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Lipoteichoic acid from an *Enterococcus faecalis* clinical strain promotes TNF-α expression through the NF-κB and p38 MAPK signaling pathways in differentiated THP-1 macrophages

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Abstract. To study the immune-inflammatory response and signaling mechanism of macrophages to purified *Enterococcus faecalis* (*E. faecalis*) lipoteichoic acid (LTA), intact LTA was obtained from an *E. faecalis* clinical strain P25RC using the butanol method and hydrophobic interaction chromatography purification. The fractions containing LTA were determined using phosphate detection. Contaminations with lipopolysaccharide and proteins were excluded using the Limulus amoebocyte lysate assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively. LTA was analyzed using nuclear magnetic resonance. Prior to LTA stimulation assays, THP-1 monocytes were pretreated with phorbol 12-myristate 13-acetate to differentiate into macrophages. Macrophages were treated with LTA in concentration gradients and cells without LTA treatment as the control. Gene expression of *TLR2*, *CD14* and *MyD88* were evaluated by quantitative polymerase chain reaction. Tumor necrosis factor-α (TNF-α) and interleukin (IL)-10 were quantified using ELISA. The activated and total nuclear factor-κB (NF-κB) p65 and three mitogen-activated protein kinases (p38, ERK1/2 and JNK) were assessed using western blot analysis. *E. faecalis* LTA induced the gene expression of *TLR2* and *MyD88* whilst it downregulated *CD14*, suggesting a *TLR2*-dependent and *CD14*-independent immune-inflammatory activity. LTA stimulated the expression of pro-inflammatory cytokine TNF-α ($P<0.05$), but not the anti-inflammatory cytokine IL-10. In conclusion, *E. faecalis* LTA stimulated the expression of TNF-α in macrophages possibly through the NF-κB and p38 pathways.

Introduction

Enterococcus faecalis (*E. faecalis*), a Gram-positive bacterium, is a predominant cause of hospital-associated infections (1). *E. faecalis* is also involved in root canal infections (2) and is considered a major pathogen associated with endodontic treatment failure (3). Several virulence attributes that promote bacterial colonization, the invasion of host tissues and the evasion of host defense mechanisms are indicated in persistent infections caused by *E. faecalis* (4). The virulence factors include lipoteichoic acid (LTA), peptidoglycan, aggregation substances, cytolysin and lytic enzymes. Among them, LTA is the key virulence factor due to its major role in pathogenicity. It has been demonstrated that *E. faecalis* LTA can stimulate leukocytes to release certain mediators associated with inflammatory response (5) and plays a critical role in biofilm formation (6). *E. faecalis* LTA is also known to inhibit the repair mechanism of periapical bone by decreasing the proliferation of human osteoblast-like cells and inducing apoptosis (7).

E. faecalis LTA belongs to classical (type 1) LTA. Its basic structure is comprised of a glycolipid moiety and 1,3-polyglycerolphosphate substituted at position C-2 with D-alanine and kojibiose residues (8). The alanyl esters with D-configured glycerophosphate influence the antibiotic action, pathogenesis, adhesion, biofilm formation and virulence of Gram-positive bacteria (6,9). The dlt operon consists of 4 genes: *dltA*, *dltB*, *dltC* and *dltD*, which are responsible for D-alanine synthesis (10). The inactivation of the *dlt* genes resulted in the absence of D-alanyl residues of LTA and further decreased the virulence features of *E. faecalis* (6).

Bacterial antigens are recognized by the pattern recognition receptors (PRRs) of immune cells and subsequently induce the production of pro-inflammatory cytokines. Toll-like receptors (TLR) are ubiquitous PRRs that play a pivotal role in the innate immune response (11). Of all the TLRs, TLR2 is known

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to specifically interact with bacterial LTA (12). Baik *et al* (5) found that *E. faecalis* LTA stimulates RAW 264.7 cells to express tumor necrosis factor-α (TNF-α). However, molecular mechanisms pertaining to the sensing and signaling of clinically derived *E. faecalis* LTA and human macrophages have not yet been studied. The cells of the THP-1 human monocytic cell line can differentiate into macrophages under stimulation by phorbol 12-myristate 13-acetate (PMA) (13). The differentiated THP-1 macrophages are widely used as human macrophages *in vitro* for studying inflammatory diseases (14,15). In order to address this research gap, highly purified, intact LTA was extracted from an *E. faecalis* clinical strain and its effect on TNF-α production was studied in differentiated THP-1 macrophages. Nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinase (MAPK) signaling pathways are activated by endodontic infectious contents (16). The present study also investigated the involvement of NF-κB and MAPK signaling pathways in the production of TNF-α.

