International Immunopharmacology 15 (2013) 646–653

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Thymosin alpha-1-transformed *Bifidobacterium* promotes T cell proliferation and maturation in mice by oral administration

Congwen Shao [a], Guojun Tian [a], Yuanjian Huang [a], Wenying Liang [a], Hang Zheng [b], Jiannan Wei [c], Cheng Wei [d], Cuilan Yang [a], Hong Wang [a], Weisen Zeng [a,*]

[a] Department of Cell Biology, Southern Medical University, Guangzhou, 510515, China
[b] Department of Oncology, Nanfang Hospital, Southern Medical University, Guangzhou, 510515, China
[c] Department of Breast Surgery, Southern Medical University, Shenzhen Maternal and Child Health Hospital, Shenzhen, 518028, China
[d] Department of Gastroenterology, Jinan University of Medical Sciences, Shenzhen Municipal People's Hospital, Shenzhen, 518020, China

ARTICLE INFO

Article history:
Received 20 March 2012
Received in revised form 30 December 2012
Accepted 31 December 2012
Available online 24 January 2013

Keywords:
Thymosin alpha-1
Bifidobacterium
Oral administration
Transformation
T cell
Maturation

ABSTRACT

Thymosin alpha-1 (Tα1) has been used as an immune potentiator for treatment of immune deficiency diseases by injection administration. However, injection is inconvenient and may cause many side effects. In order to improve the administration convenience of Tα1, a human Tα1 gene transformed *Bifidobacterium longum* (BL-Tα1) was prepared and its effects on mice immunity by oral administration were investigated. The Balb/c mice were treated with BL-Tα1, which was pre-induced with 0.2% L-arabinose, every other day for 2 weeks. The *B. longum* transformed with empty vector (BL-0) was used as the negative control, and normal saline (NS, 0.9% saline) was used as the blank control. The results shown that (1) the $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells in blood, spleen and thymus, and the $CD4^+CD8^+$ cells in thymus and spleen of BL-Tα1 group were all significantly increased than that of negative control BL-0 group respectively; (2) the interferon-γ (IFN-γ) and interleukin-12 (IL-12) in serum of BL-Tα1 group were significantly increased. No significant differences were found in the levels of tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4) between BL-Tα1 group and BL-0 group; (3) thymic hyperplasia and lymphadenectasis were observed in BL-Tα1 group after three-month treatment. In conclusion, the Tα1-transformed *B. longum* promotes thymus and lymph nodes growth, stimulates T cell proliferation and maturation, and enhances cellular immunity through Th1 pathway by oral administration.

1. Introduction

Thymosin alpha-1 (Tα1) is one of the immunomodulatory peptides isolated originally from calf thymus [13]. This peptide has a number of biological activities in the immune system. It can stimulate precursor stem cell into the $CD4^+/CD8^+$ T cells, increase T cell proliferation, differentiation and maturation [1,14,26]. It can also activate natural killer (NK) cells, dendritic cells (DCs) and macrophages, and block steroid-induced apoptosis of thymocytes ([9,15,29,35,37]. Tα1 has been reported to increase significantly the production of Th1 response cytokines such as interferon-γ, interleukin-2 (IL-2) and IL-3, decrease the production of Th2 response cytokines such as tumor necrosis factor-alpha (TNF-α), IL-4 and IL-10 [41,43]. Additionally, Tα1 can directly exert its effects on target cells. It up-regulates specific cytokine receptors such as high-affinity IL-2 cytokine receptors on mitogen-stimulated T-cells, increases the expression of major histocompatibility complex (MHC) class I and tumor antigens, directly depresses viral replication, and increases expression of viral antigens on the surface of target-infected cells [2,10,11,17].

Tα1 has been used widely either as monotherapy or in combination with conventional therapies and other cytokines for the treatment of various diseases including viral infections such as chronic hepatitis B and C, immunodeficiency diseases such as acquired immune deficiency syndrome (AIDS), severe sepsis and systemic infectious disorders [2,6,7,16,19,21,24,31,33,38].

