Bacterial Teichoic Acids Reverse Predominant IL-12 Production Induced by Certain Lactobacillus Strains into Predominant IL-10 Production via TLR2-Dependent ERK Activation in Macrophages

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J Immunol 2010; 184:3505-3513; Prepublished online 26 February 2010; doi: 10.4049/jimmunol.0901569
http://www.jimmunol.org/content/184/7/3505
Bacterial Teichoic Acids Reverse Predominant IL-12 Production Induced by Certain Lactobacillus Strains into Predominant IL-10 Production via TLR2-Dependent ERK Activation in Macrophages

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The cytokine response of macrophages to probiotic lactobacilli varies between strains, and the balance of IL-10/IL-12 production is crucial for determination of the direction of the immune response. To clarify the mechanism whereby Lactobacillus strains differentially induce production of IL-10 and IL-12, we examined the potential relationship between cytokine production and MAPK activation. In mouse peritoneal macrophages, Lactobacillus plantarum potently induced IL-10 but weakly induced IL-12 production, whereas L. casei potently induced IL-12 but weakly induced IL-10 production. Kinetic analysis of the activation of ERK, p38, and JNK showed that L. plantarum induced a more rapid and intense activation of MAPKs, especially of ERK, than L. casei. A selective blockade of ERK activation induced by L. plantarum resulted in a decrease in IL-10 production and a simultaneous increase in IL-12 production. Interestingly, when macrophages were stimulated with a combination of L. plantarum and L. casei, IL-10 production was induced synergistically. We identified cell wall teichoic acid and lipoteichoic acid as key factors for triggering the synergistic induction of IL-10 production, although these teichoic acids alone only weakly induced IL-10 production. The effect of these teichoic acids on IL-10 production was mediated by TLR2-dependent ERK activation. Our data demonstrate that activation of the ERK pathway is critical for determination of the balance of the IL-10/IL-12 response of macrophages to lactobacilli and that predominant IL-12 production induced by certain lactobacilli such as L. casei can be converted into predominant IL-10 production when stimulated in the presence of teichoic acids. The Journal of Immunology, 2010, 184: 3505–3513.

Lactobacilli are widely used in many foods as probiotics, defined as, “Live microorganisms which, when administered in adequate amounts confer a health benefit on the host” (1), and they have become the focus of much research due to their potential immunoregulatory activities. An increasing number of immunoregulatory effects of lactobacilli, including augmentation of NK cell activity, enhancement of IgA production, promotion of dendritic cell maturation and regulatory T cell differentiation, and downregulation of inflammation, have been reported (2, 3).

One of the well-researched immunoregulatory functions of probiotics is the induction of cytokine production. In particular, the induction of IL-10 and IL-12 production by probiotics has been studied intensively, because the balance of IL-10/IL-12 secreted by macrophages and dendritic cells in response to microbes is crucial for determination of the direction of the immune response. IL-10 is an anti-inflammatory cytokine and is expected to improve chronic inflammation, such as that of inflammatory bowel diseases and autoimmune diseases (4). IL-10 downregulates phagocytic and T cell functions, including the production of proinflammatory cytokines, such as IL-12, TNF-α, and IFN-γ (5), that control inflammatory responses. IL-10 promotes the development of regulatory T cells for the control of excessive immune responses (6). In contrast, IL-12 is an important mediator of cell-mediated immunity and is expected to augment the natural immune defense against infections and cancers (7). IL-12 stimulates T cells to secrete IFN-γ, promotes Th1 cell development, and, directly or indirectly, augments the cytotoxic activity of NK cells and macrophages. IL-12 also suppresses redundant Th2 cell responses for the control of allergy (8). Because of their widespread immunological effects, much attention has been paid to the ability of probiotics to induce IL-10 and IL-12 production in view of the many potential benefits of induction of these cytokines, such as defense against inflammatory bowel diseases, autoimmune diseases, allergies, infections, and cancers. Therefore, clarification of the mechanism of IL-10 and IL-12 production by macrophages and dendritic cells in response to probiotics is expected to lead to a greater understanding of their immunoregulatory activities and thus to contribute to a more effective utilization of probiotics in health maintenance and disease prevention.

There have been few reports concerning the active bacterial components of probiotics that induce IL-10 and IL-12 production. Unique oligodeoxynucleotides with or without the immunostimulatory CpG motif have been identified as components of Lactobacillus rhamnosus and L. gasseri that are active in IL-12 induction (9, 10). In contrast, we recently showed that the insoluble intact cell wall, but not the soluble one, of L. casei was required for the potent induction of IL-12 production by macrophages, suggesting that recognition of the three-dimensional structure of the cell wall of...
certain *Lactobacillus* strains is required for the production of IL-12 (11). Little information is available concerning the components that are active in the induction of IL-10 production. It has been shown that genomic DNA, isolated from bifidobacteria or lactobacilli, could induce IL-10 production by human PBMCs (12). Other reports have shown that soluble or insoluble cell preparations, obtained from centrifugation of sonicated bifidobacteria, were responsible for induction of IL-10 production (13, 14). Further studies are required to clarify the active components of probiotics that induce these cytokines.

