Immunization with SARS-CoV S DNA vaccine generates memory CD4+ and CD8+ T cell immune responses

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Abstract

An effective vaccine for severe acute respiratory syndrome (SARS) will probably require the generation and maintenance of both humoral and cellular immune responses. It has been reported that after natural infection in humans and immunization in animals with SARS-CoV vaccine, antibody is produced and persistent for a long period of time. In the present study, mice were immunized i.m. with SARS-CoV S DNA vaccine, and three different methods (ELISA, ELISPOT and FACS) were used to evaluate the immune responses when the cells were stimulated in vitro with a pool of peptides overlapping entire SARS spike protein. The results show that prime-immunization with SARS-CoV S DNA vaccine can induce both CD4+ and CD8+ T cell responses. Boosting with the same vaccine enhances CD4+ and CD8+ T cell responses in both lymphoid and nonlymphoid organs and were persistent over two months. The SARS-CoV S-specific CD4+ and CD8+ T cells were CD62L−, a marker for memory cells, and −30 to 50% of the cells expressed IL-7Rα (CD127), a marker for the capacity of effector cells to develop into memory cells. In addition, immunization with the DNA vaccine elicited high levels of antibody production. Taken together, these data demonstrate that immunization with SARS-CoV S DNA vaccine can generate antigen-specific humoral and cellular immune responses that may contribute to long-term protection.
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Keywords: SARS DNA vaccine; Cellular immune response; Memory T cells

1. Introduction

Severe acute respiratory syndrome (SARS) is a new emerging infectious disease that is caused by a novel coronavirus named as SARS-CoV [1,2]. The SARS-CoV genome consists of approximately 29,000 bp and encodes four main structure proteins including spike protein (S), membrane protein (M), envelope protein (E) and nucleocapsid protein (N). Among them spike protein is responsible for binding to specific receptor on the susceptible cells. Additionally, the gene sequence of the spike protein is comparably constant [2,3]. It has been reported that patients infected with SARS-CoV had high levels of antibodies in sera. These antibodies could inhibit the infection of cells with pseudotyped lentiviral particles bearing SARS-CoV S protein and be used to treat SARS-CoV-infected patients [4–7]. Recently, several reports including ours have demonstrated that SARS-CoV S specific memory CD8+ T cells were generated and maintained in vivo in the patients with fully recovered from SARS-CoV infection [8–10]. These data indicate that the spike protein is a good target for the vaccine to generate immune responses against the SARS-CoV infection. In animal studies, a number of increasing evidence demonstrated that immunization of animals with SARS-CoV S DNA vaccine lead to the generation of long-term neutralizing antibodies. These antibodies are effective in reducing SARS-CoV replication in lungs when mice immunized with SARS-CoV S DNA vaccine

were then challenged with SARS-CoV [5,11]. Although it has been reported that CD8^{+} T cell-mediated immune responses against SARS-CoV S protein in animals are generated after SARS-CoV S DNA vaccination, it remains unclear whether CD4^{+} and CD8^{+} T cells are able to become memory cells. In this regard, our study was mainly focused on determining SARS-CoV S specific effector/memory T cell responses after mice are immunized i.m. with SARS-CoV S DNA vaccine. Our results demonstrate that immunization of mice with SARS-CoV S DNA vaccine results in the generation of both CD4^{+} and CD8^{+} T cell responses. These CD4^{+} and CD8^{+} T cells are persisted for over a long period of time with a phenotype of memory cells. These data provide important information in animal models for analyzing antigen-specific memory CD4^{+} and CD8^{+} T cell responses and rational design effective vaccine against SARS-CoV infection based on SARS S protein since it is not known whether SARS-CoV reoccurs and infects humans through animal reservoirs.

2. Materials and methods

2.1. Mice

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

2.2. Media and reagents

Complete RPMI-1640 media was purchased from GIBCO, and supplemented with 10% heat-inactivated FCS, 0.1% 2-ME, 100 U/ml penicillin and 100 ug/ml streptomycin.

2.3. Antibodies

Purified anti-CD28 and anti-CD16/CD32, anti-CD4-PerCP, anti-CD62L-FITC, anti-CD8-APC-CY7, anti-IFN-γ-PE, anti-IL-2-APC and isotype-matched control Abs were purchased from BD/PharMingen (San Diego, CA). Anti-IL-7Rα-FITC was obtained from eBioscience (San Diego, CA).

2.4. SARS-CoV S DNA vaccine and pooled S peptides

Plasmids encoding SARS-CoV spike (S) protein was constructed as described and kindly provided by Dr. Gary J. Nabel from vaccine Research Center, NIAID, National Institutes of Health, Bethesda, MD, USA. Plasmid DNA was purified by plasmid purified kit (QIAGEN, USA). The 260/280 ratios ranged from 1.8 to 2.0. The endotoxin content from purified plasmid DNA was found below 20 units/ml. The endotoxin level within this range had no effect on the immune response.

