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Exopolysaccharide-Overproducing *Lactobacillus paracasei* KB28 Induces Cytokines in Mouse Peritoneal Macrophages *via* Modulation of NF-κB and MAPKs

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Exopolysaccharides (EPSs) are microbial polysaccharides that are released outside of the bacterial cell wall. There have been few studies on EPS-producing lactic acid bacteria that can enhance macrophage activity and the underlying signaling mechanism for cytokine expression. In the current study, EPS-overproducing *Lactobacillus (L.) paracasei* KB28 was isolated from *kimchi* and cultivated in conditioned media containing glucose, sucrose, and lactose. The whole bacterial cells were obtained with their EPS being attached, and the cytokine-inducing activities of these cells were investigated. Gas chromatography analysis showed the presence of glucose, galactose, mannose, xylose, arabinose, and rhamnose in EPS composition. EPS-producing *L. paracasei* KB28 induced the expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-12 in mouse macrophages. This strain also caused the degradation of IκBα and phosphorylation of the major MAPKs: Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK)1/2. The use of pharmacological inhibitors showed that different signaling pathways were involved in the induction of TNF-α, IL-6 and IL-12 by *L. paracasei* KB28. Our results provide information for a better understanding of the molecular mechanisms of the immunomodulatory effect of food-derived EPS-producing lactic acid bacteria.

Keywords: Exopolysaccharides, *Lactobacillus paracasei*, macrophage, cytokine, signaling

Exopolysaccharides (EPSs) are microbial polysaccharides that are released outside of the bacterial cell wall [2]. EPSs act as a surface coating for bacteria, thus protecting them from the harsh environment [9]. As a result, some EPSs form a biofilm that causes hygiene problems, whereas other EPSs from food-grade microorganisms contribute to improved mouthfeel textural properties of fermented dairy foods by increasing the viscosity and water-holding capacity [1].

With the recent increased interest in EPSs, extensive research into their presumed health benefits has been performed. Oral intake of EPSs produced by *Lactobacillus*(*L.*) *kefiranofaciens* prevented the onset and development of atherosclerosis in hypercholesterolemic rabbits and increased the IgA-positive cells in the intestine and the serum levels of interleukin (IL)-4, IL-10, and interferon (IFN)-γ [13, 14]. EPSs from *Lactococcus lactis* subsp. *cremoris* and *L. delbrueckii* susbp. *bulgaricus* were found to be B cell mitogens and produce IFN-γ and IL-1 in mouse macrophages.

Macrophages play an important role in cell-mediated innate immunity by destroying foreign substances and scavenging cellular debris [10]. Macrophages ingest microbes through a process called phagocytosis, at the same time secreting cytokines such as tumor necrosis factor (TNF)-α, IL-6, and IL-12. Cytokine expression is mainly regulated by intracellular signaling molecules such as nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs).

Some food-grade lactic acid bacteria have been applied in defective cell immunity that can cause allergy, recurrent bacterial and viral infections, and tumors [6, 8, 11]. However, there have been few studies on EPS-producing lactic acid bacteria that can enhance macrophage activity and the underlying signaling mechanism. In the current study, we isolated EPS-overproducing *L. paracasei* KB28 from *kimchi* and cultured it in conditioned media containing

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different sugars. We stimulated mouse peritoneal macrophages with this EPS-producing strain and investigated its induction of cytokines and intracellular signaling molecules.

Materials and Methods

Animals

Eight-week-old male BALB/c mice were purchased from the Korean branch of Taconic (SamTaco, Korea) and fed rodent chow and water *ad libitum* in a temperature- and humidity-controlled pathogen-free animal facility at Kyung Hee University. Mice were maintained in accordance with the Guide for the Care and Use of Laboratory Animals issued by the US National Research Council (1996), and the protocol was approved by the Kyung Hee University Institutional Animal Care and Use Committee.

Preparation of Microorganisms

Eleven kinds of *kimchi* were purchased from local markets in Cheongju, Korea for the isolation of EPS-producing lactic acid bacteria. The *kimchi* samples were grinded with a hand blender (Phillips, USA) for 10 s in saline [0.85% (w/v) NaCl; Junsei Chem., Japan] and serially diluted (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}). Diluents were spread onto modified MRS (Merck, Darmstadt, Germany) supplemented with 0.5% L-cysteine·HCl (Merck). After incubation at 37°C for 48 h under aerobic conditions, the colonies grown on the plates were grouped into pure cultures *via* biotyping by morphological analysis and chemotyping by SDS–PAGE analysis. Total amounts of EPS produced by each isolate were measured by the procedures described below. The isolate producing large amounts of EPS was identified by 16S rRNA nucleotide sequence analysis. PCR amplification of the 16S rRNA gene was performed using the universal bacterial primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Species identification was confirmed by comparing the sequence of related reference strains in the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) using the Fasta program.