The anti-tumor effects of Tα1 have also been observed in studies on both experimental and human cancers [10,36]. Synergistic effect of restoration of NK activity was observed in a murine model of immune-suppression induced by cyclophosphamide (CY) when combined treatments with Tα1 and low doses of alpha interferon (IFN-α) or IL-2. In addition, when combined with specific chemotherapy, it was able to increase the anti-tumor effect of chemotherapy while markedly reducing the general toxicity of the treatment [23,27,32].

Abbreviations: B. longum, Bifidobacterium longum; Tα1, Thymosin alpha-1; BL-Tα1, *B. longum* strain transformed with human Tα1 expression plasmid; BL-0, *B. longum* strain transformed with empty vector; CTL, cytotoxic T cell; ELISA, enzyme-linked immunosorbent assay; Th1, T helper cell type 1; GIT, gastrointestinal tract.

* Corresponding author at: No. 1023 Satai Road, Baiyun district, Guangzhou 510515, China. Tel.: +86 20 6164 7073; fax: +86 20 6164 8208.
E-mail address: zengwsyh@yahoo.com.cn (W. Zeng).

1567-5769/$ – see front matter.
http://dx.doi.org/10.1016/j.intimp.2012.12.031

However, Tα1 used in clinic is produced by solid phase peptide synthesis or genetic engineering. The isolation and purification procedures for Tα1 peptide production are very complicated. Moreover, injection is the main administration route for peptide so far. It is inconvenient and may cause many side effects [34,36]. Although the techniques of enteric micro-sphericization and encapsulation make the oral administration of thymosin feasible, the complex manufacturing processes, low loading efficiency and high cost of production still limit its application in clinic [40].

Recently, we have developed a new oral delivery system for peptide drug by using engineered *Bifidobacterium* as the host. The human oxyntomodulin (OXM), interleukin 10 (IL-10) and interferon-α2b (IFN-α2b) transformed bifidobacteria were prepared using the oral delivery system and applied in the treatment for experimental obesity, colitis and myocarditis in mice respectively [22,42,44].

In this study, the transformed *Bifidobacterium* with human Tα1 gene was selected and its effects on cellular immunity in mice by oral administration were evaluated.

2. Materials and methods

2.1. Reagents

The *Bifidobacterium longum* (*B. longum*) strain NCC2705 was kindly provided by the Nestle Research Center (Lausanne, Switzerland). The restriction endonucleases, polymerase and T4 DNA ligase were purchased from MBI Fermentas Inc. (Vilnius, Lithuania). Human Tα1 DNA was synthesized by Shanghai Sangon Biological Engineering Technology and Services Ltd. (Shanghai, China). L-arabinose was purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Tα1 monoclonal antibody was purchased from Bioss Inc. (Woburn, MA, USA). The enhanced chemiluminescence substrate was purchased from Thermo Fisher Scientific Inc. (Shaumburg, IL, USA). The Tα1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from R & D Inc. (Fremont, CA, USA). The Mouse FlowCytomix Basic Kit and Mouse cytokine FlowCytomix (IFN-γ, IL-12, TNF-α, IL-4) Simple Kits were purchased from Bender MedSystems GmbH (Vienna, Austria). The fluorescence conjugated antibodies (FITC-anti-CD3, APC-anti-CD4 and PE-anti-CD8) were purchased from Molecular Probes, Invitrogen Corporation (Camarillo, CA, USA). The Lymphocyte Separation Medium was purchased from Dakewe Ltd. (Beijing, China).

2.2. Plasmid construction, transformation of B. longum and identification

The *E. coli–Bifidobacterium* shuttle expression plasmid vector pBBADs-GFP was obtained as described previously [22]. The human Tα1 expressing vector pBBADs-Tα1 was constructed by inserting the DNA fragment of human Tα1 at the restriction sites of BpiI and XbaI in plasmid pBBADs-GFP to replace GFP gene (Fig. 1). The empty control vector (pBBADs-0) was constructed by cutting off the GFP DNA fragment from plasmid pBBADs-GFP at the two SalI sites as reported previously [42]. Both the recombinant plasmids were verified by DNA sequencing (Takara, Japan). The transformed *B. longum* was prepared by electroporation with plasmids pBBADs-0 and pBBADs-Tα1 respectively. The electroporation was carried out as described [3]. The transformed bacteria were selected with selective MRS agar plate supplemented with 0.05% L-cysteine and 60 μg ampicillin ml^{-1} (final concentration). The positive colonies were picked and inoculated in the MRS broth containing 60 μg ampicillin ml^{-1}. The identification of transformed *B. longum* clones were performed as described previously [22].

