

MKK3/6-p38 MAPK Signaling Is Required for IL-1 β and TNF- α -Induced RANKL Expression in Bone Marrow Stromal Cells

CARLOS ROSSA, JR.,^{1,*} KATHRYN EHMANN,^{2,*} MIN LIU,³
CHETAN PATIL,^{2,3} and KEITH L. KIRKWOOD³

ABSTRACT

Coupled bone turnover is directed by the expression of receptor-activated NF- κ B ligand (RANKL) and its decoy receptor, osteoprotegerin (OPG). Proinflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) induce RANKL expression in bone marrow stromal cells. Here, we report that IL-1 β and TNF- α -induced RANKL requires p38 mitogen-activating protein kinase (MAPK) pathway activation for maximal expression. Real-time PCR was used to assess the p38 contribution toward IL-1 β and TNF- α -induced RANKL mRNA expression. Steady-state RANKL RNA levels were increased approximately 17-fold by IL-1 β treatment and subsequently reduced ~70%–90% when p38 MAPK was inhibited with SB203580. RANKL mRNA stability data indicated that p38 MAPK did not alter the rate of mRNA decay in IL-1 β -induced cells. Using a RANKL-luciferase cell line receptor containing a 120-kB segment of the 5' flanking region of the RANKL gene, reporter expression was stimulated 4–5-fold by IL-1 β or TNF- α treatment. IL-1 β -induced RANKL reporter expression was completely blocked with specific p38 inhibitors as well as dominant negative mutant constructs of MAPK kinase-3 and -6. In addition, blocking p38 signaling in bone marrow stromal cells partially inhibited IL-1 β and TNF- α -induced osteoclastogenesis *in vitro*. Results from these studies indicate that p38 MAPK is a major signaling pathway involved in IL-1 β and TNF- α -induced RANKL expression in bone marrow stromal cells.

INTRODUCTION

EXCESSIVE OSTEOCLAST-MEDIATED bone resorption is a common feature of chronic inflammatory processes, for example, periodontitis, rheumatoid arthritis, and failing joint prostheses. Several lines of evidence indicate that cytokine networking and crosstalk between stromal/osteoblastic cells and monocyte/osteoclast progenitor cells dictate cellular responses involved in bone remodeling. Several mediators of bone resorption and remodeling have been identified in response to such proinflammatory cytokines, as interleukin-1 (IL-1), bacterial lipopolysaccharide (LPS), and other bone-resorptive agents. Bone marrow stromal cells respond to these agents and directly or indirectly secrete various cytokines that activate receptor activator of NF- κ B ligand (RANKL), resulting in enhanced osteoclastogenesis.^{1,2}

Bone marrow stromal cell-derived RANKL is essential for osteoclastic differentiation of monocyte precursor cells into mature multinucleated osteoclasts in the presence of macrophage colony-stimulating factor (M-CSF).^{3,4} RANKL is highly expressed on the surface of bone marrow stromal cells and pre-osteoblasts in the areas of excessive osteolysis and trabecular bone remodeling.⁵ RANKL expression is upregulated in osteoblasts or stromal cells by IL-1 β , IL-6 (in the presence of soluble IL-6 receptor), tumor necrosis factor- α (TNF- α), prostaglandin E₂ (PGE₂), parathyroid hormone (PTH), 1,25-dihydroxyvitamin D₃, and others.^{1,6–8} Through interactions with its cognate receptor (RANK) on osteoclast progenitor cells, RANKL induces differentiation and maturation of osteoclasts. Balancing this system is the decoy receptor, osteoprotegerin (OPG), a soluble member of the tumor necrosis family capable of binding to RANKL, thus decreasing RANKL functional ac-

¹Department of Diagnosis and Surgery, State University of Sao Paulo (UNESP), Araraquara, SP, Brazil.

²Department of Oral Biology, State University of New York at Buffalo, Buffalo, NY 14214.

³Department of Periodontics and Oral Medicine, University of Michigan, Ann Arbor, MI 48109.

*These authors contributed equally to this work.

tivity.⁹ It is the ratio of RANKL/OPG that eventually determines bone turnover.¹⁰

Proinflammatory cytokines stimulate multiple intracellular signaling pathways, including the p38 mitogen-activating protein kinase (MAPK) pathways, NF- κ B pathway, extracellular signal-regulated kinase (ERK) pathway, and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway (SAPK/JNK).^{11,12} Although much attention has been focused on the NF- κ B pathway of cytokine gene activation, more recent study has addressed JNK and p38 MAPK during inflammation and environmental stress-induced signaling.¹³ The p38 kinases constitute a distinct MAPK subfamily that plays a role in adaptation, homeostasis, and stress responses.¹⁴ Of the identified four splice variants, p38 α MAPK is the best characterized and perhaps the most relevant kinase involved in inflammatory responses.^{15,16} The essential role of p38 α MAPK in RANKL-induced osteoclastogenesis has been elucidated in macrophage/osteoclast precursor cells,¹⁷ although the role of p38 regulation in stromal/osteoblastic-derived RANKL expression has been addressed only recently.^{7,18,19} In this study, the role of p38 signaling was investigated during cytokine-induced RANKL expression in bone marrow stromal cells. Our results indicate that MKK3/6-p38 signaling is necessary for IL-1 β -induced RANKL expression involving a transcriptional mechanism that does not require the proximal RANKL promoter relative to the transcriptional start site.

MATERIALS AND METHODS

Tissue culture of murine ST-2, UAMS-32P cells

ST-2 bone marrow stromal cells were obtained from Riken (Wako, Japan). UAMS-32P bone marrow stromal cells were obtained from Dr. Charles O'Brien (University of Arkansas). UAMS-32P cell lines express RANKL in response to 1,25(OH)₂D₃ and constitutively express M-CSF.²⁰ Cells were cultured in α -MEM medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO). Cells were routinely serum starved in 0.3% FBS-containing medium for 6–8 h prior to cytokine stimulation.

Real-time PCR analysis

Total RNA (5 μ g) from ST-2 and UAMS-32P cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and used for cDNA synthesis with oligo(dT) 12–18 primers and Superscript II (RNase H) (Invitrogen) in reverse transcription (RT) reactions. Real-time PCR was performed with 2 μ L of the RT preparation using Taq polymerase (Invitrogen) and primers specific for mouse RANKL and GAPDH cDNAs. RANKL and GAPDH were amplified for 50 cycles using the BioRad's SYBR Green Supermix kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Real-time PCR primer sequences were as follows: RANKL, forward: 5'-agcaacg-gaaaactaagggt-3', reverse: 5'-cccccaacattatggaata-3'; GAPDH, forward: 5'-caagaccagagtccttcaga-3', and reverse: 5'-gatg-gtcttggtccttagcc-3'. Threshold values were assigned by the iCycler program (Bio-Rad) and used with amplification efficiencies to calculate RANKL expression. Q-gene (www.biotechniques.com/softlib/qgene.html) quantitative software

was used to analyze gene expression based on cycle threshold values normalized to GAPDH expression. Real-time PCR products were also verified through electrophoresis on 2% agarose gel and visualized using ethidium bromide staining to verify authenticity.

mRNA stability experiments

To determine the relative role of p38 MAPK on IL-1 β -induced RANKL mRNA stability, the rate of RANKL mRNA decay was determined in the presence and absence of the p38 inhibitor, SB203580. Actinomycin D (ActD) was used in the experiments to arrest transcription as described previously.²¹ Briefly, ST-2 and UAMS-32P cells were pretreated with SB203580 (2 μ M) for 45 min, then treated with IL-1 β for 16–18 h. Subsequently, ActD (2 μ M) was added to prevent further transcription. Total RNA was harvested and analyzed by real-time RT-PCR as described 0–8 h post-ActD addition.

