Cytokine 92 (2017) 1–11

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Lactobacillus paracasei modulates LPS-induced inflammatory cytokine release by monocyte-macrophages via the up-regulation of negative regulators of NF-kappaB signaling in a TLR2-dependent manner

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ARTICLE INFO

Article history:
Received 18 July 2016
Received in revised form 28 December 2016
Accepted 3 January 2017

Keywords:
Lactobacillus paracasei
Proinflammatory cytokine
TLR2
NF-κB
Negative regulator

ABSTRACT

The application of the probiotic lactobacillus is suggested in the treatment of some inflammatory diseases of intestines due to its potential ability to attenuate inflammation. However, the mechanism is not completely understood. In PBMCs, *Lactobacillus paracasei* (*L. Paracasei*) down-regulated the LPS-induced production of TNF-α and IL-6. Using a macrophage-like differentiated THP-1 cell line induced by PMA, we investigated the effect of *L. paracasei* on the production of pro-inflammatory cytokines by monocyte-macrophages. Treatment of the differentiated THP-1 cells with *L. paracasei* either concurrently with or before LPS challenge attenuated the LPS-induced secretion of TNF-α and IL-1β. This effect was due to a decrease in IκB phosphorylation and NF-κB nuclear translocation. Furthermore, treatment of the differentiated THP-1 cells with *L. paracasei* induced the expression of negative regulators of the NF-κB signaling pathway, including the deubiquitinating enzyme A20, suppressor of cytokine signaling (SOCS) 1, SOCS3, and IL-1 receptor-associated kinase (IRAK) 3. Pretreatment with an IRAK4 inhibitor suppressed the *L. paracasei*-induced expression of these negative regulators and further increased the LPS-mediated expressions of TNF-α and IL-1β. Moreover, treatment with an antibody against Toll-like receptor (TLR) 2 reversed the effect of *L. paracasei* on inducing negative regulators and inhibiting TNF-α and IL-1β productions. Our findings suggest that *L. paracasei* inhibits the production of pro-inflammatory cytokines by monocyte-macrophages via the induction of negative regulators of the NF-κB signaling pathway in a TLR2-IRAK4-dependent manner.

1. Introduction

Probiotics have been defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host [1]. Lactobacillus is an important member of the probiotic family, which plays an essential role in immune regulation [2]. Lactobacillus, not only in animal experiments, but also in clinical trials, has shown some prospects for the prevention and treatment of inflammation such as abating nosocomial diarrhoea of infants [3], alleviating rheumatoid arthritis [4], decreasing the risk for allergy in children [5]. The properties of probiotics are strain-dependent. Some strains of lactobacilli have been shown to reduce the production of pro-inflammatory cytokines, such as TNF-α [6]. While other strains are found to induce IL-10-producing regulatory T cells [7]. Even soluble factors from some strains of lactobacillus inhibited the nuclear translocation of NF-κB and the expression of NF-κB-dependent pro-inflammatory cytokines in mice [8]. However, the mechanisms by which probiotic lactobacilli modulate inflammation are not completely characterized. Further studies are necessary to better understand how probiotic lactobacilli can be utilized and applied in the clinic.

Monocyte-macrophages are very important cells that contact with pathogens via pattern recognition receptors (PRRs) and produce pro-inflammatory cytokines to initiate innate immunity and then activate adaptive immunity [9]. Toll-like receptor 4 (TLR4) is the main PRR of monocyte-macrophages for LPS which is a very strong triggering factor in Gram-negative bacterial infections. TLR4 binds LPS-CD14 to form the LPS-CD14-MD2-TLR4 complex, thus resulting in TLR4 polymerization and activation. Myeloid differen-

Abbreviations: LPS, lipopolysaccharides; TLR, toll like receptor; SOCS, suppressor of cytokine signaling; IRAK, IL-1 receptor-associated kinase; TOLLIP, toll-interacting protein.

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http://dx.doi.org/10.1016/j.cyto.2017.01.003
1043-4666/

tiation primary response gene 88 (Myd88) then activates and combines with IL-1R-associated kinase 4 (IRAK4) and IRAK2 to form Myddosome. TNF-α receptor-associated factor 6 (TRAF6) binds to IRAK4/2 and activates NF-κB-inducible kinase (NIK), which then activates IκB kinase α/β (IKKα/β) to phosphorylate the inhibitor of NF-κB (IκB) and deconstructs the IκB-NF-κB complex. Afterwards, NF-κB translocates into the nucleus to initiate the expression of pro-inflammatory cytokines [10–13]. On the other hand, phosphorylation of IRAK4 can form a signal that is responsible for the initiation of negative regulatory factors in the NF-κB pathway. Theses negative regulators including deubiquitinating enzyme A20, IRAK3, Toll interacting protein (TOLLIP), suppressors of cytokine signaling 1 (SOCS1) and suppressors of cytokine signaling 3 (SOCS3) are able to reduce NF-κB and JAK/STAT signal transduction [14–17]. Pretreatment with low dose LPS reduced the degree of inflammation induced by subsequent treatment with high dose LPS; this process has been referred to as endotoxin tolerance [18]. The mechanism of LPS tolerance involves the expressions of these negative regulators [19]. It is not exactly known whether lactobacillus induces a resistance to inflammatory reaction initiated by LPS stimulation through a similar mechanism.

