

## Co-expression and Immunity of *Legionella pneumophila mip* Gene and Immunoadjuvant *ctxB* Gene

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**Abstract** The *mip* gene of *Legionella pneumophila* and the *ctxB* gene of *Vibrio cholerae* were amplified by PCR respectively. The amplified cDNA was ligated to the pcDNA3.1(+ ) vector. The recombinant plasmids pcDNA3.1-*mip* and pcDNA3.1-*ctxB* were identified by restriction analysis and PCR, and further confirmed by sequencing analysis. NIH3T3 cells were transfected with pcDNA3.1-*mip* and pcDNA3.1-*ctxB* according to the Lipofection method. Transient and stable products of the co-expression of the *mip* gene and *ctxB* gene were detected by immunofluorescence and Western blotting. The results showed that NIH3T3 cells were successfully transfected, and that the transiently and stably co-expressed products can be detected in the transfected cells. To detect the humoral and cellular immune response in immunized mice induced by the co-immunization of the *mip* and *ctxB* genes, female BALB/c mice were immunized intramuscularly with pcDNA3.1-*mip* and pcDNA3.1-*ctxB*. The results showed that the specific antibody titer and the cytotoxic T-lymphocyte response for pcDNA3.1-*mip* immunization and co-immunization were increased compared with that of pcDNA3.1(+ ) immunization. Furthermore, the specific antibody titer and cytotoxic T-lymphocyte response for co-immunization were increased compared with that of pcDNA3.1-*mip* immunization. Statistical analysis using one-way analysis of variance (ANOVA) showed that there was a significant difference between the groups ( $P < 0.01$ ). The results indicated that the *ctxB* gene enhanced the humoral and cellular immune response to the *mip* gene immunization. These findings provide experimental evidence to support the development of the *L. pneumophila* DNA vaccine.

**Key words** *Legionella pneumophila*; co-expression; humoral immunity; cellular immunity

Legionnaires' disease is an infectious disease of the respiratory tract caused by *Legionella pneumophila*. The disease is an important cause of epidemic and sporadic pneumonia in humans [1]. Human infection occurs by the inhalation of aerosolized bacteria from *Legionella pneumophila*-contaminated water sources. The elderly and immunodeficient people are the most susceptible. *Legionella* infection causes diseases such as atypical pneumonia and Pontiac fever. The death rate from infection may be very high if it is incorrectly treated. *L. pneumophila* is the most common etiologic agent of Legionnaires'

disease. It is found in soil and the natural or man-made aquatic environment, and is a facultative intracellular parasite of mononuclear phagocytes [2]. With the popularity of air-conditioners and humidifiers in daily life, *Legionella* infection will become increasingly widespread and severe. In China, prevention and control of Legionnaires' disease are being emphasized as it is a newly emerging infectious disease. But researchers have not found an effective way to deal with the disease. Safe and effective vaccines may help to control the spread of Legionnaires' disease and prevent an epidemic. An important virulence factor, Mip (macrophage infectivity potentiator), encoded by the *mip* gene, has been found to contribute to the intracellular survival of *L. pneumophila* [3].

This study uses the *mip* gene of *L. pneumophila* as a candidate vaccine gene. To improve the effect of the

Received: November 23, 2004 Accepted: February 1, 2005

This work was supported by a grant from the National Natural Science Foundation of China (No. 30300302)

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vaccine, we combined the *mip* gene with the *ctxB* gene (adjuvant cholera toxin subunit B gene) of *Vibrio cholerae* (*V. cholerae*). We hope to provide more experimental evidence to support the development of the *L. pneumophila* DNA vaccine.

## Materials and Methods

### Materials

Prokaryotic recombinant plasmids pLpmip and pCTB were constructed in a previous study [4,5]. The NIH3T3 cell strain was presented by the Biomedicine Engineering Institute, Sichuan University, Chengdu, China. Rabbit serum antibodies of *L. pneumophila* and *V. cholerae* were presented by the Institute of Prevention and Control of Infection Disease, Center of Disease Control, China. LipofectAMINE reagent was purchased from Invitrogen Company (California, USA).

### Animal

Female BALB/c mice aged 6–8 weeks were purchased from the Animal Center for Medicine, Sichuan University, Chengdu, China and used throughout the immunization experiment.