A pool of 169 peptides (15–17-mer) overlapping by 10 spanning the entire SARS-CoV S protein were synthesized and kindly provided by Dr. Richard Karp from vaccine Research Center, NIAID, National Institutes of Health, Bethesda, MD, USA.

2.5. Immunizations

Female BALB/c mice were injected either i.m. (thigh) or s.c. (foot-pad) with 50 ug/mouse of SARS-CoV S plasmid DNA in 100 ul of sterile PBS. Mice were boosted at 2–3-week intervals.

2.6. Cell culture and measurement of cytokine in culture supernatants by ELISA

Mice were sacrificed. Inguinal and popliteal lymph nodes (LN), spleen and lungs from individual mice were harvested one week before and after boost vaccinations. Single-cell suspensions were prepared and plated in triplicate in a 96-well microtiter plate at 4×10^5 cells/200 ul. A pool of SARS-CoV S peptides at 1 ug/ml of the final concentration of each peptide and anti-CD28 mAb (1 ug/ml) were added to cultures. In each experiment, as a negative control, 1 ug/ml anti-CD28 was added at the absence of SARS-CoV S peptides. Seventy-two hours after incubation, cell-free culture supernatants were collected, and levels of IFN-γ and IL-2 were assessed by specific ELISA kit (BD PharMingen) according to the manufacturer's protocol. The lower limits of detection for IFN-γ and IL-2 were both 3.1 pg/ml.

2.7. Detection of SARS-S-specific antibody

The titers of mouse anti-SARS S protein antibody were measured as described previously [12]. Briefly, 96-well plates were pre-coated overnight with Transmembrane-domain-truncated SARS-CoV S protein-cell supernatants. Serum was obtained from mice before and after vaccination. The plates were washed, blocked for 1 h in 10% FBS and then washed again. The diluted serum samples were added to the wells in triplicate for 2 h at room temperature, the plates were washed, and horseradish peroxidase-conjugated anti-mouse IgG (Jakson, USA) were added at 1/2000 dilution for 1 h at room temperature. After washing, the plates were developed with tetramethylbenzidine (TMB) and hydrogen peroxide (BD PharMingen) and read it using Elx800 universal microplate reader (BIO-TEK, USA).

2.8. Assessment of SARS-CoV S-specific IFN-γ-producing cells by ELISPOT after vaccination

IFN-γ-producing cells were assessed by specific ELISPOT kit (Diaclone, France) according to the manufacturer's protocol. In brief, Single-cell suspensions were prepared from lymph nodes and spleens of mice after vaccination, and plated in 96-well microplates pre-coated

with anti-IFN-γ antibody specific for ELISPOT. Cells were incubated overnight in the presence or absence of pooled SARS-CoV S peptides (1 ug/ml) and anti-CD28 (1 ug/ml). The plates were then washed and alkaline phosphatase-conjugated anti-mouse IFN-γ antibody was added, developed with ready-to-use BCIP/NBT, and read by ChampSpotII ELISPOT reader (Sage Creation, China).

2.9. Cell surface and intracellular cytokine staining

Single-cell suspensions from the lymph nodes, spleens and lungs of mice after vaccination were stimulated with or without pooled SARS-CoV S peptides at 1 ug/ml of the final concentration of each peptide and anti-CD28 (1 ug/ml) for a total of 5 h. Brefeldin A (BFA, 10 ug/ml) was added into the culture at the end of first hour during the incubation. The cells were washed, fixed with 4% paraformaldehyde, and permeabilized with PBS containing 0.1% saponin (Fluka, Sigma, USA) plus 0.5% BSA buffer overnight at 4 °C. The cells were then blocked for 20 min with 1 ug/ml of anti-CD16/CD32 mAbs in PBS containing 0.5% BSA, and stained with anti-CD4, anti-CD8, anti-IFN-γ, anti-IL-2, anti-IL-7Rα and CD62L for 30 min at 4 °C. Cells (100,000–200,000) were acquired on a FACSCalibu flow cytometer (BD Biosciences) and FACS data were analyzed using CellQuest software (BD Biosciences). Isotype-matched controls for cytokines were included in each staining.

2.10. Statistics

Statistical evaluation of differences between means of experimental groups was done 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. Cellular immune responses elicited by prime and boost immunizations with SARS-CoV S DNA vaccine

To assess the production of cytokines following the priming injections, BALB/c mice were immunized i.m. with SARS-CoV S DNA vaccine. Seven days after vaccination, mice were sacrificed and cells were prepared from spleen and lymph nodes. The cells were stimulated with or without a pool of 169 SARS-CoV S individual peptides overlapping entire SARS S antigen and anti-CD28 mAb to assess SARS-CoV S-specific immune responses. The levels of IFN-γ and IL-2 in the cell-free culture supernatants were determined by ELISA. As a negative control, cells stimulated with anti-CD28 mAb alone did not produce any cytokines, indicating that cytokine production was specific for peptide antigen (data not shown).

