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ORIGINAL ARTICLE

Immunomodulation and signaling mechanism of *Lactobacillus rhamnosus* GG and its components on porcine intestinal epithelial cells stimulated by lipopolysaccharide

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KEYWORDS
immunomodulation;
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porcine intestinal epithelial cells

Abstract *Background/purpose:* This study aimed to evaluate the immunomodulatory effects and signaling mechanisms of *Lactobacillus rhamnosus* GG (LGG) and its components [surface-layer protein (SLP), DNA, exopolysaccharides, and CpG oligodeoxynucleotides] on lipopolysaccharide (LPS)-stimulated porcine intestinal epithelial cell (IEC) IPEC-J2.
Methods: The mRNA expressions of inflammatory cytokines and Toll-like receptors (TLRs) were measured by quantitative real-time polymerase chain reaction. Activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signaling was detected by western blot and immunofluorescence.
Results: Pretreatment of IPEC-J2 cells with LGG, SLP, or exopolysaccharides significantly alleviated LPS-induced inflammatory cytokines and TLR activation at mRNA level. LGG, SLP, and exopolysaccharides also attenuated LPS-induced MAPK and NF-κB signaling activations. CpG oligodeoxynucleotides significantly increased the interleukin 12, tumor necrosis factor α, and TLR9 mRNA levels and enhanced NF-κB signaling activation in LPS-stimulated cells.
Conclusion: LGG had immunomodulatory effects on LPS-induced porcine IECs by modulating TLR expressions and inhibiting MAPK and NF-κB signaling to decrease inflammatory cytokine

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expressions. Components of LGG exerted immunomodulatory effects on porcine IECs, especially immunostimulatory CpG oligodeoxynucleotides.
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Introduction

Intestinal epithelial cells (IECs) play an important role in the innate immune response to pathogens.[1] The porcine small intestinal epithelial cell line (IPEC-J2), which was isolated from the small intestines of neonatal piglets, has features similar to those of porcine primary IECs.[2] IPEC-J2 was used as a model *in vitro* for studying immune responses in pathogen–host interactions.[3]

Lactic acid bacteria (LAB) are one of the commensal bacteria living in the intestinal tracts of piglets.[4] LAB have beneficial effects on its host, but the immunomodulatory effects of LAB on the innate immune system vary between strains.[5–7] *Lactobacillus rhamnosus* GG (LGG) is a well-known probiotic strain isolated from healthy adult feces by Goldin and Gorbach two decades ago.[8] LGG could alleviate inflammation or pathogen-induced barrier dysfunction,[8–10] and could also prevent intestinal injuries induced by rotavirus diarrhea.[11]

Toll-like receptors (TLRs) are a group of pattern recognition receptors and play a critical role in mucosal immune responses.[12] TLRs can recognize microbe-associated molecular patterns; lipopolysaccharides (LPSs) as one of microbe-associated molecular patterns can cause inflammation in IECs through TLR4.[13] Researches have demonstrated that TLR2, TLR4, and TLR9 are involved in the LAB modulation for intestinal inflammation.[2,5,7] Receptors can activate downstream signaling. Nuclear factor kappa B (NF-κB) signaling is known to play an imperative role in immune responses.[14] In response to inflammatory signals, mitogen-activated protein kinase (MAPK) signaling is also activated by phosphorylation of p38MAPK, extracellular signal-regulated kinase (ERK1/2), and Jun N-terminal protein kinase. Previous reports have indicated that LAB modulate the immune responses in IECs through the NF-κB and MAPK signaling.[15–17] Cytokines are produced by the activation of such signaling as part of the IECs' innate immune response to stimuli.[18] Studies have demonstrated that LAB strains could trigger IECs to produce inflammatory cytokines.[2,7,15]

Components of LAB strains exert various effects on IECs, resulting in various effects of LAB strains on intestines. Surface-layer protein (SLP) from LAB had adhesive properties to prevent pathogen invasion.[19] Exopolysaccharides (EPSs) of LGG form a protective shield against inflammatory factors in the intestines.[20] LGG DNA had immunomodulatory effects on TNF-α-induced IECs.[21] The CpG-oligodeoxynucleotides (CpG-ODNs) 5′-ACTTTCGTTTTCTGCGTCAA-3′ from LGG had immunostimulatory effects on immune cells.[22]

The aims of this study are to evaluate the effects of LGG and its components on the expression of cytokines and TLRs, and to elucidate the mechanisms for comprehensive probiotic modulation of IECs by lactobacilli.

Materials and methods

Reagents and antibodies

LPSs were purchased from Sigma-Aldrich (St Louis, MO, USA). TLR4 inhibitor polymyxin B was purchased from InvivoGen (San Diego, CA, USA). NF-κB inhibitor Pyrrolidinedithiocarbamic acid,ammonium salt (PDTC), ERK inhibitor U0126, and p38MAPK kinase inhibitor SB203580 were purchased from Sigma-Aldrich. The anti-phospho-p65, anti-I-κBα, anti-phospho-p38MAPK, anti-p38MAPK, anti-phospho-ERK1/2, anti-ERK1/2, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). Horseradish peroxidase- or Fluorescein Isothiocyanate (FITC)-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA).

Bacterial strain and culture conditions

The LGG was a gift from Professor Jinru Chen at the University of Georgia, Athens, GA, USA. The bacterium was anaerobically grown at 37°C in de Man, Rogosa, and Sharp broth (MRS broth; Hope Bio, Qingdao, Shandong, China) for 18 hours, and then harvested in the logarithmic growth phase and stored at −80°C. Prior to use, the bacteria were thawed and washed with Dulbecco's Modified Eagle Medium/Ham's F-12 (1:1; DMEM/F12; Gibco, Carlsbad, CA, USA). The number of bacterial cells was determined by the plate-counting agar method. The bacterial counts were expressed as colony forming units per milliliter.

Cell culture

The IPEC-J2 cells were a generous gift from Dr Yizhen Wang (Zhejiang University, Hangzhou, P.R. China) and were originally generated in the laboratory of Dr Anthony Blikslager at North Carolina State University (Raleigh, NC, USA).[23] The cells were maintained in an incubator at 37°C in 5% CO_2 in DMEM/F12 supplemented with 10% (v/v) fetal bovine serum (Gibco). Cells (1×10^5 cells/well) were seeded n plastic six-well culture plates (Corning, Tewksbury, MA, USA) and maintained for 14 days (1×10^6 cells/well).

Preparation of components from LGG

The LGG was grown as described above. A bacterial genomic DNA extraction kit (Aidlab, Beijing, China) was used to extract genomic DNA according to previous methods.[24] SLP was obtained from LGG using ultrafiltration with 5M LiCl.[25] EPSs were extracted using trichloroacetic

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acid and ethanol according to previous methods.[26] The CpG-ODN 5′-ACTTTCGTTTTCTGCGTCAA-3′ was synthesized by Sangon Inc. (Shanghai, China).

LGG immunomodulation of LPS-stimulated IPEC-J2 cells

IPEC-J2 cells were grown and maintained as described above. The LPS concentration (1 μg/mL) and LGG multiple of infection (= 20) were optimized (Figure S1). In each of the four groups (control, LPS, LPS + LGG, and LGG), the following three independent treatments were conducted: (1) post-LPS stimulation treatment—IPEC-J2 cells were induced with 1 μg/mL LPS for 4 hours, and then LPS was removed and cells were treated with LGG for another 4 hours; (2) simultaneous LPS stimulation treatment—IPEC-J2 cells were incubated with LGG and 1 μg/mL LPS for 4 hours; and (3) pre-LPS stimulation treatment—IPEC-J2 cells were treated with LGG for 4 hours, and then LGG was removed and cells were induced with 1 μg/mL LPS for another 4 hours. The treated cells were washed with cold phosphate buffered saline twice and subjected to RNA extraction and western blot.

