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Induction of c-Fos and NFATc1 during RANKL-stimulated osteoclast differentiation is mediated by the p38 signaling pathway

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Abstract

The crucial role of p38 mitogen-activated protein kinase for osteoclast differentiation has been suggested from studies with specific pharmacological inhibitors and dominant-negative forms of p38. However, the targets through which p38 regulates osteoclast differentiation have not been clearly revealed. Here, we show that inhibition of p38 activity with SB203580 reduced osteoclastogenesis from primary precursor cells, with concomitant suppression in the induction of both c-Fos and nuclear factor of activated T cells (NFAT) c1 by receptor activator of nuclear factor κB ligand (RANKL), the key osteoclast differentiation factor. Overexpression of dominant-negative forms of p38 upstream kinases MKK3 and MKK6 elicited similar reduction in RANKL-stimulated elevation of c-Fos and NFATc1. Interestingly, overexpression of c-Fos restored RANKL-induced osteoclast differentiation from and NFATc1 expression in SB203580-treated precursor cells. Our results demonstrate a previously unknown function of the p38 pathway in up-regulating c-Fos and NFATc1 expression during RANKL-induced osteoclastogenesis.

Keywords: Osteoclasts; RANKL; p38; c-Fos; NFATc1

Bone homeostasis is maintained by the balance between bone-forming activity of osteoblasts and bone-resorbing activity of osteoclasts. Receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) plays a pivotal role in the regulation of differentiation, function, and survival of osteoclasts [1,2]. Binding of RANKL expressed on the osteoclast-interacting cells such as osteoblasts and lymphocytes to its receptor RANK on osteoclast precursor cells initiates diverse signaling cascades. Important mediators of RANKL signaling include tumor necrosis factor receptor-associated factor (TRAF) proteins, transcription factors (NFATc1, AP-1, and NF-κB), mitogen-activated protein kinases (MAPKs), and Akt signaling pathway proteins [3]. The importance of the transcription factors for osteoclastogenesis is highlighted from the in vivo and in vitro studies in which the genes encoding those transcription factors were either inactivated or overexpressed. Mice deficient in NF-κB showed osteopetrotic phenotype [4,5]. c-Fos knock-out mice also develop osteopetrosis [6]. Similarly, mice expressing dominant-negative c-Jun developed osteopetrosis, suggesting a role of AP-1 complex for osteoclastogenesis [7]. When osteoclast precursor cells were overexpressed with NFATc1, differentiation of these cells into osteoclasts occurred even in the absence of RANKL [8]. Since these transcription factors are suggested as major regulators of gene expression during osteoclast differentiation, it is of particular interest to delineate signal transduction pathways leading RANK ligation to the activation of NF-κB, AP-1 complex, or NFAT.

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The activation of MAPK pathways is one of the signaling events following RANKL stimulation of osteoclast precursor cells. All three MAPK family members, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, are rapidly phosphorylated and activated by RANKL [9–11]. Among them, role of p38 in osteoclast differentiation and function was extensively investigated, due to the availability of specific inhibitor pyridinylimidazole SB203580 [12]. SB203580 potently inhibited either RANKL- or TNF-induced differentiation of bone marrow macrophages into osteoclasts [13], but had no effect on the survival or bone resorption activity of mature osteoclasts [11]. Genetic studies employing dominant-negative forms of p38α or the upstream kinase MKK6 upheld the notion that p38 activity is critical for osteoclastogenesis [13]. However, the precise mechanism by which p38 mediates osteoclast differentiation remains to be fully understood.

In the present study, we tested the hypothesis that p38 may regulate osteoclast differentiation by modulating c-Fos and NFATc1. We found that pharmacological inhibition of p38 by SB203580 in primary osteoclast precursor cells strongly inhibited RANKL-induced osteoclastogenesis and induction of c-Fos and NFATc1. Genetic inactivation of p38 by dominant-negative forms of MKK3 and MKK6, p38 upstream kinases, had similar effects on c-Fos and NFATc1 expression. Importantly, overexpression of c-Fos restored RANKL-induced osteoclast differentiation and NFATc1 induction in the presence of the p38 inhibitor. These data indicate that p38 exerts its function by regulating c-Fos expression during osteoclast differentiation.

