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ORIGINAL ARTICLE

Lactobacillus rhamnosus GG components, SLP, gDNA and CpG, exert protective effects on mouse macrophages upon lipopolysaccharide challenge

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Significance and Impact of the Study: *Lactobacillus rhamnosus* GG (LGG) is widely used as probiotics. However, its main components are not well known for affecting immunomodulation. This study investigated the effects of pre-treatments with different components such as surface layer protein, genomic DNA and unmethylated cytosine-phosphate-guanine-containing oligodeoxynucleotides, alone or in combination on immunomodulation, and evaluated the signalling mechanism in mouse macrophage RAW264.7 cells challenged with lipopolysaccharide. Pre-incubation with components alone or in combination generally inhibited the activation of Toll-like receptor, mitogen-activated protein kinases, extracellular regulated protein kinases and nuclear factor-kappa B signalling pathways in lipopolysaccharide-stimulated cells, which generally leads to attenuated inflammatory cytokine interleukin-6 and tumour necrosis factor alpha production. These results indicate that nonviable probiotic LGG components exert an anti-inflammation effect on epithelial cells.

Keywords
CpG, gDNA, immunomodulation, *Lactobacillus rhamnosus* GG, macrophages, surface layer protein.

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Abstract

The aim of this study was to determine whether *Lactobacillus rhamnosus* GG (LGG) components (surface layer protein, SLP; genomic DNA, gDNA; unmethylated cytosine-phosphate-guanine-containing oligodeoxynucleotide, CpG-ODN), alone or in combination, could affect immunomodulation, and evaluate the signalling mechanism in mouse macrophage RAW264.7 cells challenged with lipopolysaccharide (LPS). LGG components were used to treat cells before LPS stimulation. Cytokine and Toll-like receptor (TLR) expression were assessed using real-time quantitative PCR (RT-qPCR). Mitogen-activated protein kinase (MAPK), extracellular regulated protein kinase (ERK) and nuclear factor-kappa B (NF-κB) signalling pathways were evaluated using immunoblots and immunofluorescence. SLP or SLP + gDNA pre-treatment significantly reduced the LPS-induced mRNA expression of tumour necrosis factor alpha (TNF-α). Pre-treatment with LGG single components (SLP, gDNA or CpG) or their combinations (SLP + gDNA or SLP + CpG) significantly decreased the LPS-induced interleukin-6 (IL-6) mRNA level ($P < 0{\cdot}05$). Pre-treatment with SLP or gDNA, alone or in combination, significantly suppressed LPS-induced TLR2 and TLR4 mRNA levels ($P < 0{\cdot}05$). SLP pre-treatment also significantly decreased the LPS-induced expression of TLR9 ($P < 0{\cdot}05$). Pre-treatment with LGG single components or combinations significantly suppressed the LPS-induced phosphorylation levels of ERK ($P > 0{\cdot}05$). In conclusion, pre-incubation with LGG components, singly or in combination, generally inhibited the activation of TLR, MAPK and NF-κB signalling pathways in LPS-stimulated cells, leading to attenuated inflammatory cytokine TNF-α and IL-6 production. These results indicate that nonviable probiotic LGG components exert an anti-inflammation effect on epithelial cells.

Introduction

Lactobacillus rhamnosus GG (LGG) is a probiotic that can regulate intestinal flora, promote host defences, prevent invasion of harmful bacteria and improve host immunity (Lebeer *et al.* 2010). Macrophages are an important cellular component of the immune system. They play an important role in innate immune responses, exerting functions such as phagocytosis, killing of pathogens, antigen presentation, cytokine production and tumour inhibition.

Two pathways, the mitogen-activated protein kinases (MAPK) pathway and the nuclear factor-kappa B (NF-κB) pathway, have been studied extensively. LGG reduced levels of NF-κB inhibitor alpha (IκBα) protein and restored the concentration of tight junction proteins in the duodenum of mice (Cresci *et al.* 2013). *Lactobacillus plantarum* HY7712 could activate NF-κB signalling in normal macrophages but inhibit the transcriptional activity of NF-κB in lipopolysaccharide (LPS)-stimulated macrophages (Jang *et al.* 2013; Lee *et al.* 2014). A 8·7 kDa protein in metabolites from *L. plantarum* 10hk2 was found to be able to significantly improve the expression of interleukin-10 (IL-10), and inhibit the phosphorylation of IκB and P38 MAPK(Chon *et al.* 2010). Pre-treatment with LGG prevented interferon gamma (IFN-γ) induced barrier damage by restoring occludin and ZO-1 to control levels in human intestinal cells (Han *et al.* 2019). Treatment with LGG supernatant significantly improved intestinal barrier function reflected by increased mRNA expression of tight junction (TJ) in ileum and reversal of an alcohol-reduced T_{reg} cell population, while it increased T_H17 cell population, as well as interleukin-17 (IL-17) secretion, in a mouse model of chronic-binge alcohol feeding (Chen *et al.* 2016).