The EPS-producing isolate was cultured again on modified MRS agar (Difco, Detroit, MI, USA) containing 2% sucrose, 2% glucose, and 2% lactose for 48 h at 37°C. The bacterial cells were harvested in 15 ml of sterile saline solution and heated at 100°C for 30 min. Then all samples were lyophilized and kept at −80°C until needed.

EPS Analysis

Chemically defined medium (CDM) in this study consisted of 25 g of casamino acid, 2 g of K_2HPO_4, 1 g of Tween 80, 2 g of ammonium citrate, 5 g of sodium acetate, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4 \cdot H_2O$, and 0.5 g of L-cysteine·HCl and supplemented with 20 g of glucose, 20 g of lactose, or 20 g of sucrose per liter. One percent (v/v) of the subcultures was then inoculated into CDM and incubated at 30°C for 48 h. EPS was isolated from the media according to a modified method of Yang *et al.* [15]. Ten ml of culture samples was centrifuged at 15,000 ×*g* for 15 min at 4°C. The pellet containing cells was washed in 5 ml of sterile saline and then centrifuged at 15,000 ×*g* for 15 min at 4°C. The viscous pellet was suspended in 5 ml of 1 M NaCl. EPS was dissociated from cells by sonication at 40 W for 3 min at 4°C with an ultrasonic processor (Sonicator GE 130PB, Sonics and Materials Inc., Newtown, CT, USA). Samples were then centrifuged at 6,000 ×*g* for 30 min at 4°C to eliminate insoluble material. To isolate the EPS released into the culture broth, supernatants were obtained after centrifugation, and incubated for 2 h at 4°C under gentle agitation. Precipitated proteins were removed by centrifugation at 25,000 ×*g* for 20 min at 4°C. EPS formation was monitored by thin-layered chromatography (TLC) on Whatman K6F silica gel plates with 85:15 acetonitrile–water. After irrigating three times, the TLC plate was dried and visualized by dipping into 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine, 5% (v/v) H_2SO_4 in methanol, followed by heating at 100°C for 10 min. Analysis of sugar contents after image scanning and density comparison was performed using image analysis software (Multi Gauge, Fuji, Japan). Monosaccharides were identified as alditol acetates by gas chromatography. Briefly, the samples were partially hydrolyzed with 2 M trifluoroacetic acid for 1.5 h at 121°C and converted into the corresponding alditol acetates. After acetylation, the resulting carboxyl-reduced alditol acetates were analyzed by gas chromatography at 60°C for 1 min, 60°C to 220°C (30°C /min), 220°C for 12 min, 220°C to 250°C (8°C /min), and 250°C for 20 min, using an M600D (Young-Lin Co., Korea) equipped with an SP-2380 capillary column (0.2 μm film thickness, 0.25 mm i.d. × 30 m) (Supelco, Bellefonte, PA, USA). The mole percentage was calculated from the peak areas and response factors using the flame-ionization detector.

Preparation and Stimulation of Mouse Macrophages

Mice were injected intraperitoneally with 2 ml of sterile thioglycollate medium (BD, MD, USA). Three days later, macrophages were collected by peritoneal lavage with cold Dulbecco's modified Eagle's medium (DMEM) (WelGENE, Daegu, Korea). After centrifugation, cells were suspended in DMEM with 10% fetal bovine serum (WelGENE) and 1% antibiotic–antimycotic (Invitrogen, Carlsbad, CA, USA) and incubated for 90 min in a humidified atmosphere of 5% CO_2 at 37°C. Non-adherent cells were removed for further assays. For analysis of cytokine release, macrophages at a concentration of 5×10^5 cells/ml were stimulated with heat-killed bacteria at a concentration of 2, 20, and 200 μg/ml for 24 h. For RT-PCR analysis of gene expression, macrophages at a concentration of 2.5×10^6 cells/ml were stimulated with bacteria for 24 h. For inhibition studies, macrophages were treated for 1 h with sulfasalazine (Sigma, St. Louis, MO, USA), SB203580 (Promega, Madison, WI, USA), U0126 (Sigma), or SP600125 (Sigma), before stimulation with the bacteria for 24 h. For analysis of intracellular signaling proteins, macrophages were stimulated with bacteria at a concentration of 20 μg/ml for various times.

