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Nuclear Factor-κB Dependent Induction of TNF-α and IL-1β by the *Aggregatibacter actinomycetemcomitans* Lipopolysaccharide in RAW 264.7 Cells

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Aggregatibacter actinomycetemcomitans **is an important pathogen in the development of localized aggressive periodontitis. Lipopolysaccharide (LPS) is a virulent factor of periodontal pathogens that contributes to alveolar bone loss and connective tissue degradation in periodontal disease. Our present study was designed to investigate the cytokine expression and signaling pathways regulated by *A. actinomycetemcomitans* LPS (Aa LPS). Cytokine gene expression profiling in RAW 264.7 cells was performed by microarray analyses. The cytokine mRNA and protein levels and related signaling pathways induced by Aa LPS were measured by RT-PCR, ELISA and western blotting. Microarray results showed that Aa LPS strongly induced the expression of NF-κB, NF-κB-related genes, inflammatory cytokines, TNF-α and IL-1β in RAW 264.7 cells. NF-κB inhibitor pretreatment significantly reduced the levels of TNF-α and IL-1β mRNA and protein. In addition, the Aa LPS-induced TNF-α and IL-1β expression was inhibited by p38/JNK MAP kinase inhibitor pretreatment. These results show that Aa LPS stimulates TNF-α and IL-1β expression through NF-κB and p38/JNK activation in RAW 264.7 cells, suggesting the essential role of this pathway in the pathogenesis of localized aggressive periodontitis.**

Key words: NF-κB, *Aggregatibacter actinomycetemcomitans*, lipopolysaccharide, TNF-α, IL-1β

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Introduction

Aggregatibacter actinomycetemcomitans, a gram-negative bacterium capnophilic bacillus, is an important pathogen causing the key localized aggressive periodontitis [1]. Localized aggressive periodontitis is an early-onset form of periodontitis which is characterized with rapid periodontal destruction [2]. It is also occasionally responsible for non-oral infections including endocarditis, pericarditis, pneumonia, infectious arthritis, osteomyelitis, synovitis and abscess [3].

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria and has been shown to stimulate inflammatory cytokine production from monocytes/macrophages and neutrophils [4]. Inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α have been reported to initiate and regulate subsequent inflammatory cascades leading to tissue destruction [5]. *A. actinomycetemcomitans* LPS (Aa LPS) has been suggested to play a crucial role in periodontal tissue destruction during the course of aggressive inflammatory periodontal disease [6].

Cytokines are secreted by immune cells and play essential roles in immune modulation and activation of various immune cells. Although cytokines play protective roles in elimination of infected bacteria, overproduction of proinflammatory

cytokine may be related with periodontal destruction, such as periodontal attachment loss, destruction of collagen, and alveolar bone resorption [7,8]. TNF-α and IL-1β are representative proinflammatory cytokines associated with periodontal pathogenesis [9]. TNF-α initiates a cascade of cytokines and increases the vascular permeability, which recruits macrophages and neutrophils to a site of infection. IL-1 βmediates the host inflammatory responses in innate immunity and stimulates chemokine production by endothelial cells and macrophages. However, the signaling pathway related with TNF-α and IL-1β expression induced by *A. actinomycetemcomitans* associated with aggressive periodontitis remains to be elucidated.

In this study, we examined highly expressed genes induced by Aa LPS and further investigated the regulation mechanism for TNF-α and IL-1β expression in mouse macrophage cell line, RAW 264.7 cells for identifying the pathogenesis of aggressive periodontitis and developing effective therapeutic approach.

Materials and Methods

Bacterial strain and culture condition

A. actinomycetemcomitans (ATCC 33384) was cultured in a tryptic soy broth (BD Difco, Franklin Lakes, NJ, USA) which contained 5 ㎎/㎖ hemin (Sigma, St. Louis, MO, USA)and 0.5 ㎎/㎖ of vitamin K (Sigma, St. Louis, MO, USA) at 37ºC in an anaerobic chamber in an atmosphere containing 90% N_2, 5% H_2, and 5% CO_2.

