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Immunobiotic *Lactobacillus jensenii* Elicits Anti-Inflammatory Activity in Porcine Intestinal Epithelial Cells by Modulating Negative Regulators of the Toll-Like Receptor Signaling Pathway

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The effect of *Lactobacillus jensenii* TL2937 on the inflammatory immune response triggered by enterotoxigenic *Escherichia coli* (ETEC) and lipopolysaccharide (LPS) in a porcine intestinal epitheliocyte cell line (PIE cells) was evaluated. Challenges with ETEC or LPS elicited Toll-like receptor 4 (TLR4)-mediated inflammatory responses in cultured PIE cells, indicating that our cell line may be useful for studying inflammation in the guts of weaning piglets. In addition, we demonstrated that *L. jensenii* TL2937 attenuated the expression of proinflammatory cytokines and chemokines caused by ETEC or LPS challenge by downregulating TLR4-dependent nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) activation. Furthermore, we demonstrated that *L. jensenii* TL2937 stimulation of PIE cells upregulated three negative regulators of TLRs: A20, Bcl-3, and MKP-1, deepening the understanding of an immunobiotic mechanism of action. *L. jensenii* TL2937-mediated induction of negative regulators of TLRs would have a substantial physiological impact on homeostasis in PIE cells, because excessive TLR inflammatory signaling would be downregulated. These results indicated that PIE cells can be used to study the mechanisms involved in the protective activity of immunobiotics against intestinal inflammatory damage and may provide useful information for the development of new immunologically functional feeds that help to prevent inflammatory intestinal disorders, including weaning-associated intestinal inflammation.

Intestinal epithelial cells (IECs) serve a critical function in the immune responses triggered by antigens in the gastrointestinal tract (45). Under steady-state conditions, IECs create a tolerogenic environment; however, in the presence of pathogenic microorganisms, they secrete cytokines that are crucial for the recruitment and activation of inflammatory cells. These two functions of IECs—to distinguish between the diverse elements of the intestinal flora and to respond to invading pathogens—are principally determined by pattern recognition receptors (PRRs) (45). Toll-like receptors (TLRs) are an important class of PRRs in innate immunity, and TLRs play a critical role in pathogen recognition and host defense. However, inappropriate TLR signaling can contribute to loss of tolerance and result in tissue injury (1, 13); the best example of such injury is the intestinal damage mediated by the inflammatory response triggered by the interaction between lipopolysaccharide (LPS) and TLR4. LPS present in the outer membranes of some Gram-negative pathogens such as enterotoxigenic *Escherichia coli* (ETEC) triggers the production of proinflammatory mediators that may contribute to intestinal inflammation and damage during the infection (21, 24). Thus, while TLR4 recognition of LPS is required for clearance of Gram-negative organisms, it is believed that excessive and/or prolonged proinflammatory cytokine secretion can be harmful to the host (1, 13).

Additionally, dysregulated immune responses to bacterium-derived molecules in the healthy intestine can result in excessive mucosal inflammation even in the absence of infection, especially during weaning. In piglets for example, the numbers of intestinal $CD4^+$ and $CD8^+$ cells, plasma concentrations of fibrinogen (an acute-phase protein mediating inflammation), and blood plasma interleukin 1 (IL-1) levels increase during weaning (22, 23). Moreover, weaning is associated with upregulation of IL-1, IL-6, and tumor necrosis factor alpha (TNF-α) in the intestine, and this early inflammatory response may contribute to both anatomical and functional intestinal disorders in piglets (28). Previously, we studied the expression of TLRs in porcine intestinal epithelial cells derived from neonatal pigs and found that, of the TLRs, TLR4 mRNA was preferentially expressed (24). Therefore, TLR4 expressed on IECs may have a role in the inflammatory response associated with weaning.

Lactic acid bacteria (LAB) are demonstrably beneficial in the treatment of a variety of mucosal disorders, including inflammatory diseases (2, 3, 9, 11). Studies that evaluated the effects of probiotic bacterial strains on immune responses showed that probiotic-mediated protection against pathogen-induced injury

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TABLE 1 Primer sequences used in this study

Primer	Sense primer	Antisense primer
Gene cloning		
SIGIRR (including ORF)	AGCCTCTGACCTGACTTGGA	GGACTGTGGTCCTGCTGAGT
Tollip (including ORF)	GCCGTCAGCTGACTGTGG	CAGGAGGTGGGTTTTCAGTG
A20 (including ORF)	GACTTGGACTTGGGATTTCG	GGAAAACTTGAAGAAAGCCAAC
Bcl-3 (including ORF)	GAAACCACCCTCCCGTGC	CGGGTGCCTGAGAATAAGAG
MKP-1 (including ORF)	AACGAGGGTCAGGCTTTTCC	TCCCCAATGTGCTGAGTTCAG
IRAK-M (partial)	GCTGGATGTTCGTCACATTG	AATACACTGATGCGGACGTG
Real-time PCR		
Porcine β-actin	CATCACCATCGGCAACGA	GCGTAGAGGTCCTTCCTGATGT
Porcine IL-1α	AGAATCTCAGAAACCCGACTGTTT	TTCAGCAACACGGGTTCGT
Porcine IL-6	TGGATAAGCTGCAGTCACAG	ATTATCCGAATGGCCCTCAG
Porcine IL-8	GCTCTCTGTGAGGCTGCAGTT	TTTATGCACTGGCATCGAAGTT
Porcine MCP-1	ACAGAAGAGTCACCAGCAGCAA	GCCCGCGATGGTCTTG
Porcine SIGIRR	ATGTGAAGTGTCGGCTCAATGT	TTCATCTCCACCTCCCCATACT
Porcine Tollip	TACCGTGGGCCGTCTCA	CCGTAGTTCTTCGCCAACTTG
Porcine A20	CCTCCCTGGAAAGCCAGAA	GTGCCACAAGCTTCCTCACTT
Porcine Bcl-3	CGACGCGGTGGACATTAAG	ACCATGCTAAGGCTGTTGTTTTC
Porcine MKP-1	AACGAGGGTCAGGCTTTTCC	TCCCCAATGTGCTGAGTTCAG
Porcine IRAK-M	TGGAGCAGCCTTGAATCCTT	TGGATAACACGTTTGGGAATCTT

and inflammation results, in part, from modulation of the balance of pro- and anti-inflammatory cytokines in immune cells (2, 3, 19, 32). Additionally, in recent years the interactions between probiotics and IECs have garnered substantial attention (3, 31). Some evidence indicates that probiotic LAB can stimulate IEC responses, including restitution of damaged epithelial barriers, production of antibacterial substances, and blockade of cytokine-induced intestinal epithelial cell apoptosis (9). However, it is unclear how these beneficial effects are initiated. Thus, because of increasing evidence of the beneficial effects of probiotics, these investigations should be extended to include other potential probiotic strains, to better define the benefits of probiotics, and to clarify the mechanisms of action of probiotics, which are as yet largely unknown.

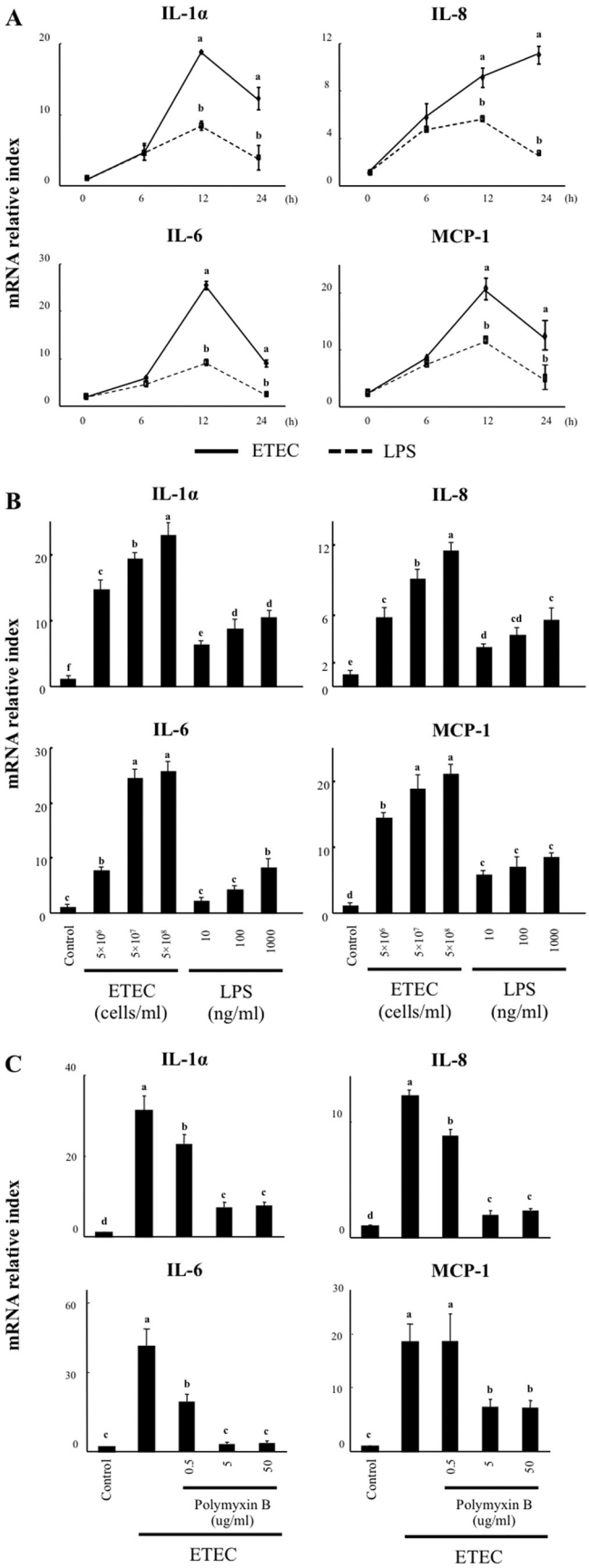
Controlling early intestinal inflammation is a major challenge in managing postweaning gut disorders in piglets. In order to study the mechanisms by which swine IECs induce an immune response to antigens and the potential immunoregulatory effect of immunobiotics, we previously established a clonal porcine intestinal epitheliocyte cell line (PIE cells) originally derived from intestinal epithelia isolated from an unsuckled neonatal swine (24). Studies of TLR1 to TLR9 and MD-2 mRNA expressions in PIE cells demonstrate that TLR4 is the most strongly expressed of these receptors. We confirmed that PIE cells, which preferentially express TLR4/MD-2, underwent inflammatory responses, specifically proinflammatory cytokine expression, in response to LPS or to ETEC, which is a major pathogen of neonatal swine (24). Thus, this PIE cell line is useful for *in vitro* study of the inflammatory responses mediated by TLR4/MD-2. Here, we characterized the inflammatory immune responses triggered by ETEC and by LPS in PIE cells by studying the expression of proinflammatory cytokines and chemokines and TLR4 signaling. In addition, we identified an immunobiotic LAB strain that mitigates the inflammatory response triggered by ETEC or LPS in PIE cells. Moreover, we clarified the mechanisms of immunobiotic action by demonstrating that the interaction of IECs with immunobiotics could influence the expression of negative regulators of TLRs in IECs.