A number of biological effects have been associated with nonviable probiotics, recently termed as 'paraprobiotics', highlighting that they could constitute an excellent option to improve health status and wellness (Almada *et al.* 2016). Lactobacillus surface layer protein (SLP) is hydrophobic and beneficial for the adhesion of the lactic acid bacteria to intestinal epithelial cells. The SLP of LGG can promote the adhesion of the bacteria to *in vitro* cultured cells (Lebeer *et al.* 2012). SLP derived from *Lactobacillus acidophilus* NCFM significantly inhibited NO (nitric oxide) and PGE2 (prostaglandin E_2) production through downregulating the expression levels of iNOS and COX-2. Furthermore, SLP was found to inhibit NF-κB p65 translocation into the nucleus (Wang *et al.* 2018). S-layer glycoprotein from *Lactobacillus kefiri* CIDCA 8348 enhances macrophage response to LPS (Malamud *et al.* 2018), and its immunostimulatory activity was realized through glycan recognition by C-type lectin receptors in host (Malamud *et al.* 2019). Treatment with LGG-derived soluble protein p40 from postnatal days 2 to 21 significantly enhanced intestinal IgA production in the early life of wild-type mice (Shen *et al.* 2018).

For a long time, DNA was thought to have only weak immunogenicity. Since DNA fragments extracted from *Mycobacterium bovis* strain BCG were found to have antitumour activity under certain conditions (Tokunaga *et al.* 1984), the immunological properties of DNA have been rediscovered. In fact, single-stranded oligodeoxynucleotides (ODNs) containing nonmethylated cytosine–guanine motifs (CpG-ODN, cytosine-phosphate-guanine-containing oligodeoxynucleotide) are recognized by the innate immune system as 'danger signals'. The CpG sequence of the nonmethylated cytosine dinucleotide in bacterial DNA was the material basis of its immune activity, and synthetic oligonucleotides with the same sequence of CpG-ODNs had the same immunogenicity and could induce the activation of a variety of immune cells (Hartmann and Krieg 2000). CpG-ODNs bind and activate Toll-like receptor 9 (TLR9), which is located in the endoplasmic reticulum of plasmacytoid dendritic cells (pDC) and B cells (Iwasaki and Medzhitov 2003). TLR-9 activation subsequently triggers a signalling cascade involving myeloid differentiation factor 88 (MyD88), interleukin-1 receptor-associated kinase (IRAK) and tumour necrosis factor receptor associated factor 6 (TRAF-6), resulting in the activation of NF-κB and interferon regulatory factor 7 (IRF7) pathways (Hemmi *et al.* 2000).

As mentioned above, LGG components showed a difference in modulating the immune response in *in vitro* cell culture or in an *in vivo* animal model. However, to our knowledge, the combination of LGG components has not been investigated until now. The purpose of this study was to determine whether different components of *L. rhamnosus* (LGG), alone or in combination, could modulate the immune response in the mouse macrophage cell line RAW264.7 upon LPS challenge.

Results and discussion

LGG components modulate the cytokine response in RAW264.7 cells

We determined the optimal time for the LPS stimulation of RAW264.7 cells and the optimal doses for LGG components. Based on a time course experiment (0, 0·5, 1, 2, 4 h) evaluating the effects of LPS on the levels of phospho-P38/P38 and IκBα (Fig. S1), LSP induced the greatest change in phospho-P38/P38 and IκBα level with stimulation for 0·5 h; therefore, we selected 0·5 h as the optimal treatment time. The relationship between bacteria utilized per single target cell is designated as the multiplicity of

infection (MOI). As the LGG pre-treatment at MOI = 10 for 2 h significantly induced phospho-P38/P38, phosphorylated extracellular signal regulated kinase (ERK)1/2 and significantly suppressed IκBα (Fig. S2), we decided to use doses equivalent to LGG at MOI = 10 for the LGG components SLP, genomic DNA (gDNA) and CpG.

To determine the protective effects of LGG components on RAW264.7 cells after LPS stimulation, the cytokine expression levels were measured using real-time quantitative PCR (RT-qPCR). LPS stimulation induced significantly higher proinflammatory cytokines tumour necrosis factor alpha (TNF-α) and IL-6 levels, whereas it had no significant effect on the IL-10 level. SLPs have been found in the outermost layer of the cell wall in many kinds of lactobacillus, which is considered an important factor in the intestinal immune response. SLP or SLP + gDNA pre-treatment significantly reduced the LPS-induced mRNA expression of TNF-α ($P < 0{\cdot}05$, Fig. 1), whereas DNA or SLP + CpG had no significant effect on the LPS-induced TNF-α ($P > 0{\cdot}05$, Fig. 1). Pre-exposure of macrophages to CpG-ODNs for short periods augments the amount of TNF-α generated after an LPS challenge (Crabtree *et al.* 2001). Similarly, CpG-ODN pre-treatment augmented the TNF-α level in RAW264.7 cells after LPS stimulation. Therefore, SLP + CpG pre-treatment did not affect the LPS-induced TNF-α although SLP showed a potential to decrease TNF-α level.