LPS purification

A. actinomycetemcomitans was grown under anaerobic conditions and harvested at the end of the logarithmic phase of growth. LPS extraction was achieved by the hot-phenol-water method [10]. Briefly, bacterial cell pellet was suspended in pyrogen-free water, and then equal volume of 90% phenol at 60ºC was added dropwise for 20 min and stirred constantly. The aqueous phase was separated by centrifugation at 7,000 rpm for 15 min at 4ºC and collected. This process was repeated, and the aqueous phase was pooled and dialyzed against deionized water for 3 days at 4ºC. The dialyzed LPS preparation was then centrifuged at 40,000 rpm for 1.5 h at 4ºC in an ultracentrifuge (Beckman, Palo Alto, CA, USA). The precipitate was suspended with 30 ml of pyrogen-free water, dialyzed against distilled water for 3 days, lyophilized and stored at 4ºC. LPS samples were separated by SDS-PAGE and stained with Coomassie blue to confirm the purity of the LPS moieties.

Cell culture and signaling inhibitors

The mouse monocyte-macrophage cell line, RAW264.7, was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco`s modified Eagle medium (DMEM, Gibco, Carlsabd, CA, USA) with 10% FBS (Life Technologies Inc., Paisley, Scotland, UK), 100 U/ml penicillin and 100 ㎍/ml of streptomycin (Gibco, Carlsabd, CA, USA), and were incubated at 37ºC in a humidified atmosphere of 5% CO_2. To study signaling pathway, pyrrolidine dithiocarbamate (PDTC), a NF-κB inhibitor, mitogen-activated protein (MAP) kinase inhibitors including PD98059 for extracellular signal-regulated kinase (ERK), SB203580 for p38 MAP kinase (p38), and SP600125 for c-Jun N-terminal protein kinase (JNK) were purchased from Calbiochem (San Diego, CA, USA).

RNA preparation

RAW 264.7 cells were treated for 2 h and 18 h with 100 ng/ml of Aa LPS. Total RNA from these cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm using spectrophotometer (Eppendorf, Hamburg, Germany).

Microarray analysis

The microarray analysis was performed using the nonradioactive signal transduction pathway finder GEArray Q series kit (SuperArray Inc., MD, USA) according to the manufacturer's instructions. This array membrane is composed of 96 signal transduction pathway gene, a plasmid pUC18 negative control, and four housekeeping genes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin A, ribosomal protein L13a, and β-actin, each gene is printed with tetra spots format. The biotin-16-dUTP-labeled cDNA probes were synthesized from 5 ㎍ of total RNA. After pre-hybridization with GEAhyb Hybridization solution (SuperArray Inc., MD, USA) containing 100 ㎍/ml of denatured salmon sperm DNA (Invitrogen, CA, USA) for 2 h at 60ºC, the array membrane was hybridized with denatured cDNA probed overnight at 60ºC. Following washing the membrane twice with 2 x SSC, 1% SDS and twice with 0.1x SSC, 0.5%

SDS for 15 min at 60ºC each, the membrane was blocked with GEAblocking Solution Q (SuperArray Inc., MD, USA) for 40 min and incubated with alkaline phosphatase-conjugated for 10 min at room temperature. Chemiluminescent detection was performed using CDP-Star chemiluminescent substrate and array image was recorded with X-ray film. The image was scanned with a scanner TouchToss SIS-3800 (Samsung, Seoul, Korea). The resulting scanned image was converted to raw data file using Scanalyse software. GEArray Analyzer software (SuperArray Inc., MD, USA) was used for data analysis. The relative expression levels of different genes were estimated by comparing its signal intensity with that of internal control of β-actin.