Cytokine production is regulated by intracellular signal transduction pathways that are activated following recognition of microorganisms or microbial components via receptors, such as TLRs, that exist on the cell and phagosomal membranes of phagocytes. One of the crucial signal transduction events that control cytokine production is activation of MAPKs, including ERK, JNK, and p38 (15). Furthermore, several reports have shown that activation of the ERK pathway following the recognition of cell components via TLRs is important for determination of the balance of IL-10/IL-12 production. ERK activation, induced by recognition of Pam3Cys, LPS, and Cpg DNA via TLR2, TLR4, and TLR9, respectively, preferentially induces IL-10 production and downregulates IL-12 production (16–19). It also has been shown that defective activation of ERK is responsible for elevated production of IL-12 by macrophages in response to *Helicobacter hepaticus* (20). As for the cytokine response of phagocytes to probiotic bacteria, the roles of MAPKs, especially ERK, have not yet been fully elucidated, although some reports showed the importance of the MAPK pathways in the regulation of cytokine production in response to probiotics as well as other bacteria (21–24). Little is known regarding the receptors and active components of probiotics involved in MAPK activation.

In this report, we aimed to clarify the mechanism of IL-10 and IL-12 production induced by lactobacilli by studying MAPK activation in mouse peritoneal macrophages following recognition of three different strains of lactobacilli: *L. plantarum*, which potently induces IL-10 but weakly induces IL-12 production, *L. casei*, which potently induces IL-12 but weakly induces IL-10 production, and *L. johnsonii*, which weakly induces the production of both IL-10 and IL-12. Additionally, we investigated the bacterial cell components and their receptors that are involved in MAPK activation. We report that ERK activation induced by TLR2 recognition of teichoic acids plays a key role in determination of the balance of IL-10/IL-12 production by macrophages in response to lactobacilli.

**Materials and Methods**

Reagents and culture medium

*N*-Acetylmuramidase SG and benzonuclease were purchased from Seikagaku Kogyo (Tokyo, Japan) and Merck (Darmstadt, Germany), respectively. DNase, RNase, trypsin, and pronase were obtained from Roche Diagnostic Systems (Somerville, NJ). U0126, a MEK1/2 inhibitor, SB203580, a p38 inhibitor, and SP600125, a JNK inhibitor, were purchased from Calbiochem (San Diego, CA). Lipoteichoic acid (LTA) from *Staphyloccocus aureus* was obtained from InvivoGen (San Diego, CA). RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 mM 2-ME was used to culture macrophages.

**Animals**

Female BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) and used for preparation of macrophages, unless otherwise specified. Male TLR2-deficient mice with a BALB/c genetic background were obtained from Oriental BioService (Kyoto, Japan), and male BALB/c mice were used as controls. Animals were used at 8–12 wk of age. Experiments were performed in accordance with the guidelines for the care and use of laboratory animals set by the Yakult Central Institute (Tokyo, Japan).

**Bacteria**

The probiotic strain, *L. casei* Shirata (YIT 9029), was originally isolated and maintained at the Yakult Central Institute. *L. johnsonii* JCM 1201 and *L. plantarum* ATCC 14917 were obtained from the Japan Collection of Microorganisms (Wako, Japan) and the American Type Culture Collection (Manassas, VA), respectively. These bacteria were cultured for 20 h at 37 °C in Lactobacilli-de Man, Rogosa, and Sharpe broth (Difco, Detroit, MI), collected by centrifugation, and washed several times with sterile distilled water. The bacteria were killed by heating at 100°C for 30 min and lyophilized. To prepare FITC-labeled lactobacilli, heat-killed lactobacilli were suspended at a concentration of 5 ng/ml in 50 mM carbonate buffer (pH 9.6), reacted with FITC isomer 1 (4.5 μg/ml; Dojindo Laboratory, Kumamoto, Japan) at 37°C for 60 min, and then washed with sterile PBS.