RT-PCR and Real-Time PCR

Total RNA was isolated using Trizol reagent (Invitrogen) and cDNA was reverse-transcribed using oligo-dT primers and Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed in an ABI PRISM 7300 sequence detector (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green master Mix (Applied Biosystems). PCR was performed in a total volume of 20 μl containing 2× Power SYBR Green master Mix, 0.2 nM each primer, and diluted cDNA. After initial heat denaturation at 95°C for 10 min, the conditions used for PCR were 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR efficiency was determined using serial dilutions of the template cDNA. For each PCR, a corresponding no-RT mRNA sample was included as a negative control. Copy numbers for each cDNA were quantified using control samples

according to the manufacturer's protocol. GAPDH was used as an endogenous control to normalize the expression of TNF-α, IL-6, and IL-12 mRNAs. The primers used were TNF-α, forward 5'-ATG ATC CGC GAC GTG GAA-3', reverse 5'-ACC GCC TGG AGT TCT GGA A-3'; IL-6, forward 5'-AGG ATA CCA CTC CCA ACA GAC CT-3', reverse 5'-CAA GTG CAT CGT TGT TCA TAC-3'; IL-12, forward 5'- CTT GCA GAT GAA GCC TTT GAA GA-3', reverse 5'-GGA ACG CAC CTT TCT GGT TAC A-3'; and GAPDH, forward 5'-GGC ATG GAC TGT GGT CAT GA-3', reverse 5'-TTC ACC ACC ATG GAG AAG GC-3'.

ELISA

Supernatant cytokine levels were measured using a Duoset ELISA development system (R&D Systems, Minneapolis, USA). The concentration of each cytokine was calculated from standard curves.

Western Blotting

Total cell extracts were prepared by resuspending the cells in lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 20 mM NaF; 0.5% NP-40; and 1% Triton X-100) containing a phosphatase inhibitor (Sigma) and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined using the Bradford assay. Cell extracts were separated on a 10% SDS gel and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h and then incubated with antibodies specific for IκBα, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-JNK, JNK, phospho-ERK1/2, ERK1/2, phospho-p38, and p38 (Cell Signaling Technology, Beverly, CA, USA) diluted 1:1,000 in 5% skim milk in TBST overnight at 4°C. The blots were washed with TBST and incubated for 1 h with anti-rabbit horseradish peroxidase-conjugated Ab diluted 1:5,000 in 5% skim milk in TBST. Immunoreactive bands were developed using the Enhanced Chemiluminescence System (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

RESULTS

Isolation of EPS-Producing *L. paracasei* KB28

About 400 colonies were grown on MRS agar plates after spreading *kimchi* samples and they were grouped into 64 pure cultures after biotyping and chemotyping analyses. Pure cultures were grown in CDM, and EPS fractions of cultures were obtained as described in Materials and Methods. EPS productivity was relatively compared by TLC analysis (data not shown) and the pure culture showing the largest amount of EPS was isolated. By using 16S rRNA gene sequence analysis, the isolate was identified as *L. paracasei*. This strain was named as *L. paracasei* KB28 and deposited in the Korean Agricultural Culture Collection with deposit number KACC 91506P.

Component Sugars of EPS Produced by *L. paracasei* KB28

We cultured *L. paracasei* KB28 in CDM containing three different sugars (glucose, sucrose, lactose) as carbon source.

Fig. 1. The component sugar of crude EPS produced by *Lactobacillus*(*L.*) *paracasei* KB28 were identified as alditol acetates by gas chromatography analysis.

The monosaccharide composition of the EPS was examined by gas chromatography. As shown in Fig. 1, the EPS consisted mainly of glucose and galactose, along with traces of mannose, xylose, arabinose, and rhamnose.

Cytokine Induction by EPS-Producing *L. paracasei* KB28

We obtained an *L. paracasei* KB28 fraction with its EPS attached, and stimulated mouse peritoneal macrophages with various concentrations of the bacterial cells for 24 h. Supernatant concentrations of cytokines were measured by ELISA. Macrophages secreted TNF-α, IL-6, and IL-12 in a dose-dependent manner (Fig. 2A–2C). Examination of these cytokine mRNAs by real-time PCR showed the same pattern (Fig. 2D–2E).