Materials and methods

Reagents. RANKL was obtained from PeproTech Inc. (Rocky Hill, NJ). M-CSF was from R&D Systems (Minneapolis, MN). Leukocyte Acid Phosphatase Assay Kit was purchased from Sigma Chemical Co. (St. Louis, MO). Lipofectamine™ 2000 was from Invitrogen Life Technologies (Carlsbad, CA). Anti-MKK3 and anti-MKK6 antibodies were purchased from Cell Signaling Technology (Beverly, MA) and Stressgen Biotechnologies Corp. (Victoria, BC, Canada), respectively. Antibodies against c-Fos, Fra2, and NFATc1 were from Santa Cruz (Santa Cruz, CA). SB203580 was obtained from Calbiochem (San Diego, CA).

Bone marrow macrophage culture and osteoclast differentiation. Bone marrow cells were obtained by flushing the marrow space of tibiae and femora from 5- to 6-week-old ICR mice with α-minimal essential medium (MEM) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were suspended in α-MEM supplemented with 10% FBS, plated on 100-mm dishes, and cultured for 16–24 h in the presence of M-CSF (5 ng/ml). After non-adherent cells were transferred to a new culture plate, cells were further cultured for 3 days in the presence of 30 ng/ml M-CSF. The adherent cells were considered bone marrow macrophages (BMMs; [14]) and used as osteoclast precursor cells. To achieve osteoclast differentiation, BMMs were cultured for 6 days with M-CSF (30 ng/ml) and RANKL (100 ng/ml) in 96-well plates at 1×10^5 cells/well or in 6-well plates at 2×10^6 cells/well. Experiments were performed in accordance with animal protocols, as approved by the Committee on the Care and Use of Animals in Research at Seoul National University.

Tartrate-resistant acid phosphatase (TRAP) staining. After osteoclast differentiation, cells were washed with PBS and fixed with 3.7% formaldehyde. Cells were then incubated with 0.1% Triton X-100 for 5 s and stained with Leukocyte Acid Phosphatase Assay kit following manufacturer's instructions. The numbers of TRAP-positive multinucleated cells ($TRAP^+$ MNC) containing three or more nuclei were counted as osteoclasts under a light microscope. Data were expressed as means ± SD of triplicate samples.

Western blotting analysis. Cells were washed twice with ice-cold PBS and lysed with a buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, and inhibitors for proteases and phosphatases [15]. Cell lysates were centrifuged at 10,000*g* for 20 min and the supernatants were collected. Twenty micrograms of cellular proteins was resolved by SDS–PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and incubated overnight at 4 °C with primary antibodies. Membranes were washed, incubated with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 h, and developed using a chemiluminescence system. Relative protein expression was calculated from band densities obtained in three experiments using UMax PowerLook 1100 scanner (Taiwan) and TotalLab gel image analysis software (Nonlinear Dynamics, New England, UK).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Cells were washed with ice-cold PBS and lysed in TRI Reagent (Sigma). Two micrograms of prepared RNA was reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen). Ten percent of the reverse-transcribed cDNA was amplified by PCR. The PCR primer sequences used are as follows: NFATc1, 5′-CAACGCCCTGACCACCGATA-3′ (forward) and 5′-GGCTGCCTTCCGTCTCATAG-3′ (reverse); c-Fos, 5′-CTGGTGCAGCCCACTCTGGT-3′ (forward) and 5′-CTTTCAGCA GATTGGCAATC-3′ (reverse); FosB, 5′-AAAAGGCAGAGCTGGA GTCGG-3′ (forward) and 5′-TGTACGAAGGGCTAACAACGG-3′ (reverse); Fra1, 5′-CTGGAGAAAGGGAGATGCAAGG-3′ (forward) and 5′-TGCGAGCAGATCAGCCCAGAG-3′ (reverse); Fra2, 5′-ATTATCCCGGGAACTTTGACAC-3′ (forward) and 5′-ATGTGCC CAGGGACTGAAGCC-3′ (reverse); GAPDH, 5′-ACCACAGTCCA TGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). The amplification cycle was comprised of a denaturation step at 94 °C for 1 min, an annealing step at 56 to 58 °C for 1 min, and an extension step at 72 °C for 30 s. The number of cycles was determined to be in a linear range of amplification (25 cycles for GAPDH and 30 cycles for the others).