Materials and methods

Bacteria and cell culture. E. faecalis P25RC was isolated from a patient's root canal at the Peking University Hospital of Stomatology (Beijing, China) by Zhu *et al* (17). *E. faecalis* P25RC was grown anaerobically (90% N_2, 5% CO_2 and 5% H_2) overnight at 37°C without agitation in the brain-heart infusion broth (Oxoid Ltd., Basingstoke, Hampshire, UK). Once the bacterial growth reached the mid-exponential phase (A_{600}=0.5), 10 liters of the bacteria were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS; pH 7.4) (Sigma-Aldrich, St. Louis, MO, USA). The bacteria were examined by Gram staining and microscopy to rule out contaminations. The bacteria were stored at -70°C until LTA extraction.

THP-1 cells (ATCC® TIB-202™) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). THP-1 cells were maintained in RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 50 μM 2-mercaptoethanol (Sigma-Aldrich), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were incubated at 37°C with 5% CO_2. The experiments were performed in 6-well plates at a density of $8x10^5$ cells/ml. Before the LTA stimulation assays, THP-1 monocytes were pretreated with 200 ng/ml PMA (Sigma-Aldrich) for 18 h to differentiate them into macrophages, which facilitates the adhesion of the cells (18).

Isolation and purification of LTA. The processes of isolation and purification of LTA were performed as described previously (19). The bacteria were defrosted and suspended in 100 ml of 0.1 M cold sodium citrate buffer (pH 4.7) (Sigma-Aldrich). Following disruption by Vibra-Cell ultrasonication (Sonic and Materials, Inc., Danbury, CT, USA) at 40% amplitude in pulse mode of 5-sec on and 5-sec off for 30 min on ice, the cell lysate was stirred with 100 ml *n*-butanol (Sigma-Aldrich) at 4°C for 30 min. Subsequently, centrifugation was carried out at 7,000 x g for 45 min. The lower aqueous phase was collected and 100 ml sodium citrate buffer was added again for re-extraction. The two aqueous phases were pooled and dialyzed overnight in deionized water using a

dialysis membrane with MWCO 1000 (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The dialyzed sample was treated with vacuum filtration (Millipore, Billerica, MA, USA) and prepared for purification. The dialyzed sample was subjected to hydrophobic interaction chromatography (HIC) on an octyl-sepharose column (2.5x10 cm) (Sigma-Aldrich). The dialyzed sample was prepared and transferred to HIC in 15% *n*-propanol in 0.1 M ammonium acetate. The bond fractions were eluted with a linear concentration gradient from 15 to 60% *n*-propanol in 0.1 M ammonium acetate (pH 4.7).

The structure of the LTA was assayed by ^{1}H nuclear magnetic resonance (NMR) spectrometry as described previously (19). The purity of the LTA was determined by measuring the protein content through Coomassie Blue G-250 staining (Bio-Rad, Hercules, CA, USA) following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in gradient gels containing 5-12% wt/vol acrylamide (Bio-Rad). Precision Plus Protein Unstained standards (Bio-Rad) were used as reference markers. The molecular weight of LTA was shown in the SDS-PAGE gel stained with Coomassie Blue dye. Endotoxin contamination was excluded using a Limulus amoebocyte lysate (LAL) assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Thus, the endotoxin was quantified and the contamination was evaluated (19,20). DNA or RNA contamination was determined by measuring ultraviolet (UV) absorption at 260 and 280 nm (NanoDrop Spectrophotometer 2000; Thermo Fisher Scientific, Wilmington, DE, USA) (21).

Extraction of the E. faecalis genome and detection of dlt genes. The genome of *E. faecalis* P25RC was extracted using the Wizard Genome DNA Purification kit (Promega Corp., Madison, WI, USA). The fragments of genes *dltA* and *dltB* were amplified using polymerase chain reaction (PCR). The sequences of primers were: *dltA* forward, 5'-TTGATGAAT GGGCACGTAAA-3' and reverse, 5'-GCCAATGCGGGA TAAATAGA-3'; and *dltB* forward, 5'-AGGTGAATGCCA CATGA-3' and reverse, 5'-GACACCACCAAGATAATGACT GA-3' (6). The PCR products were visualized on a 1.5% agarose gel stained with Redsafe Nucleic Acid Staining Solution (1:10,000) (iNtRON, Seoul, Korea).