RT-PCR

Total RNA isolated from each sample was used as a template for the cDNA synthesis. The reverse transcription (RT) was performed by AccuPower RT PreMix (Bioneer Co., Korea). cDNA was amplified by PCR in a thermocycler (denaturation for 30 sec at 95ºC, annealing for 30 sec at 60ºC, and elongation for 30 sec at 72ºC) using TNF-α (25 cycle), IL-1β (28 cycle) or β-action primers (35 cycle). The primers used in these analysis are as follows: TNF-α: 5'CCT GAT GCC CAC GTC GTA GC3' and 5'TTG ACC TCA GCG CTG AGT TG3'; IL-1β: 5'GAT ACA AAC TGA TGA AGC TCG TCA3' and 5'GAG ATA GTG TTT GTC CAC ATC CTG A3'; β-actin: 5'GGG TCA GAA CTC CTA TG3' and 5'GTA ACA ATG CCA TGT TCA AT3'.The reaction products were analyzed on 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide.

ELISA (Enzyme linked Immunosorbent Assay)

The protein levels of TNF-α and IL-1β released to the culture media after Aa LPS stimulation were analyzed by using an ELISA kit from R&D systems (Minneapolis, MN, USA). Briefly, control, standard or sample solution was added to ELISA well plate, which had been pre-coated with specific monoclonal capture antibody. After incubating for 2 h at room temperature, polyclonal anti-TNF-α and anti-IL-1β antibody conjugated with horseradish peroxidase was added to the solution, and incubated for 2 h at room temperature. Substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react for 30 min. The levels of cytokines were assessed by a micro ELISA reader at 450 nm. Each densitometric value expressed as mean ± SD was obtained from three independent experiments.

Extraction of nuclear protein

The nuclear extracts were prepared from RAW 264.7 cells after Aa LPS stimulation. The cells were washed with ice-cold PBS and pelleted. The cell pellet was resuspended in hypotonic buffer [1.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP40, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM Tris-HCl (pH 8.0), 0.1% pepstatin, 0.1% aprotinin] and incubated for 15 min on ice followed by vigorous vortex for 10 sec. The lysates were centrifuged, and supernatants were transferred to new vials. The pellet was resuspended in Extract buffer [10 mM Tris-HCl (pH 8.0), 50 mM KCl, 300 mM NaCl, 1 mM DTT, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 0.1% pepstatin, 0.1% aprotinin] and incubated for 30 min on ice followed by vigorous vortex for 10 sec. The lysates were centrifuged, and supernatants containing the nuclear proteins were transferred to new vials.

Western blot

After the amount of nuclear protein was determined by Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA), 30 μg of nuclear protein per lane, was subjected to electrophoresis under a reducing condition on a 12% SDS-PAGE gel and then transferred onto a nitrocellulose membrane at constant current of 100 V for 1.5 h in transfer buffer (20 mM Glycine, 15.6 mM Tris Base and 20% Methanol). The antibodies for NF-κB were obtained from SantaCruz Biotechnology (Dallas, TX, USA). All antibodies were diluted in 5% non-fat milk. After staining with the primary antibody, the membrane was subsequently incubated with the biotinylated secondary antibody for 1 h. After extensive wash with PBS, the membrane was coated for ECL for 1 min and the signal was captured with X-ray film. The amount of protein was determined using a scanner TouchToss SIS-3800 (Samsung, Seoul, Korea).

Results

Aa LPS highly induces NF-κB pathway genes

To investigate the signal transduction pathway induced by Aa LPS at the level of gene expression, microarray method was carried out using a GEarray. Gene array membrane (GEarray Signal Transduction Pathway Finder) contains marker genes associated with 18 signal transduction pathways and four

housekeeping genes including GAPDH, cyclophilin A, ribosomal protein L13a, and β-actin.

