The JNK-dependent CaMK pathway restrains the reversion of committed cells during osteoclast differentiation

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Summary

Osteoclastogenesis involves the commitment of macrophage-lineage precursors to tartrate-resistant acid phosphatase-positive ($TRAP^+$) mononuclear pre-osteoclasts (pOCs) and subsequent fusion of pOCs to form multinuclear mature osteoclasts. Despite many studies on osteoclast differentiation, little is known about the signaling mechanisms that specifically mediate the osteoclastic commitment. In this study, we found that inhibition of JNK at the pOC stage provoked reversion of $TRAP^+$ cells to $TRAP^-$ cells. The conversion to $TRAP^-$ cells occurred with concomitant return to the state with higher expression of macrophage antigens, and greater activity of phagocytosis and dendritic-differentiation potential. JNK inhibition at the pOC stage reduced NFATc1 and CaMK levels, and addition of active NFATc1 partially rescued the effect of JNK inhibition. In addition, the level of NFATc1 was decreased by knockdown of CaMK by RNAi and by catalytic inhibition of CaMK, which both caused the reversion of pOCs to macrophages. These data suggest that JNK activity is specifically required for maintaining the committed status during osteoclastogenesis and that the CaMK-NFATc1 pathway is the key element in that specific role of JNK.

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Key words: JNK, Osteoclast, Commitment, CaMK, NFATc1

Introduction

Osteoclasts have the specialized function of dissolving bone matrix and they play key roles in the pathology of many bone-erosive disorders, such as osteoporosis, rheumatoid arthritis and Paget disease (Boyle et al., 2003; Tanaka et al., 2005; Teitelbaum, 2007; Zaidi et al., 2003). Osteoclasts are derived from hematopoietic stem cells (HSCs), specifically from the monocyte-macrophage lineage of myeloid cells. Two cytokines play fundamental roles in osteoclast generation from the progenitors: macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) (Lacey et al., 1998; Yasuda et al., 1998). M-CSF induces expression of RANK, the receptor for RANKL, in early-stage precursors of osteoclast lineages in mouse bone-marrow cells (Arai et al., 1999). Subsequent ligation of RANK by RANKL initiates an osteoclastic differentiation program. Under normal conditions, the RANKL-RANK axis appears to be essential for osteoclastogenesis because both *RANKL*$^{-/-}$ and *RANK*$^{-/-}$ mice develop no osteoclasts (Dougall et al., 1999; Kong et al., 1999). The major signaling events triggered upon RANK ligation include recruitment of tumor necrosis factor receptor-associated factor (TRAF) adaptor proteins, activation of protein kinases (Src, Akt, TAK, IKKs and MAPKs), and elevation in the expression and/or activity of transcription factors NF-κB, Fos, AP-1 and NFATc1 (Boyle et al., 2003; David et al., 2002; Huang et al., 2006; Lee et al., 2002; Lee and Kim, 2003; Matsuo et al., 2000; Kobayashi et al., 2001; Takayanagi et al., 2002a; Takayanagi et al., 2002b; Wong et al., 1999). The activity of these transcription factors ultimately leads to the induction of genes encoding proteins related to osteoclast function, such as tartrate-resistant acid phosphatase (TRAP), cathepsin-K, MMP9 and calcitonin receptor (Ikeda et al., 2004; Kim et al., 2005; Matrumoto et al., 2004; Sharma et al., 2007).

Osteoclasts share the same progenitor cells with macrophages and dendritic cells (Akagawa et al., 1996; Udagawa et al., 1990). The molecular understanding of the signaling mechanisms responsible for the lineage diversification between osteoclasts, macrophages and dendritic cells is currently very poor. With mouse bone-marrow precursor cells, osteoclastic and dendritic cell differentiation has been shown to be reciprocally regulated by M-CSF and granulocyte monocyte colony stimulating factor (GM-CSF); osteoclastic differentiation driven by M-CSF plus RANKL was suppressed in the presence of GM-CSF, whereas dendritic cell differentiation driven by GM-CSF was inhibited by M-CSF (Miyamoto and Akashi, 2005). A prominent intracellular event that correlated with the GM-CSF-dependent inhibition of osteoclast differentiation was reduction in the Fos level (Miyamoto and Akashi, 2005), suggesting a crucial role of Fos in the fate determination of this common lineage of cells to osteoclasts versus dendritic cells. Other signaling events have also been described for lineage determination from HSCs. The MEK-ERK signaling pathway was recently reported to play a crucial role in granulocyte-macrophage lineage commitment from HSCs (Hsu et al., 2007). Also, the ERK pathway was shown to be specifically required at an early stage in retinoic-acid-dependent adipocyte-lineage commitment of

embryonic stem cells (Bost et al., 2002). By contrast, inhibition of ERK was reported to stimulate adipogenesis from adult human mesenchymal stem cells (Jaiswal et al., 2000). Therefore, it appears that the same signaling pathway can have different roles in lineage determination depending on the differentiation signals and progenitor cell types.