CpG-ODN is an oligonucleotide containing cytosine and guanine dinucleotide. As CpG islands are abundant in bacterial gDNA, we investigated whether the effects of LGG gDNA could be recapitulated using CpG-ODNs. Pre-treatment with LGG single components (SLP, gDNA or CpG) or combinations (SLP + gDNA or SLP + CpG) significantly decreased the LPS-induced IL-6 mRNA level ($P < 0{\cdot}05$, Fig. 1), whereas these pre-treatments had no significant effect on the IL-10 level, with the exception of the SLP + gDNA combination. It is worth further investigation to discover why only SLP + gDNA was observed to exert a positive effect on IL-10. In the latter situation, the SLP + gDNA combination treatment also showed more potential to restore the LPS-induced signalling pathway, such as P38MAPK and IκBα molecules. These results suggest that LGG components mostly exerted immunoregulatory effects and dampened LPS-induced inflammation.

In both *in vitro* and *in vivo* studies, *Lactobacillus helveticus* MIMLh5 and its SLP-A play an anti-inflammatory

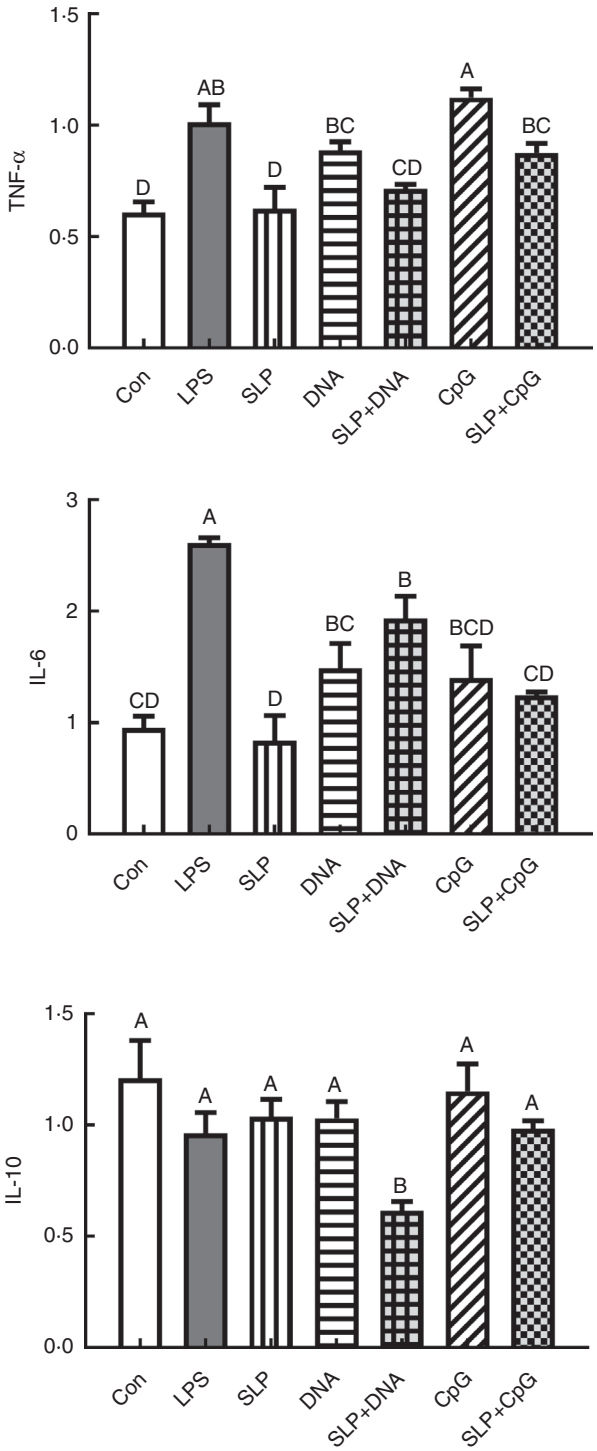

Figure 1 Effects of pre-treatments with LGG components on cytokine expression in LPS-stimulated RAW264.7 cells. Cells were pre-treated with SLP, gDNA, synthesized CpG, SLP plus gDNA or SLP plus CpG for 2 h then stimulated with LPS for 0·5 h. LGG, *Lactobacillus rhamnosus* GG; SLP, surface layer protein; CpG-ODN, unmethylated CpG oligodeoxynucleotide; gDNA, genomic DNA; LPS, lipopolysaccharide; TNF-α, tumour necrosis factor-alpha; IL-6, interleukin-6; IL-10, interleukin-10. The values are means + SD ($n = 6$). ^{A,B,C,D} Means for a variable without a common letter indicate significant differences ($P < 0{\cdot}05$)

role by reducing the activation of NF-κB in intestinal epithelial Caco-2 cells line. However, *L. helveticus* MIMLh5 and SLP-A, as stimulating factors of the innate immune system, can also trigger the expression of TNF-α and COX-2 in human macrophage U937 by binding to TLR2 (Taverniti *et al.* 2013). Therefore, SLP-A plays an important role in modulating the immunostimulatory activity of bacteria, which could help to control the host's defence against infections. In addition, SLP can activate the clearance reaction of macrophages, as indicated by the importance of SLP in the clearance of *Clostridium difficile* infection (Collins *et al.* 2014). SLP from *L. acidophilus* CICC 6074 can trigger immunomodulatory effects in RAW264.7 cells (Zhang *et al.* 2017).

