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Full Length Research Paper

Construction the recombinant BCG targeting delivering *Iprl* into macrophages: A new strategy of vaccine against tuberculosis

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This study was aimed at constructing a recombinant *Bacillus* Calmette-Guérin (BCG) that can target the delivery of the intracellular pathogen resistance I (*Ipr1*) gene into macrophages. To achieve this, a eukaryotic plasmid pBGOI was constructed, which co-expressed *Ipr1* and green fluorescent protein (GFP). Then, pBGOI was transfected into murine macrophage cell line RAW264.7 and transformed into BCG to construct the recombinant BCG, which was used to immunize C3HeB/FeJ mice intranasally. *Ipr1* expression *in vitro* and *vivo* was detected by real time-polymerase chain reaction (RT-PCR), fluorescence microscope, Western-blot and immunohistochemistry. Results on restriction enzyme digestion and sequence analysis showed that pBGOI had been constructed successfully. *Ipr1* expression was detected not only in macrophages infected with pBGOI, but also in lung and spleen tissues of C3HeB/FeJ mice that were immunized by recombinant BCG. This study therefore provides a good basis for further research on the function and mechanisms of *Ipr1* against tuberculosis.

Key words: *Ipr1* gene, *Bacillus* Calmette-Guérin (BCG), macrophage, *Mycobacterium tuberculosis*, vaccine.

INTRODUCTION

One-third of the world's population is infected with *Mycobacterium tuberculosis*. Tuberculosis (TB) is primarily a pulmonary infectious disease and remains one of the leading infectious causes of death worldwide. Factors that have recently appeared put more burdens on the treatment of TB. These factors include co-infection with HIV, prolonged treatment (at least six months), and the emergence of *M. tuberculosis* strains that are resistant to the front-line drugs (WHO, 2007; WHO Global Report Tuberculosis, 2005; Aronson et al., 2004). Therefore, new therapeutic approaches are needed.

In 2005, Pan et al. found a gene in C57BL/6J mice that could promote macrophages resisting intracellular pathogen and was named the intracellular pathogen resistance gene 1 (*Ipr1*). *Ipr1* could promote resistance of macrophages to *M. tuberculosis* infection *in vitro* (Pan et al., 2005). However, the mechanisms of how *Ipr1* prevents *M. tuberculosis* infection are still unclear. In our study, we constructed a eukaryotic plasmid pBGOI that could co-express *Ipr1* and green fluorescent protein (GFP), and transformed this plasmid into *Bacillus* Calmette-Guérin (BCG) to construct the recombinant BCG. Although this recombinant BCG is a potential vaccine candidate against *M. tuberculosis,* it could also be a good tool for further research on the mechanisms of

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Table 1. Primers and digested site used in this study.

Amplicon (bp)		Primer sequence or digested site	Template
IprI (1338)	IF	5'-CCGCTGCAG-ATGTTCACTCTGACCAAAGC-3'	pEGFP-*Ipr1*
	IR	5'-GCGGGATCC-CTAGGCACCCTTCTTTTGAGGTT-3′	
OriM(1886)		Digested with *Nhe* I	pZM03
GFP (720)	GF	5'-ATTGGTACC-GCCACCATGGTGAGCA-3'	pEGFP-C1
	GR	5'-CCGAGATCT-TTACTTGTACAGCTCGTCCAT-3'	

Ipr1 against *M. tuberculosis in vivo*.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

The *Escherichia coli* strains used were grown in Luria-Bertani (LB) broth or on LB agar (Difco) for the amplification of recombinant clones, plasmid isolation and transformation. BCG Pasteur (2003050401) was obtained from Shanghai Institute of Biological Products. BCG was routinely cultured in Middlebrook 7H9 (Difco) broth containing 10% OADC, 0.2% (v/v) glycerol and 0.05% (v/v) Tween 80, and 7H10 agar containing 10% oleic acid-dextrose-catalase (OADC). BCG was washed twice in phosphate buffered saline (PBS) and stored frozen at -80°C in 10% (v/v) sterile glycerol/PBS. When required, the following antibiotics were used at the specified concentrations: kanamycin (30 µg/ml) and Zeocin^{TM} (40 µg/ml). The plasmid pEGFP-*Ipr1* and pZM03 had been constructed and identified previously (Li et al., 2008; Yi et al., 2007).