MATERIALS AND METHODS

PIE cells. PIE cells, which are nontransformed intestinal cultured cells originally derived from intestinal epithelia isolated from an unsuckled neonatal swine (24), were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 100 mg/ml penicillin, and 100 U/ml streptomycin at 37°C in an atmosphere of 5% CO_2. PIE cells grow rapidly and are well adapted to culture conditions even without transformation or immortalization. However, the proliferative ability of PIE cells diminishes after 50 passages in culture. Therefore, we used PIE cells only between the 20th and 40th passages in these experiments.

Microorganisms. ETEC strain 987 (O9:H−:987 pilus+:heat-stable enterotoxin+) was kindly provided by M. Nakazawa at the National Institute of Animal Health (Tsukuba, Japan) (46). ETEC cells were grown in tryptic soy broth (TSB; Becton, Dickinson and Company, San Jose, CA) for 24 h at 37°C with shaking. After overnight incubation, the subcultures of bacteria were centrifuged at 5,000 × *g* for 10 min at 4°C, washed with phosphate-buffered saline (PBS), and heat killed (100°C, 30 min). All the lactobacillus strains (18 strains; see Table 2) were grown in MRS medium (Difco, Detroit, MI) for 16 h at 37°C, washed with PBS, and heat killed (56°C, 30 min). These bacterial samples were resuspended in DMEM, enumerated using a microscope and a Petroff-Hausser counting chamber, and stored at −80°C until use.

HEK^{pTLR2} immunoassay. Transfectants that stably express pTLR2 (GenBank accession no. AB072190) were isolated as previously described (38). Briefly, HEK293 cells were seeded (4×10^5 cells/well in 6-well plates) 24 h prior to transfection. The cells were transfected with the pcDNA3 vector (Invitrogen) encoding FLAG-tagged pTLR2 (1 mg/well) using Lipofectamine 2000 (Invitrogen). The pTLR2-expressing transfectants were positively selected with G418 (Invitrogen) and designated HEK^{pTLR2}. HEK293 cells receiving only the plasmid vector were designated HEK^{Cont}. The nuclear factor κB (NF-κB) reporter assay in HEK^{pTLR2} cells was performed as previously described (38). Cells (8×10^4/well in 24-well plates) were transfected with 0.2 mg of pGLM-ENH-luci vector, which included the human NF-κB gene A1 and A2 sites, using Lipofectamine reagent for 18 h. After 24 h of stimulation with LAB strains, luciferase assays were performed using transfected cells according to the manufacturer's protocol (Promega, Tokyo, Japan). The relative index (RI) was estimated from the resonance units (RU) using the following equation: RI = (RU in



HEK^{pTLR2} − RU in background)/(RU in HEK^{Cont} − RU in background). All assays were conducted in triplicate wells at least three times.

Anti-inflammatory assay in PIE cells. PIE cells were seeded at 1.5×10^4 cells/24-well plate on type I collagen-coated plates (Iwaki, Tokyo, Japan) and cultured for 3 days. After changing medium, lactobacilli (5×10^7 cells/ml) or Pam3CSK4 (200 ng/ml; EMC Microcollection, Tübingen, Germany) was added; 48 h later, each well was washed vigorously with medium at least three times to eliminate all stimulants, and then cells were stimulated with ETEC (5×10^7 cells/ml) or with LPS (1,000 ng/ml; from *Escherichia coli* O55:B5 prepared by phenol extraction followed by gel-filtration chromatography; Sigma, St. Louis, MO) for 6, 12, or 24 hours. To assess the contribution of ETEC-bound LPS to the inflammatory response observed in PIE cells, polymyxin B (Sigma) was used. Briefly, indicated amounts of polymyxin B were mixed with ETEC, and these mixtures were added to PIE cell cultures for 12 h. In experiments using p38 inhibitor (PD169316; Calbiochem, Darmstadt, Germany), PIE cells were pretreated with 10 nM PD169316 or an equal volume of dimethyl sulfoxide (DMSO) for 1 h before stimulation. PIE cells were then washed and stimulated with ETEC to evaluate the anti-inflammatory effects of p38 signaling.

Quantitative expression analysis by real-time PCR. We performed two-step real-time quantitative PCR to characterize the expression of mRNAs in PIE cells. Total RNA was isolated from each PIE cell sample using TRIzol reagent (Invitrogen). All cDNAs were synthesized using a Quantitect reverse transcription (RT) kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Real-time quantitative PCR was carried out using a 7300 real-time PCR system (Applied Biosystems, Warrington, United Kingdom) and the Platinum SYBR green qPCR SuperMix uracil-DNA glycosylase (UDG) with 6-carboxyl-X-rhodamine (ROX) (Invitrogen). The primers used in this study are listed in Table 1. The PCR cycling conditions were 2 min at 50°C, followed by 2 min at 95°C, and then 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The reaction mixtures contained 5 μl of sample cDNA and 15 μl of master mix, which included the sense and antisense primers. Expression of β-actin was used to normalize cDNA levels for differences in total cDNA levels in the samples. The relative index of a cytokine mRNA in PIE cells stimulated with lactobacilli was calculated as follows: first, the average cytokine expression levels from at least three samples stimulated with ETEC or LPS without pretreatment with lactobacilli were set to 100. Then, relative expression of lactobacillus-prestimulated samples following ETEC or LPS stimulation was calculated (10, 34).

Cloning of negative regulators. Cloning of cDNAs was carried out as described previously (37) with minor modifications. Briefly, total RNA was extracted from the spleen, ileal Peyer's patches, or the mesenteric lymph node using TRIzol reagent (Invitrogen). Polyadenylated mRNA was purified from total RNA with an Oligotex-dT30 mRNA purification kit (TaKaRa Bio Inc., Kyoto, Japan), according to the manufacturer's instructions. We designed specific primers (Table 1) for cloning negative

FIG 1 Expression of IL-1α, IL-6, IL-8, and MCP-1 mRNA in porcine intestinal epithelial (PIE) cells after inflammatory stimulation. (A) PIE cells were challenged with ETEC (5×10^7 cells/ml) (solid line) or LPS (1 μg/ml) (broken line), and the levels of IL-1α, IL-6, IL-8, and MCP-1 mRNAs were evaluated at various time points after the inflammatory challenge. Unchallenged PIE cells at the indicated time were used as controls, and statistical analyses were conducted at each indicated time point. (B) PIE cells were stimulated with ETEC or LPS at various concentrations as indicated, and the levels of IL-1α, IL-6, IL-8, and MCP-1 mRNAs were evaluated 12 h after the inflammatory challenge. Unchallenged, untreated PIE cells were used as controls. Values with different superscript letters are significantly different ($P < 0.05$). (C) PIE cells were stimulated with ETEC (5×10^7 cells/ml) pretreated with the LPS inhibitor polymyxin B at the indicated concentrations (in micrograms per milliliter). The levels of IL-1α, IL-6, IL-8, and MCP-1 mRNAs were evaluated 12 h after the inflammatory challenge. Unchallenged PIE cells were used as controls. The results represent data from three independent experiments. Values with different letters are significantly different ($P < 0.05$).

regulators (SIGIRR, Tollip, A20, Bcl-3, MKP-1, and IRAK-M) with deduced multiple alignments of the conserved sequences between human and mouse (SIGIRR, human NM-021805 and mouse NM-023059; Tollip, human NM-019009 and mouse NM-023764; A20, human NM-006290 and mouse NM-009397; Bcl3, human NM-005178 and mouse NM-033601; MKP-1, human NM-004417 and mouse NM-013642; IRAK-M, human NM-007199 and mouse NM-028679) using the software GENETYX-SV/RC version 13.0.6 (GENETYX Co., Tokyo, Japan) or by searching the pig expressed sequence tag (EST) database. The cDNA fragment containing the entire open reading frame (ORF) (SIGIRR, Tollip, A20, Bcl-3, MKP-1) and partial deduced coding sequence (IRAK-M) were subcloned into the vector pGEM-T easy DNA (Promega, Madison, WI) and sequenced using the ABI310 sequence analyzer (Applied Biosystems, Foster City, CA). GENETYX was used to analyze nucleotide and deduced amino acid sequences.