The frequency of IFN-γ-producing cells at the single cell level was determined by ELISPOT assay before and after boost immunization. As shown in Fig. 1, seven days after
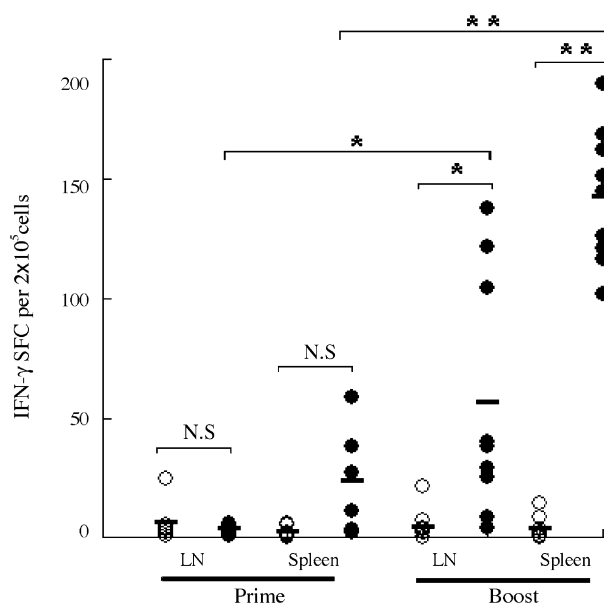

Fig. 1. Frequency of SARS-CoV S-specific IFN-γ producing cells are increased following prime and boost DNA immunization. Mice were vaccinated in a similar manner as described. One week after boost, cells from spleen and lymph node were cultured at a density of 2×10^5 cells/well with 1 ug/ml of pooled peptide and anti-CD28 in the 96 well pre-coated ELISPOT plate. After incubation for 16 h, ELISPOT assay was performed as described in Section 2. The empty circles represent the results in the absence of peptides and solid circles represent the results in the presence of peptides. $^*P < 0.05$; $^{**}P < 0.01$; n.s. $P > 0.05$.

prime immunization with SARS S DNA vaccine, cells from spleens but not from lymph nodes produced IFN-γ at a range of 25–60 spots in 2×10^5 cells following stimulation with a pool of SARS S peptides plus anti-CD28. Moreover, mice boosted with SARS S DNA vaccine exhibited a 3–30-fold increase in the frequency of IFN-γ-producing cells in spleens ($P < 0.01$) and lymph nodes ($P < 0.05$), respectively (Fig. 1), compared with the prime immunization. These data demonstrate the striking enhancement of cellular immune responses after boost immunization with SARS S DNA vaccine.