Induction of IκBα Degradation and MAPK Activation by EPS-Producing *L. paracasei*

NF-κB and MAPKs are important intracellular signaling molecules that mediate the activation of various cytokine

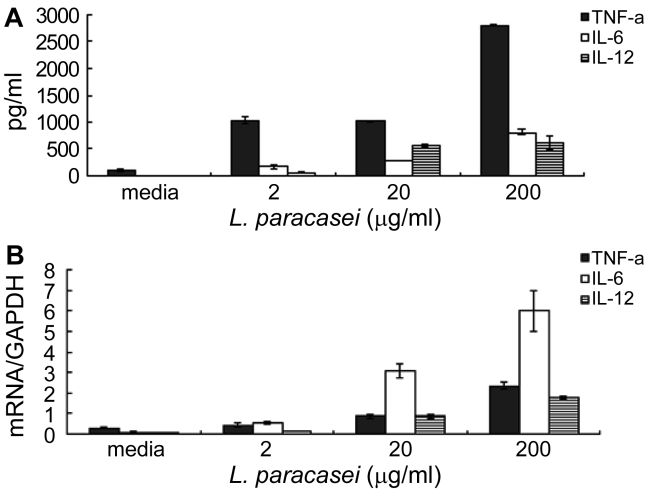

Fig. 2. Induction of TNF-α, IL-6, and IL-12 by peritoneal macrophages in response to EPS-producing *L. paracasei* KB28. Peritoneal macrophages from thioglycollate-injected mice were isolated and cultured with various concentrations of heat-killed bacteria for 24 h. (**A**) Cytokine levels were measured by ELISA. (**B**) Each mRNA expression was measured by real-time PCR. GAPDH was used as an internal control. Data represent the mean ± SD of three independent experiments.

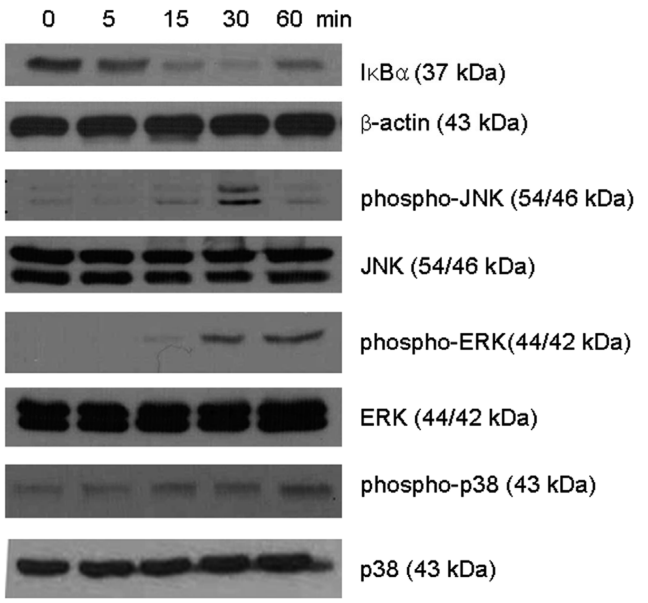

Fig. 3. IκBα degradation and activation of p38, JNK, and ERK1/2 by EPS-producing *L. paracasei* KB28.
Peritoneal macrophages were stimulated with EPS-producing *L. paracasei* KB28 (20 μg/ml) for 0, 5, 15, 30, and 60 min, and total cell lysates were analyzed by Western blotting. One of four independent experiments is shown.

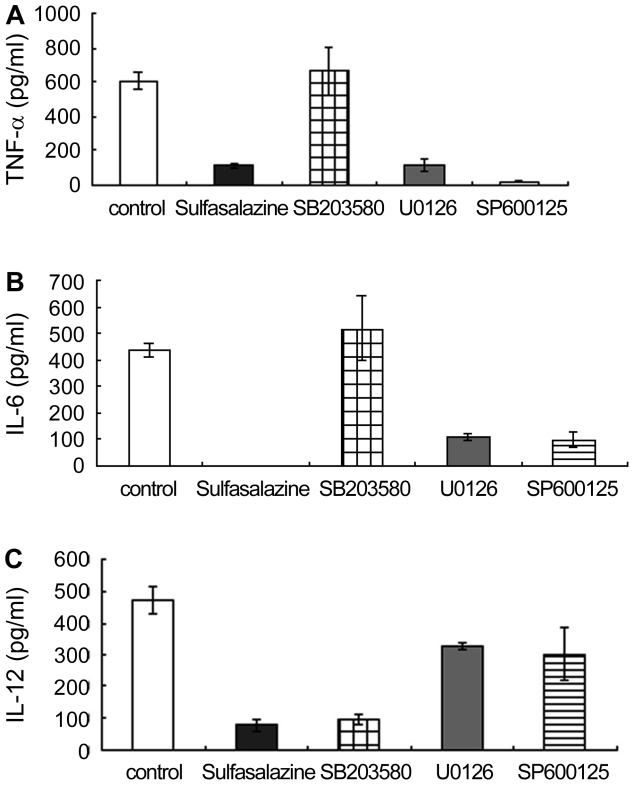

Fig. 4. The roles of NF-κB, p38, JNK, and ERK1/2 in the induction of TNF-α, IL-6, and IL-12 production by macrophages exposed to EPS-producing *L. paracasei* KB28.
Peritoneal macrophages were pretreated with sulfasalazine (200 μM), SB203580 (10 μM), U0126 (10 μM), or SP600125 (10 μM) for 1 h before culturing with bacteria for a further 24 h. Supernatants were harvested and cytokine levels were measured by ELISA. Data are presented as the mean ± SD of four independent experiments.

