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Enhanced Immune Response of a Bicistronic DNA Vaccine Expressing Fusion Antigen Hsp65-Esat-6 of *Mycobacterium Tuberculosis* with GM-CSF as a Molecular Adjuvant

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ABSTRACT

This study aimed to construct a bicistronic DNA vaccine expressing fusion antigen Hsp65-Esat-6 of Mycobacterium tuberculosis *with cytokine GM-CSF as a molecular adjuvant (pIRES-Hsp65-ESAT-6-GM-CSF, pIRHEG), and the immune response in mice. C57BL/6 mice were immunized with the recombinant plasmid to detect the titer of antibodies, lymphocyte proliferation, the ratio of $CD4^+$, $CD8^+$ T cell and IFN ~ γ□IL-2 secretion. The titer of antibody, lymphocyte proliferation, the ratio of $CD4^+$ T and $CD8^+$ T cells and IFN ~ γ, IL-2 secretion of pIRHEG group was significant higher than other recombinant plasmid groups, which significant differed by statistical mean. The bicistronic DNA vaccine could induce an effective immune response in mice and could be used as vital ingredient of a new tuberculosis vaccine candidate.*

Key words: Hsp65, Esat6, GM-CSF

INTRODUCTION

It is estimated that nearly one-third of the world's population is infected with *Mycobacterium tuberculosis* (MTB). Every year about 1,000 million new cases of its infection appear and 200 million people die of tuberculosis (Wallis et al. 2010). Vaccine represents the key tool of preventing MTB. Although the Bacillus Calmette-Guerin (BCG) vaccine has been widely used against MTB for nearly 100 years, it has not contributed significantly to the reduction in incidence of MTB, with merely only 50% rate of the success. Along with the migration and HIV infection, some new and easily-spreading MTB strains and multiple drug-resistant strains make the traditional anti-TB drugs fail to play an effective role (Hesseling et al. 2007; Azzopardi et al. 2009; Stefan et al. 2010). It is very likely that MTB may become endemic in the immunized population and cause new public problems. Therefore, it is pressing to develop a novel vaccine.

Currently, DNA vaccine has gained more attention with greater development since it maintains a long immune response and is safe as a recombinant subunit vaccine. It is effective as a live attenuated vaccine in systemic immune response, with easier cloning of DNA molecule and no requirement of protein synthesis's *in vitro*, extraction and purification (Hanif et al. 2010). The MTB hot shock protein 65 (heat shock protein 65, Hsp65), one of earliest MTB antigen studied, may have the intense protection immune response in the animal *in vivo* to MTB's infection (Yoshida et al. 2006; Sechi et al. 2006; Okada et al. 2007). It can provide stable and reliable basis protective immunity for anti-*M. tuberculosis* infection. The small-molecular dose of secretory protein ESAT-6

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is an extremely strong antigenic protein expressed in the MTB complex, in which a large quantity of B and T cell epitopes are present (Abramo et al. 2006; Van et al. 2010). It can increase the capacity of macrophage inhibition of the growth and cytotoxicity of *M. tuberculosis* intracellular level. During the long course of cultivation *in vitro*, ESAT-6 is missing from virulent *M. tuberculosis* that may lead to BCG instability of immunization effect (Brodin et al. 2004; Khera et al. 2005).
Granulocyte macrophage colony-stimulating factor (GM-CSF) as a good gene adjuvant, can increase the effectiveness of DNA vaccines by improving human body's immune level (Qiu et al. 2007; Zhang et al. 2007). This study aimed to construct a bicistronic DNA vaccine expressing fusion antigen Hsp65-Esat-6 of *Mycobacterium tuberculosis* with cytokine GM-CSF as a molecular adjuvant (pIRES-Hsp65-ESAT-6-GM-CSF, pIRHEG) and the immune response in mice, to lay the foundation for the further development of a new DNA vaccine resistance for *Mycobacterium tuberculosis* infection.