Cells pretreated by LGG components and then stimulated by LPS

Confluent monolayers of IPEC-J2 cells, as described above, were incubated for 4 hours with 1 μg/mL of one of LGG components being tested (SLP, DNA, EPS, or CpG-ODN) and then stimulated with 1 μg/mL LPS for 4 hours. IPEC-J2 cells were washed twice before being used for RNA or protein extraction.

Inhibitor treatments

Confluent monolayers of IPEC-J2 cells, as described above, were incubated with individual inhibitors (15μM PDTC, 10μM U0126, 20μM SB23058, or 50 μg/mL polymyxin B) for 30 minutes prior to their co-incubation with LPS and LGG for 4 hours, as mentioned above. The cells were washed twice before RNA extraction.

Total RNA extraction and reverse transcription

Total RNA was extracted from IPEC-J2 cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The purity and integrity of RNA samples were measured using a NanoDrop spectrophotometer (Thermo, Wilmington, DE, USA). Total RNA samples were suspended in RNase-free water and stored at −80°C until use. Reverse transcription were conducted using a reverse transcription kit (Takara Bio, Shiga, Japan), and cDNA was stored at −20°C until use.

Quantitative real-time polymerase chain reaction analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Premix Ex Taq (Takara Bio), according to the manufacturer's instructions. The reaction was conducted using an Mx3000P system (Agilent, Palo Alto, CA, USA) that was programmed to subject the samples to denaturation at 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds. The sequences for PCR primers[13] were as follows: β-actin (5′-CAGGTCATCACCATCGGCAACG-3′, 5′-GACAGCACCGTGTTGGCGTAGAGGT-3′); IL-6 (5′-TGGCTACTGCCTTCCCTACC-3′, 5′-CAGAGATTTTGCCGAGGATG-3′); IL-12 (5′-GGAGTATAAGAAGTACAGAGTGG-3′, 5′-GATGTCCCTGATGAAGAAGC-3′); TNF-α (5′-CCTCTTCTCCTTCCTCCTG-3′, 5′-CCTCGGCTTTGACATTGG-3′); TLR2 (5′-TCACTTGTCTAACTTATCATCCTCTTG-3′, 5′-TCAGCGAAGGTGTCATTATTGC-3′); TLR4 (5′-GCCATCGCTGCTAACATCATC-3, 5′-CTCATACTCAAAGATACACCATCGG-3′); and TLR9 (5′-CACGACAGCCGAATAGCAC-3′, 5′- GGGAACAGGGAGCAGAGC-3′).

The data were analyzed using the Mx3000P system software (Agilent). All gene quantifications were performed with β-actin as an internal standard, and the relative quantification of gene expression was analyzed by the cycle threshold (Ct) method as follows:

$$\Delta\Delta Ct = (Ct_{targetgene} - Ct_{housekeepinggene})\ \text{treatment} - (Ct_{targetgene} - Ct_{housekeepinggene})\ \text{control}.$$

The final data were derived from the formula $2^{-\Delta\Delta Ct}$.

Western blotting

Treated IPEC-J2 cells were lysed using lysis buffer (Sigma-Aldrich) according to the manufacturer's instructions. Concentration of protein in samples was determined using Bradford's method.[27] The total protein samples were loaded on Sodium dodecyl sulfate-Polyacrylamide gelelectrophoresis (SDS-PAGE) and then transferred to Poly vinylidene fluoride (PVDF) membranes. The membrane was blocked and incubated with primary antibody at 4°C overnight. After incubation with the horseradish peroxidase-conjugated secondary antibody, the blot was developed with electrochemiluminescence (Millipore; Merck KGaA, Darmstadt, Germany). The optical density of the bands was measured using Image J software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

According to a previous method,[28] IPEC-J2 cells were blocked with acetone for 30 minutes at 4°C. The cells were then incubated with primary antibody at 4°C overnight. After incubation with FITC-conjugated secondary antibody in a dark room, the cells were incubated with 4,6-Diamidino-2-phenylindole dihydrochlorid (DAPI) (Sigma-Aldrich), washed twice, and observed under an immunofluorescence microscope (ECLIPSE Ti; Nikon Corp., Tokyo, Japan). All micrographs were taken with identical exposure times and in the center of each well.

Statistical analysis

The data are expressed as the means ± standard deviations of the replications. The statistical significance of the differences was evaluated by using one-way analysis of

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variance [general linear model (GLM)], followed by Duncan's multiple range tests. Differences are considered significant if $p < 0.05$. Statistical analyses were performed using the SAS program (SAS Institute, Inc., Cary, NC, USA).

Results

LGG regulation of cytokines in LPS-stimulated IPEC-J2 cells

To determine the effect of LGG on the cytokine responses of IPEC-J2 cells, IPEC-J2 cells were pre-, post-, or cotreated with LGG when stimulated by LPS. The mRNA expression levels of IL-6, IL-12, and TNF-α in cells were measured using qRT-PCR (Figure 1). In all three treatments, cells pre-, post-, or costimulated with LPS elicited significantly higher cytokine mRNA expression than that in untreated cells, while LGG in combination with LPS induced significantly lower proinflammatory cytokine levels than LPS alone (Figure 1; $p < 0.05$), indicating that LGG downregulated the proinflammatory cytokines in cells induced by LPS. Meanwhile, in all three treatments, the presence of LGG induced significantly higher mRNA levels of inflammatory cytokines in cells compared with the medium control (Figure 1; $p < 0.05$).

Effect of LGG on TLR mRNA in LPS-stimulated IPEC-J2 cells

To evaluate the regulation of TLR responses by LGG in IPEC-J2 cells, cells were pre-, post-, or cotreated with LGG when stimulated by LPS. TLR2, TLR4, and TLR9 mRNA expression levels were measured by qRT-PCR (Figure 2). In all three treatments, the mRNA levels of TLR2 and TLR9 were significantly higher ($p < 0.05$) in LGG-treated cells than those in the control cells. LPS induced significantly higher levels of not only TLR4 mRNA, but also TLR2 and TLR9 mRNA (Figure 2). Meanwhile, TLR2, TLR4, and TLR9 mRNA levels were significantly lower in cells pre-, post-, or cotreated with LGG when stimulated with LPS than those in LPS-induced cells (Figure 2; $p < 0.05$).

Effects of LGG components on cytokine and TLR responses in LPS-stimulated IPEC-J2 cells

To determine the protective effects of different components (SLP, DNA, CpG-ODN, and EPS) of LGG on IPEC-J2 cells after LPS challenge, the cytokine and TLR expression levels were determined by qRT-PCR. Four components of LGG had immunoregulatory effects on the immune responses of IECs. Compared with cells stimulated with LPS alone, SLP and EPS significantly reduced the mRNA expression of cytokines IL-6, IL-12, and TNF-α in cells after LPS challenge (Figure 3; $p < 0.05$). Interestingly, LGG genomic DNA had no effects on the proinflammatory cytokine mRNA levels in the cells after LPS stimulation. Moreover, CpG-ODNs significantly increased the IL-12 and TNF-α mRNA levels in the cells after LPS challenge (Figure 3; $p < 0.05$), suggesting that CpG-ODNs and with LPS had synergistic immunostimulatory effects on IECs. EPS and SLP suppressed TLR2, TLR4, and TLR9 mRNA expression levels in the cells after LPS stimulation compared with those in cells induced with LPS alone (Figures 4A and 4B; $p < 0.05$). CpG-ODNs significantly increased the TLR9 mRNA level, which is a specific receptor for CpG-ODNs (Figure 4C; $p < 0.05$).

LGG and its components modulate NF-κB and MAPK signaling in LPS-induced IPEC-J2 cells

In order to evaluate the phosphorylation level of signaling factors, we conducted pre-LPS stimulation treatment to investigate the IEC signaling involved in the immune responses to LPS in the presence of LGG and its components, the molecules of the NF-κB and MAPK signaling were detected by western blotting and immunofluorescence. IPEC-J2 cells pretreated with LGG had significantly lower phosphorylation levels of p65, p38, and ERK, while higher levels of I-κBα after LPS stimulation, than in cells induced with LPS alone (Figure 5).

