

Actinobacillus *actinomycetemcomitans* lipopolysaccharide induces interleukin-6 expression through multiple mitogen-activated protein kinase pathways in periodontal ligament fibroblasts

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Actinobacillus actinomycetemcomitans plays a major role in the pathogenesis of aggressive periodontitis. Lipopolysaccharide (LPS) derived from *A. actinomycetemcomitans* is a key factor in inflammatory cytokine generation within periodontal tissues. In this study, we identify major mitogen-activated protein kinase (MAPK) signaling pathways induced by *A. actinomycetemcomitans* LPS, *Escherichia coli* LPS and interleukin-1 β (IL-1 β) in a murine periodontal ligament (mPDL) fibroblast cell line. Immunoblot analysis was used to assess the phosphorylated forms of p38, extracellular-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) MAPK following stimulation with *A. actinomycetemcomitans* LPS, *E. coli* LPS and IL-1 β . IL-6 mRNA induction was detected via reverse transcription-polymerase chain reaction, while protein levels were quantified via enzyme-linked immunosorbent assays (ELISA). We utilized biochemical inhibitors of p38, ERK and JNK MAPK to identify the MAPK signaling pathways needed for IL-6 expression. Additional use of stable mPDL cell lines containing dominant negative mutant constructs of MAPK kinase-3 and -6 (MKK-3/6) and p38 null mutant mouse embryonic fibroblast (MEF) cells were used to substantiate the biochemical inhibitor data. Blocking p38 MAPK with SB203580 reduced the induction of IL-6 mRNA by *A. actinomycetemcomitans* LPS, *E. coli* LPS and IL-1 β by >70%, >95% and ~60%, respectively. IL-6 ELISA indicated that blocking p38 MAPK reduced the IL-6 protein levels induced by *A. actinomycetemcomitans* LPS, *E. coli* LPS and IL-1 β by ~60%, ~50% and ~70%, respectively. All MAPK inhibitors significantly reduced the IL-6 protein levels induced by *A. actinomycetemcomitans* LPS, *E. coli* LPS and IL-1 β whereas only p38 inhibitors consistently reduced the *A. actinomycetemcomitans* LPS, *E. coli* LPS and IL-1 β induction of IL-6 mRNA steady-state levels. The contribution of p38 MAPK LPS-induced IL-6 expression was confirmed using MKK-3/6 dominant negative stable mPDL cell lines. Wild-type and p38 $\alpha^{-/-}$ MEF cells provided additional evidence to support the role of p38 α MAPK in *A. actinomycetemcomitans* LPS-stimulated IL-6. Our results indicate that induction of IL-6 by *E. coli* LPS, IL-1 β and *A. actinomycetemcomitans* LPS requires signaling through MKK-3-p38 α ERK, JNK and p38 MAPK in mPDL cells.

Key words: *Actinobacillus actinomycetemcomitans*; interleukin-6; LPS; MAPkinases; periodontal disease; periodontal ligament fibroblast

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Periodontal disease initiation and progression occur as consequences of the host immune inflammatory response to oral pathogens. Periodontal pathogens produce collagenases and proteases that facilitate extracellular matrix degradation to produce nutrients for their growth (2). In addition, *Actinobacillus actinomycetemcomitans* and other periodontal pathogens possess multiple virulence factors, such as leukotoxin, peptidoglycans, outer membrane proteins and LPS (20), which initiate the host inflammatory response, including the production of host-derived inflammatory molecules such as matrix metalloproteinases (MMPs), cytokines and prostaglandins (6, 7, 20). Although much effort has been applied in the analysis of lipopolysaccharide (LPS)-mediated destruction of the periodontium, little is known of the major intracellular signaling pathways utilized by *A. actinomycetemcomitans* LPS within resident periodontal tissue types.

Mitogen-activated protein kinases (MAPKs) are divided into three families, the extracellular signal-regulated kinases (ERK-1/2), *c-jun* N-terminal kinase (JNK) and p38 α . Mitogens and growth factors primarily activate ERK-1/2, whereas the pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), and cell stress-inducing factors, such as heat shock, ultraviolet radiation and oxygen radicals chiefly activate JNKs and p38 α . The three MAPKs control the activation of many transcription factors, including AP-1 (homo- or heterodimer of the proteins *c-fos* and *c-jun*), nuclear factor- κ B (NF- κ B), or C/EBP (ccAAAT enhances binding protein).

It is assumed that all three MAPK families are expressed in diseased periodontal tissues, although expression levels may differ depending upon the cell types activated and the levels of inflammation. Within periodontally resident cells, including tissue macrophages and other periodontal cells, MAPKs are activated chiefly by LPS, IL-1 and TNF- α (18, 22, 27). The p38 MAPK, most notably its p38 α isoform, is activated mainly within cells involved in the inflammatory process. Activation of p38 α through more proximal signaling intermediates termed MAPK kinases (MKK) induces synthesis of pro-inflammatory cytokines and prostanoids such as TNF- α , IL-1, IL-6, IL-8 and prostaglandin E₂, via direct activation of gene transcription (8, 9, 14, 15, 21). The p38 α MAPK system can also increase cytokine production through post-transcriptional mechanisms of mRNA

stabilization or translation through p38-MAPK-activated protein kinase 2 (MK2) stress-activated pathways (21, 32). In addition, p38 α MAPK controls the synthesis of other compounds, including chemokines, metalloproteinases and prostaglandins (16). LPS-induced events leading to IL-6 production in periodontal ligament (PDL) cells has not been described. These studies indicate that although all MAPK pathways are involved in LPS-induced IL-6 production, p38 α MAPK is the predominant MAPK required for maximal LPS-induced IL-6 expression.