The construction of recombinant plasmid pBGOI

Coding sequences of *Ipr1*, GFP and digested origins of replication for mycobacteria (OriM) were all cloned directionally into vector pBudCE4.1 (Invitrogen) to form the recombinant plasmid pBGOI. Briefly, *Ipr1* was amplified by polymerase chain reaction (PCR) with primers IF/IR from the pEGFP-*Ipr1* vector. The OriM was digested from the pZM03 vector. The GFP was amplified by PCR with primers GF/GR from the pEGFP-C1 vector. Recombinant plasmids were sequenced by Sanger method with universal primers (BGI, China). After sequencing, the resulting DNA fragments of *Ipr1*, GFP and OriM were subcloned directionally into pBudCE4.1 vector at *Pst* I/*BamH* I, *Nhe* I, *Kpn*I/*BglII* sites, respectively. Details of the primers used in the above-mentioned procedures are provided in Table 1.

In vitro transfection and cell culture

Murine macrophage cell line RAW264.7 was maintained in RPMI 1640 (Gibco) medium with 10% FCS (Gibco) and cultured at 37°C in 5% CO2. According to the manufacturer's instructions (GeneJammer, Merck), the day before transfection, adherent RAW264.7 cells were released with 0.1% trypsin-ethylenediaminetetraacetic acid (EDTA) and diluted in RPMI 1640-10% FCS medium to a density of 1×10^6 cells/ml. About 5×10^5cells were plated into each well containing a sterile cover slip of 24-well cell culture plates and incubated overnight under 5% CO_2 at 37°C. Diluted DNA was combined with Lipofectamine2000. For each transfection, 0.5 ml of medium without serum and antibiotics was added to the tube containing the DNA and Lipofectamine2000. The cells were incubated with the complexes for 4-6 h at 37°C in a 5% CO_2 incubator. Following incubation, 0.5 ml of growth medium containing 10% FCS was added in the dish after removing the used medium.

The GFP protein was detected by fluorescence microscope after 24 h to evaluate the transfection efficiency. The mRNA of *Ipr*1 gene in pBGOI transfected cells was detected by real time-polymerase chain reaction (RT-PCR). Slips covered with the pBGOI transfected cells and slips carrying the negative control parental-vector transfected cells were taken out after 48 h to detect the expression of *Ipr1* in cells through immunocytochemical staining. Briefly, slips were fixed by 4% paraformaldehyde at room temperature. Immunocytochemical staining was carried out according to the protocol of the SP reagent kit manufacturer. The primary antibody was Mouse Monoclonal His-Tag antibody (Santa Cruz, USA) and the second antibody was the goat anti-mouse IgG (Booster, China). The nucleus of positive cells stained brown-yellow.

Construction the recombinant BCG (BCGi) and its intracellular bioactivity assay

pBGOI was transformed into BCG by electroporation at 4°C using standard conditions of 2.5 kV, 25 µF and 1000 Ω and a 2-mm cuvette (Bio-Rad, Richmond, CA). A recombinant clone named BCGi was selected based on Zeocin resistance and confirmed with PCR. Next, BCGi (5×10^7 CFU/500 µL) of logarithmic growth phase was added to the medium of RAW264.7 cells and incubated 8 h under 5% CO_2 at 37°C. The culture was washed with serum-free RPMI1640 medium three times in order to remove the bacteria that were not swallowed by macrophage, and then RPMI1640 medium with 10% FCS and antibiotic was added in the culture to continue incubation. The Ziehl-Neelsen (acid-fast) staining was done at 12 h, and the expression of *Ipr1* was detected by RT-PCR and immunocytochemistry in RAW264.7 cells at 96 h. It was noteworthy that before reversing transcription into DNA, the RNA products were added into the column with DNAse I (RNase free DNase set, Qiagen) in order to remove the residual DNA. The primers of RT-PCR were the same with the IF and IR mentioned in Table 1.

