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Tumor necrosis factor-α and interleukin-1β expression pathway induced by *Streptococcus mutans* in macrophage cell line RAW 264.7

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SUMMARY

***Streptococcus mutans*, a major etiological agent of dental caries, frequently causes systemic disease, such as subacute bacterial endocarditis, if it enters the bloodstream. In this study, the production pathways of the proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), induced by *S. mutans* in mouse macrophage were examined using a quantitative real-time polymerase chain reaction and an enzyme-linked immunosorbent assay. The *S. mutans* stimulated the expression of TNF-α and IL-1β mRNA at a multiplicity of infection of 1 : 100, which increased at 2 and 4 h, respectively, to 24 h. It also induced the production of high levels of the TNF-α and IL-1β proteins, which increased at 2 h and reached a peak at 4 and 24 h, respectively. Nuclear factor-κB (NF-κB) was activated and reached a maximum level 30 min after the *S. mutans* treatment. The expression of TNF-α and IL-1β mRNA and protein was suppressed by the treatment with pyrrolidine dithiocarbamate, an NF-κB inhibitor. The *S. mutans*-induced TNF-α expression was suppressed by the presence of SB203580, a p38 mitogen-activated protein (MAP) kinase inhibitor, or SP600125, a Jun N-terminal kinase (JNK) MAP kinase inhibitor. On the other hand, IL-1β expression was inhibited by extracellular signal-regulated kinase (ERK)/p38/JNK MAP kinase inhibitor pretreatment. In addition, TNF-α production was suppressed more in the Toll-like receptor $2^{-/-}$ ($TLR2^{-/-}$) macrophages than in the $TLR4^{-/-}$ macrophages, whereas IL-1β production was suppressed more in the $TLR4^{-/-}$ macrophages than in the $TLR2^{-/-}$ macrophages. These results show that *S. mutans* stimulates the production of TNF-α and IL-1β in the mouse macrophage cell line, RAW 264.7, by activating ERK/p38/JNK, and NF-κB through TLR2 and TLR4, respectively.**

INTRODUCTION

Streptococcus mutans, a gram-positive bacterium, is the principal causative agent of dental caries and an important component of oral biofilms. It can contribute significantly to oral diseases such a pulpitis (Paterson & Watts, 1992). Furthermore, *S. mutans* is correlated with infective endocarditis. It was reported that oral streptococci contributed to infective endocarditis in

more than 20% of cases (Benito *et al.*, 2009). Oral streptococci include several species: *Streptococcus mitis*, *Streptococcus sanguinis*, *S. mutans*, *Streptococcus salivarius* and *Streptococcus intermedius* (*S. intermedius* and *Streptococcus anginosus*) (Tornos *et al.*, 2011). *Streptococcus mutans* and other oral streptococci can enter the bloodstream and cause transient bacteremia in humans after dental extractions, brushing teeth and chewing (Lockhart *et al.*, 2009). Transient bacteremia facilitates *S. mutans* colonization of the valve tissues, particularly in patients with pre-existing valve damage, which can lead to infective endocarditis. Specific serotypes of *S. mutans* are reported to more efficiently invade human coronary artery endothelial cells (Abranches *et al.*, 2009). Nevertheless, the precise pathogenic mechanism of the endocarditis related to *S. mutans* is not completely understood.

Bacterial recognition involves a wide variety of pattern recognition receptors (PRRs) that are specific to pathogen-associated molecular patterns (PAMPs), including Toll-like receptors (TLRs), lectins, scavenger receptors and integrins. Among them, TLRs are essential for the development of the innate immune responses (Medzhitov, 2001; Akira *et al.*, 2006). TLR signaling is mediated by the cytoplasmic toll/interleukin-1 receptor (IL-1R) domain homomeric or heteromeric associations with toll/IL-1R-containing adaptors (Takeda & Akira, 2004; Akira *et al.*, 2006). Two members of the Toll-like receptor family, TLR2 and TLR4, have been identified as signaling receptors for the bacterial cell wall components. TLR2 confers responsiveness to a variety of bacterial components, such as peptidoglycan, lipoprotein and lipoarabinomannan (Lien *et al.*, 1999; Means *et al.*, 1999; Akira *et al.*, 2006). TLR4 is the principal signal transducer of most types of lipopolysaccharide (Takeuchi *et al.*, 1999; Akira *et al.*, 2006).

The TLRs are expressed mainly on antigen-presenting cells, such as monocytes, macrophages and dendritic cells, and can discriminate between distinct molecular patterns associated with microbial components. The recognition of microbial products by TLRs leads to a variety of signal transduction pathways that regulate the nature, magnitude and duration of the inflammatory response (Meylan *et al.*, 2004). Various PAMP/PRR interactions result in mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) activation and the consequent expression of proinflammatory cytokines. However, the signaling pathways including kinases induced by *S. mutans* are not completely understood.