RT-PCR analysis

Total RNA (5 μ g) from ST-2 and UAMS-32P cells was isolated using Trizol reagent and used for cDNA synthesis with oligo(dT) 12–18 primers and Superscript II (RNase H) in RT reactions. RT product (2 μ L) was used as a template for PCR amplification of RANKL and GAPDH gene products. Primers sequences used were: RANKL [AF_019048, forward: 5'-cagcactcactgctttatagatcc-3', reverse: 5'-agctgaagatagctgtag-gtacgc-3'; GAPDH [NM_002046], forward: 5'-caccatggagaag-gccgggg 3', reverse: 5'-gacggacacattgggtag-3'. RT-PCR products were separated and analyzed by gel electrophoresis. Resulting images were captured using the Gel-Doc (Bio-Rad) imaging system equipped with UV light and a gel scanner. PCR results were quantitated using Bio-Rad's PhosphorImager system and Quantity One software to assess relative differences.

Immunoblot analysis

UAMS-32P cells were exposed to SB203580 (5 μ M) for ~30 min, then stimulated with IL-1 β (5 ng/mL) for 72 h. Cells were rinsed with ice-cold phosphate-buffered saline (PBS), then lysed in SDS-PAGE buffer (Bio-Rad). Protein concentrations were measured by Bradford's method (Bio-Rad). Each sample (10 μ g) was electrophoresed on 10% denatured SDS-PAGE gels and electrotransferred to nitrocellulose membranes (Bio-Rad). For detection of membrane-bound RANKL expression, whole cell lysates were probed with 0.2 μ g/mL rabbit antimouse RANKL IgG (R&D Systems, Minneapolis, MN) and detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and LumniGlo (Cell Signaling Technologies, Beverly, MA) chemiluminescence detection. Anti-GAPDH antibody (Chemicon, Temecula, CA) was used to verify even protein loading transferred to membranes.

RANKL-luciferase reporter cell line

RANKL-luc cells (derived from UAMS-32P cells) were generated through stable transfection of a bacterial artificial chromosome (BAC) clone containing the entire exonic/intronic sequence of the murine RANKL gene along with the 5' flanking region (–120 kb). The 3'-UTR of the RANKL gene was removed in the BAC clone and replaced with a lu-

ciferase reporter (gift from C. O'Brien, University of Arkansas²²). The dual luciferase assay kit (Promega, Madison, WI) was used to measure RANKL-luc activity in total protein normalized samples (LMaxII, Molecular Devices, Menlo Park, CA). Luciferase activity was measured in response to constructs harboring upstream constitutively active

genes encoding MKK6b and MKK3b²³ (a gift from Bhat, University of South Carolina). In other studies, MKK6 and MKK3 dominate negative (dn) constructs (a gift from J. Han, Scripps Institute) were transfected, and IL-1 β or TNF- α was added 24 h posttransfection to stimulate RANKL-luc cells.

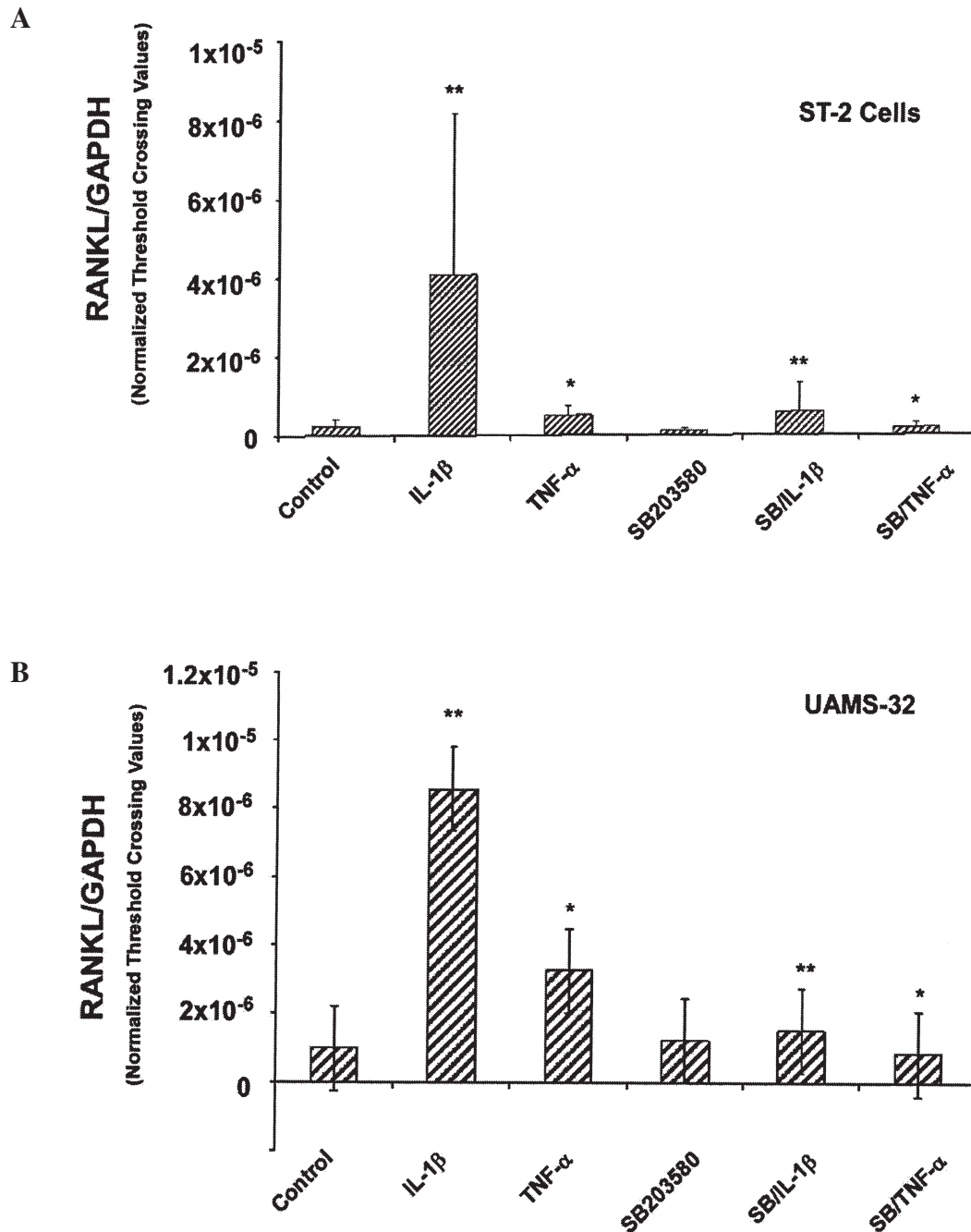


FIG. 1. IL-1 β and TNF- α -induced RANKL expression in different bone marrow stromal cells is regulated by p38 MAPK. (A) ST-2 and (B) UAMS-32P cells were treated with 10 μ M SB203580 (SB) and stimulated with 1 ng/mL IL-1 β or 5 ng/mL TNF- α for 18 h. Real-time PCR with cytokine-induced RANKL steady-state mRNA expression. Values expressed represent threshold crossing values normalized to GAPDH ($n = 4$ for each). IL-1 β and TNF- α stimulated RANKL expression: ** $p < 0.0001$ vs. control and * $p < 0.05$ vs. control, respectively. SB203580 decreased RANKL expression: ** $p < 0.0001$ for IL-1 β vs. SB/IL-1 β in ST-2 cells and UAMS-32P cells: * $p < 0.05$.