**Preparation of bacterial cell components**

Bacterial cell components were prepared from heat-killed *L. plantarum* and *L. casei*, and the preparation steps are summarized in Supplemental Figs. 1 and 2. Preparation of polysaccharide–peptidoglycan complex (PSPG) and protoplast was described previously (25). Briefly, heat-killed lactobacilli were exhaustively digested with *N*-acetylmuramidase. After centrifugation, the precipitate was washed with distilled water and then collected as the protoplast. The supernatant was treated with DNase, RNase, and trypsin. The digested supernatant was dialyzed against distilled water and used as the PSPG fraction. The preparation isolated from *L. plantarum* is described as the teichoic acid–peptidoglycan complex (TAPG) in this study.

Intact cell wall (ICW) was prepared by the method of Sekine et al. (26) as described previously (25). Briefly, heat-killed *L. casei* or *L. plantarum* was treated with 0.3% SDS and then washed with acetone. The cells were treated with pronase and delipidated by successive refluxing with methanol–chloroform–water (1:1:1) and methanol–chloroform (1:1). The preparation was treated with benzene nuclease and pronase. The insoluble material was washed with distilled water and then used as the ICW preparation. To prepare intact peptidoglycan (PGN), which was obtained by release of the polysaccharide moiety from the linkage region of ICW, the ICW was treated with 47% hydrogen fluoride at 4°C for 20 h (27). The material was washed with distilled water and then used as the PGN. 24 h.

Isolation of LTA was described previously (25). Briefly, the protoplast fraction prepared from *L. casei* was broken by sonication. The sonicated material was extracted with hot phenol and treated with benzene nuclease. LTA was purified by column chromatography over Mono-Prep High Q (Bio-Rad, Hercules, CA) and Octyl-Sepharose CL-4B (Pharmacia Biochem, Uppsala, Sweden) columns.

**Isolation of peritoneal macrophages**

Peritoneal macrophages were harvested 4 d after i.p. injection of 2 ml 4% thioglycollate broth (Difco). Cells were washed with HBSS containing 10 mM HEPES and cultured in RPMI 1640.

**Flow cytometric analysis of phagocytosis**

Peritoneal macrophages (6 × 106 cells) were cultured with FITC-labeled lactobacilli (10 μg/ml) in 1.2 ml RPMI 1640 in a 24-well culture plate for various time periods (1–24 h). Macrophages were dislodged by treatment with 10 mM EDTA/PBS for 10 min and washed with 3 mM EDTA/PBS. Analysis was performed on an EPICS Altra flow cytometer with Expo32 software (Beckman Coulter, Fullerton, CA).

**Microscopic analysis of macrophage lysis of phagocytosed lactobacilli**

Intracellular digestion of lactobacilli by macrophages was analyzed as described previously (11). Briefly, peritoneal macrophages (2 × 106 cells) growing on round, 12-mm, collagen type I-coated cover glasses (Asahi Techno Glass, Funabashi, Japan), were cultured with heat-killed lactobacilli (10 μg/ml) in a 24-well culture plate in 1.2 ml RPMI 1640 for 24 h. The macrophages were then fixed with methanol and stained with Giemsa. Lysis of the lactobacilli by macrophages was observed by light microscopy.

**Cytokine production by macrophages**

Peritoneal macrophages (1 × 106 cells) were plated in triplicate in a 96-well culture plate and cultured with heat-killed lactobacilli (1–30 μg/ml) or bacterial (yoto) components (1–10 μg/ml) in 200 μl RPMI 1640 for 24 h, unless indicated otherwise. In some experiments, the cells were pretreated with U0126 (2.5 μM), SB203580 (5 μM), or SP600125 (10 μM) for 30 min before stimulation with lactobacilli or bacterial components. The production of IL-10 and IL-12 was determined by ELISA using each cytokine-specific capture antibody and biotin-labeled detection antibody (Genzyme Diagnostic, Cambridge, MA) and biotin-streptavidin peroxidase conjugate (Amersham, Arlington Heights, IL). The absorbance was measured at 450 nm with a microtiter plate reader (Molecular Devices, Sunnyvale, CA). Data are expressed as mean ± SE of triplicate determinations. Statistical analysis was performed by unpaired Student’s t test, and p < 0.05 was considered as significant.
supernatants were measured by ELISA.

Expression of cytokine mRNA in macrophages

Peritoneal macrophages (1.2 × 10⁶ cells) were cultured in a 12-well culture plate with heat-killed lactobacilli (10 µg/ml) in 2.4 ml RPMI 1640 for various time periods (2–24 h). In some experiments, U0126 (2.5 µM) was added to the cultures 30 min before the stimulation with L. plantarum, or a rat anti-mouse IL-10 mAb (clone JES5-2A5, 10 µg/ml; BD Pharmingen, San Diego, CA) was added at the beginning of the stimulation. Total RNA was extracted from the cells using the RNeasy Mini Kit (Ambion, Austin, TX), and reverse transcription was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays for IL-10, IL-12 p35, and IL-12p40 expression (Applied Biosystems) in an ABI 7500 Real-Time PCR System (Applied Biosystems). The GAPDH gene was used as an endogenous control to normalize the expression of cytokine genes.

