

A dominant function of p38 mitogen-activated protein kinase signaling in receptor activator of nuclear factor- κ B ligand expression and osteoclastogenesis induction by *Aggregatibacter actinomycetemcomitans* and *Escherichia coli* lipopolysaccharide

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Background and Objective: Lipopolysaccharide from gram-negative bacteria is one of the microbial-associated molecular patterns that initiate the immune/inflammatory response, leading to the tissue destruction observed in periodontitis. The aim of this study was to evaluate the role of the p38 mitogen-activated protein kinase (MAPK) signaling pathway in lipopolysaccharide-induced receptor activator of nuclear factor- κ B ligand (RANKL) expression by murine periodontal ligament cells.

Material and Methods: Expression of RANKL and osteoprotegerin mRNA was studied by reverse transcription-polymerase chain reaction upon stimulation with lipopolysaccharide from *Escherichia coli* and *Aggregatibacter actinomycetemcomitans*. The biochemical inhibitor SB203580 was used to evaluate the contribution of the p38 MAPK signaling pathway to lipopolysaccharide-induced RANKL and osteoprotegerin expression. Stable cell lines expressing dominant-negative forms of MAPK kinase (MKK)-3 and MKK6 were generated to confirm the role of the p38 MAPK pathway. An osteoclastogenesis assay using a coculture model of the murine monocytic cell line RAW 264.7

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was used to determine if osteoclast differentiation induced by lipopolysaccharide-stimulated periodontal ligament was correlated with RANKL expression.

Results: Inhibiting p38 MAPK prior to lipopolysaccharide stimulation resulted in a significant decrease of RANKL mRNA expression. Osteoprotegerin mRNA expression was not affected by lipopolysaccharide or p38 MAPK. Lipopolysaccharide-stimulated periodontal ligament cells increased osteoclast differentiation, an effect that was completely blocked by osteoprotegerin and significantly decreased by inhibition of MKK3 and MKK6, upstream activators of p38 MAPK. Conditioned medium from murine periodontal ligament cultures did not increase osteoclast differentiation, indicating that periodontal ligament cells produced membrane-bound RANKL.

Conclusion: Lipopolysaccharide resulted in a significant increase of RANKL in periodontal ligament cells. The p38 MAPK pathway is required for lipopolysaccharide-induced membrane-bound RANKL expression in these cells.

Coupled bone turnover is a tightly regulated process balanced by osteoblasts and osteoclasts. Uncoupling leads towards net bone loss associated with several inflammatory bone diseases, including periodontal diseases. Initiated by pro-inflammatory stimuli (such as lipopolysaccharide) originating from dental biofilm-associated bacteria, molecular events leading towards periodontal tissue and bone destruction are set in motion in the periodontal microenvironment. Fundamental aspects of the bone resorption process have been discovered in recent years following identification of receptor activator of nuclear factor- κ B ligand (RANKL) and its decoy receptor, osteoprotegerin. These cytokines constitute the current paradigm for the processes of osteoclast differentiation and activation. The induction of coupled bone resorption by inflammatory cytokines involves increased RANKL expression on various cell types, including osteoblastic cells, bone marrow stromal cells, endothelial cells, mononuclear cells and periodontal ligament fibroblasts, which activate receptor activator of nuclear factor- κ B (RANK) on pre-osteoclastic cells to induce osteoclastogenesis and subsequent bone resorption (1–5).

The unique location of the periodontal ligament between two mineralized tissues, cementum and alveolar bone, suggests that it may have an

essential role in periodontal homeostasis. This assumption is supported by classical histological studies on periodontal regeneration (6). Thus, alveolar bone formation and resorption in the periodontium seems to be largely controlled by cells in the periodontal ligament, as also supported by studies on orthodontic movement of teeth where RANKL/osteoprotegerin expression was shown to be involved (7). Even though periodontal ligament cells were initially considered as inhibitors of osteoclastogenesis and shown to express osteoprotegerin (8), these cells were demonstrated to express RANKL in situations associated with resorption of mineralized tissue, including physiologic permanent tooth eruption and deciduous tooth root resorption (9–11). Specifically, periodontal ligament cells were shown to form bone-like tissues (12,13) and also to express RANKL and osteoprotegerin mRNA after stimulation with di-hydroxyl vitamin D₃, prostaglandin E₂, interleukin-1 β , interleukin-1 α and mechanical stress (10,14–18).

The RANKL/RANK/osteoprotegerin system as a key mediator of bone loss in periodontal disease comes from recent *in vivo* studies, both in animal models (19,20) and in humans (21,22), demonstrating the expression of higher levels of RANKL and lower levels of osteoprotegerin in the pres-

ence of alveolar bone resorption (23). Even though numerous studies have demonstrated that bacterial lipopolysaccharide has bone-resorbing capacity *in vitro* and *in vivo* (24–27), controversy still exists regarding the potency of the different pathogen-associated molecular patterns in inducing RANKL expression. Recently, periodontal ligament fibroblasts have been shown to produce RANKL mRNA induced by interleukin-1 α indirectly through prostaglandin E₂ induction, which involved extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) signaling (15). Also recently, all three MAPKs (ERK, c-Jun N-terminal kinase and p38) were shown to be involved in interleukin-1 α -induced RANKL expression by human periodontal ligament fibroblasts (28). Conversely, RANKL expression by *Porphyromonas gingivalis*-infected osteoblasts did not involve p38 or ERK MAPKs or phosphoinositol-3 kinase pathways (29). In fact, these authors (29) reported that lipopolysaccharide from *P. gingivalis* was not responsible for the induction of RANKL in infected osteoblasts, which suggests that the Toll-like receptor 2 (TLR-2) signaling pathway may not be involved in RANKL expression by these cells.

There is a lack of information on the signaling pathways involved in lipopolysaccharide-induced RANKL

expression by periodontal ligament fibroblasts. As these cells may play an important role on alveolar bone resorption, both during periodontal disease and orthodontic movement, understanding the signaling pathways involved may provide critical information towards alternative therapeutic strategies for the control of alveolar bone resorption process. Recent data from our group supports the role of novel therapeutics, which blocks p38 signaling in preventing alveolar bone loss induced by lipopolysaccharide *in vivo* (3). Considering that RANKL expression may require different signaling pathways depending on the nature of extracellular stimulation and also on the cell type, in this study we examined the role of p38 MAPK signaling on lipopolysaccharide-induced RANKL expression by periodontal ligament cells.