Enzyme-linked immunosorbent assay. The concentration of IL-6 and IL-8 secreted into the supernatant of PIE cell cultures, 48 h after ETEC stimulation, was determined using two commercially available enzyme-linked immunosorbent assay (ELISA) kits (porcine IL-6 ELISA kit [RayBio, Norcross, GA] and IL-8 immunoassay kit [Biosource, Camarillo, CA]), according to the manufacturers' instructions.

Western blotting. PIE cells cultured (1.8×10^5 cells/dish) in 60-mm dishes were simulated with *Lactobacillus jensenii* TL2937 or Pam3CSK4 in the same time schedule and using equivalent amounts of stimulants as those described in the "Anti-inflammatory assay in PIE cells" section of Materials and Methods. PIE cells were then washed and stimulated with ETEC or LPS for the indicated time period. PIE cells were then washed three times with PBS and resuspended in 200 μl of CelLytic M cell lysis reagent (Sigma) including protease and phosphates inhibitors (Complete Mini, PhosSTOP; Roche, Mannheim, Germany). Cells were transferred into Eppendorf tubes and boiled for 5 min at 95°C. Protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Total protein samples (8 μg/sample) were loaded on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred electrophoretically to a nitrocellulose membrane. Phosphorylation of p38, Jun N-terminal protein kinase (JNK), and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinases and IκBα degradation were evaluated using anti-phosphated p38, anti-phosphated JNK, anti-phosphated ERK, and anti-IκB antibodies, respectively (Cell Signaling Technology, Beverly, MA), according to the manufacturer's instructions. The optical density of each band was measured using Image J (National Institutes of Health, Bethesda, MD).

Anti-inflammatory assay in a PIE immune cell coculture system. Single-cell suspensions of immunocompetent Peyer's patches were prepared from the ileum of adult swine, as previously described (35, 39, 40). All procedures were conducted in accordance with the Guidelines for Animal Experimentation of Tohoku University, Sendai, Japan. Briefly, tissue specimens were cut into small fragments, gently pressed through a nylon mesh, and finally washed three times in complete RPMI 1640 medium (Sigma) supplemented with 10% FCS (Sigma). Residual erythrocytes were lysed by resuspension in hypotonic salt solution (0.2% NaCl), followed by hypertonic rescue of immune cells in an equal volume of 1.5% NaCl. Finally, immune cells were fractionated with Lympholyte-Mammal (Cedarlane, Hornby, Ontario, Canada) density gradient centrifugation, and the purified cells were suspended in complete DMEM (Invitrogen, Tokyo, Japan) supplemented with 10% FCS (Sigma), penicillin (50 U/ml)-streptomycin (50 μg/ml), and 50 μg/ml gentamicin (Nakalai Tesque, Kyoto, Japan).

In the Transwell culture system, PIE cells were seeded in the apical compartment at a concentration of 1.5×10^5 cells/well in 12-well tissue culture plates (Transwell-COL [PTFE]; pore size, 0.2 mm), and porcine Peyer's patch immunocompetent cells were seeded in the basolateral compartment at a concentration of 2×10^7 cells/well. For the evaluation of the anti-inflammatory activity of lactobacilli in the PIE immune cell coculture system, PIE cells in the apical compartment were stimulated with lactobacillus strains for 48 h and then washed twice with PBS. Finally, PIE cells were stimulated with ETEC for 12 h. Studies of protein expression of different cytokines were performed using the flow cytometric analysis described below.

Flow cytometric analysis. Expression levels of IL-8 and IL-6 proteins in PIE cells were determined using flow cytometry (36). After stimulation with lactobacilli and ETEC, brefeldin A (1 μg/ml) (Sigma) was added to the culture to stop vesicular protein transport. PIE cells were collected and washed with washing buffer (2% FCS, 0.01% NaN_3-PBS). Viable PIE cells were adjusted to 1×10^6 cells/tube and fixed and permeabilized with the BD Cytofix-Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Then, fixed and permeabilized cells were resuspended and labeled with primary (anti-pig IL-6 mouse IgG2b [R&D Systems, Minneapolis, MN] and anti-pig IL-8 mouse IgG2a [Abcam, Cambridge, MA]) and secondary (anti-mouse IgG2b-fluorescein isothiocyanate [FITC] [Santa Cruz Biotechnology, Santa Cruz, CA] and anti-mouse IgG2a-PerCP [Southern Biotech]) antibodies for codetection of IL-6 and IL-8. Expression levels of TLR4 were determined after 48 h of pretreatment with *Lactobacillus jensenii* TL2937. The cells were harvested using Accutase (Sigma) and stained with anti-human TLR4 polyclonal antibody (catalog no. sc-10741; Santa Cruz Biotechnology) or rabbit IgG isotype control (catalog no. 20304E; IMGENEX, San Diego, CA), followed by Alexa 488-conjugated anti-rabbit IgG (Invitrogen). Analysis of stained cells was performed using FACS-Calibur (BD, Franklin Lakes, NJ) equipped with Cell-Quest software. Data analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis. The statistical analysis was performed using GLM procedures of the SAS computer program (SAS, 1994). Mean values of relative mRNA expression and of relative protein expression were compared for four inflammatory cytokines—IL-1α, IL-6, IL-8, and monocyte chemoattractant protein 1 (MCP-1)—using the Bonferroni correction and multicomparison tests. For these analyses, an adjusted P value of <0.05 was defined as significant. Time sequential changes in MKP-1, A20, and Bcl-3 mRNA expressions in PIE cells stimulated with Pam3CSK4 were assessed using one-way analysis of variance (ANOVA).

Nucleotide sequence accession numbers. The nucleotide sequences of porcine SIGIRR, Tollip, A20, Bcl-3, and MKP-1 have been submitted to the DDBJ, EMBL, and GenBank nucleotide databases under accession numbers AB490122, AB490123, AB490119, AB490120, and AB490121, respectively.

RESULTS

Inflammatory response in PIE cells stimulated with ETEC or LPS. We evaluated the mRNA levels of IL-1α, IL-6, IL-8, and MCP-1 at different time points after stimulation with LPS or ETEC, with the aim of establishing the most appropriate time to study the inflammatory response. After the challenge with ETEC or LPS, IL-1α, IL-6, and MCP-1 mRNA increased progressively in PIE cells and reached a peak 12 h after the challenge (Fig. 1A). Similarly, IL-8 mRNA peaked 12 h after PIE cells were challenged with LPS. However, IL-8 mRNA in PIE cells peaked 24 h after stimulation with ETEC (Fig. 1A). Based on these results, the following experiments were conducted with PIE cells 12 h after challenge with LPS or ETEC. We studied the response of PIE cells to different concentrations of ETEC and LPS. The levels of proinflammatory mediators produced by PIE cells 12 h after ETEC or LPS challenge showed direct relationships to the doses of ETEC and LPS used (Fig. 1B). However, with the maximum dose of ETEC, there was also a significant injury to the PIE monolayer (data not shown); thus, in subsequent experiments, intermediate doses of ETEC were used. PIE cells challenged with ETEC and those challenged with LPS showed similar patterns of IL-1α, IL-6, IL-8, and MCP-1 mRNA expression; therefore, we hypothesized

TABLE 2 Effect of lactobacillus strains on TLR2-expressing transfectants and on expression of cytokine mRNAs in PIE cells after inflammatory stimulation[a]

Strain	*Lactobacillus* species	TLR2 activation	Cytokine expression			
			IL-1α	IL-6	IL-8	MCP-1
Control			4.8 ± 0.4	4.4 ± 2.6	11.2 ± 1.5	8.6 ± 2.4
TL2937	*L. jensenii*	High	104.8 ± 10.4	34.8 ± 6.2*	111.1 ± 4.2	92.2 ± 0.6
MEP221102	*L. reuteri*	Moderate	144.6 ± 15.9	44.7 ± 10.4*	113.3 ± 7.4	95.5 ± 12.5
MEP221113	*L. salivarius*	Moderate	120.7 ± 8.6	49.1 ± 8.6*	114.6 ± 3.7	94.7 ± 7.6
MEP221111	*L. rhamnosus*	Negative	134.2 ± 4.4	55.8 ± 11.5*	119.6 ± 16.3	95.1 ± 5.4
OLL2768	*L. casei*	Negative	95.6 ± 12.5	51.9 ± 18.3*	124.0 ± 17.8	106.5 ± 7.2
MEP221104	*L. casei*	Negative	98.1 ± 9.4	55.0 ± 14.1*	132.3 ± 26.6	112.9 ± 9.6
MEP221115	*L. casei*	Negative	102.4 ± 9.4	57.2 ± 21.2*	125.3 ± 24.7	108.8 ± 10.7
MEP221101	*L. reuteri*	Negative	104.6 ± 15.4	69.0 ± 15.0	115.3 ± 18.2	106.9 ± 5.2
MEP221109	*L. casei*	Negative	94.6 ± 15.5	66.3 ± 5.7	120.7 ± 17.9	98.0 ± 8.6
MEP221114	*L. casei*	Moderate	98.7 ± 8.7	64.1 ± 13.9	128.1 ± 5.2	96.6 ± 9.9
MEP221110	*L. rhamnosus*	Negative	91.9 ± 9.1	64.7 ± 13.7	126.8 ± 17.6	110.8 ± 12.4
MEP221108	*L. casei*	Negative	107.7 ± 14.7	66.2 ± 5.6	122.0 ± 4.7	114.0 ± 23.3
MEP221103	*L. casei*	Negative	99.2 ± 14.2	71.6 ± 10.6	111.9 ± 5.2	79.0 ± 19.5
MEP221107	*L. casei*	Negative	101.6 ± 14.7	75.6 ± 12.8	131.6 ± 14.5	133.8 ± 18.8
MEP221105	*L. casei*	Negative	151.7 ± 15.3	85.4 ± 13.1	180.1 ± 28.6*	195.2 ± 14.5*
MEP221117	*L. gasseri*	High	179.1 ± 13.2*	87.2 ± 28.8	112.4 ± 13.5	91.0 ± 9.6
MEP221106	*L. casei*	Negative	158.2 ± 22.8*	94.7 ± 14.5	150.4 ± 22.1*	140.0 ± 4.6
MEP221112	*L. rhamnosus*	Negative	123.5 ± 17.6	111.1 ± 26.1	157.9 ± 12.6*	162.1 ± 9.6