Studies have shown that bacterial DNA containing unmethylated CpG motifs or synthetic oligonucleotides are powerful immune agonists that can directly activate dendritic cells, monocytes and macrophages to release a variety of cytokines (Dalpke *et al.* 2002; Jahrsdörfer and Weiner 2009). Previous studies showed that CpG inhibits the release of cytokines in mouse macrophages and partially inhibits the production of cytokines mediated by LPS; it also activates MAPK and transcription factors (Yeo *et al.* 2003). CpG-ODN can directly activate monocytes and macrophages, it can activate macrophages to secrete IL-6, IL-12, IL-18, IL-1P, TNF-α and other cytokines (Hartmann and Krieg 2000). Germ-free BALB/c mice were treated with CpG-ODN, control-ODN or phosphate buffer saline (PBS); compared to PBS or control-ODN treatment, CpG-ODN application to germ-free donors led to decreased intestinal inflammation as indicated by histology, decreased proinflammatory cytokines and increased IL-10 secretion (Bleich *et al.* 2009).

LGG components modulate TLR upon LPS challenge of RAW264.7 cells

To determine the modulatory effect of LGG components on the initial signalling molecules in RAW264.7 cells after LPS stimulation, the TLR expression levels were measured using RT-qPCR. Pre-treatment with SLP significantly suppressed LPS-induced TLR2, TLR4 and TLR9 mRNA levels ($P < 0{\cdot}05$, Fig. 2). gDNA pre-treatment also significantly decreased the LPS-induced expression of TLR2 and TLR4 ($P < 0{\cdot}05$), but not that of TLR9, compared to LPS alone (Fig. 2). SLP plus gDNA pre-treatment also significantly decreased the level of TLR2 and TLR4 ($P < 0{\cdot}05$, Fig. 2). Regarding TLR expression, pre-treatment with CpG-ODN had no significant effect on LPS-induced TLR2, TLR4 and TLR9 mRNA levels ($P > 0{\cdot}05$, Fig. 2). However, the combination of SLP and CpG-ODN significantly decreased LPS-induced TLR4 and TLR9 expression compared to treatment with LPS alone ($P < 0{\cdot}05$, Fig. 2). These results