Material and methods

Cells and materials

Mouse periodontal ligament fibroblasts, immortalized with simian virus 40 large T antigen, were obtained from Dr Martha Somerman (University of Washington, Seattle, WA, USA). These cells were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 100 IU/mL of penicillin, 100 µg/mL of streptomycin and 10% heat-inactivated fetal bovine serum, and maintained in a humidified atmosphere at 37°C and 5% CO₂. Mouse periodontal ligament cells used were previously characterized for the expression of genes normally expressed by primary periodontal ligament cells, including bone sialoprotein, osteopontin, osteocalcin and type I collagen (30). Unless noted otherwise, all tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Lipopolysaccharide from *Escherichia coli* (serotype 0127:B8) was purchased from Sigma-Aldrich (St Louis, MO, USA) and *Aggregatibacter actinomycetemcomitans* (formerly known as *Actinobacillus actinomycetemcomitans*) lipopolysaccharide was extracted from *A. actinomycetemcomitans* strain Y4 (serotype

B) by the hot phenol-water method, as described previously (23,31). *A. actinomycetemcomitans* lipopolysaccharide used in the present study was recently characterized during other studies carried out by our laboratory group (23). Both *E. coli* and *A. actinomycetemcomitans* lipopolysaccharide were diluted in serum-free defined culture medium (Opti-MEM; Invitrogen) to 1 mg/mL. The biochemical inhibitor SB203580 was from Calbiochem (San Diego, CA, USA) and RANKL and osteoprotegerin recombinant proteins were from R & D systems (Minneapolis, MN, USA). Mouse RANKL monoclonal antibody was purchased from Stress Gen (Ann Arbor, MI, USA), and monoclonal glyceraldehyde-3-phosphate dehydrogenase antibody was from Chemicon (Temecula, CA, USA). The absence of protein in *A. actinomycetemcomitans* lipopolysaccharide preparations was checked by polyacrylamide gel electrophoresis of extract samples and subsequent staining with silver nitrate and Coomassie Blue, and confirmed by spectrophotometry (< 0.001% nucleic acid) and by a microassay for protein quantification (cat. no 500-0002; Bio-Rad Laboratory (Hercules, CA, USA)) based on the Bradford method (lower limit of detection: 1.2 µg/mL). Dominant-negative genetic constructs of mutated MAP kinase kinase-3 and MAP kinase kinase-6 were obtained from J. Han (Scripps Institute, La Jolla, CA, USA). Stable cell lines were prepared as described previously (3). Briefly, after cotransfection of the overexpression construct and of an empty vector including resistance to gentamycin, selection was carried out for several weeks in medium containing 800 µg/mL of Geneticin (Invitrogen Corp.) and a number of clones were screened by western blot to analyse the expected changes on expression of the signaling proteins.

Semiquantitative reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was used to evaluate mRNA expression, as described

recently (3). Briefly, total RNA was harvested using Trizol (Invitrogen) reagent according to the manufacturer's instructions. Complementary DNA was synthesized by reverse transcription of 500 ng of total RNA using 2.5 µM Oligo (dT) 16 primers and 1.25 U/µL of Moloney murine leukemia virus reverse transcriptase in the presence of 5.5 mM MgCl₂, 2 mM dNTPs and 0.4 U/µL of RNase inhibitor, according to the manufacturer's protocol (Applied Biosystems (Foster City, CA, USA)). Two microlitres of the RT reaction product was used in a PCR reaction mix of 25 µL total volume. The primer pair used for RANKL (accession no.: AF019048) was: sense 5'-CAGCACTCACTGCTTTTATAGAATCC-3'; antisense 5'-AGCTGAAGATAGCTGTAGGTACGC-3'; for osteoprotegerin (accession no.: NM008764) was: sense 5'-TGTAGAGAGGATAAACGG-3'; antisense 5'-CTAGTTAAGCAGCTTAT-3'; and for glyceraldehyde-3-phosphate dehydrogenase (accession no.: NM002046) was: sense 5'-CACCATGGAGAAGGCCGGGG-3'; antisense 5'-GACGGACACATTGGGGTAG-3'. A total of 50 pmol/µL of each primer was used in the PCR reactions, yielding products of 467, 503 and 418 bp for RANKL, osteoprotegerin and glyceraldehyde-3-phosphate dehydrogenase, respectively. *Taq* DNA polymerase and other PCR reagents were purchased from Invitrogen and the conditions for RANKL and osteoprotegerin were 35 cycles (32 cycles for osteoprotegerin) of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min and a final extension step at 72°C for 10 min in the presence of 2.5 mM MgCl₂, whereas for glyceraldehyde-3-phosphate dehydrogenase the conditions were 25 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 min and a final extension step at 72°C for 10 min in the presence of 1.5 mM MgCl₂. PCR products were resolved in 1.5% agarose gels, stained with 0.5 µg/mL of ethidium bromide. The images and densitometric measurements were obtained with a digital documentation system (Gel Doc XR; Bio-Rad (Hercules, CA, USA)). The quantity of RANKL and osteoproteg-

erin mRNA in each sample was subsequently normalized to the quantity of glyceraldehyde-3-phosphate dehydrogenase mRNA and expressed as fold change over the unstimulated control using MOLECULAR ANALYST software (version 4.5.2; Bio-Rad). The fold change on the expression of RANKL was divided by the fold change on the expression of osteoprotegerin to obtain the RANKL:osteoprotegerin ratio.