Next, IPEC-J2 cells were pretreated with the individual components of LGG and then stimulated by LPS. Compared with the cells stimulated by LPS alone, cells pretreated with SLP or EPS after LPS stimulation had significantly lower phosphorylation levels of p38 (Figure 6A) and p65 (Figure 6C), but significantly higher I-κBα levels (Figure 6D). DNA and CpG-ODNs had no suppressive effects on the phosphorylation levels of p38 (Figure 6A) and ERK (Figure 6B), and expression level of I-κBα (Figure 6D) in LPS-stimulated cells, but CpG-ODNs significantly increased p65 phosphorylation (Figure 6C).

Signaling activations in IPEC-J2 cells pretreated with LGG or its components for 4 hours and then induced with LPS were verified by immunofluorescence. Consistent with previous western blotting results (Figures 5 and 6), pretreatment of LGG reduced the level of p-p38 (Figure 7A) and p-ERK (Figure 7B), but increased the level of I-κBα in LPS-induced cells (Figure 7C). Compared with cells stimulated with LPS alone, pretreatment of SLP and EPS decreased the level of p-p38 (Figure 8A) while increased the level of I-κBα (Figure 8C), and SLP also decreased the level of p-ERK (Figure 8B) in cells.

Inhibition of signaling during LGG immunomodulation of IPEC-J2 cells

In order to verify the signaling pathways involved in the immunomodulation of LGG on host, we conducted experiments with LGG and LPS simultaneously to stimulate the cells that were pretreated by specific inhibitors for 30 minutes. The mRNA levels of the proinflammatory cytokines IL-6, IL-12, and TNF-α were detected by qRT-PCR. Results showed that IL-6, IL-12, and TNF-α mRNA levels significantly decreased in the LPS- and LGG-treated cells in the presence of NF-κB, p38, or ERK inhibitors (Figure 9), indicating that LGG and LPS could modulate cytokine mRNA levels via the activation of the NF-κB, p38, and ERK pathways in IECs. The ERK inhibitor decreased the levels of the proinflammatory cytokine IL-12 mRNA, but had no significant effect on IL-6 and TNF-α in the LPS-induced cells with LGG treatment.

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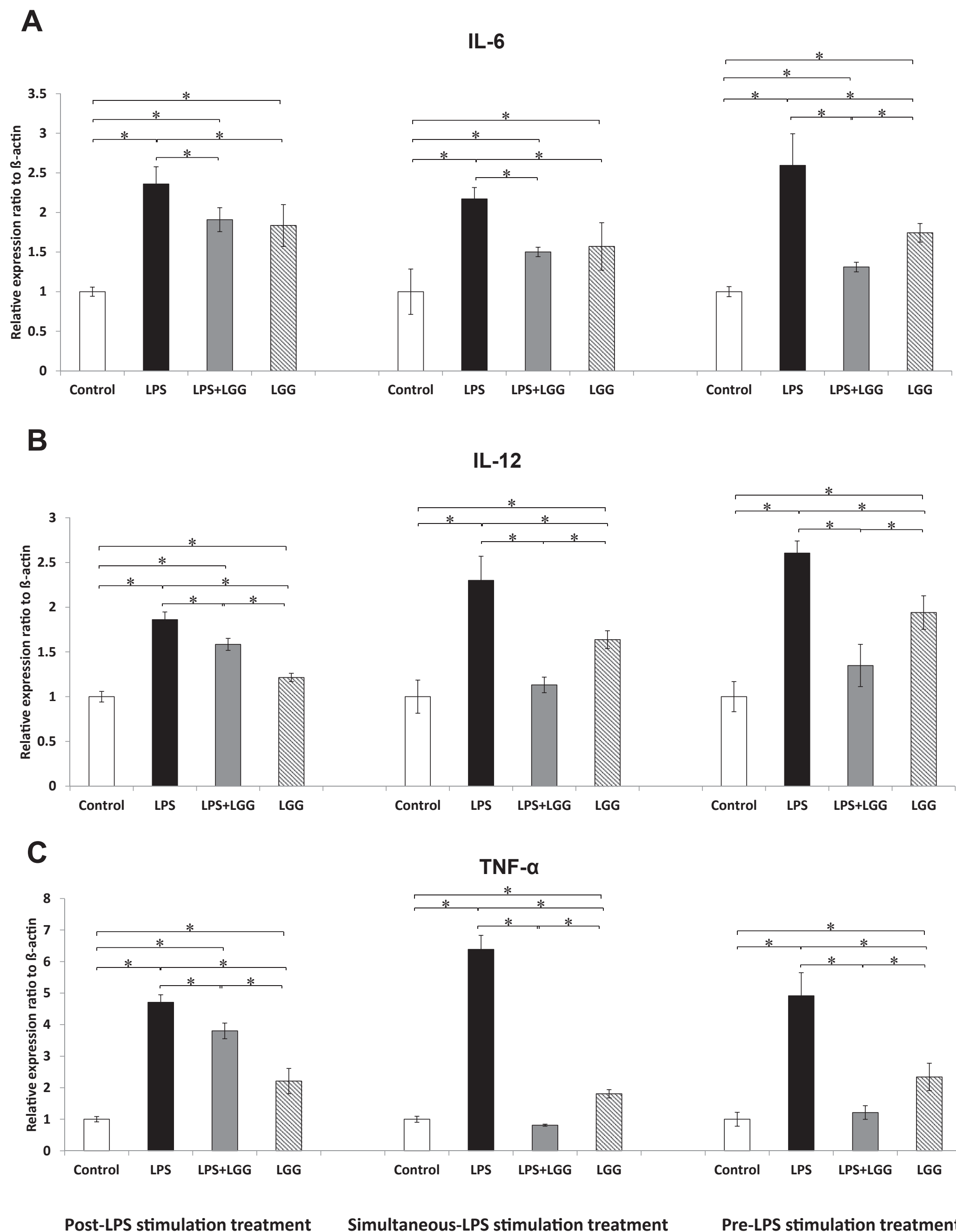

Figure 1. Probiotic effects of LGG on cytokine levels in LPS-induced IPEC-J2 cells. The monolayers of IPEC-J2 cells were induced with 1 μg/mL LPS for 4 hours prior to treatment with LGG (MOI = 20) for another 4 hours, treated with LGG prior to the LPS challenge, or simultaneously treated with both for 4 hours. The cytokine expression levels were detected by qRT-PCR. The values are expressed as the mean ± SD ($n = 3$). $*p < 0.05$. IL = interleukin; LGG = *Lactobacillus rhamnosus* GG; LPS = lipopolysaccharide; MOI = multiple of infection; qRT-PCR = quantitative real-time polymerase chain reaction; SD = standard deviation; TNF-α = tumor necrosis factor alpha.

Discussion

IECs function as the first line of defense against pathogens, and react to them by producing cytokines and chemokines to maintain gut homeostasis.[1] LGG is one of probiotics that can dose-dependently regulate immune responses in intestines.[11] LGG not only alleviates inflammation,[9] but also has an immunostimulatory effect on IECs.[2] In this study, we investigated the effects of LGG and its components on LPS-stimulated porcine IECs using the IPEC-J2 cell line.

