Lactobacillus paracasei treatment modulates mRNA expression in macrophages

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ABSTRACT

Macrophage metabolic pathways show changes in response to various external stimuli. Especially, increased lipopolysaccharide, an important bacterial component and Toll-like receptor 4 agonist, can induce activity in various macrophage metabolic pathways, including energy production and biosynthesis, as well as high immune responses due to increase in differentiated M1 macrophages. In this study, we confirmed that Lactobacillus paracasei (L. paracasei) KBL382, KBL384 and KBL385, isolated from the feces of healthy Koreans, can modulate various enzymes and membrane transporters related to glycolysis or macrophage polarization including hypoxia-inducible factor 1-alpha (HIF1A), inducible nitric oxide synthase (iNOS) and arginase in stimulated macrophages at the mRNA level, using the in vitro rodent bone-marrow-derived macrophage (BMDM) model. All L. paracasei exhibited significant down-regulatory effects on mRNAs for glycolysis-related enzymes, including lactate dehydrogenase A, solute carrier family 2 member 1, and triosephosphate isomerase. Moreover, L. paracasei treatment could lead to significant reductions in HIF1A or iNOS mRNA, and induced arginase mRNA in the BMDM model. Therefore, further extensive studies should be performed to support the application of L. paracasei, such as in probiotics or therapeutics, in controlling abnormal immune responses related to macrophage.

1. Introduction

Recently, important roles of metabolic reprogramming in immune cells have been reported [1,2]. Especially, macrophages can alter their metabolic pathways in response to various environmental stimuli [3]. For example, increases in lipopolysaccharide (LPS), an important bacterial component and Toll-like receptor 4 agonist, can trigger various metabolic pathways related to energy production and biosynthesis in M1 macrophage. Previous studies have been reported that differentiated M1 macrophages are strongly related to various immune responses [4,5].

During inflammation due to LPS, enzymes related to glycolysis and pentose phosphate pathway or inducible nitric oxide synthase (iNOS) can be induced in M1 macrophages to produce the energy sources adenosine triphosphates and nitric oxide (NO), respectively [6–8]. Moreover, induction of hypoxia-inducible factor 1-alpha (HIF1A) and arginase can affect macrophage polarization [9–11].

Probiotics such as Lactobacillus spp. have been reported to show strong immunomodulatory effects [12,13]. We previously confirmed the strong anti-inflammatory effects of Lactobacillus paracasei (L. paracasei) species, isolated from the feces of healthy Koreans, in the in vivo dextran sodium sulfate (DSS)-induced colitis animal model [12]. In this study, we investigated the modulatory effects of L. paracasei treatment on various enzymes related to glycolysis or macrophage polarization, including HIF1A, iNOS and arginase in stimulated macrophages at the mRNA level in vitro using the rodent bone-marrow-derived macrophage (BMDM) model.

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2. Materials and methods

2.1. Preparation of L. paracasei strains

Previously, we isolated three L. paracasei strains (KBL382, KBL384 and KBL385) from the feces of healthy Koreans and confirmed their strong resistance to bile salts or low pH [12]. All L. paracasei strains were cultured in anaerobic conditions using Lactobacilli MRS agar (Becton, Dickinson and Company, Sparks, MD, USA) with 0.05% L-cysteine-hydrochloride and Anaeropack (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan) at 37 °C for 24 h as described previously [12]. Bacteria were collected using centrifugation at 1200 × g and washed twice using 1 × phosphate-buffered saline (PBS). Bacterial concentrations were quantified as colony-forming units (CFUs) through the cultivation, and the bacteria were stored at −4 °C until further use.

2.2. Preparation of BMDM

BMDMs were isolated from 6-week-old female C57BL/6 mice (Central Lab Animals Inc., Seoul, Republic of Korea), as described previously [14,15]. BMDMs were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 1% penicillin/streptomycin (Gibco), 1% gentamycin (Gibco), and 10% fetal bovine serum (Gibco) at 37 °C under a humidified atmosphere with 5% CO2. To measure the total cell concentration, BMDMs were stained with trypan blue and counted using a CKX31 inverted microscope (Olympus Corp., Tokyo, Japan).