Materials and methods

Cells and materials

Murine periodontal ligament (mPDL) fibroblasts immortalized with simian virus 40 large T antigen were obtained from Dr Martha Somerman (University of Washington, Seattle, WA). Stable mPDLs containing MKK-3 dominant-negative (DN) and MKK-6 DN mPDL stable cell lines were as described previously (22). Mouse embryonic fibroblasts (MEF), both wild-type and p38 α ^{-/-}, were obtained by material transfer from Dr Angel Nebreda, Madrid, Spain. The p38 α null MEFs were obtained by targeted deletion of the p38 α gene and subsequent isolation of fibroblasts established from E11.5–E12.5 embryos (1). All cells were cultured with Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μ g/ml streptomycin in an incubator at 37°C in 5% CO₂. IL-1 β and *Escherichia coli* LPS were obtained from R & D Systems, Inc. (Minneapolis, MN) and Sigma Aldrich (St Louis, MO), respectively. The *A. actinomycetemcomitans* LPS serotype b was purified and obtained from strain Y4 as described elsewhere (29–31). Biochemical inhibitors SB203580 (water-soluble), SP600125 (dimethyl sulfoxide [DMSO] soluble), and PD98059 (DMSO soluble) were obtained from Calbiochem (San Diego, CA). The final DMSO concentration used in all experimental protocols was 0.01% (v/v). Controls used included DMSO at the final concentration used to dissolve SP600125 and PD98059.

Immunoblot analysis

Whole cell lysates were harvested by scraping the cells on SDS sample buffer (62.5 mM Tris-HCl buffer pH 6.8, 10% glycerol, 50 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue)

on ice, sonicated for 10 s and heat-denatured at 95°C for 5 min. Western blot analysis was performed as described previously (22), proteins were separated on 10% Tris-HCl polyacrylamide gels run at 150 V for 50 min and subsequently electro-transferred for another 45 min at 32 mA in a semi-dry apparatus to nitrocellulose membranes. The membranes were blocked (Tris-buffered saline with 5% non-fat dry milk, 0.1% Tween-20) for 1 h at room temperature and then probed overnight at 4°C with primary antibodies. Primary antibodies for phosphorylated p38, JNK and ERK MAPK were from Cell Signaling (Danvers, MA), as well as the secondary horseradish peroxidase-conjugated antibodies. The GAPDH primary antibody was obtained from Chemicon (Temecula, CA). The presence of the primary antibodies was detected on radiographic film by using horseradish peroxidase-conjugated secondary antibody and a chemiluminescence system (Lumi-Glo, Cell Signaling).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA was resuspended in Tris-ethylenediaminetetraacetic acid (TE, pH 7.0). RNA was quantified at an optical density of 260 nm (OD₂₆₀) using a Beckman DU-600 spectrophotometer. RT-PCR was used to analyse mRNA expression as described previously. Briefly, cDNA was synthesized by a RT kit (Applied Biosystems, Foster City, CA) using 300 ng total RNA in a 15 μ l reaction. Each reaction contained 1.5 μ l RT-buffer, 3.3 μ l 25 mM MgCl₂, 3 μ l 10 mM dNTP, 0.75 μ l oligo-dT, 0.3 μ l RNase inhibitor, 0.37 μ l of Multiscribe reverse transcriptase, and 3.77 μ l DNase/RNase-free H₂O. RT thermocycler conditions were as follows: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. A 2- μ l subsample of the RT product was then used in 25- μ l PCR. The PCR mix contained nuclease-free water, 0.125 μ l *Taq* polymerase, 1.5 mM MgCl₂, 20 pM sense/antisense primer, and 0.5 μ l 12.5 mM dTNP. The PCR thermocycler (iCycler™ Thermocycler, BioRad, Hercules, CA) conditions used were as follows: 95°C for 5 min, and 35 cycles of 95°C for 1 min, 58°C (IL-6) or 52°C (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) for 2 min, and 72°C for 1 min followed by a 10-min elongation phase at 72°C. Amplicon primers were used for murine IL-6

(accession no. NM_031168.1, forward 5'-atgaagtctctctgcaagagact-3' and reverse 5'-cactaggttgccgagtagatctc-3') and murine GAPDH (accession no. NM_008084, forward 5'-caccatggagaaggccgggg-3' and reverse 5'-gacggacacattggggtag-3'). PCR products were run on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. IL-6 levels were quantified and normalized to GAPDH levels using densitometric analysis on a BioRad Gel-Doc system and BioRad QUANTITY ONE® software.

Enzyme-linked immunosorbent assay (ELISA)

PDL cells were plated at 5×10^4 cells/well in 24-well dishes and stimulated with *A. actinomycetemcomitans* LPS (5 µg/ml), *E. coli* LPS (5 µg/ml), or IL-1β (5 ng/ml) for 24 h. To determine the MAPK pathways involved, cells were pretreated for 1 h with SB203580, SP600125 and PD98059 (all at 10 µM) and stimulated. Vehicle-only and inhibitor-only cultures served as controls. Cell culture supernatants were harvested using an mIL-6 ELISA following the manufacturer's instructions (R & D Systems Inc.).