Retroviral gene transfer. Recombinant retroviral vectors harboring dominant negative (DN) forms of MKK3 and MKK6 were generated by subcloning MKK3-DN and MKK6-DN genes (provided by Dr. Roger J. Davis, University of Massachusetts Medical School, Worcester, MA, USA) into a retroviral vector pMX (provided by Dr. Toshio Kitamura, University of Tokyo, Tokyo, Japan). The recombinant plasmids and parental pMx vector were transfected into Plat E packaging cells with 4 μg DNA and 10 μl Lipofectamine™ 2000 reagent following manufacturer's instructions. After 48 h, cell culture medium containing viral particles were collected and centrifuged at 1500*g* for 10 min. The supernatants were stored at −70 °C until use. For infection with retroviruses, BMMs in 6-well plates (1×10^6 cells/well) were incubated with the virus-containing medium (2 ml/well), polybrene (10 μg/ml), and M-CSF (30 ng/ml) for 24 h. An equal volume of α-MEM supplemented with 10% FBS and 30 ng/ml M-CSF was added and incubated for another 24 h.

Luciferase reporter assay. RAW264.7 cells (5×10^5/well in 6-well plates) were transfected with 4 μg c-Fos-dependent luciferase reporter vector (provided by Dr. Erwin F. Wagner, IMP, Vienna, Austria) using 10 μl Lipofectamine™ 2000 in serum-free DMEM. At 4 h after transfection, media were replaced by DMEM supplemented with 10% FBS. After incubation at 37 °C in a CO_2 incubator for 20 h, cells were collected by scraping, resuspended in α-MEM supplemented with 10% FBS, and replated in 96-well plates at 2×10^4/well. After stimulation with 100 ng/ml RANKL for 16 h, cells were lysed in Reporter Lysis Buffer (Promega) and luciferase activity was measured using a luminometer.

Results and discussion

p38 inhibition blocks osteoclast differentiation by down-regulating NFATc1 induction

The role of p38 during RANKL-induced osteoclast differentiation from primary mouse bone marrow-derived macrophages (BMMs) was investigated using a p38-specific inhibitor, SB203580. Fig. 1A clearly shows that SB203580 inhibited RANKL-induced differentiation of BMMs into osteoclasts. Although the effect of SB203580 at 0.1 μM concentration was marginal, incubation of BMMs with 1 μM SB203580 resulted in clear reduction of osteoclast number generated after 6-day incubation with RANKL and M-CSF. Virtually no TRAP-positive multinuclear osteoclast was observed when the cells were treated with 10 or 20 μM SB203580. SB203580 at 20 μM concentration did not affect the viability of BMMs, excluding the possibility of osteoclastogenesis inhibition due to cytotoxicity (data not shown). Since the role of NFATc1 is reported to be pivotal in osteoclastogenesis, we next examined expression of NFATc1 after SB203580 treatment to BMMs. The protein level of NFATc1 significantly increased at 24 h after RANKL stimulation of BMMs (Fig. 1B). However, at concentrations that evidently inhibited RANKL-induced osteoclast differentiation (1 μM or higher), SB203580 dramatically reduced NFATc1 protein expression (Fig. 1B). RT-PCR analysis showed that SB203580 also reduced NFATc1 mRNA levels following RANKL stimulation in a similar manner (Fig. 1C). The addition of 1 μM SB203580 reduced NFATc1 mRNA expression close to the basal level. These results suggest that p38 is involved in the induction of NFATc1 by RANKL at the mRNA level during osteoclast differentiation.

