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# Recombinant *Mycobacterium smegmatis* expressing Hsp65-hIL-2 fusion protein and its influence on lymphocyte function in mice

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ABSTRACT

**Objective:** To construct a strain of recombinant *Mycobacterium smegmatis* expressing the heat shock protein 65(Hsp65) and human interleukin 2 (IL-2) fusion protein (rMS-Hsp65/IL-2) and to explore the effect of this construct on lymphocyte function in mice. **Methods:** The fusion gene encoding Hsp65-hIL-2 was cloned into shuttle vector pSMT3. The recombinant plasmid pSMT3-Hsp65-hIL-2 was transferred to *Mycobacterium smegmatis* by electroporation. Positive clones were selected by hygromycin and identified by PCR. The expression of fusion protein Hsp65-hIL-2 was verified using indirect immunofluorescence staining. Mice were immunized for two times by subcutaneously injection with $1\times10^6$ CFU rMS-Hsp65/IL-2 at a three-week interval. Two weeks after the second immunization, mice were sacrificed and the serum samples were collected for determination of anti-Hsp65 specific IgG. Splenic lymphocytes were isolated and treated with the rMS-Hsp65/IL-2 to determine lymphocytic proliferation activity by MTT assay. IFN-γ and IL-2 in the medium of the treated cells were also determined by ELISA. **Results:** Successful construction of rMS-Hsp65/IL-2 was verified by PCR and immunofluorescence staining. Compared to the splenic lymphocytes isolated from mice immunized with Bacille Calmette-Guerin or mice immunized with *Mycobacterium smegmatis* alone, the splenic lymphocytes isolated from mice immunized with rMS-Hsp65/IL-2 showed a marked increase in the proliferation of lymphocytes, together with an increased production of important cytokines such as IFN-γ and IL-2. **Conclusions:** rMS-Hsp65/IL-2 markedly enhances lymphocyte function. Therefore, the fusion protein generated by rMS-Hsp65/IL-2 may be of potential value in generating an effective vaccine against tuberculosis.

## 1. Introduction

Combinatorial use of immunomodulatory agents with antituberculosis drugs holds a greater therapeutic potential for *Mycobacterium tuberculosis* (MTB). Therapeutic vaccines can be combined with antituberculosis agents to overcome immune tolerance and to selectively induce the body's specific and nonspecific immune response, so that a better therapeutic goal may be achieved[1]. Heat shock protein 65(Hsp65) is a highly immunogenic protective antigen that is important in MTB infection. The gene vaccine of Hsp65 has been widely used in MTB therapy[2]. human interleukin 2 (IL-2) plays a key role in cell-mediated immunity for intracellular bacteria. It is able to induce and activate NK and T cells to produce IFN-γ[3]. IL-6 can also promote the proliferation of NK and T cells, enhance NK cell cytotoxicity, and stimulate the maturity of cytolytic T lymphocyte cells[4]. In addition, IL-2 can activate macrophages and enhance their killing effect on MTB[5]. As such, IL-2 has been successfully used as an adjuvant treatment for TB[6]. Based on these published data, we hypothesized that a fusion protein containing

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Hsp65 and IL-2 would provoke a stronger immune response against MTB infection.

In this study, we constructed a Hsp65/IL-2 fusion protein and made this protein expressed in recombinant *Mycobacterium smegmatis* (rMS). The ability of the fusion protein expressing live vector vaccine to induce immune response in mice was examined. The insights from this study will lay a foundation for novel treatment approaches against TB.

