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# Lipoteichoic acid enhances IL-6 production in human synovial fibroblasts via TLR2 receptor, PKCδ and c-Src dependent pathways☆

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ABSTRACT

Patients with rheumatoid arthritis (RA) are at increased risk of developing infections and appear to be particularly susceptible to septic arthritis. Lipoteichoic acid (LTA), a cell wall component of Gram-positive bacteria is an amphiphilic, negatively charged glycolipid. However, the effects of LTA on human synovial fibroblasts are largely unknown. We investigated the signaling pathway involved in IL-6 production stimulated by LTA in rheumatoid arthritis synovial fibroblasts (RASF). LTA caused concentration- and time-dependent increases in IL-6 production. LTA-mediated IL-6 production was attenuated by Toll-like receptor 2 (TLR2) monoclonal antibody or siRNA. Pretreatment with PKCδ inhibitor (rottlerin), c-Src inhibitor (PP2), AP-1 inhibitor (tanshinone IIA) and NF-κB inhibitor (PDTC and TPCK) also inhibited the potentiating action of LTA. However, focal adhesion kinase (FAK) mutant and siRNA did not affect LTA-mediated IL-6 production. Stimulation of cells with LTA increased the PKCδ and c-Src phosphorylation and kinase activity. LTA increased the accumulation of p-c-Jun and p-p65 in the nucleus, as well as AP-1 and NF-κB luciferase activity. LTA-mediated increase of AP-1 and NF-κB luciferase activity was inhibited by rottlerin and PP2 or TLR2 and PKCδ siRNA or c-Src mutant. Our results suggest that LTA-increased IL-6 production in human synovial fibroblasts via the TLR2 receptor, PKCδ, c-Src, AP-1 and NF-κB signaling pathways.



## 1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by robust infiltration of leukocytes into the synovium, resulting in hyperplasia of the synovial lining, progressive cartilage destruction, and erosion of the underlying bone [1]. In response to the proinflammatory cytokines produced by macrophages, such as interleukin-1β and tumor necrosis factor-α (TNF-α), RA synovial fibroblasts produce chemokines that promote inflammation, neovascularization, and cartilage degradation via activation of matrix-degrading enzymes, such as matrix metalloproteinases [2].

Endotoxin, a component of the outer membrane of Gram-negative bacteria, has been identified as the prime initiation of Gram-negative bacterial septic shock. In contrast to endotoxic shock, we know relatively little about the mechanisms of Gram-positive bacteria-induced septic shock. Lipoteichoic acid (LTA), a cell wall component of Gram-positive bacteria is an amphiphilic, negatively charged glycolipid [3]. Recently, a number of studies have indicated that LTA shares many inflammatory properties of LPS and plays a role in the pathogenesis of septic shock or severe inflammatory response induced by Gram-positive bacterial infection [4]. Most of these effects are due to the activation of macrophages and generation of proinflammatory cytokines, such as TNF-α, interleukin (IL)-6, and IL-8 [5,6]. LTA binds Toll-like receptor 2 (TLR2) [7], which activates transcription factors and induces gene expression [7,8].

IL-6 is a multifunctional cytokine that plays a central role in both innate and acquired immune responses. It is the predominant mediator of the acute phase response, an innate immune

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*Abbreviations:* LTA, lipoteichoic acid; PGN, peptidoglycan; siRNA, small interference RNA; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; RA, rheumatoid arthritis; RASF, rheumatoid arthritis synovial fibroblasts; TLR, Toll-like receptor; ODN, oligonucleotide; qPCR, quantitative real-time PCR.

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mechanism which is triggered by infection and inflammation [9,10]. IL-6 also plays multiple roles during the subsequent development of acquired immunity against incoming pathogens, including regulation of the expressions of cytokine and chemokine, stimulation of antibody production by B cells, regulation of macrophage and dendritic cell differentiation, and the response of regulatory T cells to microbial infection [9,10]. In addition to these roles in pathogen specific inflammation and immunity, IL-6 levels are elevated in chronic inflammatory conditions, such as RA [11,12]. Several consensus sequences, including those for NF-κB, CREB, NF-IL-6, and activator protein-1 (AP-1) in the 5′ promoter region of the IL-6 gene, have been identified as regulatory sequences that induce IL-6 in response to various stimuli [13,14]. AP-1 is commonly activated in response to inflammatory stimuli and has been implicated in cytokine expression and cellular immune responses [15,16]. Moreover, c-Jun/AP-1 has been implicated in RA [17]. NF-κB, the other key transcription factor that regulates IL-6 expression, is a dimer of either transcription factor p65 or transcription factor p50 (22). NF-κB activation has been shown to be involved in IL-6 expression in synovial fibroblasts [18,19].

Patients with RA are at increased risk of developing infections and appear to be particularly susceptible to septic arthritis. IL-6 plays an important role in acute and chronic inflammatory mediator in RA. LTA, a component of the membrane of Gram-positive bacteria, induces IL-6 expression in macrophages [20,21]. Although a role for LTA in IL-6 induction has been implied for some cell types, the signaling pathway for LTA in IL-6 production in synovial fibroblasts has not been extensively studied. In the present study, we explored the intracellular signaling pathway involved in LTA-induced IL-6 production in human synovial fibroblast cells. The results showed that LTA activates TLR2 receptor and results in the activation of PKCδ, c-Src, AP-1 and NF-κB pathways, leading to up-regulation of IL-6 expression.

## 2. Materials and methods

### 2.1. Materials

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, TLR2, TLR4, FAK, PKCδ, c-Src, c-Jun, p-c-Jun, p65, lamin B, and the small interfering RNAs (siRNAs) against TLR2, c-Jun, p65 and control for experiments using targeted siRNA transfection (each consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ON-TARGET smart pool FAK and PKCδ siRNA and ON-TARGET plus siCONTROL Nontargeting pool siRNA were purchased from Dharmacon (Lafayette, CO, USA). Rabbit polyclonal antibodies specific for FAK phosphorylated at $Tyr^{397}$, PKCδ phosphorylated at $Thr^{505}$, c-Src phosphorylated at $Tyr^{416}$, and p65 phosphorylated at $Ser^{536}$ were purchased from Cell Signaling and Neuroscience (Danvers, MA, USA). GF109203X, rottlerin, PP2, PDTC and TPCK were purchased from Calbiochem (San Diego, CA, USA). The Tanshinone IIA was purchased from BIOMOL (Butler Pike, PA, USA). IL-6 enzyme immunoassay kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). The AP-1 and NF-κB luciferase plasmid were purchased from Stratagene (La Jolla, CA, USA). The phosphorylation site mutant of FAK(Y397F) was a gift from Dr. J.A. Girault (Institut du Fer a' Moulin, Moulin, France). The c-Src dominant negative mutant was a gift from Dr. S. Parsons (University of Virginia Health System, Charlottesville, VA, USA). The human IL-6 promoter construct pIL6-luc651(−651/+1), AP-1 site mutation (pIL6-luc651ΔAP1), NF-κB site mutation (pIL6-