3.2. Characterization of T cell responses following prime–boost immunization

We next analyzed the immune responses of T cell subsets generated after prime and boost immunization using flow cytometry. To determine T cell responses after prime vaccination, mice were immunized i.m. with SARS-CoV S DNA vaccine. Seven days after immunization, cells from lymph nodes, spleens and lungs were prepared and stimulated with a pool of SARS-CoV S peptides. Intracellular cytokines and cell surface markers were stained. Initially, a lymphocyte-enriched population was selected from total cells by forward/side scatter gating, CD8$^+$ and CD4$^+$ T cells (Fig. 2A) were then gated and analyzed for their expression of IFN-γ and IL-2. As shown in Fig. 2B, SARS-CoV S-specific CD4$^+$ and CD8$^+$ IFN-γ-producing T cells were detected in
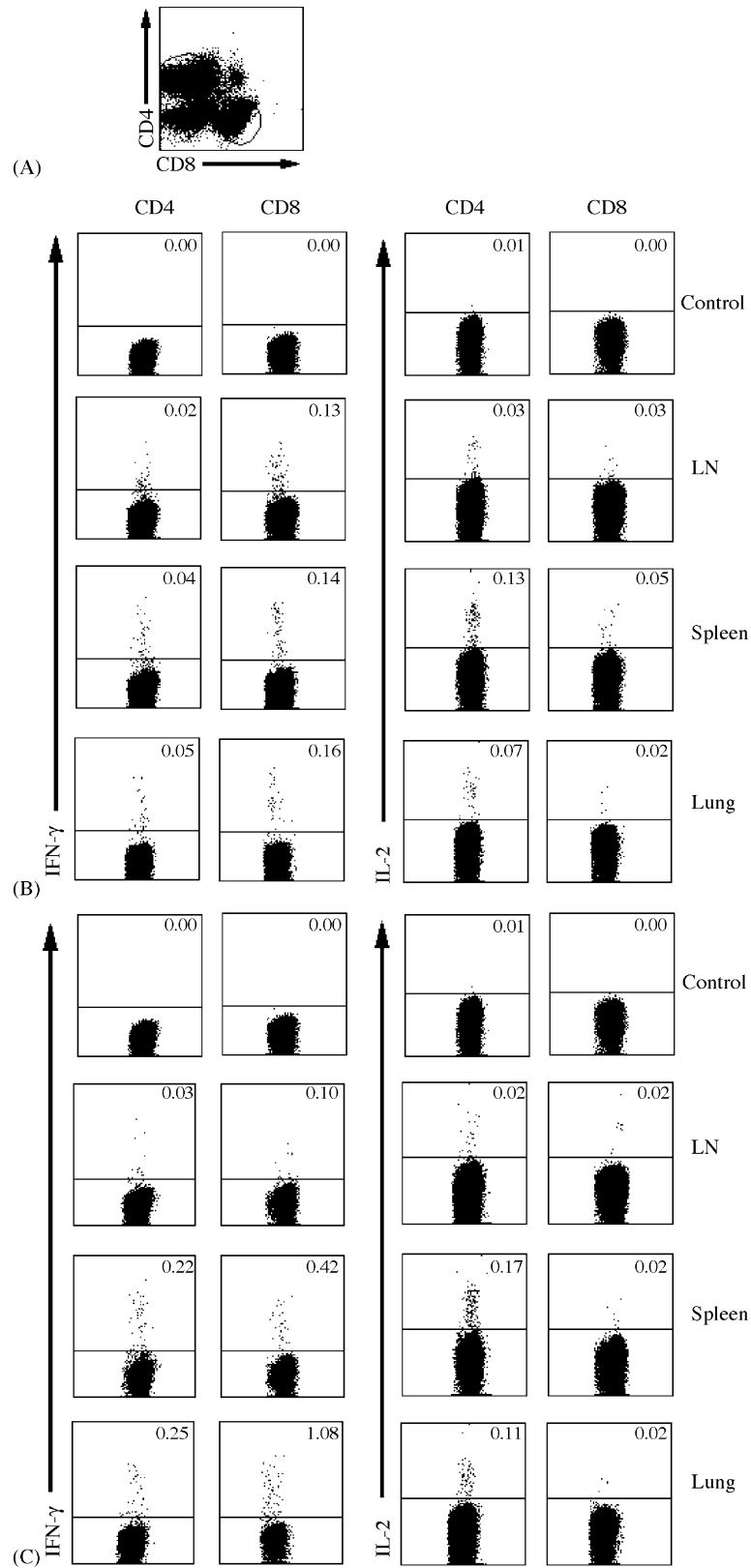
Fig. 2. SARS-CoV S-specific CD4+ and CD8+ T cells are generated following prime and boost vaccination. Mice were vaccinated as described in Fig. 1. Cells were prepared from lymph nodes (LN), spleen and lungs, and incubated with a pool of SARS-CoV S peptides and anti-CD28 for 5 h. CD4+ and CD8+ T cells were first gated (A), IFN-γ and IL-2-producing CD4+ and CD8+ T cells were determined by intracellular cytokines staining as described in Section 2 following prime (B) and boost (C) vaccination. The frequency of IFN-γ+ and IL-2+ cells was indicated as percentage of CD4+ and CD8+ T cells.

lymph nodes, spleens and lungs after prime immunization. The frequency of IFN-γ⁺ cells in the CD8⁺ T cell population was markedly higher than that in the CD4⁺ T cells in each of the organs tested. In the same experiment, the frequency of IL-2-producing cells in the CD4⁺ and CD8⁺ T cell populations were analyzed in a similar manner. As shown in Fig. 2B, IL-2⁺ cells were detected in CD4⁺ T cells but very few IL-2-producing cells were detected in CD8⁺ T cells.

To further ascertain whether the frequency of SARS-CoV S specific CD4⁺ and CD8⁺ T cell responses was increased after boost vaccination, mice were boosted i.m. with SARS-CoV S DNA vaccine, seven days after injection, IFN-γ- and IL-2-producing CD4⁺ and CD8⁺ T cells were determined in lymph nodes, spleen and lungs. As shown in Fig. 2C, the vaccine boost dramatically increased the frequency of CD4⁺ and CD8⁺ IFN-γ-producing T cells in spleens and lungs but not in lymph nodes. In the same experiment, the frequencies of CD4⁺ and CD8⁺ IL-2-producing T cells did not change in any of the tissues tested.

Moreover, we compared the route of immunization with SARS-CoV S DNA vaccine side by side. In this regard, mice were immunized and boosted either s.c. or i.m. and the frequency of antigen-specific IFN-γ-producing CD4⁺ and CD8⁺ T cells were determined by FACS. The results showed that s.c. immunization induced the highest immune responses of CD4⁺ T cells in lymph nodes than in spleen and lungs (Fig. 3A), whereas there was no markedly different responses of CD8⁺ T cells among all organs (Fig. 3B). Immunization of mice by i.m. injection elicited the highest frequency of antigen-specific IFN-γ expression of CD8⁺ T cells in lung than in lymph nodes and spleen. Similar results were obtained for CD4⁺ T cell responses. In general, vaccination via i.m. induced better immune responses than s.c. injection. Thus, we choose i.m. immunization for further study.