LPS trigger inflammation in IECs through TLR4,[13] whereas LAB strains can interact with IECs via various TLRs. A previous study showed that host TLR2, TLR4, and TLR9 mRNA levels were increased as a result of *Salmonella* exposure, but decreased in the presence of LGG in HT-29 cells.[7] Consistent with a previous study, although LGG

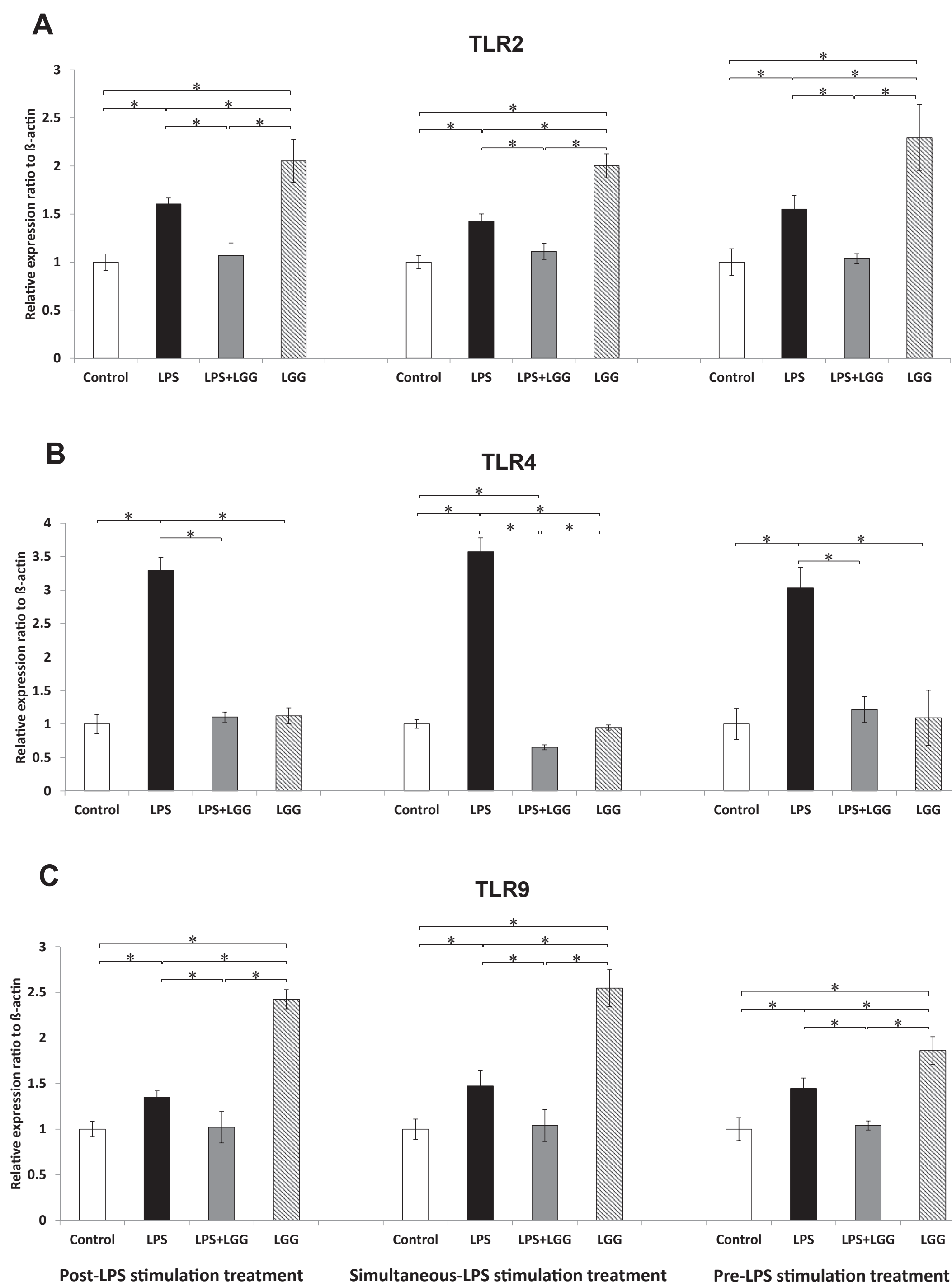

Figure 2. Probiotic effects of LGG on TLR levels in LPS-induced IPEC-J2 cells. The monolayers of IPEC-J2 cells were induced with 1 μg/mL LPS for 4 hours prior to treatment with LGG (MOI = 20) for another 4 hours, treated with LGG prior to the LPS challenge, or simultaneously treated with both for 4 hours. The TLR expression levels were detected by qRT-PCR. The values are expressed as the mean ± SD ($n = 3$). *$p < 0.05$. LGG = *Lactobacillus rhamnosus* GG; LPS = lipopolysaccharide; MOI = multiple of infection; qRT-PCR = quantitative real-time polymerase chain reaction; SD = standard deviation; TLR = Toll-like receptor.

upregulated significantly higher TLR2 and TLR9 mRNA levels in cells, LGG downregulated TLR2, TLR4, and TLR9 mRNA levels in cells after LPS stimulation (Figure 2). Accordingly, decreased proinflammatory cytokine responses at the transcriptional level appeared in LPS-stimulated cells treated with LGG (Figure 1). These results indicate that LGG may exert probiotic activities through a TLR2- or TLR9-dependent pathway. TLR2 is involved in the *Lactobacillus amylovorus*-mediated inhibition of TLR4 inflammatory signaling in Caco-2 cells.[29] The *Lactobacillus plantarum*-induced inhibition of the TNF-α signaling is accompanied by the suppressed mRNA expression of TLR2, TLR4, and

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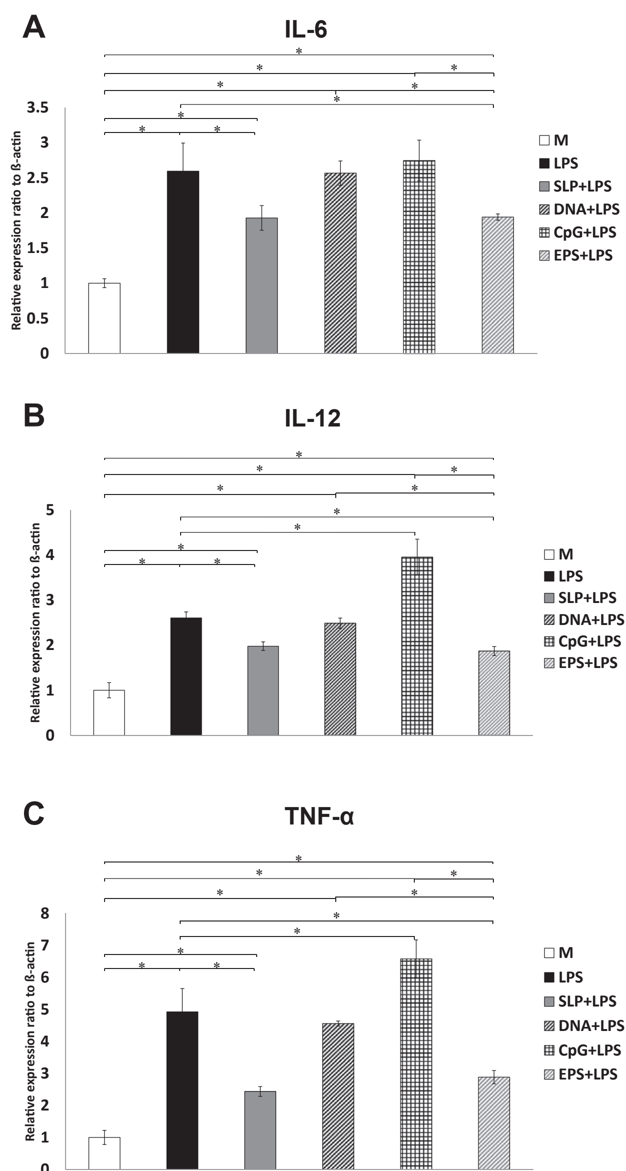

Figure 3. The mRNA expression of cytokines in IPEC-J2 cells treated with LPS and LGG components. Cells were preincubated with SLP, DNA, CpG-ODN, or EPS for 4 hours and then stimulated with LPS for 4 hours. The cytokine mRNA expression levels were measured using qRT-PCR. The values are expressed as the mean ± SD ($n = 3$). Each experiment was repeated twice with similar results. *$p < 0.05$. CpG-ODN = CpG oligodeoxynucleotide; EPS = exopolysaccharide; LGG = *Lactobacillus rhamnosus* GG; LPS = lipopolysaccharide; qRT-PCR = quantitative real-time polymerase chain reaction; SD = standard deviation; SLP = surface-layer protein.