LTA treatment. LTA-containing fractions were pooled and lyophilized. LTA was weighed using an analytical balance (Sartorious, Goettingen, Germany), reconstituted using PBS (pH 7.4) to a concentration of 1 mg/ml and stored at -70°C until use. *E. faecalis* LTA was added into RPMI-1640 culture medium and mixed thoroughly in plastic tubes. PMA pre-activated THP-1 cells were treated with *E. faecalis* LTA at a gradient concentration between 0.1 and 50 μg/ml at 37°C for 18 h. Cells without LTA treatment were used as controls. All the experiments were performed in triplicate.

ELISA assays. Following the stimulation of the cells with *E. faecalis* LTA, supernatants were collected and assayed for inflammatory and anti-inflammatory cytokines using sandwich ELISA with their well-matched antibodies. Cytokines, such as TNF-α and interleukin (IL)-10, were detected using ELISA kits (Invitrogen Life Technologies, Frederick, MD, USA) according to the manufacturer's instructions (22).

Quantitative PCR (qPCR). The cells were washed twice with PBS and harvested by scraping using cell scrapers (Costar, Corning, NY, USA) following incubation with LTA. The mRNA of each sample was extracted and purified using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) and quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). The cDNA was obtained through SuperScript® VILO™ MasterMix (Invitrogen Life Technologies) using 1.5 μg mRNA as a template for the final reaction volume of 20 μl. The qPCR was performed using a StepOne qPCR System (Applied Biosystems Inc., Carlsbad, CA, USA) in standard mode and the PCR products were detected with SYBR-Green (Life Technologies, Austin, TX, USA). The signaling pathway messengers, including *TLR2*, *CD14* and *MyD88*, were amplified using qPCR. Primer sequences were as follows: *TLR2* forward, 5'-TTATCCAGCACACGAATACACAG-3' and reverse, 5'-AGGCATCTGGTAGAGTCATCAA-3'; *CD14* forward, 5'-GACCTAAAGATAACCGGCACC-3' and reverse, 5'-GCAATGCTCAGTACCTTGAGG-3'; *MyD88* forward, 5'-GGCTGCTCTCAACATGCGA-3' and reverse, 5'-CTG TGTCCGCACGTTCAAGA-3'; and GAPDH forward, 5'-ATGTGTCCGTCGTGGATCTGA-3' and reverse, 5'-ATG CCTGCTTCACCACCTTCT-3'. The qPCR reaction conditions were performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The experiment was performed in triplicate. The relative expression of the mRNA was normalized with GAPDH using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis. The phosphorylation of NF-κB and MAPKs, including ERK1/2, JNK1/2 and p38, was detected by western blot analysis, as previously described (21). The nuclear and cytoplasmic proteins were extracted using NE-PER™ Nuclear and Cytoplasmic Extraction reagents (Thermo Fisher Scientific, Rockford, IL, USA). The protein was quantified with a BCA™ protein assay kit (Thermo Fisher Scientific). Briefly, cell lysates containing 20 μg of protein were boiled for 5 min in Lane Marker Sample buffers (Thermo Fisher Scientific) and the volume ratio of sample buffer in the protein sample was 1:4. The protein was subsequently separated by 5-12% SDS-PAGE (Bio-Rad) and blotted onto polyvinylidene fluoride membranes (GE Healthcare, Uppsala, Sweden). After blocking for 1 h in 20 mmol/l Tris-HCl, 150 mmol/l NaCl and 0.05% Tween-20 (TBS-T) containing 5% skimmed milk, the membranes were washed three times in 20 ml TBS-T and probed overnight at 4°C with rabbit anti-human antibodies for phospho-NF-κB p65 (monoclonal; 3033), NF-κB p65 (monclonal; 4764), phospho-p38 (polyclonal; 9211), p38 (polyclonal; 9212), phospho ERK1/2 (monoclonal; 4377), ERK1/2 (monoclonal; 4695), phospho-SAPK/JNK (monoclonal; 4671), SAPK/JNK (polyclonal; 9252) (Cell Signaling, Beverly, MA, USA) in TBS-T with 0.5% skimmed milk. Following three washes in 20 ml TBS-T, the membranes were incubated with secondary horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) for 2 h and washed three times in 20 ml TBS-T. Bands were detected using enhanced chemiluminescence reagents (Thermo Fisher Scientific, Wilmington, DE, USA). The proteins were normalized with β-actin (Santa Cruz Biotechnology Inc., Dallas, TX, USA). The band densities were measured using Quantity One 4.6.9 software (Bio-Rad).
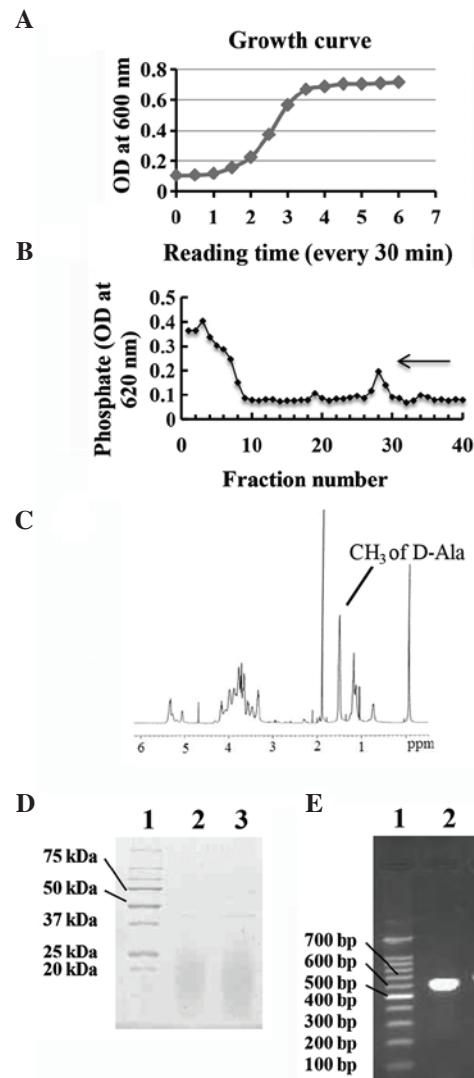