Tumor necrosis factor-α (TNF-α) is a potent proinflammatory cytokine that initiates a cascade of cytokines and increases the vascular permeability, which recruits macrophages and neutrophils to a site of infection. IL-1β is a cytokine produced mainly by activated mononuclear phagocytes, whose principal function is to mediate the host inflammatory responses in innate immunity. It has several biological effects, including the induction of endothelial cell adhesion molecules and the stimulation of chemokine production by endothelial cells and macrophages. This study examined the production of TNF-α and IL-1β, as well as the signaling pathways through TLR stimulation induced by *S. mutans* Ingbritt in mouse macrophages. The results showed that *S. mutans* Ingbritt induces TNF-α and IL-1β production by stimulating TLR2-dependent and TLR4-dependent NF-κB activation through p38/c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinases (ERK)/p38/JNK activation, respectively.

METHODS

Reagents

The Mouse TNF-α and IL-1β enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN). The Mouse NF-κB transcription factor assay kit was obtained from Chemicon (Billerica, MA). Pyrrolidine dithiocarbamate (PDTC) was acquired from Calbiochem (San Diego, CA). The MAPK inhibitors, PD98059 for ERK, SB203580 for p38 MAPK (p38), and SP600125 for JNK, were supplied by Calbiochem.

Mice

BALB/c mice deficient for TLR2 and TLR4 ($TLR2^{-/-}$ and $TLR4^{-/-}$, respectively) were a kind gift from Dr S. Akira (Department of Host Defense, Research Institute for Microbial Disease, Osaka University, Osaka, Japan). Six-week-old BALB/c mice for normal controls were purchased from the Hana Company in Pyeongtaek, Korea. The mice were bred under specific pathogen-free conditions and processed

according to the guidelines of the Animal Care Committee of Pusan National University, Korea.

Bacterial culture and infection conditions

Streptococcus mutans Ingbritt was cultured in brain–heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C in humidified air containing 5% CO_2. Viable *S. mutans* cells were counted on BHI agar according to their optical density at 595 nm (OD_{595}). To prepare the bacteria for infection, overnight cultures were diluted to an OD_{595} of 0.1 (2.69×10^7 colony-forming units) in BHI broth. The bacteria were washed once, resuspended in phosphate-buffered saline and used to infect the macrophages at a set macrophage to *S. mutans* ratio of (1:0, 1:1, 1:10, 1:20, 1:50 or 1:100). After reverse transcription-polymerase chain reaction (RT-PCR) analysis for TNF-α and IL-1β, the appropriate ratio of bacterial infection was determined. The infection ratio used in this study was 100 bacteria per macrophage (1 : 100).

Cell culture

The mouse macrophage cell line, RAW 264.7, was purchased from the American Type Culture Collection (Rockville, MD). The cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Life Technologies Inc., Paisley, UK), 100 U penicillin/ml and 100 μg streptomycin/ml, and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Isolation of peritoneal macrophages

Thioglycollate-elicited peritoneal macrophages were obtained from TLR2 and TLR4 knockout mice according to the procedure described previously (Sato *et al.*, 2002). Briefly, the mice were injected intraperitoneally with 2 ml of 3% thioglycollate broth. Three days after the injection, the peritoneal elicited cells were harvested by lavage with 10 ml serum-free RPMI-1640 medium and added to the wells of 24-well tissue culture plates (Nunc, Roskilde, Denmark). After 3 h of incubation at 37°C in an atmosphere containing 5% CO_2, the non-adherent cells were removed by vigorous washing (three times) with warm serum-free medium and the adherent cells were incubated overnight in complete RPMI-1640 medium. The cells were then stimulated with *S. mutans*. The culture supernatants were collected at 2, 4, 8 or 18 h. The levels of TNF-α and IL-1β production in the culture supernatants were measured by ELISA.

Quantitative RT-PCR

The RAW 264.7 cells (5×10^5 cells per well) were grown in six-well plates for each time. The total RNA was prepared from the RAW 264.7 cells using a TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The total RNA (1 μg) isolated from each sample was used as a template for cDNA synthesis. Quantitative RT-PCR was performed with an ABI-Prism 7500 Sequence Detector System (Life Technologies, Carlsbad, CA) using a Syber Green kit (Promega, Madison, WI, USA). The results were expressed as relative amounts of the target gene using β-actin as the inner reference gene. The sequences of primers were as follows: TNF-α, 5′-GCC TAT GTC TCA GCC TCT TC-3′ and 5′-CAC TTG GTG GTT TGC TAC GA-3′; IL-1β, 5′-GAT ACA AAC TGA TGA AGC TCG TCA-3′ and 5′-GAG ATA GTG TTT GTC CAC ATC CTG A-3′; and β-actin, 5′-GGG TCA GAA CTC CTA TG-3′ and 5′-GTA ACA ATG CCA TGT TCA AT-3′.