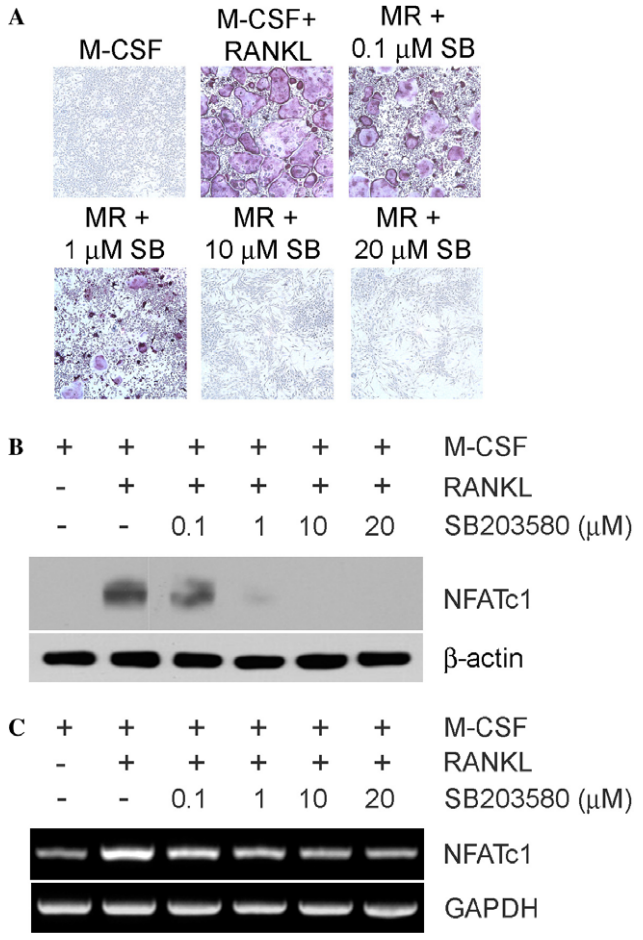

Fig. 1. Inhibition of p38 blocks RANKL-induced osteoclast differentiation and NFATc1 expression. (A) BMMs were stimulated with 30 ng/ml M-CSF alone (M-CSF), M-CSF and 100 ng/ml RANKL (MR), or M-CSF, RANKL, and increasing concentrations of SB203580 (MR+SB). After 6-day culture, cells were fixed and stained for TRAP activity. (B,C) BMMs were treated with 30 ng/ml M-CSF and 100 ng/ml RANKL in the presence of SB203580. After 24 h incubation, cells were harvested and subjected to either Western blotting (B) or RT-PCR analysis (C) for NFATc1 expression.

p38 MAPK is critical for c-Fos induction by RANKL in osteoclastogenesis

It has been established that the activity of AP-1 complex component c-Fos is essential for RANKL-induced osteoclast differentiation [6]. To test if p38 MAPK signaling pathway is also involved in the Fos family member expression during osteoclast differentiation, we examined mRNA expression of Fos family members after stimulating BMMs with RNAKL. Among the tested Fos family members, mRNA expression of c-Fos, Fra1, and Fra2 significantly increased at 24 h following RANKL treatment (Fig. 2A). However, incubation of BMMs with p38 inhibitor SB203580 at 10 μM or higher concentrations completely blocked the induction of c-Fos, Fra1, and Fra2 mRNAs in response to RANKL (Fig. 2A). Treatment of SB203580 at 1 μM, which evoked significant reduction in osteoclast differentiation by RANKL, reduced c-Fos and Fra2 mRNA expression. On the other hand, mRNA expression of FosB was neither increased by RANKL treatment nor affected by incubation with SB203580 at the concentrations tested. RANKL induced dramatic increase in c-Fos and Fra2 protein levels after 24 h, consistent with the changes in the mRNA level (Fig. 2B). As low as 1 μM SB203508 inhibited RANKL-induced elevation of c-Fos and Fra2 protein expression. The effect of SB203580 on c-Fos transcription activity was further investigated. When RAW264.7 cells were transfected with c-Fos promoter/luciferase reporter construct and stimulated with RANKL, c-Fos transcription activity increased significantly (Fig. 2C). Again, SB203580 completely inhibited RANKL-induced c-Fos transcription activity (Fig. 2C). These data suggest that p38 has a role in c-Fos induction by RANKL.