Figure 1. Determination of lipoteichoic acid (LTA). (A) The growth kinetic curve of *Enterococcus faecalis* (*E. faecalis*) P25RC at an optical density (OD) at 600 nm. (B) Phosphate detection at an OD at 620 nm. The black arrow points to the fractions containing highly purified LTA. (C) The ^{1}H-nuclear magnetic resonance spectrum of LTA from *E. faecalis* P25RC (600 MHz). (D) The image of SDS-PAGE. The molecular weight of LTA is ~20 kDa. Lane 1, protein ladder; lane 2, LTA (5 μg); lane 3, LTA (10 μg). (E) The image of the *dltA* and *dltB* gene fragments on the agarose gel. Lane 1, DNA ladder; lane 2, *dltA* fragment (598-bp); lane 3, *dltB* fragment (649-bp).

Statistical analysis. The qPCR, ELISA and western blot analysis data were analyzed by analysis of variance using IBM SPSS software version 20 (IBM Corp., Armonk, NY, USA). The Bonferroni method was used to compare the difference between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Extraction, purification and identification of LTA. E. faecalis P25RC was grown and reached an optical density of 0.5 at 600 nm for LTA extraction (Fig. 1A). LTA was extracted using the *n*-butanol method and purified by HIC on octyl-sepharose. The bond fractions were assayed for total phosphorus (Fig. 1B). The fractions corresponding to the peak on the curve, which were considered the ones containing LTA, were
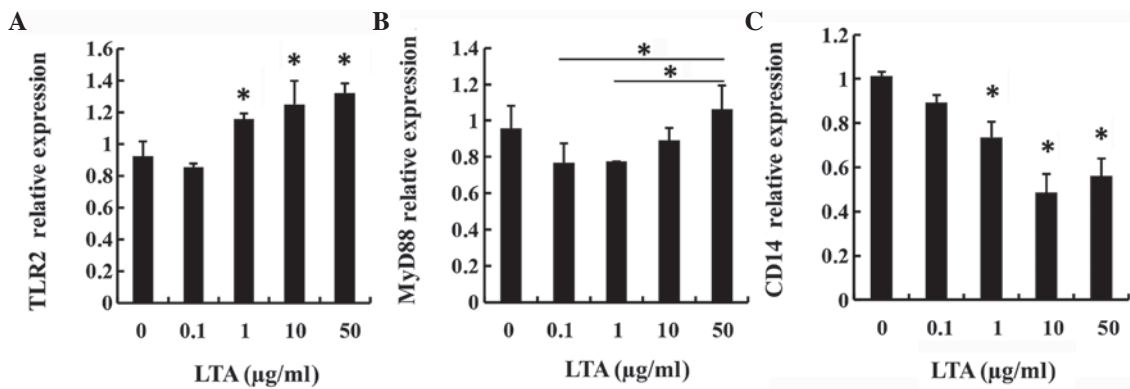
Figure 2. Relative mRNA expression of signaling mediators, including (A) *TLR2*, (B) *MyD88* and (C) *CD14*, was assayed using quantitative polymerase chain reaction. Data are presented as mean ± standard deviation. $^*P<0.05$ compared to the untreated cells. LTA, lipoteichoic acid.

