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Improved immunogenicity of a tuberculosis DNA vaccine encoding ESAT6 by DNA priming and protein boosting

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Abstract

The study evaluated the immune response elicited by a DNA vaccine encoding ESAT6 protein of *Mycobacterium tuberculosis* by DNA prime-protein boost protocol. BALB/c mice were respectively vaccinated with plasmid DNA encoding ESAT6 protein, with ESAT6 protein in IFA adjuvant, or a combined DNA prime-protein boost regimen. While DNA immunization induced Th_1-polarized immune response, protein–in-adjuvant vaccination elicited a Th_2-dominant response. When animals were primed with DNA and boost with protein, both antibodies and Th-cell proliferative response were significantly enhanced. Moreover, production of Th_1-type cytokine (IFN-γ) was increased significantly by DNA priming-protein boosting. This protocol also resulted in an increased relative ratio of IgG_{2a} to IgG_1 and the cytotoxicity of T cells. Thus, this study demonstrated that the formation of ESAT6 DNA prime-protein boost inoculation could improved antigen-specific cellular immune responses, which are important for protection against *TB* infection.

Keywords: DNA prime-protein boost; *Mycobacterium tuberculosis*; ESAT6 antigen; DNA vaccine

1. Introduction

Infection with *Mycobacterium tuberculosis* remains to be a major cause of morbidity and mortality throughout the world, resulting in 3 million death and over 8 million new case of tuberculosis each year [1]. Apparently, BCG vaccine used for 80 years, has failed to control *TB* epidemic [2]. In recent years, increased emergency of multi-drug-resistant (MDR) strains of *M.TB* and co-infection with HIV complicated the situation. Hunting for improved *TB* vaccines is urgently needed.

The development of new vaccines requires an understanding of the protective immune response against tuberculosis. The crucial factor of protective immunity against *TB* is a T-cell mediated response characterized by the secretion of IFN-γ and other cytokines [3]. Hence, we are in desperate need of enhancing understanding of how to develop a new generation of *TB* vaccines which are able to provoke potent and long-lasting protective cell-mediated immunity.

DNA vaccine offers a promising alternative to conventional vaccines. It has been shown to elicit both humoral as well as cellular immune responses, and confer protection against some viral, bacterial and parasitic pathogens [4–6]. In the past few years, DNA vaccines have been also studied against *M. tuberculosis*. These vaccines including Ag85A/B/C, ESAT6, MPT64, PST1/2/3, 38 kDa and HSP70 [7–10], when used individually or combination, have demonstrated impressive immunogenicity in mice, but the level of conferred immune protection against subsequent *M.TB* infection has never exceeded that by BCG. Therefore, new strategies are needed to improve the immunogenicity and protective efficacy of these DNA vaccines. ESAT6 protein is one of the secreted antigens and is expressed only in virulent *Mycobacterium bovis* strains and *M. tubrerculosis*. Of importance, a large proportion of memory T cells in *M.TB*-infected mice are ESAT6-reactive. Hence, ESAT6 is a promising candidate antigen. In experimental mouse models, ESAT6 DNA vaccine conferred the protective efficacy inferior to that by BCG [11].

Here, we reported an attempt to improve the immunogenicity of this ESAT6 DNA vaccine by a DNA prime-protein boost immunization regimen. i.m. DNA

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inoculation can trigger a Th_1-type immune response, but the low amount of actual proteins expressed in the host cells is a serious limitation of this type of immunization. Prime-boost strategies of consecutive DNA priming followed by boosting with proteins have the potential to enhance the immune response elicited by DNA vaccines. A number of studies have reported the effect of DNA prime-protein boost regimen in viral, protozoan infection [12,13]. Little is depicted in bacterial disease. In the present study, we demonstrated that protein boosting of mice vaccinated with ESAT6 DNA vaccine for *M. tuberculosis* was capable of improving the immune response primarily elicited by the DNA vaccine.