Dominant-negative forms of p38 upstream proteins MKK3 and MKK6 inhibit c-Fos and NFATc1 induction by RANKL

It has been established that activation of MKK3 and MKK6 is essential for downstream phosphorylation and

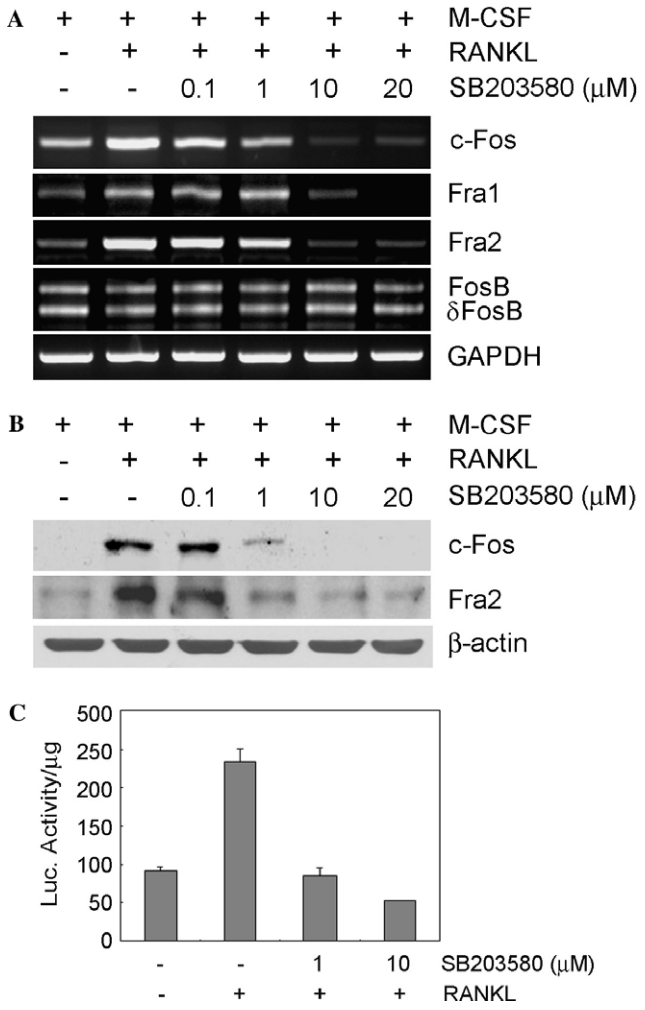

Fig. 2. SB203580 reduces RANKL-induced c-Fos expression. (A) BMMs were treated with 30 ng/ml M-CSF and 100 ng/ml RANKL in the presence of increasing concentrations of SB203580. After 24 h incubation, RT-PCR analyses were performed to determine the expressions of c-Fos, Fra1, Fra2, and FosB. (B) BMMs were treated as in (A) and subjected to Western blotting for c-Fos and Fra2. (C) RAW264.7 cells were transfected with c-Fos-dependent luciferase reporter construct and stimulated with 100 ng/ml RANKL in combination with SB203580 for 40 h. Cells were lysed and luciferase activity was measured.

