed that boosting with peptides plus CpG ODN could dramatically increase the frequency of antigen specific IFN-γ producing cells in both lymph nodes and spleens (Fig. 6A and B). In addition, the higher frequency of IFN-γ$^+$ and/or IL-2$^+$ CD4$^+$ T cells was detected in spleens (Fig. 6C and D). However, percentage of IFN-γ producing cells in the lung was about 4% in CD8$^+$ T cells, which was

two-folds more than in the spleen (about 1.8% in CD8$^+$ T cells).

3.7. Measurement of serum antibodies against N50 and N60

To assess the ability of N50 and N60 to induce specific antibody production, sera from SARS CoV S DNA immunized BALB/c mice were collected. N50, N60 and truncated SARS CoV S protein specific IgG were detected in serum by ELISA. *Lister innocua* CTL epitope (KYGVSVQVI, K9I) was used as a negative control. As shown in Fig. 7, the optical density (OD) value of antibody specific to truncated S protein was detected in a dose-dependent manner, but there was no or very low levels of antibody for N50 and N60.

4. Discussion

In this report, four H-2^d restricted T cell epitopes, N50, N60, P141 and P152 of SARS CoV S protein have been identified by scanning a 17–19-mer peptide library for induction of IFN-γ response in BALB/c mice. N50 and N60 were located in the region of RBD, which mediated SARS CoV S protein binding to receptors (ACE-2) on the surface of susceptible cells. N50 and N60 seemed to be dominant epitopes for CD8$^+$ and CD4$^+$ T cells, respectively. The sequences of these two epitopes were similar to those reported by Zhi et
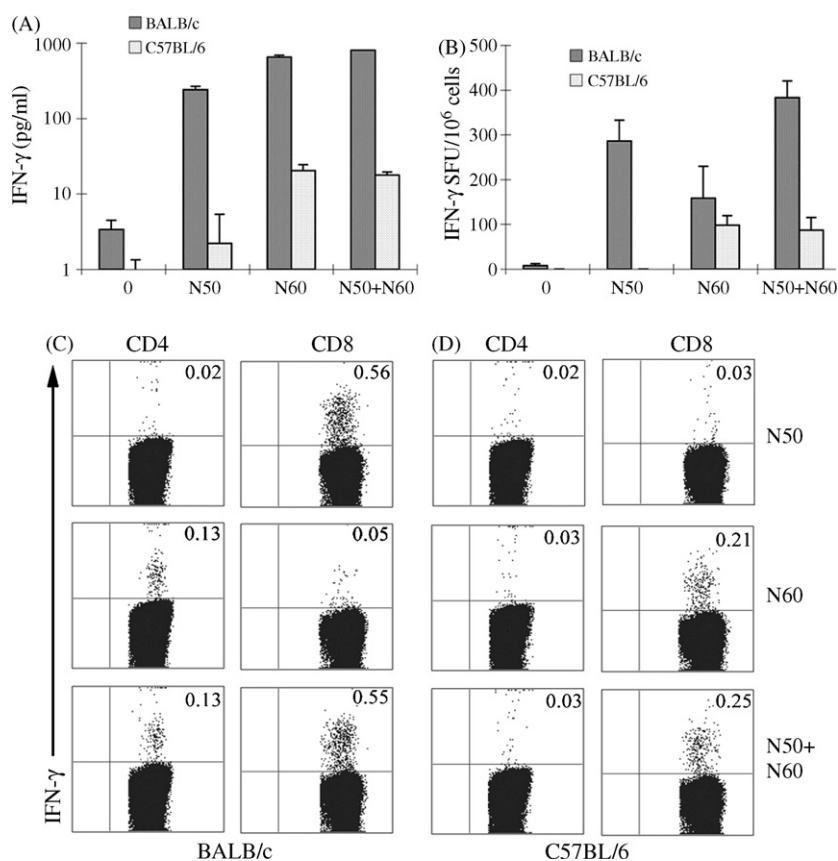
Fig. 4. Synergistic roles of peptide N50 and N60 in H-2^b and H-2^d restricted mice. BALB/c and C57BL/6 mice were immunized by SARS CoV S DNA vaccine as described previously. One to two weeks after final boost vaccination, N50 and N60 were administrated alone or combined to stimulate splenocytes from both kinds of heterogeneous mice at the same times. ELISA (A), ELISPOT (B) and FACS (C) were performed to detect IFN-γ. "0" represents non-peptide contained negative control. Experiments were done in duplicate and representative results were shown.