2. Materials and methods

2.1. Mice

BALB/c female mice, 6–8-weeks old, were bred in the animal facilities of the Second Military Medical University.

2.2. Cell transfection

The recombinant plasmid pcDNA3-ESAT6 was transfected into P815 (H-2^d a lymphoma cell line, from Type Culture Collection of Chinese Academy of Sciences) cells by liposome (Roche Molecular Biochemicals) according to the manufacture's instruction. After selection in medium supplemented with G418 (800 μg/ml), stable transfectants were subcloned by limiting dilution and then determined by RT-PCR and immunochemistry methods.

2.3. Immunocytochemistry

The expression of ESAT6 protein was detected by immunocytochemistry. P815 stable transfectants were fixed in 4% paraformaldehyde for 10 min and placed on a poly-L-lysine-treated microslides. And were then air-dried for 30 min. Slides were redehydrated and blocked using 1% BSA in PBS plus 0.1% Triton X-100 (pH 7.2) for 1 h. Then slides were incubated overnight at 4 °C in a humid chamber with appropriate sera diluted at 1:20 in PBS from the patients infected with *M. tuberculosis* (provided by Dr Qiaomin Zhang). After washing in PBS (three times for 10 min), the bound human immunogloblins were detected by incubation for 24 h at 4 °C with goat anti-human-HRP-conjugated second antibody (Southern Biotechnology Associates, SBA) diluted 1:100 in PBS plus 1% goat serum. After washed in PBS (three times for 10min), the interest antigen was coloured by DAB substrate and the slides were counterstained with hematoxylin.

2.4. Plasmid construction and preparation

The DNA vaccine pcDNA3-ESAT6 was constructed by cloning the cDNA of ESAT6 [14] into the *Hin*dIII and *Xba*I restriction sites of pcDNA3 plasmid (Invitrogen), downstream of the CMV early promoter. Plasmids used in this study were prepared with alkaline lysis method followed by TritonX-114 treatment to remove endotoxin [15].

2.5. Vaccination protocol

For DNA vaccination, mice were injected with pcDNA3-ESAT6 into both quadriceps with 2×50 μg DNA three times at 3 weeks intervals. Mice inoculated with pcDNA3 was as a negative control. For protein vaccination, mice were injected subcutaneously (s.c.) in the back with 100 μg of ESAT6 protein expressed and purified in *E. coli* [16]. The protein was emulsified in IFA. For the DNA prime-protein boost, mice were immunized i.m. with ESAT6 DNA vaccine once and s.c. with 100 μg of ESAT6 protein twice. All mice were immunized three times at 3 weeks intervals. To enhance muscle cells uptake of plasmid DNA [17], 25% sucrose was injected into the muscles of both quadriceps 15 min before plasmid inoculation.

2.6. Enzyme-linked Immunoabsorbent assay (ELISA)

Anti-ESAT6 IgG, IgG_1, IgG_{2a} were measured by ELISA in individual serum sample from vaccinated mice. The method was as described previously [6], using recombinant ESAT6 protein (1 μg per well) and anti-mouse IgG, IgG_1 or IgG_{2a} coupled to horseradish peroxidase (HRP) (Southern Biotechnology Associates, SBA). The antibody titers were determined according to the optical density (OD 450 nm). Finally, the relative ratio of IgG_{2a} to IgG_1 was calculated.

2.7. Lymphocytes proliferation assay

Mice were sacrificed 3 weeks after the last immunization. Spleens from each group were pooled and analyzed. Th-cell proliferation assay was performed as previously described. Briefly, the isolated spleen cells were resuspended to a concentration of 5×10^6 cells/ml. A volume of 100 μl of cell suspension was added to 96-well plates and the ESAT6 protein was added to the wells in triplicate at the final concentration of 5 μg/ml. The plates were incubated at 37 °C in an atmosphere of 5% CO_2 for 66 h. Then the proliferation responses were detected by MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] (5 mg/ml, Sigma) method and the stimulation index (SI) was calculated. The stimulation index was determined from the formula: stimulation index (SI) = experimental OD/negative OD. To assure that cells were healthy, 10 μg/ml ConA was used as a polyclonal stimulator for positive control.