Measurement of TNF-α and IL-1β

The amounts of TNF-α and IL-1β released into the culture media after *S. mutans* stimulation were analysed using an ELISA kit purchased from R&D Systems. Briefly, a standard or sample solution was added to the precoated ELISA well plate for 2 h. Polyclonal anti-TNF-α and anti-IL-1β antibodies conjugated with horseradish peroxidase were incubated at room temperature. A substrate solution containing chromogen was added and allowed to react for 30 min. The levels of cytokine expression were assessed using an ELISA reader (TECAN Inc., Mannedorf, Switzerland) at 450 nm. Each densitometric value expressed as mean ± SD was obtained from three independent experiments.

NF-κB activation assay

The nuclear extracts were prepared from the RAW 264.7 cells after *S. mutans* stimulation for

10 min, 20 min, 30 min, 1 h, 2 h, 4 h and 8 h. Briefly, the cells were washed with ice-cold phosphate buffered saline. The cell pellets were suspended in a hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5–5 mM dithiothreitol, 0.1% Triton X-100, protease inhibitor cocktail) and incubated for 15 min on ice. The lysates were centrifuged, and the supernatants were discarded. The pellets were then suspended in a hypotonic lysis buffer, and the lysates were slowly drawn into a syringe and ejected with a single stroke through a small gauge needle. The lysates were centrifuged and the supernatants were transferred to new vials. The nuclear pellets were suspended in an extract buffer (20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5–5 mM dithiothreitol, 1.0% Igepal CA-630, 25% (volume/volume) glycerol, protease inhibitor), incubated for 30 min on ice, and stirred by vigorous vortexing for 10 s. The lysates were centrifuged and the supernatants containing the nuclear proteins were transferred to new vials.

An NF-κB activation assay was carried out using an NF-κB p50/p65 transcription factor assay. Briefly, the samples prepared above, negative control sample, positive control sample, and competitive oligonucleotide control sample were added to each plate well coated with the capture probe, which is a double-stranded biotinylated oligonucleotide containing the flanked DNA binding consensus sequence for NF-κB (5′-GGGACTTTCC-3′). After incubation for 2 h at room temperature, the supernatant in each well was discarded. The active NF-κB protein that was immobilized on the capture probe bound to the streptavidin plate well was detected using the specific primary antibody, rabbit anti-NF-κB p65. In particular, rabbit anti-NF-κB p65 was added to each assay well and incubated for 1 h at room temperature. A horseradish peroxidase-conjugated anti-rabbit antibody was added to each well as the secondary antibody and incubated for 30 min at room temperature. A pre-equilibrated 3,3′,5,5′-tetramethybenzidine substrate solution was added to each well. The plate was incubated at room temperature for 10 min to allow color development. The absorbance of each well was measured at 450 nm using a microplate reader (TECAN). Each value is expressed as the mean ± SD obtained from three independent experiments.

RESULTS

Streptococcus mutans induces messenger RNA expression and protein synthesis of TNF-α and IL-1β in RAW 264.7 cells

The RAW 264.7 cells were infected with *S. mutans* at different ratios (1:0, 1:10, 1:20, 1:50 or 1:100) for 2 h. The total RNA was extracted and quantitative RT-PCR was performed for TNF-α and IL-1β. As shown in Fig. 1, TNF-α and IL-1β messenger RNA (mRNA) expression was optimally induced at a multiplicity of infection 1 : 100. Hence, bacterial stimulation with approximately 100 bacteria per macrophage was selected for the following experiments.

To clarify the time kinetics of TNF-α and IL-1β mRNA expression, the RAW 264.7 cells were

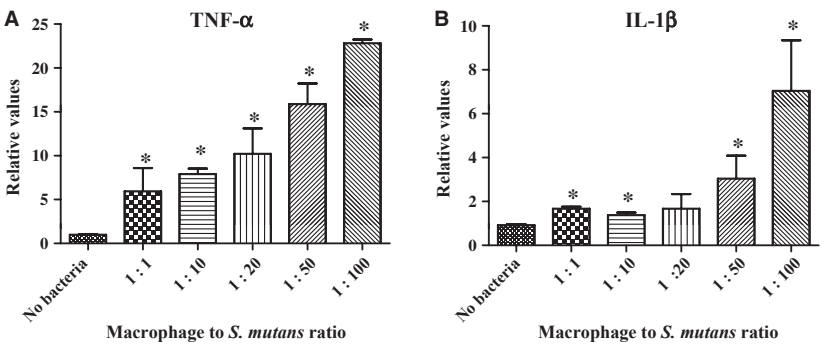

Figure 1 Tumor necrosis factor-α (TNF-α) (A) and interleukin-1β (IL-1β) (B) messenger RNA expression after stimulation with various infection ratios of macrophages to live *Streptococcus mutans*. The RAW 264.7 cells were treated with the bacteria at different macrophage to bacteria ratios, and the total RNA was prepared after 2 h. Reverse transcription-polymerase chain reaction was performed as described in the Methods. **P* < 0.05 compared with untreated control as determined by Mann–Whitney test.