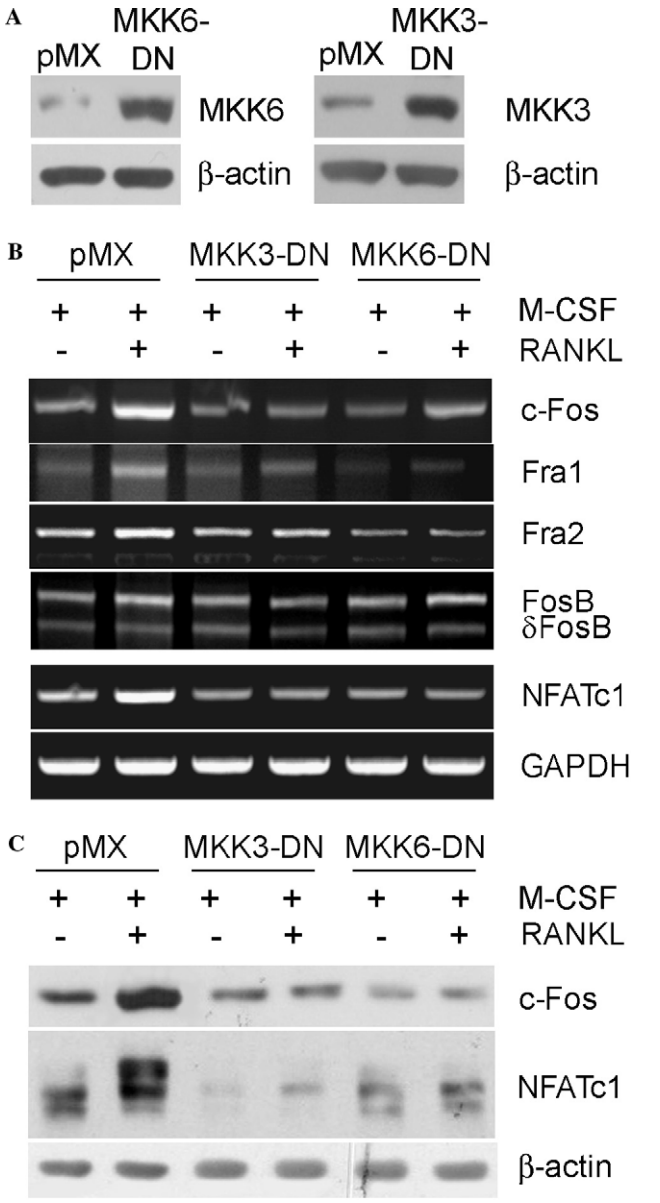

Fig. 3. Dominant-negative MKK3 or MKK6 inhibits RANKL-induced increase in c-Fos and NFATc1 expression. (A) BMMs were infected with control (pMX), MKK3-DN or MKK6-DN retroviruses. The levels of overexpressed proteins were confirmed by Western blotting at 24 h after infection. (B) Retrovirus-infected cells were stimulated with 30 ng/ml M-CSF and 100 ng/ml RANKL for 24 h. The mRNA levels of c-Fos, Fra1, Fra2, FosB, and NFATc1 were examined by RT-PCR analysis. (C) Cells treated as in (B) were lysed and subjected to Western blotting analysis to examine NFATc1 and c-Fos expression.

activation of p38 [16,17]. To test the effect of inactivation of MKK3 and MKK6 on RANKL-induced expression of Fos family transcription factors and NFATc1, dominant-negative forms of MKK3 and MKK6 were introduced to BMMs by retroviral infection. Fig. 3A confirms overexpression of dominant-negative MKK3 and MKK6 by immunoblotting. The infected cells were cultured in the presence of RANKL and M-CSF for 24 h and mRNA expression of c-Fos family members and NFATc1 was examined. RANKL strongly induced mRNA expression of c-Fos, Fra1, Fra2, and NFATc1, but not that of FosB in control virus-infected cells (Fig. 3B). Similar to the results obtained by pharmacological inhibition of p38 by SB203580, introduction of MKK3 and MKK6 dominant-negative forms clearly reduced RANKL-induced mRNA expression of c-Fos, Fra1, Fra2, and NFATc1. Next, effect of p38 inactivation by dominant-negative MKK3 or MKK6 on c-Fos and NFATc1 protein expression was examined. Consistent with the changes in mRNA level, overexpression of dominant-negative MKK3 and MKK6 blocked RANKL-induced up-regulation of c-Fos and NFATc1 proteins (Fig. 3C). Taken together, these data suggest that stimulation of osteoclast precursors with RANKL induces transcription factors NFATc1 and

c-Fos family of AP1 complex by activating p38 through MKK3/MKK6.

Overexpression of c-Fos restores NFATc1 expression and osteoclastogenesis in the presence of SB203580

It was previously reported that NFATc1 induction is regulated by c-Fos during osteoclastogenesis [18]. Therefore, if c-Fos is ectopically overexpressed, NFATc1 induction and osteoclastogenesis might be recovered even under p38 inhibitory conditions. To test this possibility, we infected BMMs with retrovirus harboring c-Fos expression construct containing green fluorescence protein (GFP) tag. Both control- and c-Fos virus-infected cells showed expression of GFP, suggesting that viral infection was effective (Fig. 4A, left panel). The transduction efficiency of the retroviral system was 60–70% (Fig. 4A, right panel). When the expression of c-Fos protein was examined by immunoblotting, cells infected with viruses containing c-Fos construct showed significantly higher c-Fos expression compared with that of control-infected macrophages (Fig. 4B). Next, the effect of c-Fos overexpression on the suppression of RANKL-induced NFATc1 expression by SB203580 was examined. While 10 μM SB203580 completely prevented induction of NFATc1 at 24 h after RANKL stimulation in control-infected cells, NFATc1 was substantially induced in c-Fos-overexpressing cells by RANKL in the presence of 10 μM SB203580 (Fig. 4C). The effect of c-Fos overexpression on the suppression of RANKL-induced osteoclast differentiation by SB203580 was also