METHODS

Construction and identification of pIRES-Hsp65-ESAT-6-GM-CSF plasmids

The whole-genome was extracted from MTB *H37Rv* by *M. tuberculosis* genomic DNA extraction kit (beads) (Shanghai DOBIO, China), and used it as a template to amplify the *Hsp65* and *Esat6* gene. the sequences of gene-specific primers for *Hsp65* forward: 5'-CGGCTAGC ATGGCCAAGACAATTGCGTACG-3', including restriction enzyme cutting site *Nhe*I (TaKaRa BIO Inc, Japan) and initiation codon, and reverse: 5′-CAGAATTCGTCACCTTTACCGCATCG -3′, including restriction enzyme cutting site *Eco*RI and without termination codon. *Esat6* forward: 5′-CAGAATTCGGTGGCGGTGGAAGCGGCGGT GGCGGAAGCGGCGGTGGCGGCAGCACAGA GCAGCAGTGGAATTTC-3′, including restriction enzyme cutting site *Eco*RI and a hydrophobic peptide consisted of 15 amino acids, and reverse: 5'-GCACGCGTCTATGCGAACATCCCAGTGAC G-3′, including restriction enzyme cutting site *Mlu*I and termination codon. CDS forward: 5'-CATCTAGAATGTGGCTGCAGAGCCTGCT-3', including restriction enzyme cutting site *Xba*I and initiation codon ATG, and reverse: 5'-ATGCGGCCGCTCACTCCTGGACTGGCTC-3', including restriction enzyme cutting site *Not*I and termination codon TGA. Vector pORF-GM-CSF was used as a template. The two gene fragments *Hsp65-ESAT-6* and *GM-CSF* were cloned into pIRES vector with the two multiple cloning sites (MCSA and MCSB). Finally a recombinant plasmid named pIRHEG, which was identified by PCR, double digested with *Xba*I and *Not*I restriction enzyme analysis and sequencing, was obtained.

Detection of recombinant plasmids expression

The recombinant plasmids (pIRES-Hsp65-ESAT-6-GM-CSF (pIRHEG), pIRES-Hsp65-ESAT-6 (pIRHE), pIRES-Hsp65 (pIRH), pIRES-ESAT-6 (pIRE)) and empty plasmid vector pIRES were transfected into HepG-2 cells, then the expression of proteins was detected by Western-blot and enzyme-linked immunosorbent assay (ELISA). The expressed proteins were separated by 10% SDS-PAGE, electro-transferred onto a nitrocellulose membrane. The membrane was blocked with nonfat milk, then incubated with 1:1000 dilution of mouse anti-human Hsp65 or 1:500 dilution of mouse anti-human ESAT6, and then incubated with corresponding a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. The immunochemical detection was performed by an enhanced chemiluminescence (ECL) system (Thermo Fisher Scientific Inc. USA) to visualize specific immune-reactive bands. Transfected HepG-2 cells culture supernatants were collected separately after 24 and 48 h, using Double-antibody sandwich ELISA to detect of the GM-CSF protein expression level according to the instruction of kit (R&D Systems, USA).

Animal immunization

Female C57BL/6 mice (6-8 weeks old) were randomly divided into seven groups (total 140): pIRES-Hsp65 group (pIRH); pIRES-ESAT-6 group (pIRE); pIRES-Hsp65-ESAT-6 group (pIRHE); pIRES-Hsp65-ESAT-6-GM-CSF group (pIRHEG) (Endotoxin-free plasmids were prepared using an EndoFree plasmid purification mega prep kit, Qiagen); BCG group; pIRES group; and PBS group, twelve mice per group. Mice were immunized three times at 2-week intervals bilateral anterior tibial muscle injection with 100 μg pIRH, pIRE, pIRHE, pIRHEG or pIRES. The

control animals (PBS group) were sham-immunized with 100 μl PBS. The BCG group was vaccinated subcutaneously with 0.5 mg once at the first immunization. Mice were sacrificed to conduct the analysis of immune responses at 2, 4 and 6 weeks after the immunization. Blood samples were collected and kept at – 20°C for usage. The study was carried was approved by the Ethics and Animal Care Committee of First Affiliated Hospital of Jinan University for all animal procedures and the experimental protocol.

