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Lactobacillus sakei K040706 evokes immunostimulatory effects on macrophages through TLR 2-mediated activation

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ABSTRACT

Lactobacillus sakei K040706 is the most populous lactic acid bacteria (LAB) in over ripened *Doenjang*, a traditional Korean fermented soybean paste. In this study, we investigated the immunostimulating effects of *L. sakei* K040706 (K040706) in macrophages and in cyclophosphamide-induced immunosuppressed mice. Upon exposure to K040706, significant increases in phagocytic activity and in the productions of nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) were observed in rIFN-γ-primed RAW 264.7 macrophages. K040706 also increased the expressions of inducible nitric oxide synthase (iNOS) at the protein and promoter binding levels, and the expressions of iNOS, TNF-α, and IL-6, at the mRNA level. In addition, K040706 significantly increased the transcriptional activities and DNA binding of nuclear factor-κB (NF-κB), which was accompanied by parallel enhancement of the nuclear translocation of p65 *via* the phosphorylations inhibitory kappa B-α (IκB-α) and IκB-kinase (IKK). On the other hand, pretreatment with NF-κB inhibitors reduced K040706-induced NO production in IFN-γ-primed RAW 264.7 macrophages. Furthermore, K040706 induced-NO production was completely abolished by anti-Toll-like receptor 2 (TLR2) antibody. In our cyclophosphamide-induced immunosuppressed mouse model, administration of K040706 restored thymus and spleen indices. Taken together, our findings suggest that K040706 improves immune function by regulating immunological parameters, such as, the productions of NO, TNF-α, and IL-6 *via* NF-κB activation, and by activating TLR2 in rIFN-γ-primed macrophages. In parallel, K040706 restored immunological parameters in cyclophosphamide-induced immunosuppressed mice and may warrant further evaluations as potential immunomodulatory agent.

1. Introduction

Lactic acid bacteria (LAB) are a member of the order *Lactobacillales* and distinctively produce lactic acid through their metabolism. There are 13 genera of LAB which include *Enterococcus*, *Lactococcus*, *Lactobacillus*, and *Leuconostoc* [1], and of these, the genus *Lactobacillus* comprises a large heterogeneous group of low G + C content, Gram-positive, non-sporulating, and facultative anaerobes [2]. LAB are normal components of the healthy human intestinal microflora and are frequently used as probiotics for the fermentation of food products [3]. Lactobacilli are known to play important roles in the enhancement of immunity, the maintenance of intestinal microbial balance, and the prevention of gastrointestinal infections [4]. Previous research suggests that Lactobacilli can be used to stimulate the immune system and increase early lines of defense against invading pathogens [5].

Macrophages and lymphocytes play key roles in innate and adaptive immunity [6]. In particular, macrophages prevent invasion of pathogens by releasing inflammatory and cytotoxic molecules, such as, nitric oxide (NO) and reactive oxygen species (ROS), and by secreting pro-inflammatory cytokines, such as, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [7,8]. NO is an important intra- and inter-cellular regulatory molecule with many biological functions. It is synthesized by inducible nitric oxide synthase (iNOS) and mediates many biological functions, such as, vasodilatation, neurotransmission, immunoresponse and the inhibitions of platelet aggregation [9]. Cytokines, such as, TNF-α and IL-6, are also related to immune and inflammatory responses and have been implicated in host defense against pathogenic bacteria and parasites. These molecules are involved in the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways. On the other hand, lymphocytes are a type of white blood cell in the vertebrate immune system and are classified as natural killer (NK) cells, T cells, and B cells. NK cells are part of the innate immune system

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and play an important role defending the host from both tumors and viral infections, whereas T and B cells are the major cellular components of adaptive immune response [10]. Lactobacilli have been shown to enhance immunity in dendritic cells, T cells, and in mice [11–13], and exert immuno-enhancing effects on the phagocytic activities of monocytes and polymorphonuclear leukocytes cells and on IgA, chemokine, and cytokine production [14]. These responses are mediated in part by the recognition of microbe-associated molecular patterns (MAMPs) through pattern recognition receptors (PRRs) present on immune cells [15].

Lactobacillus sakei K040706 (K040706) was found in the traditional Korean fermented food *Doenjang*, which is prepared by fermenting moldy cooked soybeans (Meju) in brine. This process results in the degradation of soy proteins and in the production of organic acid [16]. Although immunological activities of some LAB have been reported [14], no report has been issued on immunostimulatory activities of K040706 or on the molecular mechanisms involved. Therefore, as a part of our on-going screening program to evaluate the immunostimulatory potentials of new probiotic strains, we investigated the molecular mechanisms underlying the immunostimulatory properties of K040706 in rIFN-γ-primed RAW 264.7 macrophages and in a cyclophosphamide-induced mouse model of immunosuppression.