### Construction and identification of eukaryotic expression vectors of *mip* and *ctxB* genes

The *mip* gene was amplified by PCR from the DNA template pLpmip, digested with *Bam*HI and *Xho*I, and ligated into the *Bam*HI/*Xho*I-cut pcDNA3.1(+) vector. The *ctxB* gene was amplified by PCR from the DNA template pCTB, digested with *Xho*I and *Apa*I, and ligated into the *Xho*I/*Apa*I-cut pcDNA3.1(+) vector. The constructed plasmids were used to transform *Escherichia coli* JM109. The constructed plasmids were prepared and purified from *E. coli* transformant, identified by PCR, restriction analysis and sequencing analysis, and were then named pcDNA3.1-*mip* and pcDNA3.1-*ctxB*.

### Transfection of NIH3T3 cells using the Lipofection method

The recombinant plasmids pcDNA3.1-*mip* and pcDNA3.1-*ctxB* were prepared, purified and transfected into NIH3T3 cells using the Lipofection method [6]. About  $1 \times 10^5$  cells were seeded in every well containing 2 ml of Dulbecco's minimum essential medium (DMEM) complete growth medium (10% calf serum, 100 U/ml penicillin and streptomycin) in a 6-well tissue culture plate. The cells

were incubated at 37 °C in a CO<sub>2</sub> incubator that was 50%–80% confluent. Solutions A and B were prepared in 12 mm×75 mm sterile tubes, with Solution A containing 1.5 μg of DNA dissolved into 50 μl of transfection buffer, and Solution B containing 5 μl of Lipofectamine reagent diluted into 50 μl of transfection buffer. Solutions A and B were then combined together and mixed gently. The mixture was incubated at room temperature for 30 min to allow the formation of DNA-liposome complexes, and 0.8 ml of serum-free and antibiotic-free medium was added to the complexes. The diluted solution containing the complexes was mixed gently and overlaid onto the rinsed cells and incubated at 37 °C in a CO<sub>2</sub> incubator for 5 h. Following incubation, 1 ml of growth medium containing 20% calf serum was added without removing the transfection mixture. At 18 h following the start of transfection, fresh medium was added to replace the old medium.

### Detection of the transient products using immunofluorescence analysis

Slides were prepared by depositing the cells onto them. At 72 h following the start of transfection, the transient products were detected with immunofluorescence. *L. pneumophila* rabbit serum antibody (1:100, V/V), *V. cholerae* rabbit serum antibody (1:200, V/V) and goat anti-rabbit IgG (1:100, V/V) conjugated with fluorescein isothiocyanate (FITC) were used in the experiments.

### Detection of the stable products by Western blotting

At 72 h following the start of transfection, the cells were passaged with 1:10 (V/V) into the G418 selective medium. The positive cell clones containing the recombinant pcDNA3.1-*mip* and pcDNA3.1-*ctxB* were screened and multiplied. The cells were harvested and total protein samples were prepared. The protein samples were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a nitrocellulose membrane. The membrane was pre-equilibrated with buffer solution containing 5% (W/V) skimmed milk for 1 h and then incubated with rabbit serum antibody for 1 h at room temperature, and further incubated for 30 min at room temperature with anti-rabbit IgG conjugated with horseradish peroxidase (HRP). The blot signal was observed with a DAB kit.

### DNA vaccination

Female BALB/c mice aged 6–8 weeks were divided into three groups randomly, with nine mice in each group. The mice in group 1 were given intramuscular injections with 50 μg pcDNA3.1-*mip* and pcDNA3.1-*ctxB*. The mice in

group 2 were given intramuscular injections with 50 µg pcDNA3.1-*mip*. The mice in group 3 were given intramuscular injections with 50 µg pcDNA3.1(+). Two weeks later, enhanced immunization was performed with the same DNA dose.

### Preparation of *L. pneumophila* soluble mixed antigen

Bacteria of *L. pneumophila* serogroup 1 were grown on buffered charcoal-yeast extract agar with BCYE- $\alpha$  ( $\alpha$ -ketoglutarate) in a candle urn at 34 °C and harvested with PBS. The bacterial mixture was sonicated for about 10 min. The cell suspension was centrifuged and the supernatant was collected. The supernatant was used as the antigen mixture.

### Measurement of the specific antibody of humoral immunity

Mice serum samples were collected from the mice by eye bleeding on the 7th, 14th and 21st day after enhanced immunization and analyzed by indirect ELISA for the presence of specific antibodies. Microtiter plates were coated with 100 µl of mixed antigen in carbonate-bicarbonate buffer and incubated overnight at 4 °C. The plates were washed three times and blocked for 3 h at room temperature with 5% skimmed milk in PBS. Diluted serum samples were incubated for 2 h at 37 °C. Each sample was performed in triplicate. After washing the plates three times, they were incubated with HRP-conjugated goat anti-mouse IgG (1:1000, V/V) for 1.5 h at 37 °C. The wells were washed and tetramethylbenzidine was added. After 30 min, the color development was stopped with H<sub>2</sub>SO<sub>4</sub> and A<sub>460</sub> was measured.