Coculture assays

UAMS-32P bone marrow stromal cells (1×10^4) were cultured in 6-well dishes for 24 h, deinduced for 6 h in 0.3% serum containing medium, and stimulated for 72 h as described for immunoblot analysis. Following the 72-h incubation period, stromal cells were washed extensively with PBS, and RAW264.7 macrophages were added at 2×10^4 cells/well and cocultured with adherent stromal cells in α -MEM containing 10% serum for 6 days, with the medium replaced on day 3. For control cultures, RAW264.7 cells only were cultured with or without RANKL (50 ng/mL). Following coculture, cells were cytochemically stained for tartrate-resistant acid phosphatase (TRAP), using a commercially available kit (Sigma). TRAP-positive multinucleated cells containing more than three nuclei were identified as osteoclasts and counted by light microscopy. All experiments were carried out three times in triplicate measurements. Images of cultured cells were digitally captured using a Nikon TS100 inverted scope and Nikon 5.1 megapixel camera (Nikon, Tokyo, Japan).

Statistical analysis

Pairwise comparisons between experimental groups were performed using the Student's *t*-test with Welch's correction for unequal variances or one-way ANOVA analysis where indicated. Significance level was set at 5% unless otherwise noted. All calculations were performed using Prism 4 software (GraphPad, Inc., San Diego, CA).

RESULTS

SB203580 inhibits IL-1 β and TNF α -induced RANKL mRNA steady-state levels in bone marrow stromal cells

Real-time PCR was used to quantitate stromal cell-derived RANKL expression. By comparison of normalized threshold

crossing values (Fig. 1), results indicate that steady-state RNA levels were increased approximately 17-fold by IL-1 β treatment ($p < 0.0001$) and subsequently reduced 70%–90% by pretreatment with SB203580 (Fig. 1) ($p < 0.0001$ in ST-2 cells and $p < 0.05$ in UAMS-32P cells). Inhibition of TNF- α -stimulated RANKL expression was observed following addition of a p38 inhibitor; however, the level of stimulation with TNF- α was consistently less than the stimulation observed with IL-1 β in both ST-2 and UAMS-32P cells ($p < 0.05$ vs. control).

Inhibition of p38 MAPK decreases IL-1 β and TNF- α -induced membrane-bound RANKL expression

Immunoblot analysis was used to determine if p38 MAPK signaling affected IL-1 or TNF-induced RANKL protein expression. Whole cell extracts from cells stimulated for 72 h in the presence or absence of the p38 inhibitor indicated that IL-1 β -induced RANKL expression was partially inhibited by SB203580 (Fig. 2A). A graphic depiction of RANKL:GAPDH on the immunoblot is shown in Figure 2B. Data from three experiments indicated significant inhibition of IL-1 β -stimulated RANKL protein expression in the presence of the p38 inhibitor ($p < 0.01$).

p38 MAPK does not increase RANKL mRNA half-life

p38 signaling is a critical determinant of the mRNA stability of several cytokine and chemokine mRNAs.²⁴ To determine if p38 MAPK was involved in IL-1 β -induced RANKL stability, mRNA decay experiments were performed using ActD to arrest transcription. Following stimulation with IL-1 β with or without SB203580, ActD was added to ST-2 and UAMS-32P cells for the indicated times. RANKL mRNA levels were analyzed by real-time RT-PCR, as in Figure 1. Mean results of two independent experiments yielding identical results are presented in Figure 3. Following treatment with SB203580, no significant alteration in mean rate of mRNA decay was observed in IL-

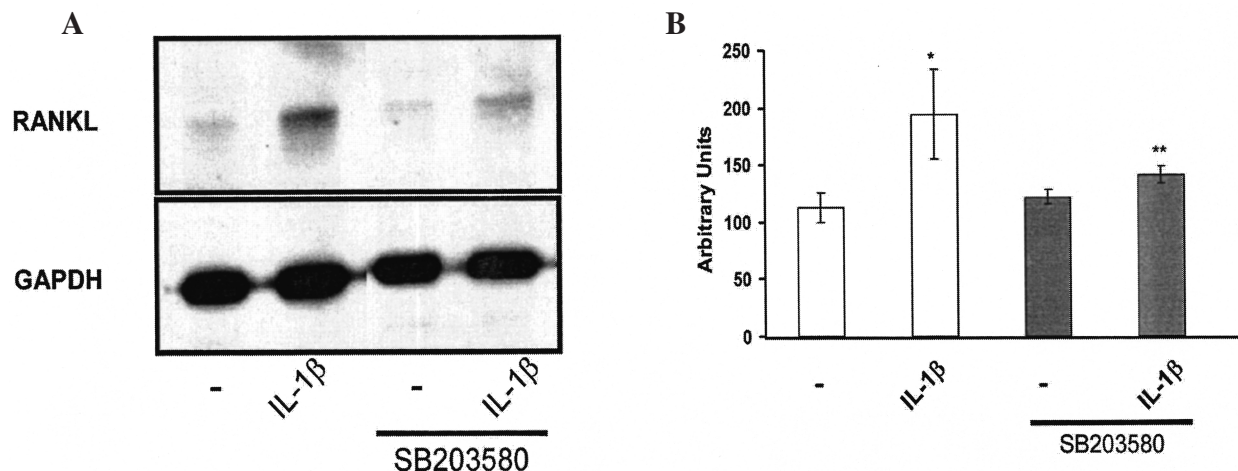


FIG. 2. Membrane-bound RANKL protein expression in bone marrow stromal cells is dependent on p38 signaling. (A) UAMS-32P cells were stimulated with 1 ng/mL IL-1 β in the presence or absence of 10 μ M SB203580 for 72 h. Medium with new cytokines and p38 inhibitors was added after 36 h of culture. Immunoblot analysis of whole cell lysates indicates that membrane-bound RANKL protein expression is increased by IL-1 β treatment (lane 2) but partially inhibited in the presence of SB203580. (B) Graphic representation of data in A. * $p < 0.05$ for IL-1 β -stimulated RANKL vs. control; ** $p < 0.01$ for SB/IL-1 β vs. IL-1 β only.