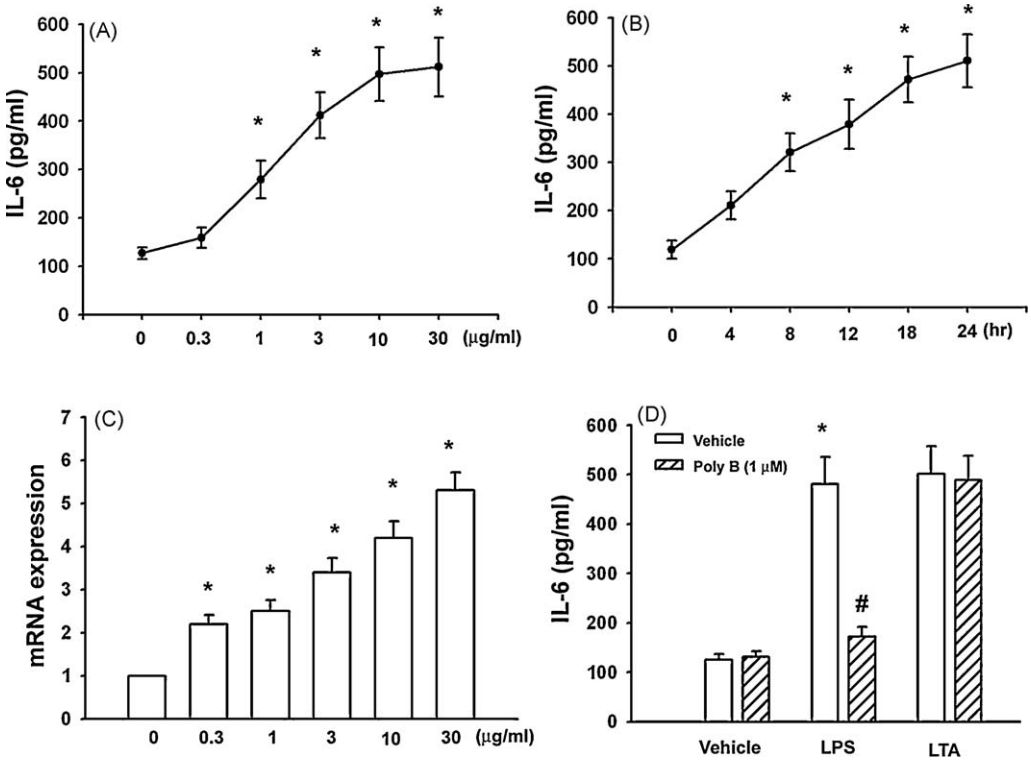


**Fig. 1.** Concentration- and time-dependent increases in IL-6 production by LTA. Human synovial fibroblasts were incubated with various concentrations of LTA for 24 h (A) or with LTA (10 μg/ml) for 4, 6, 12 or 24 h (B). Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. $^{*}p < 0.05$ as compared with basal level. (C) RASF cells were incubated with LTA (0.3–30 μg/ml) for 24 h, the mRNA expression of IL-6 was examined by qPCR. (D) RASF cells were pretreated with polymyxin B (poly B, 1 μM) for 30 min followed by stimulation with LPS (1 μM) or LTA (10 μg/ml) for 24 h. Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. $^{*}p < 0.05$ as compared with basal level. $^{\#}p < 0.05$ as compared with LPS or LTA-treated group.

luc651ΔNF-κB) and C/EBP-β site mutation (pIL6-luc651ΔC/EBP-β) were gifts from Dr. Oliver Eickelberg (Department of Medicine II, University of Giessen, Giessen, Germany). The pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA, USA). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. Cell cultures

Human synovial fibroblasts were isolated using collagenase treatment from synovial tissues obtained from ten patients with RA after approval by the hospital's ethics committee. Patients with RA fulfilled the diagnostic criteria of the American College of Rheumatology [22]. Fresh synovial tissues were minced and digested in a solution of collagenase, and DNase. Isolated fibroblasts were filtered through 70 μM nylon filters. The cells were grown on plastic cell culture dishes in 95% air–5% $CO_2$ with RPMI 1640 (Gibco, Grand Island, NY, USA) which was supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) (pH adjusted to 7.6). Fibroblasts from passages four to nine were used for the experiments [23].

### 2.3. Measurements of IL-6 production

Human synovial fibroblasts were cultured in 24-well culture plates. After reaching confluence, cells were treated with LTA, and then incubated in a humidified incubator at 37 °C for 24 h. For examination of the downstream signaling pathways involved in LTA treatment, cells were pretreated with various inhibitors for 30 min before LTA (10 μg/ml) administration. After incubation, the medium was removed and stored at −80 °C until assay. IL-6 in the medium was assayed using the IL-6 enzyme immunoassay kits, according to the procedure described by the manufacturer.

### 2.4. Quantitative real-time PCR

Total RNA was extracted from synovial fibroblasts using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using oligo(dT) primer. The quantitative real-time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA, USA). 100 ng of total cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (β-actin was used as internal control) (Applied Biosystems, CA, USA). qPCR assays were carried out in triplicate on an StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted $C_T$).

### 2.5. Western blot analysis

The cellular lysates were prepared as described previously [23]. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against PKCδ, p-PKCδ, c-Src or p-c-Src (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY, USA).

### 2.6. Kinase activity assay

PKCδ or c-Src activity was assessed by PKC Kinase Activity Assay Kit (Assay Designs, Ann Arbor, MI, USA) or c-Src Kinase Activity Assay Kit (Abnova, Taipei, Taiwan) according to manufacturer's instructions. Kinase activity kit is based on a solid-phase ELISA that uses a specific synthetic peptide as a substrate for PKCδ or c-Src and a polyclonal antibody that recognized the phosphorylated form of the substrate.

### 2.7. Synthesis of NF-κB and AP-1 decoy ODNs

We used a phosphorothioate double-stranded decoy oligonucleotide ODN carrying the NF-κB-consensus sequence 5′-CCT-TGAAGGGATTTCCCTCC-3′/3′-GGAACTTCCCTAAAGGGAGG-5′. The AP-1 decoy ODN sequence was 5′-TGTCTGACTCATGTC-3′/3′-ACA-GACTGAGTACAG-5′. The mutated (scrambled) form 5′-TTGCCG-TACCTGACTTAGCC-3′/3′-AACGGCATGGACTGAATCGG-5′ was used as a control. ODN (5 μM) was mixed with Lipofectamine 2000 (10 μg/ml) for 25 min at room temperature, and the mixture was added to cells in serum-free medium. After 24 h of transient transfection, the cells were used for the following experiments.

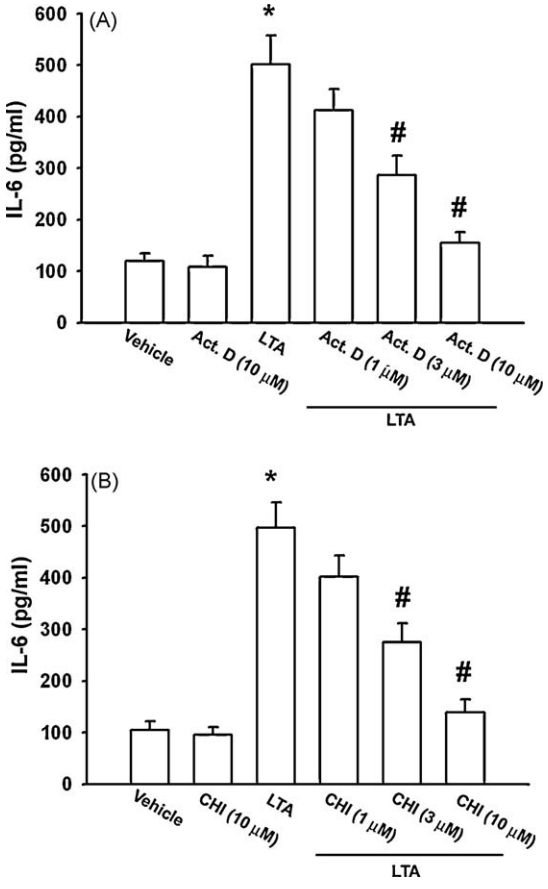


**Fig. 2.** Inhibition of LTA-induced IL-6 production by actinomycin D and cycloheximide. Cells were pretreated with or without actinomycin D (Act. D, 1–10 μM; A) or cycloheximide (CHI, 1–10 μM; B) for 30 min, and then incubated in the absence or presence of LTA (10 μg/ml) for 24 h. Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. $^{*}p < 0.05$ as compared with basal level. $^{\#}p < 0.05$ as compared with LTA-treated group.

### 2.8. Transfection and reporter gene assay

Human synovial fibroblasts were co-transfected with 0.8 μg luciferase plasmid and 0.4 μg β-galactosidase expression vector. RASF cells were grown to 80% confluence in 12 well plates and were transfected on the following day by Lipofectamine 2000 (LF2000; Invitrogen; Carlsbad, CA, USA). DNA and LF2000 were premixed for 20 min and then applied to the cells. After 24 h transfection, the cells were incubated with the indicated agents. After a further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI, USA) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector.

### 2.9. Statistics

For statistical evaluation, Mann–Whitney *U*-test for non-Gaussian parameters and Student's *t*-test for Gaussian parameters (including Bonferroni correction). The difference is significant if the *P* value is $<0.05$.