2.8. Evaluation of cytokines production in vitro

Single splenocyte suspensions from immunized mice were diluted in 10% bovine calf serum-supplemented RPMI

1640 to 5×10^6 cells/ml. One hundred microliters cell suspensions were placed in each well of a 96-well plate (Costar) and stimulated with ESAT6 protein (5 μg/ml). After 72 h incubation, cell-free supernatants were harvested and were screened for the presence of IFN-γ and IL-4 with an ELISA detection system (Jingmei, Biotech) according to the manufacture's instruction.

2.9. Cytotoxicity assay of T cell

Spleen cells adjusted to a concentration of 10^7/ml from in vivo-primed mice were cocultured with mitomycin (10 μg/ml)-treated target cells (P815-ESAT6, 5×10^5/ml) in a 10 ml cell suspension in RPMI 1640 for 5 days at 37 °C in 5% CO_2. Twenty unit per milliliter recombinant murine IL-2 (Biosource) was also added to the cell solution for 5 days. The P815 cell was used as a negative control.

To measure the specific lysis of the target cells, we used the lactate dehydrogenase (LDH) release assay. This assay yields highly similar results as the standard chromium release assay, but does not require the use of radioisotopes [18]. In 96-well round-bottom plates, effector cells were incubated with target cells at different E/T ratio for 4 h in phenol red-free RPMI 1640 containing 2% BSA, 2mM glutamine, and 1% penicillin and streptomycin. After centrifuging the plates at $250 \times g$ for 10 min, 100 μl per well of the supernatant was then transferred to a 96-well plates, and lysis was determined by measuring LDH release using cytotocxicity detection kit (Roche Molecular Biochemicals). The released LDH converted the added substrate tetrazolium salt into red formazan product, and the amount of color is proportional to the number of lysed cells. The absorbance values from supernatants were recorded at *OD* 492 on an ELISA microplate reader. The percent lysis was calculated as follows: [(sample release − spontaneous release) × 100%/(total release − spontaneous release) × 100%.

2.10. Statistical analysis

The statistical significance of differential findings between experimental groups was determined by Student's test. Data were considered statistically significant at $P < 0.05$.

3. Results

3.1. Construction of ESAT6 DNA vaccine

The cDNA of ESAT6 antigen was inserted into the pcDNA3 vector, and the recombinant pcDNA3-ESAT6 plasmid was confirmed by *Hin*dIII and *Xba*I digestion. After a large scale of preparation, the plasmids were suspended in endotoxin-free PBS. DNA was quantified by spectrophotometry at 260 nm and the final concentration of the solution was adjusted to 1 μg/μl of DNA in PBS.

3.2. The stable expression of ESAT6 protein in P815 cells

After selection by G418 (800 μg/ml) for 25 passages, clones of transfected cells were screened for ESAT6-mRNA by reverse transcription-PCR. Positive clones were further examined for expression of ESAT6 by immunocytochemistry. The imunostaining was restricted to the cytoplasm of the cells transfected with pcDNA3-ESAT6 plasmid (data not shown). However, no staining was detected in P815 cells. These results demonstrated that ESAT6 antigen could be expressed stably in P815 cells, and that the obtained cells could be used as target cells in the cytotoxicity assay.

3.3. Production of antibodies induced by different vaccines

To determined the level of ESAT6-specific IgG by different inoculations, mice of different groups were immunized three times at 3 weeks intervals. Three weeks after the last immunization, the sera from mice were collected by retro-orbital bleeding and antigen-specific antibodies were detected. As shown in Fig. 1, compared to the pcDNA3 group, sera from DNA–DNA group, DNA–protein group or protein–protein group elicited a significantly higher level of IgG ($P < 0.01$). DNA prime-protein boost vaccination induced a higher IgG titer than DNA–DNA vaccination ($P < 0.05$). Protein prime-protein boost inoculation elicited the highest titer of IgG.