Cytokine determination by ELISA

Cytokine concentrations in culture supernatants were assayed by sandwich ELISA. IL-10 levels were determined using a rat anti-mouse IL-10 mAb (clone JES5-5X1C1) as the capture Ab and a biotinylated rat anti-mouse IL-10 mAb (clone JES5-2A5) as the detection Ab. To determine IL-12p70 levels, rat anti-mouse IL-12p35 (clone 9A5) and biotinylated rat anti-mouse IL-12p40 (clone C17.8) mAbs were used. All of these Abs and recombinant cytokines were purchased from BD Pharmingen.

Western blot analysis

Peritoneal macrophages (1 × 10⁶) were stimulated with heat-killed lactobacilli (10 µg/ml) or bacterial components (3 µg/ml) in 2 ml RPMI 1640 in a 24-well culture plate for various time periods (0.17–24 h). The cells were lysed with 1% NP-40 in 25 mM Tris-HCl buffer (pH 7.5) supplemented with 150 mM NaCl, 10% glycerol, 1% protease inhibitor mixture (Sigma-Aldrich), 1 mM NaF, and 0.005% protease inhibitor mixture, the membranes were blocked with anti-phosphorylated ERK, anti-phosphorylated p38, anti-phosphorylated JNK, and anti-ERK polyclonal Abs (Cell Signaling Technology, Beverly, MA) overnight at 4 °C. The membranes were incubated with a secondary HRP-conjugated anti-rabbit IgG Ab (Cell Signaling Technology) for 1 h. Proteins were visualized using ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, U.K.) according to the manufacturer’s instructions.

Results

Characteristic patterns of macrophage IL-10 and IL-12 production induced by Lactobacillus strains

To analyze the effect of Lactobacillus strains on macrophage cytokine production, mouse peritoneal macrophages were cultured with various concentrations of L. plantarum, L. casei, or L. johnsonii for 24 h, and IL-10 and IL-12p70 concentrations in the supernatants were measured by ELISA. L. plantarum induced high levels of IL-10 in a dose-dependent manner but relatively low levels of IL-12, which had an optimal dose of 3 µg/ml (Fig. 1A). In contrast, L. casei induced high levels of IL-12 and lower levels of IL-10 in a dose-dependent manner, whereas L. johnsonii induced very low levels of both IL-10 and IL-12. We examined the kinetics of cytokine production. When macrophages were stimulated with 10 µg/ml lactobacilli, L. plantarum induced earlier production of IL-10 and IL-12 than L. casei (Fig. 1B). The level of IL-12 induced by L. plantarum increased until 12 h and continued to be of a similar degree until 24 h, whereas that induced by L. casei continued to increase until the end of the culture.

The effect of these strains on macrophage expression of cytokine mRNAs was also examined. L. plantarum potently, and L. casei moderately, induced IL-10 mRNA expression, and that induced by L. plantarum increased earlier than that induced by L. casei (Fig. 2). L. plantarum also induced potent and rapid expression of IL-12p35 and IL-12p40 mRNA, whereas IL-12p35 mRNA expression was only moderately induced by L. casei and L. johnsonii. IL-12p40 mRNA expression was only weakly induced by L. johnsonii. In contrast, L. casei induced potent expression of IL-12p40; however, this expression increased at a later time than that induced by L. plantarum.

Phagocytosis and lysis of lactobacilli by macrophages

To determine the phagocytosis of lactobacilli by macrophages, peritoneal macrophages were cultured with FITC-labeled L. plantarum, L. casei, or L. johnsonii, and bacterial uptake was analyzed by flow cytometry. The time course of phagocytosis varied depending on the Lactobacillus strain (Fig. 3A). Over 50% of macrophages had phagocytosed L. plantarum within 2 h, and ~50% of macrophages had phagocytosed L. johnsonii by 4 h. However, phagocytosis of L. casei by 50% of the macrophages required a period of 16 h.

To analyze the macrophage intracellular digestion of lactobacilli, macrophages were cultured with lactobacilli for 24 h, stained with Giemsa, and then observed under a light microscope. Phagocytosed L. casei cells maintained their cell morphology during the culture period, whereas L. johnsonii cells lost the morphology and only their digested fragments were observed (Fig. 3B). L. plantarum showed an intermediate pattern of digestion between L. casei and L. johnsonii. Some of the L. plantarum cells that were phagocytosed by macrophages retained their cell morphology, whereas others appeared to be digested.
Intracellular lysis of bacteria was observed by light microscopy. Similarly, results were obtained when FITC-positive cells were analyzed by flow cytometry. The percentage of lactobacilli and bacterial uptake was analyzed by flow cytometry. The percentage of L. plantarum Peritoneal macrophages were cultured with FITC-labeled lactobacilli, and the expression of IL-10, IL-12p35, and IL-12p40 mRNAs in the cells was analyzed by quantitative RT-PCR. The relative expression of cytokine mRNA was normalized to GAPDH. Data are means ± SD of three independent experiments.