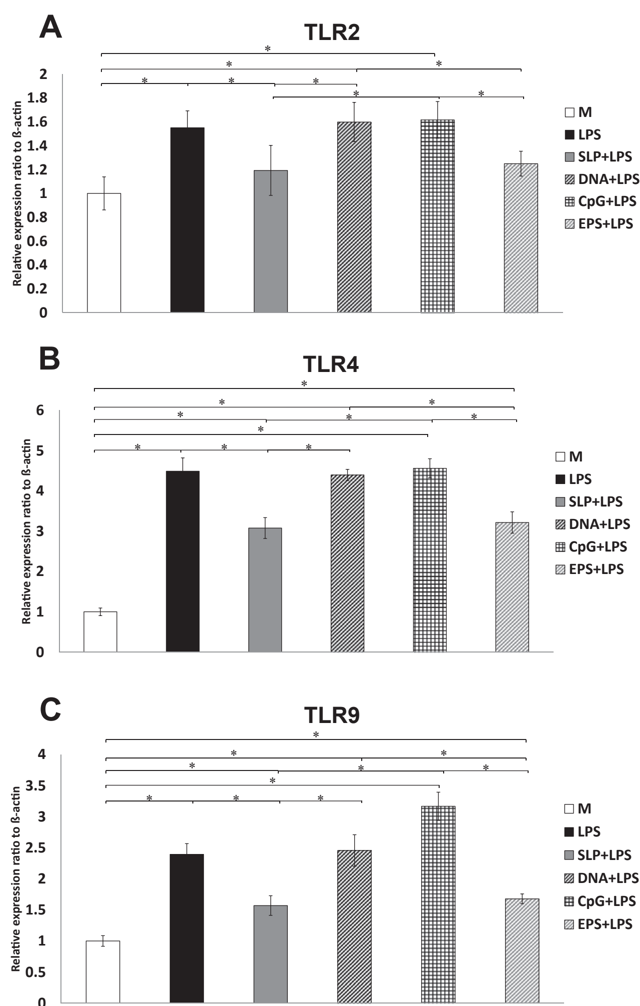

Figure 4. The mRNA expression of TLRs in IPEC-J2 cells treated with LPS and LGG components. The cells were preincubated with SLP, DNA, CpG-ODN, and EPS for 4 hours and then stimulated with LPS for 4 hours. The TLR mRNA expression levels were measured using qRT-PCR. The values are expressed as the mean ± SD ($n = 3$). Each experiment was repeated twice with similar results. *$p < 0.05$. CpG-ODN = CpG oligodeoxynucleotide; EPS = exopolysaccharide; LGG = *Lactobacillus rhamnosus* GG; LPS = lipopolysaccharide; qRT-PCR = quantitative real-time polymerase chain reaction; SD = standard deviation; SLP = surface-layer protein; TLR = Toll-like receptor.

TLR9.[30] For determining whether LGG interferes with LPS-specific TLR4 signaling by exerting effects on the TLR2 or TLR9 pathways, further studies using porcine-specific TLR2 or TLR9 antagonists are needed.

LGG interacts with IECs through TLRs and other receptors cooperatively, thereby activating or inhibiting downstream cell signaling, including NF-κB and MAPK signaling, to modify the transcription of extracellular stimulus-response genes. Stimuli can activate p38MAPK, which is always associated with NF-κB signaling in LAB—host immune responses. NF-κB and MAPK signaling are activated via phosphorylation, while it is hard to identify dynamic changes in the phosphorylation status when microbe—host cell interactions take place. Therefore, we used LGG or its components before LPS stimulation treatment to investigate the effects of LGG and its components on cell signaling activations.

Previous evidences revealed that lactobacilli stimulated innate immune responses by upregulating cytokines in IECs.[7] *Lactobacillus acidophilus* induces cytokine and chemokine productions via NF-κB and p38MAPK signaling in IECs.[15] Consistent with the above studies, we found that

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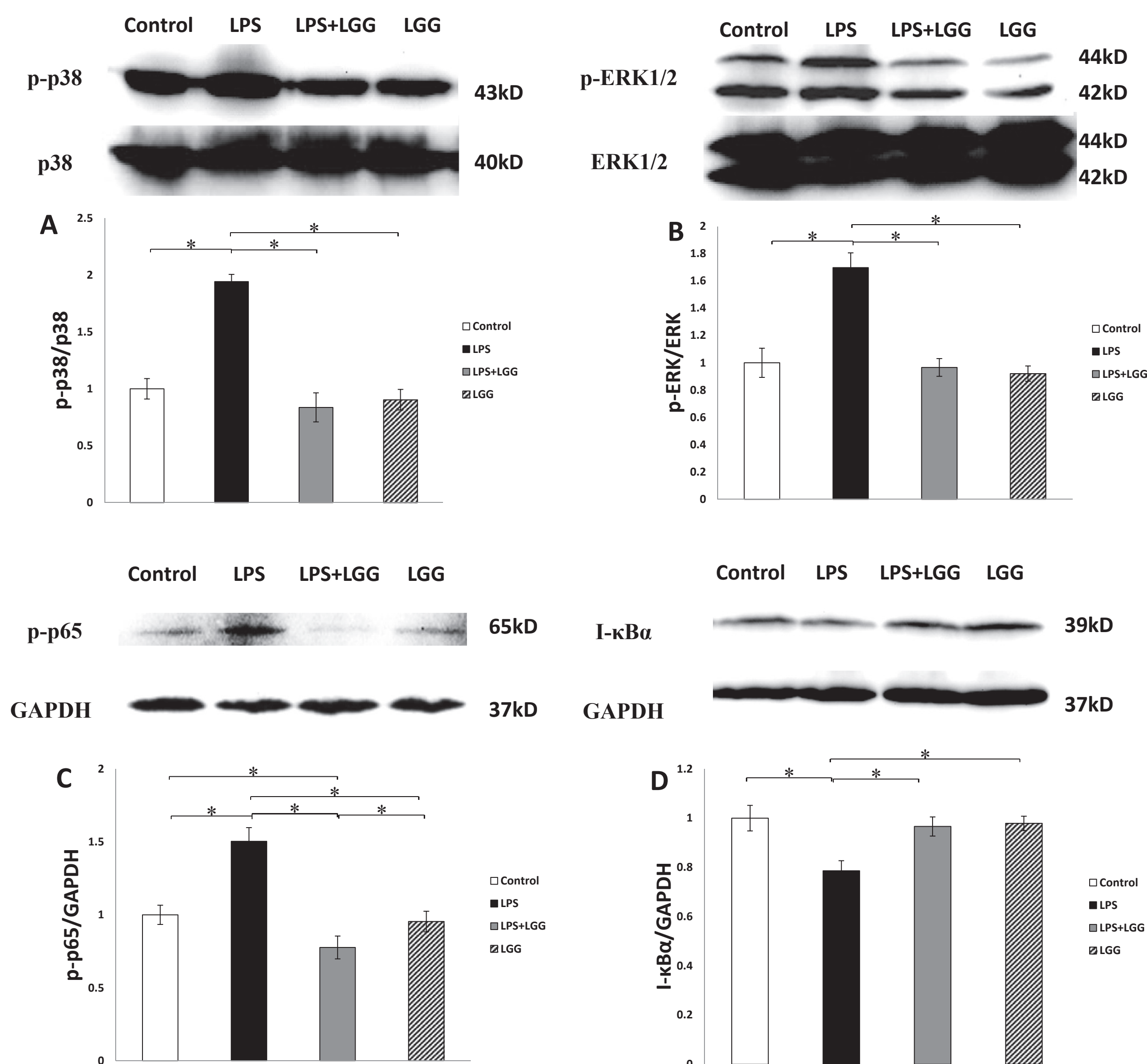