The results of microarray spots induced by Aa LPS are shown in Fig. 1 and summarized in Table 1. Aa LPS increased mRNA levels of TGF-β pathway gene (Cdkn1a), p53 pathway gene (Cdkn1a), NF-κB pathway genes (Nfkb1, Nfkbia, Icam1, Tnf), Estrogen pathway gene (Cdkn1a), Phospholipase C pathway genes (Junb, Icam1, Ptgs2), and LDL pathway genes (Csf2, Ccl2). Among various pathways, expression of Nfkbia and Tnf which belong to NF-κB pathway was highly up-regulated over 10 fold increase at 2 hr and 18 hr. The expression of Junb, Ptgs2 and Ccl2, which are associated with inflammatory response, was also increased. This microarray result suggests that Aa LPS can strongly induce inflammatory response mediated by NF-κB and other pathways.

NF−κB activation

To elucidate whether NF-κB is involved in Aa LPS-induced TNF-α and IL-1β expression, nuclear extracts was prepared at 10min, 20min, 30 min, 1 h, 2 h, 4 h and 8 h after RAW 264.7 cells were treated with 100 ng/ml of Aa LPS. The induction of NF-κB protein in nucleus was analyzed by Western blot (Fig. 1A). The NF-κB protein was strongly detected at 20 and 30 min after Aa LPS treatment.

Aa LPS induces TNF−α and IL−1β mRNA and protein expression

To clarify whether Aa LPS can induce TNF-α and IL-1β gene expression, RAW 264.7 cells were treated with 100 ng/ml of Aa LPS. Total RNA was prepared at 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h after Aa LPS treatment, respectively. The expression levels of TNF-α and IL-1β mRNA were measured by RT-PCR (Fig. 1B). After treatment with Aa LPS, TNF-α mRNA was showed two expression peaks at 2 h and 24 h and IL-1β mRNA was continually increased from 1 h.

Production of TNF-α and IL-1β protein in culture supernatants was measured by ELISA (Fig. 1C & 1D). TNF-α protein was detected at 2 h and reached to the maximum after treatment at 2 h. The production of IL-1β protein was detected at 4 h and increased in a time-dependent manner.