Western blot analysis

A total of 5×10^4 periodontal ligament cells were grown for 24 h in each well of six-well plates, de-induced by incubation for 12 h in culture medium containing 0.3% fetal bovine serum and stimulated with either *E. coli* or *A. actinomycetemcomitans* lipopolysaccharide for 72 h, both with and without a 30-min pretreatment with $10 \mu\text{M}$ SB203580. Whole-cell lysates were harvested by scraping the periodontal ligament cells with sodium dodecyl sulfate 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, followed by sonication for 10 s and heat-denaturation at 95°C for 5 min. Total protein content was quantified by a Bradford-based microassay. For western blotting, 30 μg of total proteins were separated on 10% Tris-HCl polyacrylamide gels run at 100 V for 60 min and subsequently electrotransferred for another 60 min at 110 mA in a semidry apparatus to nitrocellulose membranes. The membranes were blocked (Tris-buffered saline with 5% nonfat dry milk, 0.1% Tween-20) for 1 h at room temperature and then probed overnight at 4°C with primary antibodies. The presence of the primary antibodies was detected by using horseradish peroxidase-conjugated secondary antibody and a chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA). Digital images and quantification of the membranes were obtained and analyzed on a chemiluminescent documentation system (ChemiDoc XRS; Bio-Rad).

Osteoclastogenesis assays

A total of 5×10^4 periodontal ligament cells were plated on six-well dishes and, 24 h later, the medium was changed to 0.2% fetal bovine serum-containing medium and the cells were stimulated with *E. coli* and *A. actinomycetemcomitans* lipopolysaccharide for 72 h. As preliminary experiments indicated a similar ability of both *E. coli* and *A. actinomycetemcomitans* lipopolysaccharide to induce RANKL mRNA in periodontal ligament cells, as well as in their capacity to support osteoclastogenesis, only lipopolysaccharide from periopathogenic *A. actinomycetemcomitans* was used to stimulate the stable cell lines MKK3 dominant negative-periodontal ligament and MKK6 dominant negative-periodontal ligament. Lipopolysaccharide was added to the cocultures at 0 and 32 h. Conditioned medium was removed at 72 h, the cell layer was washed with phosphate-buffered saline twice and then scraped off the wells and resuspended in 0.5 mL of fresh 0.2% fetal bovine serum-containing medium. A total of 3×10^4 cells were then transferred to each well of a six-well plate, which already contained RAW 264.7 cells (initial plating density: 5×10^4 cells/well), and cultured for 24 h. Then, 0.5 mL of conditioned medium was added to the RAW 264.7 cells to determine the contribution of soluble RANKL by the periodontal ligament cells. RANKL (50 ng/mL) and osteoprotegerin (100 ng/mL) were used as controls for investigating the capacity of RAW 264.7 cells to differentiate into osteoclasts in response to RANKL, as well as for studying the ability of osteoprotegerin to block this process. The cocultures were maintained for 6 d, with one change of medium on day 3, and then submitted to tartrate-resistant acid phosphate (TRAP) staining (as described in Technical Bulletin no. 445; BD Biosciences, Franklin Lakes, NJ, USA). All reagents used for this assay were from Sigma. Briefly, culture medium was removed and the cells were washed twice with phosphate-buffered saline. The cells were then fixed with 10% glutaraldehyde for 15 min (37°C),

washed twice with prewarmed phosphate-buffered saline and stained for 5–10 min at 37°C with the staining buffer (50 mM sodium acetate buffer, 30 mM sodium tartrate, 0.1 mg/mL of naphthol AS-MX phosphate, 0.1% Triton X-100 and 0.3 mg/mL of Fast Red Violet LB stain), pH 5.0. After removal of the staining buffer, the cells were washed three times with phosphate-buffered saline. These cells were observed on an inverted microscope at $\times 40$ magnification, depending on the cell density, using bright field and the numbers of stained cells containing three or more nuclei were counted.

Statistical analysis

Pairwise comparisons between experimental groups were performed using the *t*-test with Welch's correction for unequal variances. Comparison between fold changes on mRNA expression between lipopolysaccharide-stimulated and untreated cells was performed with the one-sample *t*-test. The significance level was set to 5% and all calculations were performed using PRISM 4 software (GraphPad, Inc., San Diego, CA, USA).

Results

P38 MAPK regulates preferentially RANKL mRNA expression in lipopolysaccharide-stimulated periodontal ligament cells

Preliminary experiments indicated that the maximum expression of RANKL mRNA occurred after 18–24 h of stimulation with lipopolysaccharide from either *E. coli* or *A. actinomycetemcomitans* (Fig. 1A,B). The 18-h period of lipopolysaccharide stimulation was therefore chosen to enable the study of lipopolysaccharide on RANKL gene expression in periodontal ligament cells. Semiquantitative RT-PCR indicated that the inhibition of p38 MAPK with SB203580 before stimulation with lipopolysaccharide from *E. coli* or *A. actinomycetemcomitans* resulted in a significant decrease in the expression of RANKL mRNA (Fig. 2A). Interestingly, osteoprotegerin mRNA expression was not consistently affected by