Evaluation of antibody responses

An indirect enzyme-linked immunosorbent assay (ELISA) was used for determining the specific antibody. Serum was collected every week after the immunization by tail bleeding and stored at −70°C for further analysis. Plates were coated with PPD protein at a final concentration of 10 μg / ml at 4°C overnight. After blocking with 1% BSA-PBS, serum (1:100 dilution) was added in duplicate at 37°C after 1.5 h. HRP-conjugated goat anti-mouse IgG was added, followed by OPD substrate addition. The absorbance was measured in a microplate reader at 490 nm. Monitor was used to detect the levels of serum antibody in immunized mice for eight weeks duration.

Proliferation of splenocyte

Lymphocyte proliferation of immunized mice were measured by MTT assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide]. At 2, 4, 6 weeks after the first immunization, mice were scarified and their spleens were aseptically removed and splenocyte were prepared as single cell suspensions. The cells were plated in 96-well flat-bottomed microtitre plates in triplicate at 2×10^7 cells per well. RPMI 1640 (GIBCO, UK) supplemented with 10% fetal calf serum was added to each well and stimulated with 50 μl PPD (the Ministry of Health, Chengdu Institute of Biological Products) at 1 mg/l. Lymphocytes stimulated with the medium alone were used as a negative control. The cells were incubated for 48 h at 37°C in humid atmosphere of 5% CO_2 and then 20 ml of MTT (5 mg/ml) (Sigma) was added to each well. After additional incubation for 4 h, the supernatant were carefully aspirated and 200 μl of DMSO was added into each well and absorbance of the soluble formazan was measured at 570 nm by automatic microplate reader (Bio-Rad, USA).

Percentages of splenocyte subsets

The splenocyte from mice were prepared and cultured. Then they were seeded in 6-well flat-bottom plates at a concentration of 5×10^6 cells per well and in duplicate for culture with 1.5 ml PPD (1 mg/l), The plate was incubated for 72 h at 37°C in humid atmosphere of 5% CO_2. Then the cells were washed with PBS, then rabbit anti-mouse $CD4^+$-PE (eBioscience, San Diego, CA, USA) and rabbit anti-mouse $CD8^+$-fluorescein isothiocyanate (eBioscience) were added and incubated for 1 h at room temperature. Subsequently the cells were washed three times and the proportions of $CD4^+$ T and $CD8^+$ T cells were determined by flow cytometry.

IFN-γ and IL-2 concentration of the lymphocyte secreted

The splenocyte of immunized mice were cultured by the same operation in the proliferation assays for 72 h. Following incubation, the supernatant from each well were removed and kept at – 20°C for the evaluation of secreted IFN-γ and IL-2 level. The concentrations of IFN-γ and IL-2 in the culture supernatant were measured by murine cytokine enzyme-linked immunosorbent assay kits (R&D systems, USA).

Statistical analysis of data

All the measurement data represented the mean ± standard errors (x±s). Statistical software SPSS was used to perform the statistical analysis. Differences among the groups were analysed with stochastic analysis of variance. Differences were considered significant when a probability value of < 0.05 was obtained.

RESULTS

Detection of recombinant vaccine expression

When pIRHEG, pIRHE, pIRH, pIRE and pIRES plasmide were transfected into HepG-2 cells, the expression proteins were detected by the Western-blot. The results showed that protein expression was 75 KD of pIRHEG and pIRHE groups as expected. Protein expression ESAT6 was 10 KD and Hsp65 was 65 KD as the same as anticipated (Fig. 1).

The levels of cytokine GM-CSF were measured by using Microplate reader in pIRHEG group. In two different periods, compared with the pIRES group,

the levels of GM-CSF cytokine of pIRHEG group had a statistically significant difference ($P < 0.01$); compared with the pIRES-GM group, the levels of GM-CSF cytokine of pIRHEG group had no significant difference ($P > 0.05$). The results showed that the expression of Hsp65-Esat6 fusion gene in the upstream multiple cloning sites of the vector pIRES did not significantly affect the downstream cloning site of GM-CSF protein expression (Fig. 2).