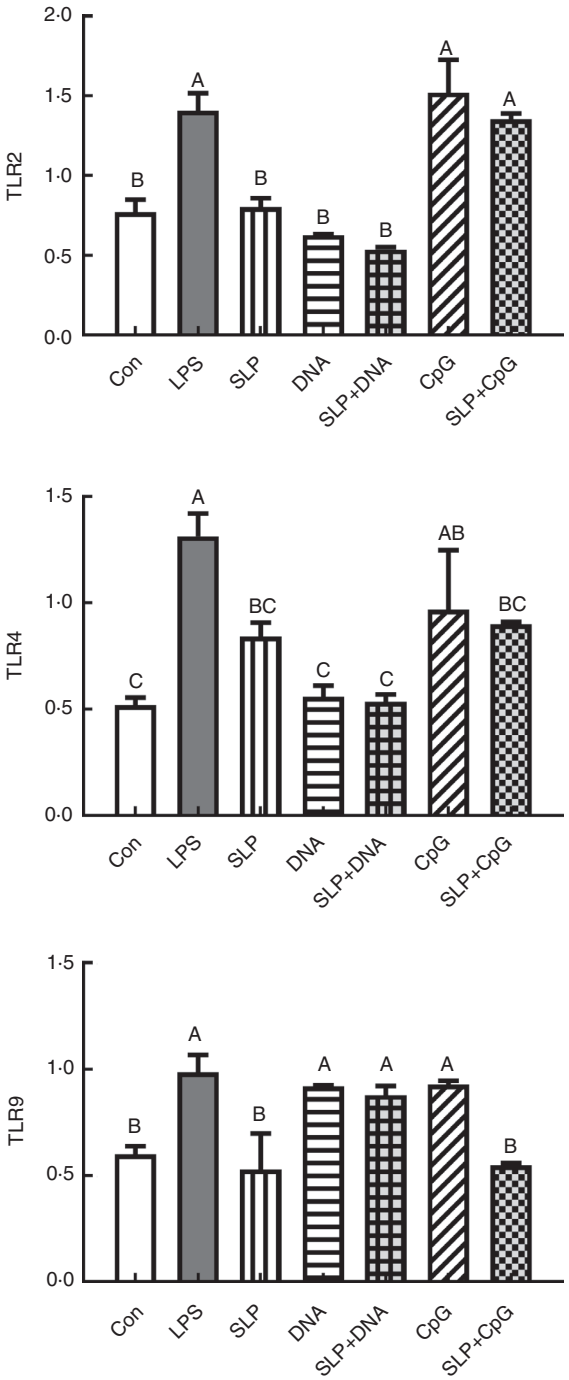

Figure 2 Effects of pre-treatments with LGG components on Toll-like receptor expression in LPS-stimulated RAW264.7 cells. Cells were pre-treated with SLP, gDNA, synthesized CpG, SLP plus gDNA or SLP plus CpG for 2 h, then stimulated with LPS for 0·5 h. LGG, *Lactobacillus rhamnosus* GG; SLP, surface layer protein; CpG-ODN, unmethylated CpG oligodeoxynucleotide; gDNA, genomic DNA; LPS, lipopolysaccharide; TLR, Toll-like receptor. The values are means + SD ($n = 6$). ^{A,B,C} Means for a variable without a common letter indicate significant differences ($P < 0{\cdot}05$)

suggest that CpG-ODN partially recapitulated the regulatory effects of LGG gDNA.

A previous study showed that oral administration of *Lactobacillus casei* CRL431 significantly decreased the mRNA levels of inflammatory cytokines (TNF-α and IFN-γ) by inhibiting TLR2, TLR4 and TLR9 in the intestine of mice challenged with *Salmonella* (Castillo *et al.* 2011). Like most TLRs, TLR9 is expressed both by immune cells including dendritic cells, macrophages and B cells, and by intestinal epithelial cells (Krieg 2002; Abreu 2010). In immune cells, TLR9 is expressed intracellularly in the endosomes (Barton *et al.* 2006). The upregulation of TLR9 expression coincided with significantly increased production of TNF-α induced by LPS plus CpG ODN (An *et al.* 2010). *L. rhamnosus* strains used as probiotics have high counts of unmethylated CpG motifs that can be recognized by TLR9 (Kant *et al.* 2014). LGG pretreatment decreased TLR2, TLR4 and TLR9 mRNA levels in intestinal epithelial cells after LPS stimulation indicating that LGG may exert probiotic activities through a TLR2- or TLR9-dependent pathway (Gao *et al.* 2017). In this study, CpG-ODN had an inhibitory effect on IL-6 in LPS-stimulated RAW264.7 cells compared with LPS stimulation alone. At the same time, CpG-ODN treatment led to increased IL-10 and TNF-α secretion, and TLR2, TLR4 and TLR9 expression.

LGG components modulate signalling pathway molecules upon LPS challenge of RAW264·7 cells

Lipopolysaccharide treatment significantly stimulated the phosphorylation of P38 and ERK while downregulating IκBα ($P > 0{\cdot}05$, Fig. 3). Pre-treatment with LGG single components (SLP, gDNA or CpG) or their combinations (SLP + gDNA or SLP + CpG) significantly decreased the LPS-induced phosphorylation levels of ERK ($P > 0{\cdot}05$, Fig. 3). All of these pre-treatments had a tendency to inhibit the LPS-induced phosphorylation levels of P38 and counteracted the LPS-induced inhibition of IκBα. *L.*