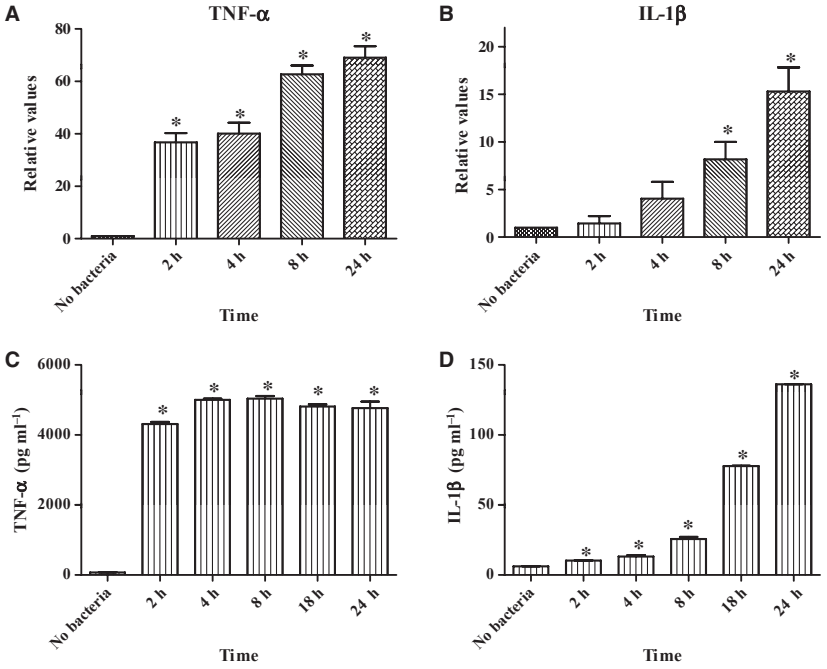

Figure 2 Time kinetics of *Streptococcus mutans*-induced tumor necrosis factor-α (TNF-α) (A, C) and interleukin-1β (IL-1β) (B, D) expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (A, B) and enzyme-linked immunosorbent assay (ELISA) (C, D). The RAW 264.7 cells were treated with *S. mutans*, and the total RNA was prepared at the indicated times. The qRT-PCR was performed as described in the Methods. The levels of TNF-α and IL-1β production in the culture supernatants were measured by ELISA. The values are reported as the mean ± SD obtained from three independent experiments. $^*P < 0.05$ compared with untreated control as determined by Mann–Whitney test.

treated with *S. mutans* for 2, 4, 8 and 24 h. The total RNA was prepared, and TNF-α and IL-1β mRNA expression was examined by quantitative RT-PCR. The TNF-α mRNA was detected at 2 h after the *S. mutans* treatment and gradually increased (Fig. 2A). Interleukin-1β mRNA was detected at 4 h, and also increased gradually up to 24 h (Fig. 2B). To determine the time kinetics for TNF-α and IL-1β protein production, the RAW 264.7 cells were challenged with *S. mutans*. The culture supernatants were collected at the indicated time points and the level of TNF-α and IL-1β protein production was analysed by ELISA. Production of TNF-α was evident at 2 h, increasing gradually and reaching a peak 4 h after *S. mutans* treatment (Fig. 2C). The IL-1β production was evident at 2 h. The level increased gradually, and reached a peak 24 h after the *S. mutans* treatment (Fig. 2D). This suggests that *S. mutans* can induce the mRNA expression and protein production of TNF-α and IL-1β in the mouse macrophage cell line, RAW 264.7 cells.

Streptococcus mutans-induced TNF-α and IL-1β expression mediates the activation of NF-κB in RAW 264.7 cells

To determine the possible involvement of NF-κB, a transcription factor, in *S. mutans* -induced TNF-α and IL-1β gene expression, the activation of NF-κB in *S. mutans* -treated RAW 264.7 cells at various time intervals was measured using the method described in the Methods. *Streptococcus mutans* markedly enhanced NF-κB activation. It reached its maximum level at 30 min but declined gradually thereafter (Fig. 3).