### Measurement of the cytotoxic T-lymphocyte (CTL) response of cellular immunity

On the 7th, 14th and 21st day after enhanced immunization, spleen lymphocytes were separated from immunized mice in sterilized conditions, and they were counted and adjusted to a cell density of 5×10<sup>6</sup> cells/ml with RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA). The spleen lymphocytes were used as effector cells for the detection of the CTL response. The positive clone cells containing recombinant pcDNA3.1-*mip* were designated as the target cells, and the density was adjusted to 5×10<sup>5</sup> cells/ml with RPMI 1640 medium. Then, 100 µl of effector cell suspension and target cell suspension were added into the same well, and the ratio of effector cells to target cells was 10:1. Effector lymphocyte control, target control and blank control groups were also set up. The CTL response was detected using the methyl thiazolyl te-

trazolium (MTT) method [7]. The cells were incubated at 37 °C in a CO<sub>2</sub> incubator for 20 h, after which 20 µl MTT was added in every well and the incubation continued for another 4 h. The culture plate was centrifuged for 5 min at 3400 g. The medium was then discarded, and 100 µl of dimethyl sulfoxide (DMSO) was added and mixed completely. The cell culture plate was incubated for 30 min at 37 °C until the lasureous particles were dissolved completely. Then, A<sub>460</sub> was measured and the CTL response was calculated as follows:

$$CTL = \frac{1 - [A_{460}(\text{exp}) - A_{460}(\text{effectorcontrol})]}{A_{460}(\text{targetcontrol})} \times 100\%$$

One-way analysis of variance (ANOVA) was performed to analyze the CTL response. The differences were considered to be statistically significant when  $P < 0.05$ .

## Results

### Detection of the transient products

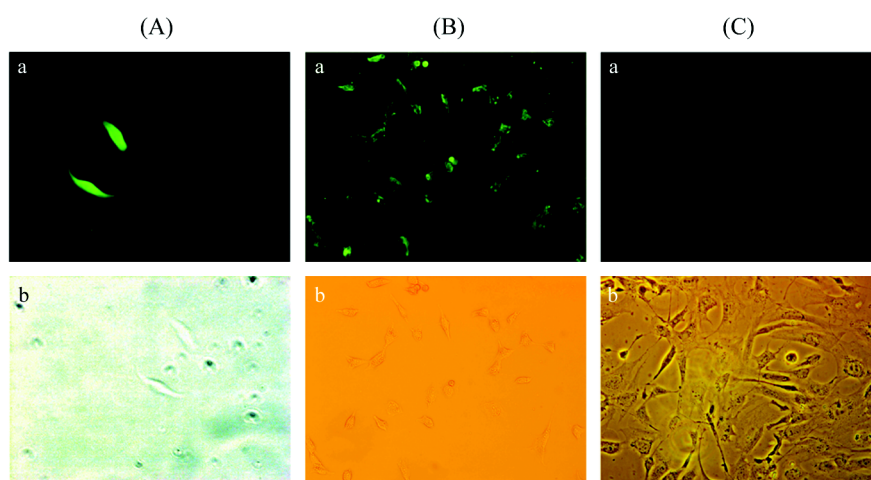
NIH3T3 cells transfected by pcDNA3.1-*mip* and pcDNA3.1-*ctxB* were detected with the rabbit serum antibody of *L. pneumophila* and *V. cholerae*, respectively. Strong green fluorescence was detected in the cell membrane and cytoplasm. The results show that NIH3T3 cells were transfected by pcDNA3.1-*mip* and pcDNA3.1-*ctxB* successfully and transient products can be detected in the transfected cells. There was no green fluorescence in NIH3T3 cells transfected by pcDNA3.1(+) (**Fig. 1**).