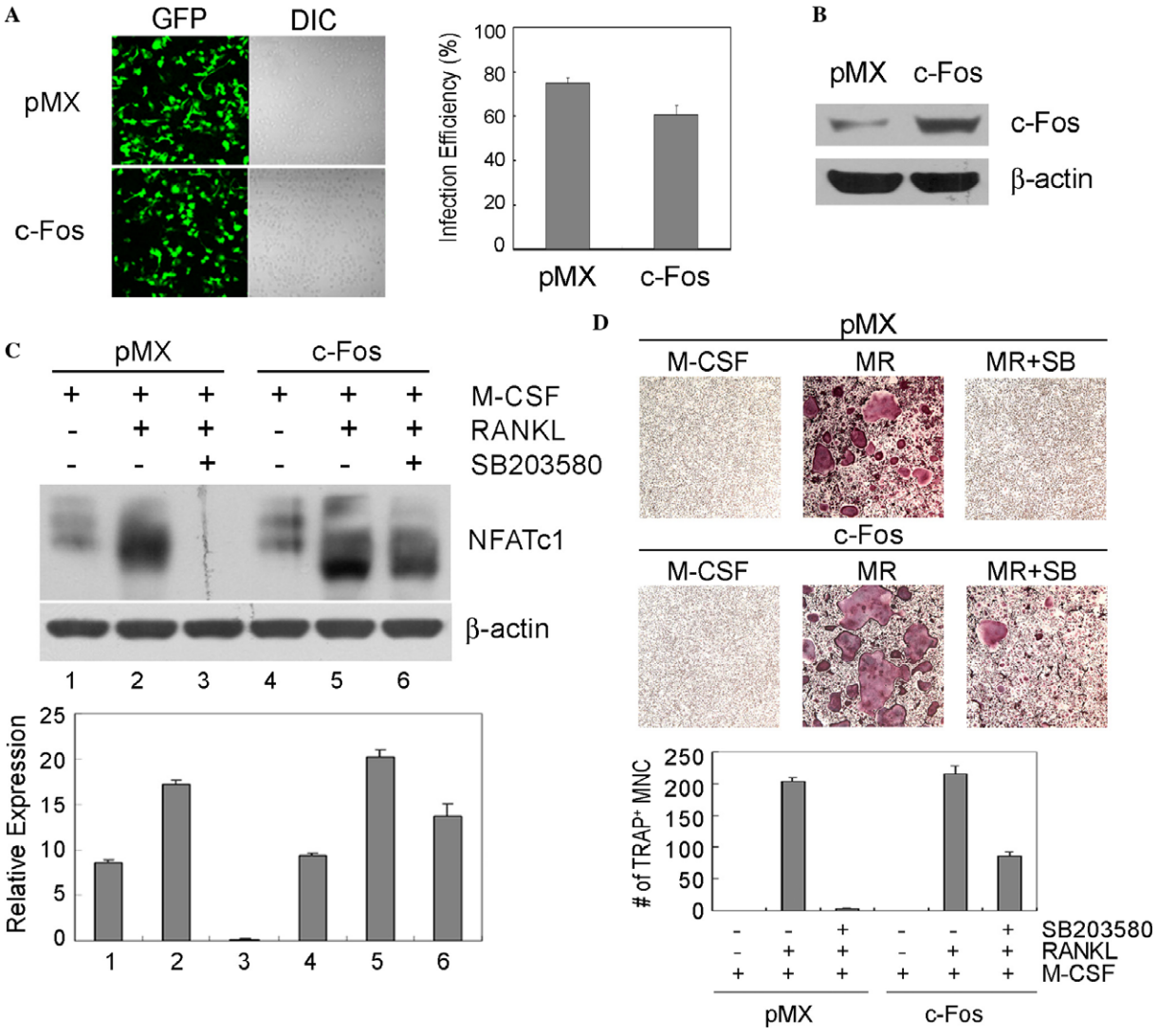

Fig. 4. Overexpression of c-Fos restores osteoclastogenesis in SB203580-treated BMMs. (A) BMMs were infected with retroviruses harboring control GFP (pMX) or c-Fos expression construct with GFP tag. After 24 h incubation, infection efficiency was examined by counting cells expressing GFP under fluorescence microscope. (B) Western blotting was performed to verify overexpression of c-Fos in virus-infected cells. (C) BMMs infected with control or c-Fos retroviruses were treated with 30 ng/ml M-CSF and 100 ng/ml RANKL in combination with 10 μM SB203580 for 24 h. Cells were then lysed and Western blotting was performed. (D) BMMs infected with control or c-Fos retroviruses were treated with 30 ng/ml M-CSF alone (M-CSF), M-CSF plus 100 ng/ml RANKL (MR), or M-CSF, RANKL, and 10 μM SB203580 (MR+SB). After 6-day incubation, cells were fixed and stained for TRAP activity. The numbers of $TRAP^+$ MNCs are presented (lower panel).

investigated. Although overexpression of c-Fos by itself did not significantly affect the number of TRAP-positive multinuclear osteoclasts following incubation with RANKL, increased c-Fos expression reduced the suppressive effect of SB203580 on osteoclastogenesis by approximately 50% (Fig. 4D). Considering that c-Fos restored approximately 50% of RANKL-induced NFATc1 expression in the presence of SB203580 (Fig. 4C, lanes 4–6), these results suggest that p38 may exert its role during osteoclast differentiation by modulating c-Fos-mediated NFATc1 expression.

Although several groups reported that p38 activity is crucial for osteoclast differentiation, the precise mechanism by which p38 modulates osteoclastogenesis is still unclear. Furthermore, the possibility that p38 may regulate the expression of transcription factors during osteoclastogenesis has not been tested. In the present study, we showed that either pharmacological inhibition or genetic inactivation by upstream signaling molecules of p38 resulted in dramatic suppression in the induction of c-Fos and NFATc1 mRNA and protein following RANKL treatment. We further demonstrated that p38-dependent NFATc1 expression is downstream of c-Fos by showing that overexpression of c-Fos could rescue NFATc1 expression in cells treated with