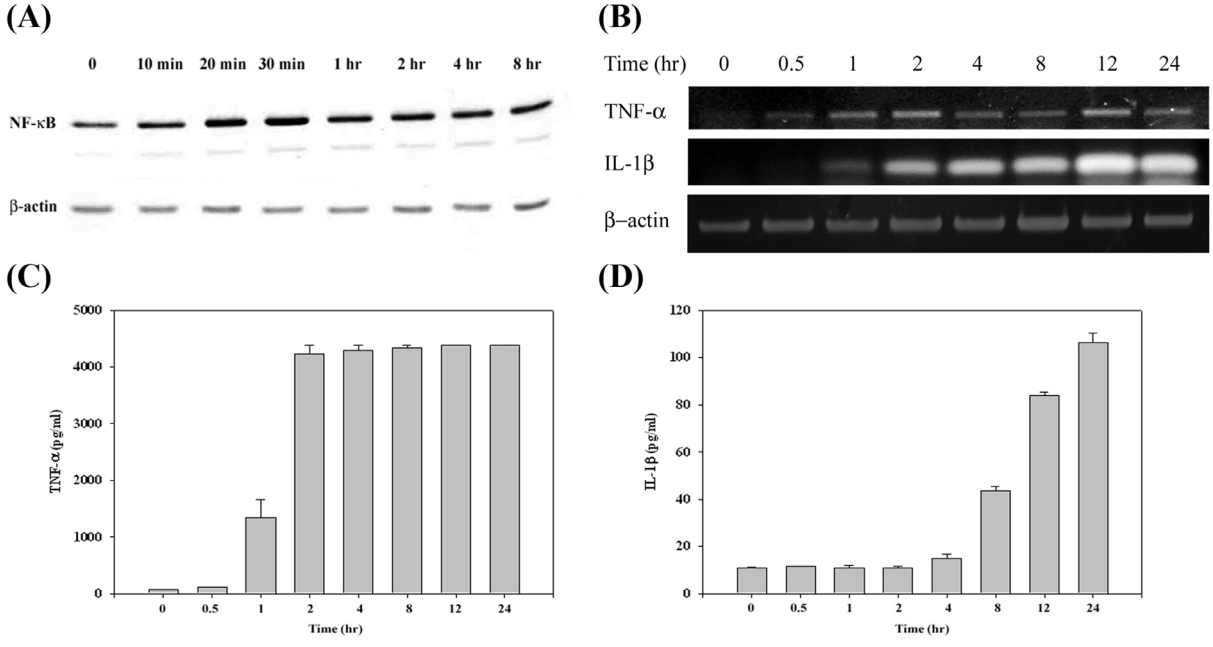

Fig. 1. (A) Western blot analysis of NF-κB activation induced by Aa LPS. RAW 264.7 cells were treated with 100 ng/ml of Aa LPS and nuclear extracts was prepared at 10 min, 20 min, 30 min, 1 h, 2 h, 4 h and 8 h after Aa LPS treatments. The induction of NF-κB protein in nucleus was analyzed by Western blot. The expression of β-actin protein was used as a control. **(B)** Expression levels of TNF-α and IL-1β mRNA induced by Aa LPS. RAW 264.7 cells were treated with 100 ng/ml of Aa LPS and total RNA was prepared at 30 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h after Aa LPS treatment. The expression levels of TNF-α and IL-1β mRNA were measured by RT-PCR. The expression of β-actin gene was used as a control gene. Production of TNF-α **(C)** and IL-1β **(D)** protein after treatment with Aa LPS. RAW 264.7 cells were treated with 100 ng/ml of Aa LPS and culture supernatants were prepared at 30 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h after Aa LPS treatment. The level of TNF-α and IL-1β protein production in culture supernatants was measured by ELISA. Values are expressed as mean ± SD obtained from three independent experiments.

These data indicate that Aa LPS strongly induce both TNF-α and IL-1β mRNA expression and protein production.

Aa LPS induced TNF−α and IL−1β expression through NF− κB pathway

To elucidate whether NF-κB is involved in Aa LPS- induced TNF-α and IL-1β expression, RAW 264.7 cells were pretreated with 50 mM pyrrolidine dithiocarbamate (PDTC), NF-κB inhibitor, and stimulated with Aa LPS for 2 hr. The expression of TNF-α and IL-1β was examined by RT-PCR (Fig. 2A) andELISA (Fig. 2B), respectively. PDTC pretreatmentcompletely inhibited both the mRNA expressionand protein production of TNF-α and IL-1β (Fig. 2). These data indicated that the expression of TNF-α and IL-1β induced by Aa LPS was mediated by NF-κB activation.