Figure 5. Western blot analysis of MAPK and NF-κB activation in IPEC-J2 cells following LGG treatment and LPS challenge. (A) Ratio of p-p38/p38 in IPEC-J2 cells treated with LGG for 4 hours, followed by with LPS for 30 minutes. (B) Ratio of p-ERK/ERK in IPEC-J2 cells treated with LGG for 4 hours, followed by with LPS for 30 minutes. (C) Ratio of p-p65/GAPDH in IPEC-J2 cells treated with LGG for 4 hours, followed by with LPS for 30 minutes. (D) Ratio of I-κBα/GAPDH in IPEC-J2 cells treated with LGG for 4 hours, followed by with LPS for 30 minutes. The values are expressed as the mean ± SD ($n = 3$). *$p < 0.05$. ERK = extracellular signal-regulated kinase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; LGG = *Lactobacillus rhamnosus* GG; LPS = lipopolysaccharide; MAPK = mitogen-activated protein kinase; NF-κB = nuclear factor kappa B; qRT-PCR = quantitative real-time polymerase chain reaction; SD = standard deviation; SLP = surface-layer protein; TLR = Toll-like receptor.

LGG alone also could upregulate mRNA level of cytokines in IECs (Figure 1), although LGG alone did not increase phosphorylation levels of cell signaling factors (p38 and ERK) in cells after 4 hours of incubation (Figure 5). This interesting phenomenon might be due to the tolerance of cell signal activation or the negative feedback regulation by the increased cytokines such as IL-6, IL-12, and TNF-α. In fact, in our pilot experiment, LGG could activate time-point-dependent phosphorylation of p38 and ERK. Meanwhile, LGG could alleviate LPS-induced inflammation by suppressing NF-κB and p38MAPK signaling (Figures 1 and 5). In line with our findings, *L. plantarum* suppresses proinflammatory cytokine production by inhibiting both NF-κB and p38MAPK.[17] *Lactobacillus jensenii* attenuates proinflammatory responses by modulating NF-κB and p38MAPK signaling.[16] Previous studies also showed that LGG alleviates inflammation in epithelial barriers by inhibiting NF-κB signaling.[9,31] These studies were confirmed by using specific signaling inhibitors, which decreased cytokine mRNA levels in LPS-induced cells (Figure 9). The results indicated that LGG could enhance host immune tolerance and have beneficial effects on host via inhibition of NF-κB and p38MAPK signaling. By contrast, LGG could also trigger cytokine productions by activating inflammatory signaling to maintain the intestinal homeostasis of host.

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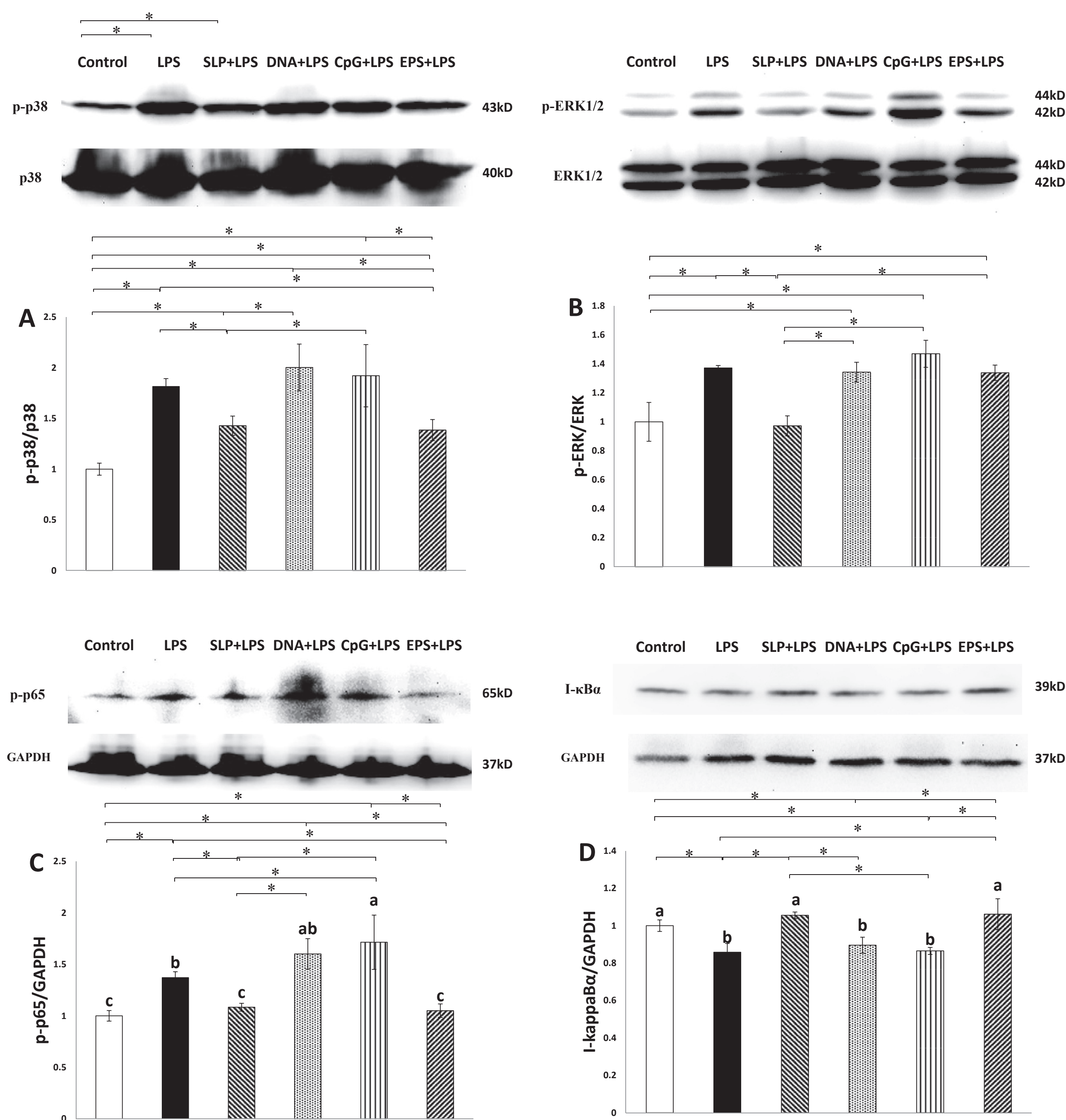

Figure 6. Western blot analysis of MAPK and NF-κB activation in IPEC-J2 cells following LGG component treatment and LPS challenge. (A) Ratio of p-p38/p38 in IPEC cells pretreated with LGG components for 4 hours, followed by with LPS for 30 minutes. (B) Ratio of p-ERK/ERK in IPEC cells pretreated with LGG components for 4 hours, followed by with LPS for 30 minutes. (C) Ratio of p-p65/GAPDH in IPEC cells pretreated with LGG components for 4 hours, followed by with LPS for 30 minutes. (D) Ratio of I-κBα/GAPDH in IPEC cells pretreated with LGG components for 4 hours, followed by with LPS for 30 minutes. The values are expressed as the means ± SD ($n = 3$). *$p < 0.05$. ERK = extracellular signal-regulated kinase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; LGG = *Lactobacillus rhamnosus* GG; LPS = lipopolysaccharide; MAPK = mitogen-activated protein kinase; NF-κB = nuclear factor kappa B; qRT-PCR = quantitative real-time polymerase chain reaction; SD = standard deviation; SLP = surface-layer protein; TLR = Toll-like receptor.