## 3. Results

### 3.1. LTA induces IL-6 production in human synovial fibroblasts

The typical pathology of RA includes chronic inflammation of the synovium, which is characterized by infiltrations of inflammatory cells and synovial hyperplasia, especially fibroblast-like synoviocytes. Therefore, we decided to use human synovial fibroblasts to investigate the signaling pathways of LTA in the production of IL-6, an inflammatory response gene. Treatment of RASF with LTA (0.3–30 μg/ml) for 24 h induced IL-6 production in a concentration- (Fig. 1A) and time-dependent manner. After LTA (10 μg/ml) treatment for 24 h, the amount of IL-6 released had increased in RASF cells (Fig. 1B). In addition, stimulation of cells with LTA also increased mRNA expression of IL-6 concentration dependently (Fig. 1C). To further confirm this stimulation-specific mediation by LTA without LPS contamination, we used polymyxin B, an LPS inhibitor. We found that polymyxin B (1 μM) completely inhibited LPS (1 μM)-induced IL-6 release. However, it had no effect on LTA (10 μg/ml)-induced IL-6 release in RASF cells (Fig. 1D). To further determine whether LTA-induced IL-6 expression required transcription or translation, RASF cells were stimulated with LTA in the absence or presence of a transcriptional

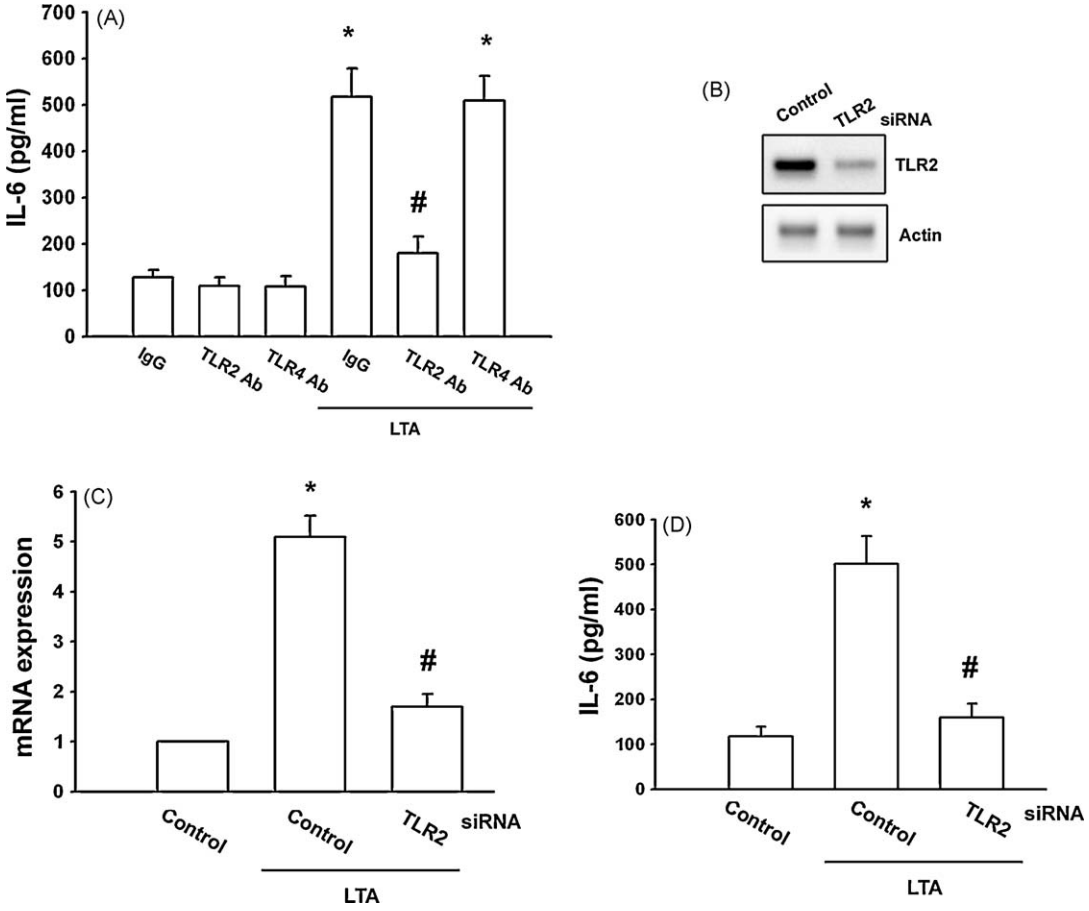


**Fig. 3.** Involvement of TLR2 receptor in LTA-mediated IL-6 production in synovial fibroblasts. (A) RASF cells were pretreated for 30 min with TLR2 Ab (5 μg/ml), TLR4 Ab (5 μg/ml) or IgG (5 μg/ml) followed by stimulation with LTA (10 μg/ml) for 24 h. Media were collected to measure IL-6. (B) RASF cells were transfected with TLR2 or control siRNA for 24 h, the protein level of TLR2 was determined using Western blot analysis. RASF cells were transfected with TLR2 or control siRNA for 24 h followed by incubation with LTA (10 μg/ml) for 24 h to analyze the mRNA and protein expression, respectively. Total RNA and media were collected, and the expressions of IL-6 were analyzed by qPCR and ELISA (C and D). Results are expressed of four independent experiments performed in triplicate. $^{*}p < 0.05$ as compared with basal level. $^{\#}p < 0.05$ as compared with LTA-treated group.

level inhibitor, actinomycin D or a translational level inhibitor, cycloheximide, and IL-6 expression was determined by ELISA. As shown in Fig. 2, LTA-mediated induction of IL-6 expression was abolished by either actinomycin D or cycloheximide. Taken together, these findings demonstrate that the induction of IL-6 by LTA depends on *de novo* protein synthesis in human synovial fibroblasts.

### 3.2. Involvement of TLR2 receptor in LTA-mediated increase of IL-6 production

It has been reported that LTA exerts its effects through interaction with a specific TLR2 receptor [8]. To investigate the role of the TLR2 receptor in LTA-mediated increase of IL-6 production, anti-TLR2 Ab was used. RASF were pretreated with anti-TLR2 or anti-TLR4 Ab for 30 min, and then stimulated with LTA for 24 h. Fig. 3A shows that LTA-induced IL-6 production was inhibited by pretreatment with anti-TLR2 Ab, but not by anti-TLR4 Ab, indicating the implication of TLR2 in these responses. Next, we used TLR2 siRNA to confirm the result of TLR2 Ab, specific inhibition of TLR2 receptor expression was accomplished with siRNA (Fig. 3B). By using qPCR and ELISA, we found that TLR2 receptor-specific siRNA, but not control siRNA significantly blocked LTA-mediated increase of IL-6 production in human RASF cells (Fig. 3C and D). These results suggest that TLR2 may be involved in LTA-induced IL-6 expression and release in human synovial fibroblasts.

### 3.3. The signaling pathways of PKCδ and c-Src are involved in the potentiating action of LTA

It has been reported that focal adhesion kinase (FAK) is involved in TLR2-mediated inflammatory response [24,25]. Our previous study also demonstrated that peptidoglycan (PGN)-induced IL-6 expression through FAK activation in RASF [26]. We then examine whether FAK activation also involved in LTA-induced IL-6 production. Phosphorylation of tyrosine 397 of FAK has been used as a marker of FAK activity. As shown in Fig. 4A, stimulation of cells with LTA did not affect the phosphorylation of FAK at $Tyr^{397}$. Transfection of cells with FAK(Y397F) mutant or FAK siRNA also did not affect LTA-mediated IL-6 production (Fig. 4B). Therefore, FAK did not mediate LTA-induced IL-6 production in RASF cells. Previous studies have shown that PKC-δ plays a crucial role in the regulation of gene expression such as IL-6 and IL-8 [27,28]. To determine whether PKC isoforms were involved in LTA-triggered IL-6 production, RASF cells were pretreated with either GF109203X, a pan-PKC inhibitor, or rottlerin, a selective PKC-δ inhibitor [29] for 30 min and then incubated with LTA for 24 h. As shown in Fig. 5A and B, pretreatment with GF109203X and rottlerin reduced LTA-induced IL-6 production and expression, suggesting that PKC-δ plays a potential role in LTA-induced IL-6 expression in RASF. Transfection with a PKCδ siRNA specifically blocked protein expression of PKCδ (Fig. 5C). In addition, PKCδ siRNA also reduced LTA-induced IL-6 expression (Fig. 5C). We then directly measured PKCδ phosphorylation in response to LTA. Stimulation of RASF cells led to a significant increase of phosphorylation of PKCδ (Fig. 5D). In addition, PKCδ activity was also increased by LTA treatment in RASF cells time dependently (Fig. 5E). Pretreatment of cells with TLR2 Ab or transfection of cells with TLR2 siRNA also reduced LTA-mediated PKCδ kinase activity (Fig. 5F). Based on these results, it appears that LTA acts through the TLR2- and PKCδ-dependent signaling pathway to enhance IL-6 production in human synovial fibroblasts. It has been reported that PKCδ dependent c-Src activation in the regulation of COX-2 expression [30]. We then investigated the role of Src in mediating LTA-induced IL-6 expression using the specific Src inhibitor PP2. As shown in Fig. 6A and B, LTA-induced IL-6