3.3. Cell subsets of IFN-γ and IL-2-producing CD4⁺ and CD8⁺ T cells

To explore cell subsets of IFN-γ and IL-2-producing CD4⁺ and CD8⁺ T cells at the single cell level, the intracellular cytokine staining were performed and analyzed. As shown in Fig. 4, based on the expression of IFN-γ and IL-2, the CD4⁺ T cells could be divided into three subpopulations: IFN-γ⁺, IL-2⁺ and IFN-γ⁺IL-2⁺. However, in the CD8⁺ T cells majority of the cells were IFN-γ⁺, only a small population of the IFN-γ⁺IL-2⁺ CD8⁺ T cells could be detected in the spleen and very few single IL-2⁺ CD8⁺ T cells were observed. These data demonstrate that the IFN-γ-and IL-2-producing cells constitute distinct, but over-lapping subsets of CD4⁺ T and CD8⁺ effector/memory populations.

In addition, it is noted that in Figs. 1 and 2, before DNA vaccine boost immunization, the frequency of IFN-γ⁺ cells in CD4⁺ and CD8⁺ T cell population was no different in lymphoid organs (nodes and spleen) and non-lymphoid organ (lungs). However, after boost vaccination, the frequency of IFN-γ⁺ T cell population was dramatically increased in the
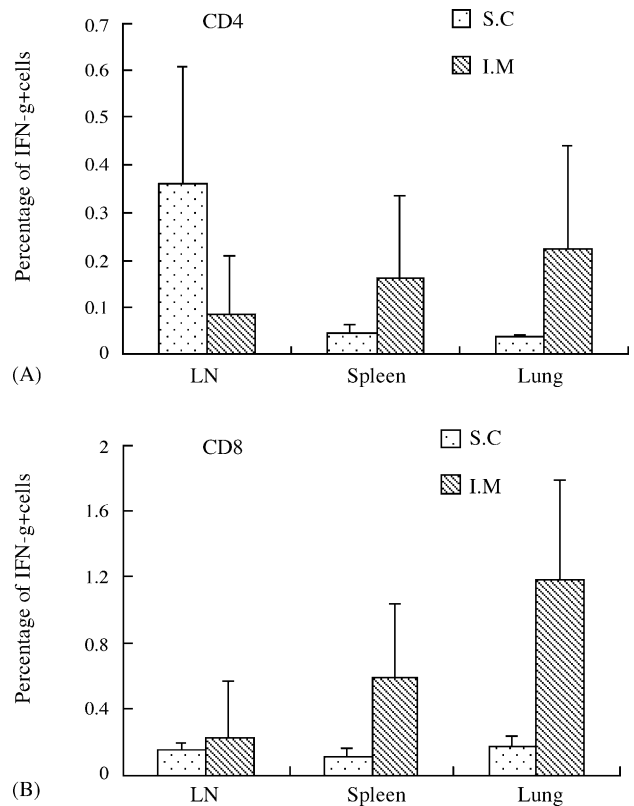

Fig. 3. Immunization via i.m. results in higher frequency of SARS-CoV S-specific IFN-γ producing cells than that of s.c. Mice were immunized via either i.m. or s.c. with SARS-CoV S DNA vaccine. One week after boost, cells from lymph nodes, spleen and lungs were cultured and stained as described in Fig. 2. The results were expressed as a mean percentage (three to five mice) of IFN-γ-producing cells in CD4⁺ T cells (A) and CD8⁺ T cells (B).

spleens and lungs, especially in the lungs for the CD8⁺ T cells population. In the same experiment, SARS-CoV S antigen-specific CD4⁺ T cells expressed higher frequency of IL-2-producing cells than CD8⁺ T cells in spleens and lung, consist with enrichment of effector/memory T cells from spleen to non-lymphoid organs.