Ipr1 expression *in vivo* in mice after intranasal vaccination with BCGi

Specific pathogen-free female C3HeB/FeJ mice used at 6 - 8 weeks

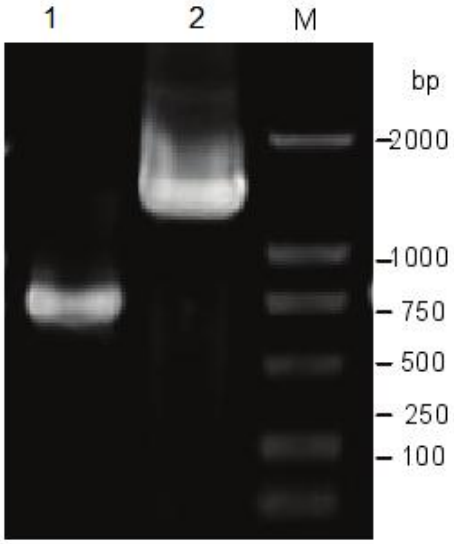

Figure 1. PCR results of pBGOI (1) PCR product of GFP; (2) PCR product of *Ipr1*; (M) DNA marker DL2000.

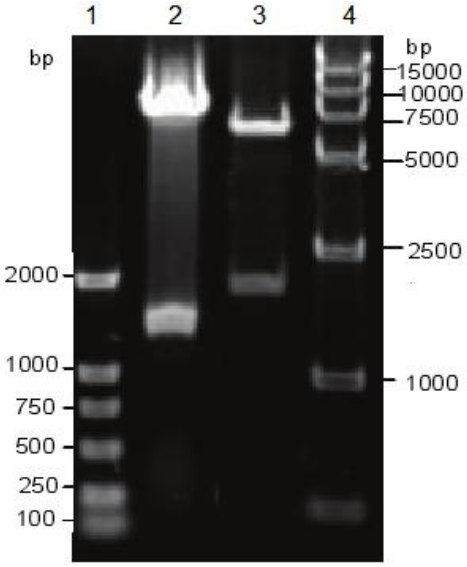

Figure 2. Digestion results of pBGOI. (1) DNA marker DL2000; (2) pBGOI was digested with *Pst*I and *BamH*I; (3) pBGOI was digested with *Nhe*I; (4) DNA marker DL15000.

of age, were obtained from the local breeding facility at the Chongqing Medical University. All animals were housed in cages and kept on a daily 12 h cycle of light and dark. Mice were provided with sterilized food and water in SPF conditions. Mice (n = 6) were intranasally vaccinated with 1×10^5 CFU/50 µL BCGi or BCG. Mice were treated on a total of three occasions with viable bacteria at 0, 3 and 6 days. Subsequently, animals were sacrificed 9 days after the first inoculation and their lungs and spleens were removed for *Ipr1* detection by RT-PCR and immunohistochemistry analysis.

RESULTS

PCR and digestion identification of pBGOI

The eukaryotic co-expression plasmid pBGOI containing the GFP and *Ipr1* genes was successfully constructed as confirmed by restriction analysis and sequencing. Figure 1 shows the PCR results of the two genes, with sizes 720 and 1338 bp, respectively. Additionally, Figure 2 shows the digestion results of the pBGOI. Lane 2 indicated that pBGOI was digested with *Pst*I and *BamH*I, and Ipr1 DNA fragment (1338 bp) was obtained. Meanwhile, lane 3 indicated that pBGOI was digested with *Nhe*I, OriM DNA fragment (1896 bp) was obtained.

Ipr1 expression in RAW264.7 cell transfected with pBGOI

pBGOI was transfected into murine cell line RAW264.7. GFP expression both in the nuclei and cytoplasm of RAW264.7 cells was observed by inverted fluorescence microscope at 24 h, while the negative control transfected with pBudCE4.1 was not fluorescent (Figure 3). The expression of *Ipr1* was detected with RT-PCR (Figure 4) and Western blot (Figure 5) at 48 h.