To determine if NF-κB is essential for TNF-α and IL-1β gene expression by *S. mutans*, the RAW 264.7 cells were pretreated with the 50 μM PDTC, NF-κB inhibitor and stimulated with *S. mutans* for 30 min (Biswas *et al.*, 2007). Expression of TNF-α and IL-1β mRNA was suppressed markedly by the presence of PDTC at a concentration of 50 μM (Fig. 4A, B). The production of the TNF-α and IL-1β proteins induced by *S. mutans* was also inhibited greatly by pretreat-

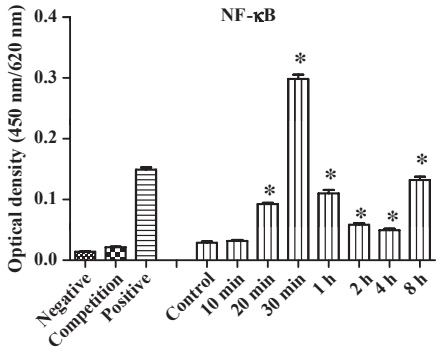

Figure 3 *Streptococcus mutans*-induced nuclear factor-κB (NF-κB) activation in RAW 264.7 cells. The cells were stimulated with *S. mutans* for various times. The nuclear extracts from the RAW 264.7 cells after the *S. mutans* treatment were prepared, and an NF-κB activation assay was carried out, as described in the Methods. Negative, competition and positive controls provided by the manufacturer were used as controls for NF-κB activation. The values are reported as the mean ± SD obtained from three independent experiments. **P* < 0.05 compared with untreated control as determined by Mann–Whitney test.

ment with 50 μM PDTC (Fig. 4C, D). This indicates that the production of TNF-α and IL-1β by *S. mutans* can be regulated at the transcriptional level by NF-κB.

MAP kinase inhibitors suppress *S. mutans* -induced TNF-α and IL-1β expression

Involvement of the MAP kinase pathway in TNF-α and IL-1β upregulation by *S. mutans* was investigated. The RAW 264.7 cells were pretreated with three MAPK inhibitors 30 min before *S. mutans* stimulation at a concentration of 10, 20 or 30 μM (Kang *et al.*, 2004). The *S. mutans* -induced TNF-α gene expression and IL-1β gene expression was suppressed in the presence of each dose of inhibitor, PD98059, an ERK MAPK inhibitor, or SB203580 (30 μM), a p38 MAPK inhibitor, or SP600125 (20 or 30 μM), a JNK MAPK inhibitor (Fig. 5A, B). The TNF-α protein production induced by *S. mutans* was inhibited by all three inhibitors, but less affected by the

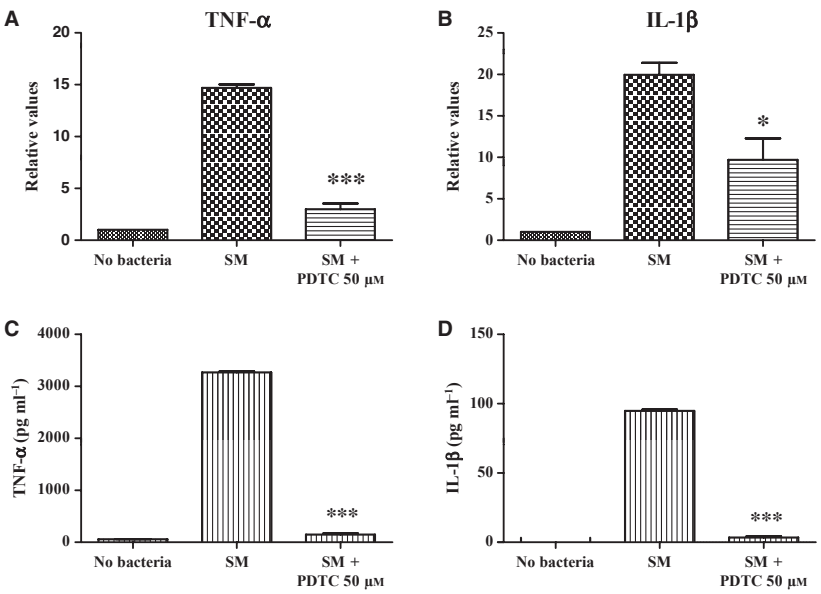

Figure 4 Effects of pyrrolidine dithiocarbamate (PDTC) on *Streptococcus mutans*-induced tumor necrosis factor-α (TNF-α) (A, C) and interleukin-1β (IL-1β) (B, D) expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (A, B) and enzyme-linked immunosorbent assay (ELISA) (C, D). The RAW 264.7 cells were pretreated with PDTC (50 μM) for 30 min before the *S. mutans* treatment. The total RNA was prepared 2 h for TNF-α and 4 h for IL-1β after the *S. mutans* treatment, and qRT-PCR was performed as described in the Methods. The RAW 264.7 cells were pretreated with PDTC (50 μM) for 30 min before the *S. mutans* treatment and the culture supernatants were collected 4 h for TNF-α and 24 h for IL-1β after *S. mutans* treatment. The levels of TNF-α and IL-1β production in the culture supernatants were measured by ELISA. Values are reported as the mean ± SD obtained from three independent experiments. **P* < 0.05 or ****P* < 0.001 compared with untreated control as determined by Mann–Whitney.