2.3. Induction of gene expression in vitro

The induction method of exogenous gene expression in transformed *B. longum* has been described previously in detail [22]. Briefly,

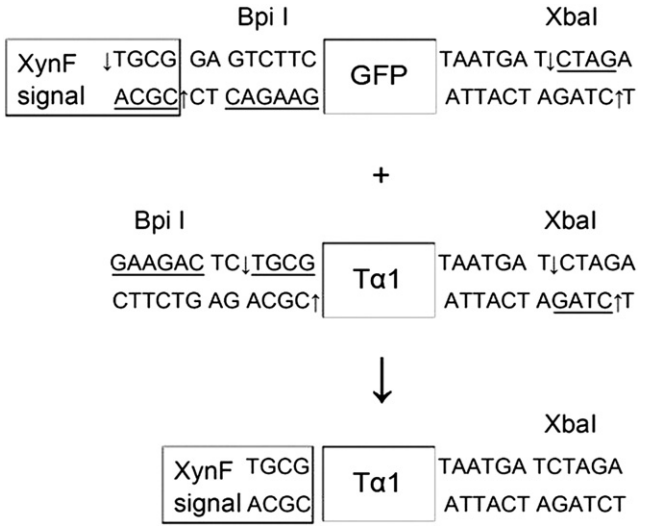

Fig. 1. Cloning strategy of pBBADs-Tα1 expression vector. The expression vector pBBADs-Tα1 was constructed by inserting the human Tα1 gene into plasmid pBBADs-GFP to replace the GFP gene at the BpiI and XbaI sites. Small arrows indicate BpiI and XbaI cleavage sites.

transformed bacteria were cultured in MRS broth containing 60 μg ampicillin ml^{-1} (final concentration) until the absorption of bacterium suspension at OD_{695nm} reached about 0.8, then L-arabinose was added to 0.2% (w/v) final concentration to induce the expression of target gene. The culture supernatants and pallets were collected and stored at −70 °C at 12 h, 24 h and 36 h continuous cultivation respectively after induction.

2.4. Animal experiment protocol

All animal procedures undertaken abided by the experimental animal administration measures of South Medical University (Guangzhou, China). Six-week-old male Balb/c mice weighing 18–20 g were purchased from the Laboratory Animal Center of Southern Medical University (PR China). Mice were housed in standard cages with the climate and temperature (22 ± 1 °C) controlled and a 12 h light/dark cycle. Mice were fed with standard laboratory chow and water. The mice (total 24) were randomly divided into three groups and treated as follows: the BL-Tα1 group (n = 8) was treated with 0.1 ml (6×10^9cells ml^{-1}) pBBADs-Tα1 transformed *B. longum* (referred to as BL-Tα1), which had been induced with 0.2% L-arabinose for 24 h, by intragastric administration every other day; the BL-0 group (n = 8) was treated with 0.1 ml (6×10^9cells ml^{-1}) pBBADs-0 transformed *B. longum* (referred to as BL-0) used as the negative control; the NS group (n = 8) was treated with 0.1 ml NS (0.9% saline) used as blank control. Animals were sacrificed 2 weeks later. The intestinal contents of the ileocecum, peripheral blood, spleens and thymuses were obtained. Lymphocytes were separated from spleens and thymuses respectively with lymphocyte separation medium. To investigate the effect of BL-Tα1 for a long period time on lymph nodes and thymus growth, another 24 mice were divided into 3 groups and administrated orally 0.1 ml (6×10^9cells ml^{-1}) pre-inducted BL-Tα1 with 0.2% L-arabinose for 24 h every other day for 3 months. The BL-0 and the NS were used as the negative control and blank control respectively. The sizes of thymus, spleen and lymph nodes in mice were surveyed after being sacrificed. The tissues were fixed immediately in 4% phosphate-buffered paraformaldehyde. The sections of tissue were stained by hematoxylin-eosin (HE) staining method.

2.5. Enzyme-linked immunosorbent assay

The levels of Tα1 in culture supernatant, serum and intestinal content samples were detected with ELISA kits. Intestinal content