The IgG subclasses give an indication of the Th_1 versus Th_2 nature of the immune responses. We further analyzed the relative ratio of IgG_{2a}/IgG_1 from different groups. As shown in Fig. 2, DNA-protein vaccination increased the ratio significantly more than did DNA–DNA vaccination. When the animals were immunized with only protein, lower ratio was observed, compared to DNA–DNA vaccination. These

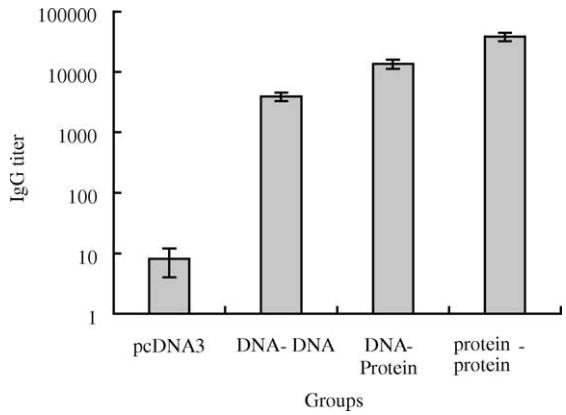

Fig. 1. The ESAT6-specific IgG titer in mice immunized by different protocols. Each group of mice ($n = 8$) was immunized respectively by DNA–DNA, DNA–protein or protein–protein at 0, 3, and 6 weeks. Mice were bled at 3 weeks after the last immunization and ESAT6-specific IgG titer was detected by ELISA. Optical density was measured at 450 nm. Data shown represented geometric mean titers (GMT) and standard errors for each group of animals.

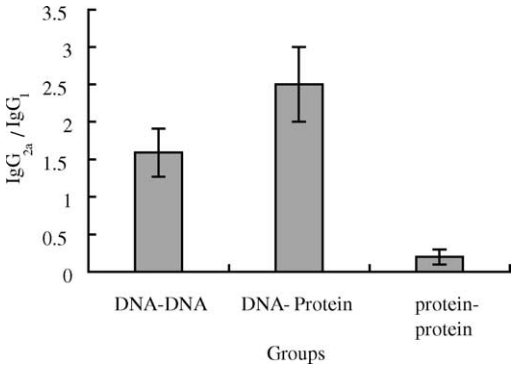

Fig. 2. The ratio of IgG_{2a} titer to IgG_1 titer in different groups. The mice were immunized as described in Fig. 1. The titers of IgG_{2a} and IgG_1 were detected respectively. The ratio of IgG_{2a}/IgG_1 was obtained by dividing the titer of IgG_{2a} by that of IgG_1.

results indicated that DNA prime-protein boost inoculation did not convert the type of immune response (Th_1-type) elicited by the primary DNA vaccine.

3.4. Lymphocyte proliferation response and cytokine production by splenocytes from vaccinated mice

T helper cells play an important role in eliciting both humoral and cellular immune responses via expansion of antigen-stimulated B cells and expansion of $CD8^+$T cells respectively. Hence, it is important to measure proliferation of T cells after immunization with vaccines when stimulated in vitro with a specific antigen. In our experiment, ESAT6 (5 μg/ml) and ConA (10 μg/ml) were used as a specific stimulator and a polyconal stimulator of T cells respectively. As shown in Fig. 3, a low background level of T-cell