al. [19]. Moreover, two novel subdominant H-2^d restricted SARS CoV S epitopes P141 and P152 were identified in our study, which was specific for CD8$^+$ and CD4$^+$ T cells, respectively.

For the identification of potential T cell epitopes, ELISA, ELISPOT and FACS were used to detect the production of IFN-γ after peptide stimulation. We found that the frequencies of IFN-γ producing cells elicited by the CD4$^+$ T cell epitope N60 were similar to those induced by CD8$^+$ T cell epitope N50. However, levels of IFN-γ induced by N60 in the supernatant of cultured cells were about 20–30 times higher than those induced by N50. The discrepancies of the results from ELISPOT and ELISA might be due to the production of many other cytokines, such as IL-2, IL-4 and IL-10 secretion by CD4$^+$ T cells after stimulation with peptides. These cytokines could help expansion and survival of T cells in the course of culture [26].

Besides IFN-γ, IL-2 is another important functional cytokine secreted by antigen specific T cells, especially by CD4$^+$ T cells, after antigenic stimulation. It was reported that, based on the expression of IFN-γ and IL-2, CD4$^+$ T cells could be divided into three subpopulations, IFN-γ$^+$ IL-2$^-$, IFN-γ$^-$IL-2$^+$ and IFN-γ$^+$IL-2$^+$ [18]. To determine the subpopulation of antigen specific T cells, both IFN-γ and IL-

2 were simultaneously detected in CD4$^+$ and CD8$^+$ T cells. We happened to found that although few of IFN-γ producing cells were detected after P152 stimulation, some of the CD4$^+$ T cells apparently expressed IL-2. It indicates that P152 might be a CD4$^+$ T cell epitope.

Interestingly, when N50 and N60 were used to stimulate cells from SARS CoV S DNA-immunized C57BL/6 (H-2^b) mice, we found that N60 that was specific for CD4$^+$ T cells in BALB/c mice (H-2^d) switched to be a CD8$^+$ T cells epitope, and N50 lost its effect on both CD4$^+$ and CD8$^+$ T cells. It is due to the difference in MHC molecule in these two strains of heterogeneous mice [27].