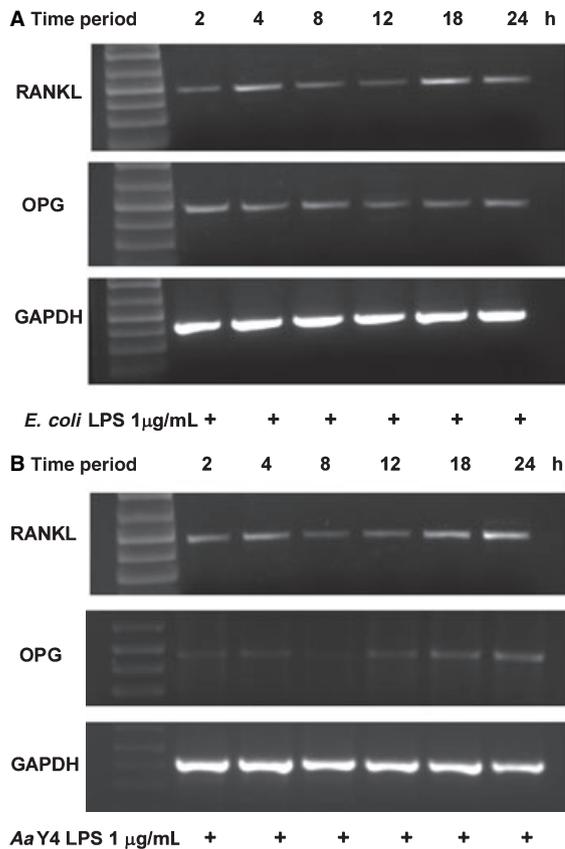


Fig. 1. Time course of receptor activator of nuclear factor- κ B ligand (RANKL) mRNA expression induced by *Escherichia coli* and *Aggregatibacter actinomycetemcomitans* lipopolysaccharide in periodontal ligament cells. Reverse transcription-polymerase chain reaction shows that RANKL mRNA expression induced by lipopolysaccharide is biphasic, reaching an early peak after 4 h of stimulation and reached the maximum after 18–24 h (A,B). *E. coli* lipopolysaccharide was a more potent inducer of osteoprotegerin expression (A), which was already noticeable after 2 h of stimulation and remained essentially constant throughout the 24-h period. *A. actinomycetemcomitans* Y4 lipopolysaccharide increased osteoprotegerin mRNA modestly, and this effect was delayed, because it took 18 h of stimulation to be more evident. Stimulation for periods longer than 24 h did not induce further increases of either RANKL or osteoprotegerin mRNA expression (data not shown). Also, we did not observe noticeable levels of RANKL mRNA expression by periodontal ligament cells in the absence of any stimulation (data not shown). Representative images of three independent experiments. *Aa*, *Aggregatibacter actinomycetemcomitans*; *E. coli*, *Escherichia coli*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; OPG, osteoprotegerin.

lipopolysaccharide stimulation and also not significantly changed by inhibiting p38 MAPK (Fig. 2B). Nevertheless, the decrease of RANKL expression achieved with SB203580 was of sufficient magnitude to reduce the RANKL:osteoprotegerin ratio (Fig. 2C). As it is this ratio that will ultimately indicate the net effects on osteoclast differentiation and activation, these results indicate that blocking p38 MAPK might impair osteoclastogenesis induced by lipo-

polysaccharide-stimulated periodontal ligament cells. However, in spite of being significant, the lipopolysaccharide-induced stimulation on RANKL expression by periodontal ligament cells was somewhat modest, represented by a 40% increase over the level observed with unstimulated cells. On the other hand, the decrease of RANKL mRNA expression after blocking p38 MAPK activity with SB203580 was clear when *E. coli* lipopolysaccharide or *A. actinomycetem-*

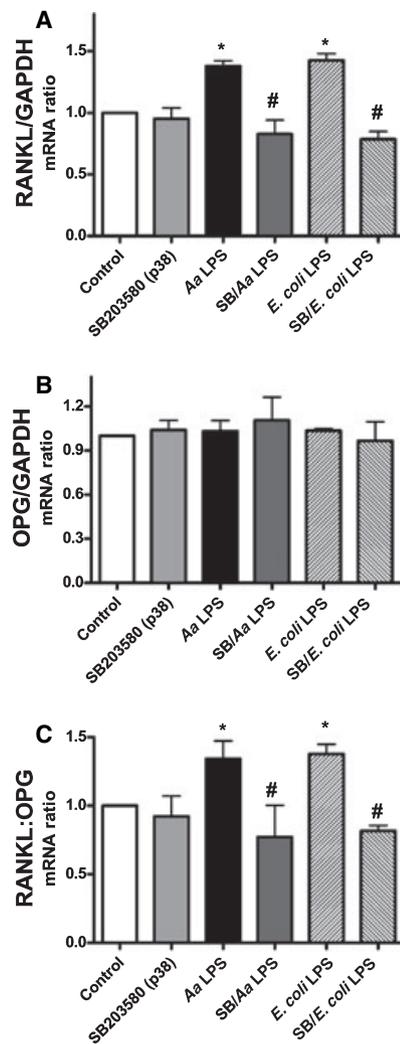
comitans Y4 lipopolysaccharide was used for stimulation.

RANKL protein expression induced by lipopolysaccharide also requires p38 MAPK activity

Expression of RANKL at the protein level after stimulation with lipopolysaccharide from either *E. coli* or *A. actinomycetemcomitans* confirmed the importance of p38 MAPK, as blocking this pathway with the biochemical inhibitor SB203580 resulted in a significant decrease of protein expression (Fig. 3A,B). Also, protein expression is probably indirect as we could not find detectable levels of RANKL after 24 or 48 h of stimulation (data not shown). We were also unable to detect RANKL protein on concentrated cell culture supernatants.

Lipopolysaccharide-stimulated periodontal ligament cells support osteoclastogenesis by membrane-bound RANKL expression

Morphological changes (size and multinucleation) and expression of TRAP are indicative of terminal osteoclast differentiation. The RAW 264.7 cell line is capable of differentiation into osteoclasts in the presence of exogenous RANKL (Figs 4A and 5) (32). The osteoclastogenesis assay indicates the functionality of RANKL produced by lipopolysaccharide-stimulated periodontal ligament cells (Fig. 5). The results mimicked the findings for RANKL mRNA expression, as a significant ($p < 0.05$) increase in the number of TRAP⁺ multinucleated cells was observed after coculture with periodontal ligament cells stimulated with lipopolysaccharide (Fig. 4B). A reduction in the number of osteoclasts occurred when osteoprotegerin (100 ng/mL) was added to the cocultures, indicating that osteoclast differentiation was associated with RANKL expression by periodontal ligament cells. This decrease was significant ($p < 0.05$) for *E. coli* lipopolysaccharide stimulation, almost reaching statistical significance ($p = 0.056$) after *A. actino-*



mycetemcomitans Y4 lipopolysaccharide stimulation. As conditioned medium from lipopolysaccharide-stimulated periodontal ligament cells had no effect on osteoclast formation (data not shown), it is concluded that residual lipopolysaccharide used for stimulating the periodontal ligament cells was not a confounding factor and that lipopolysaccharide-stimulated cells did not produce soluble RANKL.