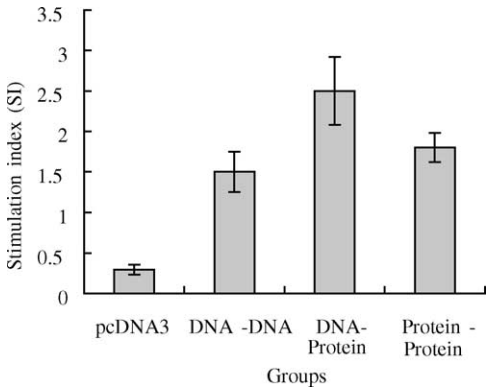

Fig. 3. The proliferation of splenocytes after in vitro stimulation with ESAT6 protein. Different group of mice ($n = 8$) were immunized by DNA–DNA, DNA–protein or protein–protein three times at 3 weeks interval. Three weeks after the last immunization, spleen cells from sacrificed mice were pooled and stimulated with 5 μg/ml ESAT6 protein, 10 μg/ml ConA as a positive control. After 66 h stimulation, samples were assayed in triplicate. The ConA control sample showed a stimulation index of 8.36 ± 2.4. Values and bars represent the mean and S.D. This experiment was repeated, with the expected results.

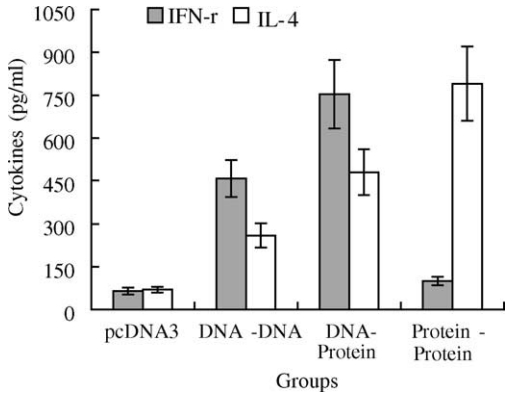

Fig. 4. Production of IFN-γ and IL-4 from splenocytes of different groups. The immunization protocol was the same as that in Th proliferation response. Splenocytes were stimulated with 5 μg/ml ESAT6 protein for 72 h. Samples were assayed in triplicate. Values and bars represent the mean of released cytokine concentrations and S.D.

proliferation was observed. DNA priming-protein boosting enhanced Th-cell proliferation responses more significantly than did either DNA–DNA or protein–protein vaccination ($P < 0.05$).

Th_1 cytokines (IL-2, IFN-γ) and Th_2 cytokines (IL-4, IL-5 and IL-10) are major parameters in our understanding of the polarization of immune responses. Th_1 immune responses are thought to drive induction of cellular immunity, whereas Th_2 immune responses preferentially drive humoral immunity. We next examined the cytokine production profiles in our research. As demonstrated in Fig. 4, the level of IFN-γ was significantly increased by DNA prime-protein boosting protocol, compared to DNA–DNA vaccination group. However, the level of IFN-γ was lower in protein–protein immunization than that in DNA–DNA group or DNA–protein group. DNA prime-protein boost immunization also enhanced IL-4 production compared to DNA–DNA group, but a lesser degree than protein–protein vaccination. Th_1-type responses were observed in both DNA–DNA group and DNA–protein group; however, a Th_2-dominant response was induced in protein–protein immunization group. Those results supported the conclusion that DNA prime-protein boost inoculation influenced both Th_1 and Th_2 phenotype in vivo, but did remain Th_1-profile immune response.

3.5. Improved CTL induction by DNA prime-protein boost immunization

Cytotoxic T-cell responses were determined with a LDH release assay, after in vitro restimulation, against the target cell line P815-ESAT6, which stably expressed the ESAT6 protein. P815 cell was used as a negative control. As the results shown in Fig. 5, DNA immunization with pcDNA3-ESAT6 plasmids resulted in a strong CTL response, which was significantly above the background level ($P < 0.01$). DNA prime-protein boost immunization