3.4. Memory T cell responses after prime–boost immunization with SARS S DNA vaccine

In previous experiment, we assessed the cellular immune responses seven days after prime or boost vaccination. Here, we extended our study to further characterize SARS-CoV S specific long-persistent memory T cell response 8 weeks after the final immunization. CD4⁺ and CD8⁺ T cells from lymph nodes, spleens and lungs were divided into IL-7Rα (CD127)⁺ and CD62L⁺ subpopulations which have been shown in mice to distinguish naïve and memory T cells [13,14]. As shown in Fig. 5A, IFN-γ-producing CD4⁺ T cells from lymph nodes, spleen and lungs were IL-7Rα⁺. In contrast, IFN-γ-producing CD4⁺ T cells were CD62L⁻. Similar results were also obtained for IL-2-producing CD4⁺ T cells (Fig. 5B). In addition, IFN-γ-producing CD8⁺ T cells could
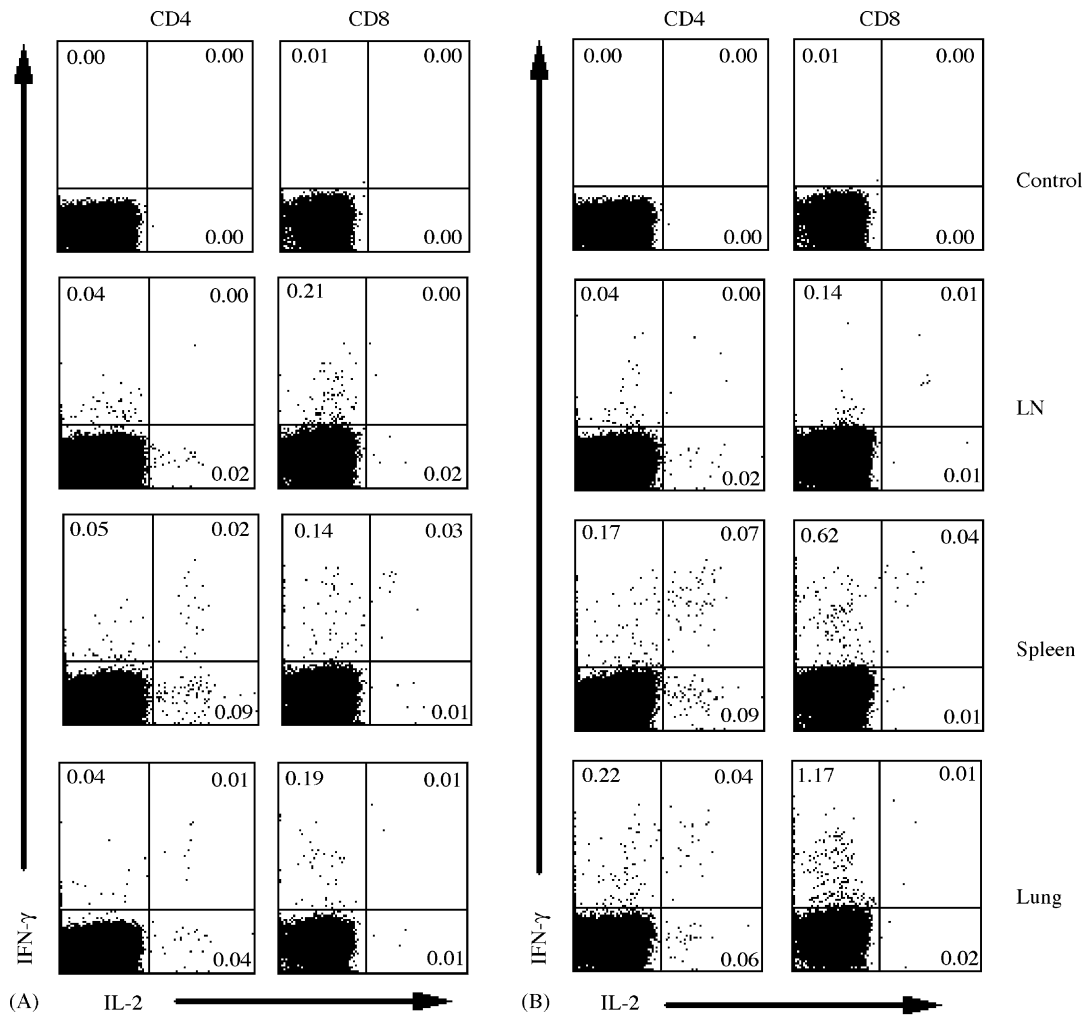
Fig. 4. Distinct populations of SARS-CoV S specific IFN-γ and IL-2-producing CD4⁺ and CD8⁺ T cells are persistent in lymphoid and non-lymphoid organs following prime and boost DNA vaccination. Mice were vaccinated as indicated in Fig. 2 following prime (A) and boost (B) DNA vaccination. Cells were prepared from lymph nodes (LN), spleens and lungs, and incubated with a pool of SARS-CoV S peptides and anti-CD28 for 5 h. CD4⁺ and CD8⁺ T cells were first gated, the frequency of IFN-γ and/or IL-2-producing cells were analyzed within the population of CD4⁺ and CD8⁺ T cells. Results are representative of three separate experiments with similar results. The numbers at the corner in each sample represent the percentage of cytokine-producing cells.

be separated into IL-7Rα⁺ and IL-7Rα⁻ subsets (Fig. 5A). However, consistent with IFN-γ-producing CD4⁺ T cells, IFN-γ-producing CD8⁺ T cells were CD62L⁻. Interestingly, the expression of IL-7Rα and CD62L in IL-2-producing CD8⁺ T cells were similar to IL-2-producing CD4⁺ T cells (Fig. 5B). These data demonstrate that long-lasting CD4⁺ and CD8⁺ T cells specific for SARS-CoV S antigen in vivo were memory phenotype.

3.5. Antibody responses following prime–boost immunization

We finally assessed the production of antigen specific antibody induced by the SARS-CoV S DNA vaccination. As shown in Fig. 6, the sera obtained from the immunized mice but not from non-immunized mice had significantly higher amount of SARS-CoV S specific antibody, indicating that SARS-CoV S DNA vaccine could induce strong

humoral immune response that is consistent with the previous report.