2.3. Measurement of mRNA expression in the in vitro BMDM model

Approximately 3 × 10^5 cells of BMDMs were incubated with 100 ng/ml LPS (Sigma-Aldrich Corp., St. Louis, MO, USA) and 20 ng/ml interferon-γ (IFN-γ) or interleukin-4 (IL-4) (PeproTech Inc., Rocky Hill, NJ, USA) in each well of a 48-well plate (SPL Life Sciences Co., Ltd., Pocheon-si, Gyeonggi-do, Republic of Korea) at 37 °C for 24 h as previously described with some modification [16,17]. Approximately 3 × 10^5 CFUs of each L. paracasei strain were co-incubated with the BMDMs. The cytotoxicity of Lactobacillus spp. for BMDM were confirmed using Lactate dehydrogenase (LDH) Assay Kit (Cytotoxicity) (Abcam Inc., Cambridge, MA, USA) following the manufacturer’s protocol. To measure mRNA expression, total RNA was extracted using an easy-spin Total RNA Extraction Kit (iNtRON biotechnology Inc., Seongnam-si, Gyeonggi-do, Republic of Korea) following the manufacturer’s protocol. Complementary DNA (cDNA) was synthesized using a High-Capacity RNA-to-cDNA Kit (Thermo Scientific). Then, real-time polymerase chain reaction (RT-PCR) was performed using a Power SYBR Green PCR Master Mix (Thermo Scientific) with 0.01 mM primers [16–18] (Table 1). PCR was carried out with an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s using a Step-One-Plus Real-Time PCR System (Applied Biosystems Inc., Forster City, CA USA). The expression levels of target mRNAs were normalized to hypoxanthine-guanine phosphoribosyl transferase (HPRT) [19].

2.4. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) with at least three independent experiments. When appropriate, the Mann-Whitney U test was used, and P-values less than 0.05 were considered statistically significant. GraphPad Prism 5.04 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analyses and visualizations.

### Table 1

| Target | Sequence Reference | Reference |
|--------|--------------------|-----------|
| Arginase | Fw: 5'-CAG AAT AAG GGA AGA GTC AG-3' | [18] |
| Enolase 1 | Fw: 5'-GGC TTC CAC TGG CAT CTA C-3' | [16] |
| | Rev: 5'-CAG ATC TGG GAC GAG TCA CC-3' | [16] |
| HIF1A | Fw: 5'-AAC TGT TGT TAT GAG GCT CAC C-3' | [16] |
| | Rev: 5'-TCT GCT CAT GGT CAT CTT C-3' | [16] |
| HPRT | Fw: 5'-TTA TGG ACA GGA CTG AAA GCC-3' | [19] |
| | Rev: 5'-GCT TTA AGT TAA TCC ACC AGG T-3' | [19] |
| INOS | Fw: 5'-TGG GCT TGG AGG GCT TTA AAG GCC TCG C-3' | [18] |
| | Rev: 5'-AGG ACC AGG AAC AGG AGT TTA C-3' | [18] |
| LDHA | Fw: 5'-CAT TGT CAA GTA CAG TCC ACA CT-3' | [16] |
| | Rev: 5'-TTT CAA TTA CTC GGT TGT TGG GA-3' | [16] |
| MCT4 | Fw: 5'-TCA GGG TCT TCC CCT ACC C-3' | [16] |
| | Rev: 5'-GGC AAA GGC GTC CAC ACA C-3' | [16] |
| PFK1 | Fw: 5'-GGC GGC GAC AAC ATC AAG CC-3' | [16] |
| | Rev: 5'-GGG OCT TCC CTC GTC GGA A-3' | [16] |
| PKM2 | Fw: 5'-GCC CCG TGG ACA TGG ACT C-3' | [16] |
| | Rev: 5'-CCA TGA GAG AAA TTC ACC CGA G-3' | [16] |
| SLC2A1 | Fw: 5'-CAG TTC CAC TAT AAC ACT GGT G-3' | [16] |
| | Rev: 5'-GCC CCC GAC GAG GAA GAT G-3' | [16] |
| TPI | Fw: 5'-CCA GGA AGT TCT TCG TTT GGC-3' | [16] |
| | Rev: 5'-CAA AGT CCA TGT AAG GGG TGG-3' | [16] |

aFw represents sequences of forward primers.

bRv represents sequences of reverse primers.

3. Results

3.1. Modulation of mRNA expressions related to glycolysis with L. paracasei treatment in the in vitro BMDM model

Fig. 1 shows the modulation of mRNA expressions related to glycolysis in BMDM with L. paracasei treatment. L. paracasei concentration had no cytotoxic effects in BMDM (data not shown). Overall, treatment with all L. paracasei strains had significant down-regulatory effects on the mRNAs of glycolysis-related enzymes or membrane transporters. mRNA levels of lactate dehydrogenase A (LDHA), solute carrier family 2 member 1 (SLC2A1), and triosephosphate isomerase (TPI) were significantly reduced after treatment of L. paracasei strains compared to control without treatment (P < 0.05) (Fig. 1 B and C). Moreover, L. paracasei KBL385 exhibited strong down-regulatory effects on enolase 1, monocarboxylate transporter 4 (MCT4), phosphofructokinase 1 (PFK1), and pyruvate kinase (PKM2). In addition, L. paracasei KBL382 and KBL384 showed significant down-regulatory effects at mRNA levels of MCT4 and PFK1 (Fig. 1C and E). L. paracasei KBL384 also modulated Enolase 1 and PKM2 significantly (Fig. 1A and E).