[a] Transfectants stably expressing porcine TLR2 (designated HEK^{pTLR2} cells) were isolated as previously reported, and the NF-κB reporter assay in HEK^{pTLR2} cells was performed as previously described (38). The capacity of the strains to activate TLR2 was evaluated using this immune assay and by stimulating HEK^{pTLR2} cells with the different lactobacillus strains for 24 h. Strains were classified into three functional groups according to the relative index (RI) values as follows: high TLR2 activation (RI > 5), moderate TLR2 activation (RI between 1 and 5), and negative TLR2 activation (RI < 1). For the anti-inflammatory evaluation of lactobacillus, porcine intestinal epithelial (PIE) cells were pretreated with different strains for 48 h and then stimulated with ETEC (5×10^7 cells/ml). The levels of IL-1α, IL-6, IL-8, and MCP-1 mRNAs were evaluated 12 h after the inflammatory challenge. PIE cells without lactobacillus treatment and challenged with ETEC were used as controls. Cytokine mRNA expression in this group was set to 100 and used for calculating the changes in lactobacillus-treated PIE cells as mean ratio ± standard deviation (SD) against the ETEC control. In addition, values of cytokine expression in PIE cells without lactobacillus treatment and no challenge with ETEC are shown (control). The results represent three independent experiments. Asterisks indicate significant differences ($P < 0.05$) compared with the cytokine mRNA expression levels of PIE cells without lactobacillus treatment and challenged with ETEC.

that the inflammatory response triggered by ETEC was mediated principally by ETEC-associated LPS. Therefore, we evaluated the effects of ETEC pretreated with the LPS inhibitor polymyxin B. IL-1α, IL-6, IL-8, and MCP-1 mRNA expression was significantly lower in PIE cells challenged with polymyxin B-treated ETEC than in those challenged with untreated ETEC; moreover, the effect on mRNA expression correlated directly with the dose of polymyxin B (Fig. 1C). These findings confirmed that the inflammatory response triggered by ETEC was mediated principally by LPS.

Identification of lactobacillus strains that modulate the proinflammatory response in PIE cells. The anti-inflammatory effects of 18 lactobacillus strains were evaluated in PIE cells challenged with ETEC (Table 2). Individual PIE cell cultures were stimulated with a single LAB strain for 48 h and then challenged with ETEC, and the levels of IL-1α, IL-6, IL-8, and MCP-1 mRNAs were studied 12 h after the challenge. Challenge with the intestinal pathogen resulted in significant increases of proinflammatory cytokine expression in lactobacillus-treated and untreated control PIE cells (Table 2). However, IL-6 mRNA levels in PIE cells stimulated with any one of seven strains—TL2937, MEP221102, MEP221113, MEP221111, MEP221104, MEP221115, or OLL2768—were significantly lower than those observed in the untreated control. In contrast, IL-1α, IL-8, and MCP-1 mRNA in the PIE cells treated with most of the lactobacillus strains were not different from the control group. We evaluated the effect of lactobacillus strains on unchallenged PIE cells. Therefore, PIE cell cultures were separately stimulated with each of the 18 lactobacillus strains for 48 h, and the levels of IL-1α, IL-6, IL-8, and MCP-1 mRNA expression were determined (Fig. 2). Lactobacilli did not modify PIE cell viability, because 94% of PIE cells were viable in all cases (data not shown). We observed that each strain studied had specific effects on the expression of the cytokines in PIE cells. Strains TL2937, MEP221111, and MEP221101 caused an increase in IL-1α expression, but strains MEP221109 and MEP221107 significantly reduced this cytokine in PIE cells (Fig. 2). IL-8 mRNA levels were upregulated by strains TL2937, MEP221111, and MEP221112 and downregulated by MEP221104 and MEP221109. In contrast, IL-6 and MCP-1 mRNA levels were upregulated by some strains, but no strain downregulated both cytokines. Several strains, including TL2937, upregulated MCP-1 mRNA, and MEP221101 induced the largest increase of IL-6 mRNA expression (Fig. 2).

We previously established pTLR2-transfected cells (HEK^{pTLR2}) using lipofection and a pcDNA-FLAG vector containing pTLR2 (38). Using RT-PCR, confocal laser microscopy, and two-dimensional (2-D) microscopy of HEK^{pTLR2} cells treated with FITC-zymosan, we confirmed that pTLR2 was expressed and functional in HEK^{pTLR2} cells (38). Moreover, TLR2-mediated immune activation via a wide range of LAB strains, including the 18 strains used in the present study, was evaluated using these pTLR2-expressing cells (38). As shown in Table 2, several lactobacillus strains, including *L. jensenii* TL2937, *L. reuteri* MEP221102, and *L. gasseri* MEP221117, significantly induced the NF-κB-dependent promoter that drives expression of luciferase. In contrast, no increase in NF-κB activity was observed compared with that of other lactobacillus strains, including *L. rhamnosus*

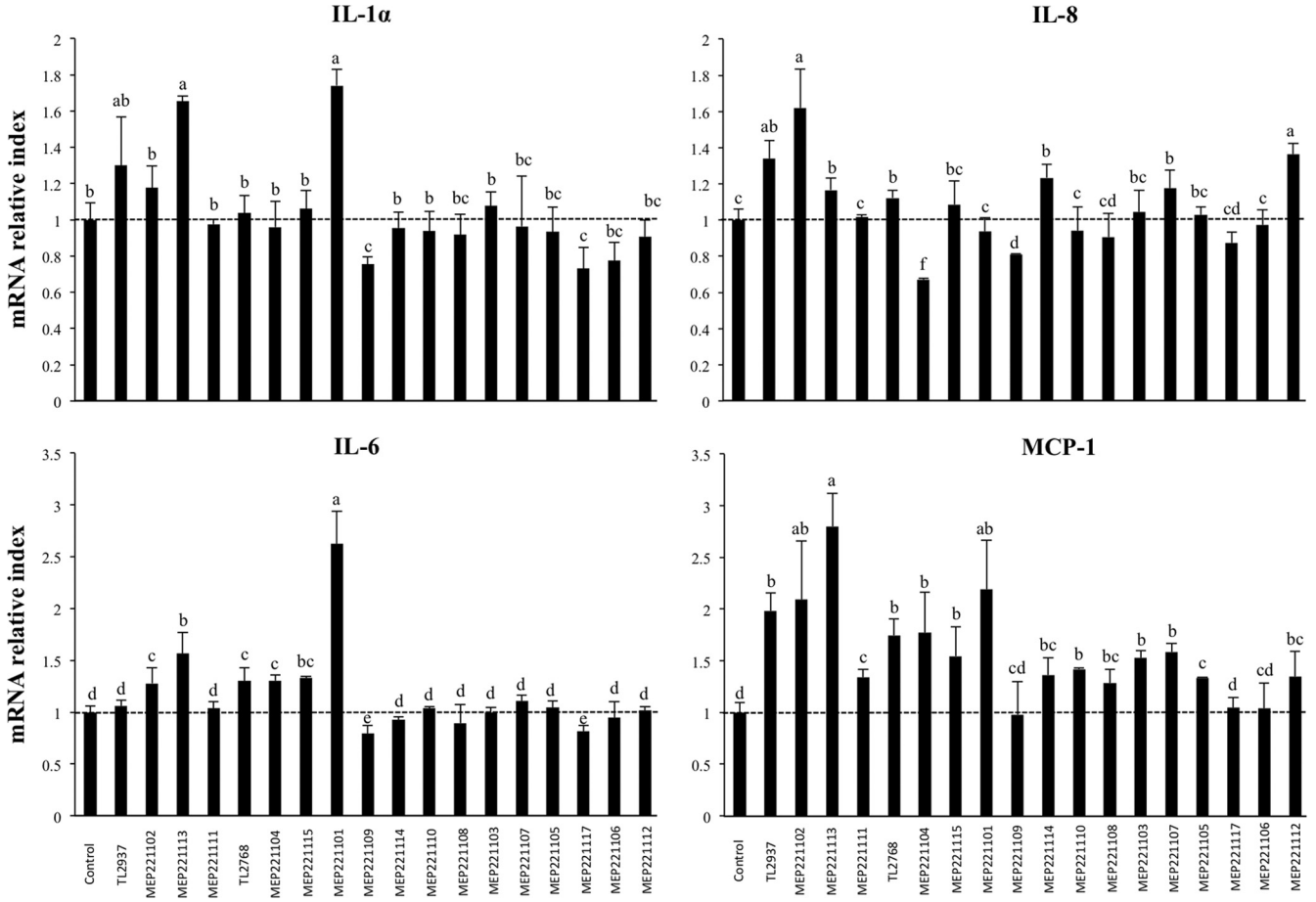


FIG 2 Expression of IL-1α, IL-6, IL-8, and MCP-1 mRNAs in porcine intestinal epithelial (PIE) cells after treatment with different lactobacillus strains. PIE cells were pretreated with lactobacilli for 48 h, and subsequently the levels of IL-1α, IL-6, IL-8, and MCP-1 mRNAs were evaluated. The results represent three independent experiments. Values with different letters are significantly different ($P < 0.05$).