**Activation of MAPK pathways by lactobacilli**

Macrophages were stimulated with L. plantarum, L. casei, or L. johnsonii, and the activation profiles of ERK, p38, and JNK were compared. L. plantarum induced intense phosphorylation of ERK and JNK, whereas L. casei and L. johnsonii induced weaker phosphorylation of ERK and JNK (Fig. 4). All three lactobacilli induced comparable levels of p38 phosphorylation.

ERK activation induced by L. plantarum peaked 1 h after stimulation, whereas that induced by L. johnsonii peaked between 4 and 8 h after stimulation. The latest induced peak of ERK activation was that induced by L. casei, which peaked after 8 h of culture, following which intense phosphorylation was maintained over 24 h. The time course of p38 and JNK activation induced by the lactobacilli showed a similar tendency to that of ERK activation. The kinetics of MAPK phosphorylation induced by each Lactobacillus strain appeared to be related to the time course of their phagocytosis by macrophages.

**Effects of MAPK inhibitors on cytokine production**

To investigate the functional role of ERK, p38, and JNK pathways in cytokine production, macrophages were pretreated with a selective inhibitor of the ERK (U0126), p38 (SB203580), or JNK (SP600125) pathway, prior to induction of cytokine production by stimulation with L. plantarum, L. casei, or L. johnsonii. As shown in Fig. 5A, IL-10 secretion induced by all three strains dramatically declined in the presence of inhibitors of the ERK, p38, and JNK pathways, indicating that all three pathways are important for IL-10 production.

In contrast, the potent IL-12 production induced by L. casei was markedly suppressed in the presence of SB203580 and SP600125, but only slightly suppressed by U0126. Intriguingly, the weak production of IL-12 that was induced by L. plantarum, but not that induced by L. johnsonii, was dramatically increased by the addition of U0126. Next, we examined the effects of U0126 on the expression of IL-12 mRNAs induced by L. plantarum. Inhibition of the ERK pathway both enhanced the peak of IL-12p40 mRNA expression at 8 h and diminished the decrease of its expression at 16 h, whereas neutralization of IL-10 with a specific Ab only reduced the decrease at 16 h, suggesting that activation of the ERK pathway inhibited IL-12p40 mRNA expression through IL-10-dependent and IL-10-independent mechanisms (Fig. 5B). The data obtained using selective inhibitors of MAPK pathways suggest that activation of both p38 and JNK pathways is required for both IL-10 and IL-12 production, whereas the ERK pathway is critical for determination of the balance of IL-10/IL-12 production.

**L. plantarum synergizes with L. casei to enhance IL-10 production**

Our data showed that activation of the ERK pathway by lactobacilli may favor IL-10 production and that L. plantarum is a potent activator of this pathway. Therefore, we hypothesized that the potent ERK activation induced by L. plantarum may modulate the balance of macrophage IL-10/IL-12 production induced by other lactobacilli when macrophages are stimulated with a combination of L. plantarum and these other lactobacilli. To test this possibility, macrophages were cultured with L. casei or L. johnsonii in the presence of L. plantarum, and cytokine production was analyzed. L. plantarum synergized with L. casei to enhance IL-10 production and simultaneously inhibited the IL-12 production that can be potently induced by L. casei in the absence of L. plantarum (Fig. 6). Addition of a neutralization Ab against IL-10 to the cultures diminished the inhibitory effect of L. plantarum on secretion of IL-12 induced by L. casei, suggesting that the increased production of IL-10 induced by L. plantarum played a role in the inhibition of IL-12 secretion (Supplemental Fig. 3). The synergistic induction of IL-10 production was more clearly observed when low doses (1 and 3 μg/ml) of L. plantarum were added. Individual treatment with these low doses of L. plantarum was not sufficient to induce effective IL-10 production. In contrast, L. plantarum did not synergize with L. johnsonii or with latex beads for IL-10 production. This result suggests that L. plantarum induces potent activation of ERK, which may synergize with certain stimuli derived specifically from L. casei to enhance IL-10 production.
Bacterial components that include teichoic acid induce ERK activation and promote synergistic induction of IL-10 production with L. casei