3.2. Modulation of mRNA expressions related to macrophage polarization with L. paracasei treatment in the in vitro BMDM model

Treatment with L. paracasei KBL382, KBL384, or KBL385 had significant down-regulatory effects on the M1 macrophage-related marker HIF1A and iNOS (P < 0.05) (Fig. 2A and B). Moreover, L. paracasei KBL382 or KBL385 showed up-regulatory effects on the M2 macrophage–related marker arginase in the BMDMs stimulated with LPS and IFN-γ (P < 0.05) (Fig. 2C). However, mRNA levels of arginase did not change significantly with L. paracasei KBL384 treatment (Fig. 2D). Moreover, mRNA levels of HIF1A changed significantly with L. paracasei KBL384 treatment in BMDMs stimulated with LPS and IL-4 (P < 0.05) (Fig. 3A). The high arginase levels were observed in BMDMs stimulated with LPS and IL-4 after L. paracasei KBL382 treatment (P < 0.05) (Fig. 3B).
4. Discussion

In this study, we demonstrated that treatment with *L. paracasei* KBL382, KBL384, or KBL385 clearly modulated the expression levels of various mRNA related to glycolysis and macrophage polarization in the *in vitro* BMDM model. Especially, *L. paracasei* treatment significantly reduced the mRNA levels of glycolysis-related enzymes or membrane transporters in BMDMs treated with 100 ng/mL lipopolysaccharide (LPS) and 20 ng/mL interferon-γ (IFN-γ) (Fig. 1). Increased enolase 1 can contribute to the production of pro-inflammatory cytokines and prostaglandins [20]. Moreover, previous study has been reported that LDHA in macrophages can boost the T Helper 17 (Th17) cell-dependent immune responses and exacerbate autoimmune diseases [21]. When glycolysis is suppressed, pyruvate production and oxidative phosphorylation also decrease [22]. Then, chain reduction of MCT4, which is involved in the conversion of pyruvate to lactate, and PFK1 can occur [22,23]. Decreases in the mRNA levels of PKM2, SLC2A1 and TPI, which are important enzyme or membrane transporter in the glycolytic pathway, were also exhibited in BMDM with *L. paracasei* treatment (Fig. 1E) [16].

\[ \text{LDHA} \] 

**Fig. 1. Effects of *L. paracasei* treatment on mRNA expressions related to glycolysis.** A) Enolase 1, B) lactate dehydrogenase A (LDHA), C) monocarboxylate transporter 4 (MCT4), D) phosphofructokinase 1 (PFK1), E) Pyruvate Kinase M2 (PFK2), F) solute carrier family 2 member 1 (SLC2A1), G) triosephosphate isomerase (TPI). Approximately 3 \( \times \) 10^5 cells of rodent bone-marrow-derived macrophage cells (BMDMs) treated with 100 ng/mL lipopolysaccharide (LPS) and 20 ng/mL interferon-γ (IFN-γ) were co-incubated with 3 \( \times \) 10^6 colony-forming units of each *L. paracasei* strain at 37°C for 24 h. Total RNA was extracted using an easy-spin Total RNA Extraction Kit and cDNA was synthesized using a High-Capacity RNA-to-cDNA Kit. PCR reaction was performed and the expression levels were normalized to hypoxanthine-guanine phosphoribosyl transferase (HPRT). Samples without *L. paracasei* treatment were used as a control. Data are expressed as mean ± SEM of three independent experiments. Asterisks indicated a statistical significance (*, P < 0.05; **, P < 0.01).

\[ \text{HIF1A} \]

**Fig. 2. Effects of *L. paracasei* treatment on mRNA expression related to macrophage polarization in BMDM stimulated with LPS and IFN-γ.** A) Hypoxia-inducible factor 1-alpha (HIF1A), B) inducible nitric oxide synthase (iNOS), C) arginase. PCR was performed using cDNA and the expression levels were normalized to HPRT. Samples without *L. paracasei* treatment were used as a control. Data are expressed as mean ± SEM of three independent experiments. Asterisks indicated a statistical significance (*, P < 0.05).
Our data suggested the important effects of environmental stimuli, can be related to various autoimmune diseases [26].

phages, further longitudinal studies with multi-omics approaches in treatment on the expression patterns and differentiation of macrophage polarization in BMDM stimulated with LPS and interleulin-4. Moreover, associated with the increased iNOS expression and down-regulation of arginase [25]. Our study confirmed that L. paracasei treatment can modulate the reduction of iNOS mRNA and induction of arginase mRNA, especially with KBL382 or KBL385 treatment (Fig. 2B and C). However, to elucidate the mechanisms including metabolites underlying the effects of L. paracasei treatment on the expression patterns and differentiation of macrophages, further longitudinal studies with multi-omics approaches in vivo models need to be performed.

Over-polarization of M1 macrophages, which is caused by the environmental stimuli, can be related to various autoimmune diseases [26]. Our data suggested the important effects of L. paracasei treatment to mRNA levels of macrophages in the in vitro conditions. Therefore, in conclusion, further extensive studies should be performed to support the application of L. paracasei, such as in probiotics or therapeutics, in controlling abnormal immune responses related to macrophage.

Declaration of competing interest

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