Ipr1 expression in RAW264.7 cells infected with BCGi

We selected the BCGi colony based on Zeocin resistance and detected the *Ipr1* and OriM gene in the strain by PCR (Figure 6). According to the Acid-fast staining, for the RAW264.7 cells infected with BCGi at 12 h, most of the BCGi that was red and thin rod-shape entered into the RAW264.7 cells whose color was blue and exist in the cytoplasm; only a few BCGi scattered in the cell gap (Figure 7). This proves that BCGi is a good transport tool that could target the delivering the target gene into macrophages. *Ipr1* expression was detected with RT-PCR (Figure 8) and immunohistochemistry assay in RAW264.7 cells infected with BCGi at 96 h. Particularly, the result of immunohistochemistry assay indicated that *Ipr1* protein whose color was brown was mainly located in the nucleus of RAW264.7 cells (Figure 9).

Ipr1 expression in C3HeB/FeJ mice vaccinated with BCGi

Nine days after the first treatment, *Ipr1* mRNA was detected with RT-PCR in mice lung and spleen tissues of

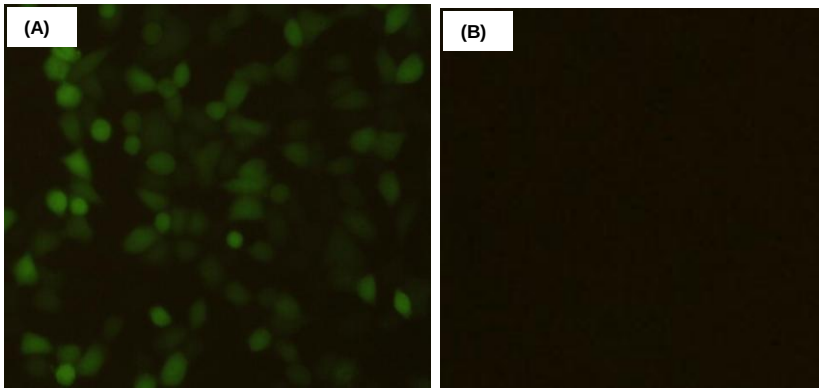

Figure 3. GFP expression in RAW264.7 transfected by pBGOI under fluorescence microscope (×200). (A) RAW264.7 transfected with pBGOI; (B) negative control of RAW264.7 transfected with pBudCE4.1.

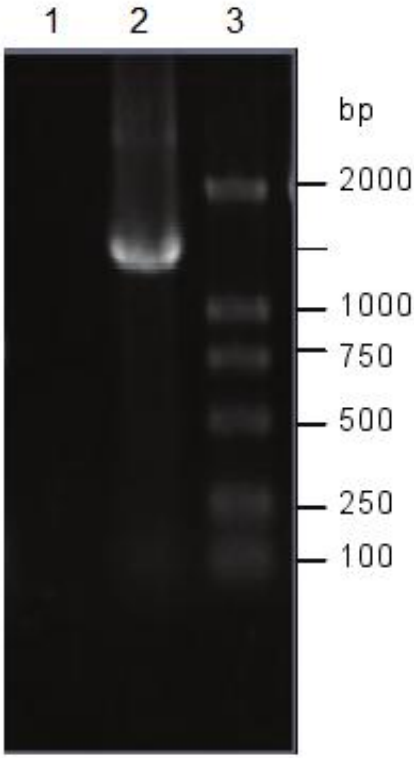

Figure 4. The RT-PCR result of *Ipr1* in RAW264.7 transfected with pBGOI. (1) Negative control: RAW264.7 infected by BCG, which was transfected with pBudCE4.1 (2) RAW264.7 infected by BCGi; (M) DNA marker DL2000.