Recently, it has been found that IL-7 plays an important role in cell survival [28], and antigen specific T cells that expressed CD127 (IL-7Rα) develop into long-term memory T cells [29]. In addition, CD62L was regarded as a marker not only for naïve T cells but also for central memory T cells, which could live longer but could not immediately exhibit an effective function compared to effector memory T cells [30]. Effector memory T cells predominantly persist in peripheral sites, which are key portals of entry for pathogens [31]. In this study, two dominant SARS CoV S peptides were used to stimulate lymphocytes from BALB/C mice after immunization. Results indicated that epitopes N50 and N60 could
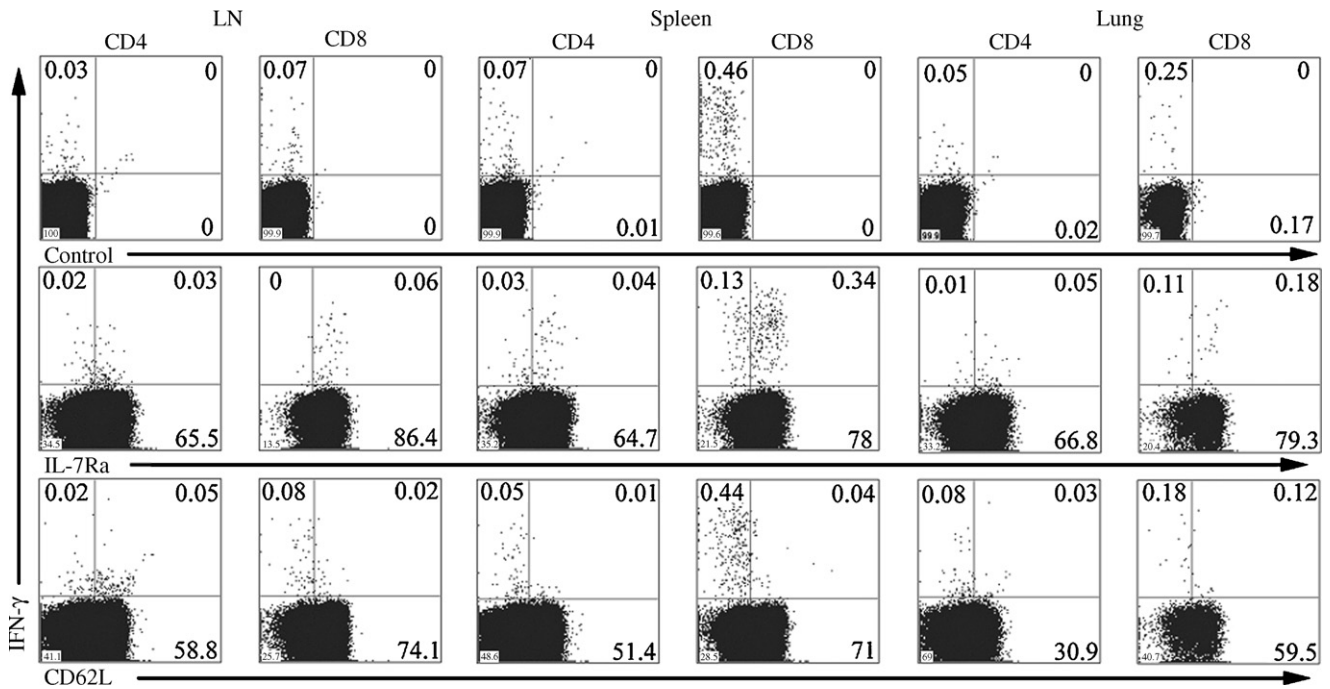
Fig. 5. Characterization of N50 and N60 specific effector/memory CD4+ and CD8+ T cells. Mice were vaccinated as described previously [18]. Two months after the final boost vaccination, cells were prepared from lymph node (LN), spleen and lung, and incubated with N50 and N60 plus anti-CD28 for 5 h. FACS was performed. CD4+ and CD8+ T cells were first gated and frequencies of IFN-γ+ CD4+ and CD8+ T cells were analyzed in the population of IL-7R+ and CD62L+ cells. Numbers at the corner in each sample represent the percentage of positive cells. Experiments were done in triplicate and representative results were shown.

effectively induce the responses of memory CD8+ and CD4+ T cells, respectively. Additionally, most of the IFN-γ producing T cells expressed high levels of IL-7Rα, but not CD62L. These data were consistent with our previous results when stimulation of T cells with a pool of SARS S peptides [18].

Peptides could be used not only to detect antigen specific T cells, but also to immunize animals for specific pathogen infection. Injection of animals with epitope vaccines could effectively elicit humoral and cellular immunity with less side effects [32]. It has been reported that vaccination with cytotoxic T lymphocyte (CTL) epitope could protect against human metapneumovirus infection in mouse study [33]. At present, prime-boost strategy has been shown to generate high levels of memory T cells in animal models. Several reports have highlighted the power of prime-boost strategies in eliciting protective cellular immunity against a variety of pathogens [34]. In SARS vaccine studies, different prime-boost immune strategies have been compared by use of several different SARS vaccine candidates, such as DNA vaccine, adenovirus vaccine, inactive virus vaccine and recombind S proteins [35,36]. Generally, DNA vaccine appears to be more effective at priming than boosting immune responses. It is well known that SARS is a kind of pulmonary infection and lung is the portal of entry for SARS CoV infections. In the case of pulmonary infections, there is evidence that vaccines need to elicit mucosal immunity or effector memory T cell pools in the lung [37]. After priming with