## 2. Materials and methods

### 2.1. Animals and materials

Female BALB/c mice of SPF level with a body weight of 18-22 g were obtained from the Experimental Animal Center of Fourth Military Medical University (Xi´an, China). MTB strain H37Rv was provided by Dr. Rui Wang (Institute of Tuberculosis Prevention and Control of Shaanxi Province, Xi´an, China). Bacille Calmette Guerin (BCG) vaccine strain was provided by the Lanzhou Institute of Biological Products (Lanzhou, China). The prokaryotic vector pPRO-Hsp65-hIL-2 containing human IL-2 and Hsp65 was supplied by the Fourth Military Medical University Experimental Animal Center (Xi´an, China)[7]. Restriction enzymes and DNA ladder DL2000 were purchased from Takara Company (Dalin, China). ADC (Albumin-dextrose-catalase) enrichment and 7H9 medium were purchased from Difco Company (Detroit, MI USA). Anti-HSP65 monoclonal antibody was purchased from Perfe Scientific (Los Angles, USA). Anti-human IL-2 monoclonal antibody was purchased from Lab Vision Corporation (Kalamazoo,USA). FITC-labeled goat anti-mouse IgG was purchased from Beyotime biology and technology (Shanghai, China). *Mycobacterium smegmatis* $mc^2$ 155 strain was provided by the Department of Microbiology, the Fourth Military Medical University (Xi´an, China). *Escherichia coli* (*E. coli*)-*Mycobacterium* shuttle vector pSMT3 was a gift from Professor Marcus A. Horwitz (School of Medicine, the University of California, Los Angles, USA). All animal experiments were approved by the Animal Ethics Committee of the Gansu College of Traditional Chinese Medicine (Approval ID: 12002).

### 2.2. Construction of pSMT3-Hsp65-hIL-2

The pPRO-Hsp65-hIL-2 plasmid was digested with *Eco*RⅤ and *Hin*dⅢ. The target gene fragments (Hsp65 and hIL-2) were purified and sub-cloned into the *E. coli-Mycobacterium* expression vector pSMT3 following treatment with restriction enzymes *Eco*RⅤ and *Hin*dⅢ. The target genes containing pSMT3 vectors were transformed into *E. coli* DH5α competent cells by electroporation (2.5 KV, capacitance 25 μF, resistance 1 000 Ω). The bacteria were spread on hygromycin-containing LB plates for growth. Clones were picked, cut with restriction enzymes, and amplified. The purified plasmids were digested with *Eco*RⅤ and *Hin*dⅢ and the expression of target genes were verified usinging 1.5% agarose gel electrophoresis. Clones with confirmed expression of the target gens were further electroporated into $5\times10^6$ CFU (colony forming units) of competent cells for amplification. The resultant vector designated pSMT3-Hsp65-hIL-2 was used in the subsequent experiments.

### 2.3. Generation and identification of recombinant Mycobacterium smegmatis strain

The above-obtained rMS strain were expanded in 7H9 media at 37 ℃ for 3 h, and then grown at 37 ℃ for 3 h on 7H10 plates which were pre-coated with ADC (containing glucose, bovine serum albumin factor v, and catalase). The recombinant *Mycobacterium smegmatis* containing pSMT3-Hsp65-IL-2 gene (thereafter designated rMS-Hsp65/IL-2) was obtained by hygromycin selection. The positive clones identified by PCR were further cultured for 2 days, centrifuged at 10 000 *g* for 3 min, and collected after three washes in sterile saline. Bacterial cells were counted using flow cytometry and diluted to a concentration of $1\times10^8$ CFU per 300 μL media containing 50 μL normal calf serum. Cells were then stained with anti-Hsp65 (diluted at 1:1 000) and anti-IL-2 (diluted at 1:1 000) monoclonal antibodies at 37 ℃ for 30 min. Cells were centrifuged at 10 000 *g* for 2 min, washed with sterile normal saline, and stained with FITC-labeled goat anti-mouse IgG (1:4 000) at room temperature for 15 min in the dark. After washing for three times in sterile normal saline, three μL bacterial suspension was taken onto a glass slide for observation under the fluorescence microscope (Olympus Fluoview BX61, Japan).

### 2.4. Animal immunization

Ten female BALB/c mice were subcutaneously injected in the dorsal flank with $1\times10^6$ CFU of one of the following: bacterial cells containing rMS-Hsp65/IL-2 ($1\times10^6$ CFU), *Mycobacterium smegmatis* only, BCG alone, and normal saline. Booster injections were given after 3 weeks.