MEP221111 (Table 2). Considering the stimulating effect of some lactobacillus strains on the HEK^{pTLR2} system (38) (Table 2) and the anti-inflammatory effect in PIE cells studied in this work, we defined three functional types of lactobacilli: strains with the capacity to downregulate IL-6 mRNA in PIE cells in response to ETEC and (i) high HEK^{pTLR2} activity (strain TL2937), (ii) moderate HEK^{pTLR2} activity (strain MEP221102), or (iii) no HEK^{pTLR2} activity (strain MEP221111). Thus, *L. jensenii* TL2937, *L. reuteri* MEP221102, and *L. rhamnosus* MEP221111 were selected for the following experiments.

Characterization of the anti-inflammatory effect of three lactobacillus strains. We next examined whether a shorter prestimulation of PIE cells with selected lactobacillus strains was able to exert any effect on the production of inflammatory mediators. For this purpose, PIE cells were stimulated with *L. jensenii* TL2937, *L. reuteri* MEP221102, or *L. rhamnosus* MEP221111 for 3 or 12 h prior to challenge with ETEC. Levels of proinflammatory cytokines in cells stimulated for 3 h with lactobacilli were not different from those of the control (Fig. 3A). In addition, there were no significant changes in IL-1α or MCP-1 mRNA expression in cells treated for 12 h with any lactobacillus strain. However, the IL-6 mRNA expression was significantly lower in cells prestimulated for 12 h with *L. reuteri* MEP221102 or *L. rhamnosus* MEP221111 than in the control (Fig. 3A). Because induction of IL-8 mRNA was highest 24 h after challenge with ETEC, we evaluated whether the anti-inflammatory effect of lactobacilli persisted 24 h after the challenge. As was observed 12 h after challenge with ETEC (Table 2), the IL-6 mRNA level was lower in PIE cells treated with any one of the three strains than in control cells (Fig. 3B). Additionally, all three strains induced downregulation of IL-8 mRNA within 24 h of ETEC challenge, but only *L. reuteri* MEP221102 also induced downregulation of MCP-1 mRNA expression. Interestingly, the three strains induced upregulation of IL-1α mRNA in PIE cells within 24 h of ETEC challenge (Fig. 3B). To further confirm the anti-inflammatory effects of these strains, we conducted ELISAs and found that IL-6 and IL-8 proteins were significantly downregulated 48 h after ETEC challenge (Fig. 3C).

***L. jensenii* TL2937 and TLR2 ligation modulate TLR4-mediated inflammatory responses in PIE cells.** We investigated whether the anti-inflammatory effect of lactobacillus strains was related to their influence on the LPS-TLR4 interaction. PIE cells, prestimulated with one of the lactobacillus strains, were challenged with LPS, and 12 h later, changes in expression of inflammatory mediators were assessed. Stimulation with *L. jensenii* TL2937 downregulated levels of IL-6, IL-8, and MCP-1 mRNA in PIE cells in response to LPS challenge (Fig. 4A). There was no

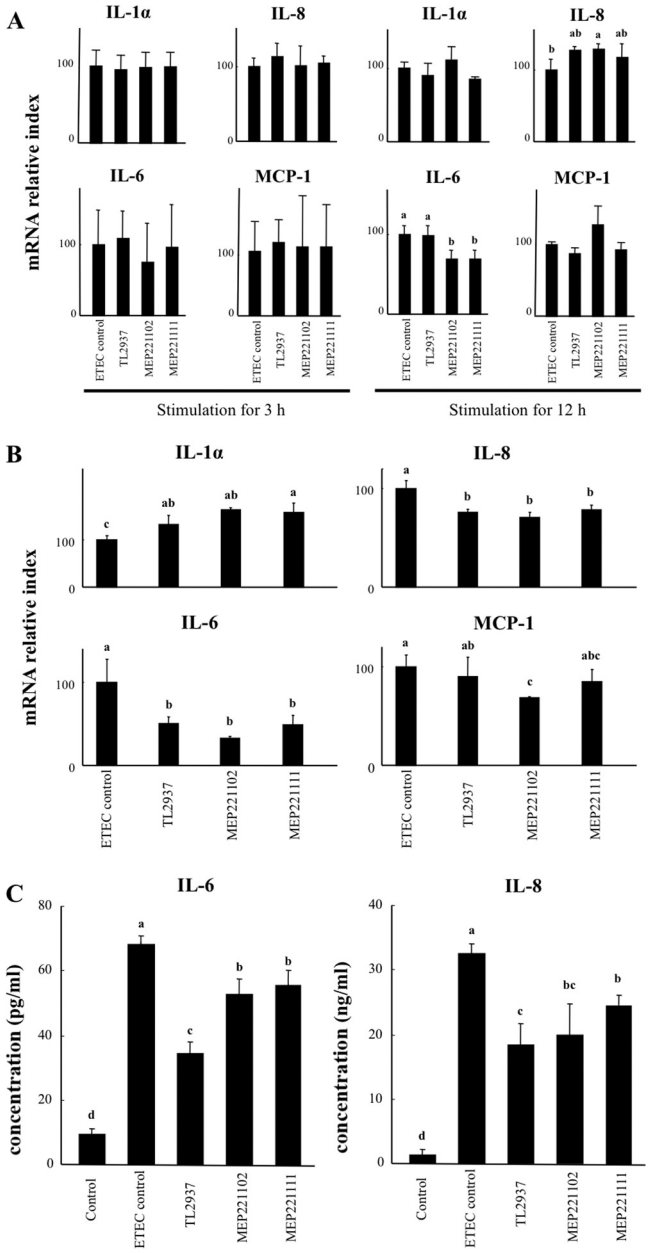


FIG 3 Expression of IL-1α, IL-6, IL-8, and MCP-1 mRNAs in porcine intestinal epithelial (PIE) cells after lactobacillus treatment and inflammatory challenge. (A) PIE cells were pretreated with *L. jensenii* TL2937, *L. reuteri* MEP221102, or *L. rhamnosus* MEP221111 for 3 or 12 h and then challenged with ETEC (5×10^7 cells/ml). The levels of IL-1α, IL-6, IL-8, and MCP-1 mRNAs were evaluated 12 h after the inflammatory challenge. ETEC-challenged PIE cells that were not pretreated with lactobacilli were used as the ETEC controls. (B) PIE cells were pretreated with *L. jensenii* TL2937, *L. reuteri* MEP221102, or *L. rhamnosus* MEP221111 for 48 h and then challenged with ETEC (5×10^7 cells/ml). The levels of IL-1α, IL-6, IL-8, and MCP-1 mRNAs were evaluated 24 h after the inflammatory challenge. ETEC-challenged PIE cells that were not pretreated with lactobacilli were used as the ETEC controls. (C) PIE cells were pretreated with *L. jensenii* TL2937, *L. reuteri* MEP221102, or *L. rhamnosus* MEP221111 for 48 h and then challenged with ETEC (5×10^7 cells/ml). The concentration of IL-6 and IL-8 protein in culture supernatants was determined 48 h after the inflammatory challenge. Unchallenged, untreated PIE cells were used as controls. The results represent data from two independent experiments. Values with different superscript letters were significantly different ($P < 0.05$).

change in the expression of these cytokines when PIE cells were prestimulated with the other two strains. Notably, IL-1α mRNA levels were not significantly different in lactobacillus-treated and control cells (Fig. 4A). *L. jensenii* TL2937 is a potent stimulator of TLR2 (38); therefore, we stimulated PIE cells with Pam3CSK4, a synthetic TLR2 ligand and a potent activator of NF-κB. As shown in Fig. 4A, prestimulation with Pam3CSK4 reduced the expression of IL-1α, IL-8, and MCP-1 mRNA, but there were no changes in IL-6 mRNA expression. We also evaluated whether the factors released by *L. jensenii* TL2937 into the culture medium contributed to the anti-inflammatory effect of the strain. Supernatant from *L. jensenii* TL2937 cultures was collected after 48 h of incubation. PIE cells were pretreated with this supernatant for 12 h and then challenged with ETEC. Although this pretreatment slightly reduced IL-8 mRNA expression, the expression of the other inflammatory cytokines was unchanged (Fig. 4B). Additionally, the expression of TLR4 protein was not significantly different in *L. jensenii* TL2937-treated PIE cells and control cells (Fig. 4C).

Effect of *L. jensenii* TL2937 on MAPK and NF-κB pathways in PIE cells. We next evaluated whether *L. jensenii* TL2937 was able to attenuate the proinflammatory response by modulating the NF-κB pathway. Challenge of PIE cells with ETEC significantly reduced the levels of the IκBα. In contrast, PIE cells prestimulated with *L. jensenii* TL2937 showed less reduction in IκBα following ETEC challenge (Fig. 5A and C). We also examined the relationship between mitogen-activated protein kinase (MAPK) activation and regulation of proinflammatory cytokines in PIE cells. PIE cells were stimulated with heat-killed *L. jensenii* TL2937 or control medium, and the activation profiles of ERK, p38, and JNK were compared after ETEC challenge. ETEC induced intense phosphorylation of ERK, p38, and JNK; this phosphorylation reached the peak between 12 and 25 min following the challenge. The time course of ERK and JNK activation induced by ETEC in PIE cells treated with *L. jensenii* TL2937 was similar to that in the control. Except, the phosphorylation of p38 was significantly lower in PIE cells treated with strain TL2937 (Fig. 5A and C). Because the inflammatory activity of ETEC was primarily mediated by LPS and the anti-inflammatory effect of *L. jensenii* TL2937 was partially mediated by interactions with TLR2, we assessed the effect of *L. jensenii* TL2937 and Pam3CSK4 on the MAPK and NF-κB pathways in LPS-challenged PIE cells. LPS challenge, like ETEC challenge, activated the NF-κB pathway in PIE cells and increased the phosphorylation of p38, ERK, and JNK (Fig. 5B and C). None of the treatments induced changes in the kinetics of ERK or JNK phosphorylation, and only *L. jensenii* TL2937 reduced phosphorylation of p38. However, both Pam3CSK4 and *L. jensenii* TL2937 did inhibit NF-κB activation (Fig. 5B and C). To investigate the role of the p38 pathway in cytokine production, PIE cells were pretreated with a selective inhibitor of the p38 pathway, PD169316, prior to challenge with ETEC (Fig. 5D). No modifications in the expression of IL-8 or MCP-1 mRNA were observed in PIE cells treated with the p38 inhibitor. In contrast, IL-1α and especially IL-6 mRNA expression declined dramatically in the presence of the p38 inhibitor, indicating that the p38 pathway was important for the production of IL-1α and IL-6 in PIE cells (Fig. 5D).