### Detection of the stably expressed products

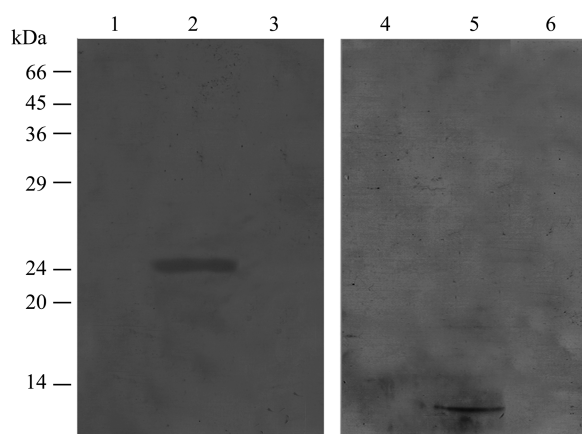
The culture supernatants and the cells transfected by pcDNA3.1-*mip* and pcDNA3.1-*ctxB* were collected and the stable expression products were detected by Western blotting. The results show that cell lysate can bind with antibodies to form a strip in the hybridization membrane, while the culture supernatants of the transfected cell have no blot signal. In the cell lysate, 24 kDa and 11 kDa proteins were detected with the rabbit serum antibody of *L. pneumophila* and *V. cholerae*, respectively. The molecular weight of the strip was in accordance with expectation. There was no strip in the NIH3T3 cells transfected by pcDNA3.1(+) (**Fig. 2**).

### Measurement of specific antibodies

On the 7th, 14th and 21st day after enhanced immunization, the presence of specific antibodies in the serum was detected by ELISA in the two experimental



**Fig. 1 Immunofluorescence analysis of the transient expression products of pcDNA3.1-mip and pcDNA3.1-ctxB in NIH3T3 cells**  
 (A) NIH3T3 cells transfected by pcDNA3.1-mip and pcDNA3.1-ctxB and detected with the rabbit serum antibody of *L. pneumophila*. a, fluorescence; b, ordinary light.  
 (B) NIH3T3 cells transfected by pcDNA3.1-mip and pcDNA3.1-ctxB and detected with the rabbit serum antibody of *V. cholerae*. a, fluorescence; b, ordinary light.  
 (C) NIH3T3 cells transfected by pcDNA3.1(+). a, fluorescence; b, ordinary light. Magnification, 10×10.



**Fig. 2 Western blot analysis of the expression products of pcDNA3.1-mip and pcDNA3.1-ctxB**

1, culture supernatant of NIH3T3 cells transfected by pcDNA3.1-mip and pcDNA3.1-ctxB; 2, cell lysate of NIH3T3 cells transfected by pcDNA3.1-mip and pcDNA3.1-ctxB; 3, cell lysate of NIH3T3 cells transfected by pcDNA3.1(+); 4, culture supernatant of NIH3T3 cells transfected by pcDNA3.1-mip and pcDNA3.1-ctxB; 5, cell lysate of NIH3T3 cells transfected by pcDNA3.1-mip and pcDNA3.1-ctxB; 6, cell lysate of NIH3T3 cells transfected by pcDNA3.1(+). Expression products in Samples 1, 2 and 3 were detected with the rabbit serum antibody of *L. pneumophila*, and expression products in Samples 4, 5 and 6 were detected with the rabbit serum antibody of *V. cholerae*.

groups, while specific antibodies were not detected by ELISA in the pcDNA3.1(+) group. Furthermore, in the co-immunization group of pcDNA3.1-mip and pcDNA3.1-

ctxB, the serum sample had an antibody titer of 1:64 or more. These titers were increased compared with that of the pcDNA3.1-mip group (Table 1).

#### Measurement of the cytotoxic T-lymphocyte response

On the 7th, 14th and 21st day after enhanced immunization, cytotoxic T-lymphocyte responses were detected by the MTT method. The CTL responses of the pcDNA3.1-mip group and the co-immunization group of pcDNA3.1-mip and pcDNA3.1-ctxB were increased compared with that of the pcDNA3.1(+) control group. Furthermore, the CTL response of the co-immunization group of pcDNA3.1-mip and pcDNA3.1-ctxB was increased compared with that of the pcDNA3.1-mip group. Statistical analysis using one-way ANOVA showed that there was a significant difference between the groups ( $P < 0.01$ ) (Table 2).

#### Discussion

Since Legionnaires' disease was first reported in 1976, there have been many studies conducted on the *L. pneumophila* vaccine, which has progressed through several forms, including an inactivated whole bacterial vaccine, a live attenuated vaccine and a protein subunit vaccine. The development of a DNA vaccine provides a new direction for researchers working on the *L. pneumophila* vaccine. When inoculated into the body, the DNA vaccine

**Table 1** Titer of the specific antibody of mice immunized with DNA vaccine against *L. pneumophila*

Number of days after enhanced immunization	Titer of specific antibody	
	pcDNA3.1-mip group	pcDNA3.1-mip and pcDNA3.1-ctxB group
7	1:4	1:64
14	1:32	1:256
21	1:16	1:64