### 2.5. Detection of anti-Hsp65 serum specific IgG

Blood samples were taken at 2, 4, 6, and 8 weeks after the initial immunization and the level of antibody against anti-Hsp65 was determined by ELISA. Briefly, 96-well ELISA plates were coated with 100 μL of purified Hsp65 protein (10 μg/mL) at 4 ℃ overnight. Plates were then washed with sterile normal saline followed by blocking with 1% bovine serum albumin at 37 ℃ for 1 h. Into each well, 100 μL of serum sample from each mouse was added and the plates were incubated at 37 ℃ for 1

h. Unconjugated antibodies were removed by washing the wells with normal saline. Goat anti-mouse IgG-HRP (1:5 000) was added to each well and the plates were incubated at 37 ℃ for 1 h. The plates were then incubated with the substrate solution (O-phenylenediamine) (100 μg/mL per well). All the measurements were obtained at $A_{450}$ nm and presented as $OD_{450}$ nm.

### 2.6. Isolation of splenic lymphocytes from mice

Two weeks following the second immunization, six mice from each group were sacrificed by cervical dislocation. The spleen was dissociated with several times of syringe needle core grinding, and the resultant splenic tissues were re-suspended in RPMI-1640 medium. The cell suspension was gently added to lymphocyte separation medium (at 1:2 ratio) and centrifuged at 1 000 rpm for 20 min. The lymphocyte rich middle layer was collected and washed twice with RPMI-1640.

### 2.7. Specific lymphocyte proliferation test by MTT assay

The effect of rMS-Hsp65/IL-2 on the proliferation of mouse lymphocytes was examined by standard MTT assay. Briefly, the above isolated lymphocytes were cultured at a concentration of $1\times10^5$/well in 96-well plates in RPMI-1640 supplemented with 10% fetal calf serum (FCS). Fifty μL of purified Hsp65 protein (25 mg/L in PBS) was then added to each well and the plates were incubated at 37 ℃ at 5% $CO_2$ for 68 h. Four hours after addition of 20 μL/well of MTT (5 mg/mL in PBS, pH7.2), 150 μL of DMSO was added to each well and incubated for 10 min. Cell proliferation was measured by taking the OD reading at 490 nm. The results were expressed as stimulation index (SI), calculated as: $SI=OD_{490}$ of experimental condition/$OD_{490}$ of respective control group.

### 2.8. Determination of induced cytokines from lymphocytes

Approximately $4\times10^6$ mouse splenic lymphocytes were cultured in 24-well plates in RPMI-1640 supplemented with 10% FCS. Cells in each well were incubated with 200 μL of purified Hsp65 at 5% $CO_2$, 37 ℃ for 72 h. The culture medium was collected and centrifuged at 5 000 rpm for 5 min. The supernatant was then stored at -20 ℃ for subsequent measurement for IFN-γ and IL-2 by ELISA kits, according to the manufacturer´s instructions (Jinmei Biotech Ltd, Shenzhen, China).

### 2.9. Statistical analysis

Data in different treatment groups were compared using factorial analysis of variance and were expressed as mean±standard deviation (SD). A $P$ value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. Verification of successful construction of rMS-Hsp65/IL-2 by immunofluorescence microscopy and PCR

We successfully sub-cloned the mycobacterium Hsp65-IL-2 fusion gene into the expression vector pSMT3. Staining of these clones by using specific antibody against mouse Hsp65 (Figure 1A) and mouse IL-2 (Figure 1B) revealed strong green immunofluorescence, indicating that these clones express Hsp65 and IL-2, respectively. By PCR, the size of the gene product (2 067 bp) by the pSMT3-Hsp65-hIL-2 vector is equivalent to that of the gene encoding Hsp65-IL-6 fusion protein (Figure 2A). Clones containing the pSMT3-Hsp65-hIL-2 vector were further cultured in 7H10 plate medium and positive clones expanded in 7H9 medium. Analysis of the extracted DNA from these clones by PCR showed successful expression of the Hsp65 gene fragment (1 620 bp) and IL-2 fragment (447 bp) (Figure 2B), confirming that pSMT3-Hsp65-hIL-2 expresses the Hsp65-hIL-2 fusion gene.