To identify the active components responsible for the synergistic induction of IL-10 production, ICW, TAPG/PSPG, PGN, and protoplast were isolated from L. plantarum and L. casei, and their effect on macrophage IL-10 production was assayed following culture of the macrophages in the presence or absence of intact L. casei cells. Only treatment with ICW from L. plantarum could induce IL-10 production in the absence of L. casei cells. However, ICW, TAPG, and protoplast, but not PGN, prepared from L. plantarum could synergize with L. casei cells in the induction of IL-10 production. ICW and its soluble form, TAPG, from L. plantarum contain cell wall teichoic acid (WTA), and protoplast from both L. plantarum and L. casei contains LTA. However, no teichoic acid is present in the other cell components that we tested. These findings suggest that teichoic acids play a critical role in the synergism with L. casei for the induction of IL-10 production, although teichoic acids alone only weakly induce IL-10 production.

Because the ERK pathway plays a significant role in IL-10 production as shown in Fig. 5A, we therefore examined the effect of the isolated cell components on ERK activation. As expected, ICW, TAPG, and protoplast from L. plantarum and protoplast from L. casei, all of which contain teichoic acid, induced strong phosphorylation of ERK (Fig. 7B). Unexpectedly, PGN from either L. plantarum or L. casei, which contains no teichoic acid, also induced phosphorylation of ERK, suggesting that ERK activation alone may not be sufficient to induce the synergistic production of IL-10.

**ERK plays a critical role in synergistic production of IL-10 induced by teichoic acid**

The results shown in Fig. 7 suggested that WTA extending from PGN of L. plantarum, and LTA extending from the plasma membrane of L. plantarum and L. casei, are responsible for the synergistic production of IL-10. Thus, we tested the ability of LTA, purified from L. casei, and TAPG from L. plantarum, which is composed of WTA and a small residue of PGN, to synergize with L. casei cells in the induction of IL-10 production. The ability of LTA purified from nonlactobacillus bacterium S. aureus was also tested in this assay. All of these teichoic acids synergized with L. casei for enhancement of the production of IL-10 and for simultaneous decrease in IL-12 production, although, when treated alone, they induced very little IL-10 production (Fig. 8). Addition of the selective inhibitor of the ERK pathway abrogated the synergistic effect of these teichoic acids, indicating that teichoic acid-induced ERK activation plays a critical role in the synergism with L. casei for the production of IL-10.

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production was observed when *L. rhamnosus*, but not *L. johnsonii*, was substituted for *L. casei* (data not shown). We have previously shown that *L. rhamnosus* is similar to *L. casei* but different from *L. johnsonii* in that it is resistant to macrophage intracellular digestion and can effectively induce IL-12 production (11).

**TLR2 is involved in the synergistic production of IL-10 induced by teichoic acids and *L. casei***

It has been shown that LTA is recognized by TLR2 (28). To verify the involvement of TLR2 in the synergistic production of IL-10 induced by teichoic acids in the presence of *L. casei*, macrophages prepared from TLR2-deficient or wild-type mice were cultured with combinations of teichoic acids and *L. casei*, and the concentrations of cytokines in the supernatants were measured. TAPG and LTA failed to stimulate TLR2-deficient macrophages to affect IL-10 and IL-12 production (Fig. 9A). Furthermore, neither TAPG nor LTA induced potent phosphorylation of ERK in TLR2-deficient macrophages, although they could do so in wild-type macrophages (Fig. 9B). In TL4-deficient macrophages, these teichoic acids could induce potent ERK activation to lead synergistic induction of IL-10 production with *L. casei* (data not shown). Taken together, these data indicated that teichoic acids were recognized by TLR2, activated the ERK pathway, and then synergized with *L. casei* to induce IL-10 production (Fig. 10).

**Discussion**

We examined the relationship between MAPK activation and regulation of IL-10 and IL-12 production in mouse peritoneal macrophages in response to lactobacilli. *L. plantarum*, which potently induces the production of IL-10, strongly activated ERK, whereas *L. casei*, which potently induces the production of IL-12, showed a weak ability to activate ERK. WTA, which is a characteristic cell wall component of *L. plantarum*, was responsible for the strong activation of ERK induced by *L. plantarum*. Furthermore, specific inhibition of the ERK pathway by the addition of U0126 converted *L. plantarum* into a potent inducer of IL-12 production, whereas ERK activation by the addition of WTA converted *L. casei* into a potent inducer of IL-10 production. These data suggest that activation of the ERK pathway is crucial for determination of the balance of IL-10/IL-12 production induced by lactobacilli.

In contrast, neither stimulation of the ERK pathway in macrophages by WTA alone nor stimulation of macrophages with a combination of WTA and *L. johnsonii*, which weakly induces IL-12 production, could induce effective production of IL-10.