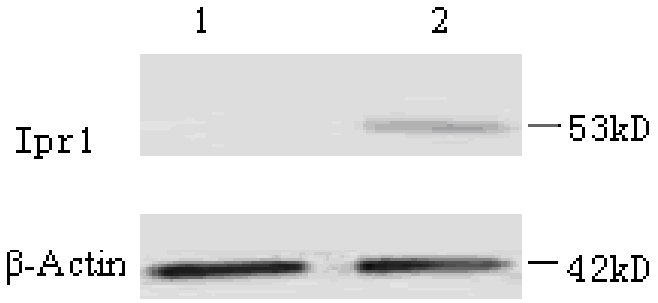

Figure 5. Western-blot result of *Ipr1* in RAW264.7. (1) RAW264.7 transfected with pBudCE4.1; (2) RAW264.7 transfected with pBGOI.

C3HeB/FeJ mice intranasally vaccinated with BCGi (Figure 10). The result of immunohistochemistry also indicated *Ipr1* expression in lung tissues of mice (Figure 11).

Cell distributed around the bronchiole whose nucleus was brown-yellow were stained positive for Ipr*1*.

DISCUSSION

The long duration of conventional chemotherapy for tuberculosis, compliance, drug resistance and co-infection with HIV point to an urgent need for additional prevention and treatment strategies against tuberculosis. In this study, we expressed *Ipr1,* which promotes macrophages resistance to intracellular pathogens such as *M. tuberculosis* (MTB) in BCG. The *Ipr1* gene has been identified at the *sst1* (super-susceptibility to tuberculosis 1) locus on mouse chromosome 1. As *Ipr1* plays a major role in mediating innate immunity in a mouse model of *M. tuberculosis* infection, the human *Ipr1* homologue, *SP110* is a recognized candidate gene for control *M. tuberculosis* infection. Studies have demonstra-

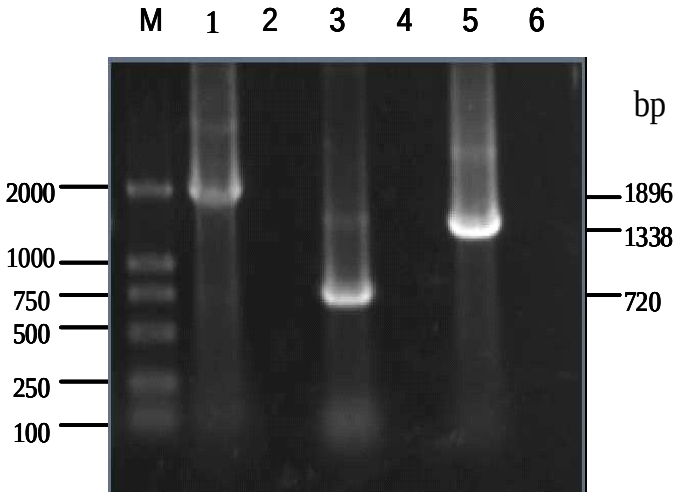

Figure 6. PCR result of BCGi. (M) DNA marker DL2000; (1) PCR product of OriM; (2) negative control of OriM PCR; (3) PCR product of GFP; (4) negative control of GFP PCR; (5) the PCR product of *Ipr1*;(6) negative control of *Ipr1* PCR.

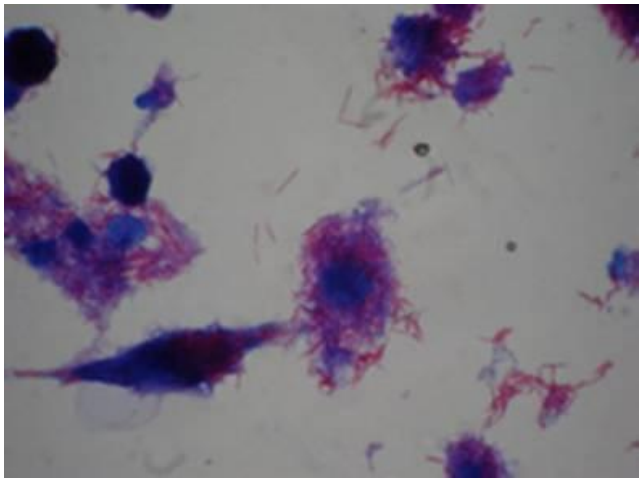

Figure 7. Acid-fast staining of RAW264.7 infected by BCGi at 12 h (×1000). RAW264.7 cells were blue and its nucleus was black; BCGi was red and rod in shape, and the space between the cells was white.