pooled. The structure of LTA was detected using ^{1}H NMR spectrometry (Fig. 1C). The purity of LTA was determined by measuring the protein contamination through Coomassie Blue staining following SDS-PAGE and the molecular weight of LTA showed ~20 kDa (Fig. 1D). Endotoxin contamination was excluded through an LAL assay and endotoxin content was <0.3 EU/mg in the lyophilized LTA. DNA and RNA contaminations were determined by measuring UV absorption at 260 and 280 nm. DNA and RNA accounted for 5.4 and 3.4% (wt/wt), respectively. Conclusively, purified LTA was prepared. The D-alanine residue was confirmed by amplifying the fragments of the *dltA* and *dltB* operon genes using PCR (Fig. 1E). The presence of PCR products on the agarose gel showed the *dltA* (598 bp) and *dltB* (649 bp) fragments. Preliminarily, LTA from *E. faecalis* P25RC could be confirmed as D-alanyl LTA.

LTA promotes the gene expression of TLR2 and MyD88. LTA from the *E. faecalis* P25RC clinical strain was used to treat macrophages that were differentiated from THP-1 cells. Cells without LTA treatment were used as controls. At the concentration of 50 µg/ml LTA, the expression of *TLR2* was enhanced ≤1.4-fold compared to the control and additionally, the expression of *MyD88* was enhanced ≤1.4-fold compared to 0.1 and 1 µg/ml LTA treatment. The expression of *CD14* was decreased by 0.5-fold compared to the control. Based on the results of qPCR, D-alanyl LTA from *E. faecalis* P25RC promoted the gene expression of *TLR2* (P<0.05, Fig. 2A) and *MyD88* (P<0.05, Fig. 2B), but LTA downregulated *CD14* (P<0.05, Fig. 2C). In particular, LTA from *E. faecalis* P25RC induced an inflammatory response through *TLR2*-dependent and *CD14*-independent signaling transduction.

LTA promotes the expression of TNF-α. The cytokines, such as TNF-α and IL-10, were detected using ELISA sandwich kits. LTA stimulated the expression of pro-inflammatory cytokine TNF-α (P<0.05, Fig. 3A), but not the anti-inflammatory cytokine IL-10 (P>0.05, Fig. 3B).

LTA promotes p38 and NF-κB signaling pathways. The three activated total MAPKs (p38, ERK1/2 and JNK) (Fig. 3C) and NF-κB p65 (Fig. 3D) were assayed using western blot analysis. In the present study, the results also suggest that MAPKs, particularly p38, may play an important role in signal

transduction for TNF-α production. There was a dose-dependent increase in p-p38 (P<0.05, Fig. 3E) and p-NF-κB activity (P<0.05 compared to the control, Fig. 3I). By contrast, *E. faecalis* LTA did not induce ERK1/2 or JNK activity. The total ERK1/2 (Fig. 3G) and JNK (Fig. 3H) were upregulated, but the activation was not detected. Therefore, the p-p38 and p-NF-κB signaling transduction pathways likely play a major role in *E. faecalis* LTA-induced TNF-α expression in differentiated THP-1 macrophages.