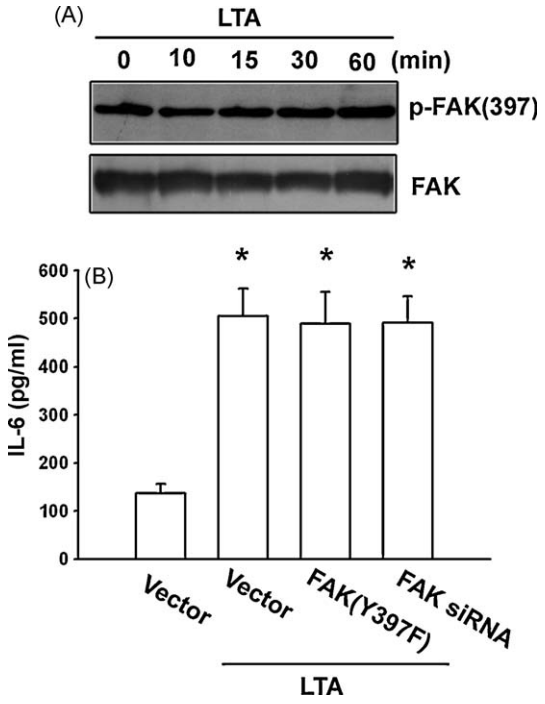


**Fig. 4.** FAK activation is not involved in LTA-induced IL-6 production. (A) Cells were incubated with LTA (10 μg/ml) for indicated time intervals, cell lysates were then immunoblotting with an antibody specific for phosphor-FAK. (B) RASF cells were transfected with FAK mutant, FAK siRNA or vector for 24 h, and then stimulated with LTA (10 μg/ml) for 24 h. Media were collected to measure IL-6. $^{*}p < 0.05$ as compared with control. $^{\#}p < 0.05$ as compared with LTA-treated group.

expression was markedly attenuated by pretreatment of cells for 30 min with PP2 or transfection of cells for 24 h with c-Src mutant. The major phosphorylation site of c-Src at the $Tyr^{416}$ residue results in activation from c-Src autophosphorylation [31]. To directly confirm the crucial role of Src in IL-6 expression, we measured the level of Src phosphorylation at the $Tyr^{416}$ in response to LTA. As shown in Fig. 6C, treatment of fibroblasts with LTA resulted in a time-dependent phosphorylation of c-Src at $Tyr^{416}$. Next, we directly examined c-Src kinase activity in response to LTA. Stimulation of cells with LTA also increased the kinase activity of c-Src time dependently (Fig. 6D). To determine the relationship among TLR2, PKCδ and c-Src in the LTA-mediated signaling pathway, we found that pretreatment of cells for 30 min with TLR2 Ab and rottlerin markedly inhibited the LTA-induced c-Src kinase activity (Fig. 6E). Based on these results, it appears that LTA acts through TLR2 receptor, PKCδ and c-Src-dependent signaling pathway to enhance IL-6 production in human synovial fibroblasts.

### 3.4. Involvement of AP-1 and NF-κB in LTA-induced IL-6 production

The promoter region of human IL-6 contains three known *cis*-regulatory elements including AP-1, C/EBP-β and NF-κB-binding sites [32]. Three different IL-6 promoter constructs containing mutations at NF-κB, AP-1, or C/EBP-β sites, respectively, were generated by site directed mutagenesis. We found that LTA-stimulated luciferase activity was abolished by AP-1 or NF-κB-binding site mutation, but not by C/EBP-β site mutations (Fig. 7A). The role of AP-1 and NF-κB were further established using the AP-1 inhibitor (tanshinone IIA), NF-κB inhibitor (PDTC) and IκB protease inhibitor (TPCK) and showed that these inhibitors blocked the enhancement of IL-6 production induced by LTA (Fig. 7B). Furthermore, the increase of IL-6 production by LTA was antagonized by *cis* element decoy agonist AP-1-binding site (decoy AP-1 ODN) or NF-κB-binding site (decoy NF-κB ODN) but not