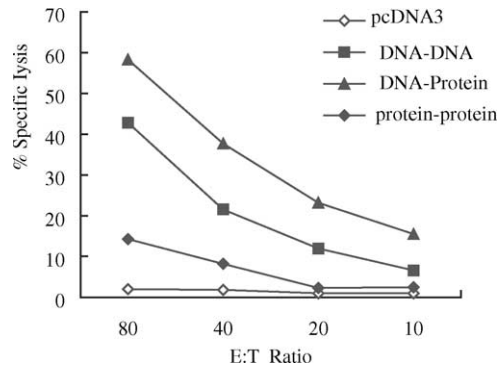

Fig. 5. Induction of $CD8^+$ CTL following immunization with different vaccines. Mice were inoculated as above. The percentage of specific lysis was measured after in vitro restimulation of the in vivo-primed cells with mitomycin-treated P815-ESAT6 cells. Effector cells [E] were incubated with P815-ESAT6 (target cells [T]) in a LDH release assay. Samples were assayed in triplicate. Results are representative of three independent experiments.

enhanced the cytotoxic T-cell response, compared to DNA–DNA inoculation ($P < 0.05$). Protein–protein injection group elicited a much lower CTL response, compared to both DNA–DNA and DNA–protein group. The blank vector immunization did not induce CTL response. The spontaneous release was below 10%.

4. Discussion

It has been reported that DNA vaccines preferentially induced Th_1-dominant immune response [19]. The exact mechanism of driving Th_1 or Th_2 type response has not been well known, but it has been suggested that CpG motifs from a bacterial plasmid might be responsible for driving immune responses toward Th_1-type [20–23]. Th_1-type response has been reported to correlated with protective immunity in certain tumor, bacterial or viral infection, as well as some parasitic disease [24,25]. Protective immunity against tuberculosis mainly depends on cellular immune responses and some cytokines of Th_1-type, such as IFN-γ. Studies on DNA vaccines against *TB* have been reported in the past few years [7–10]. However, results showed that the efficacy of *TB* DNA vaccines needed more improvement.

DNA prime-protein boost immunization protocol is well known for its capacity to modulate immune responses elicited by DNA vaccines. Sin's study [25] showed that in a murine herpes simplex virus-2 gD vaccine model, DNA prime-protein boost immunization enhanced both antigen-specific antibody and Th_1-type cellular immune responses. Another report [26] demonstrated that mice vaccinated with mapI gene followed by protein boost augmented protection against the challenge with *Cowdria ruminantium*. The augmented protection by the prime-boost regimen correlated with the improved Th_1-type immune response which was induced by the DNA vaccine. Those responses were characterized by production of IFN-γ, IL-2 and anti-map I antibodies of predominantly IgG_{2a} isotype. Haddad et al. [27] studied the change of IgG isotype by DNA prime-protein boost protocol against *Plasmodium falciparum*. However, Their results demonstrated that after protein boosting, immunoglobulin (Ig)G subclass profile skewed to the IgG_1 isotype, which indicated a Th_2 type immune response.

In our experiment, we aimed to observe the effect of DNA prime-protein boost immunization formation on the immune responses elicited by ESAT6 DNA vaccine of *TB* in mice model. The combination prime/boost strategy has the potential to overcome possible deficiencies that may arise using a single vaccination modality against *TB* (for example employing a DNA, protein subunit vaccine alone). ESAT6 DNA vaccine elicited a Th_1-type immune response. After one injection of DNA and two inoculations with recombinant ESAT6 protein, the ratio of IgG_{2a} to IgG_1 was considerably enhanced, compared to DNA immunization. The level of IgG_{2a} was lower than that of IgG_1 in protein inoculation group, which indicated that the immune response was Th_2 type. Similarly, the lymphocyte proliferation response and IFN-γ secretion in DNA vaccination mice were significantly increased by the boost of ESAT6 protein. In protein immunization group, the IFN-γ level was lower than that of IL-4, which also indicated a Th_2-biased response. In the case of cytotoxicity of T-cell assay, DNA prime-protein boost protocol induced stronger cytotoxic T cell activity than DNA immunization. All these results indicated that the immune responses, Th_1 or Th_2 type was related to the types of priming immunogen. Those suggested that the ESAT6 DNA vaccine primed the correct pathway of the immune response (Th_1-type) and the ESAT6 protein boost enhanced the DNA–primed memory responses. Priming by the DNA vaccine was crucial for the type of protection immune response, since the ESAT6 protein on its own induced a different pathway (Th_2 type) of the immune response. Our findings in agreement with Audrey's reports [28], which showed that a boost injection of protein in mice which were given a DNA vaccine encoding Ag85A was capable of dramatically enhancing the Th_1-type immune response primed with this DNA vaccine.