**FIGURE 7.** Effect of bacterial components on IL-10 production and ERK activation. A, Peritoneal macrophages were cultured with ICW, PSPG/TAPG, protoplast, or PGN (3 ng/ml) prepared from *L. plantarum* (p) or *L. casei* (c) in the presence (+Lc) or absence of *L. casei* cells (10 ng/ml) for 24 h. The levels of IL-10 in supernatants were determined by ELISA. Data are means ± SD of triplicate cultures. Experiments were repeated twice with similar results. The dash represents medium only or *L. casei* for determination of the balance of IL-10/IL-12 production in wild-type mice.

**FIGURE 8.** Importance of ERK activation in teichoic acid-mediated regulation of cytokine production. Peritoneal macrophages were pretreated (+ERKi) or not treated (−) with U0126 (2.5 μM) for 30 min and cultured with *L. plantarum* TAPG (3 μg/ml), *L. casei* LTA (LTAc), or *S. aureus* LTA (LTAs) in the presence (+Lc) or absence of *L. casei* (10 ng/ml) for 24 h. The levels of IL-10 and IL-12p70 in supernatants were determined by ELISA. Data are means ± SD of triplicate cultures. Experiments were repeated twice with similar results.

**FIGURE 9.** Requirement of TLR2 recognition of teichoic acids for both synergistic production of IL-10 and ERK activation. A, Peritoneal macrophages from wild-type (WT) or TLR2-deficient (TLR2-KO) mice were cultured with *L. plantarum* TAPG or *L. casei* LTA (3 μg/ml) in the presence (+Lc) or absence of *L. casei* (10 μg/ml) for 24 h. The levels of IL-10 and IL-12p70 in supernatants were determined by ELISA. Data are means ± SD of triplicate cultures. Experiments were repeated twice with similar results. B, Peritoneal macrophages from wild-type or TLR2-deficient mice were cultured with the bacterial components for 0.5, 1, and 2 h. Whole-cell lysates were prepared and analyzed by immunoblotting using specific Abs to phosphorylated (p) and total ERK. Experiments were repeated twice with similar results. M represents unstimulated macrophages.

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Moreover, addition of the ERK inhibitor could not result in the induction of IL-12 production by L. johnsonii. Taken together, all of these data suggest that production of IL-10 and IL-12 by macrophages in response to lactobacilli requires the activation of common signal transduction pathways and that additional potent activation of ERK leads to IL-10 production but that insufficient ERK activation leads to IL-12 production (Fig. 10). L. johnsonii does not appear to be able to activate the common signaling pathways required for IL-10 and IL-12 production, whereas L. plantarum and L. casei appear to activate such signaling pathways. The difference between L. johnsonii and the other two strains in their ability to activate the common signaling pathways may be related to their difference in sensitivity to macrophage intracellular digestion. Thus, we showed in Fig. 3B that L. johnsonii is susceptible to intracellular digestion, L. casei is resistant to digestion and retains its cell morphology following ingestion, and a subpopulation of the L. plantarum cells phagocytosed by macrophages also maintained their cell morphology. We have previously shown that the Lactobacillus cell wall structure that is resistant to intracellular digestion is responsible for effective induction of IL-12 production by macrophages (11). We therefore speculate that cell wall structure-derived stimuli could lead to activation of the signaling pathways common to IL-10 and IL-12 production (Fig. 10). Further studies will be necessary to clarify this issue.

Our data indicated that recognition of WTA and LTA by TLR2 leads to ERK activation, which is the key to regulation of the balance of IL-10/IL-12 production by macrophages in response to lactobacilli. Several reports have already revealed the importance of the ERK pathway in the regulation of IL-10 and IL-12 production. Dillon et al. (18) clearly showed that Pam3Cys, which is a ligand for TLR2, strongly induces ERK phosphorylation in mouse dendritic cells and effectively induces IL-10 production. That group also observed that selective blockade of the ERK pathway results in a decrease in IL-10 production and a simultaneous increase in IL-12 production in response to Pam3Cys. Similar results were obtained in studies using LPS and CpG DNA, which are ligands for TLR4 and TLR9, respectively (17, 19). Although these previous findings correspond well with our results, an interesting difference between those findings and ours is that Pam3Cys, LPS, and CpG DNA alone were able to induce IL-10 production, whereas WTA and LTA alone could not. These data suggest that ERK activation is necessary, but not sufficient, for IL-10 production and that the first three TLR ligands, but not the latter two, can additionally activate other signaling pathways required for IL-10 production and also probably for IL-12 production. When these teichoic acids stimulate macrophages in the presence of L. casei, teichoic acid-induced ERK activation could lead to IL-10 production in collaboration with stimuli derived from L. casei that induce IL-12 production (Fig. 10). Such signals derived from L. casei have been suggested to be independent of recognition by TLR2 (11), in contrast to the TLR2-dependent signals induced by teichoic acids. Collaboration between TLR2-derived signals and other receptor-derived signals in IL-10 production has also been observed in the response of dendritic cells to yeast zymosan (29). In that case, dectin-1, a C-type lectin receptor for β-glucans, was shown to act as the second receptor. It is of great interest that TLR2-dependent and TLR2-independent signals cooperatively regulate IL-10 production.