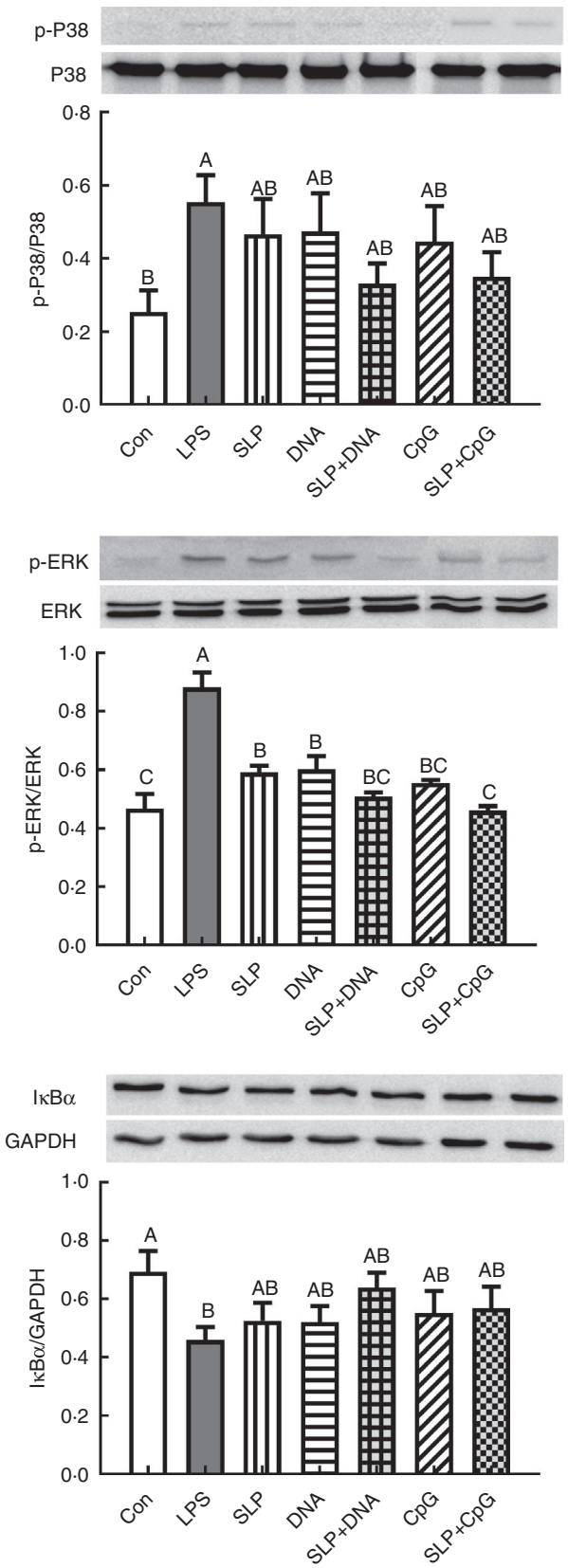

Figure 3 Effects of pre-treatments with LGG components on MAPK and NF-κB activation in LPS-stimulated RAW264.7 cells. Cells were pre-treated with SLP, gDNA, synthesized CpG, SLP plus gDNA or SLP plus CpG for 2 h, then stimulated with LPS for 0·5 h. The ratio of phospho-P38 to total P38, the ratio of phospho-ERK to total ERK and the ratio of IκBα to GAPDH are shown. LGG, *Lactobacillus rhamnosus* GG; SLP, surface layer protein; CpG-ODN, unmethylated CpG oligodeoxynucleotide; gDNA, genomic DNA; LPS, lipopolysaccharide; P38, mitogen-activated protein kinase; ERK, extracellular regulated protein kinases; IκBα, NF-κB inhibitor alpha. The values are means + SD ($n = 6$). ^{A,B,C} Means for a variable without a common letter indicate significant differences ($P < 0{\cdot}05$)

acidophilus NCFM SLP attenuates the production of TNF-α, IL-1*β* and reactive oxygen species (ROS) by inhibiting the MAPK and NF-κB signalling pathways (Wang *et al.* 2018). Our study showed that SLP had an inhibitory effect on P38MAPK and ERK signalling pathways in LPS-stimulated RAW264.7 cells compared with LPS stimulation alone. At the same time, SLP had the tendency to inhibit the degradation of IκBα, thus, the NF-κB pathway.

Immunofluorescence was used to verify the regulatory effect of LGG components on RAW264.7 cells stimulated by LPS. In accordance with the previous western blotting results, the phospho-P38 and phospho-ERK levels were reduced, whereas the IκBα was increased in RAW264.7 cells pre-treated with SLP plus gDNA or SLP plus CpG-ODNs compared to LPS stimulation alone (Fig. 4). These results suggest that the combination of SLP and gDNA/CpG-ODNs may have inhibited MAPK and NF-κB signalling pathways to exert the anti-inflammatory functions observed earlier.

It is deemed that the role of CpG DNA depends on P38 MAPK and STATl signalling (Takauji *et al.* 2002). CpG-DNA inhibits the release of cytokines in mouse macrophages, and partially inhibits the production of LPS-induced cytokines, through dampening the MAPK pathway (Yeo *et al.* 2003). The P38 MAPK signalling pathway is an important part of the MAPK family, also known as the MAPK emergency signalling pathway, which plays an important role in inflammatory response. In this study, CpG-ODN was used to pre-treat the macrophages of the abdominal cavity of mice before LPS stimulation, which could significantly inhibit the ERK1/2 signalling pathway but have only a certain inhibitory effect on the P38 MAPK signalling pathway and IκBα