In conclusion, the data presented above suggested that DNA prime-protein boost mainly enhanced Th_1-type cellular immune response. As it drive cytotoxic T-cell response, production of IgG_{2a} as well as IFN-γ were all enhanced. In contrast to infection with viral and protozoan pathogens, infection with *M. tuberculosis* remains largely confined to an intracellular localization. Thereby, it is greatly accepted that cell-mediated immune response is essential for control of the infection. However, antibody-dependent cytotoxicity through antigen-specific IgG_{2a} immunoglobulin, IFN-γ cytokines should also play a role in the initial control of *TB* infection. Taken together, our results demonstrated that DNA prime-protein boost protocol could be as a new strategy to improve the efficacy of *TB* DNA vaccine.

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References

[1] Kaufmann SH. Is the development of a new tuberculosis vaccine possible? Nat Med 2000;6(9):955–60.
[2] Roche PW, Triccas JA, Winter N. BCG vaccination against tuberculosis: past disappointments and future hopes. Trend Microbiol 1995;3(10):397–401.
[3] Cardona PJ, Cooper A, Luquin M, Ariza A, Filipo F, Orme IM, et al. The intravenous model of murine tuberculosis is pathogenic than the aerogenic model owing to a more rapid induction of systemic immunity. Scand J Immunol 1999;49(4):362–6.
[4] Fuller DH, Rajakumar PA, Wilson LA, Trichel AM, Fuller JT, Shipley T, et al. Induction of mucosal protection against primary, heterologous simian immunodeficiency virus by a DNA vaccine. J Virol 2002;76(7):3309–17.
[5] Roche PW, Neupane KD, Failbus SS, Kamath A, Britton WJ. Vaccination with DNA of the *Mycobacterium tuberculosis* 85B antigen protects mouse foot pad against infection with *M. leprae*. Int J Lepr Other Mycobact Dis 2001;69(2):93–8.
[6] Wang QM, Sun SH, Hu ZH, Wu D, Wang ZC. Immune response and protection elicited by DNA immunization against *Taenia cysticercosis*. Vaccine 2003;21(15):1672–80.
[7] Ulmer JB, Liu MA, Montgomery DL, Yawman AM, Deck RR, Dewitt CM, et al. Expression and immunogenicity of *Mycobacterium tuberculosis* antigen 85 by DNA vaccination. Vaccine 1997;15(8):792–4.
[8] Kamath AT, Feng CG, Macdonald M, Briscoe H, Britton WJ. Differential protective efficacy of DNA vaccines expressing secreted proteins of *Mycobacterium tuberculosis*. Infect Immun 1999;67(4):1702–7.
[9] Tanghe A, Lefevre P, Denis O, D'Souza S, Braibant M, Lozes E, et al. Immunogenicity and protective efficacy of tuberculosis DNA vaccine encoding putative transport receptors. J Immun 1999;162(2):1113–9.
[10] Zhu X, Venkataprasad N, Tangouaj HS, Hill M, Singh M, Ivanyi J, et al. Function and specificity of T cells following nucleic acid vaccination of mice against *Mycobacterium tuberculosis* infection. J Immun 1997;158(12):5921–6.
[11] Delogu G, Howard A, Collins FM, Morris SL. DNA vaccination against tuberculosis: expression of a ubiquitin-conjugated tuberculosis protein enhances antimycobacterial immunity. Infect Immun 2000;68(6):3097–102.
[12] Kang Y, Calvo PV, Daly TM, Long CA. Comparison of humoral immune responses elicited by DNA and protein vaccines based on merozoite surface protein-I from *Plasmodium yoelii*, a rodent malaria parasite. J Imunol 1998;161(8):4211–9.