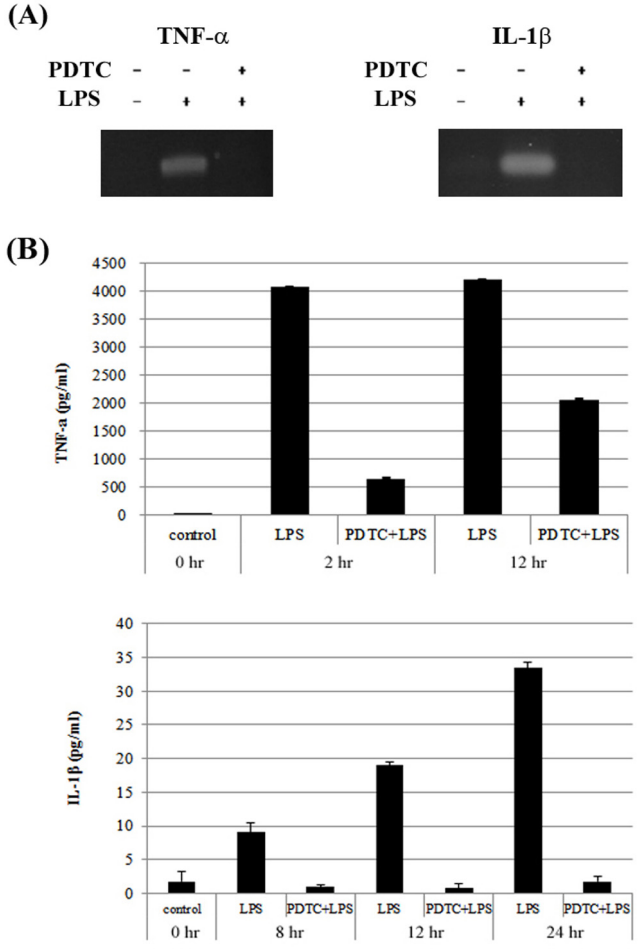

Fig. 2. Effect of inhibiting NF-k (A) mRNA expression pattern of ELISA treatment with PDTC. (B) Production of TNF-α and IL-1β protein after treatment with PDTC. RAW 264.7 cells were pretreated with 50 uM PDTC for 30 min and then treated with 100 ng/ml of Aa LPS for 2 h. The expression patterns of TNF-α and IL-1β mRNA were examined by RT-PCR. The levels of TNF-α and IL-1β protein production in culture supernatants were measured by ELISA. Values are expressed as mean ± SD obtained from three independent experiments.

MAP kinase inhibitors suppress Aa LPS−induced TNF−α and IL−1β expression

To determine whether mitogen-activated protein (MAP) kinase pathways are involved in Aa LPS-induced TNF-α and IL-1β expression, RAW 264.7 cells were pretreated with MAP kinase inhibitors 30 min before Aa LPS treatment. PD98059, SB203580 and SP600125 were used to inhibit extracellular signal-regulated kinases (ERK), p38 and c-Jun N-terminal kinase (JNK), respectively. The expression of TNF-α mRNA and protein induced by Aa LPS was inhibited by pretreatment of JNK inhibitor, while IL-1β expression was down-regulated by p38 inhibitor pretreatment (Fig. 3). ERK inhibitor did not greatlyaffect the TNF-α and IL-1β expression. Taken together, these findings suggest that JNK and p38 pathway maybe the

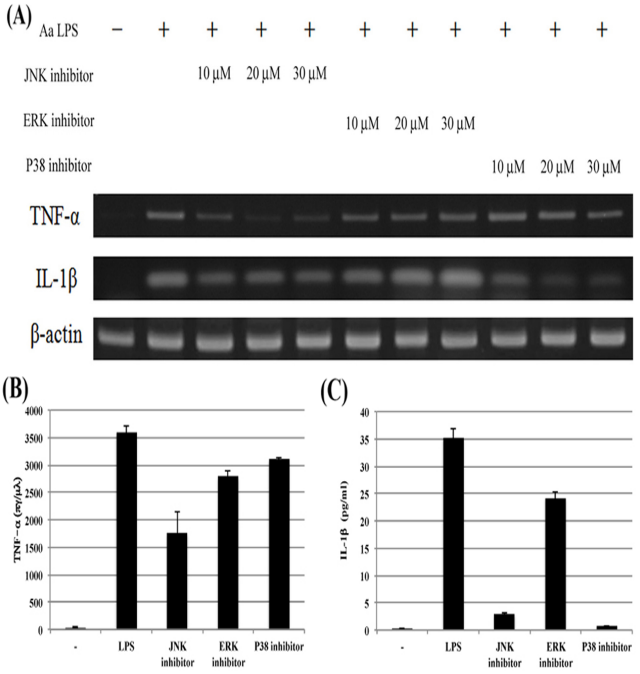

Fig. 3. Effect of inhibiting MAPK signaling pathway. (A) mRNA expression pattern of TNF-α and IL-1β mRNA after treatment with MAPK kinase inhibitors. Production of TNF-α (B) and IL-1β (C) protein after treatment with MAPK kinase inhibitors. RAW 264.7 cells were pretreated with MAPK kinase inhibitors for 30 min and then treated with 100 ng/ml of Aa LPS for 2 h. The expression patterns of TNF-α and IL-1β mRNA were examined by RT-PCR. The levels of TNF-α and IL-1β protein production in culture supernatants were measured by ELISA. Values are expressed as mean ± SD obtained from three independent experiments.

major pathway in the TNF-α and IL-1β production induced by Aa LPS, respectively.