DNA vaccine, BALB/c mice were boosted with N50 and N60 plus CPG ODN, which dramatically increased the frequencies of antigen specific CD4+ and CD8+ T cells, especially in the lung. Therefore, DNA prime-peptide boost regimen could be an effective strategy in eliciting SARS specific immunity. Recently, it has been reported that injection of SARS CoV S protein into mice would result in an acute lung failure in vivo through the pathway of ACE-2 to which RBD served as the receptor [38]. These two dominant epitopes (N50 and N60) were just located in the region of RBD. Thus, inoculation of animals with these two epitopes based vaccine seemed to be more effective than SARS CoV S protein or RBD based vaccines.

It has been reported that SARS CoV S DNA vaccine could induce neutralizing antibodies, which played important roles in protective immune responses to virus infection. Furthermore, several B cell epitopes, such as S471–503, S803–828, S1061–1093, S335–352 and S442–458, in SARS CoV S protein were identified [39–41]. However, T cell epitopes N50 and N60 could not generate antibodies in our study, indicating that N50 and N60 could not be B cell epitopes.

Taken together, in the present study we identified four epitopes for T cells in SARS CoV S protein in BALB/c mice. N50 and N60 were located in the RBD and were the dominant T cell epitopes for CD8+ and CD4+ T cells, respectively. Boosting animals with N50 and N60 could enhance memory T cell responses in vivo. Our study provides valuable information for the design of vaccine for SARS study.