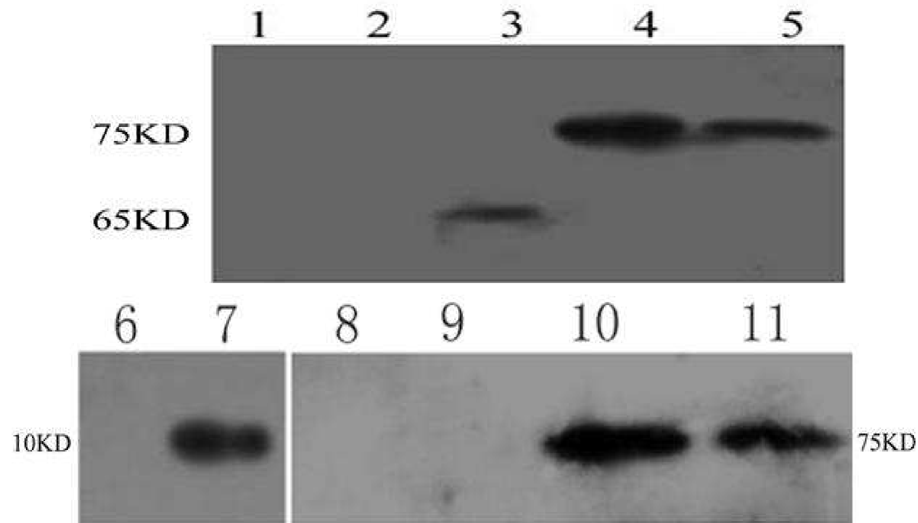

Figure 1 - Identification of fusion protein Hsp65- ESAT-6 by Western blot. The fusion protein expressed in HepG-2 cell line could be recognized by anti-Hsp65 MoAb and anti-ESAT-6 MoAb, respectively.

Line1: Lysate supernatant from transfected pIRES HepG-2 cells with anti-Hsp65 MoAb
Line2: Lysate supernatant from transfected pIRE HepG-2 cells with anti-Hsp65 MoAb
Line3: Lysate supernatant from transfected pIRH HepG-2 cells with anti-Hsp65 MoAb
Line4: Lysate supernatant from transfected pIRHE HepG-2 cells with anti-Hsp65 MoAb
Line5: Lysate supernatant from transfected pIRHEG HepG-2 cells with anti-Hsp65 MoAb
Line6: Lysate supernatant from transfected pIRES HepG-2 cells with anti-ESAT-6 MoAb
Line7: Lysate supernatant from transfected pIRE HepG-2 cells with anti-ESAT-6 MoAb
Line8: Lysate supernatant from transfected pIRES HepG-2 cells with anti-ESAT-6 MoAb
Line9: Lysate supernatant from transfected pIRH HepG-2 cells with anti-ESAT-6 MoAb
Line10: Lysate supernatant from transfected pIRHE HepG-2 cells with anti-ESAT-6 MoAb
Line11: Lysate supernatant from transfected pIRHEG HepG-2 cells with anti-ESAT-6 MoAb

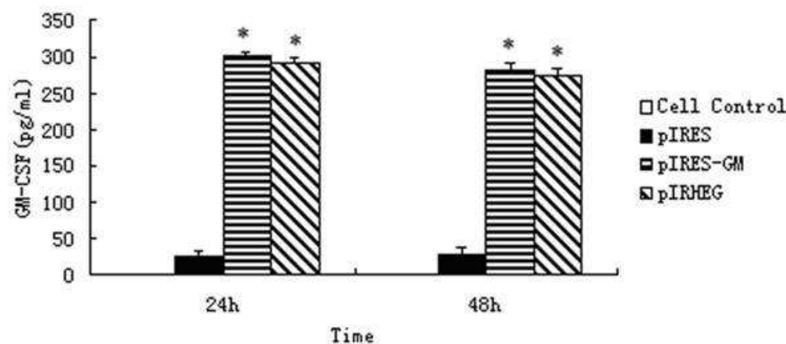

Figure 2 - ELISA analysis of GM-CSF protein in HepG-2 cells. Collected the transfected cells culture supernatants separately at 24 and 48 h, then detect the expression by using GM-CSF protein ELISA examination reagent box according to the instruction of box. *, $P < 0.05$ compared to the negative control groups (Cell Control, pIRES).