Discussion

Periodontitis, an inflammatory disease of the tooth-supporting tissues involving complex host-bacteria interactions, is initiated by an overgrowth of specific Gram-negative anaerobic bacteria. *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* are considered as major periodontopathogens [11] and their numbers in subgingival plaque increase significantly during the active phase of periodontitis [12]. More specifically, *A. actinomycetemcomitans* has been implicated as a key etiological agent of localized aggressive periodontitis [1,13] and possesses virulence factors which allow them to colonize oral epithelium, invade host cells, evade host defenses, and promote connective tissue destruction [14]. The lipopolysaccharide (LPS) of *A. actinomycetemcomitans* has been proposed to contribute to alveolar bone loss and connective tissue degradation in periodontal disease [15,16]. It has been reported that *A. actinomycetemcomitans* induces the expression of several cytokines in periodontal inflammatory response [17]. Low concentration of Aa LPS stimulates macrophage to produce interleukins and tumor necrosis factor [18]. However, Aa LPS-induced signal transduction pathway is not well studied. In this study, we examined the signaling pathways activated by Aa LPS in mouse macrophages.

At first, we analyzed which signaling pathways or genes were activated by Aa LPS using microarray. As the result of microarray analysis using Signal Transduction Pathway Finder GEarray, the expression of Cdkn1a, Nfkb1, Nfkbia, Icam1, tnf, Junb, Ptgs2, and Ccl2 was up-regulated by Aa LPS at transcription level in RAW 264.7 cells (Table 1). While the expression of Ccnd1, Bax, Hsf1, Cdk2, and Fasn was down-regulated (Table 2). Among these genes, the expression of NF-κB pathway-related genes including Nfkb1,

Table 1. Genes expression increased in RAW264.7 cells stimulated by *A. actinomycetemcomitans* LPS

Pathway	Symbol	Gene Name	2 hr	18 hr
Mitogenic Pathway	Jun	c-Jun	1.1	2.7
Wnt Pathway	Jun	c-Jun	1.1	2.7
TGF-B Pathway	Cdkn1a	p21Waf1/p21cip	4.28	4.61
P13kinase/AKP Pathway	Jun	c-Jun	1.1	2.7
Jak/Src Pathway	Bcl2l1	Bcl-x	1.07	1.43
p53 Pathway	Cdkn1a	p21Waf1/p21cip	4.28	4.61
	Mdm2	Mdm2	1.45	1.66
NF-κB Pathway	Bcl2a1d	Bfl-1	4.44	5.69
	Nfkb1	NfkB1	3.37	4.53
	Nfkbia	ikBa/Mad3	10.89	11.33
	Icam1	ICAM-1	8.86	6.43
	Tnf	TNFa	14.14	14.71
Jak-Stat Pathway	Irf1	IRF-1	1.2	2.2
Estrogen Pathway	Cdkn1a	p21Waf1/p21cip	4.28	4.61
Calcium and protein Kinase C Pathway	Csf2	GM-CSF	0.83	4.06
	Jun	c-Jun	1.1	2.7
	Odc	Ornithine decarboxylase	2.49	2.86
Phospholipase C Pathway	Jun	c-Jun	1.1	2.7
	Junb	Jun-B	10.29	11.57
	Icam1	ICAM-1	8.86	6.43
	Ptgs2	Cox-2	3.11	11
LDL Pathway	Csf2	GM-CSF	0.83	4.06
	Ccl2	Scya2	4	22
Insulin Pathway	Cebpb	C/EBP beta	1.28	1.16
Retinoic Acid Pathway	Ctsd	Cathepsin D	0.78	1
	Stra6	Stra6	1.61	1.26

Table 2. Genes expression decreased in RAW264.7 cells stimulated by *A. actinomycetemcomitans* LPS

