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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 extensively, 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 benzonase nuclease were purchased from Seikagaku Kogyo (Tokyo, Japan) and Merck (Darmstadt, Germany), respectively. DNase, RNase, trypsin, and pronase 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).

**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 Shirota (YIT 9029), was originally isolated and maintained at the Yakult Central Institute. L. johnsonii JCM 1292T and L. plantarum ATCC 14917T 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 mg/ml in 50 mM carbonate buffer (pH 9.6), reacted with FITC isomer I (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–chloform–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 for 4°C for 20 h (27). The material was washed with distilled water and then used as the PGN.

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 Macro-Prep High Q (Bio-Rad, Hercules, CA) and Octyl-Sepharose CL-4B (Pharmacia Bio-chem, 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 × 10^6 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 × 10^6 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 × 10^6 cells) were plated in triplicate in a 96-well culture plate and cultured with heat-killed lactobacilli (1–30 μg/ml) or bacterial supernatants (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
supernatants were measured by ELISA. L. plantarum low levels of IL-12, which had an optimal dose of 3 μg/ml; BD Pharmingen, San Diego, CA) was added at the beginning of the stimulation. Total RNA was extracted from the cells using the RNAsefree 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-SXC1) as the capture Ab and a biotinylated rat anti-mouse IL-10 Ab (clone JES5-2A5) as the detection Ab. To determine IL-12p70 levels, rat anti-mouse IL-12p75 (clone 9A5) and biotinylated rat anti-mouse IL-12p70/23 (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 cells) were cultured 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 Na3VO4, and 50 mM NaF. The cell lysates were treated with SDS sample buffer containing 4.5% SDS, 125 mM Tris, 20% glycerine, 0.02% bromphenol blue, and 10% 2-ME, boiled for 5 min, separated by SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane (Pall, East Hills, NY). After being blocked for 1 h with 4% BSA in 20 mM Tris-HCl buffer (pH 7.5) supplemented with 0.1% NaN3, 50 mM NaF, and 0.005% protease inhibitor mixture, the membranes were blotted 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.

**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. (10 FITC-positive cells is shown. Experiments were repeated twice with h, and bacterial uptake was analyzed by flow cytometry. The percentage of (Lp, 10 L. plantarum Peritoneal macrophages were cultured with FITC-labeled dependent experiments. mRNA was normalized to GAPDH. Data are means ± SD of three independent experiments.

**FIGURE 2.** Expression of cytokine mRNA in macrophages stimulated with lactobacilli. Peritoneal macrophages were cultured with L. plantarum (Lp, 10 μg/ml), L. casei (Lc), or L. johnsonii (Lj) for 2, 4, 8, 16, and 24 h, 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.

**FIGURE 3.** Phagocytosis and lysis of lactobacilli by macrophages. A, Peritoneal macrophages were cultured with FITC-labeled L. plantarum (Lp, 10 μg/ml), L. casei (Lc), or L. johnsonii (Lj) for 1, 2, 4, 8, 16, and 24 h, and bacterial uptake was analyzed by flow cytometry. The percentage of FITC-positive cells is shown. Experiments were repeated twice with similar results. B, Peritoneal macrophages were cultured with L. plantarum (10 μg/ml), L. casei, or L. johnsonii for 24 h and stained with Giemsa. Intracellular lysis of bacteria was observed by light microscopy.
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 to enhance IL-10 production (Fig. 7A). Interestingly, protoplast from L. casei could also 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 tectioic 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 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. The effect of teichoic acids on IL-10 production was also tested with latex beads (La) in the presence or absence of L. plantarum (Lp, 1–10 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 four times with similar results. A. Peritoneal macrophages were pretreated with U0126 (ERK inhibitor, 2.5 μM), SB203580 (p38 inhibitor, 5 μM), or SP600125 (JNK inhibitor, 10 μM) for 30 min and then stimulated with L. plantarum (Lp, 10 μg/ml), L. casei (Lc), or L. johnsonii (Lj) 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 four times with similar results.
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 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.

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 TLR4-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.
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 potentially 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

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**FIGURE 10.** Summary of possible mechanisms of regulation of IL-10 and IL-12 production by lactobacilli. i. L. casei (Lc) is phagocytosed by macrophages and stimulates them through the cell wall structure to produce IL-12. ii. L. plantarum (Lp) potently activates the ERK pathway via recognition of cell surface WTA by TLR2, in addition to the signals derived from the cell wall structure that would lead to IL-12 production, thereby inducing potent IL-10 production and a simultaneous decrease of IL-12 production. iii. When L. casei stimulates macrophages in the presence of WTA or LTA, the signals derived from L. casei cell wall structure and the potent ERK activation induced through TLR2 cooperatively induce potent production of IL-10.
induced by \textit{L. casei} in mouse dendritic cells was inhibited by \textit{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 \textit{L. acidophilus} was inhibited by \textit{L. reuteri}, \textit{L. delbrueckii} spp. \textit{bulgaricus}, and \textit{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 \textit{L. plantarum}, which induces weak production of IL-12 but potent production of IL-10, leads to synergistic induction of IL-10 with \textit{L. casei} and simultaneous inhibition of IL-12 production. The difference between our results and those of the previous studies on the effect on IL-10 production may be due to differences in the bacterial strains or immune cells used in each study. Our findings that certain lactobacilli or bacterial components can convert IL-12-inducing lactobacilli into potent IL-10 inducers may be of great interest in the application of probiotics for the regulation of the host immune response.

The strain of \textit{L. casei} used in this study is a probiotic strain, \textit{L. casei} Shirota, for which several health-promoting functions have been reported in human clinical trials and experimental animal models. Oral administration of \textit{L. casei} Shirota to humans restored NK cell activity (38), reduced the risk of cancers (39), and decreased allergen-specific plasma IgE levels (40). Additionally, animal studies have shown that this strain prevented infections (41) and improved autoimmune diseases (42–44) and inflammatory bowel diseases (45). Although upregulation of IL-12 production and downregulation of proinflammatory cytokines such as IL-6 have been implicated in these functions (38, 41, 45), the mechanisms by which this probiotic bacterium exerts such multifunctional activities are largely unknown. Multifunctional activities have also been observed for other probiotic strains such as \textit{L. rhamnosus} GG (46). It is generally accepted that IL-12-inducing probiotics might be useful for augmentation of the host immune system and that IL-10-inducing probiotics might be useful for downregulation of inflammation. Indeed, Foligne et al. (47) reported that strains that induce higher levels of IL-10 and lower levels of IL-12 in human PBMC cultures offer the best protection in a mouse colitis model. However, it is difficult to explain the mechanisms whereby a single probiotic strain can both augment natural immune defenses and control inflammatory responses. Our findings show that potent IL-12-inducing strains can convert into potent IL-10 inducers in the presence of certain lactobacilli or bacterial components. This observation could 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 secrete low levels of proinflammatory cytokines, such as IL-12, in response to such bacteria. Various mechanisms, including constitutive expression of inhibitory transcription factors in the mucosal phagocytes and a supply of suppressive mediators by intestinal epithelial cells, have been proposed to explain the mechanism by which proinflammatory cytokine responses are controlled (48, 49). In the current study, we clearly show that LTA and WTA can convert a predominant IL-12 response to specific bacteria into a predominant IL-10 response. Because these teichoic acids are widely distributed in Gram-positive bacteria, this teichoic acid-mediated potential anti-inflammatory mechanism could be important for the maintenance of intestinal homeostasis. This speculation should be assessed in in vivo models in future studies.

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Disclosures

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