Discussion

The present study examined the molecular mechanism behind the interaction of a highly purified LTA derived from the *E. faecalis* clinical strain with differentiated THP-1 macrophages. The *n*-butanol method and HIC on octyl-sepharose were used to extract and purify LTA. The butanol method can be used to extract structurally intact LTA and has been widely used to extract LTA from numerous types of Gram-positive bacteria, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *E. faecalis* and *Streptococcus gordonii* (6,19,23,24). Furthermore, SDS-PAGE and Coomassie Blue G-250 staining were used to detect protein contamination. Coomassie Blue G-250 staining provides a detection limit of 1 ng protein and is sensitive enough to exclude protein contamination. Of note, LTA could be exhibited on the gel following staining by Coomassie Blue, while LTA was previously examined using silver stain (25,26). LTA showed a clear broad band of ~20 kDa. The LTA image was in agreement with a previous study regarding LTA derived from other Gram-positive bacteria (27). Therefore, SDS-PAGE analysis with Coomassie Blue staining could become a rapid and easy way to detect LTA. The NMR spectrum was highly analogous to those reported in a previous study regarding *E. faecalis* LTA (6). Taken together, highly purified LTA was successfully isolated from an *E. faecalis* clinical strain.

LTA is an amphiphile that is anchored to the cell membrane via its glycolipid and extends its long chains of glycerophosphate into the wall (20). The *dlt* genes are involved in the production of D-alanine residues and the inactivation of the *dlt* genes can lead to the absence of D-alanine linked on the LTA glycerolphosphate backbone. Furthermore, D-alanine is closely associated with the formation of biofilm (9).
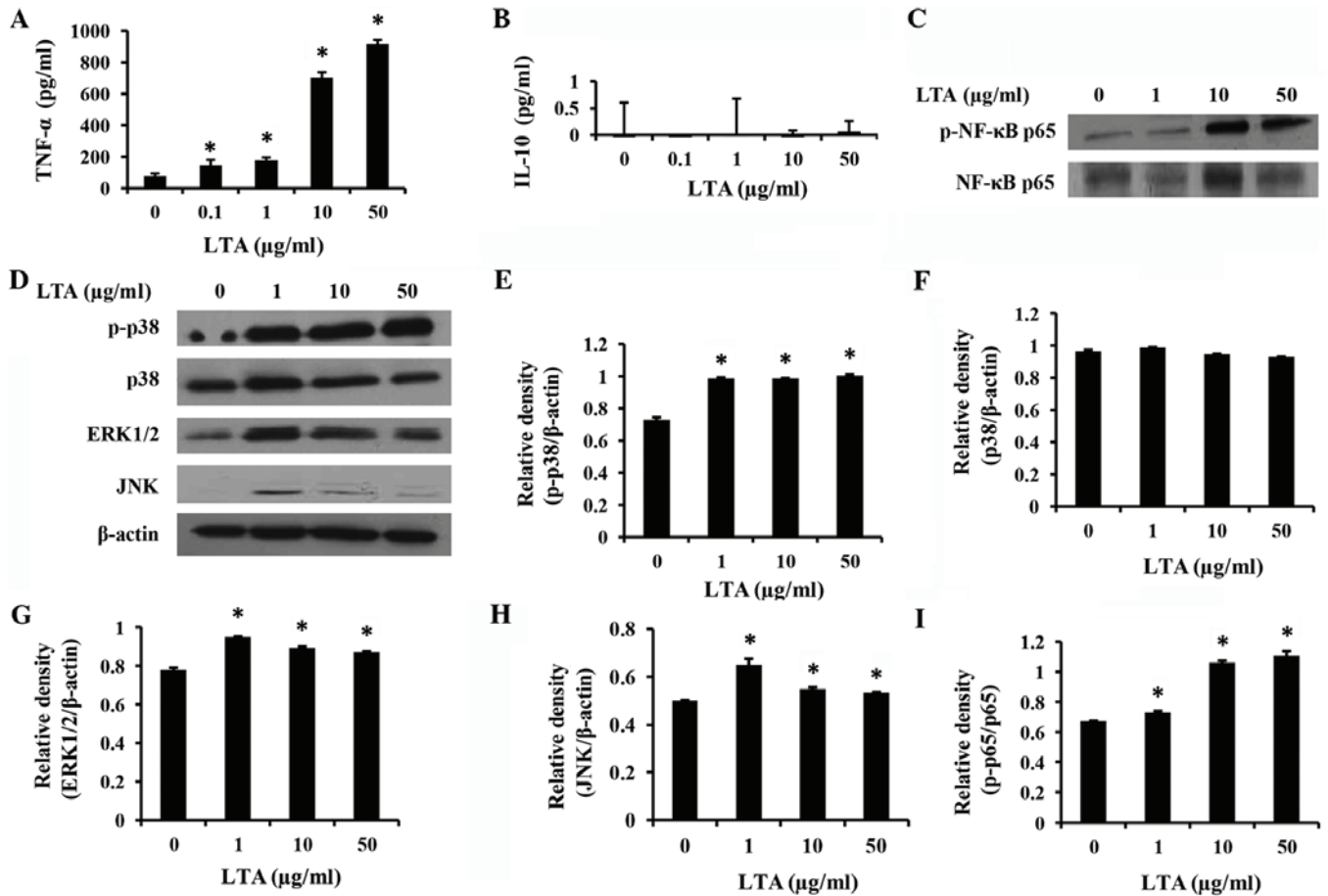
Figure 3. Release of (A) tumor necrosis factor-α (TNF-α) and (B) interleukin (IL)-10 was assayed using ELISA. The expression of (C) nuclear factor-κB (NF-κB) and (D) mitogen-activated protein kinases was assayed using western blot analysis. The band densities of (E) p-p38, (F) p38, (G) ERK1/2, (H) JNK and (I) p-p65 were measured using Quantity One 4.6.9 software. Data are presented as mean ± standard deviation. *P<0.05 compared to the untreated cells. LTA, lipoteichoic acid.