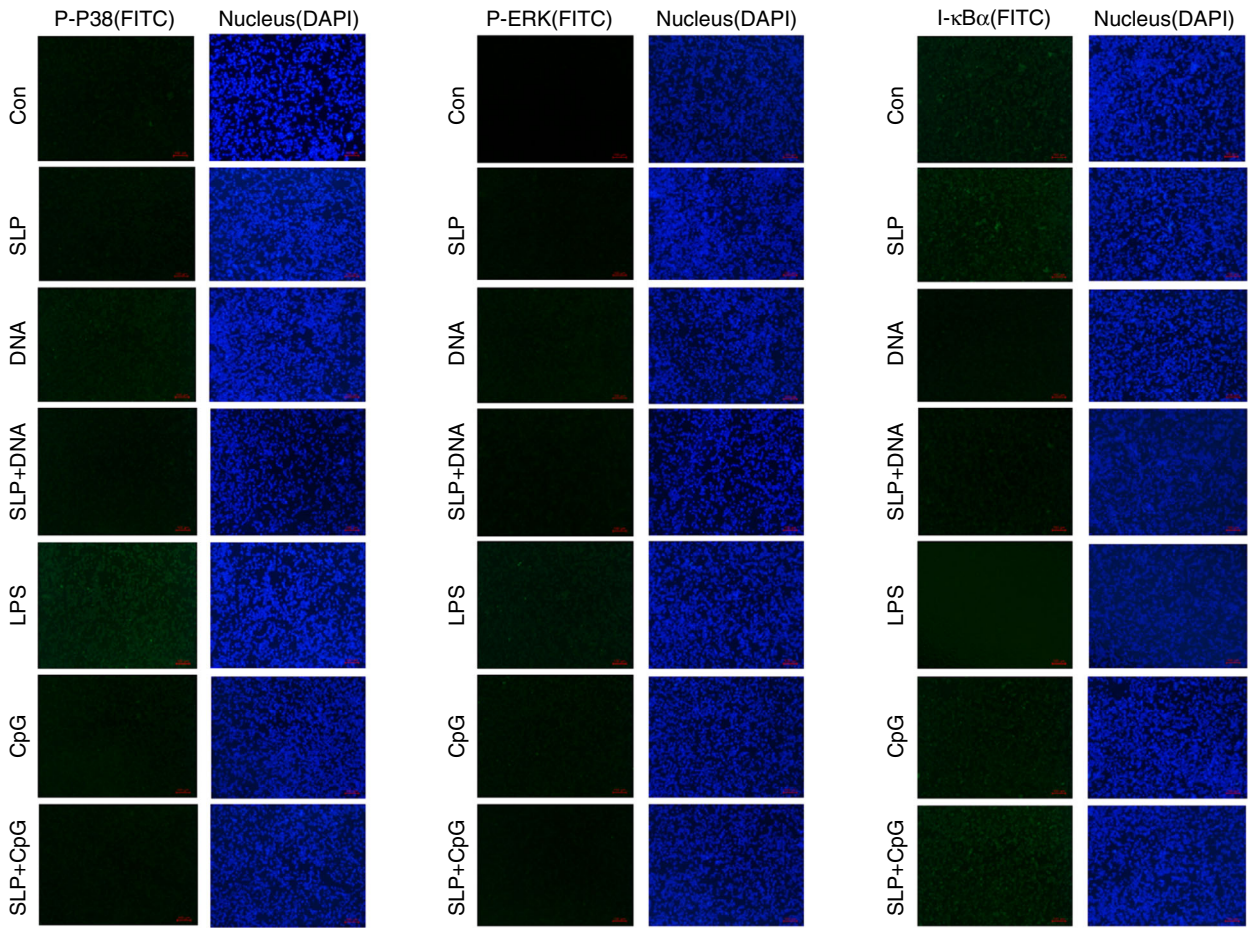

Figure 4 Immunofluorescence analysis of MAPK and NF-κB activation in LPS-stimulated RAW264.7 cells after the pre-treatments with LGG components. Cells were pre-treated with SLP, gDNA, synthesized CpG, SLP plus gDNA or SLP plus CpG for 2 h then stimulated with LPS for 0·5 h. LGG, *Lactobacillus rhamnosus* GG; SLP, surface layer protein; CpG, unmethylated CpG oligodeoxynucleotide; gDNA, genomic DNA; LPS, lipopolysaccharide; P38, mitogen-activated protein kinase; ERK, extracellular regulated protein kinases; IκBα, NF-κB inhibitor alpha. All images were taken at the centre of the wells, with the same exposure time (magnification, 40×; bar, 100 μm). [Colour figure can be viewed at wileyon linelibrary.com]

protein expression. Relevant articles are sufficient to show that SLP has an immunomodulatory effect on the inflammatory system, and that CpG-ODN has an immune stimulation effect, while gDNA does not play an obvious role (Gao *et al.* 2017). Similarly, we found that gDNA pretreatment had no significant modulating effect on signalling molecules P38MAPK, ERK1/2 and IκBα in RAW264.7 cells stimulated by LPS in this study.

In conclusion, pre-incubation with SLP, gDNA and CpG-ODN generally inhibited the activation of the MAPK and NF-κB signalling pathways, and then downregulated TNF-α and IL-6, whereas they upregulated IL-10 level in RAW264.7 cells induced by LPS. *L. rhamnosus* gDNA did not completely function like CpG in immunomodulation.

Materials and methods

Reagents and antibodies

EDTA was obtained from Sangon Biotech (Shanghai, China). Trypsin was purchased from VWR (AMRESCO). LPS was acquired from Sigma-Aldrich (St. Louis, MO). High-glucose Dulbecco's Modified Eagle's medium (DMEM) was obtained from Genome Biomedical Technologies (Hangzhou, China). Dimethyl sulfoxide (DMSO) was purchased from MP Biomedicals (Solon, OH). The total protein extraction kit and Bradford protein quantitative kit were obtained from KeyGen Biotech (Nanjing, China). The RNA pure total RNA kit and DNA extraction kit were obtained from Tiangen (Beijing, China). The PrimeScript reagent kit and SYBR Premix Ex Taq Tli RNase H Plus were acquired from Takara (Japan). Phospho-ERK1/2, ERK1/2, phospho-P38MAPK, P38MAPK, IκBα and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibodies were acquired from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP)- or fluoresceine isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Jackson Immuno Research (West Grove, PA).