Statistical analysis

All data were subjected to statistical analysis through appropriate analysis parameters. Student's *t*-tests were used to obtain individual *P*-values when appropriate and two-way analysis of variance (ANOVA) was used when multifactorial statistical analysis was needed. Two-way ANOVA with Bonferroni post-tests and two-tailed paired Student's *t*-tests were performed using GRAPHPAD PRISM version 4.03 for Windows, Graphpad Software, San Diego, CA.

Results

MAPK pathways are rapidly activated in mPDL cells

Treatment with *A. actinomycetemcomitans* LPS, *E. coli* LPS and IL-1β resulted in phosphorylation of the ERK and p38 MAPK pathways (Fig. 1); *A. actinomycetemcomitans* LPS and IL-1β additionally activated JNK MAPK. These data indicate the rapid kinetics (within 10 min) of MAPK activation in mPDL cells.

Inflammatory stimuli induce IL-6 mRNA production in mPDL cells

Initial studies were conducted in mPDL cells to determine the time-course and

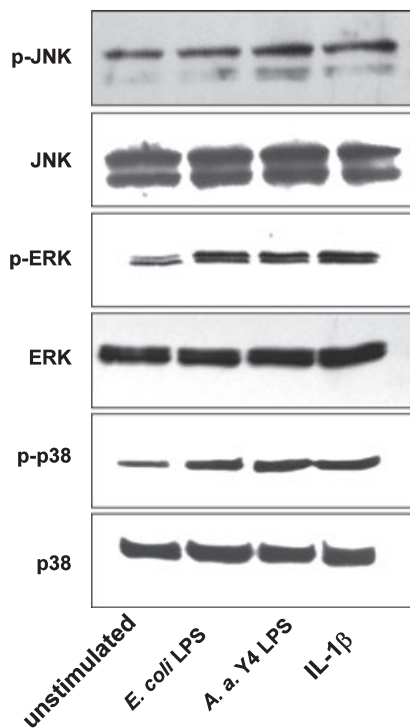


Fig. 1. IL-1β, *Escherichia coli* and *Actinobacillus actinomycetemcomitans* (Aa) LPS stimulation rapidly activates ERK, p38 and JNK MAPK. Mouse PDL cells were stimulated with IL-1β (5 ng/ml), *E. coli* LPS (5 µg/ml) or *A. actinomycetemcomitans* LPS (5 µg/ml) for 30 min. Immunoblot analysis using whole cell lysates (15 µg) indicated that ERK and p38 MAPK pathways are activated by LPS whereas *A. actinomycetemcomitans* LPS and IL-1β activate JNK. A representative experiment shown from five experiments.

dose-response of IL-6 mRNA expression in response to *A. actinomycetemcomitans* LPS, *E. coli* LPS, and IL-1β. Time-course of IL-6 mRNA induction experiments revealed that the optimal induction time for IL-6 mRNA was 18 h poststimulation (Fig. 2A). All inflammatory stimuli produced a bi-phasic induction of IL-6 mRNA, however, *E. coli* LPS and IL-1β induced similar temporal kinetic expression profiles of IL-6 mRNA. At the 18-h time-point the mean ($n = 3$) fold induction of IL-6 was 2.7 with IL-1β ($P = 0.0267$; paired *t*-test), 2.23 with *E. coli* LPS ($P = 0.0055$; paired *t*-test) and 2.01 with *A. actinomycetemcomitans* LPS stimulation ($P = 0.001$; paired *t*-test). Consistent with the IL-6 mRNA levels, IL-6 protein expression increased in a similar time-dependent manner (Fig. 2B). At the 18-h and 24-h time-points, significant elevations in IL-6 protein were observed from time 0 in response to IL-1β, *E. coli* LPS and *A. actinomycetemcomitans* LPS stimulation ($n = 3$).

Dose-response analysis revealed the optimal stimulus concentration to be 5 µg/ml for both *A. actinomycetemcomitans* LPS and *E. coli* LPS while it was 5 ng/ml for IL-1β ($n = 3$) (Fig. 2C). Interestingly, *A. actinomycetemcomitans* and *E. coli* LPS 10 µg/ml provided saturated expression of IL-6 mRNA. This effect was also observed for IL-1β at 10 ng/ml. Optimal time and dose were utilized in all subsequent experiments.

MAPK signaling pathways involved in LPS-induced IL-6 expression

Next, we evaluated the MAPK signaling pathways involved in the induction of IL-6 production by IL-1β, *E. coli* LPS and *A. actinomycetemcomitans* LPS at the mRNA and protein levels. Major MAPK signaling pathways evaluated included ERK, JNK and p38α MAPK. The pathways were inhibited using specific biochemical inhibitors PD98059 (ERK), SP600125 (JNK) and SB203580 (p38 MAPK). The RT-PCR was performed on RNA isolated 18 h post treatment ($n = 3$). SB203580 (10 µM) inhibited all inflammatory stimuli greatly, while SP600125 (10 µM) and PD98059 (10 µM) inhibition varied among inflammatory stimuli (Fig. 3A,B). SB203580 was able to reduce IL-6 mRNA production by >70% for *A. actinomycetemcomitans* LPS treatment ($P < 0.001$; two-way ANOVA), >95% for *E. coli* LPS treatment ($P < 0.05$; two-way ANOVA, and ~60% from IL-1β treatment ($P < 0.05$; two-way ANOVA). SP600125 and PD98059 had the greatest inhibitory effects (~50%; $P < 0.05$; two-way ANOVA) with *E. coli* LPS and IL-1β treatments.