Inhibition of MKK3 and MKK6 decrease osteoclastogenesis induced by lipopolysaccharide-stimulated periodontal ligament cells

Coculture experiments using stable cell lines over-expressing dominant-negative forms of upstream activators of p38 MAPK, MKK3 (MKK3 dominant negative-periodontal ligament) and

Fig. 2. p38 mitogen-activated protein kinase (MAPK) regulates preferentially receptor activator of nuclear factor- κ B ligand (RANKL) in lipopolysaccharide-stimulated periodontal ligament cells. Murine periodontal ligament cells were grown to near confluency and de-induced in medium containing 0.3% fetal bovine serum for 8 h. The specific inhibitor for p38 MAPK (SB203580) was added to the culture medium at 10 μ M 30 min before the 18-h stimulation with 1 μ g/mL of lipopolysaccharide from *Escherichia coli* and *Aggregatibacter actinomycetemcomitans* lipopolysaccharide. Total RNA was harvested and reverse transcription-polymerase chain reaction was performed and quantified using the GelDoc System. The results indicate that the inhibition of p38 MAPK reduced lipopolysaccharide-induced RANKL mRNA expression, especially after *E. coli* lipopolysaccharide stimulation (A). No significant regulation of osteoprotegerin mRNA expression was observed following p38 inhibition (B). The decrease of RANKL expression was sufficient to reduce the RANKL:osteoprotegerin ratio (C). The figures are representative of three independent experiments and bar graphs indicate mean \pm standard deviation of normalized fold changes of normalized gene expression. *Indicates a significant difference ($p < 0.05$) on mRNA expression in comparison to unstimulated cells. #Indicates a significant difference ($p < 0.05$) on mRNA expression in comparison to lipopolysaccharide-treated cells. Aa, *Aggregatibacter actinomycetemcomitans*; E. coli, *Escherichia coli*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; OPG, osteoprotegerin; SB, SB203580.

MKK6 (MKK6 dominant negative -periodontal ligament), resulted in a significant ($p < 0.05$) decrease in the number of TRAP⁺ cells in comparison to a nontransformed murine periodontal ligament cell line (Fig. 4C). This indicates that upstream activators of p38 MAPK are involved in lipopolysaccharide-induced RANKL expression by periodontal ligament cells. Furthermore, MKK3 dominant negative-periodontal ligament and MKK6 dominant negative-periodontal ligament had the same magnitude of effect on the inhibition of osteoclast differentiation, suggesting that both kinases are important for lipopolysaccharide sign-

aling through the p38 MAPK pathway. This finding might also suggest that the α isoform of p38, which is activated only by MKK6, does not play a relevant role in lipopolysaccharide-induced RANKL expression.

Discussion

In periodontal disease pathogenesis, microbial virulence factors or microbial-associated molecular patterns induce an immune/inflammatory response that will result in bone resorption (33,34). Understanding the molecular mechanisms involved in coupled bone turnover can have a profound impact on the development of therapeutic approaches aiming at the control of bone resorption associated with infections. Periodontal ligament cells have been shown to express RANKL in response to interleukin-1 α , interleukin-1 β , *A. actinomycetemcomitans* lipopolysaccharide, vitamin D3, dexamethasone, prostaglandin E₂ and mechanical stress (10,15,16,35,36). These findings illustrate the role of periodontal ligament cells in alveolar bone resorption; their relevance in bone formation during periodontal regenerative therapies is also well established (37–40).

Osteoclast differentiation was shown to be related to the activation of various intracellular signaling pathways, including c-Jun N-terminal kinase, p38 and ERK MAPKs, as well as phosphoinositol-3-kinase and protein kinase A pathways (41–43). However, the role of different signaling pathways on gene expression may differ according to the nature of extracellular stimulation, cell type and even cell differentiation state (44). Supporting this concept, bacterial-induced RANKL expression by osteoblasts was found not to require p38, ERK MAPKs or the phosphoinositol-3-kinase pathway (29), whereas p38 MAPK was recently shown to regulate RANKL mRNA and protein expression by osteoblasts stimulated by adiponectin (45). In this study we showed that p38 MAPK and its upstream activators MKK3 and MKK6 are required for lipopolysaccharide-induced RANKL expression by periodontal ligament cells. More-

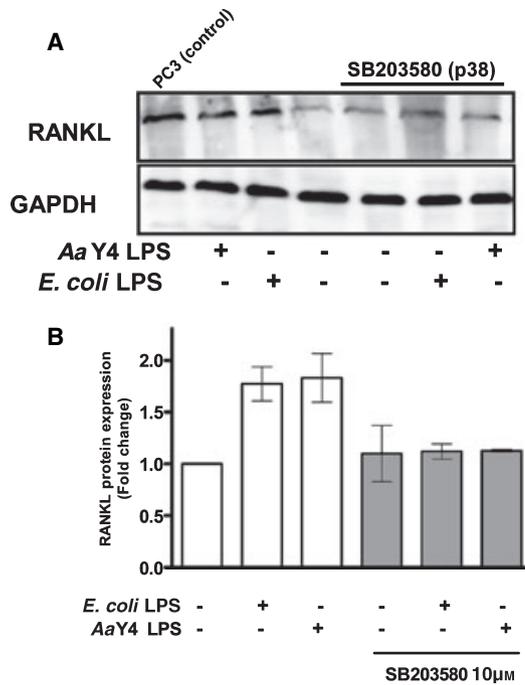


Fig. 3. Receptor activator of nuclear factor- κ B ligand (RANKL) protein expression by lipopolysaccharide-stimulated periodontal ligament cells is dependent on p38 mitogen-activated protein kinase (MAPK) activity. Periodontal ligament cells grown on six-well plates were de-induced for 12 h in culture medium containing 0.3% fetal bovine serum and were then stimulated with lipopolysaccharide from either *Escherichia coli* or *Aggregatibacter actinomycetemcomitans* for 72 h. The specific inhibitor for p38 MAPK (SB203580; 10 μ M) was added to the culture medium 30 min before stimulation with lipopolysaccharide (1 μ g/mL). (A) Western blot analysis of RANKL expression from periodontal ligament whole-cell lysates. The positive control for RANKL expression is cell lysate from the human prostate cancer cell line (PC-3). (B) Density analysis of data from three independent experiments showed significant inhibition of lipopolysaccharide-induced RANKL protein by SB203580 (* $p < 0.05$). Aa, *Aggregatibacter actinomycetemcomitans*; E. coli, *Escherichia coli*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide.

over, the results of the osteoclastogenesis assays support a role for the membrane-bound form of RANKL expressed by these cells in response to lipopolysaccharide stimulation.