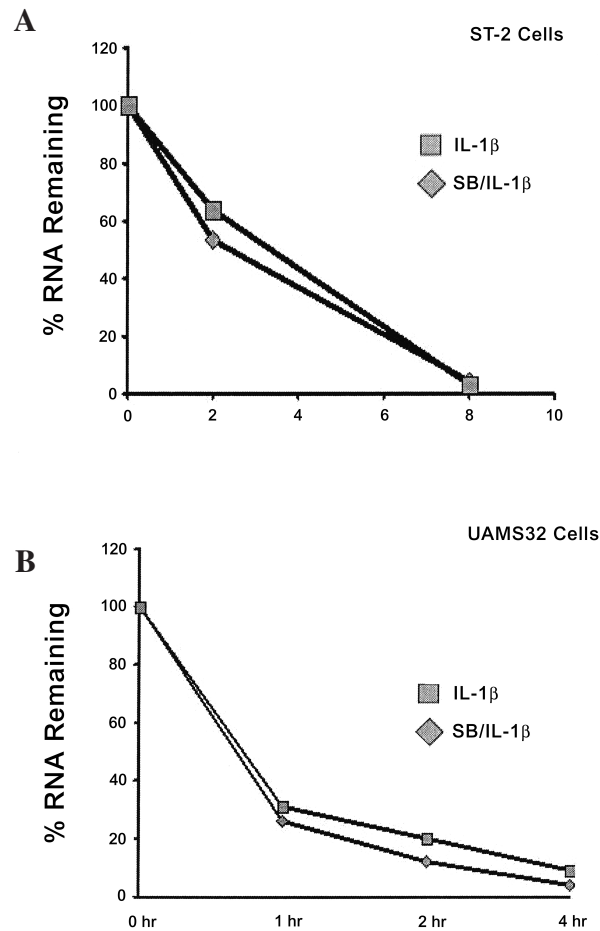


FIG. 3. p38 MAPK does not affect RANKL mRNA stability in stimulated cells. (A) ST-2 or (B) UAMS-32P cells were treated as in Figure 1. In the last 4–8 h, cells were treated with 2 μ M ActD to arrest transcription. Total RNA was analyzed by real-time PCR as described in Figure 1. Mean decay values are expressed as a percentage of RANKL mRNA expression normalized to GAPDH at 0 time in ST-2 cells (A) or UAMS-32 cells (B) from two independent experiments yielding similar results.

1 β -treated cells. Very little RANKL mRNA could be measured in untreated and SB203580-treated cultures ($n = 2$) (data not shown). Results indicate that RANKL mRNA half-life is ~ 3.8 h in ST2 cells and < 1 h in UAMS-32P cells following IL-1 β stimulation.

IL-1 β -induced RANKL-luc reporter cells through MKK3/6-p38 MAPK signaling

To determine if p38 MAPK exerts its regulatory effect on elements other than the proximal promoter of RANKL, a reporter cell line containing a 120-kb region of the murine RANKL 5'-flanking region plus the entire coding sequence along with a luciferase reporter gene was inserted into the 3'-UTR of this construct (generous gift from C. O'Brien, University of Arkansas). With this mRANKL reporter cell line (RANKL-luc), a 4.5-fold increase in luciferase reporter activ-

ity was demonstrated after 18 h of stimulation with IL-1 β or TNF- α (Fig. 4A). Pretreatment with SB203580 completely inhibited IL-1 β or TNF- α -stimulated RANKL-luc reporter expression (IL-1 β , $p = 0.0007$; TNF- α , $p < 0.0001$). To confirm the role of p38 signaling in IL-1 β or TNF- α -induced RANKL expression, RANKL-luc cells were transfected with MKK3/6 dn constructs to block p38 signaling and then stimulated with IL-1 β to stimulate RANKL-luc cells. In Figure 4B, both MKK3dn and MKK6dn completely inhibited IL-1 β -stimulated RANKL-luc reporter expression ($p = 0.0004$, IL-1 $\beta \times$ IL-1/MKK3b; $p = 0.0003$, IL-1 $\beta \times$ IL-1/MKK6b). Mock control (empty vector) did not have any effect on RANKL-luc expression. To clarify the role of p38 involvement with RANKL RANKL-luc expression, MKK3bE or MKK6bE both active mutants of the upstream p38 activators (MKK3 or MKK6) were transfected into RANKL-luc reporter cell lines (Fig. 4C). Using these vectors, a modest but significant enhancement of RANKL-luc reporter expression was observed, indicating p38 signaling activates RANKL reporter cell line expression ($p = 0.005$, mock \times MKK3ca; $p = 0.012$, mock \times MKK6ca).

MKK3/6dn constructs partially block cytokine-induced endogenous RANKL expression

As MKK3dn and MKK6dn transfected plasmids were able to partially attenuate RANKL-luc expression, it was important to determine if similar effects were observed with endogenous RANKL mRNA expression. UAMS-32P cells were transfected with MKK3/6dn constructs and 24 h later stimulated with IL-1 β for an additional 18-h period. Data presented in Figure 5 indicate that both MKK3dn and MKK6dn partially inhibit both IL-1 β -induced and TNF- α -induced RANKL expression. Significant levels of inhibition were observed with MKK3dn and MKK6dn blocking IL-1 β -stimulated RANKL in ST-2 cells ($p < 0.01$) and TNF- α -induced RANKL expression ($p < 0.01$, MKK3dn/TNF vs. TNF only; $p < 0.001$, MKK6dn/TNF vs. TNF only). In UAMS-32P cells, significant inhibition was observed only with MKK3dn blocking TNF- α -induced RANKL mRNA expression ($p < 0.05$), although the trend was observed with IL-1 β -treated cells as well.

Inhibition of p38 signaling partially blocks IL-1 β and TNF- α -induced osteoclastogenesis

To better understand the significance of p38 signaling in RANKL expression, we performed mouse osteoclastogenesis assays. Using the coculture design, we stimulated stromal cells with cytokines for 72 h, changing medium with fresh reagents after 36 h. After extensive rinsing of stromal cells, RAW264.7 macrophages were added for an additional 5–6 days of culture. In these assays, we show that IL-1 β and TNF- α both induced TRAP-positive cell formation in coculture (Fig. 6). Treatment of bone marrow stromal cells with SB203580 (10 μ M) reduced the numbers of IL-1 β and TNF- α -induced TRAP-positive cells formed in culture. Unstimulated UAMS-32P cells minimally supported osteoclast formation. In additional studies, conditioned medium from treated stromal cells did not induce osteoclast formation (data not shown), indicating that most RANKL expression was membrane bound and not secreted. RAW cells stimulated with exogenous RANKL consistently stimulated osteoclast formation (TRAP $^{+}$ cell formation) better