[13] Putkonen P, Quesada-Rolander M, Leandersson AC, Schwartz S, Thorstensson R, Okuda K, et al. Immune responses but no protection against SIV by gene-gun delivery of HIV-I DNA followed by recombinant protein boosts. Virology 1998;250(2):293–301.
[14] Qingmin W, Zhenlin H, Shuhan S. Construction of *Mycobacterium tuberculosis* protective antigens-ubiquitin system. Acad J Sec Med Univ 2003;24(1):61–3.
[15] Cotten M, Baker A, Saltik M, Wagner E, Buschle M. Lipopolysaccharide is a frequent contaminant of plasmid DNA preparations and can be toxic to primary human cells. Gene Ther 1994;1(4):293–6.
[16] Qingmin W, Zhenlin H, Shuhan S, et al. Cloning and expression of ESAT6 cDNA coding region of *Mycobacterium tuberculosis* in *E. coli*. Chin J Infect Dis 2003;21(1):303–33.
[17] Danko I, Fritz JD, Jiao S, Hogan K, Latendresse JS, Wolff JA. Pharmacological enhancement of in vivo foreign gene expression in muscle. Gene Ther 1994;1(2):114–21.
[18] Decker T, Lonmann-matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods 1998;115(1):61–9.
[19] Raz E, Tighe H, Sato Y, Corr M, Dudler JA, Roman M, et al. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. Proc Natl Acad Sci USA 1996;93(10):5141–5.
[20] Freidag BL, Melton GB, Collins F, Klinman DM, Cheever A, Stobie L, et al. CpG oligodeoxynucleotides and interleukin-12 improve the efficacy of *Mycobacterium bovis* BCG vaccination in mice challenged with *M. tuberculosis*. Infect Immun 2000;68(5):2948–53.
[21] Klinman DM, Yamshchirov G, Ishigatsubo Y. Contribution of CpG motifs to the immunogenicity of DNA vaccines. J Immunol 1997;158(8):3635–9.
[22] Jakob T, Walker PS, Krieg AM, Udey MC, Vogel JC. Activation of cutaneous dentritic cells by CpG containing oligonucleotides: a role for dentritic cells in the augmentaion of Th1 response by immunostimulatory DNA. J Immunol 1998;161(6):3042–9.
[23] Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligonucleotides act as adjuvants that switch on T helper I (Th1) immunity. J Exp Med 1997;186(10):1623–31.
[24] Chow YH, Chiang BL, Lee YL, Chi WK, Lin WC, Chen YT, et al. Developing of Th1 and Th2 population and the nature of immune response to hepatitis B virus DNA vaccines can be modulated by codelivery of various cytokine genes. J Immunol 1998;160(3): 1320–9.
[25] Sin JI, Kim JJ, Boyer JD, Ciccarelli RB, Higgins TJ, Weiner DB. In vivo modulation of vaccine-induced immune responses toward a Th1 phenotype increase potency and vaccine effectiveness in a herpes simplex virus type 2 mouse model. J Virol 1999;73(1):501–9.
[26] Nyika A, Barbet AF, Burridge MJ, Mahan SM. DNA vaccination with *map I* gene followed by protein boost augments protection against challenge with *Cowdria ruminantium*, the agent of heartwater. Vaccine 2002;20(7-8):1215–25.
[27] Haddad D, Liljeqvist S, Stahl S, Hansson M, Perlmann P, Ahlborg N, et al. Characterization of antibody response to a *Plasmodium falciparum* blood-stage antigen induced by a DNA prime/protein boost imunnizaton protocol. Scand J Immunol 1999;49(5):506–14.
[28] Tanghe A, D'Souza S, Rosseels V, Denis O, Ottenhoff TH, Dafemans W, et al. Improved immunogenicity and protective efficacy of a tuberculosis DNA vaccine encoding Ag85 by protein boosting. Infect Immun 2001;69(5):3041–7.