IL-6 ELISA was performed to determine if the effects of the biochemical inhibitors translated to the protein level (Fig. 4). Murine PDL cells were treated as described above. Cell culture supernatants were harvested 24 h post-treatment and IL-6 ELISA was performed ($n = 4$). Stimulation with each of the inflammatory stimuli produced copious IL-6 when compared to untreated cultures. Specifically *A. actinomycetemcomitans* LPS and IL-1β induced >500 pg/ml IL-6, while *E. coli* induced >450 pg/ml IL-6. SB203580 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor) provided the greatest inhibition of inducible IL-6 production in response to inflammatory agonists. PD98059 (ERK inhibitor) provided comparable inhibition when cells were stimulated with *E. coli* LPS or IL-1β. SB203580 (p38 MAPK inhibitor) reduced IL-6 production by all

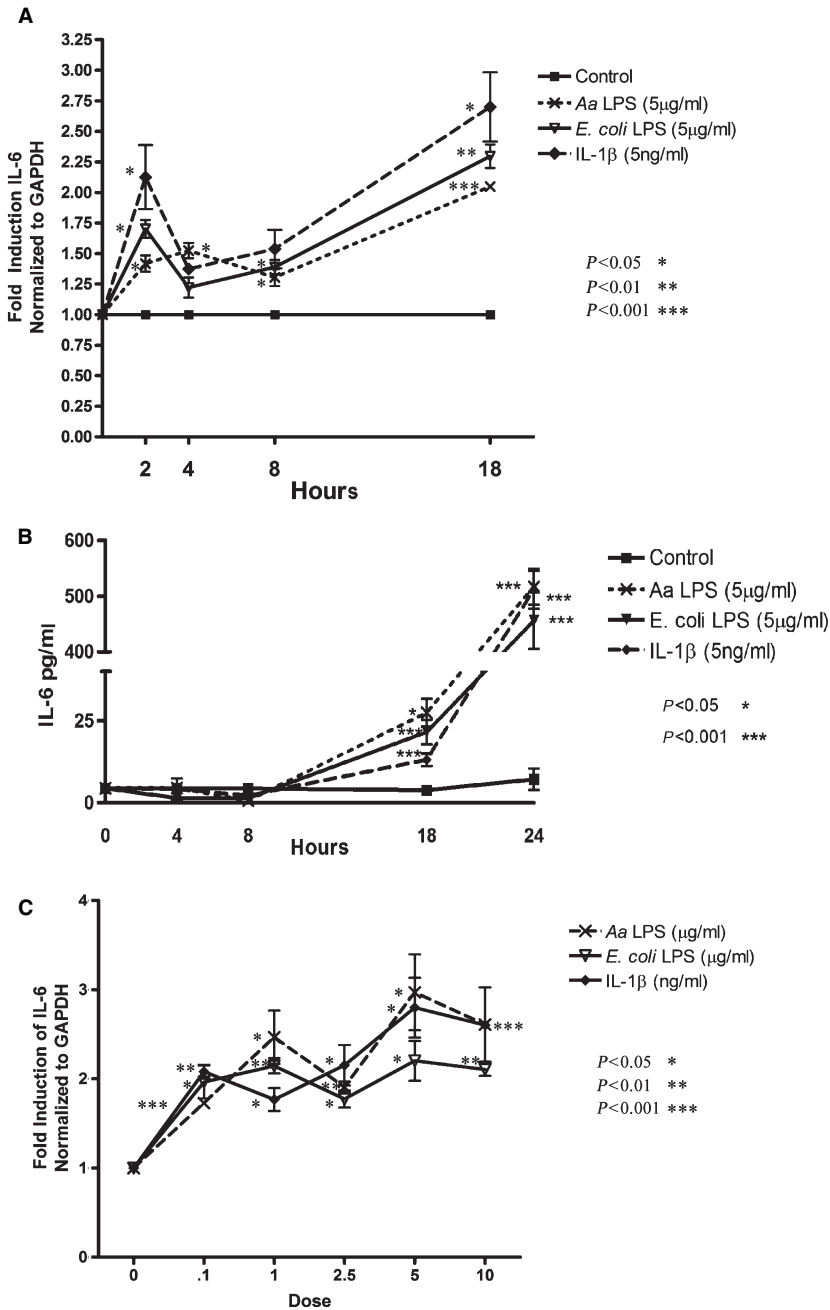


Fig. 2. IL-1 β , *Escherichia coli* and *Actinobacillus actinomycetemcomitans* (Aa) LPS induce IL-6 mRNA. (A) Murine PDL cells were grown to near confluence and serum starved in 0.3% fetal bovine serum media for 6 h. Cells were then stimulated with *A. actinomycetemcomitans* LPS, *E. coli* LPS, and IL-1 β for 2, 4, 8 and 18 h at doses as high as 10 ng/ml for IL-1 β , and 10 μ g/ml for both *A. actinomycetemcomitans* LPS and *E. coli* LPS. Total RNA was isolated and RT-PCR was performed measuring IL-6 normalized to GAPDH. Maximum IL-6 mRNA production plateaued at 5 ng/ml for IL-1 β , and 5 μ g/ml for both *A. actinomycetemcomitans* LPS and *E. coli* LPS. (B) Murine PDL cells were grown in the same manner as in (A) with cell culture supernatants harvested at the indicated time-points for IL-6 protein determination by ELISA. The results represent the mean pg/ml \pm SE ($n = 3$). Significant induction of IL-6 was observed at 18 and 24 h (C). The results represent the mean fold induction \pm SE ($n = 3$) for both panels. P -values were obtained using paired two-tailed Student's t -tests. P -values were derived comparing stimulant to control (no treatment), * $P < 0.05$ was considered statistically significant.

agonists by >60%, but the greatest decrease was observed in cells treated with *A. actinomycetemcomitans* LPS. The