Immunomodulatory activity of *L. jensenii* TL2937 in an *in vitro* Peyer's patch model culture system. Although IECs are the first line of intestinal defense, the interaction between IECs and

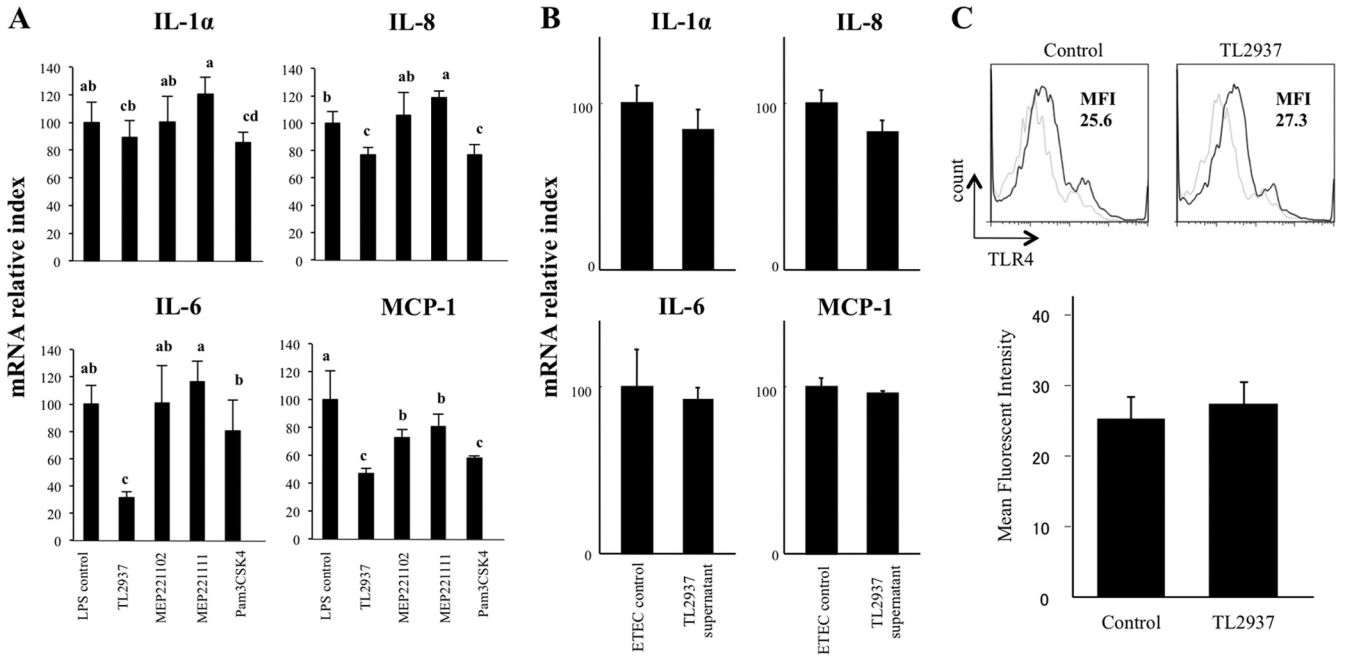


FIG 4 Expression of IL-1α, IL-6, IL-8, and MCP-1 mRNAs in porcine intestinal epithelial (PIE) cells after lactobacillus treatment and inflammatory challenge. (A) PIE cells were pretreated with *L. jensenii* TL2937, *L. reuteri* MEP221102, or *L. rhamnosus* MEP221111 for 48 h and then stimulated with LPS (1 μg/ml). The levels of IL-1α, IL-6, IL-8, and MCP-1 mRNAs were evaluated 12 h after the inflammatory challenge. LPS-challenged PIE cells that were not pretreated with lactobacilli were used as the LPS controls. (B) PIE cells were pretreated with the supernatant from cocultures of PIE cells and *L. jensenii* TL2937 and then challenged with ETEC (5×10^7 cells/ml). The levels of IL-1α, IL-6, IL-8, and MCP-1 mRNAs were evaluated 12 h after the inflammatory challenge. (C) Expression of the TLR4 protein was determined by flow cytometry after 48 h of pretreatment with *L. jensenii* TL2937. PIE cells without any treatments were used as controls. Histograms show flow cytometric analysis as follow: PIE cells stained with anti-TLR4 antibody (black line) and isotype-matched controls (gray line). Values of mean fluorescence intensity (MFI) are also shown. The results represent data from three independent experiments. Values with different letters are significantly different ($P < 0.05$).

immune cells directs the type of immune response in the gut. Therefore, we used an *in vitro* Peyer's patch model culture system to evaluate the anti-inflammatory effect of *L. jensenii* TL2937 more precisely. The analysis of IL-6 and IL-8 proteins expressed on PIE cells by flow cytometry analysis revealed that levels of both cytokines increased significantly after the challenge with ETEC in *L. jensenii* TL2937-treated and control cells (Fig. 6). PIE cells challenged with ETEC expressed significantly higher levels of IL-6 and IL-8 than those observed in unchallenged cells. However, the PIE cells pretreated with *L. jensenii* TL2937 showed reduced levels of IL-6 and IL-8 following ETEC challenge than the ETEC control group (Fig. 6).

Effect of *L. jensenii* TL2937 on negative regulators of the TLR signaling pathway in PIE cells. We also studied regulators that inhibit the TLR signaling pathway. To assess the expression of these negative regulators of TLRs, we first cloned cDNAs corresponding to these proteins from swine. Comparison of the nucleic acid sequences and the deduced amino acid of porcine SIGIRR, Tollip, A20, Bcl-3, MKP-1, and a partial porcine IRAK-M ORF with those from humans and from mice are shown in Table 3.

We also evaluated expression of SIGIRR, Tollip, A20, Bcl-3, MKP-1, and IRAK-M mRNAs in PIE cells. PIE cells were stimulated for 36 h with *L. jensenii* TL2937, *L. reuteri* MEP221102, or *L. rhamnosus* MEP221111, and the expression levels of SIGIRR, Tollip, A20, Bcl-3, MKP-1, and IRAK-M mRNA were determined using real-time PCR. None of the lactobacillus strains induced changes in expression of SIGIRR, Tollip, or IRAK-M (data not shown). However, all three strains caused upregulation of MKP-1 and A20 mRNA, but only TL2937 and MEP221102 caused upregulation of Bcl-3 mRNA (Fig. 7A). We examined the role of TLR2 in the lactobacillus-mediated modulation of these negative regulators of TLRs. Specifically, we investigated whether stimulation of PIE cells with Pam3CSK4 induced the same effect as the lactobacilli on expression of Bcl-3, MKP-1, and A20 mRNAs. Stimulation with Pam3CSK4 induced an increase in the expression of A20 mRNA within 12 h of stimulation, but upregulation of the Bcl-3 mRNA expression was not observed until 48 h after stimulation. Notably, no significant changes in MKP-1 mRNA expression were observed (Fig. 7B).

DISCUSSION

Weaning involves multiple changes for piglets; they switch from a liquid to a solid diet, they are taken away from their mothers, and they are moved to an unfamiliar building where they may be exposed to new environmental antigens. These changes can trigger transit inflammatory responses in the gut that can contribute to anatomical and functional intestinal disorders in piglets (22, 23, 28). IECs can produce a variety of inflammatory factors in response to antigens, and they may be important in weaning-associated intestinal inflammation (24, 29). Here, we observed a significant upregulation of proinflammatory mediators in cultured PIE cells challenged with ETEC. This finding was consistent with findings from a previous study that showed that PIE cells induce inflammatory responses by upregulating cytokines and chemokines in response to LPS (24). Recently, Devriendt et al. (6) demonstrated that infection of IPEC-J2 cells, which are derived