Studies have shown that ERK1/2 plays an important role in the maintenance of IEC homeostasis. *L. plantarum* inhibits ERK1/2 activation in TNF-α-treated IECs.[32] Our research confirmed that LGG could inhibit the activation of ERK1/2 signaling in LPS-induced IPEC-J2 cells (Figure 5B). The ERK1/2 inhibitor significantly downregulated IL-12 mRNA level in the cells induced with LPS and LGG together (Figure 9B). The results indicated that ERK1/2

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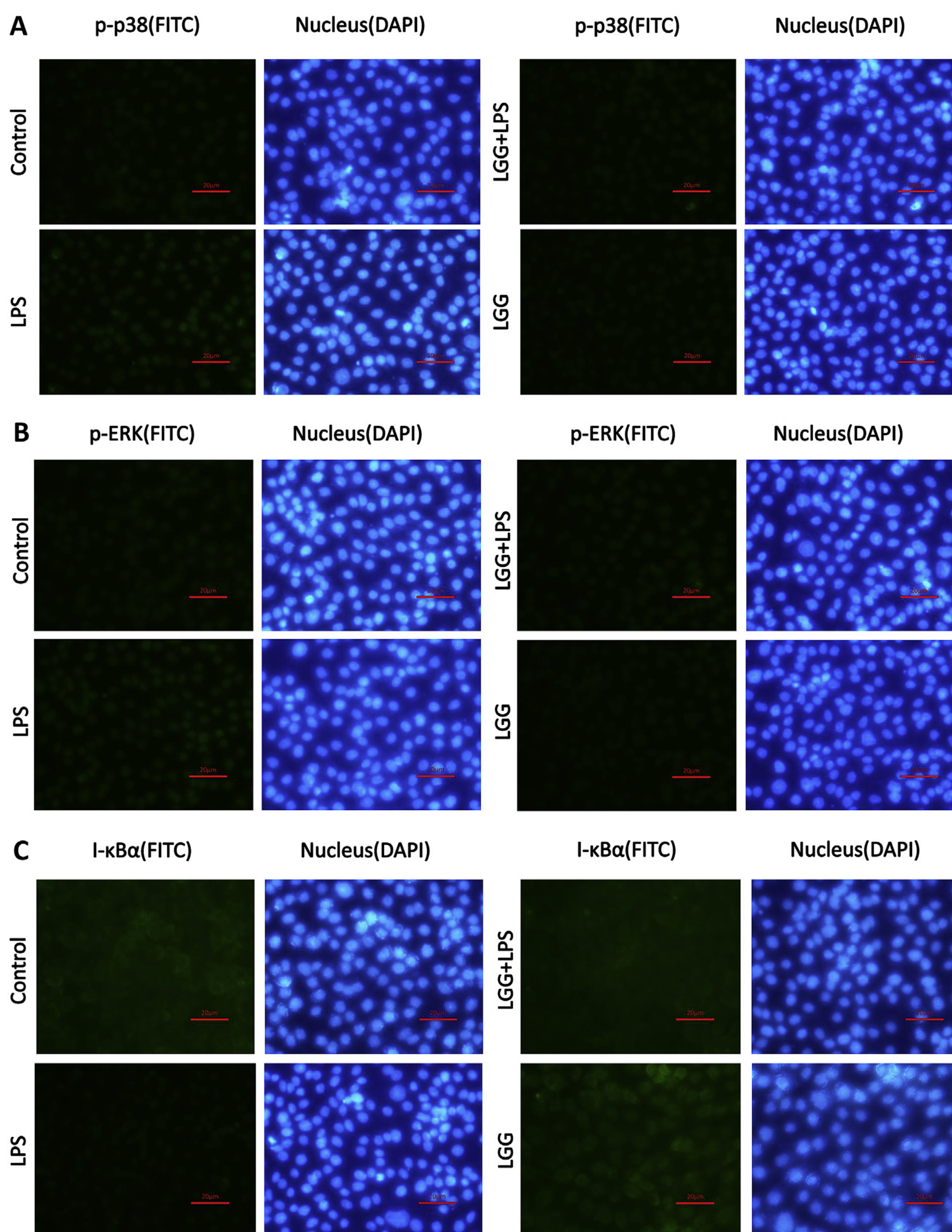

Figure 7. Immunofluorescence analysis of the signaling in LGG-pretreated IPEC-J2 cells stimulated with LPS: (A) p-p38, (B) p-ERK1/2, and (C) I-κBα. The cells were incubated with LGG for 4 hours, followed by with LPS for 30 minutes. Then, the expression of p-p38, p-ERK1/2, or I-κBα (green light) was observed by IF using the same exposure times. All images were taken in the center of the well. ERK = extracellular signal-regulated kinase; IF = immunofluorescence; LGG = *Lactobacillus rhamnosus* GG; LPS = lipopolysaccharide.

might collaborate with p38 and NF-κB to maintain intestinal homeostasis.

Components of lactobacilli have various effects on host immune responses to maintain gut homeostasis. In addition to adhesive properties of SLP that inhibit pathogenic invasions,[19] we focused on other properties of SLP in the LAB–host interaction. In the current study, SLP from LGG inhibited p38MAPK signaling in IPEC-J2 cells after LPS stimulation (Figures 6 and 8), resulting in decreased transcription of inflammatory cytokines (Figure 3). These findings are consistent with previous reports that SLP from LAB had protective roles in IECs by blocking adhesion-dependent inflammatory signalling.[33] In our study, SLP also inhibited ERK1/2 signaling in cells after LPS stimulation (Figure 6B); thus, varied effects of SLP on intestines need further study.

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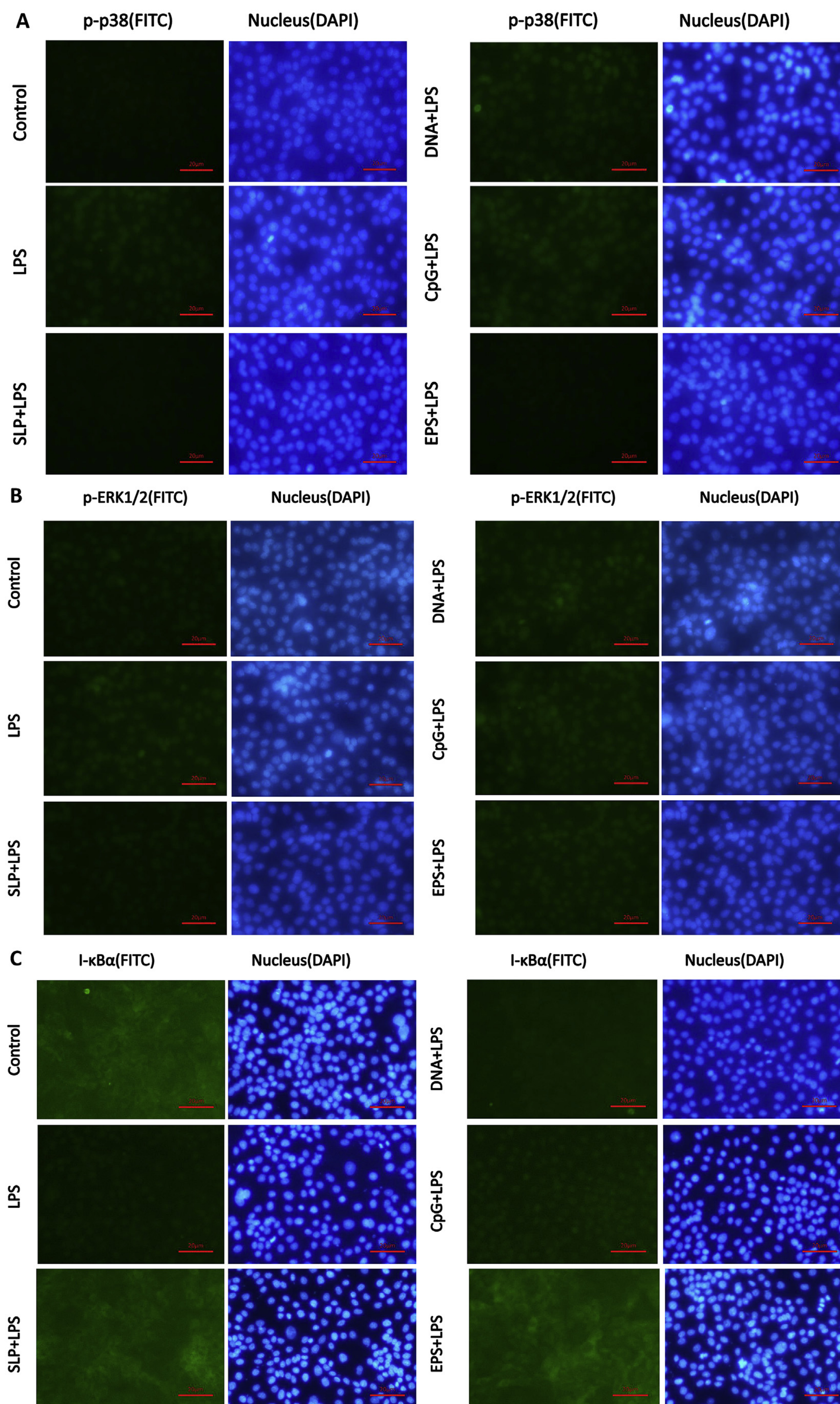