Despite a substantial amount of studies on the factors that stimulate osteoclast differentiation and on the intracellular signaling events triggered by the stimulators, when and how osteoclastic-lineage commitment from the common precursor cells is achieved are not known. A study suggested that macrophagic differentiation is the default pathway in the fate determination of the common precursor cells and that RANKL actively suppresses the default pathway for osteoclastogenesis (Arai et al., 1990). However, molecular mechanisms to explain the observation were not provided in the study. Dynamic and temporal regulation of transcription factors is pivotal to cell-fate determination during hematopoiesis (Iwasaki et al., 2006; Evans et al., 2003; Terskikh et al., 2003; Wang et al., 2006). In support of the importance of transcription factors in the commitment and maintenance of specific lineages, alterations in the level and timing of expression of transcription factors were shown to change the lineage of already committed cells (Akashi, 2005). Genetic studies have suggested that the transcription factors Fos and NF-κB are crucial to osteoclastogenesis by demonstrating that mice deficient in these transcription factors display an osteopetrotic phenotype (Franzoso et al., 1997; Grigoriadis et al., 1994). More recently, expression of Fos and NFATc1 were shown to be induced by RANKL during osteoclastogenesis (Matsuo et al., 2000; Takayanaki et al., 2002a).

Defining the cells that are committed to the osteoclastic lineage is still obscure because of a lack of unique marker(s) to distinguish between uncommitted and committed cells. In osteoclastogenic culture, two distinctive steps occur: conversion of TRAP-negative ($TRAP^-$) precursor cells to $TRAP^+$ mononuclear cells (pre-osteoclasts; pOCs) and fusion of $TRAP^+$ mononuclear pOCs to generate multinuclear $TRAP^+$ mature osteoclasts. Although the precise point of osteoclastic-lineage commitment is not known, $TRAP^+$ pOCs are likely to be at or beyond the commitment point. Thus, TRAP positivity is so far the best distinguishable feature between cells that have undergone osteoclastic commitment and those at the precursor stage. The induction of Fos and NFATc1 by RANKL occurs before the fusion point of $TRAP^+$ mononuclear cells (Takayanaki et al., 2002a), suggesting the possible involvement of these transcription factors in osteoclastic-lineage commitment. NFATc1 binds to the *TRAP* promoter (Ikeda et al., 2004) and activates – probably as a complex with other transcription factors, coactivators and chromatin remodeling proteins (Sharma et al., 2007) – the transcription of the *TRAP* gene.

In this study, we show that the JNK pathway was specifically required for the maintenance of the osteoclastic-commitment status. The $TRAP^+$ phenotype established by RANKL at the pOC stage was lost with concomitant gain of macrophagic features upon the blockade of JNK activity, even in the continuous presence of RANKL. The reversion of pOC by JNK inhibition was associated with depression of calcium/calmodulin-dependent protein kinase (CaMK) expression and subsequent decrease in NFATc1 level. This is the first report to demonstrate that osteoclastic-differentiation steps could be reversed at a certain stage by blockade of a specific signaling pathway.

Results

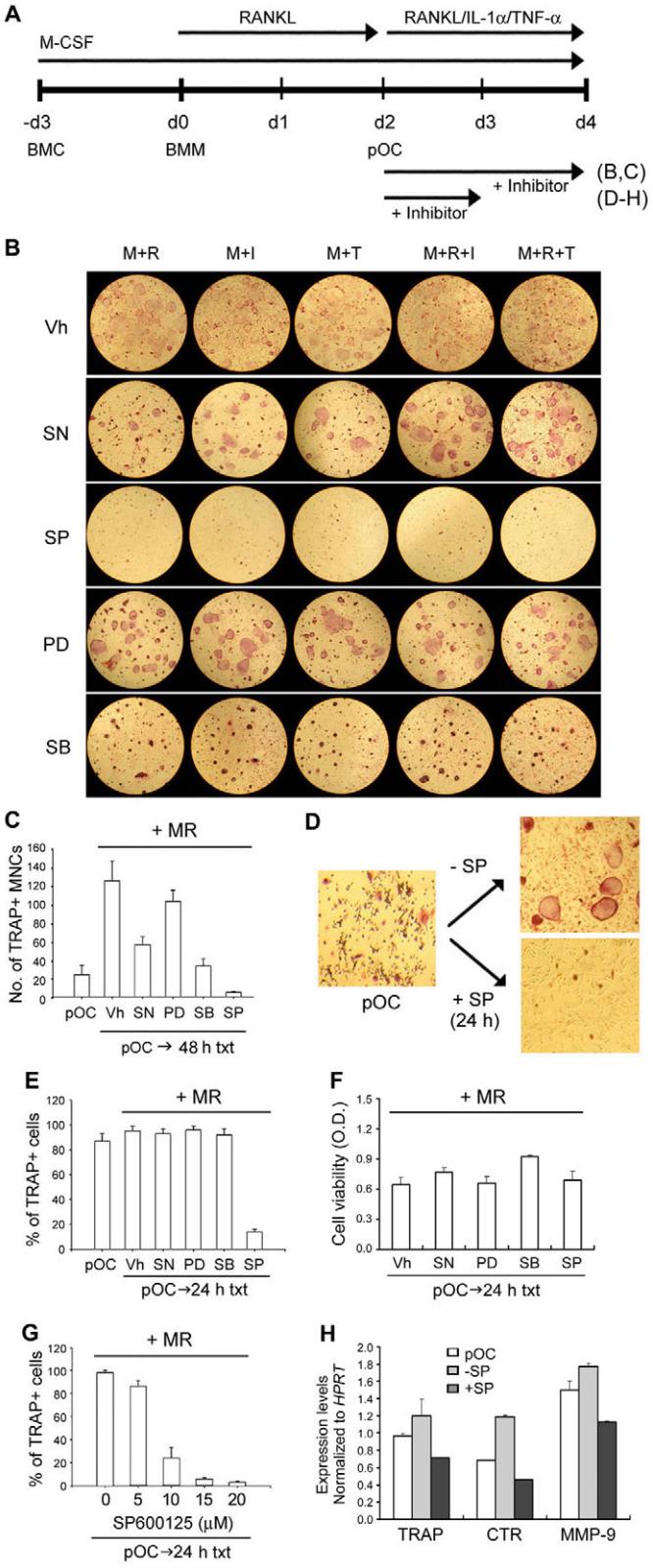
Reversion of committed pre-fusion osteoclasts by JNK inhibition