Evaluation of antibody responses

Humoral immune responses were determined by measuring the total IgG in sera collected from the immunized mice at 2, 4, 6 and 8 weeks after the immunization. The IgG level was increased with the prolongation of immune time. Figure 3 illustrates the levels of antibody response in the sera of mice immunized with recombinant plasmids and PBS in different time point, using TB-PPD as the antigen. The IgG titre of pIRHEG group was higher than other recombinant plasmid groups and BCG group ($P < 0.05$). The IgG titres of BCG group had no significant difference between other experimental groups (pIRHE, pIRH, pIRE) ($P > 0.05$). Three weeks after the first immunization, there were significant difference ($P < 0.05$) between the experimental groups (pIRHEG, pIRHE, pIRH, pIRE) and the control group (pIRES, PBS) in the titer of antibody. Compared with the two control groups, pIRHEG group showed significant difference ($P < 0.01$). However, there was no significant difference between the two control groups ($P > 0.05$).

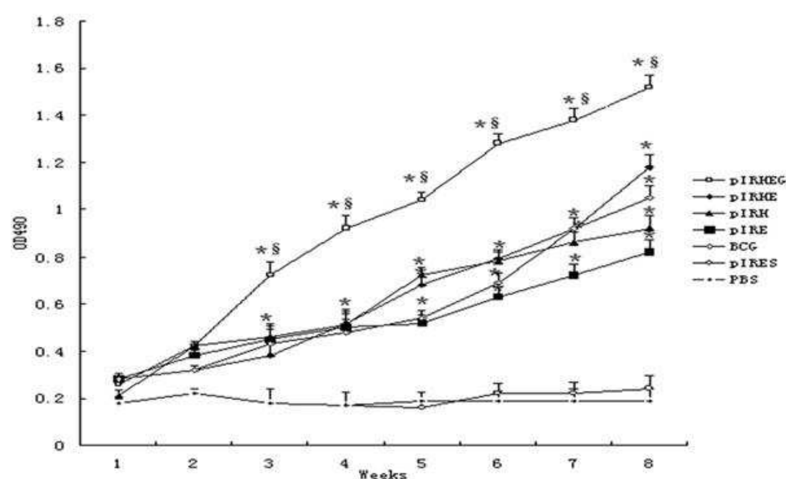

Figure 3 - Analysis of antibody responses against tuberculin-purified protein derivative (TB-PPD). Every week after immunization, mice immunized with pIRHEG, pIRHE, pIRH, pIRE, pIRES, BCG and PBS were killed. Sera were obtained and tested for specific antibody levels, using an indirect enzyme-linked immunosorbent assay. Plates were coated with PPD protein, added HRP-conjugated goat anti-mouse IgG. Results are expressed as the mean ± standard errors. *, $P < 0.05$ compared to the negative control groups (cell control, pIRES); §, $P < 0.05$ compared to BCG group.

Proliferation of splenocyte

The SI value was calculated as the ratio of absorbent value at 570 nm of splenocyte pools incubated with the TB-PPD to that of pools incubated with medium only. Figure 4 showed that the SI values in the experimental groups were always higher than the control group. At two weeks after the first immunization, the SI values between the experimental group and in the control groups had no significant difference ($P > 0.05$). At six weeks after the first immunization, the SI values in single gene experimental groups decreased than at four weeks. But the SI values in fusion gene experimental groups kept increasing. The SI values in the pIRHEG group and BCG group were significantly higher than any other recombinant plasmid groups ($P < 0.05$). However, there was no significant difference in the SI value between the pIRHEG group and BCG group ($P > 0.05$).

Percentages of splenocyte subsets

The proportions of splenocyte subsets stimulated by the TB-PPD were determined by flow cytometry. Figure 5 showed that $CD4^+$ T cells and $CD8^+$ T cells increased significantly in the immunization groups. At two weeks after the first immunization, the ratio of $CD4^+$ and $CD8^+$ T cell between the experimental groups had no significant difference ($P > 0.05$). At four and six weeks after the first immunization, the ratio of $CD4^+$ and $CD8^+$ T cells in the pIRHEG group and

BCG group were significantly higher than any other recombinant plasmid groups (P < 0.05). However, there was no significant difference in the ratio of $CD4^+$ and $CD8^+$ T cell between the pIRHEG group and BCG group (P > 0.05).