**Table 2** Cytotoxic T-lymphocyte response of spleen-derived lymphocytes from mice immunized with DNA vaccine against *L. pneumophila*

Number of days after enhanced immunization	Cytotoxic T-lymphocyte response of different group		
	pcDNA3.1(+)	pcDNA3.1-mip	pcDNA3.1-mip and pcDNA3.1-ctxB
7	1.80±0.01	34.78±0.01 <sup>#</sup>	50.00±0.37 <sup>#*</sup>
14	5.43±0.04	69.02±0.01 <sup>#</sup>	78.26±0.02 <sup>#*</sup>
21	8.70±0.01	24.46±0.02 <sup>#</sup>	39.13±0.10 <sup>#*</sup>

<sup>#</sup>*P*<0.01 compared with the control; <sup>\*</sup>*P*<0.01, multiple comparison.

is expressed to the corresponding antigen protein, which can mimic natural infection and stimulate the body to produce a full-scale immune response. Compared with traditional vaccines, the DNA vaccine has more extensive application prospects [8]. A comparison of the responses elicited by immunization with a *Legionella*-species common lipoprotein delivered as naked DNA or recombinant protein supports the potential use of the t-rPAL protein and, in particular, DNA vaccines against *Legionella* infections [9]. However, only a few related studies on the *L. pneumophila* DNA vaccine have been reported.

The Mip protein is a virulence factor that contributes to the intracellular survival of *L. pneumophila*. It is a 24 kDa protein and it is expressed on the surface of *Legionella* cells. It possesses an N-terminal signal sequence, which is cleaved off while the protein is transported through the cytoplasmic membrane, exhibits peptidylprolyl *cis/trans* isomerase (PPIase) activity and belongs to the enzyme family of FK506-binding proteins (FKBP) [10]. The addition of a PPIase inhibitor, such as FK506 or rapamycin, leads to reduced infectivity of organisms. The Mip protein is encoded by the *mip* gene. Analysis of the *mip* gene and Mip protein from 35 strains of *Legionella* shows that the sequence homology of the *mip* gene is 69%–97%, and the amino acid sequence homology of the Mip protein is 82%–99% [11]. The results indicate that the *mip* gene and Mip protein have high conservatism in *Legionella*. Therefore, we chose the *mip* gene as a candidate gene of the DNA

vaccine in our study.

Immunoadjuvants are usually used to improve the effect of a DNA vaccine. CTB is the major protective antigen of *V. cholerae* and is encoded by the *ctxB* gene. It has been previously found that CTB can be used as an immunoadjuvant with some antigens [12]. As a new type of immunoadjuvant, CTB has been the center of attention for a number of years. We have previously constructed the *mip/ctxB* fusion gene and investigated its immunogenicity [13]. The results show that the immunogenicity of pcDNA3.1-mip/ctxB is stronger than that of pcDNA3.1-mip.

In this study, we co-immunized mice with the *mip* gene and *ctxB* gene, with the aim of obtaining a better immunization effect, as well as providing experimental data to evaluate the effect and potential applications of CTB as an immunoadjuvant in the *Legionella* DNA vaccine. NIH3T3 cells were transfected with pcDNA3.1-mip and pcDNA3.1-ctxB. Transient and stable expression products were then detected in the transfected cells. We found that the *mip* gene and *ctxB* gene were successfully co-transferred into and expressed in the same cell. To detect the humoral and cellular immune responses of co-immunization, female BALB/c mice were immunized intramuscularly with pcDNA3.1-mip and pcDNA3.1-ctxB. We discovered that the specific antibody titer and the CTL response of the pcDNA3.1-mip immunized group and the co-immunized group were increased compared with that of the

pcDNA3.1(+) immunized group, and the specific antibody titer and the CTL response of the co-immunized group were increased compared with that of the pcDNA3.1-mip immunized group.

From all of these results, we can conclude that the humoral immunity and CTL response induced by the *mip* gene combined with the immunoadjuvant *ctxB* gene are increased compared with that of *mip* gene immunization alone. It has been shown that the *ctxB* gene enhances the humoral and cellular immunity response of *mip* gene immunization. Comparing the results of pcDNA3.1-mip/*ctxB* immunization with that of co-immunization with pcDNA3.1-mip and pcDNA3.1-*ctxB*, it can be concluded that a stronger CTL response is induced by immunization with the *mip/ctxB* fusion gene, while a higher humoral immunity is induced by co-immunization with the *mip* gene and *ctxB* gene. These conclusions provide more experimental proof of the potential of the *L. pneumophila* DNA vaccine.

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Edited by  
Min YE