We have previously shown that lipopolysaccharide induces activation of p38 MAPK in periodontal ligament cells (3), and in this study we demonstrated that inhibiting the p38 MAPK pathway not only decreases lipopolysaccharide-induced RANKL expression but inhibits periodontal ligament cell-induced osteoclastogenesis. It is suggested that the decrease of RANKL expression caused by inhibiting p38, and the subsequent shift on the RANKL:osteoprotegerin ratio, affects the osteoclastogenesis process. These results also agree with our previous data regarding RANKL expression and induction of osteoclastogenesis by

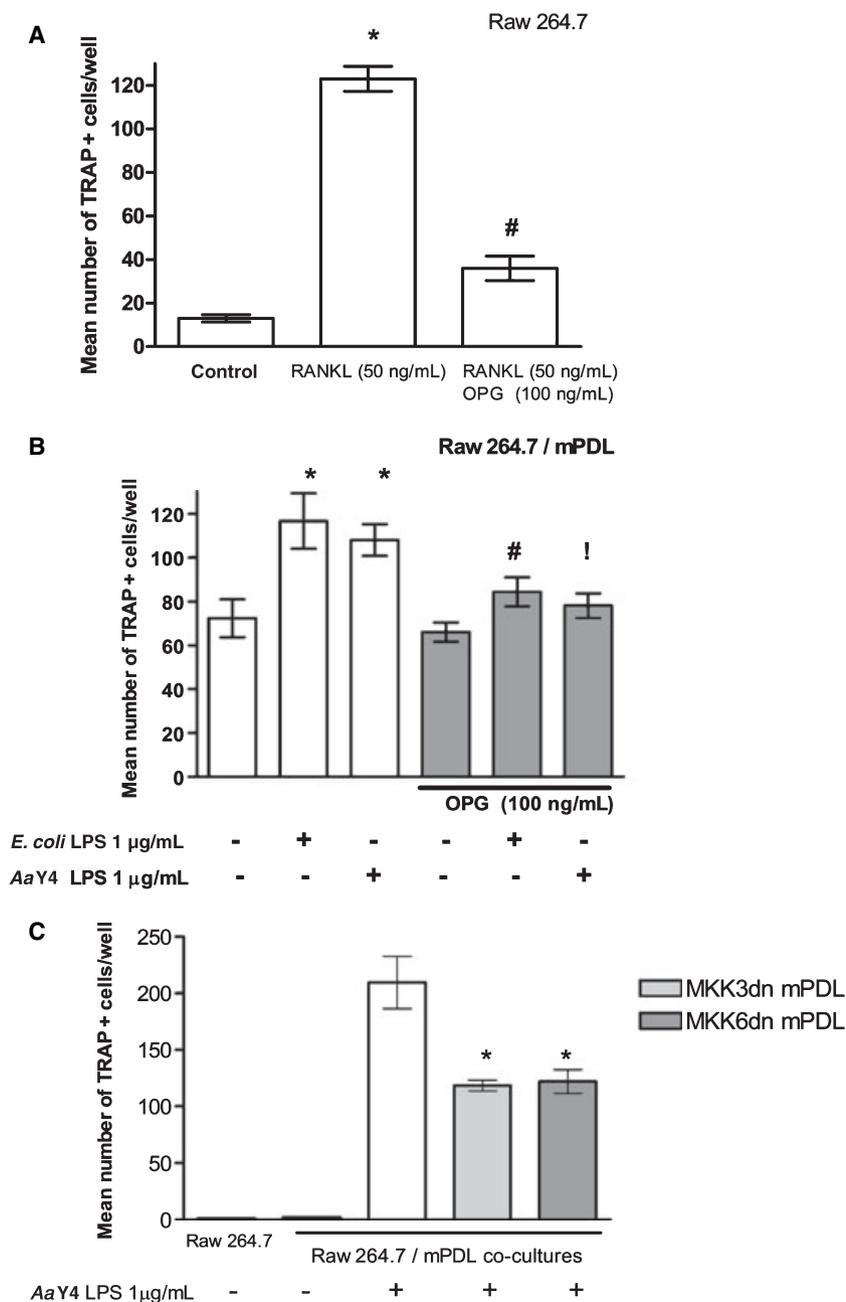
bone marrow stromal cells stimulated with interleukin-1 α and tumor necrosis factor- α (3). Importantly, we have also shown that the physiological cues of coupled bone, namely vitamin D₃ and parathyroid hormone, induced RANKL expression and were not affected by p38 inhibition. Other investigators have reported decreased osteoclastogenesis induced by RANKL-stimulated bone marrow-derived cells with p38 inhibitors (46,47), and recently all three MAPKs were shown to be involved in interleukin-1 α -induced RANKL expression by periodontal ligament fibroblasts (28).

As we evaluated only p38 MAPK, we cannot rule out a significant role for other signaling pathways in lipopolysaccharide-induced RANKL expression by periodontal ligament cells. Nevertheless, further confirmation for

the role of the p38 MAPK pathway in osteoclastogenesis supported by lipopolysaccharide-stimulated periodontal ligament cells was provided in this study by the use of genetic constructs for over-expression of dominant-negative forms of MKK3 and MKK6, upstream activators of p38 MAPK. Interestingly, MKK3 and MKK6 were recently shown to have differential effects on osteoclast differentiation induced by RANKL in bone marrow stromal cells. Even though both MKK3 and MKK6 were important for p38 MAPK activation after RANKL stimulation, only MKK6 played a role in osteoclast differentiation induced by RANKL in bone marrow cells (48). Our finding of a similar role for MKK3 and MKK6 on osteoclast differentiation supported by lipopolysaccharide-stimulated periodontal ligament cells probably reflects the role of both kinases on RANKL expression by these cells, whereas RANKL signaling in osteoclast precursor cells may be affected primarily by MKK6.