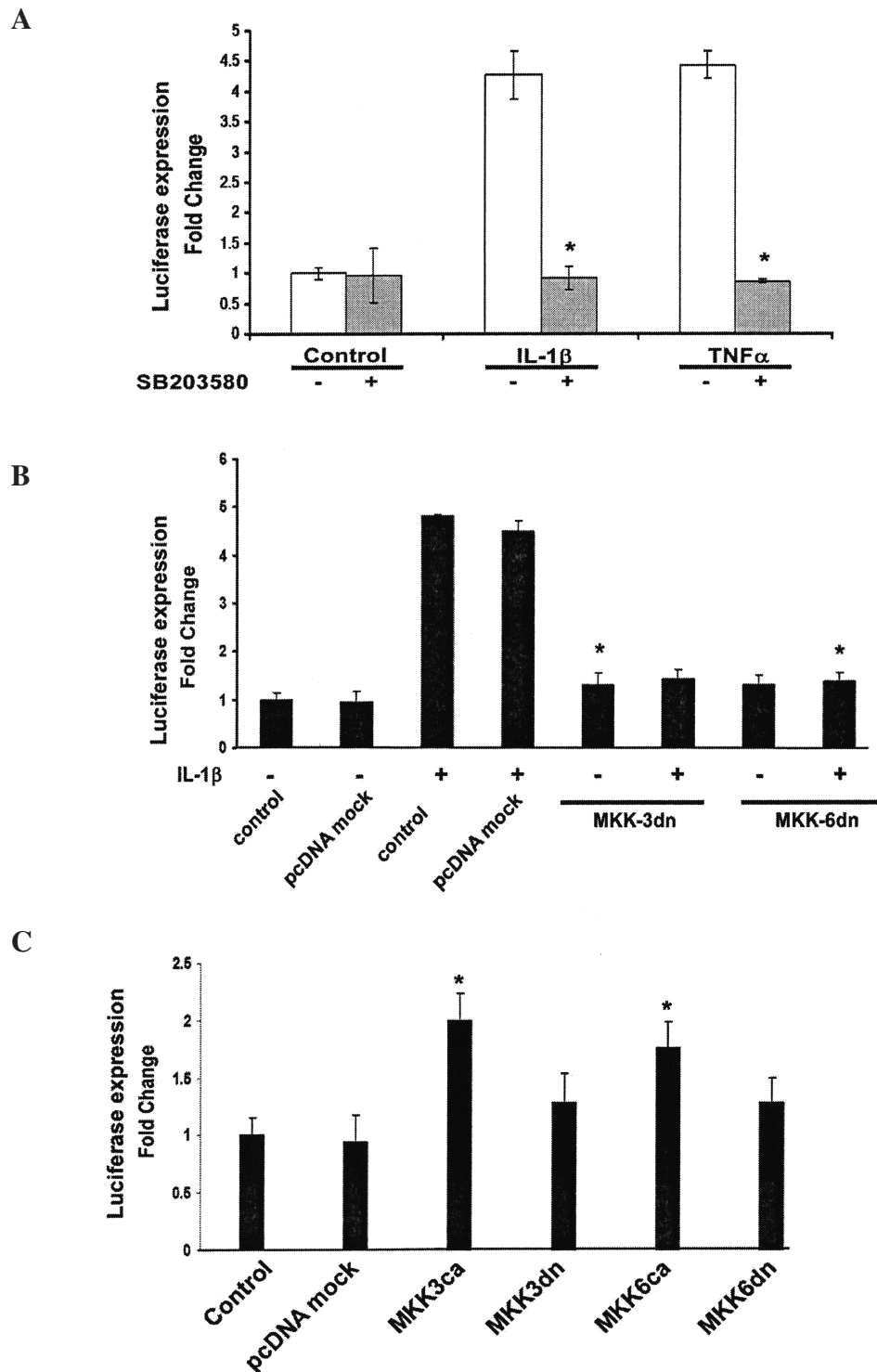


FIG. 4. RANKL-luc reporter cell lines are regulated by p38 MAPK similar to endogenous gene expression. **(A)** RANKL-luc reporter cells were treated with SB203580 (10 μ M) for 30 min and then stimulated for 18 h with IL-1 β (1 ng/mL) or TNF- α (5 ng/mL) or stimulated only with cytokines. Cell lysates were harvested and used to determine luciferase activity in RANKL-luc reporter cells. Mean luciferase activity normalized to total protein is shown ($n = 4$). SB203580 significantly blocked IL-1 β and TNF- α -induced RANKL-luc activity. **IL-1 β , $p = 0.0007$; TNF- α , $p < 0.0001$. **(B)** RANKL-luc reporter cells were transiently transfected with MKK3dn or MKK6dn and then stimulated with IL-1 β ($n = 3$). MKK3dn or MKK6dn constructs significantly blocked IL-1 β -induced RANKL luciferase reporter expression. ** $p = 0.0004$, IL-1 $\beta \times$ IL-1/MKK3b; $p = 0.0003$, IL-1 $\beta \times$ IL-1/MKK6b. **(C)** Transiently transfected MKK3 or MKK6 constitutively active (ca) vectors increase RANKL 5' flanking region reporter expression compared with empty vector-transfected cells. * $p = 0.005$, mock \times MKK3ca; $p = 0.012$, mock \times MKK6ca; $n = 3$.

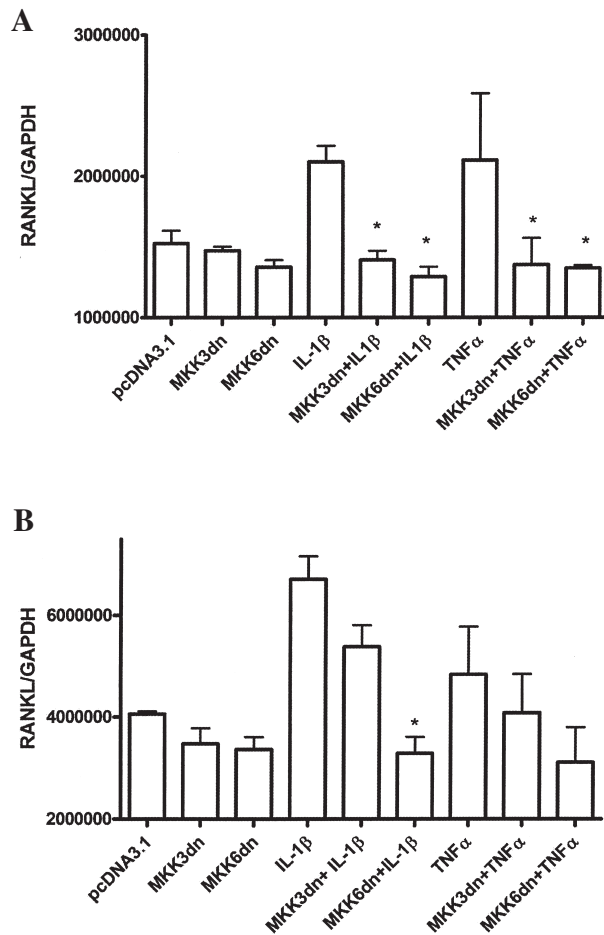


FIG. 5. MKK3/6-p38 signaling is required for IL-1 and TNF-induced RANKL mRNA expression. (A) ST-2 and (B) UAMS-32P cells were transiently transfected with MKK3dn or MKK6dn expression plasmids for 24 h and then stimulated for an additional 18 h with IL-1 β or TNF- α . Empty vector (mock transfection) was used as controls. RT-PCR analysis was used to assess endogenous RANKL mRNA normalized to GAPDH and expressed as fold change relative to mock transfected control. * $p < 0.05$, stimulated vs. MKK3dn or MKK6dn transfected and stimulated cells.

than IL-1 β or TNF- α in all experiments. Similar data were obtained with ST-2 cells, although the number of osteoclasts formed was significantly less than that observed with UAMS-32P cells (data not shown).

DISCUSSION

The crucial role of RANKL in osteoclastogenesis has been clearly demonstrated in RANKL-deficient murine studies.^{25,26} Stromal/osteoblast-derived RANKL expression is a key component leading toward osteoclastogenesis. This fact is highlighted by studies in which osteoclasts were not formed when osteoblasts from RANKL^{-/-} mice were cocultured with osteoclast precursors from wild-type mice.²⁵ Many hormones and cytokines regulate stromal cell and osteoblastic cell-derived

RANKL expression. Cellular signaling mechanisms governing PTH, vitamin D₃, and IL-6 family member-induced RANKL expression have received much of the attention because of their role in stromal cell-derived cytokine-mediated osteoclast formation.^{6,27,28} PTH-induced RANKL expression was shown to be dependent on cyclic AMP binding protein (CREB) but not c-Fos or Cbfa1.²⁷ Conditional expression of dn mutant forms Stat3 or gp130 in UAMS-32P stromal cells suppressed osteoclast-supporting activity of oncostatin M (OSM) and IL-11 but not PTH or vitamin D₃,²⁹ indicating that Stat3 is a crucial signal in RANKL expression with IL-6 family members. Inflammatory mediators, including IL-1 β and TNF- α have been shown to mediate their bone resorptive properties, in part, through RANKL induction in stromal/osteoblastic cells.^{18,30} In these studies, signaling mechanisms involved in IL-1 β or TNF- α -induced RANKL expression indicate that p38 MAPK is a critical component.