Pathway	Symbol	Gene Name	2 hr	18 hr
Wnt Pathway	Ccnd1	Cyclin D1	0.84	0.09
P13 kinase/AKP Pathway	Ccnd1	Cyclin D1	0.84	0.09
	Pten	PTEN	1.54	0.75
p53 Pathway	Bax	Bax	0.52	0.31
Stress Pathway	Hsf1	Hsf1 (tcf5)	0.6	0.28
	HSP90-Rik	HSP90/CDw52	0.77	0.31
	Trp53	p53	1.94	0.75
NF-κB Pathway	Il2	IL-2	1.27	0.65
NFAT Pathway	Il2	IL-2	1.27	0.65
Estrogen Pathway	Cdk2	Cdk 2L	0.75	0.2
Calcium and protein Kinase C Pathway	Il2	IL-2	1.27	0.65
	Tfrc	Tfrc/Trfr/Tfr1	2.91	1
Insulin pathway	Fasn	fatty acid synthase	0.92	0.11

Nfkbia, Icam1, and tnf was strongly enhanced by Aa LPS (Table 1). These data suggest that Aa LPS induces NF-κB activation at transcription level.

NF-κB is a multi-unit transcription factor, which plays a central role in induction of pro-inflammatory cytokines and other many immunoregulatory genes. Thus, the activation of NF-κB results in the production of inflammatory cytokines. *A. actinomycetemcomitans*induces the production of inflammatory cytokines including TNF-α, IL-1β, MIP-1, MCP-1 and IL-6 [9,17,20,21]. To determine NF-κB activation, NF-κB translocation was detected. Aa LPS treatment induced the NF-κ B translocation from cytosol into nucleus at 20 min. (Fig. 1A). The expression of TNF-α and IL-1β induced by Aa LPS was inhibited by the pretreatment of NF-κB inhibitor (Fig. 2). The levels of activated NF-κB in KB cells were also increased by Aa LPS with peak expression occurring at 30 min [19]. Taken together, Aa LPS induced the inflammatory cytokines through NF-κB activation.

TNF-αand IL-1βare representative pro-inflammatory cytokines associated with periodontitis. TNF-α and IL-1β play important role in aggressive inflammatory processes including the initiation and exacerbation of periodontal diseases by promoting osteoclast formation and alveolar bone resorption [9, 22]. Aa LPS have increased inflammatory infiltrate, inflammatory cytokines including IL-6, IL-1β and TNF-α expression, and TRAP-positive osteoclasts [23]. *P. gingivalis*–induced osteoclastogenesis was reduced in TNF receptor–deficient mice compared to wild-type controls, indicating that osteoclast formation was resulted from stimulation of the host response [24]. The exogenous application of recombinant human IL-1β in a rat ligature model accelerated alveolar bone destruction and inflammation [25].

In addition to NF-κB activation, LPS also stimulate mitogen-activated protein kinase (MAPK) signaling pathway. MAPKs are a family of serine/threonine proteases, and include p38, extracellular signal-related kinases (ERK 1/2) and c-Jun N-terminal kinases (JNK) [26]. The expression of TNF-α and IL-1β induced by Aa LPS was inhibited by the pretreatment with JNK and JNK/p38 inhibitors, respectively (Fig. 3). Dunmyer *et al.* have reported that *A. actinomycetemcomitans* stimulated production of TNF-α, IL-6 and IL-10 through sustained activation of MAPK pathways in macrophages [27]. Bodet *et al.* reported that Aa LPS may induce both JNK and p38 alpha activation leading to NF-κB activation in human gingival fibroblasts and have suggested that this may contribute to periodontal connective tissue destruction [6].

Taken together with these data, Aa LPS induced TNF-α and IL-1β gene expression through the activation of NF-κB and MAPK signaling. Understanding the signal transduction pathways and the functions of those genes involved in immune response induced by Aa LPS may facilitate the improvement therapy or vaccines for periodontal diseases caused by *A. actinomycetemcomitans.*

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