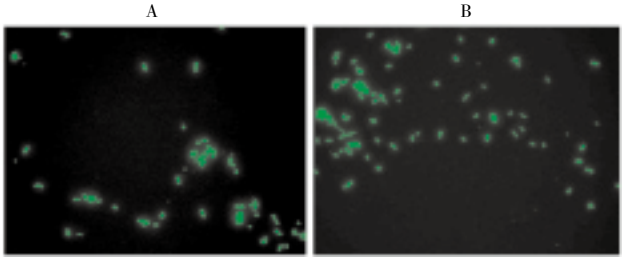


**Figure 1.** Construction and verification of the Hsp65/IL-2 fusion gene in shuttle vector pSMT3.
Positive clones from the fusion gene transfected bacterial cells were stained for Hsp65 (A) and IL-2 (B). The results were observed under fluorescence microscope (magnification: ×100).

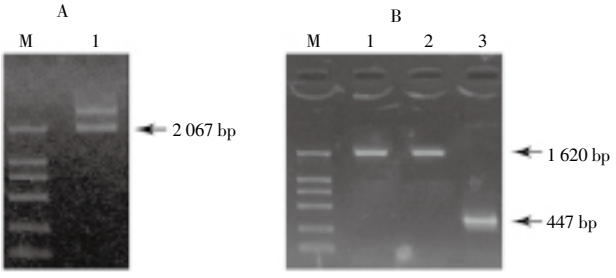


**Figure 2.** Confirmation of Hsp65-IL-2 fusion gene.
A. The amplified gene products from the sub-cloned pSMT3 vector were analyzed by PCR after being cut with the restriction enzyme *Eco*RⅤ and *Hind*Ⅲ. M: DNA marker DL2000; Lane 1: pPRO-Hsp65-IL-2 digested with *Eco*RⅤ and *Hind*Ⅲ.
B. The 2 067 bp fragment was extracted and further cut with *Eco*RⅤ and *Hind*Ⅲ, and two gene products with the size of 1 620 bp (lanes 1, 2) and 447 bp (lane 3) were generated. These two gene products correspond to Hsp65 and Il-2, respectively. M: DNA marker DL2000; 1: pPRO-Hsp65-IL-2 digested with *Eco*RⅤ and *Hind*Ⅲ. Lanes 1, 2: the PCR products for Hsp65 gene; lane 3, the PCR product for IL-2.

### 3.2. Immunization of mice with rMS-Hsp65/IL-2 induced the production of specific antibodies

Mice immunized with rMS-Hsp65/IL-2 produced much higher level of circulating anti-Hsp65-specific IgG, and a time-dependent response was observed (data not shown). Four weeks after the second immunization, higher level of antibody was detected in the serum of mice receiving pSMT-Hsp65-hIL-2 compared to mice receiving *Mycobacterium smegmatis* only or BCG (1:8 000, 1:2 400, and 1:3 200, respectively, as determined by immunohistochemical staining for the respective proteins) ($P$<0.05 between each group).

### 3.3. rMS-Hsp65/IL-2 increased lymphocyte proliferation

When spleen lymphocytes isolated from mice immunized with rMS-Hsp65/IL-2 positive clones were stimulated with Hsp65 antigen, a high stimulation index of 3.51 was achieved, which was significantly higher than the levels in animals immunized by *Mycobacterium smegmatis* only (stimulation index 1.51) or BCG (stimulation index 1.94) ($P$<0.05, rMS-Hsp65/IL-2 vs. *Mycobacterium smegmatis* group; and $P$<0.05, rMS-Hsp65/IL-2 *vs.* BCG group). These data indicate an increased proliferative ability of lymphocytes in response to rMS-Hsp65/IL-2.

### 3.4. rMS-Hsp65/IL-2 enhanced the production of interferon gamma (IFN-γ) and IL-2 by splenic lymphocytes

We next examined the ability of rMS-Hsp65/IL-2 to induce the production of cytokines that are important for immune response during anti-tuberculosis therapy. Splenic lymphocytes from each group of mice were cultured in vitro, and the production of IFN-γ and IL-2 were determined by ELISA assay as described in the Materials and Methods. Highest level of IFN-γ was observed in the splenic lymphocytes from mice immunized with rMS-Hsp65/IL-2 [(1 808.00±190.11) pg/mL], compared to those from *Mycobacterium smegmatis* immunized mice [(800.00±120.12) pg/mL], BCG immunized mice [(1 480.00±155.43) pg/mL], and saline control group [(120.00±30.83) pg/mL] ($P$<0.05), rMS-Hsp65/IL-2 *vs.* *Mycobacterium smegmatis* group; and $P$<0.05, rMS-Hsp65/IL-2 *vs.* BCG group) (Figure 3A).