strated that genotypes and haplotypes of *SP110* might be associated with susceptibility to tuberculosis in Chinese population (Liang et al., 2011). Macrophages are one of the most important cells in innate immunity against tuberculosis (Sundaramurthy and Pieters, 2007; Fremond et al., 2004). The macrophages that express *Ipr1* gene tend to undergo apoptosis which control MTB multiply, while others without *Ipr1* gene would undergo necrosis which helps the mycobacteria multiply and spread (Pan, et al., 2005). However, the mechanisms of how *Ipr1* gene

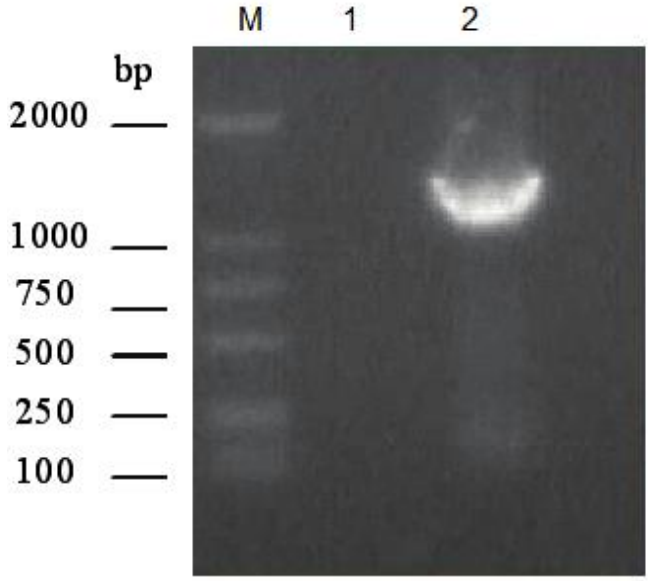

Figure 8. RT-PCR result of *Ipr1* in RAW264.7 infected with BCGi or BCG at 96 h. (M) DNA marker DL2000; (1) negative control of *Ipr1* in RAW264.7 infected by BCG, which was transfected with pBudCE4.1; (2) RT-PCR product of *Ipr1* RAW264.7 infected with BCGi.

enhances macrophage resistance to MTB remain poorly understood.

In our study, we constructed *Ipr1* eukaryotic expression plasmid pBGOI which contained the origin of replication for mycobacteria (OriM), and transformed it into BCG to select the recombinant BCG (BCGi). We found that BCGi could target deliver *Ipr1* into macrophages *in vitro*. Expression of *Ipr1* within macrophages could potentially play a role in resisting MTB infection. This is potentially a new recombinant BCG vaccine against tuberculosis. In addition, the mechanism by which Ipr1 promotes macrophage resistance to MTB is unclear, and BCGi may be a tool to study these mechanisms *in vivo*. The pBGOI did not only express *Ipr1*, but also GFP simultaneously in eukaryotic cells. GFP is recognized as a marker gene, so we can judge whether the transfection is success or not. In a previous work, we constructed the *Ipr1* prokaryotic express plasmid and transformed it into E. *coli* BL21, in order to use the expression of *Ipr1* to produce antibody in rats. However, the antibody was not detected. Therefore, in order to detect *Ipr1* expression in bacterial cells, we chose the pBudCE4.1 plasmid which has a His tag that can express *Ipr1* and His fusion protein. His-tagged fusion protein has very high specificity and sensitivity, and is not cross-reactive with protein of the bacteria, yeast or mammalian source. We used mouse anti-His tag monoclonal antibody to detect the *Ipr1* expression by Immunohistochemistry and western-blot. We then added an OriM in the *Nhe1* site of pBGOI so that pBGOI could