Consistent with work presented here, a recent study indicated that IL-1/TNF-mediated RANKL expression requires p38 activation in stromal cells.¹⁸ Earlier studies indicate that stromal/osteoblastic cells both increased or decreased OPG expression in response to IL-1 or TNF.³⁰ Our data with ST-2 and UAMS-32P bone marrow stromal cells is in agreement with these earlier studies. Importantly, regardless of the cell type studied, treatment with SB203580 decreased RANKL in both stromal cell lines. In addition, our coculture data indicate that blocking p38 signaling decreases IL-1 β and TNF- α -induced osteoclast formation. Although these later studies did not reach statistical significance, likely because of the long-term culturing needed after the p38 inhibitor was removed, these results support the role of p38 inhibitors in reducing proinflammatory cytokine-induced RANKL expression in osteolytic bone diseases.

These data strongly implicate p38 as a major signaling pathway involved in IL-1 β and TNF- α -induced RANKL mRNA expression. An indirect activation of RANKL by these cytokines has been supported by studies using cycloheximide to block protein synthesis (data not shown). This would be consistent with previous reported data suggesting that other cytokines, for example, IL-6, provide a link between IL-1-induced RANKL expression in bone marrow stromal cells.^{27,31} Other data, however, suggest that PTH directly activates RANKL expression, and more recent data indicate that this mechanism does not require IL-6 *in vivo*.^{20,29,32} Blocking p38 signaling did not block PTH or vitamin D₃-induced RANKL expression as measured by RT-PCR (data not shown), indicating that inflammatory cytokines activate RANKL from a pathway clearly distinct from that previously described for RANKL activation. As IL-6 may be mediating the effects of IL-1 on RANKL expression and osteoclast formation, we may be observing multiple points of regulation of p38 signaling in this system. Our laboratory has shown that IL-1 β induces IL-6 in osteoblasts primarily through an mRNA stability mechanism.³³ Ongoing studies in our laboratory are addressing the significance of IL-6 in LPS and IL-1-induced osteoclastogenesis.

Through Western blot analysis, membrane-bound RANKL expression was seen to be significantly downregulated compared with IL-1 β -stimulated stromal cells when pretreatment with SB203580. In other studies, we were not able to measure RANKL expression by ELISA or show that conditioned

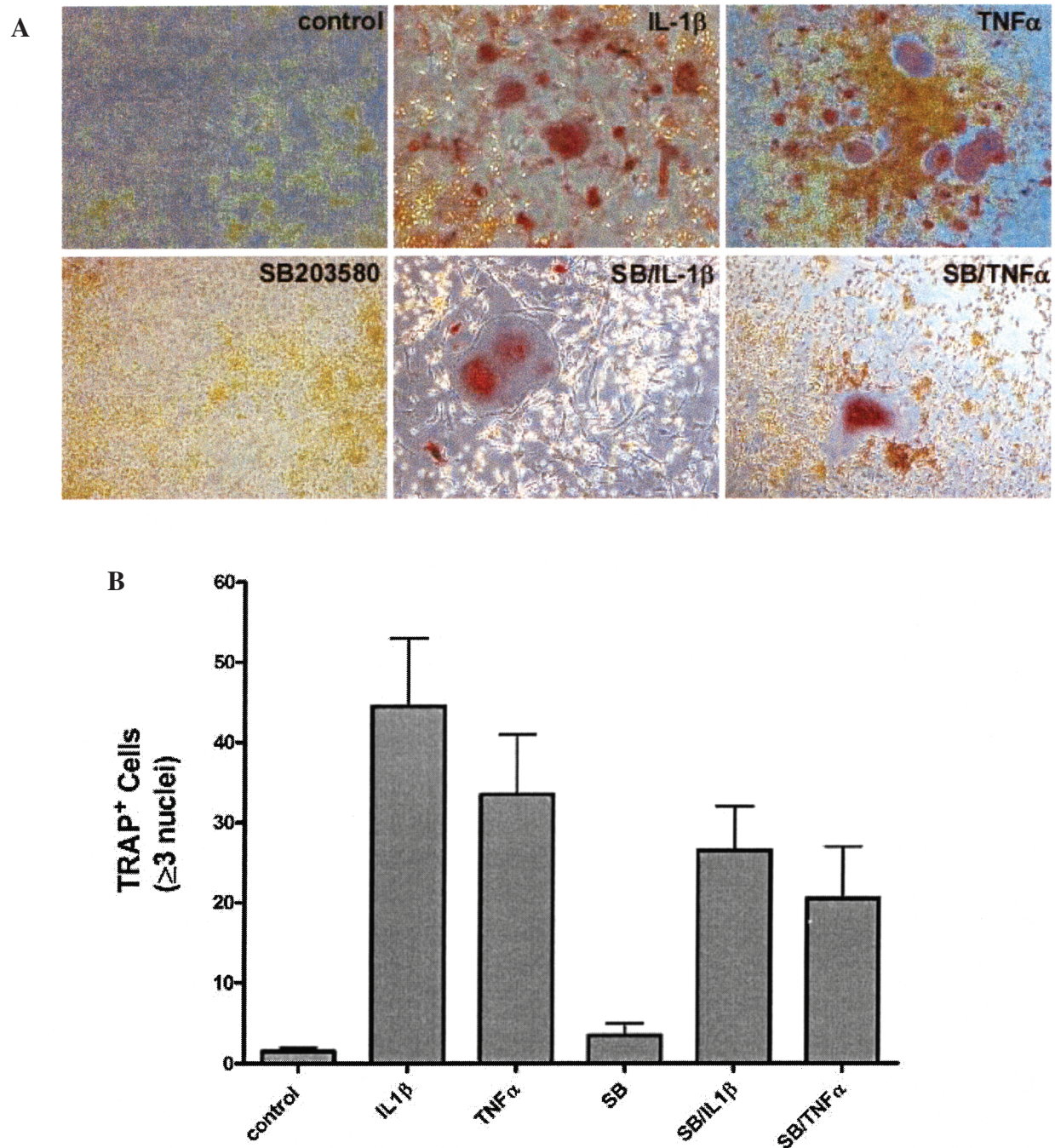


FIG. 6. p38 is required for IL-1 β and TNF- α -induced osteoclastogenesis. UAMS-32P bone marrow stromal cells were stimulated with IL-1 β (1 ng/mL) or TNF- α (5 ng/mL) for 72 h or in the presence of SB203580 (10 μ M). Stromal cells were washed extensively, and RAW264.7 macrophages were cultured with stromal cells for an additional 6 days. (A) Representative images from 6-day coculture experiments (100 \times) are shown. (B) Graphic representation of enumerated TRAP-positive multinucleated formation (\geq three nuclei) in each culture is presented, with the mean \pm SEM data from the two independent experiments.

medium from stromal cells supported osteoclastogenesis (data not shown), suggesting that most stromal cell-derived RANKL is membrane bound and not shed through enzymatic cleavage, as reported for other RANKL-expressing cells.^{34,35} Thus, expression of both mRNA and protein of stromal cell-derived RANKL was inhibited when p38 signaling was attenuated.