Similarly, higher level of IL-2 was observed in the splenic lymphocytes from mice immunized with rMS-Hsp65/IL-2 [(152.75±12.12) pg/mL] and BCG [(148.24±11.88) pg/mL], compared to those from mice immunized by *Mycobacterium smegmatis* [(56.73±12.34) pg/mL] ($P$<0.05) (Figure 3B).

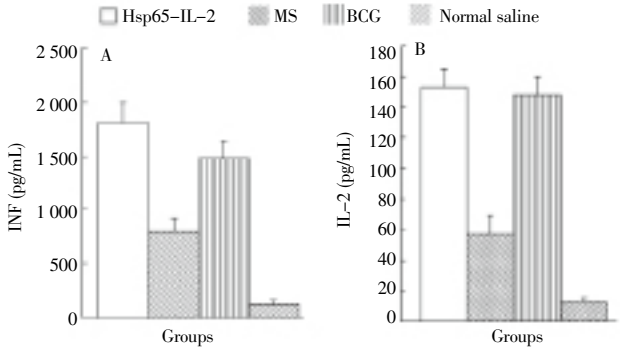


**Figure 3.** Generation of IFN-γ and IL-2 by the splenic lymphocytes isolated from mice immunized with different constructs.
A:INF, B: IL-2.

## 4. Discussion

*Mycobacterium smegmatis* is a non-toxic mycobacterium and has been successfully used in the United States as a vaccine adjuvant in the prevention and treatment of MTB. The safety and reliability of vector vaccine have been documented[8]. *Mycobacterium smegmatis* grows 10 times faster than BCG and the MTB-associated proteins expressed by *Mycobacterium smegmatis* are identical to the natural MTB proteins in terms of biochemical and immunological properties[9]. Single inoculation of the *Mycobacterium smegmatis* live vaccine has been shown to provoke sustained induction of target antigens. Recombinant *Mycobacterium smegmatis* live vaccine generated by sub-cloning exogenous genes can be used for the treatment of MTB infection. The underlying mechanisms of recombinant *Mycobacterium smegmatis* therapy include restoration of protective immunity, increased generation of $H_2O_2$ and nitric oxide by monocyte-macrophage cells, and a shift of the immune response from Th2 type to Th1-type. These changes may facilitate clearance of MTB, particularly the drug-resistant MTB[10,11].

In this study, we have demonstrated that a vector expressing Hsp65 and human IL-2 fusion protein (rMS-Hsp65/IL-2) can enhance the body´s immune response and an immune response shift from Th2-type to Th1 type[12].

Our generated rMS-Hsp65/IL-2 vector was found to stably overexpresses hIL-2 and Hsp65. When injected into mice, this construct was found to induce the production of anti-Hsp65-specific IgG antibody. The

protein product generated by this vector was shown to stimulate the proliferation of the mouse splenic lymphocytes. Compared to the classical BCG, rMS-Hsp65/IL-2 induces a significantly higher proliferation index of lymphocytes than BCG. The increased lymphocyte proliferative ability was associated with increased production of IFN-γ and IL-2, two important cytokines in the MTB induced immunity. Importantly, IFN-γ is considered to be a key cytokine that mediates resistance to MTB infection. Increased production of IFN-γ by the splenic lymphocytes provoked by the rMS-Hsp65/IL-2 may indicate that the Hsp65/IL-2 fusion protein may be of value in overcoming the MTB resistance.

As IFN-γ is also an important mediator for Th1-type cellular immune response[9], increased IFN-γ production by the rMS-Hsp65/IL-2 may likely contribute to the shift of Th2-type to Th1-type in response to MTB infection. More studies are needed to confirm the direct impact of rMS-Hsp65/IL-2 on Th2-type to Th1-type shift.

In conclusion, our study shows that recombinant live vaccine may hold a strong potential to overcome MTB resistance. Further exploration of these findings in animal models of MTB is warranted.

## Conflict of interest statement

We declare that we have no conflict of interest.

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