Bacterial strain

LGG (ATCC 53103) was inoculated in MRS broth medium, cultured for 18 h at 37°C under anaerobic condition to exponential phase, then harvested and stored at −80°C.

Cell culture

RAW264.7 cells were cultured in high-sugar DMEM culture medium containing 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in an incubator with 5% CO_2. When the cells reached 80% confluency, they were transferred to six-well culture plates at 1×10^7 cells/well and cultured for an additional 24 h. To determine an optimal time to stimulate the cultured cells, P38 MAPK and IκB were measured in cells treated with LPS (200 ng ml^{-1}) for 0, 0·5, 1, 2 and 4 h, respectively. Three treatment groups were included: control, where cells were cultured in medium only; LPS, where cells were stimulated with 200 ng LPS ml^{-1} for 0·5 h; LPS + LGG, where cells were pre-treated with LGG (Multiplicity of Infection, MOI = 10) for 2 h, then stimulated with LPS (200 ng ml^{-1}) for 0·5 h.

Preparation of LGG components

Surface proteins of LGG were purified using 5 mol LiCl l^{-1} ultrafiltration (Zhang *et al.* 2010). LGG gDNA was extracted with bacterial gDNA extraction kit (Aidlab, Beijing, China). Unmethylated CpG-ODN with the gene sequence 5′-ACTTTCGTTTTCTGCGTCAA-3′ were synthesized at Invitrogen (Shanghai, China).

Immunomodulation of LPS-stimulated RAW264.7 cells by LGG components

RAW264.7 cells were incubated with different components of LGG (equivalent to MOI = 10) for 2 h, followed by 200 ng LPS ml^{-1} for 0·5 h. Cells were washed twice then used for subsequent tests. The experiment was repeated at least six times for each group.

Preparation of RNA and RT-qPCR

Total RNA was extracted from RAW264.7 cells with an RNA pure total RNA kit (Aidlab, Beijing, China), resuspended in RNase-free water, and stored at −80 until use. The RNA samples were converted to cDNA using a PrimeScript reagent kit (Takara Bio, Japan), and cDNA was stored at −20 . RT-qPCR was performed with SYBR Premix Ex Taq (Takara Bio, Japan). The reaction was run using a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The primer sequences were described previously (Collado-Romero *et al.* 2010).

Western blotting

Lysis buffer (Sigma-Aldrich) was used to lyse RAW264.7 cells according to the manufacturer's instructions. The concentration of the extracted protein was measured using the Bradford method (Kruger 2009). The protein samples were loaded on to an SDS-PAGE gel then transferred to a polyvinylidene fluoride (PVDF) membrane. The

membrane was incubated with primary antibody overnight at 4°C. After removal of primary antibody, the membrane was incubated with horseradish peroxidise (HRP)-conjugated secondary antibody. The PVDF membrane was exposed and bands were revealed by electrochemiluminescence (ECL) (Millipore, Merck KGaA, Darmstadt, Germany). Band intensity values were measured with IMAGE J SOFTWARE (National Institutes of Health, Bethesda, MD).

Immunofluorescence

RAW264.7 cells were blocked with acetone for 30 min at 4°C. The cells were then incubated with primary antibody (1 : 1000) at 4°C overnight. After incubation with FITC-conjugated secondary antibody (1 : 5000) in a dark room, the cells were incubated with DAPI (Sigma-Aldrich), then washed twice and observed under an immunofluorescence microscope (ECLIPSE Ti, Nikon Corp., Tokyo, Japan). All micrographs were taken in the centre of the wells with identical exposure time.

Statistical analysis

Statistical significance was evaluated using one-way analysis of variance (ANOVA, general linear model), followed by Duncan's multiple range test using the SAS program (SAS Institute, Cary, NC). Differences are considered significant if $P < 0{\cdot}05$.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Western blot analysis of MAPK and NF-κB activation in RAW264.7 cells stimulated by LPS (lipopolysaccharide) at different time points. P38,

mitogen-activated protein kinase; IκBα, NF-κB inhibitor alpha. Values are means ± SD ($n = 6$). Different letters indicate the change between each group is statistically significant ($P < 0{\cdot}05$).

Figure S2. Western blot analysis of MAPK and NF-κB activation in RAW264.7 cells following LGG treatment and LPS (lipopolysaccharide) stimulus. Ratio of p-P38/P38, p-ERK/ERK and IκBα/GAPDH in RAW264.7 cells pre-treated with LGG for 2 h and treated with LPS for 30 min. LGG, *Lactobacillus rhamnosus* GG; P38, mitogen-activated protein kinase; ERK, extracellular regulated protein kinases; IκBα, NF-κB inhibitor alpha. Values are means ± SD ($n = 6$). Different letters indicate the change between each group is statistically significant ($P < 0{\cdot}05$).