effect of SP600125 was observed mostly with *E. coli* LPS and IL-1 β s the inhibitor reduced IL-6 production $\geq 70\%$. PD98059

was capable of inhibiting IL-1 β - and *E. coli* LPS-induced IL-6, but was unable to inhibit *A. actinomycetemcomitans* LPS-induced levels equivalent to that of SB203580.

MKK-3/6-p38 α MAPK pathway is required for IL-6 expression

Biochemical inhibitors provide relatively specific inhibition of their respective signaling pathways. However, to verify the role of p38 MAPK in IL-6 production, stable mPDL cells over-expressing DN MKKs, which directly phosphorylate p38, were employed. MKK-3 and MKK-6 DN stable mPDL cells were utilized, along with mock-transfected (empty vector) mPDL cells ($n = 3$). Cells were stimulated with *A. actinomycetemcomitans* LPS, *E. coli* LPS and IL-1 β as described above (Figs 4B and 5A). Eighteen hours post stimulation, total RNA was isolated and IL-6 steady-state mRNA levels were measured. In MKK-3 DN cells IL-6 mRNA expression was statistically reduced in all stimulated cultures ($P < 0.05$ for *A. actinomycetemcomitans* LPS and $P < 0.001$ for *E. coli* LPS and IL-1 β ; two-way ANOVA). MKK-6 DN cells significantly reduced the fold change of IL-6 mRNA in *E. coli* LPS ($P < 0.01$; two-way ANOVA) and IL-1 β only ($P < 0.001$; two-way ANOVA). In both MKK-3 and MKK-6 DN cells, the greatest reduction of IL-6 mRNA was observed when cells were treated with IL-1 β . These data suggest that while IL-1 β and *E. coli* LPS activate p38 MAPK-induced IL-6 mRNA through MKK-3 and MKK-6, *A. actinomycetemcomitans* LPS activates p38 MAPK predominantly through MKK-3.

To further validate the need for an intact p38 signaling system for *A. actinomycetemcomitans* LPS-induced IL-6 expression, MEFs with targeted disruption of the p38 α gene were used. The *A. actinomycetemcomitans* LPS induced over 300 pg/ml ($P < 0.001$) of IL-6 in wild-type MEFs while in p38 $\alpha^{-/-}$ MEFs, IL-6 production was not significant ($P < 0.001$) compared to the untreated controls (Fig. 6). Lack of p38 α reduced the amount of IL-6 induced by *A. actinomycetemcomitans* LPS by $\sim 90\%$ ($P < 0.001$; two-way ANOVA).

Discussion

Periodontal diseases are characterized by loss of alveolar bone, a process mediated by osteoclasts. Locally abundant LPS derived from gram-negative organisms is