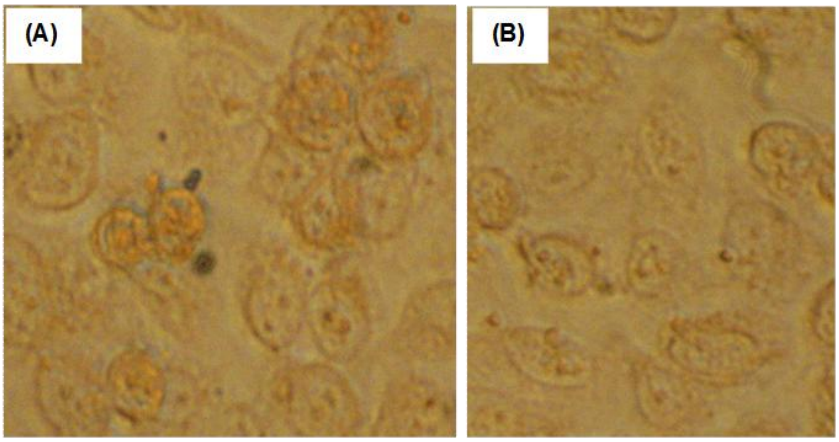

Figure 9. Immunohistochemistry result of RAW264.7 infected by BCGi at 96 h (×400). (A) *Ipr1* expression in RAW264.7 infected by BCGi; (B) negative control: RAW264.7 infected by BCG, which was transfected with pBudCE4.1.

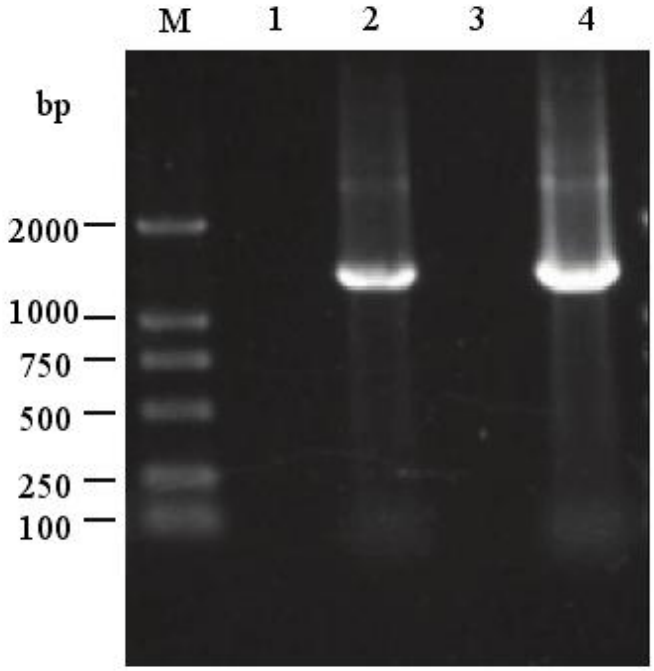

Figure 10. RT-PCR result of *Ipr1* in lung and spleen tissues of mouse infected by BCGi. (M) DNA marker DL2000; (1,3) negative control of *Ipr1* RT-PCR: mouse infected by BCG, which was transfected with pBudCE4.1; (2) spleen RT-PCR product of *Ipr1:* mouse infected by BCGi; (4) lung RT-PCR product of *Ipr1:* mouse infected by BCGi.

replicate not only in E. *coli*, but also in *Mycobacterium*. This design was fundamental for the study described in this paper, as pBGOI needs to replicate in BCG in order to deliver more *Ipr1* to macrophages.