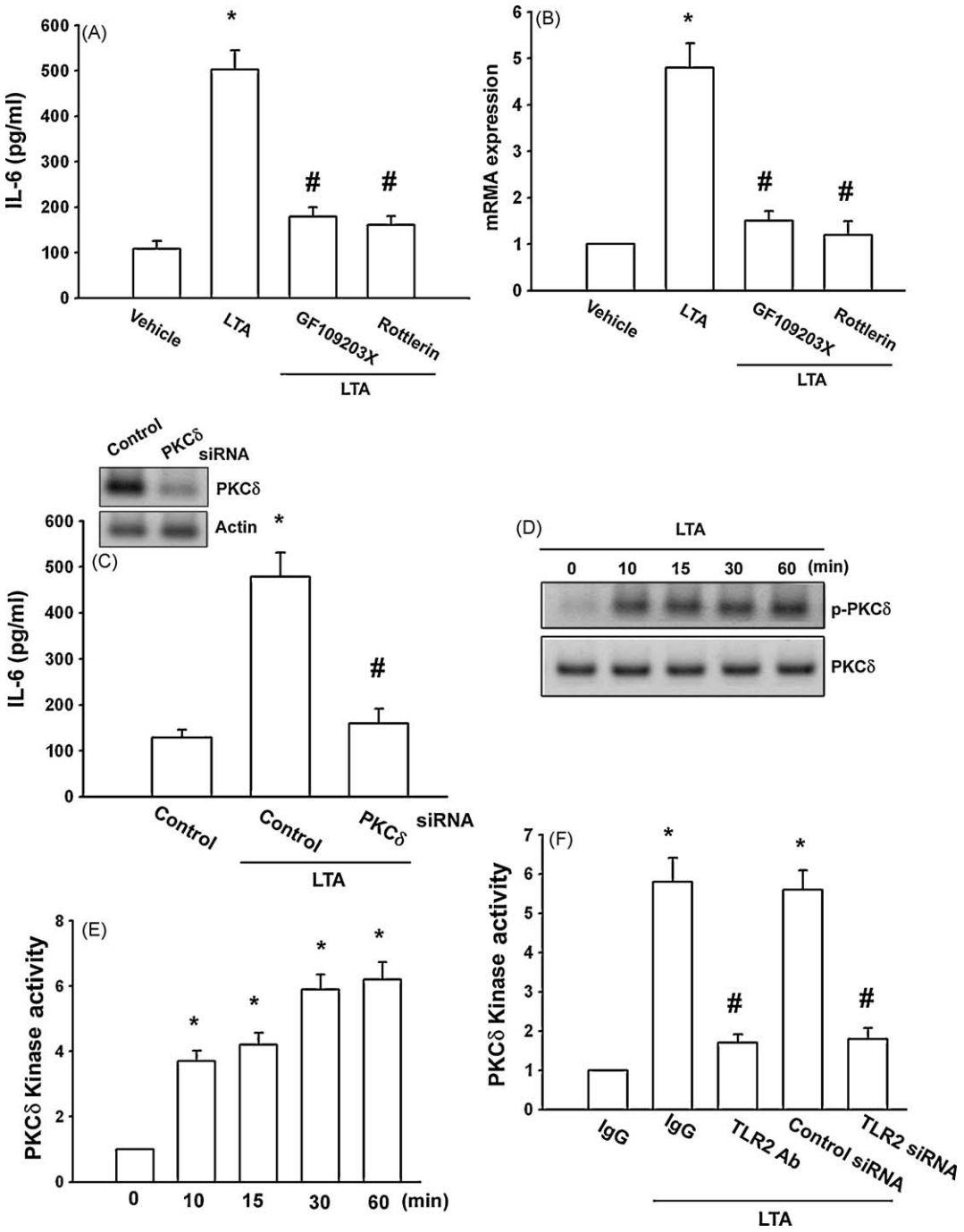


**Fig. 5.** PKCδ is involved in LTA-induced IL-6 production. RASF cells were pretreated for 30 min with GF109203X (3 μM) or rottlerin (3 μM) followed by stimulation with LTA (10 μg/ml) for 24 h to analyze the mRNA and protein expression, respectively. Media and RNA were collected, and the expressions of IL-6 were analyzed by ELISA and qPCR (A and B). RASF cells were transfected with PKCδ or control siRNA for 24 h, the protein levels of PKCδ was determined by using Western blot analysis (C; upper panel). RASF cells were transfected with PKCδ siRNA or control siRNA for 24 h, and then stimulated with LTA (10 μg/ml) for 24 h. Media were collected to measure IL-6 (C; lower panel). Cells were incubated with LTA (10 μg/ml) for indicated time intervals, and PKCδ phosphorylation was examined by Western blot (D). Cells were incubated with LTA (10 μg/ml) for indicated time intervals (E) or pretreated for 30 min with TLR2 Ab or transfected with TLR2 siRNA for 24 h, followed by stimulation with LTA (10 μg/ml) for 60 min, and PKCδ activity was determined by the PKCδ kinase kit (F). $^{*}p < 0.05$ as compared with control. $^{\#}p < 0.05$ as compared with LTA-treated group.

scrambled decoy (ODN) (Fig. 7C). AP-1 and NF-κB activation were further evaluated by analyzing the accumulation of phosphorylated c-Jun and phosphorylated p65 in the nucleus. Treatment of cells with LTA resulted in a marked accumulation of p-c-Jun and p-p65 in the nucleus (Fig. 7D). Transfection of cells with c-Jun and p65 siRNA suppressed the expression of c-Jun and p65, respectively (Fig. 7E). LTA-induced IL-6 expression was also inhibited by c-Jun and p65 siRNA but not by control siRNA (Fig. 7F). To further confirm that the AP-1 and NF-κB elements are involved in the action of LTA-induced IL-6 expression, we performed transient transfection using the AP-1 and NF-κB promoter-luciferase constructs. Synovial fibroblasts incubated with LTA led to increase in AP-1 and NF-κB promoter activity (Fig. 8A and B). The increase of AP-1 and NF-κB activities by LTA were antagonized by rottlerin and PP2 or TLR2 and PKCδ siRNA or c-Src mutant (Fig. 8A and B). Taken together, these data suggest that the activation of the TLR2, PKCδ, c-Src, AP-1 and NF-κB pathways are required for the LTA-induced increase of IL-6 in human RASF cells.

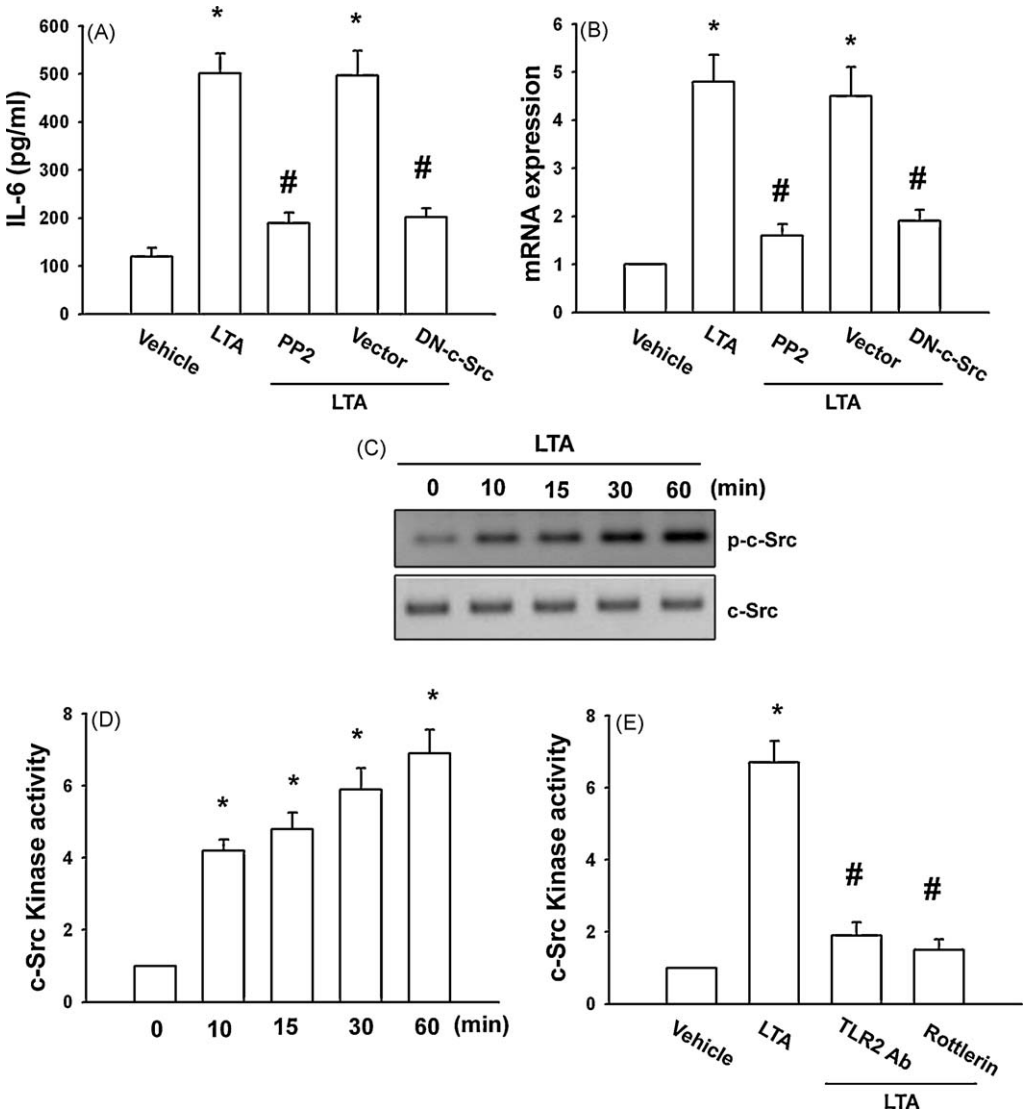


**Fig. 6.** c-Src is involved in LTA-mediated IL-6 production in synovial fibroblasts. RASF cells were pretreated for 30 min with PP2 (3 μM) or transfected for 24 h with c-Src mutant followed by stimulation with LTA (10 μg/ml) for 24 h to analyze the mRNA and protein expression, respectively. Total RNA and media were collected, and the expressions of IL-6 were analyzed by qPCR and ELISA (A and B). Cells were incubated with LTA (10 μg/ml) for indicated time intervals, and c-Src phosphorylation was examined by Western blot (C). Cells were incubated with LTA (10 μg/ml) for indicated time intervals (D) or pretreated 30 min with TLR2 Ab or rottlerin for 30 min, followed by stimulation with LTA (10 μg/ml) for 60 min, and c-Src kinase activity was determined by the c-Src kinase kit (E). $^{*}p < 0.05$ as compared with control. $^{\#}p < 0.05$ as compared with LTA-treated group.

## 4. Discussion

Bacteria and their cell wall components may initiate the host immune response, which includes innate and adaptive immunities. LTA, the component of the cell wall of Gram-positive bacteria, activates the innate immune system of the host and induces the release of cytokines and chemokines [33]. It has been reported that LTA induces the expression of inflammatory cytokines through activation of the transcription factor AP-1 and NF-κB pathways [34,35]. Here we further identified IL-6 as a target protein for the LTA signaling pathway that regulates the cell inflammatory response. We also showed that potentiation of IL-6 by LTA requires activation of the TLR2 receptor, PKCδ, c-Src, AP-1 and NF-κB signaling pathways. These findings suggest that LTA acts as an inducer of inflammatory cytokines such as IL-6 and enhance the inflammatory response in RA.