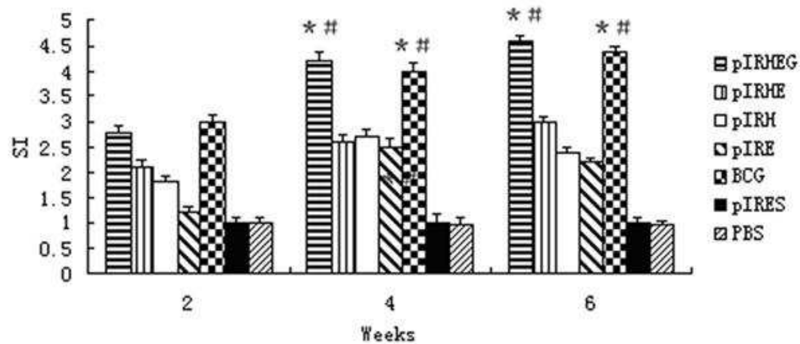

Figure 4 - Lymphoproliferative response to these types of vaccines. Lymphocytes isolated from the spleens of the mice immunized with pIRHEG, pIRHE, pIRH, pIRE, pIRES, BCG and PBS were cultured in 96-well flat bottom plates. The cells were stimulated with tuberculin-purified protein derivative (TB-PPD) (1 mg/l). Lymphocytes stimulated with the medium alone were used as a negative control. The proliferative response was measured by MTT way. Stimulation index value was calculated as the ratio of absorbent value at 570 nm of pools incubated with TB-PPD to that of pools incubated with medium only. The data is presented as means ± standard errors. *, P < 0.05 compared to the negative control groups (PBS, pIRES); #, P < 0.05 compared to any other recombinant plasmid groups (pIRHE, pIRH, pIRE).

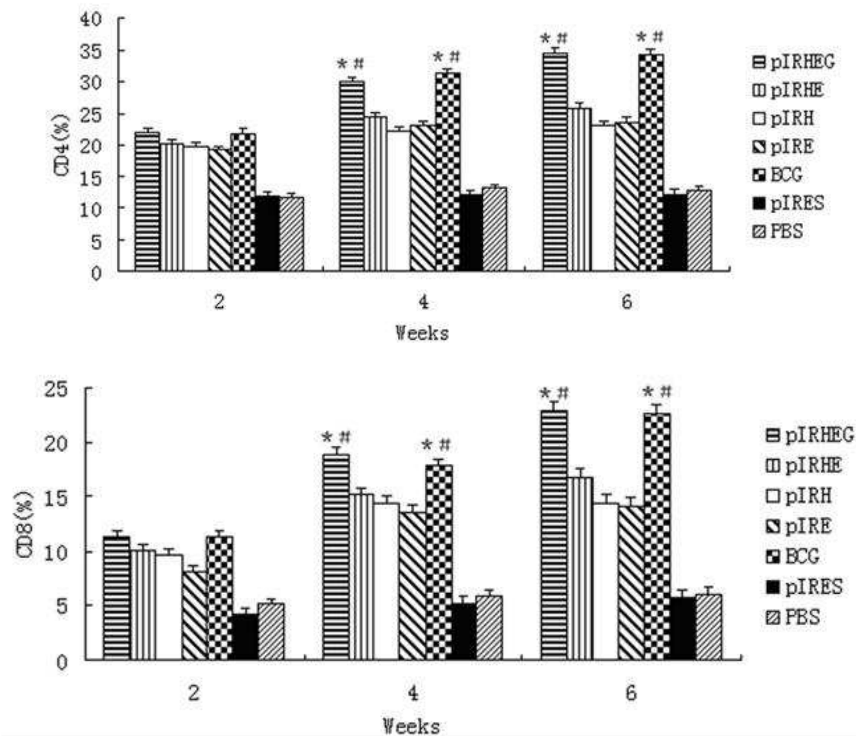

Figure 5 - The percentages of $CD4^+$ and $CD8^+$ subsets in different time point (%). The splenocyte from immunized mice were stimulated with tuberculin-purified protein derivative (TB-PPD) (1 mg / l) and were stained immunocytochemically by rabbit anti-mouse $CD8^+$-fluorescein isothiocyanate. The proportions of splenocyte subsets were determined by flow cytometry 2, 4, 6 weeks after vaccination. Results are presented as mean ± standard errors. *, P < 0.05 compared to the negative control groups (PBS, pIRES); #, P < 0.05 compared to any other recombinant plasmid groups (pIRHE, pIRH, pIRE).