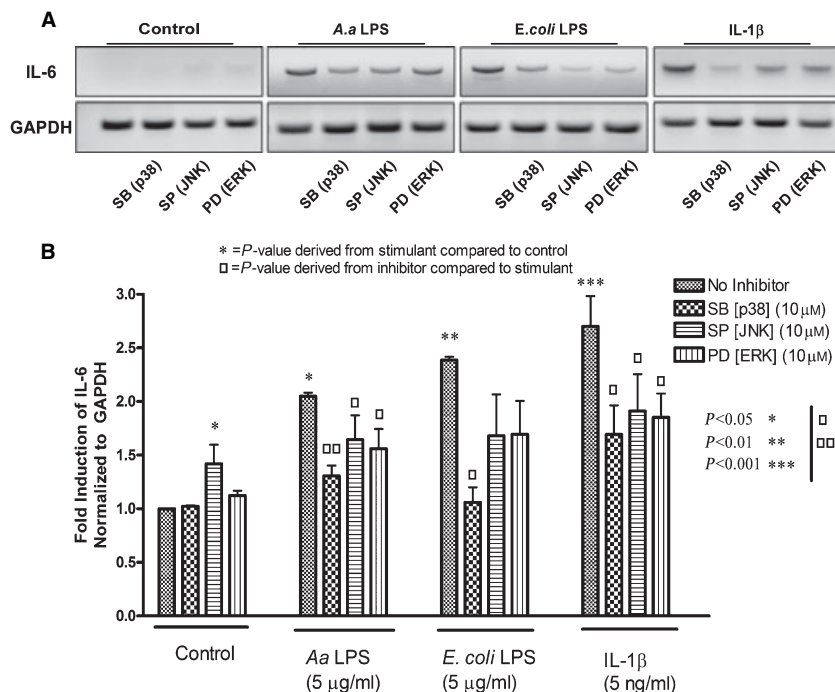


Fig. 3. IL-6 requires predominantly p38 α MAPK when stimulated by IL-1 β , *Actinobacillus actinomycetemcomitans* (Aa) and *Escherichia coli* LPS. Murine PDL cells were grown to near confluence and serum starved with 0.3% FBS media for 6 h. Cells were pretreated with inhibitors specific for ERK (PD98059), JNK (SP600125), and p38 α MAPK (SB203580) pathways at a concentration of 10 μ M and stimulated with IL-1 β (5 ng/ml) and LPS (5 μ g/ml) from *A. actinomycetemcomitans* and *E. coli*. Total RNA was harvested after 18 h and RT-PCR was performed measuring IL-6 fold induction compared to GAPDH. (A) Representative gel from RT-PCR analysis. (B) Graphical representation of results showing mean fold induction \pm SE ($n = 3$). Using two-way ANOVA, P -values were derived comparing stimulant to control (no treatment), * $P < 0.05$ was considered statistically significant.

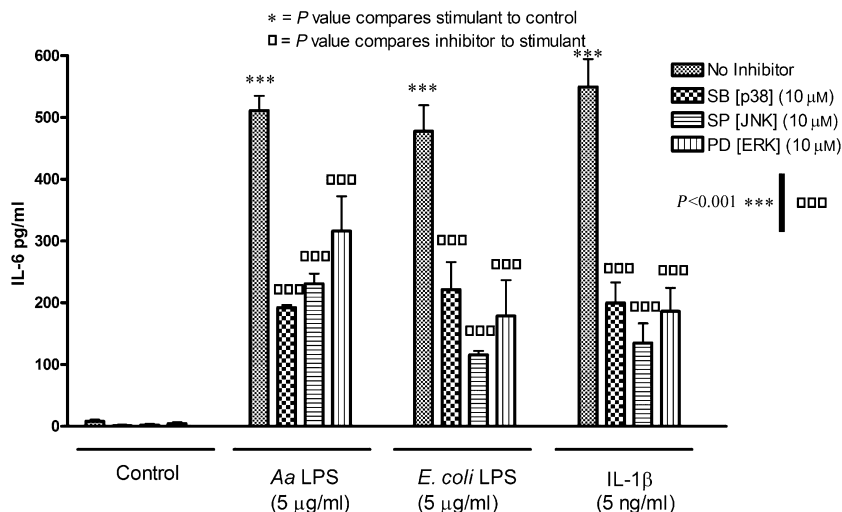


Fig. 4. p38 α MAPK and JNK pathway are needed for *Actinobacillus actinomycetemcomitans* (Aa) LPS secretion of IL-6. Murine PDL cells were grown to near confluence then serum starved with 0.3% fetal bovine serum media for 6 h. Cells were treated with inhibitors and after 1 h cells were treated with inflammatory stimuli as described in Fig. 2. Twenty-four hours after treatment, supernatants were collected and ELISA detection of IL-6 protein was measured. The results represent the mean pg/ml \pm SE ($n = 3$). Two-way ANOVA was utilized to generate P -values. P -values compare inhibitor to stimulant (no inhibitor), * P values compare stimulant to control (no treatment), or * $P < 0.05$ was considered statistically significant.

largely responsible for the increased resorption of bone. LPS can stimulate the production of osteoblastic IL-1 β , TNF- α , IL-6 and receptor activator of NF- κ B ligand (RANKL) directly or indirectly (11, 13, 19). Sources of IL-6 in the periodontium include periodontal and gingival fibroblasts, macrophages, lymphocytes and osteoblasts (25, 26). However, the PDL fibroblast is poised in position to play a significant role in IL-6 production in chronic periodontitis. Inhibition of IL-6 expression presents an attractive target for the treatment of chronic inflammatory bone disease states. Thus, understanding IL-6 regulation in PDL fibroblasts may be critical for inflammatory-cytokine-based therapies in the management of periodontal diseases. In this report, MAPK signaling pathways induced by LPS that were required for IL-6 expression in PDL fibroblasts were analysed through biochemical inhibitors, MKK DN stable cell lines, and p38 knock-out cell models. The data indicate that *A. actinomycetemcomitans* LPS induction of IL-6 uses multiple MAPK pathways but predominantly the MKK-3-p38 pathway in mPDL fibroblasts.

Numerous studies have analysed periodontal pathogens and their constituents to produce pro-inflammatory cytokines. Extracts from *A. actinomycetemcomitans* have been able to induce IL-8 in gingival epithelial cells independent of LPS (24). Comparison studies evaluating the ability of *E. coli* LPS and *Porphyromonas gingivalis* to induce IL-6, IL-8, IL-10 and IL-12 in dendritic cells found *E. coli* LPS to be the more potent agonist (12). In the present study, the prototypical *E. coli* LPS was used as a relative comparison to *A. actinomycetemcomitans* LPS. Along with *E. coli*, the *A. actinomycetemcomitans*-derived LPS has been demonstrated to induce IL-1 β , IL-6 and TNF- α in human monocytes (3).