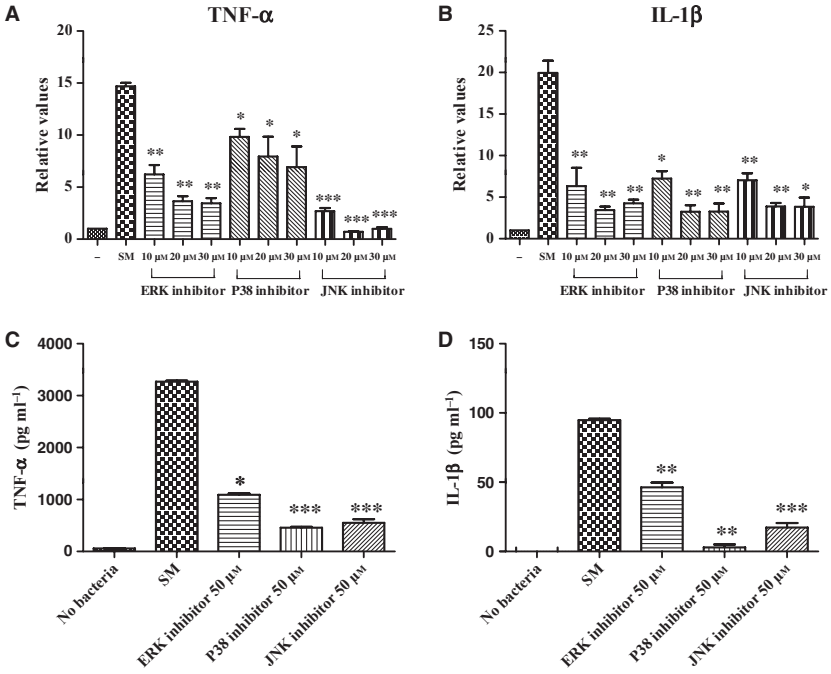

Figure 5 Effects of the mitogen-activated protein kinase (MAPK) inhibitors on the *Streptococcus mutans*-induced tumor necrosis factor-α (TNF-α) (A, C) and interleukin-1β (IL-1β) (B, D) expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (A, B) and enzyme-linked immunosorbent assay (ELISA) (C, D). The RAW 264.7 cells were pretreated with each MAPK inhibitor (+, 10 μM; ++, 20 μM; +++, 30 μM) for 30 min before the *S. mutans* treatment. The total RNA was prepared 2 h for TNF-α and 4 h for IL-1β after the *S. mutans* treatment and qRT-PCR was performed as described in the Methods. RAW 264.7 cells were pretreated with each MAPK inhibitor (50 μM) for 30 min before the *S. mutans* challenge, and the culture supernatants were collected 4 h for TNF-α and 24 h for IL-1β after the *S. mutans* treatment. The levels of TNF-α and IL-1β production in the culture supernatants were measured by ELISA. The values are expressed as the mean ± SD obtained from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ or $^{***}P < 0.001$ compared with untreated control as determined by Mann–Whitney.

ERK inhibitor, PD98059 (Fig. 5C). Similar to the result of mRNA expression, the protein production of IL-1β was inhibited by all three inhibitors, but less affected by ERK inhibitor (Fig. 5D). Overall, these results indicate that the p38 and JNK kinase pathways are involved in TNF-α and IL-1β expression by *S. mutans*, and the ERK pathway to a lesser extent.

Streptococcus mutans induces TNF-α and IL-1β expression through the TLR2 and TLR4 receptor, respectively

To determine if TLRs play a role in the signal transduction evoked by *S. mutans* in macrophages, this study first focused on TLR2 and TLR4 because they recognize the bacterial ligands expressed by most gram-positive and gram-negative bacteria. Using TLR2 and TLR4 knockout mice as a source of macrophages, the peritoneal macrophages were prepared and treated with *S. mutans* for 2, 4, 8 and 18 h. The production of the TNF-α and IL-1β proteins was analysed by ELISA. As shown in Fig. 6A, TNF-α production was suppressed more in the $TLR2^{-/-}$ peritoneal macrophages than in the $TLR4^{-/-}$ peritoneal macrophages. On the other hand, IL-1β production was suppressed more in the $TLR4^{-/-}$ peritoneal macrophages than in the $TLR2^{-/-}$ peritoneal macrophages, as shown in Fig. 6B. In addition, to confirm these results, TNF-α and IL-1β production profiles were investigated using the peritoneal macrophages from normal BALB/c mice (Fig. 6C, D). Five mice for control and five for experimental group were used. Patterns and amounts of TNF-α and IL-1β production were similar to those of RAW 264.7 cells treated with *S. mutans*. Production of TNF-α was greater in normal mouse peritoneal macrophages than in $TLR2^{-/-}$ mouse peritoneal macrophages, but there was little difference in comparison to $TLR4^{-/-}$ mouse peritoneal macrophages. As for IL-1β, when *S. mutans* were treated, normal mouse peritoneal macrophages