In the present study, we aimed to examine the mechanism of *Lactobacillus paracasei* (*L. paracasei*) to attenuate pro-inflammatory cytokines secretion triggered by LPS. We used an *in vitro* model of PMA induced differentiated THP-1 cells cocultured with LPS, which mimicked the interaction of Gram-negative bacteria and macrophages in gut mucosal environment. The results demonstrated that *L. paracasei* inhibited the production of TNF-α and IL-1β through inducing the productions of negative regulators, including A20, SOCS1, SOCS3 and IRAK3, to suppress NF-κB activation and nuclear translocation. The results also showed that TLR2 is required for the anti-inflammatory activity of *L. paracasei*.

2. Materials and methods

2.1. Bacterial preparations

L. paracasei was isolated from human origin by Cultech Limited (UK), and then presented to our laboratory to use in this experiment, which was subjected to biochemical identification using API 50CHL test kits (Bio Merieux, France). From frozen stocks (−80°C), bacteria were suspended in Man-Rogosa-Sharpe liquid medium (MRS broth; Oxoid) and plated in MRS agar. After culturing anaerobically at 37°C for 24 h, bacteria were inoculated in fresh MRS broth and grown at 37°C under anaerobic conditions for 48 h. Total counts were performed by viable count. Freshly cultured bacteria were diluted in RPMI 1640 with 10% FBS (Sigma) to a final concentration of 1×10^{10} CFU/ml as viable lactobacilli. Viable lactobacilli were then killed by incubations in a water bath at 60°C for 1 h. The viable count was performed to make sure no viable bacteria survived. Bacteria were stored at −80°C as heat-killed lactobacilli.

2.2. Cell preparation, culture, and treatment

PBMCs were isolated from the peripheral blood of healthy donors. Briefly, after a Histopaque 1077 (Sigma) gradient centrifugation, mononuclear cells were collected, washed, and adjusted in RPMI 1640 medium supplemented with 10% FBS (Sigma). PBMCs (2×10^5 cells/ml) were then plated in duplicate in 96-well culture plates and stimulated with overnight-cultured viable lactobacilli (at a final concentration of 1×10^7 CFU/ml), heat-killed lactobacilli (at a final concentration of 1×10^9 CFU/ml), and LPS (10 μg/ml, Sigma) separately. In some experiments, PBMCs were stimulated with LPS and viable or heat-killed lactobacilli. After 24 h of stimulation at 37°C in a humidified atmosphere with 5% CO_2, the supernatants were stored at −70°C for cytokine assays.

THP-1 cells (Cell bank of Chinese Academy of Sciences, Shanghai) were derived from human monocytic leukemia. The differentiation of these cells was achieved by culturing with 10 ng/ml PMA (Sigma) in RPMI 1640 (with 10% FBS, Sigma) at a density of 5×10^5 cells/ml in the beginning for 48 h. The medium was then removed, and the cells were washed with 37°C RPMI 1640 three times and then replaced with FBS-free RPMI 1640. The cells were continuously rested for 24 h to put them in a differentiated macrophage-like state. These PMA-induced differentiated THP-1 cells were then stimulated with heat-killed lactobacillus alone or in combination with LPS (10 μg/ml). Supernatants were harvested for cytokine measurements, and RNA and protein were extracted.

In other experiments, the differentiated THP-1 cells were pretreated with low-dose LPS (10 ng/ml), heat-killed *L. paracasei*, or Pam3CSK4 (Sigma) for 24 h. After washing three times with PBS, 1 μg/ml LPS was added into the culture medium and incubated for 3 h. Supernatants were collected, and cells were stored. In some experiments, the IRAK1/4 inhibitor (25 mM) (Millipore, USA) was added into the cultures 2 h before pretreatment with *L. paracasei* or Pam3CSK4. To investigate the role of TLR2, a rat anti-human TLR2 antibody (pAbhTLR2, InvivoGen, USA) was added 2 h prior to pretreatments.

2.3. Cytokine assays

Levels of TNF-α, IL-1β, and IL-6 in supernatants were measured by sandwich ELISA kits (BD Biosciences, USA), according to the manufacturer's protocols.

2.4. IκB analysis by western blotting

The differentiated THP-1 cells were stimulated with heat-killed *L. paracasei* alone or in combination with LPS for various time periods (15–240 min). After treatment, the THP-1 cells were lysed in whole-cell extract buffer (Beyotime, China). After centrifugation, supernatants were separated by electrophoresis on a 12% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes on ice. After blocking for 2 h with 4% BSA in TBSA, the membranes were blotted with rabbit anti-human IκB-α (1:500) (Santa Cruz Biotechnology, USA) and mouse anti-human p-IκBα (1:1000) (Cell Signaling Technology, USA) polyclonal antibodies overnight at 4°C. The membranes were incubated with a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) (Proteintech Group, USA) and goat *anti*-mouse IgG (1:5000) (Immunology Consultants Laboratory, USA) antibodies for 2 h. Immunoreactive proteins were detected using the enhanced chemiluminescence protocol (Millipore, USA).

2.5. NF-κB detection by indirect immunofluorescence and trans-nuclear activity assay

Differentiated THP-1 cells were treated with heat-killed *L. paracasei* alone or in combination with LPS for 2 h. The cells were washed with PBS (37°C) and then fixed with 4% paraformaldehyde solution in PBS at room temperature for 30 min. The fixed cells were washed with PBS and treated with 0.2% Triton X-100 in PBS for 5 min. The washed cells were then incubated with bovine serum albumin in PBS for 1 h, after which they were incubated with mouse *anti*-human NF-κB/P65 monoclonal antibody (1:100) at 37°C in a humidified atmosphere with 5% CO_2 overnight. The intracellular NF-κB/P65-antibody complexes were subsequently detected with PE-labeled goat anti-mouse IgG (MultiSciences Biotech, China) after a 2-h incubation. Cell nuclei were stained with