To understand the mechanism of p38-dependent RANKL mRNA expression, posttranscriptional regulation was explored. RANKL does not possess a classic AU-rich 3'-UTR, a common feature of several unstable cytokine mRNAs; however, previous studies using UAMS-32P cells indicated that PTH enhanced RANKL mRNA stability.²⁷ Moreover, p38 signaling is

the major signaling mechanism linked to enhanced mRNA stability of several cytokine genes,²⁴ including IL-1 β -induced IL-6 mRNA stability in osteoblasts.³³ Surprisingly, RANKL mRNA degradation studies performed in both ST-2 and UAMS-32P bone marrow stromal cell lines indicated that SB203580 did not alter IL-1 β -induced RANKL mRNA decay rates. The half-life of RANKL varied significantly between cell lines. The results indicate that p38-induced RANKL expression does not significantly involve posttranscriptional mRNA stability mechanisms. These findings indicate that most of the regulation must occur through RANKL transcriptional activity.

Although the proximal 0.7 kb of the RANKL promoter contains a Cbfa1/Runx2 binding site, this site was shown to be insufficient to drive RANKL expression in stromal cell lines.²⁰ Other studies using bone-derived endothelial cells have indicated that transforming growth factor- β (TGF- β) can induce RANKL expression through a p38/PKA-dependent mechanism involving ATF-2/CREB within the proximal promoter.⁷ Although two CRE sites are located in the RANKL proximal promoter, TGF- β -induced promoter activity was stimulated only 2-fold, whereas transfection with the wt-CREB overexpression construct greatly enhanced RANKL mRNA levels. Preliminary promoter regulation experiments with ST-2 cells using a murine promoter construct (-1014 to +111) failed to show any regulation with IL-1 β or TNF- α (data not shown). These data were consistent with proximal promoter data obtained using the -700 to +111 and -7000 to +111 RANKL proximal promoter constructs, where promoter activity in bone marrow stromal cells was higher compared with other cell types, but no significant regulation was observed with OSM, which increased RANKL gene expression in bone marrow stromal cells. Collectively, these data suggested that RANKL promoter regulation may require additional 5' flanking elements or an intronic sequence to mediate transcriptional activation.²⁰

Because these data indicated that the proximal RANKL promoter region was not the major target for IL-1 β -induced RANKL expression, a larger segment of the 5' flanking region of the RANKL promoter along with the entire RANKL intronic/exonic sequence (minus the 3'-UTR where the reporter gene was inserted) was obtained to analyze the transcriptional activity and responsiveness to p38-induced signals. Using this reporter cell line (RANKL-luc), IL-1 β and TNF- α induced RANKL promoter reporter activity nearly 5-fold. This fold activation mimicked endogenous RANKL mRNA stimulation, suggesting that within this 120-kb flanking region, or possibly the intronic sequence of the RANKL gene, was the regulatory element(s) required for RANKL expression. As recent data indicated that TNF-mediated RANKL stimulation required a functional IL-1 signaling system, the IL-1 β response element may be the functionally relevant target within this 120-kb region.¹⁸ Critical to these studies, we show that SB203580 can completely abolish both IL-1 β and TNF- α -induced RANKL-luc expression. Rather than addressing the exact *cis*-acting elements within this 120-kb region or intronic sequences, we focused our attention on p38-signaling intermediates that may impact RANKL expression.

p38 MAPK is activated by phosphorylation via upstream kinases, such as MKK3 and MKK6. Using dn constructs of MKK3 and MKK6, both constructs completely blocked IL-1 β -induced RANKL-luc activity. Similarly, endogenous RANKL

expression was blocked with MKK3dn and MKK6dn constructs, although significant levels of inhibition were not observed in all experiments due, in part, to transfection efficiency in these cells. These data confirmed p38 inhibitor data and suggest that p38 α is a major signaling intermediate required for IL-1 β or TNF- α -induced RANKL expression in stromal cells because SB203580 is specific for p38 α / β isoforms and both MKK3 and MKK6 can phosphorylate p38 α .^{36,37} Whereas MKK6 can phosphorylate all p38 isoforms, MKK3 phosphorylates only the p38 α isoform.³⁸ A constitutively active mutant MKK3 also increased RANKL-luc activity, providing additional support the p38 α MAPK pathway as a central signaling pathway controlling RANKL expression at the distal promoter level.

Other recent studies have focused on the RANKL 5' flanking region. One recent abstract indicated that this 120-kb 5' flanking region was stimulated by cAMP in stromal/osteoblastic cells and not in hepatic cells.²² Deletion analysis of this region indicated that an 8-kb region located 74 kb upstream of the transcriptional start site was necessary for cAMP, PTH, 1,25(OH) $_2$ D $_3$, or OSM induction. This 8-kb region was found to be conserved across many species and, similar to other studies where some regulation was observed with the proximal promoter, two conserved CRE-binding sites were found. When these sites were mutated, cAMP responsiveness was lost. As p38 can increase ATF-2/CREB activity, it is possible that p38 can regulate the RANKL region through this CRE-containing 8-kb conserved region. In addition, other studies suggest that IL-1 β -induced p38 MAPK can activate Stat3, with delayed kinetics.³⁹ Ongoing studies are pursuing the possibility that p38 may regulate RANKL regulation through an indirect mechanism whereby p38 activates Stat3 to stimulate IL-6 family members that, in a paracrine manner, feed back on the stromal/osteoblastic cells to induce RANKL expression.

ACKNOWLEDGMENTS

We thank Drs. Renny Franceschi, Laurie K. McCauley, and Cun-Yu Wang for their critical reading of the manuscript and Dr. Charles A. O'Brien for cell lines, plasmid constructs, and critical discussions. We also thank Dr. Wade J. Sigurdson, SUNY Buffalo, for his help with real-time PCR.

This work was supported by NIH DE14460 and DOD W81XWH-05-0075 to K.L.K., NIH T35DE07106 to K.E., and T32 DE07034 to C.P.

REFERENCES

1. Horwood NJ, Elliott J, Martin TJ, Gillespie MT. Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells. *Endocrinology* 1998; 139:4743-4746.
2. Karin M. Mitogen-activated protein kinase cascades as regulators of stress responses. *Ann. NY Acad. Sci.* 1998;851:139-146.
3. Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, Miyata T, Anderson DM, Suda T. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. *J. Exp. Med.* 1999;190:1741-1754.