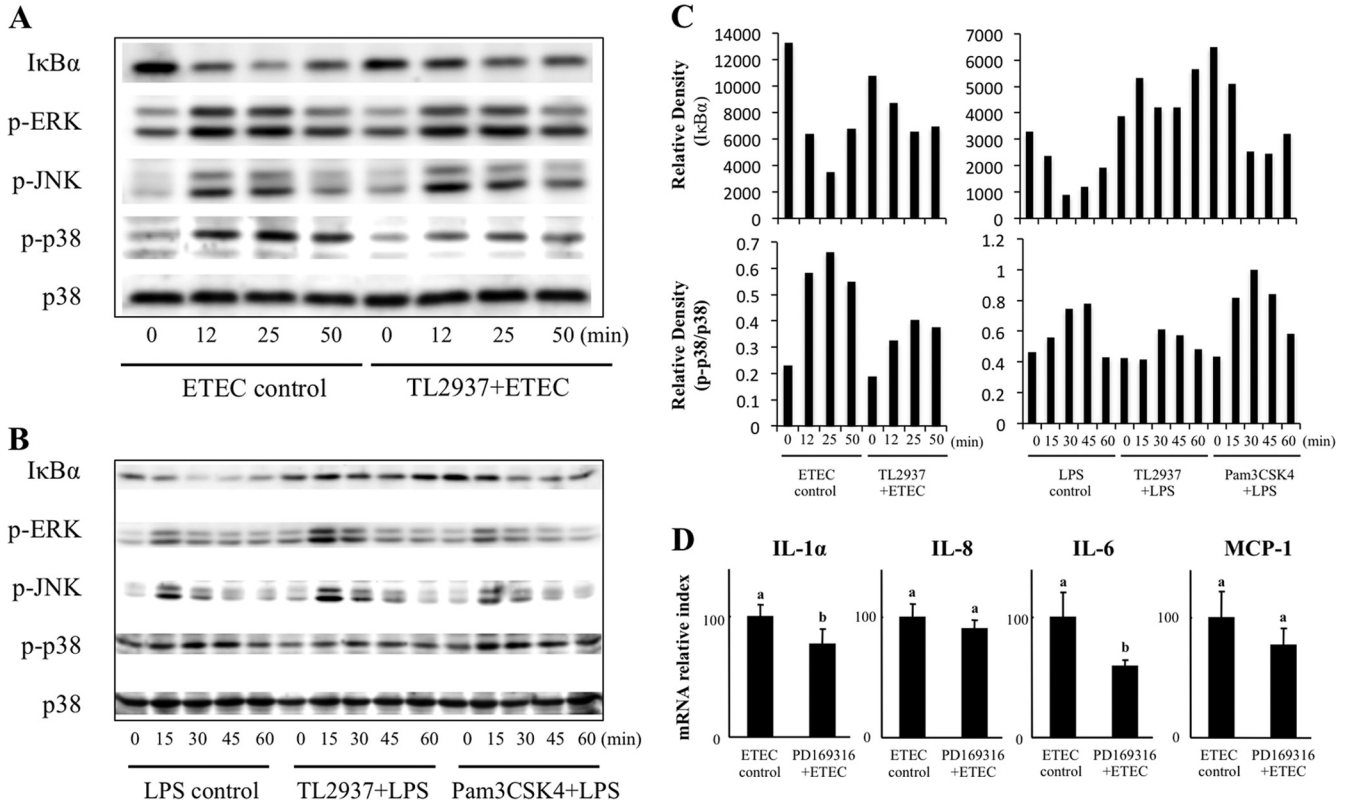


FIG 5 Western blot analysis of MAPK and NF-κB activation in PIE cells following inflammatory challenge. PIE cells were pretreated with lactobacilli for 48 h and then challenged with ETEC (A) or LPS (B). Samples were collected at the indicated time points after ETEC or LPS challenge. (C) The intensities of the IκBα, p-p38, and p38 bands were determined using image analysis software (Image J; Research Services Branch, NIMH, NIH, Bethesda, MD). The densitogram of each band was determined, and the relative density was expressed as the area of the peak in the densitogram. (D) Expression levels of IL-1α, IL-6, IL-8, and MCP-1 mRNA in PIE cells treated with p38 inhibitor PD169316 (10 nM) for 1 h and then stimulated with ETEC for 12 h. Values with different letters are significantly different ($P < 0.05$).

from the porcine jejunum, with the wild-type $F4^+$ ETEC strain enhanced IL-6 and IL-8 secretion via stimulation of TLR5 by flagellin. Our findings were consistent with these findings in that we demonstrated that PIE cells also upregulated the expression of IL-6 and IL-8 after ETEC stimulation; however, the 987P strain used in this study was heat killed and does not express flagellin. Moreover, we found that ETEC strain 987P triggered an inflammatory response via TLR4 through activation of the NF-κB and MAPK cascades.

TLR4 activation and cytokine production by IECs can induce recruitment and activation of inflammatory cells. Although this mechanism represents an important primary line of host defense, prolonged or dysregulated proinflammatory cytokine production may lead to tissue damage and epithelial barrier dysfunction (9, 13). We found that the damage to PIE cells correlated with the levels of proinflammatory cytokine mRNAs produced after ETEC or LPS challenge (data not shown); this finding was consistent with findings demonstrating that challenge of human intestinal Caco-2 cells with ETEC causes a strong upregulation of proinflammatory mediators that led to membrane damage (30, 31).

Some studies have shown that probiotics can mitigate damaging immune responses during ETEC infection (30, 48). A recent study in Caco-2 cells demonstrated that *L. rhamnosus* GG is able to counteract ETEC-induced upregulation of IL-1β and TNF-α and the downregulation of TGF-β1 expression, and consequently it prevents cytokine deregulation (31). In addition, comparative studies between *L. rhamnosus* GG and *Bifidobacterium animalis* MB5 (31) and between different bifidobacterial strains (8) demonstrated that the effects of individual probiotic strains on the inflammatory responses in IECs differ (8, 31). These findings indicate that some probiotic LAB strains may be beneficial for preventing inflammatory response-mediated damage during weaning. Thus, we attempted to identify lactobacillus strains that modulate the proinflammatory response induced by ETEC or LPS in PIE cells. Intestinal bacteria may be in contact with IECs for long periods of time; therefore, we prestimulated PIE cells for 48 h. Several lactobacillus strains did cause downregulation of the inflammatory response, and these effects changed over time. Interestingly, of the three functional types of lactobacilli defined by the anti-inflammatory effect on PIE cells and by their stimulating effect on the HEK^{pTLR2} system (38), we found that the strain with a high capacity to induce the NF-κB-dependent promoter in HEK^{pTLR2} cells, *L. jensenii* TL2937, was also the strain with the highest capacity to downregulate IL-6 and IL-8 production by PIE cells in response to ETEC and LPS. For this reason, we were particularly interested in *L. jensenii* TL2937 and decided to investigate the mechanisms behind the anti-inflammatory effects caused by this strain.

Studies comparing the anti-inflammatory activities of supernatant from *L. jensenii* TL2937-prestimulated cultures and the

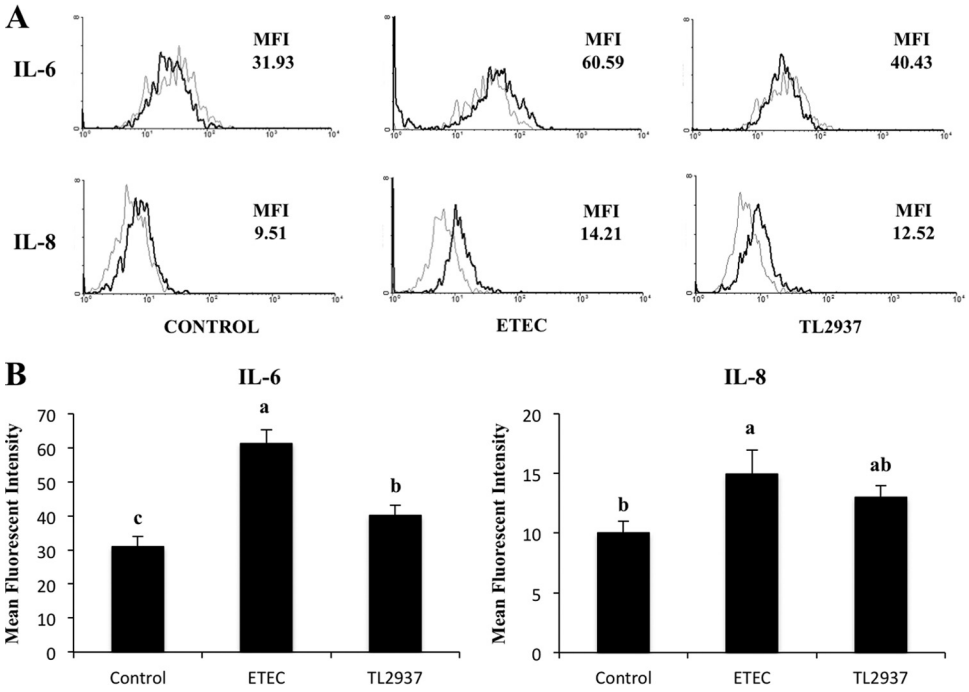


FIG 6 Expression of IL-6 and IL-8 proteins on porcine intestinal epithelial (PIE) cells following inflammatory challenge. PIE cells were cocultured with Peyer's patch immunocompetent cells, pretreated with *L. jensenii* TL2937 for 48 h, and then challenged with ETEC (5×10^7 cells/ml). The levels of IL-6 and IL-8 proteins were determined by flow cytometry 12 h after the inflammatory challenge. PIE cells without any treatments were used as controls. (A) Histograms show flow cytometric analyses as follows: PIE cells stained with anti-IL-8 or anti-IL-6 antibodies (black lines) and isotype-matched controls (gray lines). (B) Values of mean fluorescence intensity (MFI) are shown for each group. The results represent data from three independent experiments. Values with different letters are significantly different ($P < 0.05$).

immunobiotic strain indicate that the immunomodulatory effect of *L. jensenii* TL2937 was primarily mediated by factors in or on the microorganism and not by factors released into the medium during the culture period. We also found that treatment of PIE cells with this strain in the absence of inflammatory challenge did not cause downregulation of the expression of inflammatory cytokines. Moreover, we found that *L. jensenii* TL2937 could inhibit NF-κB and MAPK signaling pathways. Probiotics can inhibit excessive NF-κB induced by proinflammatory cytokine secreted from IECs. Immunobiotics can suppress TNF- or *Salmonella enterica* serovar Typhimurium-induced IL-8 mRNA expression and secretion by IECs in an NF-κB-dependent manner (25, 49).

TABLE 3 Comparison of the nucleic acid sequences and the deduced amino acid sequences of the complete ORFs of cloned porcine SIGIRR, Tollip, A20, Bcl-3, and MKP-1 and a partial porcine IRAK-M ORF with those from humans and mice

	% homology[a]			
	Nucleic acid sequence to:		Deduced amino acid sequence to:	
Cloned porcine cDNA	Human	Mouse	Human	Mouse
SIGIRR (ORF)	86.8	80.5	87.3	81.5
Tollip (ORF)	88.0	82.9	89.8	92.0
A20 (ORF)	85.5	80.7	88.5	80.7
Bcl-3 (ORF)	89.6	79.6	89.9	79.7
MKP-1 (ORF)	92.5	88.0	96.7	94.8
IRAK-M (partial ORF)	86.9	73.6	NT	NT

[a] NT, not tested.