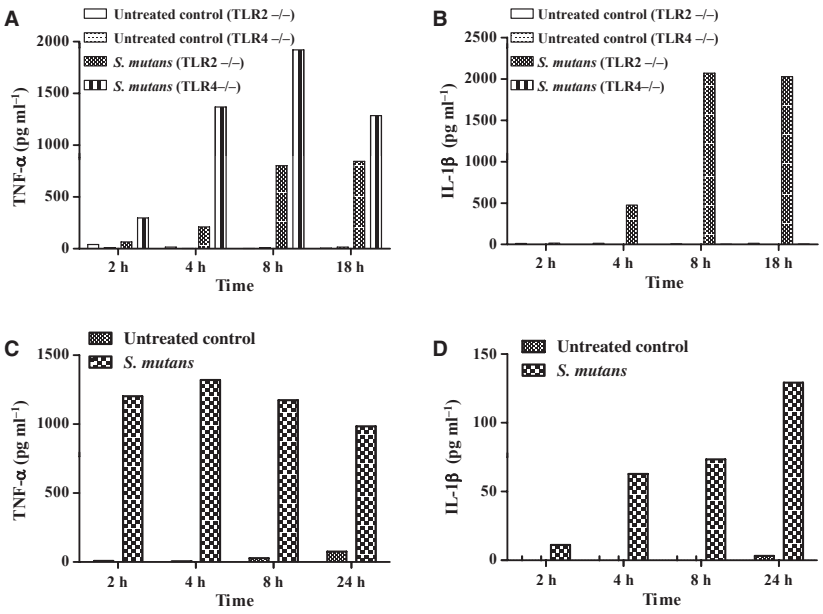

Figure 6 Time kinetics of *Streptococcus mutans*-induced tumor necrosis factor-α (TNF-α) (A, C) and interleukin-1β (IL-1β) (B, D) protein production in the peritoneal macrophages of TLR2-deficient mice, TLR4-deficient mice and normal BALB/c mice. The peritoneal macrophages were prepared and treated with *S. mutans,* as described in the Methods. The culture supernatants were collected at the indicated times. The levels of TNF-α and IL-1β production in the culture supernatants were measured by enzyme-linked immunosorbent assay. The values are expressed as the mean ± SD obtained from three independent experiments.

produced more IL-1β than TLR4$^{-/-}$ mouse peritoneal macrophages, but much less than those of TLR2$^{-/-}$ mouse peritoneal macrophages. These results suggest that TLR2 is involved in *S. mutans* -induced TNF-α production, whereas *S. mutans* induces IL-1β production through TLR4 in mouse macrophages.

DISCUSSION

In this study, it was demonstrated that *S. mutans* Ingbritt stimulates TNF-α and IL-1β production in the mouse macrophage cell line, RAW 264.7 cells, by activating p38/JNK and ERK/p38/JNK, and NF-κB through TLR2 and TLR4, respectively. Initially, the effect of increasing concentrations of *S. mutans* on TNF-α and IL-1β expression was examined in RAW 264.7 cells. The expressions of TNF-α and IL-1β mRNA were induced in a dose-dependent manner (Fig. 1). The *S. mutans* also induced high levels of TNF-α and protein production (Fig. 2). These results are similar to other reports showing that *S. mutans* stimulates the production of TNF-α and IL-1β in human peripheral blood mononuclear cells or human monocytes, or the *S. mutans* OMZ175 protein I/II induces the production of TNF-α and IL-1β in human synoviocytes (Benabdelmoumene *et al.*, 1991; Soell *et al.*, 1994; Jiang *et al.*, 1999; Gourieux *et al.*, 2001).

Nuclear factor-κB is a multiunit transcription factor that plays a key role in the induction of genes for proinflammatory cytokines (Collart *et al.*, 1990; Ziegler-Heitbrock *et al.*, 1993; Neff *et al.*, 2001) as well as many other immunoregulatory genes (Baeuerle, 1991). However, the involvement of NF-κB in *S. mutans* -induced TNF-α and IL-1β expression is not completely understood. This study examined whether or not NF-κB might be related to TNF-α and IL-1β expression in RAW 264.7 cells. *Streptococcus mutans* induced the activation of NF-κB, which reached a maximum at 30 min after the *S. mutans* treatment (Fig. 3). Next, PDTC, NF-κB inhibitor, was used to determine if NF-κB activation is critical for TNF-α and IL-1β expression. As expected, both TNF-α and IL-1β mRNA expression and protein synthesis were suppressed by PDTC (Fig. 4). This suggests that *S. mutans* induces TNF-α and IL-1β expression through the activation of NF-κB.

