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Bifidobacterium as an oral delivery carrier of interleukin-12 for the treatment of Coxsackie virus B3-induced myocarditis in the Balb/c mice

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ABSTRACT

IL-12 plays an important role in the treatment of many infectious diseases by being administered intravenously or intramuscularly. However, intravenous or intramuscular administration is difficult and inconvenient and may cause side effects. The aim of this study is to develop a novel oral delivery system for IL-12 using genetically engineered *Bifidobacterium longum* as the carrier and further investigate the efficacy of IL-12-expressed *B. longum* on the coxsackie virus B3 (CVB3)-induced myocarditis in mice. A mIL-12 gene expression vector pBBADs-IL-12 for *B. longum* was constructed and transformed into *Bifidobacterium*. Subsequently, the expression of mIL-12 in the engineered *B. longum* was identified in vitro by western blot and enzyme-linked immunosorbent assay (ELISA) after L-arabinose induction. Moreover, our data indicated that oral administration of IL-12-expressed *B. longum* for two weeks after CVB3 infection in the Balb/c mice could downregulate the severity of virus-induced myocarditis, markedly reduce the virus titers in the heart and induce a Th1 pattern in the spleen and heart compared with the controls. In conclusion, a novel oral delivery system of *Bifidobacterium* for murine IL-12 has been successfully established. Oral administration of mIL-12-transformed *B. longum* may play a therapeutic role in the treatment of CVB3-induced myocarditis in the mice.

1. Introduction

IL-12 is a heterodimeric cytokine composed of two disulfide-linked peptides, p35 and p40. Mounting evidences have demonstrated that IL-12 can upregulate IFN-γ production from NK and T cells and promotes the differentiation of T cells to a Th1 phenotype [1]. In addition, early induction of IL-12 in infectious diseases has shown a broad range of effects on both innate and adaptive immunity in vivo [2,3]. Recently, multiple studies indicate that IL-12 plays a critical role in the treatment of many infectious diseases, including poxvirus, hepatitis B virus, myxoma virus and mycoplasma infection [4–10]. Especially in Coxsackie virus B3 (CVB3)-induced myocarditis, IL-12 can protect the BALB/c mice from myocarditis development by increasing IFN-γ and macrophage and neutrophil populations in the heart [11]. Moreover, exogenous IL-12 treatment during group B coxsackieviruses infection is able to suppress the immunopathological mechanisms that lead to chronic disease [12]. Thus, early administration of IL-12 in the course of CVB3-induced infection provides an alternative way to improve the development of immunopathology.

However, injection is still the main approach for IL-12 delivery. It is not easy for patients to receive intravenous or intramuscular administration for a long time. Recently, Elías-López et al. reported that transgenic tomato expressing interleukin-12 has a therapeutic effect to reduce lung tissue damage during early and late mycobacterial infection [13]. Salvatore et al. also suggested intranasal IL-12 therapy could be used to treat mycoplasma pneumonia [14]. These studies indicate that the new delivery approach, such as oral or intranasal administration, can be successfully developed for IL-12 delivery. The genera *Bifidobacterium* is the dominant probiotic bacteria inhabiting the distal jejunum, ileum and large intestine of human and other warm-blooded animals. *Bifidobacterium* spp. have many beneficial effects on human health, including prevention of infection, immunomodulation and protection against colon cancer. Recently, genetically engineered *Bifidobacterium* has been used as an exogenous gene delivery carrier of several cytokines for cancer gene therapy and bowel disease [15–19]. These studies suggest that *Bifidobacterium*

Abbreviations: IL-12, interleukin-12; CVB3, Coxsackie virus B3; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; GFP, green fluorescent protein; MNCs, mononuclear cells.

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may be the suitable carriers for IL-12 gene expression and secretion in the intestinal tract.

In this study, we described an oral delivery system of *Bifidobacterium longum* for IL-12 delivery by constructing an *Escherichia coli–Bifidobacterium* shuttle vector. The expression of murine IL-12 (mIL-12) in *B. longum* in vitro was analyzed, and the therapeutic effects of IL-12-transformed *B. longum* after oral administration on CVB3-induced myocarditis in mice by immunomodulation were evaluated and confirmed.

2. Methods and materials

2.1. Vector construction

The *E. coli–Bifidobacterium* shuttle expression plasmid vector pBBAD/Xs was constructed as reported previously [18]. Briefly, pBBAD/Xs contains an extracellular exo-xylanase (XynF) signal peptide (Xs) sequence and the replicon amplified from a *B. longum* strain. The green fluorescent protein (GFP) report gene expression vector pBBADs-GFP was constructed by inserting GFP from pEGFP-N1 plasmid (Mountain View, CA, USA, Clontech) into NcoI and XbaI sites in pBBAD/Xs. The mIL-12 expression vector pBBADs-IL-12 was constructed by inserting mIL-12 gene into plasmid pBBADs-GFP to replace the GFP gene at the BpiI and XbaI sites. The mIL-12 gene is synthesized in Invitrogen Company and expressed as a single polypeptide chain with a linker sequence between the p35 and p40 subunits, Val–Pro–Gly–Val–Gly–Val–Pro–Gly–Val–Gly[13]. These plasmids were transformed into *E. coli* Top10 and subsequently all constructs were verified by double-enzyme digestion and sequencing. Target gene expression of pBBADs-IL-12 can be induced by L-arabinose.