Our studies also demonstrated that *L. jensenii* TL2937 inhibited inflammatory responses in PIE cells by downregulating the MAPK pathway, specifically p38. Although we found that a p38 pathway inhibitor reduced IL-1α and IL-6 mRNA expression and *L. jensenii* reduced only IL-6 expression in ETEC-challenged PIE cells, the p38 inhibitor had a larger effect on IL-6 than on IL-1α. The difference could be due to p38-independent effects (16, 17) or to the differences of inhibitory mechanisms. These findings indicate that the p38 signaling pathway is responsible for reduction of IL-6 expression in the PIE cells.

In the gut, cross-talk between IECs and immune cells directs the type of immune response (29). Therefore, we evaluated whether PIE cells responded differently to ETEC challenge when cocultured with immunocompetent cells. Results confirmed that the challenge with ETEC increased the production of IL-6 and IL-8 by PIE cells and that the pretreatment of these cells with *L. jensenii* TL2937 was able to reduce levels of both proinflammatory cytokines.

Regulatory proteins that restrict the duration and intensity of TLR signals can modulate cellular responses, thereby helping to determine whether TLR activation leads to homeostasis or inflammation (4, 20). To dissect the mechanism(s) that underlies the anti-inflammatory effect of *L. jensenii* TL2937, we assessed the effect of this strain on expression of negative regulators of TLRs in PIE cells. The expression levels of SIGIRR, Tollip, A20, Bcl-3, MKP-1, and IRAK-M were evaluated, and we found that MKP-1, A20, and Bcl-3 mRNA expression was upregulated in PIE cells stimulated with *L. jensenii* TL2937. MKP-1 plays a role in the inhibition of proinflammatory mRNA expression, because it can

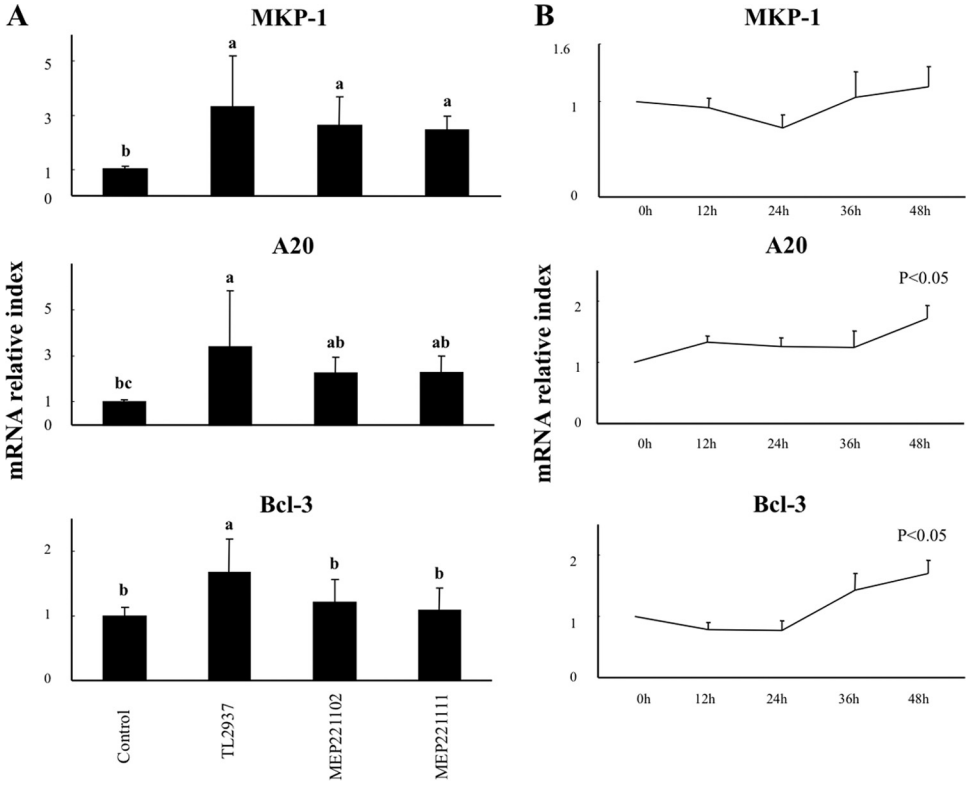


FIG 7 Expression of three negative regulators of Toll-like receptors (TLRs): A20, Bcl-3, and MKP-1 mRNAs in porcine intestinal epithelial (PIE) cells. (A) PIE cells were pretreated with *L. jensenii* TL2937, *L. reuteri* MEP221102, or *L. rhamnosus* MEP221111 for 36 h, and then the levels of A20, Bcl-3, and MKP-1 mRNA were evaluated. The results represent data from three independent experiments. Values with different letters are significantly different ($P < 0.05$). (B) PIE cells were stimulated with Pam3CSK4 (200 ng/ml), and then the levels of A20, Bcl-3, and MKP-1 mRNAs were evaluated at the indicated time points. A20 and Bcl-3 mRNA expression in PIE cells stimulated with Pam3CSK4 was significantly higher at 48 h than at 0 h ($P < 0.05$).

inactivate MAPK. An MKP-1 deficiency leads to hyperresponsiveness to LPS stimulation in innate immune cells (50). Moreover, MKP-1 desensitizes enterocytes to TLR ligands by inactivating the p38 signaling pathway (42), and MKP-1 was not induced by TLR2 stimulation, although ligands of TLR3, TLR4, TLR5, and TLR9 induced MKP-1. These finding are consistent with our finding that the TLR2 ligand, Pam3CSK4, did not induce the expression of MKP-1. A20 has an essential role in the termination of NF-κB signaling in response to TNF-α and microbial products, such as LPS (5, 43). An A20 deficiency in enterocytes reportedly renders mice sensitive to a TNF-α-induced inflammatory response that leads to disruption of the epithelial barrier, infiltration of commensal bacteria that initiate a more systemic inflammatory response, and ultimately death (41). The Bcl-3 protein functions as an inhibitor of NF-κB activity by stabilizing repressive NF-κB homodimers in a DNA-bound state and preventing binding of dimers that activate transcription. In fact, stabilization of repressive complexes through the induction of Bcl-3 expression is proposed to function in the process of LPS tolerance (44). Moreover, treatment of macrophages with IL-10 induces the expression of Bcl-3, and Bcl-3 expression leads to inhibition of LPS-induced TNF-α production (18). Thus, the induction of negative regulators by *L. jensenii* TL2937 in PIE cells may be important for establishing NF-κB- and MAPK-mediated tolerance of LPS and ETEC.

Our results also suggested that TLR2 may have an important role in the anti-inflammatory activity of *L. jensenii* TL2937, because both this strain and Pam3CSK4 increased expression of A20 and Bcl-3 mRNA and reduced the expression of IL-8 mRNA in ETEC- and LPS-challenged PIE cells. Lactobacilli can stimulate TLR2 on immune cells and lead to secretion of cytokines that are able to improve gut immunity and protect against intestinal pathogens (3, 15, 32). However, TLR2 also seems to be involved in some anti-inflammatory effects of probiotics. Immunobiotics can inhibit IL-12 production by macrophages (33) and dendritic cells (47) by TLR2-mediated recognition of lipoproteins and insoluble cell walls. Moreover, TLR2 can induce tolerance of LPS that is referred to as heterotolerance or cross-tolerance (26). Lipoteichoic acid isolated from *L. plantarum* reduces LPS-induced TNF-α production in THP-1 human monocyte-like cells in a TLR2-dependent manner (14). Therefore, it is possible that *L. jensenii* TL2937 may induce cross-tolerance in IECs by interacting with TLR2. However, more detailed studies are necessary to establish the exact role of TLR2 in *L. jensenii* TL2937-mediated immunomodulatory effects. In addition, we observed that *L. jensenii* TL2937, but not Pam3CSK4, increased expression of MKP-1 and decreased phosphorylation of p38 and IL-6 secretion; these finding indicated that a PRR(s), other than TLR2, may mediate the anti-inflammatory effects of *L. jensenii* TL2937. Identification of the unknown receptor or receptors is another interesting topic for future investigations.

In summary, we have demonstrated that *L. jensenii* TL2937 modulated the intestinal epithelial cells by attenuating LPS- and

ETEC-induced NF-κB and MAPK activation and expression of proinflammatory cytokines. When PIE cells encounter *L. jensenii* TL2937, TLR2 may act synergistically and cooperatively with one or more other PRRs, and these interactions may result in a coordinated sum of signals that induce the upregulation of some negative regulators of TLRs, including A20, Bcl-3, and MKP-1. The upregulation of these negative regulators could have an important physiological impact on maintaining or reestablishing homeostatic TLR signals in PIE cells. A20, Bcl-3, and MKP-1 regulate TLR-induced inflammatory signaling and exert a more definitive impact on the transcriptional outcome of TLR signals. Therefore, PIE cells pretreated with the immunobiotic strain produce lower concentrations of IL-8 and IL-6 in response to TLR4 activation. The lower levels of IL-8 and IL-6 help to regulate the recruitment and activity of inflammatory cells and limit the inflammatory damage. In addition, we showed that our PIE cell *in vitro* system can represent the situation of increased responsiveness to TLR4 activation in piglets; therefore, this system is useful for studying TLR4-mediated inflammatory responses in the gut. PIE cells can be used to study mechanisms involved in the protective activity of immunobiotics against intestinal inflammatory damage, providing useful information for the development of new immunologically functional feeds that could help to prevent inflammatory intestinal disorders, including weaning-associated intestinal inflammation.

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