The cytoplasmic portion of TLRs shows high similarity to that of the IL-1 receptor family and is now called the Toll/IL-1 receptor (TIR) domain [36]. Upon recognizing respective ligands, the TIR domain recruits MyD88/IRAK/TRAF6 and activates downstream signaling molecules such as MAPK and AP-1 [37]. It has been reported that LTA binds TLR2, which activates transcription factors and induces gene expression [7,35,38]. Our previous study revealed that RASF cells expressed the TLR2 receptor by RT-PCR analysis [26]. TLR2 has also been shown to contribute to IL-6 expression in synovial fibroblasts [26,39]. In our study, we found that LTA-mediated induction of IL-6 expression was inhibited by pretreatment with anti-TLR2 Ab, but not by anti-TLR4 Ab, which is consistent with the finding that LTA-induced activation of signaling transduction pathways was mediated through TLR2 in various cell types [38,40]. This hypothesis was further supported by the finding that LTA-induced IL-6 expression was attenuated by transfection with a siRNA against TLR2. These results suggest that TLR2 is an upstream receptor in LTA-induced IL-6 release.

It has been shown that FAK is involved in TLR2-mediated inflammatory response [24,25]. Our previous study demonstrated that PGN (a cell wall component of Gram-positive bacteria is an alternating β-linked *N*-acetylmuramyl and *N*-acetylglucosaminyl

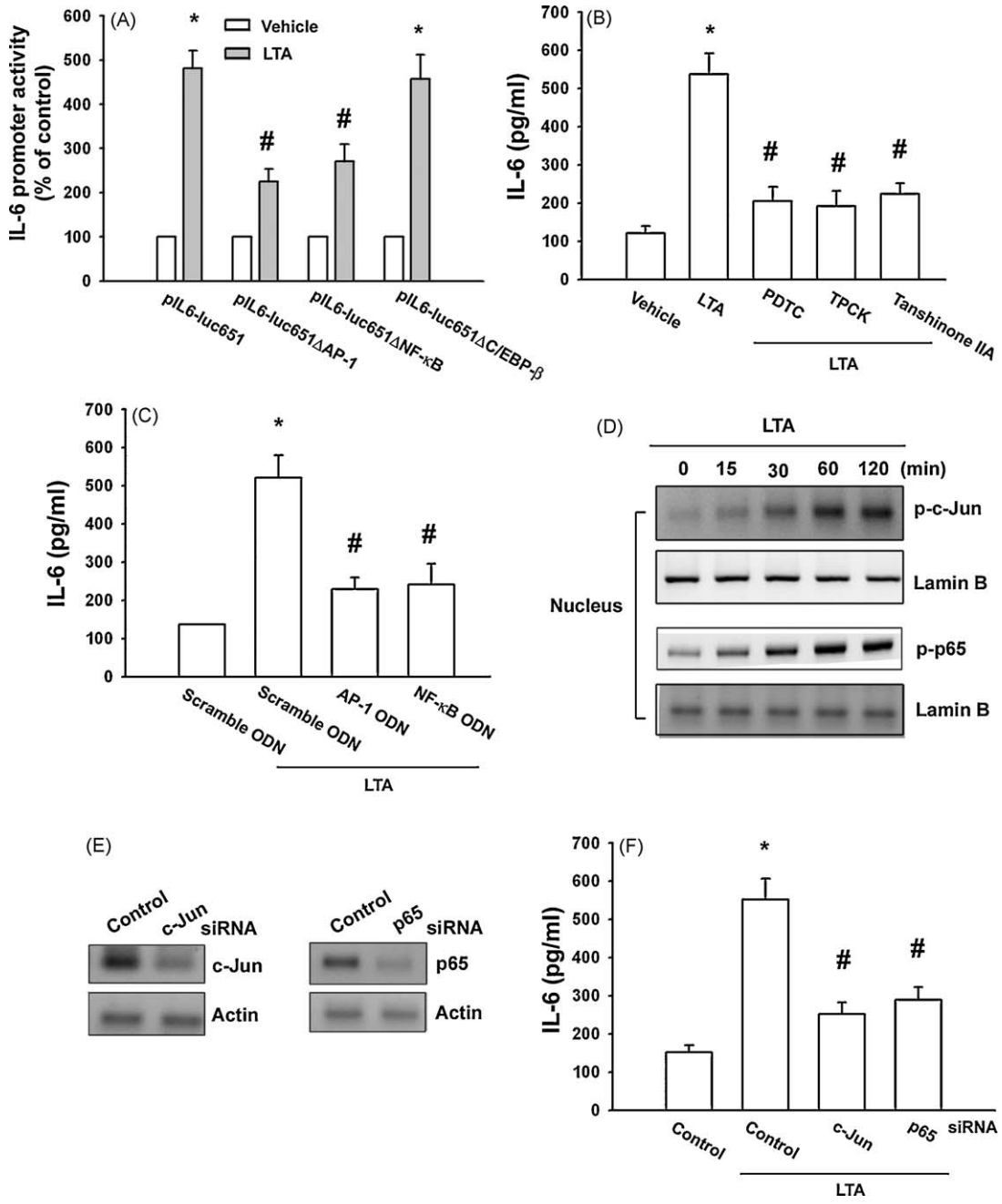


**Fig. 7.** AP-1 and NF-κB are involved in the potentiation of IL-6 production by LTA. (A) RASF cells were transfected with IL-6 luciferase plasmids before incubation with LTA (10 μg/ml) for 24 h. Luciferase activity was then assayed. (B) RASF cells were pretreated for 30 min with PDTC, TPCK, tanshinone IIA followed by stimulation with LTA (10 μg/ml) for 24 h. Media were collected to measure IL-6. (C) RASF cells were transfected with NF-κB ODN, AP-1 ODN or scramble ODN before incubation with LTA (10 μg/ml) for 24 h. Media were collected to measure IL-6. (D) RASF cells were incubated with LTA (10 μg/ml) for indicated time intervals, and c-Jun and p65 phosphorylation in nucleus were determined by Western blot. RASF cells were transfected with c-Jun, p65 or control siRNA for 24 h, the protein levels of c-Jun and p65 were determined by using Western blot analysis (E). RASF cells were transfected with c-Jun, p65 or control siRNA for 24 h, and then stimulated with LTA (10 μg/ml) for 24 h. Media were collected to measure IL-6 (F). $^{*}p < 0.05$ as compared with control. $^{\#}p < 0.05$ as compared with LTA-treated group.

glycan) induced IL-6 production through TLR2-dependent FAK activation [26]. However, stimulation of RASF cells with LTA did not affect the phosphorylation of FAK at $Tyr^{397}$. Furthermore, the FAK(Y397F) mutant and FAK siRNA also did not affect the LTA-mediated potentiation of IL-6. Therefore, FAK activation is not required for LTA-induced IL-6 production. There results indicate that FAK activation is not a common event in Gram-positive cell wall compound-induced IL-6 expression.

Several isoforms of PKC have been characterized at the molecular level and these have been found to mediate several cellular molecular responses [41]. We demonstrated that PKC inhibitor GF109203X antagonized the LTA-mediated potentiation of IL-6 expression, suggesting that PKC activation is an obligatory event in LTA-induced IL-6 production in these cells. In addition, rottlerin (a specific PKCδ inhibitor) also inhibited LTA-induced IL-6 production. This was further confirmed by the result that the PKCδ siRNA inhibited the enhancement of IL-6 production in synovial fibroblasts. Incubation of RASF cells with LTA also increased PKCδ phosphorylation and kinase activity. On the other hand, TLR2 Ab and TLR2 siRNA reduced LTA-mediated PKC kinase activity. These data suggest the TLR2 and PKCδ pathways are required for LTA-induced IL-6 production. Src, a tyrosine kinase, plays a critical role