genes in response to microbial products. In the cytosol, the nuclear localization sequence of NF-κB is hidden by IκBα, but upon stimulation degradation of IκBα occurs, allowing NF-κB to transmigrate to the nucleus where it binds to the promoters of cytokine genes [5]. Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK)1/2, the major mammalian MAPKs, converge diverse extracellular signals and modulate nuclear transcription factors or regulate cytokine mRNA stability [3]. We obtained cellular protein extracts at 0, 5, 15, 30, and 60 min after stimulation of macrophages with EPS-producing *L. paracasei* KB28. As shown in Fig. 3, degradation of IκBα was observed and this result strongly showed that *L. paracasei* KB28 activated the NF-κB pathway. At the same time, phosphorylation of JNK, p38, and ERK1/2 in response to the bacteria was detected (Fig. 3).

Next, we investigated whether these signaling pathways were directly involved in the production of cytokines using pharmacological inhibitors. Macrophages were pretreated for 1 h with sulfasalazine (NF-κB inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), and U0126 (ERK1/2 inhibitor), followed by incubation with EPS-producing *L. paracasei*. Secretion of TNF-α and IL-6 was strongly suppressed in response to sulfasalazine, SP600125, and U0126, but not SB203580, indicating that NF-κB, JNK, and ERK1/2 are essential for induction of TNF-α and IL-6. On the other hand, blocking of NF-κB and p38 strongly impaired the synthesis of IL-12 by *L. paracasei* (Fig. 4).

Discussion

The outer layer of Gram-positive bacteria forms a carbohydrate zone, which may be categorized into capsular polysaccharides, wall polysaccharides, and extracellular polysaccharides (EPSs) [2]. Capsular polysaccharides, often found in pathogenic bacteria, are tightly associated with, and often covalently bound to, the peptidoglycan of the cell wall and make bacteria resistant to phagocytosis, thus acting as a virulence factor [12]. Wall polysaccharides are covalently or noncovalently linked to the cell wall. EPSs are not attached to the cell wall but, depending on the conditions, can be tightly associated with the peptidoglycan. The structure of lactic acid bacteria EPS is an assembly of oligosaccharides consisting mainly of glucose, galactose, and rhamnose. Once the sugar, most often monosaccharides and disaccharides, is transported from the surrounding medium into the cytoplasm of bacteria, it is converted into either sugar-6-phosphates, which are consumed in catabolic pathways, or sugar-1-phosphates, which are used for synthesis of polysaccharides including EPSs [9].

Induction of cytokines by lactic acid bacteria mostly relies on the carbohydrate or lipid-based structure of their cell wall [4, 7]. A recent study has demonstrated that mutant types of *L. casei* strain Shirota with defective cell wall-associated high molecular mass polysaccharides are potent inducers of IL-12, TNF-α, and IL-6, indicating that high molecular mass polysaccharides may give suppressor signals [16]. Comparing these results with ours, it appears that *L. paracasei* KB28 may produce EPSs that contain a higher ratio of low molecular mass polysaccharides to high molecular mass polysaccharides in structure.

NF-κB and MAPKs are important transcription factors activated by various receptors that recognize microbial products and share multiple points along the downstream pathways. We used pharmacological inhibitors to confirm the contributions of the NF-κB and MAPK pathways to cytokine induction by *L. paracasei*. According to our results, NF-κB, JNK, and ERK1/2 were necessary for TNF-α and IL-6 production, whereas NF-κB and p38 were required for IL-12 production. Given these results, it became clear that *L. paracasei* KB28 used different signaling pathways to provoke cytokine expression in macrophages. However, it should be noted here that as we did not separate EPS from *L. paracasei* KB28, we did not clarify whether the observed responses were merely due to EPS or a combined effect of EPS and the bacteria. Further studies are required to examine the precise role of EPSs.

In conclusion, treatment of macrophages with EPS-producing *L. paracasei* caused induction of TNF-α, IL-6, and IL-12 *via* modulation of the NF-κB, JNK, p38, and ERK1/2 pathways. Our results provide information for a better understanding of the molecular mechanisms of the immunomodulatory effect of food-derived lactic acid bacteria.

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