Fabretti et al (6) found that the dltA deletion mutant of a strong biofilm producer (E. faecalis 12030) was not as potent as the wild-type strain in the production of biofilm. In the present study, the dltA and dltB genes were detected in E. faecalis P25RC. They are responsible for the production of D-alanine, suggesting that E. faecalis P25RC LTA contains D-alanine. The E. faecalis P25RC strain came from an infected root canal of a patient suffering from persistent periapical periodontitis. Therefore, understanding the immune-inflammatory activity of E. faecalis P25RC may enhance our understanding of the apical resorption process observed in endodontic infections.

As a surface molecule of Gram-positive bacteria, LTA plays an important role in the stimulation of inflammatory responses, in vivo and in vitro (21,28). In the present study, gene expression of the signaling mediators was detected using qPCR. LTA from the clinical strain used in the present study induced an inflammatory response through TLR2-MyD88-dependent, but CD14-independent signaling transduction. There are 10 TLRs in human cells, each of which can recognize different pathogen-associated molecular patterns (26). LTA is a ligand against TLR2 and partially dependent on TLR1, TLR6 and CD14 (27). TLR2 may bind easier to E. faecalis LTA than other TLRs in infected dental pulps, as an overwhelming majority of TLR2-positive macrophages are found in pulp inflammation (29).

The interaction of LTA with TLR2-dependent pathways can activate intracellular messengers, such as MyD88 and MAPKs, and subsequently transcription factors, including NF-κB, to stimulate the expression of inflammatory cytokines (28,30). In the present study, the release of cytokines was quantified using ELISA and the protein expression of cells was assessed using western blot analysis. The dose-dependent increment of TNF-α corresponded to the activation of NF-κB at the LTA concentration of 10 μg/ml. NF-κB is an important transcription factor that promotes the rapid release of cytokines (31). The present results show that NF-κB is responsible for the production of TNF-α, in terms of the effect of the E. faecalis clinical strain LTA on macrophages. The p38 was also activated, albeit not simultaneously with NF-κB. Martinho et al (16) also reported that the phosphorylation of NF-κB and p38 did not reach their peaks at the same time in the study of signaling pathways associated with primary endodontic infectious contents. In the signaling pathways, the upstream adaptors could be responsible for this inconsistent phenomenon regarding the activation of NF-κB and p38 (32).

In the present study, highly purified LTA was successfully isolated from a clinical E. faecalis strain derived from the infected root canal of a patient with persistent apical periodontitis. E. faecalis LTA was also demonstrated to promote the expression of pro-inflammatory cytokine TNF-α through

activating the p38 MAPK and NF-κB signaling pathways, but did not influence the expression of anti-inflammatory cytokine IL-10. Therefore, the present study provides a new insight into the pathogenic mechanisms of *E. faecalis* in endodontic infections and may possibly lead to the development of novel therapeutic options targeting signaling pathways.

Acknowledgements

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