Our results support other studies that have shown a similar plateau in IL-6 expression by *E. coli* LPS in monocytes and bone-marrow-derived macrophages (17, 18). The *A. actinomycetemcomitans* LPS also had the same kind of response as *E. coli* LPS. This effect may emphasize a conserved mechanism of activation during LPS-induced inflammation. Although IL-6 mRNA production was statistically significant as early as 2 h with *A. actinomycetemcomitans* LPS, *E. coli* LPS and IL-1 β , maximal steady-state IL-6 expression was not reached until 18 h in mPDL cells. The ability of inflammatory stimuli to induce IL-6 after 2 h suggests that there is direct stimulation of IL-6 because *de novo* protein

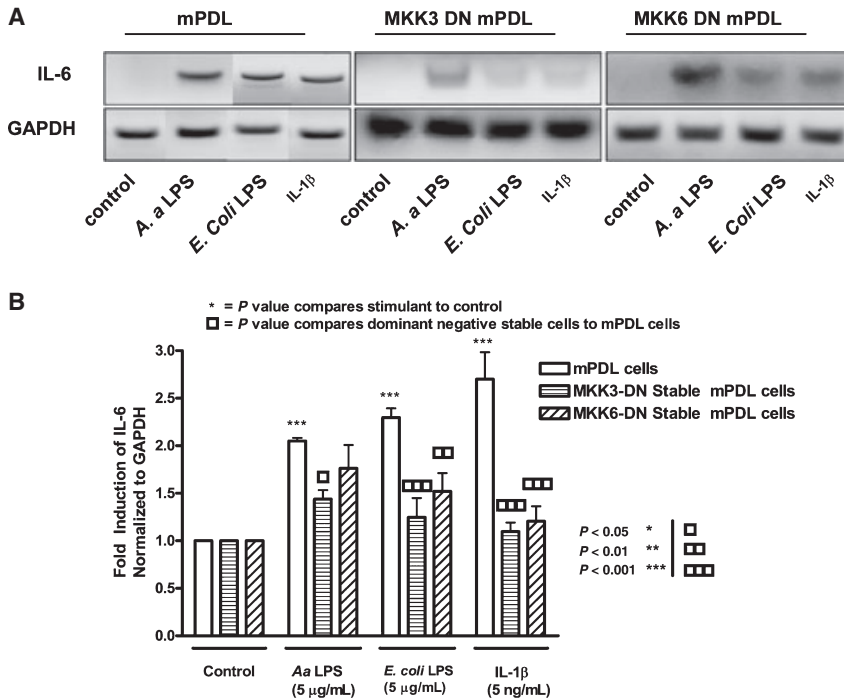


Fig. 5. MKK-3-induced-p38 α MAPK is utilized in IL-1 β , *Actinobacillus actinomycetemcomitans* (Aa) LPS, and *E. coli* LPS induction of IL-6 mRNA. Wild-type, MKK-3 and MKK-6 DN stable mouse PDL cells were serum starved with 0.3% fetal bovine serum media for 6 h then stimulated with IL-1 β 5 ng/ml, *A. actinomycetemcomitans* LPS (5 µg/ml), and *E. coli* LPS (5 µg/ml). Total RNA was isolated 18 h later and RT-PCR was performed. IL-6 was compared to GAPDH and IL-6 fold induction was determined. (A) Representative gel from RT-PCR analysis. (B) Graphical representation of results showing the mean fold induction \pm SE ($n = 3$). Two-way ANOVA was utilized to generate P -values. P -values compare DN stable cell lines to mPDL cells. * P -values compare stimulant to control (no treatment), or * $P < 0.05$ was considered statistically significant.

synthesis has not occurred. At 18 h, IL-6 may be stimulated by multiple cytokines, acting in an autocrine manner, thus indirectly activating and amplifying inflammatory stimulus-induced IL-6 expression.

Surprisingly, when PDL cells were treated with biochemical inhibitors alone, the data indicate that SP600125 (the JNK inhibitor) significantly increased IL-6 mRNA induction when compared to vehicle control. In previous experiments, we have observed that SP600125 (JNK inhibitor) and PD98059 (ERK inhibitor) increase basal phosphorylated p38 MAPK levels in mPDL cell lysates (22). IL-6 mRNA appeared to be induced predominantly through p38 MAPK for all inflammatory stimuli. However, p38 and JNK appeared to be equally involved in *A. actinomycetemcomitans* LPS secretion of IL-6. All biochemical inhibitors reduced IL-1 β and *E. coli* LPS-induced IL-6 secretion. The discrepancies observed between IL-6 mRNA and protein inhibition are most likely the result of differences in IL-6 protein and mRNA half-lives (14).