2.2. Bifidobacteria transformation and identification

B. longum NCC 2705 were transformed with recombinant plasmids pBBADs-IL-12 or pBBADs-GFP by electroporation. Competent *B. longum* cell preparation and electroporation were carried out using a Gene Pulser and Pulse Controller apparatus (Hercules, CA, USA, Bio-Rad) as described previously [18]. Bacteria were plated on MRS agar (1.5%, w/v) supplemented with 0.05% L-cysteine and 100 mg L^{-1} ampicillin. Plates were incubated anaerobically at 37 °C for 2 days and then colonies of transformed bacteria were selected and cultured in MRS broth. The transformed bacteria were identified by using PCR to amplification of target genes (IL-12 or GFP) and sequencing.

2.3. Gene expression induction and identification in vitro

Transformed *B. longum* were cultured in MRS broth with 100 mg L^{-1} ampicillin and L-arabinose was added to induce target gene expression when the optical density (OD) at 695 nm of bacterial culture reached 0.6. Culture supernatants and bacterium pellets were collected at 6, 12, 18, 24 and 48 h after 0.2% L-arabinose induction and then stored at −70 °C for IL-12 ELISA detection and western blotting. IL-12 concentration was determined using the murine IL-12 ELISA kit (Rapidbio) according the manufacturer's manual. The detection limit of the ELISA assay for IL-12 was 600 pg ml^{-1}.

Proteins from the supernatants and pellets of bacteria lysates were separated by SDS–PAGE and electroblotted onto nitrocellulose membranes (GE Healthcare) and recombinant mIL-12 was detected by western blot analysis. Mouse anti-murine monoclonal IL-12 antibody (Prepro Tech) was used as the primary antibody (1:1,000).

2.4. Recombinant B. longum administration and virus

Transformed *B. longum* were anaerobically cultured at 37 °C to the middle-log phase in MRS broth and then switched to MRS liquid culture medium for 24–48 h. Subsequently, the *B. longum* culture was concentrated at 4,000 rpm and 4 °C. The resulting sediment was collected, resuspended, and diluted to 2.5×10^7–3.0×10^7 bacilli ml^{-1} with MRS broth containing 0.2% L-arabinose. To determine the actual number of viable bacilli in the inoculum, a 10 μl suspension of bacteria was seeded onto BL agar plates (Nissui) containing 100 mg L^{-1} ampicillin. Colonies were counted after 24 h of anaerobic incubation.

CVB3 (Nancy strain) was obtained from Prof. Yifei Wang, the Department of Biotechnology of Jinan University [20]. African green monkey kidney (Vero) cells were cultured in DMEM containing 8% fetal calf serum (Gibco, Life Technologies, Rockville, MD). Confluent cultures of Vero cells were infected with CVB3 and incubated at 37 °C until an extensive cytopathic effect was observed (generally 3 to 5 days postinfection). Subsequently, the culture medium was collected, aliquoted and stored at −80 °C after the cell debris were pelleted by centrifugation and removed. Viral titration from the culture medium was determined by plaque-forming assay. Briefly, the viral medium was absorbed and diluted with RPMI 1640 medium in serial 10-folds. Vero cells were grown to confluency in microtiter trays and infected with serial dilutions of the homogenates, incubated for 72 h at 37 °C; the monolayers were then fixed in 10% of phosphate-buffered formalin for 10 minutes and stained in crystal violet (Invitrogen), and the numbers of plaques were counted.

2.5. Preparation and interventions of the mice

This study was approved by the ethics committee of Southern Medical University (Guangzhou, China). Four-week-old male Balb/c mice (weight, 15 g ± 0.5 g; Southern Medical University) were inoculated i.p. with a dose of CVB3 of 5×10^6 of 50% infection in cell culture (as determined by plaque assay on Vero cells). The Balb/c mice infected with this viral dosage survived for at least 6 months post-infection. We investigated the efficacy of pBBADs-IL-12-transformed *B. longum* on the CVB3-induced myocarditis. The 30 Balb/c mice were inoculated with the virus and divided into 3 groups (IL-12 group, GFP group and Saline group, 10 mice per group); the "IL-12 group" and "GFP group" were orally administered with pBBADs-IL-12 or pBBADs-GFP transformed *B. longum* for two weeks respectively after the inoculation of the virus. "Saline group" was administered i.p. once daily with sterile PBS after viral infection until day 14 post-infection. Recombinant bacteria were given orally once every 2 days using a tuberculin syringe attached to a 20-gauge olive-tip steel feeding tube, passed through the oral cavity and esophagus. All animals were killed in day 14 post-infection (following ether anesthesia). Until day 14 post-infection, half of the murine hearts were dissected aseptically for viral titration and RNA extraction for cytokine quantification. The other section of the heart was used for hematoxylin–eosin (H&E) staining and examined by light microscopy. Spleens were surgically removed for the isolation of mononuclear cells (MNCs). Mouse blood and fecal samples were collected as well from each mouse.

2.6. Quantification of mIL-12 in feces and serum

Mouse blood samples were collected and concentrated at 5,000 g and 4 °C. Sera were removed and stored at −20 °C for further analysis. To obtain fecal samples, the small intestine and ascending colon were removed, and wash solution containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, and 1.5 μM aprotinin in 2 ml of PBS) were passed through and incubated in the entire intestine for 10 min at room temperature (Alignani et al. 2005). Fecal Samples were collected and centrifuged (4,000*g*, 30 min, 4 °C), and the supernatants were stored at −20 °C. The mIL-12 concentration in the fecal supernatants was measured using the mIL-12 ELISA kit (Rapidbio).