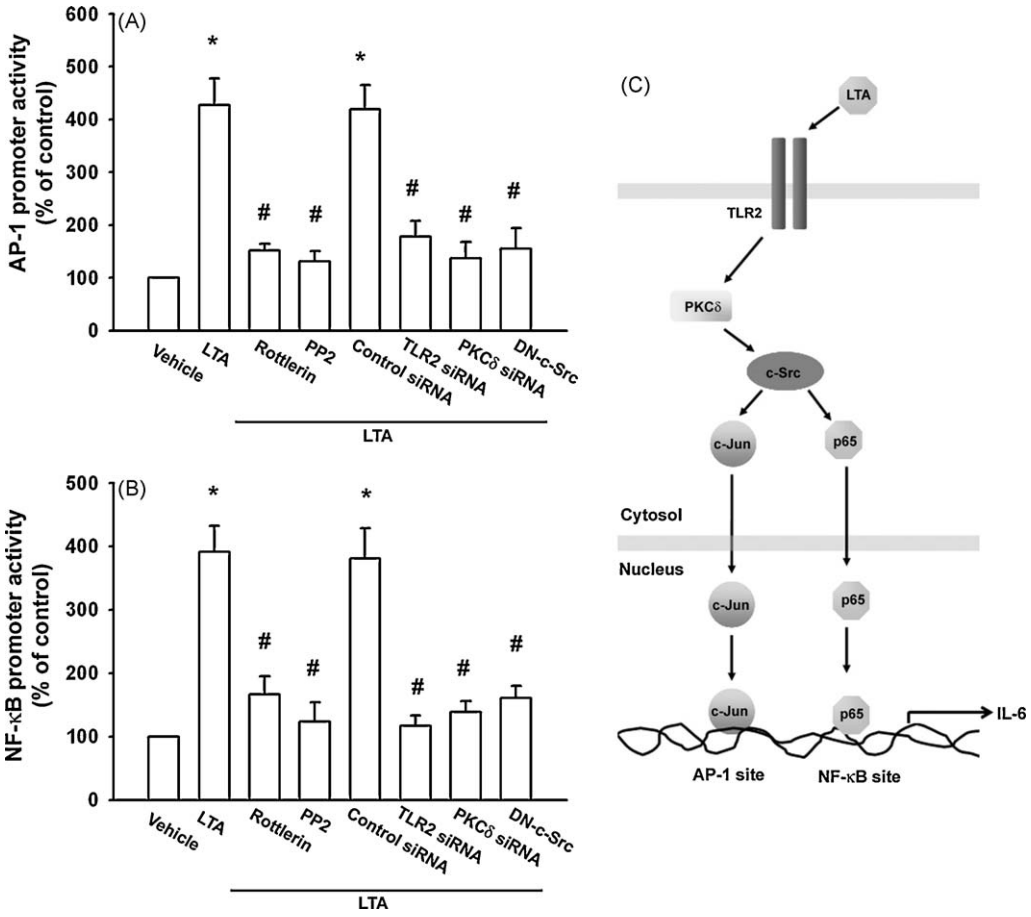


**Fig. 8.** TLR2/PKCδ/c-Src pathway is involved in LTA-induced AP-1 and NF-κB activation. RASF cells were transfected with AP-1 (A) or NF-κB (B) luciferase expression vector and then pretreated with rottlerin and PP2 or co-transfected with TLR2 and PKCδ siRNA or c-Src mutant before incubation with LTA (10 μg/ml) for 24 h. Luciferase activity was then assayed. $^{*}p < 0.05$ as compared with control. $^{\#}p < 0.05$ as compared with LTA-treated group. (C) Schematic diagram of the signaling pathways involved in LTA-induced IL-6 production in synovial fibroblasts. LTA increases IL-6 expression by binding to the TLR2 receptor and activation of PKCδ, c-Src which enhances binding of c-Jun and p65 to the AP-1 and NF-κB site, resulting in the transactivation of IL-6 expression.

in the induction of chemokine transcription [42]. In human synovial fibroblasts, thrombin induced IL-6 expression through a c-Src activation [19]. Because c-Src has been reported to be a downstream effector of PKCδ [30], we examined the potential role of c-Src in the signaling pathway of LTA-induced IL-6 expression. Treatment of cells with c-Src inhibitor PP2 or transfection of cells with c-Src mutant reduced LTA-mediated IL-6 production. In addition, we also found that treatment of synovial fibroblasts with LTA induced increases in c-Src phosphorylation at $Tyr^{416}$ and in c-Src kinase activity. These effects were inhibited by TLR2 Ab and rottlerin, indicating the involvement of TLR2, PKCδ-dependent c-Src activation in LTA-mediated IL-6 induction. Taken together, our results provide evidence that LTA up-regulates IL-6 in human synovial fibroblasts via the TLR2/PKCδ/c-Src signaling pathway.

There are several binding sites for a number of transcription factors including NF-κB, CREB, NF-IL-6, and AP-1 box in the 5′ region of the IL-6 gene [13,14]. Recent studies on the IL-6 promoter have demonstrated that IL-6 induction by several transcription factors occurs in a highly stimulus-specific or cell-specific manner [43]. The results of this study show that AP-1 and NF-κB activation contributes to LTA-induced IL-6 production in synovial fibroblasts, and deletion of AP-1 and NF-κB site reduced LTA-mediated IL-6 promoter activity. Pretreatment of cells with AP-1 inhibitor and NF-κB inhibitors also reduced LTA-increased IL-6 production. This was further confirmed by the result that the ODNs of AP-1 and NF-κB inhibited the enhancement of IL-6 production by LTA. The c-Jun and p65 are the major transcription factors of AP-1 and NF-κB. The results of this study show that LTA induced p-c-Jun and p-p65 nuclear accumulation. In addition, c-Jun and p65 siRNA also abolished the LTA-induced IL-6 production in RASF cells. Therefore, the c-Jun and p65 activation are mediated by LTA-increased IL-6 expression. Using transient transfection with AP-1 and NF-κB luciferase as an indicator of AP-1 and NF-κB activity, we found that LTA-induced an increase in AP-1 and NF-κB activity. In addition, rottlerin and PP2 or TLR2 and PKCδ siRNA or c-Src mutant reduced LTA-increased AP-1 and NF-κB promoter activity. These results indicate that LTA might act through the TLR2, PKCδ, c-Src, AP-1 and NF-κB pathways to induce IL-6 activation in human RASF cells.

Patients with RA are at increased risk of developing infections and appear to be particularly susceptible to septic arthritis, osteomyelitis, and skin and soft tissue infections [44], which are mostly caused by *Staphylococcus aureus*. It has been shown that *S. aureus* and other Gram-positive bacteria are potent inducers of TNF-α secretion from macrophages [45]. In response to TNF-α, RASF cells produce chemokines that promote inflammation neovascularization, and cartilage degradation. To simply observe the effects of Gram-positive bacteria infection in RA patients, we used the component of the cell wall of Gram-positive bacteria, LTA, to examine the proinflammatory cytokine (IL-6) in human RASF cells. We found that LTA-increased IL-6 production in RASF cells. Furthermore, the discovery of a LTA-mediated TLR2/AP-1 and NF-κB-dependent pathways helps us to understand the mechanism of RA and may help us to develop more effective therapies in the future.

In conclusion, we explored the signaling pathway involved in LTA-induced IL-6 production in human synovial fibroblasts. We found that LTA increases IL-6 production by binding to TLR2 receptor and activating of PKCδ, c-Src which enhances binding of c-Jun and p65 to the AP-1 and NF-κB site and results in the transactivation of IL-6 production (Fig. 8C).