Collectively, this information suggests that p38 signaling is important not only for RANKL gene expression but also for RANKL signaling. Regulation of osteoprotegerin expression seems to be more complex, as we did not observe consistent changes associated with either *E. coli* or *A. actinomycetemcomitans* Y4 lipopolysaccharide stimulation. This agrees with the findings of Tiranathanagul *et al.* (36), who used human primary periodontal ligament fibroblasts and a noncommercial preparation of lipopolysaccharide from *A. actinomycetemcomitans*. Moreover, Okahashi *et al.* (29) also did not report regulation of osteoprotegerin mRNA expression by osteoblasts after infection with *P. gingivalis*. On the other hand, Lossdorfer *et al.* (11) have shown increased osteoprotegerin mRNA expression by periodontal ligament cells after stimulation with parathyroid hormone, whereas RANKL mRNA expression was not detected.

The results of the osteoclastogenesis assay indicate that the increase of RANKL expression induced by lipopolysaccharide was of sufficient



magnitude to alter the RANKL:osteoprotegerin ratio, shifting the bone turnover towards resorption. This is in spite of no significant changes in osteoprotegerin mRNA expression induced by lipopolysaccharide in these cells. However, the increase of osteoclast numbers induced by lipopolysaccharide-stimulated cells was somewhat small (40 and 20% for *E. coli* and *A. actinomycetemcomitans* Y4 lipopolysaccharide, respectively), paralleling our findings for regulation of RANKL expression at the mRNA and protein levels. Yet, these results indi-

cate that periodontal ligament cells can have relevant consequences on bone turnover upon lipopolysaccharide stimulation. Wada *et al.* (8) have shown that unstimulated periodontal ligament cells inhibit osteoclastogenesis and attributed this effect to production of osteoprotegerin. In this study we show that the osteoclastogenesis induced by lipopolysaccharide-stimulated periodontal ligament cells can be attributed to membrane-bound RANKL expression, as osteoprotegerin blocked this effect completely and conditioned medium from lipopolysaccharide-sti-

Fig. 4. Lipopolysaccharide-stimulated periodontal ligament cells stimulate osteoclastogenesis, which is regulated by the p38 mitogen-activated protein kinase (MAPK) pathway. (A) Stimulation with receptor activator of nuclear factor- κ B ligand (RANKL) induces RAW 264.7 cells to differentiate into multinucleated tartrate-resistant acid phosphate (TRAP)⁺ cells, whereas pretreatment with osteoprotegerin inhibits this effect. *Indicates a significant ($p < 0.05$) difference from unstimulated cells and #indicates a significant decrease in the number of osteoclasts with osteoprotegerin treatment. (B) Murine periodontal ligament cells were stimulated with lipopolysaccharide from *Escherichia coli* and *Aggregatibacter actinomycetemcomitans* Y4 and cultured for 3 d. These cells were then cocultured with RAW 264.7 cells for an additional 6 d and the number of multinucleated TRAP⁺ cells was counted. Stimulation with lipopolysaccharide increased significantly the number of TRAP⁺ cells and this effect was inhibited by osteoprotegerin *Indicates a significant ($p < 0.05$) difference from unstimulated cells and #indicates a significant decrease on the number of osteoclasts with osteoprotegerin treatment. !Indicates $p = 0.056$ for the significance of the decrease in the number of osteoclasts with osteoprotegerin treatment in *A. actinomycetemcomitans* Y4 lipopolysaccharide-stimulated cells. (C) Coculture of RAW cells with stable periodontal ligament cell lines over-expressing dominant-negative mutants of MKK3 and of MKK6, upstream activators of p38 MAPK, significantly decreased the number of TRAP⁺ cells. *Indicates a significant ($p < 0.05$) difference from nontransfected periodontal ligament cells. Bar graphs indicate mean \pm standard deviation of the number of TRAP⁺ multinucleated cells counted in each well. *Aa*, *Aggregatibacter actinomycetemcomitans*; *E. coli*, *Escherichia coli*; LPS, lipopolysaccharide; MKK3dn mPDL, murine periodontal ligament cell line over-expressing dominant-negative mutants of MKK3; MKK6dn mPDL, murine periodontal ligament cell line over-expressing dominant-negative mutants of MKK6; OPG, osteoprotegerin.

mulated cells did not induce osteoclastogenesis. This requirement of direct cell-to-cell contact between periodontal ligament and osteoclast precursor cells was first reported by