Although our data suggest that it is likely that ERK activation together with stimuli derived from L. casei lead to IL-10 production, one apparent contradiction of this hypothesis is that PGN, which can induce ERK phosphorylation, did not induce IL-10 production even in the presence of L. casei (Fig. 7). One possible explanation for this contradiction is the following: PGN might promote inhibitory mechanisms in IL-10 production, in addition to activation of the ERK pathway. Watanabe et al. (30) have revealed that muramyl dipeptide (MDP), which is a by-product of PGN digestion, inhibits IL-12 production by macrophages through its recognition by nucleotide-binding oligomerization domain 2. The same group also showed that MDP-derived stimuli inhibited IL-10 production by dendritic cells (31). We recently reported that PGN isolated from lactobacilli including L. plantarum and L. casei inhibits IL-12 production by macrophages through TLR2-dependent and TLR2-independent mechanisms (32). Furthermore, we found here that PGN and 6-O-stearoyl-MDP, which is a derivative of MDP and is more effectively internalized into cells, inhibited the production of IL-10 as well as IL-12 by macrophages stimulated with a combination of L. casei and L. plantarum (Supplemental Fig. 4). Such inhibitory stimuli might occur in macrophages that have been cultured with a combination of PGN and L. casei. Alternatively, ERK activation might be no more than one of the essential factors to convert IL-12 production to IL-10 production, and PGN, but not WTA and LTA, may lack the ability to turn on the essential factors other than ERK activation.

A second apparent contradiction contained within our data are that, although L. casei contains LTA as a cellular component, this bacterium could not lead to either potent activation of ERK or effective production of IL-10. In contrast, LTA purified from L. casei potently activated ERK and induced IL-10 production in the presence of L. casei cells. The following explanation is proposed to explain this contradiction. L. casei expresses uncharged polysaccharides that are densely associated with PGN (33), which may sterically hinder the access of TLR2 to LTA extending from the plasma membrane through PGN (Supplemental Fig. 2). This assumption is supported by the result that the protoplast that was prepared by removal of PGN and associated polysaccharides from L. casei could activate ERK. However, L. plantarum has PGN-associated WTA on its cell surface (34), which is likely to be easily recognized by TLR2 (Supplemental Fig. 1). This may be the reason for the difference in the abilities of L. casei and L. plantarum to induce IL-10 production.

There have been some reports showing that certain strains of lactic acid bacteria modulate cytokine production induced by other strains. Christensen et al. (35) reported that strong IL-12 production...
induced by L. casei in mouse dendritic cells was inhibited by L. reuteri, which is a weak inducer of IL-12. Similarly, it has been shown that IL-12 production by human dendritic cells or PBMCs that was potently induced by L. acidophilus was inhibited by L. reuteri, L. delbrueckii ssp. bulgaricus, and Bifidobacterium bifidum, all of which weakly induce IL-12 production (36, 37). All of these reports indicate that lactic acid bacteria that induce a weak IL-12 response inhibit IL-12 production induced potently by other strains but that IL-10 production induced by other strains is not affected.

However, the current study shows that L. plantarum, which induces weak production of IL-12 but potent production of IL-10, leads to potent IL-10 inducers in the presence of certain lactobacilli or bacterial components. This observation may provide a theoretical basis for understanding the multifunctional activities of certain probiotics.

The human intestine hosts some hundreds of bacterial species and >100 trillion commensal bacteria, and some of these bacteria may potentially elicit proinflammatory responses. Under normal conditions, intestinal macrophages and dendritic cells are regulated to convert a predominant IL-10 response to specific bacteria into proinflammatory responses. Under normal conditions, intestinal macrophages and dendritic cells are regulated to convert a predominant IL-10 response to specific bacteria into proinflammatory responses. Under normal conditions, intestinal macrophages and dendritic cells are regulated to convert a predominant IL-10 response to specific bacteria into proinflammatory responses. Under normal conditions, intestinal macrophages and dendritic cells are regulated to convert a predominant IL-10 response to specific bacteria into proinflammatory responses. Under normal conditions, intestinal macrophages and dendritic cells are regulated to convert a predominant IL-10 response to specific bacteria into proinflammatory responses.