The present study evaluated only the contribution of MAPK towards LPS-

induced IL-6 expression; however, IL-6 contains AP-1 (activator protein 1) and NF-IL-6 transcription factor binding regions, which directly implicates NF- κ B in the production of IL-6. In the light of this, we cannot rule out the involvement of NF- κ B in the production of IL-6. Initial studies using a NF- κ B inhibitor indicated partial inhibition of LPS-induced IL-6 (data not shown). In mouse fibrosarcoma cells, TNF- α -induced, NF- κ B-stimulated IL-6 gene expression requires both p38 α MAPK and ERK. When activated, p38 α MAPK can activate NF- κ B pathways through TNF receptor-associated factor-6 (27). It is well established that p38 is phosphorylated by upstream kinases MKK-3 or MKK-6 (5). Dominant negative isoforms of both MKK-3 and -6 have been shown to have reduced kinase activity on p38 α MAPK substrates (4).

The *A. actinomycetemcomitans* LPS preferentially utilized MKK-3/p38 α activation for IL-6 mRNA expression. Interestingly, preferential activation of p38 α by either MKK-3 or MKK-6 has been observed by others. Inoue et al. observed that MKK-3 plays a greater role in cytokine

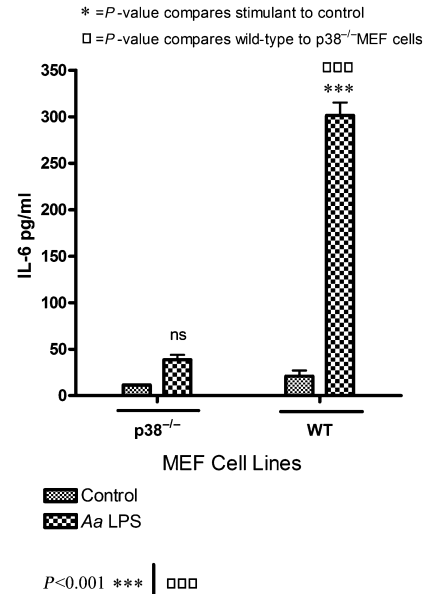


Fig. 6. p38 α MAPK is necessary for IL-6 protein expression in MEF cells. Wild-type (WT) and p38 α $^{-/-}$ MEF cells were grown plated and grown to near confluence. Cells were then serum starved with 0.3% fetal bovine serum media for 6 h followed by treatment with *Actinobacillus actinomycetemcomitans* LPS (5 µg/ml). After 24 h, supernatants were isolated and ELISA was performed measuring IL-6 protein. The results represent the mean pg/ml \pm SE ($n = 3$). Two-way ANOVA was utilized to generate P -values. P -values compare wild-type to p38 α MAPK $^{-/-}$ MEF3T3 cells. * P -values compare stimulant to control (no treatment), or * $P < 0.05$ was considered statistically significant.

and MMP expression in fibroblast-like synoviocytes from patients with rheumatoid arthritis (10). Osmotic shock and TNF-induced expression of cytokines has been reduced in MKK-3 $^{-/-}$ MEF (33). Our group has recently shown that MKK-6/p38 is important for LPS-induced MMP-13 expression in mPDL fibroblasts (Carlos Rossa, Jr., Paul M. Bronson, Min Liu, Keith L. Kirkwood, unpublished data). However, in the present study IL-1 β and *E. coli* LPS utilized both MKK-3 and MKK-6 pathways with no statistically significant preference between either MKK.

One limitation of our stable cells is that either MKK-3 DN or MKK-6 DN is expressed, not both. As a consequence of blocking MKK-3 or MKK-6 activation of p38 α MAPK can still occur through MKK-3 or MKK-6 phosphorylation. To address this, the role of p38 α in *A. actinomycetemcomitans* LPS induction of IL-6 was extended with the use of MEF wild-type and p38 α $^{-/-}$ cells. In p38 α MAPK $^{-/-}$ MEF cells, *A. actinomycetemcomitans* LPS-induced IL-6 was signifi-

cantly reduced when compared to wild-type cells, suggesting that p38 α is a vital signaling intermediate required for IL-6 expression. Interestingly, *E. coli* LPS was unable to induce IL-6 protein expression in either wild-type or p38 α ^{-/-} cells but IL-1 β effects were similar to those observed with *A. actinomycetemcomitans* LPS (data not shown). An additional limitation is that the data presented are from using p38 α ^{-/-} MEF cells, which are clearly not PDL cells. PDL cells cannot be obtained from p38 α ^{-/-} mice because these mice die *in utero*. The MEF cells were harvested between E11.5–12.5 and PDL tissue fibers that support the tooth and bone form approximately day 27 post-conception, so PDL cells from the p38 α ^{-/-} mice cannot be obtained (M. Somerman, personal communication).

In summary, *A. actinomycetemcomitans* LPS-induced IL-6 expression requires p38 α MAPK pathway signaling in PDL fibroblasts. IL-6 is a key pro-inflammatory cytokine capable of stimulating osteoclastogenesis in coupled bone turnover via induction of RANKL. Our group has recently established that TNF- α and IL-1 β -induced RANKL in bone marrow stromal cells requires p38 MAPK (23). Importantly, parathyroid hormone and 1,25(OH)₂-vitamin D₃-induced RANKL are unaffected by p38 inhibitors, supporting the role of p38 antagonists to block inflammation-stimulated bone loss while sparing physiological bone turnover. Thus, because p38 MAPK is a key signaling intermediate utilized by LPS and IL-1-induced IL-6 expression in PDL fibroblasts, targeting p38 MAPK may have therapeutic importance for the management of chronic periodontitis.

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