4. Discussion

It has been reported that DNA vaccination is a practical and effective way to induce humoral and cellular immune responses and has shown great promise for protective immune responses against several diseases in experimental animal models including HIV, tuberculosis and malaria [15–17]. In the present study, we have demonstrated that prime–boost immunization of mice with SARS-CoV spike DNA vaccine induces antigen-specific cellular and humoral immune responses. The cellular immune responses are mediated by both CD4⁺ and CD8⁺ T cell. Functional study indicates that antigen-specific cells produced cytokines IFN-γ and IL-2 from CD4⁺ and CD8⁺ T cells after short stimulation in vitro
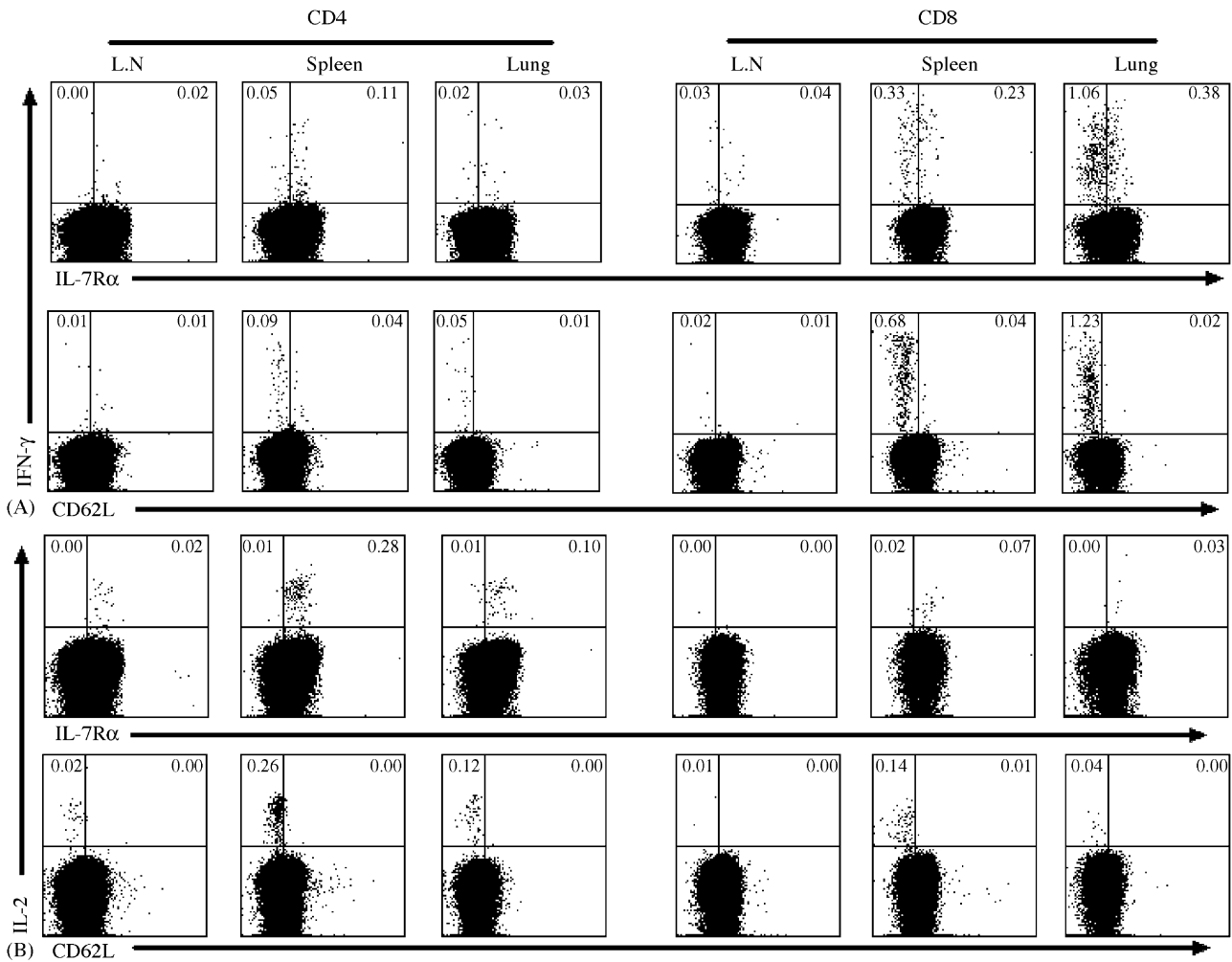
Fig. 5. Long-term persistence of effector/memory CD4^{+} and CD8^{+} T cells following DNA vaccination. Mice were vaccinated as indicated in Fig. 2. Cells were prepared from lymph nodes (LN), spleens and lungs 8 weeks after final boost vaccination, and incubated with a pool of SARS-CoV S peptides and anti-CD28 for 5 h. CD4^{+} and CD8^{+} T cells were first gated. The frequency of IFN-γ^{+}-CD4^{+} and CD8^{+}-T cells (A) or IL-2^{+}-CD4^{+} and CD8^{+}-T cells (B) were analyzed within the population of IL-7Rα^{+} and CD62L^{+} cells. The numbers at the corner in each sample represent the percentage of positive cells.