IFN-γ and IL-2 concentration of the lymphocyte secreted

IFN-γ and IL-2 secretion is indicative of Th1-biased responses. Murine cytokine enzyme-linked immunosorbent assay kit was used to detect the levels of IFN-γ and IL-2 secreted by splenocyte in all immunized mouse groups. Results clearly showed that after stimulation with the TB-PPD *in vitro*, the concentrations of IFN-γ in the experimental group and BCG group were maintained in a high level. At two weeks after the first immunization there were no significant differences between the groups ($P > 0.05$). At four and six weeks after the first immunization, the levels of IFN-γ in the pIRHEG group and the BCG group were significantly higher than any other groups ($P < 0.05$). However, there was no significant difference between the pIRHEG group and BCG group ($P > 0.05$). Other experimental groups had no significant difference ($P > 0.05$). At six weeks after the first immunization, the levels of IFN-γ in single gene experimental groups decreased than at four weeks (Fig. 6).

At two weeks after the first immunization, the concentrations of IL-2 in the groups had no significant difference ($P > 0.05$). At four and six weeks after the first immunization, the levels of IL-2 in the pIRHEG group and the BCG group were significantly higher than any other groups ($P < 0.05$). However, there was no significant difference between the pIRHEG group and BCG group ($P > 0.05$) (Fig. 6).

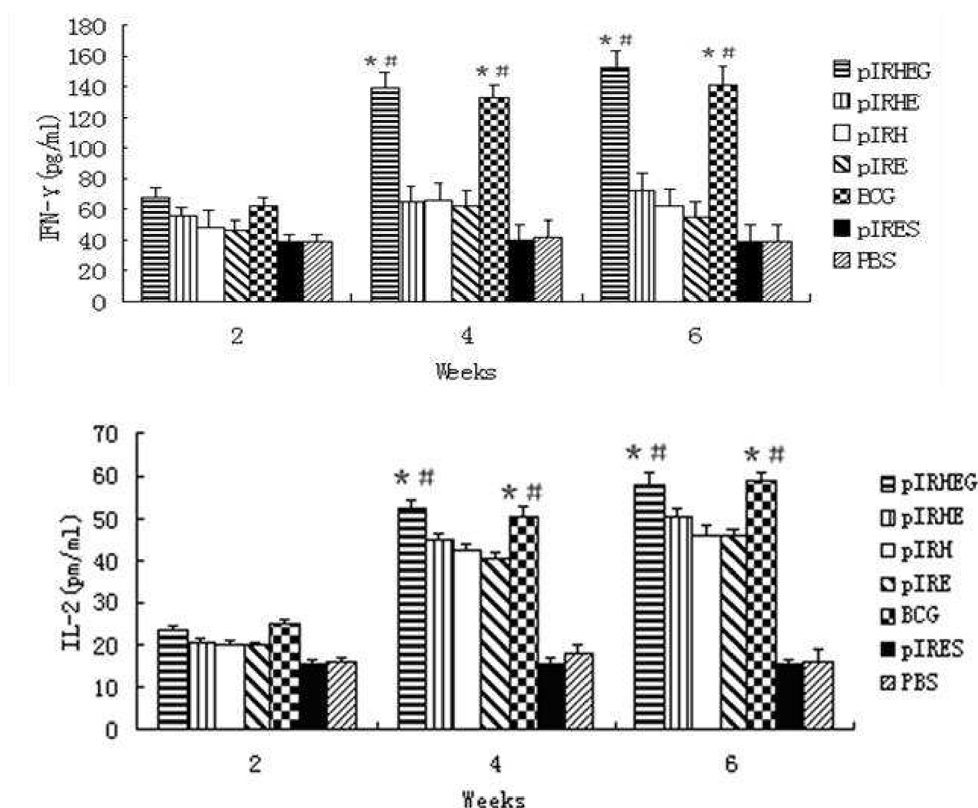

Figure 6 - ELISA analysis of IFN-γ and IL-2 concentration of the lymphocyte secreted. The splenocyte collecting from the mice immunized with pIRHEG, pIRHE, pIRH, pIRE, pIRES, BCG and PBS were seeded in 96-well flat-bottom plates and stimulated with TB-PPD (1 mg / l), and the plate was incubated at 37°C for 60h with 5% CO_2, IFN-γ and IL-2 concentration of the lymphocyte secreted into the medium was measured by ELISA kit according to the manufacture's protocol. *, $P < 0.05$ compared to the negative control groups (PBS, pIRES); #, $P < 0.05$ compared to any other recombinant plasmid groups (pIRHE, pIRH, pIRE).