Attenuated *Listeria* and *Salmonella* have been used to deliver eukaryotic expression vectors into specific cells (Qin et al., 2011). Li Hua and other scholars also proved that attenuated *Salmonella typhimurium* can act as a

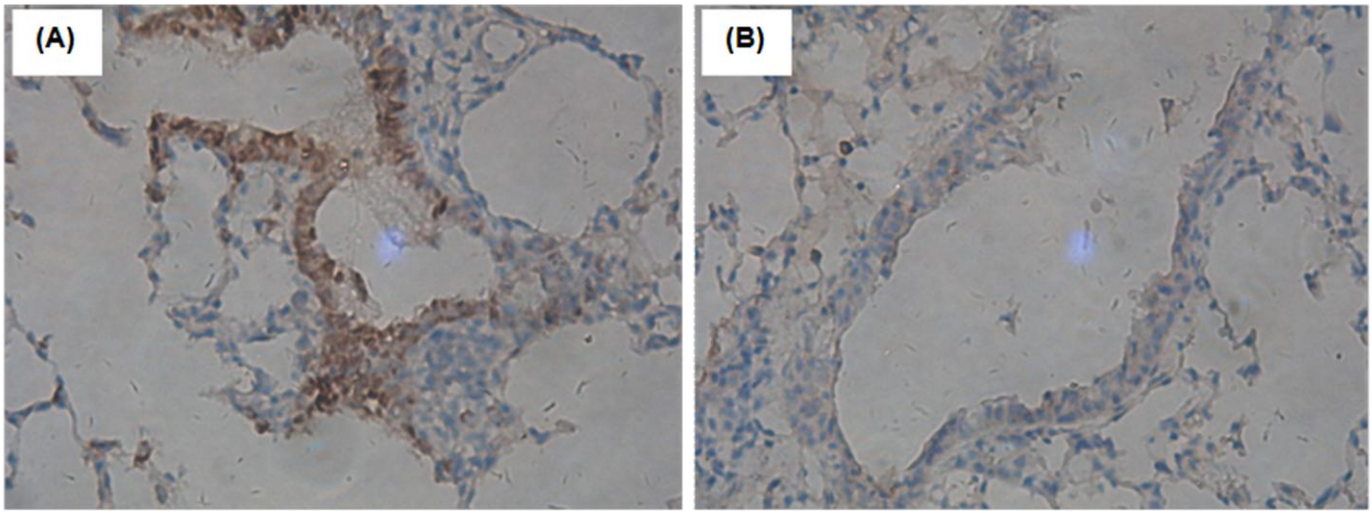

Figure 11. Immunohistochemistry result of lung tissues of C3HeB/FeJ mice (×400). **(A)** *Ipr1* expression in lung tissues of mice vaccinated by BCGi. **(B)** Negative control of *Ipr1* expression in lung tissues of mice vaccinated by BCG, which was transfected with pBudCE4.1.

carrier targeting plasmid to macrophages (Liu et al., 2011). *Bacillus* Calmette-Guerin (BCG) is a vaccine against tuberculosis and is also an attractive gene carrier (Christy et al., 2012). Moreover, BCG is safe. The World Health Organization (WHO) recommended vaccination at birth since 1948, and nearly three billion people have been vaccinated with it, with low rate of adverse reaction. Also, BCG is an intracellular organism that persists for extended periods in macrophages. In addition, BCG production is low-cost, and the vaccine has good thermal stability. Finally, and most importantly, BCG can play both the role of gene carrier and vaccine simultaneously.

C3HeB/FeJ mice are susceptible to MTB, and do not have an *Ipr1* gene in their genome (Pan et al., 2005), while C57BL/6J mice, which are resistant to MTB, have the *Ipr1* gene. Therefore, we chose C3HeB/FeJ as the experimental animals to detect *Ipr1* expression *in vivo*. Different animals have different means of vaccination. Mice can be vaccinated through the tail vein injection, intraperitoneal injection, intra-tracheal injection, aerosol spray and intranasal infection. We chose intranasal vaccination for reasons of accurate dosage and simulation of the natural way of MTB infection. All in all, we constructed the eukaryotic co-expression plasmid pBGOI, and transformed it into BCG to construct BCGi. BCGi was shown to infect macrophages. Expression of IprI was detected by RT-PCR, Western-blot, and immunohistochemistry *in vitro* and/or *in vivo*. BCGi is a potential novel recombinant vaccine against tuberculosis, but it needs further study. In future studies, we hope to study the protective effect of BCGi during MTB infection *in vivo* and obtain information on the mechanisms of protection using gene microarrays.

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