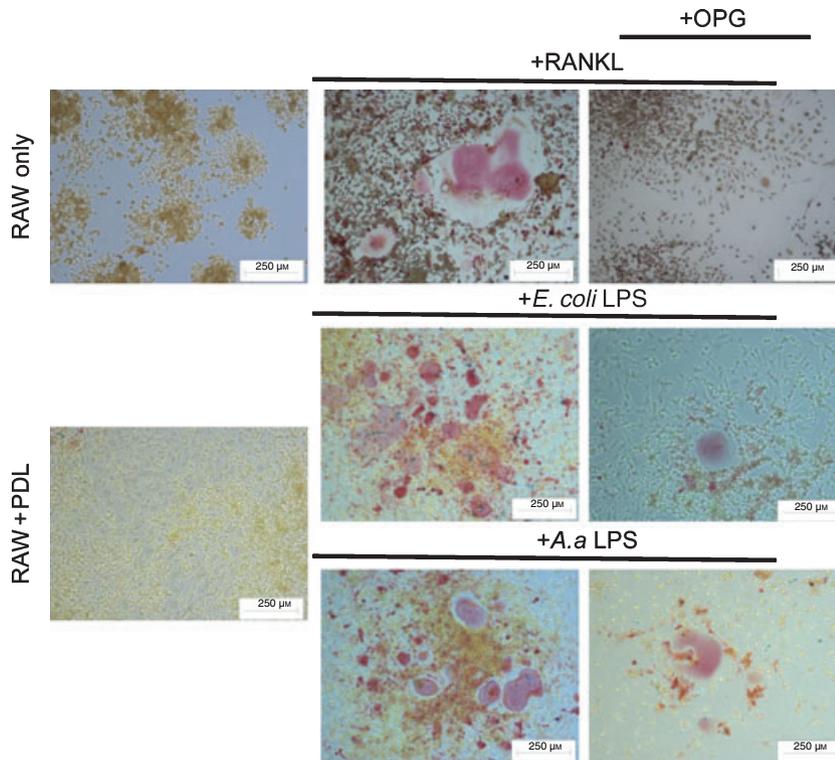


Fig. 5. RAW 264.7 differentiated into tartrate-resistant acid phosphate (TRAP)⁺ multinucleated osteoclast-like cells in response to exogenous receptor activator of nuclear factor- κ B ligand (RANKL) or coculture with lipopolysaccharide-stimulated periodontal ligament cells. The top panel depicts RAW 264.7 cells only (5×10^4) cultured for 6 d without or with RANKL (50 ng/mL) and RANKL/osteoprotegerin (100 ng/mL). The middle panel shows the results of coculture experiments where vehicle control or lipopolysaccharide (1 μ g/mL)-treated murine periodontal ligament cells were stimulated for 72 h, then 5×10^4 cells were added to each well containing 5×10^4 RAW 264.7 cells. Cocultures were maintained for 6 d, both in the presence and absence of osteoprotegerin (100 ng/mL) (right panels). All images are representative of three independent experiments. *A.a.*, *Aggregatibacter actinomycetemcomitans*; *E. coli*, *Escherichia coli*; LPS, lipopolysaccharide; OPG, osteoprotegerin; PDL, periodontal ligament.

Kanzaki *et al.* (14); however, they did not use any extracellular stimulation to induce RANKL expression. There are other important methodological differences in the osteoclastogenesis model used that may account for differences between our results and those of others (17), including the nature of stimulation used to induce RANKL (mechanical compression vs. lipopolysaccharide), the osteoclast precursor cells used (peripheral blood monocytes vs. monocytic/macrophage cell line) and the time of coculture (3 wk vs. 5–6 d). In our model, by scraping off the periodontal ligament cells after stimulation with lipopolysaccharide we were able to adjust the number of cells plated onto the precursor cells to avoid influences on cell proliferation that the

lipopolysaccharide stimulation might have had on the periodontal ligament cells.

Our findings on the osteoclastogenesis assay are contrary to a report in which lipopolysaccharide-stimulated periodontal ligament cells did not induce osteoclast formation in a coculture with precursor cells (49). This might be because of the different experimental models used. We used a continuous lineage of mouse periodontal ligament cells, whereas Wada *et al.* (49) used primary human periodontal ligament cells. In the primary cells, osteoprotegerin expression was shown to be more affected than RANKL after lipopolysaccharide stimulation. Moreover, significant variability was shown at the level of basal

and stimulated RANKL expression among the primary periodontal ligament cells, which might be responsible for the striking differences between the results of Wada *et al.* (49) and those of Tiranathanagul *et al.* (36), who reported increased RANKL expression induced by *A. actinomycetemcomitans* lipopolysaccharide in primary periodontal ligament fibroblasts, whereas osteoprotegerin expression was not affected. Also note that we experienced some variability in osteoclastogenesis assays where periodontal ligament cells cultured with RAW macrophages resulted in osteoclast formation on occasion. These data may be a result of the passage number with the RAW cells, rather than the periodontal ligament cell line. Despite this experimental baseline difference, significant stimulation of TRAP-positive multinucleated cells with lipopolysaccharide was observed. Interestingly, the periodontal ligament cells and gingival fibroblasts seem to have contrasting responses to lipopolysaccharide regarding osteoclastogenesis, as Nagasawa *et al.* (50) have shown increased expression of osteoprotegerin (and not RANKL) mRNA. Cell culture supernatants of lipopolysaccharide-stimulated gingival fibroblasts also inhibited differentiation of precursor hematopoietic cells in response to RANKL stimulation. It is important to note that the biochemical composition of purified lipopolysaccharide may influence the results and explain some of the conflicting results in the literature. Even though we evaluated our *A. actinomycetemcomitans* Y4 lipopolysaccharide preparation to ensure that it was not contaminated by nucleic acid or protein, the lower limits of detection of the procedures cannot absolutely rule out minor protein contamination.

Recent *in vivo* data from our laboratory group support the role of p38 signaling which is required for periodontal bone loss in an experimental rat model (3). In this model, *A. actinomycetemcomitans* lipopolysaccharide-induced alveolar bone loss was blocked by an orally active p38 inhibitor. Reduced numbers of osteoclasts were found adjacent to the areas of active

bone resorption, including the periodontal ligament area. Other recent studies have suggested that T- and B-cell-derived RANKL are the predominant sources of RANKL in human periodontal diseased tissue compared with healthy sites (22). However, this study utilized only biopsied soft tissue, which did not include the periodontal ligament or associated bony tissue from diseased sites. Recently, Taubman *et al.* (20) discussed the relevance of immune cells, specifically B and T cells, to bone resorption in periodontal disease. Although the role of B and T cells in bone resorption is compelling, it is not possible to rule out the role of resident cells, such as endothelial cells of periodontal ligament fibroblasts, in bone resorption, because they are also capable of expressing functional RANKL. In the complexity of the *in vivo* host response to the antigens from the subgingival biofilm, it is more than likely that cytokines, chemokines and growth factors produced by resident cells will play a role in the network of events that ultimately can modulate the activity of immune cells. As evidence supporting this assumption, *A. actinomycetemcomitans* infection was shown to induce bone loss in severe combined immunodeficiency mice. Even though this bone loss was approximately half of that observed in severe combined immunodeficiency mice transplanted with T cells from humans with aggressive periodontitis, it was significantly greater than found in sham-infected control severe combined immunodeficiency mice (19). Thus, the periodontal ligament may still play a prominent source of local RANKL production within the periodontal microenvironment, especially when one considers the dynamics of the homeostasis of the periodontal ligament and the role of the cells in this tissue to regulate alveolar bone turnover. These findings suggest that p38 signaling plays a major role in controlling cytokine expression in the periodontal ligament, which can contribute towards inflammatory bone loss associated with periodontal diseases.

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