DISCUSSION

Although, a separate gene encoding Hsp65 or Esat6 antigen resistance to *M. tuberculosis* in the role of infection has been confirmed, the role of single gene expression antigen is less than the BCG (Morris et al. 2000; Agger et al. 2006). Trail to add other immune stimuli, such as cytokines or other protective antigens have shown the beneficial results. As an adjuvant, GM-CSF has been used in gene vaccine. If the DNA vaccines were mixed injection with the constructed encoding GM-CSF plasmid, it might interfere with the immune function (Hu et al. 2004; Dou et al. 2005). The adjuvant and target gene were cloned into upstream and downstream two multiple cloning sites of bicistronic pIRES elements to make both the target gene and adjuvant has the same local micro-environment and their expression is noninterference and respectively (Martínez et al. 2001). Under this guidance, our study constructed recombinant plasmid pIRHEG with *Hsp65-Esat-6* fusion antigen gene and *GM-CSF* co-expression. By PCR, restriction enzyme digestion and sequencing confirmed that vector was successfully constructed. Western blot and ELISA confirmed the stable expression of recombinant antigen proteins with the control group pIRHE, pIRH and pIRE.

M. tuberculosis is the intracellular parasite bacterial. The body's immune response to MTB depends on the cellular immune response and evaluation of humoral immune response of MTB vaccine is an important indicator. The success of a vaccine against the intracellular pathogens as MTB mainly depends on the regulated activation of two types of T lymphocyte: $CD4^+$ and $CD8^+$ T cells. Studies have shown that mycobacterial infection can induce the spleen lymphocyte proliferation, IFN-γ, IL-2/As Th1-type cytokines can activate the macrophages to kill mycobacteria, the amount of its expression has a direct relationship with the protective effect of the vaccine (Vordermeier et al. 2002).

At four and six weeks after the immunization, the value of spleen lymphocyte proliferation, the ratio of $CD4^+$ and $CD8^+$ T cell and the level of IFN-γ, IL-2 in pIRHEG group were significantly higher than the other recombinant DNA vaccine groups. At six weeks after the immunization, the value of spleen lymphocyte proliferation and the level of IFN-γ in single-gene DNA vaccine groups were decreased than at four weeks. The values of spleen lymphocyte proliferation and the level of IFN-γ in the fusion gene DNA vaccine groups were increased after the immunization. The results indicated that the single-gene DNA vaccine induced cellular immune response were earlier than the fusion gene. The proliferation of $CD4^+$ T cell producing IFN-γ could activate the macrophages to fight against the early infection and the proliferation of $CD8^+$ T cell could promote the bacterial schizolysis by secreting porforin, granulysin and extracellular enzyme. Further investigation showed the capability of humoral immune response of the constructed DNA vaccine. The results showed that the DNA vaccine groups and BCG group had better performance in varying degrees, especially the level of antibodies of pIRHEG group was significantly higher than other recombinant DNA group and BCG group. The study showed that the pIRHEG DNA vaccine could induce strong cellular and humoral immune responses in mice, but its immune responses was not improved obviously compare with the BCG.

CONCLUSIONS

In summary, the use of the single-gene and fusion gene DNA vaccine involved in this study of immunize mice could activate the humoral immune response and cellular immune responses against *M. tuberculosis*. The adjuvant and fusion gene co-expression pIRHEG vaccine are significantly better than single-gene vaccine and fusion gene DNA vaccine pIRHE group. The GM-CSF can enhance the function and increase the number of antigen presenting cells, regulate the intensity of immune response in the immune response against *M. tuberculosis*. The results of this study provide a new approach for the development of new tuberculosis vaccines. It should have a good application prospect in tuberculosis DNA vaccines development if further studies on the role of fusion DNA vaccine in the immune protection *in vivo* are undertaken.

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