with a pool of SARS-S peptides. IFN-γ is an effector cytokine that is able to activate macrophage cells and inhibit viral replication [18], and IL-2 is a growth factor that probably maintains memory cells and mediates expansion of both CD4^{+} and CD8^{+} T cells [19]. Based on IFN-γ and IL-2 expression, memory T cells are able to divide into three subpopulations including IFN-γ^{+}, IFN-γ and IL-2^{+}, IL-2^{+} cells, indicating that memory T cells are composed of distinct functional subsets. Phenotype analysis indicates that SARS-CoV S specific CD4^{+} and CD8^{+} T cells are CD62L^{−}. Some of the memory CD4^{+} and CD8^{+} T cells express IL-7Rα. These populations of the cells may have the potential differentiate into long-term memory cells [14]. It has been reported that IL-7 is mainly secreted by the stromal cells and plays a key role in sustaining the homeostasis of memory CD8^{+} T-cell in vivo [20,21]. In line with other studies, we also find most of the memory CD4^{+} and CD8^{+} T cells were identified in non-lymphoid tissues (lungs) in which these memory T cells are called effector memory cells [22,23]. Thus, by their location, effector mem-

ory T cells can respond rapidly when re-exposed to a pathogen and may play the most important role in the defence when re-encountering a pathogen. Furthermore, the function of non-lymphoid effector memory cells has been demonstrated in a study in which Sendai virus specific memory CD4^{+} T cells persist in the lungs, and transfer of these cells into β2-microglobulin-dificient mice confers a significant level of protection against re-infection [24]. Consistent with our observations, several reports demonstrate that natural infection in humans induces memory CD8^{+} T cells responses [10]. Moreover, immunization of mice with adno-vector encoding codon-optimized SARS-CoV S protein generates two H-2[b]-restricted epitopes and one H-2[d]-restricted epitopes of CD8^{+} T cells [25]. In addition, Yang et al., reported that vaccination of mice with SARS-CoV S DNA generated cellular and humoral immunity to SARS-CoV S glycoprotein. The neutralizing antibody can inhibit SARS-CoV replication and results in protective immunity in a murine challenge model. In adoptive transfer study, they found that antibodies but not
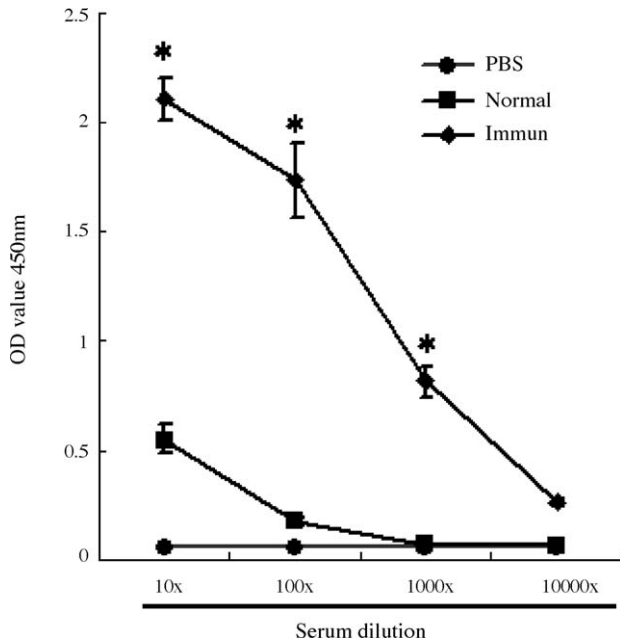
Fig. 6. SARS-CoV S protein-specific antibody responses following DNA vaccination. BALB/c mice were immunized i.m. as described in Section 2. Sera from normal and immunized mice were collected at day 7 after boost vaccination. Antibody titers in sera were determined at different dilutions by ELISA specific for SARS-S protein. Data are expressed as the mean value ± S.D. for each group. A non-parametric two-tailed t-test was used for statistical analysis with similar result; $^*P < 0.01$.

T cells play a critical role in protection against SARS-CoV challenge [5]. Thus, it is still unclear whether T cells may play a role in the generation of protective immune responses. We assume that antigen-specific $CD8^+$ T cells reveal a direct killing of virus-infected cells and $CD4^+$ T cells may provide help for antibody isotype switching and generation of memory B cells and memory $CD8^+$ T cells [23,26]. In support of our expectation, other studies of animal coronavirus have suggested that both cellular and humoral immunity contribute to protection during persistent infection [27–31].

Taken together, our results show that immunized mice with SARS-CoV S DNA vaccine can generate both cellular and humoral immunity that may be useful for preventive and therapeutic vaccine for SARS-CoV infection.

Acknowledgments

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