p38-specific inhibitor. It has been reported that c-Fos can bind to the promoter sequence of *Nfatc1* gene [18], suggesting that c-Fos may directly regulate NFATc1 expression. It has also been suggested that c-Fos might be involved in the NFATc1 autoamplification loop [8]. Thus, it is likely that modulation of c-Fos is a crucial step for p38 to regulate RANKL-induced osteoclast differentiation, in view of the master role of NFATc1 during osteoclastogenesis [19]. There are two possible ways for p38 to regulate c-Fos. First, p38 may phosphorylate c-Fos, facilitating its translocation into nucleus, thus enhancing its activity. Tanos et al. reported that p38 could phosphorylate c-Fos both in vitro and in vivo [20]. However, our data suggest that p38 may regulate c-Fos expression, both in mRNA and protein level (Fig. 2). Although we cannot exclude the possibility that p38 indeed regulates osteoclast differentiation by directly phosphorylating c-Fos, it is more likely that the indirect transcriptional regulation of c-Fos by p38 may have major role in RANKL-induced osteoclastogenesis. This second possibility includes p38-downstream protein(s) mediating the transcription of c-Fos. In LPS-stimulated glial cells, p38 pathway-dependent activation of Elk1 was responsible for c-Fos transcription [21]. In H_2O_2-stimulated vascular smooth muscle cells, induction of c-Fos was dependent on the p38-mediated activation of cyclic AMP response element-binding protein (CREB) [22]. In osteoclast precursors, further studies are required to fully elucidate the p38-dependent signaling mechanism leading to the expression of c-Fos upon RANKL stimulation.

In summary, we have identified that inactivation of p38 signaling pathway completely blocked the induction of c-Fos and NFATc1 with concomitant inhibition of RANKL-induced osteoclastogenesis. The regulation of c-Fos expression by p38 was a key step since overexpression of c-Fos could rescue both NFATc1 expression and osteoclast differentiation in the presence of p38 inhibitor. These data provide evidence for the previously unappreciated role of p38 during osteoclastogenesis, which modulates c-Fos and NFATc1 expression in response to RANKL stimulation.

Acknowledgments

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