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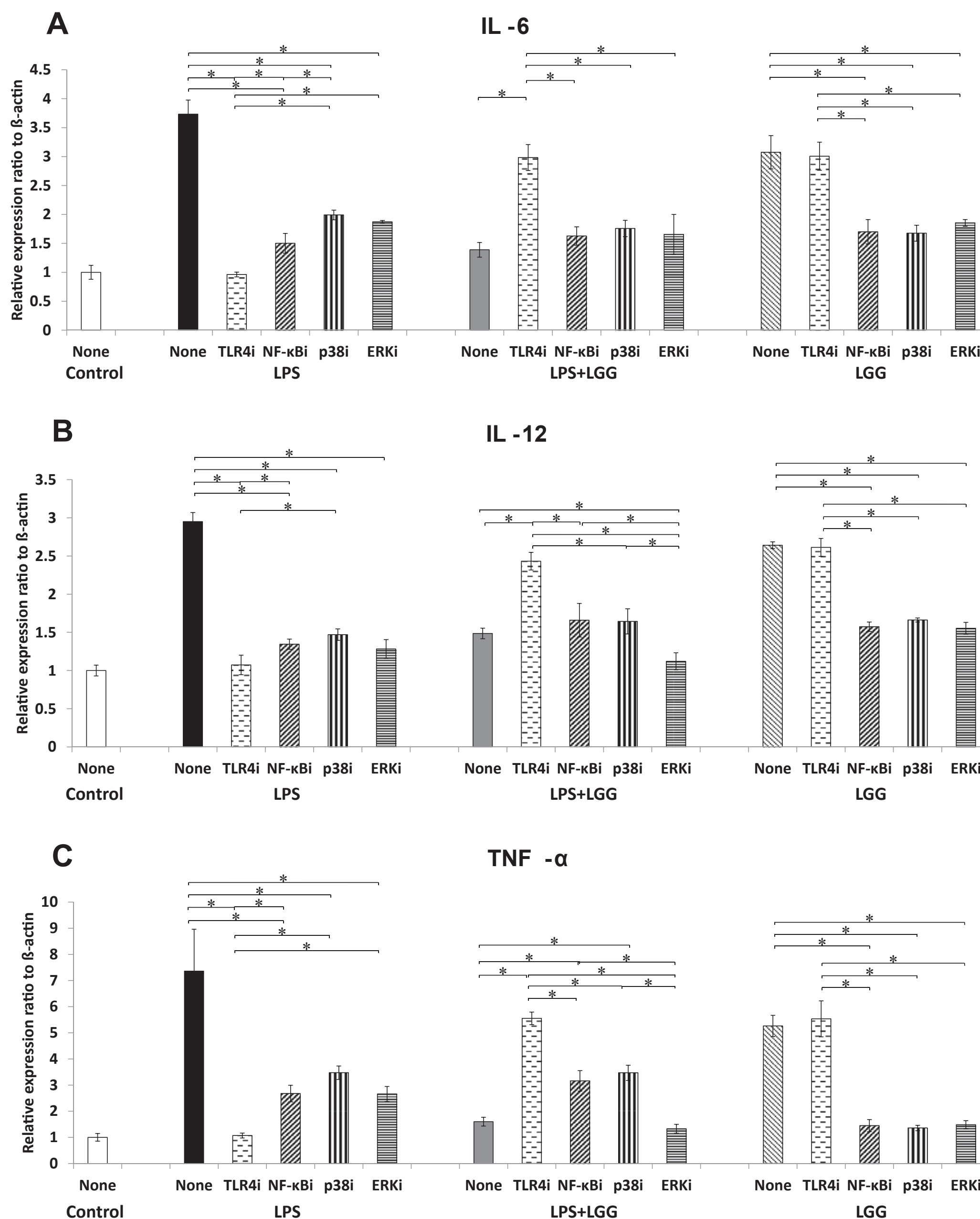

Figure 9. Cytokine expression in IPEC-J2 cells pretreated with specific inhibitors (50 μg/mL PMB, 20μM PDTC, 20μM U0126, or 15μM SB203580) before coincubation with LPS and LGG for 4 hours. Cytokine levels were detected using qRT-PCR. The values are expressed as the mean ± SD ($n = 3$). *$p < 0.05$. ERK = extracellular signal-regulated kinase; IL = interleukin; LGG = *Lactobacillus rhamnosus* GG; LPS = lipopolysaccharide; NF-κB = nuclear factor kappa B; qRT-PCR = quantitative real-time polymerase chain reaction; SD = standard deviation; TLR = Toll-like receptor; TNF-α = tumor necrosis factor alpha.

EPSs from LGG form a protective shield against innate immune factors in the intestine.[20] EPSs from *Lactobacillus* RW-9595M induced immunosuppression by decreasing inflammatory cytokines in macrophages.[34] Consistent with these reports, we found that EPS reduced inflammatory cytokine mRNA levels in LPS-induced cells by inhibiting p38MAPK and NF-κB signaling. These results indicate that EPS exerts immunomodulatory effects in IECs to alleviate inflammation.

Figure 8. Immunofluorescence analysis of the phosphorylation of (A) p38 and (B) ERK1/2 as well as the degradation of (C) I-κBα in IPEC-J2 cells pretreated with LGG components. The cells were incubated with the individual LGG components for 4 hours, followed by with LPS for 30 minutes. Then, the expression of p-p38 (green light, A), p-ERK1/2 (green light, B) or I-κBα (green light, C) was observed by IF using the same exposure times. All images were taken in the center of the well. ERK = extracellular signal-regulated kinase; IF = immunofluorescence; LGG = *Lactobacillus rhamnosus* GG; LPS = lipopolysaccharide.

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Strains of *L. rhamnosus* that are marketed as probiotics have a high count of unmethylated CpG motifs that can be recognized by TLR9.[35] The upregulation of TLR9 expression coincides with significantly increased TNF-α production induced by LPS plus CpG-ODNs.[36] Pre-exposure of macrophages to CpG-ODNs for short periods augments the amount of TNF-α generated after an LPS challenge.[37] Consistent with past studies, our results show that IPEC-J2 cells pretreated with CpG-ODNs after LPS stimulation enhanced cytokine transcription by activating MAPK and NF-κB signaling (Figures 3 and 6), indicating that CpG-ODNs exert immunostimulatory effects on IECs.

In conclusion, the results of this study improve our understanding of the mechanisms underlying the probiotic effects of LGG, demonstrating that LGG alleviates inflammation in LPS-stimulated porcine IECs by modulating TLRs and inhibiting MAPK and NF-κB signaling. Components of LGG exert immunomodulatory effects on the immune responses of porcine IECs. Further studies are needed to evaluate the immunomodulatory effects and mechanisms of LGG and its purified components in IECs.

Conflicts of interest

The authors declare no financial conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jmii.2015.05.002.

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