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## References

[1] Pope RM. Apoptosis as a therapeutic tool in rheumatoid arthritis. Nat Rev Immunol 2002;2:527–35.
[2] Mor A, Abramson SB, Pillinger MH. The fibroblast-like synovial cell in rheumatoid arthritis: a key player in inflammation and joint destruction. Clin Immunol 2005;115:118–28.
[3] Sutcliffe IC, Shaw N. Atypical lipoteichoic acids of gram-positive bacteria. J Bacteriol 1991;173:7065–9.
[4] Su SC, Hua KF, Lee H, Chao LK, Tan SK, Yang SF, et al. LTA and LPS mediated activation of protein kinases in the regulation of inflammatory cytokines expression in macrophages. Clin Chim Acta 2006;374:106–15.
[5] Deininger S, Traub S, Aichele D, Rupp T, Baris T, Moller HM, et al. Presentation of lipoteichoic acid potentiates its inflammatory activity. Immunobiology 2008;213:519–29.
[6] Aulock SV, Deininger S, Draing C, Gueinzius K, Dehus O, Hermann C. Gender difference in cytokine secretion on immune stimulation with LPS and LTA. J Interferon Cytokine Res 2006;26:887–92.
[7] Lee IT, Wang SW, Lee CW, Chang CC, Lin CC, Luo SF, et al. Lipoteichoic acid induces HO-1 expression via the TLR2/MyD88/c-Src/NADPH oxidase pathway and Nrf2 in human tracheal smooth muscle cells. J Immunol 2008;181:5098–110.
[8] Henneke P, Dramsi S, Mancuso G, Chraibi K, Pellegrini E, Theilacker C, et al. Lipoproteins are critical TLR2 activating toxins in group B streptococcal sepsis. J Immunol 2008;180:6149–58.
[9] Graeve L, Baumann M, Heinrich PC. Interleukin-6 in autoimmune disease. Role of IL-6 in physiology and pathology of the immune defense. Clin Invest 1993;71:664–71.
[10] Grimble RF. Nutritional modulation of cytokine biology. Nutrition 1998;14:634–40.
[11] Jones SA. Directing transition from innate to acquired immunity: defining a role for IL-6. J Immunol 2005;175:3463–8.
[12] Lo SF, Huang CM, Lin HC, Chen WC, Tsai CH, Tsai FJ, et al. (IL-6) and chemokine (IL-8) gene polymorphisms among rheumatoid arthritis patients in Taiwan. Clin Exp Rheumatol 2008;26:632–7.
[13] Grassl C, Luckow B, Schlondorff D, Dendorfer U. Transcriptional regulation of the interleukin-6 gene in mesangial cells. J Am Soc Nephrol 1999;10:1466–77.
[14] Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, et al. Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc Natl Acad Sci USA 1993;90:10193–7.
[15] Wagner EF, Eferl R. Fos/AP-1 proteins in bone and the immune system. Immunol Rev 2005;208:126–40.
[16] Hasselblatt P, Rath M, Komnenovic V, Zatloukal K, Wagner EF. Hepatocyte survival in acute hepatitis is due to c-Jun/AP-1-dependent expression of inducible nitric oxide synthase. Proc Natl Acad Sci USA 2007;104:17105–10.
[17] Shiozawa S, Tsumiyama K. Pathogenesis of rheumatoid arthritis and c-Fos/AP-1. Cell Cycle 2009;8:1539–43.
[18] Lee CH, Shieh DC, Tzeng CY, Chen CP, Wang SP, Chiu YC, et al. Bradykinin-induced IL-6 expression through bradykinin B2 receptor, phospholipase C, protein kinase Cdelta and NF-kappaB pathway in human synovial fibroblasts. Mol Immunol 2008;45:3693–702.
[19] Chiu YC, Fong YC, Lai CH, Hung CH, Hsu HC, Lee TS, et al. Thrombin-induced IL-6 production in human synovial fibroblasts is mediated by PAR1, phospholipase C, protein kinase C alpha, c-Src, NF-kappa B and p300 pathway. Mol Immunol 2008;45:1587–99.
[20] Draing C, Traub S, Deininger S, Mang P, Moller HM, Manso M, et al. Polypropylene glycol is a selective binding inhibitor for LTA and other structurally related TLR2 agonists. Eur J Immunol 2008;38:797–808.
[21] Dahle MK, Overland G, Myhre AE, Stuestol JF, Hartung T, Krohn CD, et al. The phosphatidylinositol 3-kinase/protein kinase B signaling pathway is activated by lipoteichoic acid and plays a role in Kupffer cell production of interleukin-6 (IL-6) and IL-10. Infect Immun 2004;72:5704–11.
[22] Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, et al. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis Rheum 1986;29:1039–49.
[23] Tang CH, Chiu YC, Tan TW, Yang RS, Fu WM. Adiponectin enhances IL-6 production in human synovial fibroblast via an AdipoR1 receptor, AMPK, p38, and NF-kappa B pathway. J Immunol 2007;179:5483–92.
[24] Cheung PF, Wong CK, Ip WK, Lam CW. FAK-mediated activation of ERK for eosinophil migration: a novel mechanism for infection-induced allergic inflammation. Int Immunol 2008;20:353–63.
[25] Zeisel MB, Druet VA, Sibilia J, Klein JP, Quesniaux V, Wachsmann D. Cross talk between MyD88 and focal adhesion kinase pathways. J Immunol 2005;174:7393–7.
[26] Chiu YC, Lin CY, Chen CP, Huang KC, Tong KM, Tzeng CY, et al. Peptidoglycan enhances IL-6 production in human synovial fibroblasts via TLR2 receptor, focal adhesion kinase, Akt, and AP-1-dependent pathway. J Immunol 2009;183:2785–92.
[27] Tokuda H, Hosoi T, Hayasaka K, Okamura K, Yoshimi N, Kozawa O. Over-expression of protein kinase C-delta plays a crucial role in interleukin-6-producing pheochromocytoma presenting with acute inflammatory syndrome: a case report. Horm Metab Res 2009;41:333–8.
[28] Page K, Li J, Zhou L, Iasvovskaia S, Corbit KC, Soh JW, et al. Regulation of airway epithelial cell NF-kappa B-dependent gene expression by protein kinase C delta. J Immunol 2003;170:5681–9.
[29] Basu A, Adkins B, Basu C. Down-regulation of caspase-2 by rottlerin via protein kinase C-delta-independent pathway. Cancer Res 2008;68:2795–802.
[30] Hsieh HL, Sun CC, Wang TS, Yang CM. PKC-delta/c-Src-mediated EGF receptor transactivation regulates thrombin-induced COX-2 expression and PGE(2) production in rat vascular smooth muscle cells. Biochim Biophys Acta 2008;1783:1563–75.
[31] Roskoski Jr R. Src kinase regulation by phosphorylation and dephosphorylation. Biochem Biophys Res Commun 2005;331:1–14.
[32] Eickelberg O, Pansky A, Mussmann R, Bihl M, Tamm M, Hildebrand P, et al. Transforming growth factor-beta1 induces interleukin-6 expression via activating protein-1 consisting of JunD homodimers in primary human lung fibroblasts. J Biol Chem 1999;274:12933–8.
[33] Draing C, Sigel S, Deininger S, Traub S, Munke R, Mayer C, et al. Cytokine induction by Gram-positive bacteria. Immunobiology 2008;213:285–96.
[34] Chang YC, Li PC, Chen BC, Chang MS, Wang JL, Chiu WT, et al. Lipoteichoic acid-induced nitric oxide synthase expression in RAW 264.7 macrophages is mediated by cyclooxygenase-2, prostaglandin E2, protein kinase A, p38 MAPK, and nuclear factor-kappaB pathways. Cell Signal 2006;18:1235–43.
[35] Yang J, Ryu YH, Yun CH, Han SH. Impaired osteoclastogenesis by staphylococcal lipoteichoic acid through Toll-like receptor 2 with partial involvement of MyD88. J Leukoc Biol 2009.
[36] O'Neill L. The Toll/interleukin-1 receptor domain: a molecular switch for inflammation and host defence. Biochem Soc Trans 2000;28:557–63.
[37] Vasselon T, Detmers PA. Toll receptors: a central element in innate immune responses. Infect Immun 2002;70:1033–41.
[38] Lee CW, Chien CS, Yang CM. Lipoteichoic acid-stimulated p42/p44 MAPK activation via Toll-like receptor 2 in tracheal smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2004;286:L921–30.
[39] Brentano F, Kyburz D, Gay S. Toll-like receptors and rheumatoid arthritis. Methods Mol Biol 2009;517:329–43.
[40] Into T, Kanno Y, Dohkan J, Nakashima M, Inomata M, Shibata K, et al. Pathogen recognition by Toll-like receptor 2 activates Weibel-Palade body exocytosis in human aortic endothelial cells. J Biol Chem 2007;282:8134–41.
[41] Berk BC, Taubman MB, Cragoe Jr EJ, Fenton II JW, Griendling KK. Thrombin signal transduction mechanisms in rat vascular smooth muscle cells. Calcium and protein kinase C-dependent and -independent pathways. J Biol Chem 1990;265:17334–40.
[42] Yeh M, Gharavi NM, Choi J, Hsieh X, Reed E, Mouillesseaux KP, et al. Oxidized phospholipids increase interleukin 8 (IL-8) synthesis by activation of the c-src/signal transducers and activators of transcription (STAT)3 pathway. J Biol Chem 2004;279:30175–81.
[43] Persson E, Voznesensky OS, Huang YF, Lerner UH. Increased expression of interleukin-6 by vasoactive intestinal peptide is associated with regulation of CREB, AP-1 and C/EBP, but not NF-kappaB, in mouse calvarial osteoblasts. Bone 2005;37:513–29.
[44] Doran MF, Crowson CS, Pond GR, O'Fallon WM, Gabriel SE. Frequency of infection in patients with rheumatoid arthritis compared with controls: a population-based study. Arthritis Rheum 2002;46:2287–93.
[45] Keller R, Fischer W, Keist R, Bassetti S. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. Infect Immun 1992;60:3664–72.