The possible involvement of MAPK in *S. mutans* -induced TNF-α and IL-1β expression was examined. The MAPK pathway is central to many host

responses. In addition, it is one of the major signaling pathway transmitting signals to the immediate early genes involved in regulating the cytokine responses. There are at least three main groups of MAPK, ERK, p38 and JNK, which can be activated in response to a variety of extracellular stimuli in many types of cells (Firestein & Manning, 1999). In this study, using the inhibitors of these MAPK, it was shown that p38 and JNK play a role in the induction of TNF-α and IL-1β mRNA by *S. mutans* in RAW 264.7 cells. PD98059, which blocks the ERK 1/2 signaling cascade, had less effect on TNF-α and IL-1β expression. This indicates that TNF-α and IL-1β induction is mostly dependent on the p38 and JNK signaling pathways. These results are supported by a previous report showing that activation of the p38 MAPK pathway plays a key role in the production of proinflammatory cytokines, such as IL-1β, TNF-α, IL-6 and IL-8, by various cells in response to either cytokines or pathogens or pathogen components (Ono & Han, 2000). Similarly, it was reported that the p38 and JNK pathways are involved in the signal transduction of lipopolysaccharide-induced TNF-α production, whereas an inhibitor of the ERK pathway did not affect the production of TNF-α by Kupffer cells (Shen *et al.*, 2005). It was also reported that the specific inhibitors of JNK and p38 MAPK, SP600125 and SB203580, suppressed the lipopolysaccharide-induced increase in IL-1β gene expression in RAW 264.7 cells (Kang *et al.*, 2004).

Toll-like receptors are important PRRs that initiate the innate immune responses in response to bacterial or viral stimuli. Upon exposure to microorganisms, these PRRs trigger distinct intracellular signaling pathways that activate a set of inflammatory genes. Until now, 11 human TLRs and 13 mouse TLRs have been identified, and each TLR appears to recognize distinct PAMPs derived from various microorganisms (Akira *et al.*, 2006). Among the TLRs, TLR2 and TLR4 are the most important PRRs for bacterial recognition in the innate immune response. Therefore, this study examined whether or not TLR2 and TLR4 are involved in *S. mutans*-induced production of TNF-α and IL-1β. As shown in Fig. 6, TNF-α production was suppressed more in the $TLR2^{-/-}$ macrophages than in the $TLR4^{-/-}$ macrophages. This contrasts with previous reports showing that TLR2 is not related to the release of TNF-α by the protein I/II of *S. mutans* in mouse macrophages (Hajishengallis *et al.*, 2002) and the ability of *S. mutans* protein Ag I/II to stimulate TNF-α secretion is independent of TLR2 and TLR4 (Zeisel *et al.*, 2005). However, these results are supported by other reports suggesting that TLR2 and TLR4 play specific roles in the recognition of gram-positive and gram-negative bacteria, respectively (Lien *et al.*, 1999; Means *et al.*, 1999; Akira *et al.*, 2006; Tietze *et al.*, 2006; Takeuchi & Akira, 2007). In contrast to TLR2-related TNF-α release, IL-1β production was depressed more in the $TLR4^{-/-}$ macrophages than in the $TLR2^{-/-}$ macrophages. This finding is in contrast to one report showing the TLR2-related recognition of gram-positive bacteria (Tietze *et al.*, 2006). However, this result is similar to another report showing that the ability of *S. mutans* protein Ag I/II to stimulate the release of a proinflammatory cytokine is partially dependent on TLR4 (Gourieux *et al.*, 2001). Overall, this study suggests that *S. mutans* induces TNF-α and IL-1β production through TLR2 and TLR4, respectively.

Streptococcus mutans is a commensal oral bacterium that contributes to an oral biofilm by producing tenacious extracellular polysaccharides, such as glucan and fructan (Hamada & Slade, 1980; Munro *et al.*, 1995). It is an important cariogenic bacterium because of its acidogenic and aciduric properties. However, when *S. mutans* gains entrance into sites that are normally sterile, it can adhere to the heart causing endocarditis (Nomura *et al.*, 2006). Nevertheless, the precise mechanism of *S. mutans* in such diseases is not completely understood. This study demonstrated that *S. mutans* stimulates the expression of TNF-α and IL-1β through the activation of p38/JNK and ERK/p38/JNK MAPK, and NF-κB by triggering TLR2 and TLR4, respectively. These results should provide insight into the inflammatory response of *S. mutans* and its capacity to stimulate the host cells in oral and non-oral infections, such as endocarditis (Que & Moreillon, 2011). Further studies of the involvement of additional kinase players in the MAPK signaling pathways through TLR will be needed to better understand the molecular mechanisms involved in *S. mutans* infections.

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