2. Materials and methods

2.1. Materials and chemicals

Dulbecco's modified Eagle medium (DMEM), Rosewell Park Memorial Institute (RPMI) medium, fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies (NY, USA). iNOS, p65, poly[ADP-ribose] polymerase-1 (PARP-1), and β-actin monoclonal antibodies were purchased from Santa Cruz Biotechnology (CA, USA). CD3 and CD28 antibodies were purchased from BD Pharmingen (CA, USA). Enzyme linked immunosorbent assay (ELISA) kits for TNF-α, and IL-6 were obtained from R&D Systems (MN, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), sulfanilamide, phenylmethylsulfonylflouride (PMSF), dithiothreitol (DTT), murine recombinant IFN-γ, lipopolysaccharide (LPS), sodium bicarbonate, HEPES, sodium dodecyl sulfate (SDS), polymyxin B sulfate, and all other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Preparation of autoclaved L. sakei K040706 (K040706)

The strain used in this experiment was *L. sakei* K040706 (KCCM11472P) separated from *Doenjang* and kept at Korean Culture Center of Microorganisms (Seoul, Seodaimu-gu, South Korea). *L. sakei* K040706 was inoculated onto the medium of MRS broth (Difco Laboratories, MI, USA) with 2% sucrose and incubated for 24 h. After incubation, the pellet was then obtained after centrifugation (3000 ×*g* for 15 min, 4 °C), washed two times with PBS. It was subsequently lyophilized and autoclaved at 121 °C for 15 min for further experiment. The number of *L. sakei* K040706 (about 4.8×10^9 CFU/g of freeze-dried lactobacilli) was determined by counting on MRS plates using automated cell counter TC20™ (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.3. Cell culture and sample treatment

RAW 264.7 macrophages were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM containing 10% FBS, penicillin, and streptomycin sulfate at 37 °C in a 5% CO_2 atmosphere. Cells were pretreated with recombinant rIFN-γ (10 ng/ml) and then stimulated with various concentrations (100, 250, or 500 ng/ml) of K040706 or LPS (10 ng/ml) for the indicated time in figure legends.

2.4. MTT assay

Cell viability was assessed by MTT assay. RAW 264.7 macrophages were seeded in 96-well plates containing 100 μl of DMEM medium with 10% FBS and incubated overnight. After overnight incubation, the K040706 (100, 250, or 500 ng/ml) was added, and the plates were incubated for 24 h. Cells were incubated with a MTT solution (5 mg/ml stock solution in PBS) for 4 h at 37 °C under 5% CO_2. The medium was discarded, and the formazan blue, which formed in the cells, was dissolved with 200 μl DMSO. Absorbance of each well at 540 nm was measured using a microplate reader (Molecular Devices Inc., CA, USA).

2.5. Phagocytosis assay

RAW 264.7 macrophages were pretreated with rIFN-γ (10 ng/ml) for 1 h, followed by stimulation with K040706 (100, 250, or 500 ng/ml) for 3 h. Nonopsonized zymosan particles (10 × μl/sample) are added and incubated for 2 h. The amount of engulfed zymosan particles was determined by using CytoSelect 96-well phagocytosis assay from Cell Biolabs (San Diego, CA, USA). The absorbance was measured by 405 nm using a 96-well microtiter plate reader (Molecular Devices Inc., CA, USA).

2.6. Nitrite assay

RAW 264.7 macrophages were cultured in 24-well plates and stimulated with the K040706 (100, 250, or 500 ng/ml) in the presence of rIFN-γ (10 ng/ml). After 24 h, culture supernatants were collected and nitrite was measured using Griess reagent. Equal volumes of Griess reagent (1:1 of 0.1% *N*-1 naphthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) and sample were incubated together at room temperature for 10 min. Absorbance at 540 nm was measured using a microplate reader. For antibody inhibition experiments, cells were pre-incubated for 1 h with 20 μg/ml anti-TLR2 antibody (Invivogen, San Diego, CA, USA), isotype IgG antibody (Biolegend, San Diego, CA, USA) or medium alone. And then, cells were treated with K040706 (500 ng/ml) or peptidoglycan (20 μg/ml) for 24 h. For carbohydrate inhibition assay, cells were pre-incubated for 1 h with various concentrations of D-mannose, and then treated with K040706 (500 ng/ml) for 24 h. Culture supernatants were collected for nitrite determination.

2.7. Determination of TNF-a and IL-6 productions

TNF-α and IL-6 levels in cell culture media were quantified using ELISA kits, according to the manufacturer's instructions.

2.8. Western blot analysis

The cells were collected by centrifugation and washed once with phosphate buffered saline (PBS). Washed cell pellets were resuspended in protein extraction solution PRO-PREP (Intron Biotechnology, Seoul, Korea) and then incubated for 15 min at 4 °C. Cell debris was removed by microcentrifugation and supernatants were quick frozen. The protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories Inc., CA, USA) according to the manufacturer's instruction. Proteins (40 μg) were electroblotted onto a PVDF membrane following separation on a 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated for 1 h with blocking solution (5% skim milk) at room temperature, and then incubated overnight with a 1:1000 dilution of primary antibody at 4 °C. Blots were washed three times with Tween 20/Tris-buffered saline (T/TBS) and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., CA, USA) for 2 h at room temperature. Blots were again washed three times with T/TBS and