4. Filvaroff E, Derynck R. Bone remodelling: a signalling system for osteoclast regulation. *Curr. Biol.* 1998;8:R679–682.
5. Ikeda T, Utsuyama M, Hirokawa K. Expression profiles of receptor activator of nuclear factor kappaB ligand, receptor activator of nuclear factor kappaB, and osteoprotegerin messenger RNA in aged and ovariectomized rat bones. *J. Bone Miner. Res.* 2001;16:1416–1425.
6. Palmqvist P, Persson E, Conaway HH, Lerner UH. IL-6, leukemia inhibitory factor, and oncostatin M stimulate bone resorption and regulate the expression of receptor activator of NF-kappa B ligand, osteoprotegerin, and receptor activator of NF-kappa B in mouse calvariae. *J. Immunol.* 2002;169:3353–3362.
7. Ishida A, Fujita N, Kitazawa R, Tsuruo T. Transforming growth factor-beta induces expression of receptor activator of NF-kappa B ligand in vascular endothelial cells derived from bone. *J. Biol. Chem.* 2002;277:26217–26224.
8. Hofbauer LC, Heufelder AE. Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. *J. Mol. Med.* 2001;79:243–253.
9. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Boyle WJ, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997;89:309–319.
10. Thomas GP, Baker SU, Eisman JA, Gardiner EM. Changing RANKL/OPG mRNA expression in differentiating murine primary osteoblasts. *J. Endocrinol.* 2001;170:451–460.
11. Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 1989;59:675–680.
12. Haskill S, Beg AA, Tompkins SM, Morris JS, Yurochko AD, Sampson-Johannes A, Mondal K, Ralph P, Baldwin AS Jr. Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity. *Cell* 1991;65:1281–1289.
13. Schett G, Tohidast-Akrad M, Smolen JS, Schmid BJ, Steiner CW, Bitzan P, Zenz P, Redlich K, Xu Q, Steiner G. Activation, differential localization, and regulation of the stress-activated protein kinases, extracellular signal-regulated kinase, c-JUN N-terminal kinase, and p38 mitogen-activated protein kinase, in synovial tissue and cells in rheumatoid arthritis. *Arthritis Rheum.* 2004;43:2501–2512.
14. Mansky KC, Sankar U, Han J, Ostrowski MC. Microphthalmia transcription factor is a target of the p38 MAPK pathway in response to receptor activator of NF-kappa B ligand signaling. *J. Biol. Chem.* 2002;277:11077–11083.
15. Branger J, van den Blink B, Weijer S, Madwed J, Bos CL, Gupta A, Yong CL, Polmar SH, Olszyna DP, Hack CE, van Deventer SJ, Peppelenbosch MP, van der Poll T. Anti-inflammatory effects of a p38 mitogen-activated protein kinase inhibitor during human endotoxemia. *J. Immunol.* 2002;168:4070–4077.
16. Kumar S, Boehm J, Lee JC. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat. Rev. Drug Discov.* 2003;2:717–726.
17. Matsumoto M, Sudo T, Saito T, Osada H, Tsujimoto M. Involvement of p38 mitogen-activated protein kinase signaling pathway in osteoclastogenesis mediated by receptor activator of NF-kappa B ligand (RANKL). *J. Biol. Chem.* 2000;275:31155–31161.
18. Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL. IL-1 mediates TNF-induced osteoclastogenesis. *J. Clin. Invest.* 2005;115:282–290.
19. Dai JC, He P, Chen X, Greenfield EM. TNFalpha and PTH utilize distinct mechanisms to induce IL-6 and RANKL expression with markedly different kinetics. *Bone* 2006;38:509–520.
20. O'Brien CA, Kern B, Gubrij I, Karsenty G, Manolagas SC. Cbfa1 does not regulate RANKL gene activity in stromal/osteoblastic cells. *Bone* 2002;30:453–462.
21. Kirkwood K, Martin T, Andreadis ST, Kim YJ. Chemically modified tetracyclines selectively inhibit IL-6 expression in osteoblasts by decreasing mRNA stability. *Biochem. Pharmacol.* 2003;66:1809–1819.
22. Fu Q, Manolagas SC, O'Brien CA. Parathyroid hormone controls receptor activator of NF-kappa B ligand gene expression via a distant transcriptional enhancer. *Mol. Cell Biol.* 2006;26:6453–6468.
23. Bhat NR, Feinstein DL, Shen Q, Bhat AN. p38 MAPK-mediated transcriptional activation of inducible nitric-oxide synthase in glial cells. Roles of nuclear factors, nuclear factor kappa B, cAMP response element-binding protein, CCAAT/enhancer-binding protein-beta, and activating transcription factor-2. *J. Biol. Chem.* 2002;277:29584–29592.
24. Tebo J, Der S, Frevel M, Khabar KS, Williams BR, Hamilton TA. Heterogeneity in control of mRNA stability by AU-rich elements. *J. Biol. Chem.* 2003;278:12085–12093.
25. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM. OPG is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 1999;397:315–323.
26. Kong YY, Boyle WJ, Penninger JM. Osteoprotegerin ligand: a common link between osteoclastogenesis, lymph node formation and lymphocyte development. *Immunol. Cell. Biol.* 1999;77:188–193.
27. Fu Q, Jilka RL, Manolagas SC, O'Brien CA. Parathyroid hormone stimulates receptor activator of NF-kappa B ligand and inhibits osteoprotegerin expression via protein kinase A activation of cAMP-response element-binding protein. *J. Biol. Chem.* 2002;277:48868–48875.
28. Kitazawa S, Kajimoto K, Kondo T, Kitazawa R. Vitamin D₃ supports osteoclastogenesis via functional vitamin D response element of human RANKL gene promoter. *J. Cell. Biochem.* 2003;89:771–777.
29. O'Brien CA, Gubrij I, Lin SC, Saylors RL, Manolagas SC. Stat3 activation in stromal/osteoblastic cells is required for induction of the receptor activator of NF-kappaB ligand and stimulation of osteoclastogenesis by gp130-utilizing cytokines or interleukin-1 but not 1,25-dihydroxyvitamin D₃ or parathyroid hormone. *J. Biol. Chem.* 1999;274:19301–19308.
30. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S. Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. *Bone* 1999;25:255–259.
31. Devlin RD, Reddy SV, Savino R, Ciliberto G, Roodman GD. IL-6 mediates the effects of IL-1 or TNF, but not PTHrP or 1,25(OH)₂D₃, on osteoclast-like cell formation in normal human bone marrow cultures. *J. Bone Miner. Res.* 1998;13:393–399.
32. O'Brien CA, Jilka RL, Fu Q, Stewart S, Weinstein RS, Manolagas SC. IL-6 is not required for parathyroid hormone stimulation of RANKL expression, osteoclast formation, and bone loss in mice. *Am. J. Physiol. Endocrinol. Metab.* 2005;289:E784–E793.
33. Patil C, Zhu X, Rossa C Jr, Kim YJ, Kirkwood KL. p38 MAPK regulates IL-1beta induced IL-6 expression through mRNA stability in osteoblasts. *Immunol. Invest.* 2004;33:213–233.
34. Horiki M, Nakase T, Myoui A, Sugano N, Nishii T, Tomita T, Miyaji T, Yoshikawa H. Localization of RANKL in osteolytic tissue around a loosened joint prosthesis. *J. Bone Miner. Metab.* 2004;22:346–351.
35. Miyamoto N, Higuchi Y, Mori K, Ito M, Tsurudome M, Nishio M,

- Yamada H, Sudo A, Kato K, Uchida A, Ito Y. Human osteosarcoma-derived cell lines produce soluble factor(s) that induces differentiation of blood monocytes to osteoclast-like cells. *Int. Immunopharmacol.* 2002;2:25–38.
36. Cuenda A, Cohen P, Buee-Scherrer V, Goedert M. Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6): comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO J.* 1997;16:295–305.
37. Kumar S, McDonnell PC, Gum RJ, Hand AT, Lee JC, Young PR. Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem. Biophys. Res. Commun.* 1997;235:533–538.
38. Shi Y, Gaestel M. In the cellular garden of forking paths: how p38 MAPKs signal for downstream assistance. *Biol. Chem.* 2002;383:1519–1536.
39. Ng DC, Long CS, Bogoyevitch MA. A role for the extracellular signal-regulated kinase and p38 mitogen-activated protein kinases in interleukin-1 beta-stimulated delayed signal transducer and activator of transcription 3 activation, atrial natriuretic factor expression, and cardiac myocyte morphology. *J. Biol. Chem.* 2001;276:29490–29498.

Address reprint requests or correspondence to:
Dr. Keith L. Kirkwood
Department of Periodontics and Oral Medicine
University of Michigan
1011 N. University Avenue
Ann Arbor, MI 48109-1078
Tel: (734) 763-7120
Fax: (734) 763-5503
E-mail: klkirk@umich.edu

Received 10 February 2006/Accepted 20 April 2006