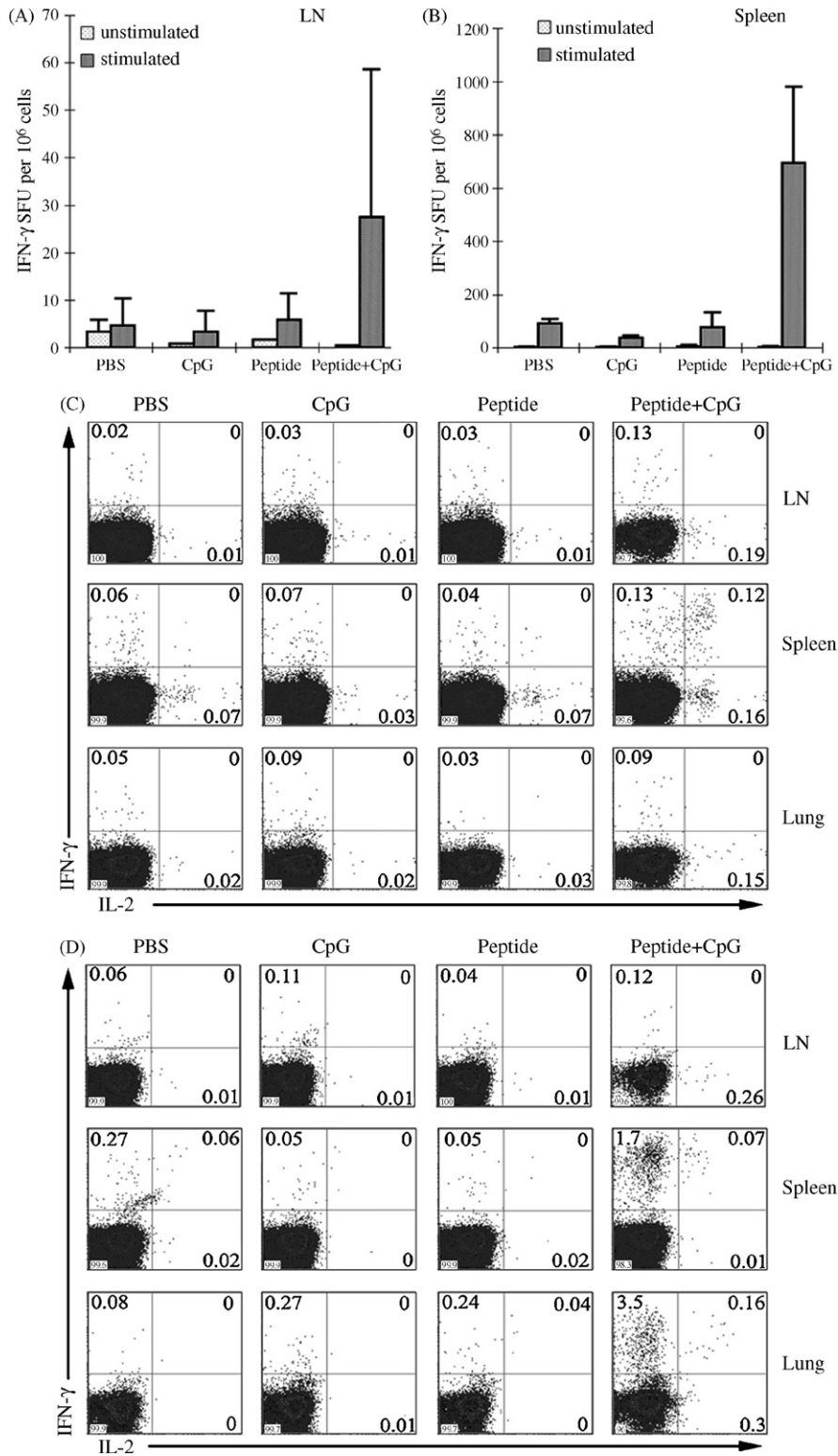
Fig. 6. N50 and N60 can elicit antigen specific immune responses in vivo. BALB/c mice were primed twice with the DNA vaccines. Three weeks later, mice were divided into four groups ($n = 4$), injected by peptides (N50 and N60, 50 μg of each) plus CpG ODN (25 μg), peptide, CpG ODN and PBS, respectively. One to two weeks after boost vaccination, single cell suspensions were prepared from lymph node (LN), spleen and lung. Cells were stimulated by peptide N50 and N60 plus anti-CD28. ELISA (A) and ELISPOT (B) were performed to detect the antigen specific IFN-γ levels and cells in each group. FACS was performed to detect the frequencies of antigen specific IFN-γ and/or IL-2 producing cells in CD4^{+} (C) and CD8^{+} (D) T cell populations, respectively. Experiments were done in duplicate and representative results were shown.
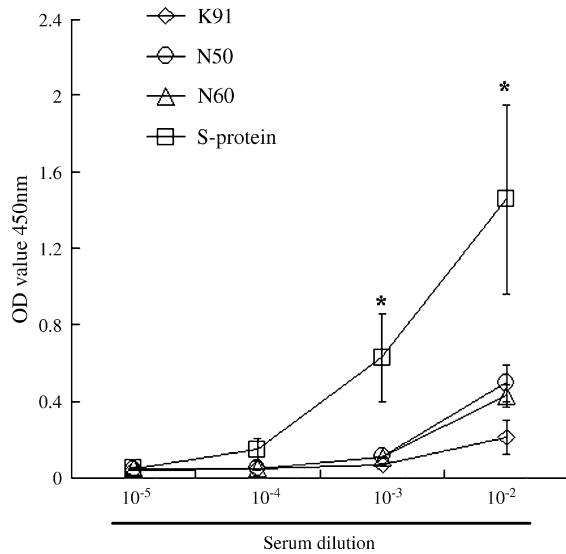
Fig. 7. Production of N50 and N60 specific IgG. Pre-coating the 96 well plate with peptide N50 (open circle), N60 (open triangle), K9I (open diamond, negative control) and truncate S protein (open squad, positive control), sera from SARS CoV S DNA immunized BALB/c mice were 10-folds dilutions and added to wells. Optical density (OD) values under 450 nm were detected. The result represents one of three independent experiments.

Acknowledgments

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