Bone-marrow-derived macrophage (BMM) cells become morphologically rounder and cytochemically $TRAP^+$ when cultured in the presence of the osteoclast differentiation factor RANKL. Under our experimental conditions, more than 80% of cells cultured for 2 days with RANKL were $TRAP^+$ and mononuclear, with the presence of a few small $TRAP^+$ multinuclear cells (MNCs). These $TRAP^+$ mononuclear cells (pOCs) are considered committed to osteoclastic lineage. pOCs progressively undergo fusion to generate large multinucleated $TRAP^+$ mature osteoclasts upon stimulation with RANKL or inflammatory cytokines such as IL1 and TNFα. In an effort to understand the mechanism for osteoclast-lineage commitment, we set up an experiment in which pOCs were treated with the NF-κB inhibitor SN50, the JNK inhibitor SP600125, the MEK inhibitor PD98059 or the p38 inhibitor SB203580, in the presence of one of the cytokines (RANKL, IL1α or TNFα) or combinations of the cytokines (Fig. 1A). M-CSF was included during the whole culture as a survival factor. When $TRAP^+$ MNC generation was assessed after 48 hours of incubation with the inhibitors and cytokines, little effect of PD98059 was observed, whereas SN50 and SB203580 showed a significant suppression (Fig. 1B,C). SP600125 almost completely blocked $TRAP^+$ MNC formation in all combinations of the cytokines (Fig. 1B,C). Strikingly, SP600125 also decreased the percentage of $TRAP^+$ mononuclear cells (Fig. 1D,E) without reducing the number of total cells and the viability of cells, which was determined by measuring the metabolic activity of cells using CCK-8 (Fig. 1F). This was clearly distinct from the effects of SN50 and SB203580, which reduced MNC formation with no effects on the $TRAP^+$ nature of pOCs (Fig. 1E). The reducing effect of SP600125 on the percentage of $TRAP^+$ cells was dose dependent (Fig. 1G). Because the blockade in JNK activity caused the conversion of $TRAP^+$ cells to $TRAP^-$, we examined the effect of SP600125 on the expression levels of TRAP and other genes related to osteoclastogenesis. The mRNA expression of *TRAP*, calcitonin receptor (CTR) and *MMP9* were significantly reduced by SP600125 treatment at the pOC stage (Fig. 1H).

We next investigated whether the reversion of $TRAP^+$ cells to $TRAP^-$ characteristics by JNK inhibition is restricted up to a certain stage. The JNK inhibitor SP600125 was added at different days of the osteoclastogenic culture of BMMs. Addition of SP600125 at day 2 of the culture elicited reversion of $TRAP^+$ cells to $TRAP^-$, whereas addition at day 3 did not reduce the percentage of $TRAP^+$ cells (Fig. 2A). Similar results were obtained with the RAW264.7 cell line, which undergoes osteoclastic differentiation upon culturing in the presence of RANKL alone. The effect of SP600125 was also observed in these cells when the inhibitor was added at the pOC stage (day 2), but not at the MNC-forming stage (day 3) (Fig. 2B). Next, we blocked JNK activity by overexpressing a dominant-negative form of JNK (JNK-DN) in RAW264.7 cells to further examine the role of JNK in maintaining the $TRAP^+$ pOC characteristics. Transfection of JNK-DN at day 2 caused a prominent reduction in $TRAP^+$ cell percentage, whereas wild-type JNK (JNK-WT) transfection did not have a significant effect (Fig. 2C). JNK-DN transfection at day 3 had no effect on the percentage of $TRAP^+$ cells (Fig. 2C). The data shown in Figs 1 and 2 suggest that JNK activity is specifically required to keep the $TRAP^+$ mononuclear cells in the osteoclast-lineage-committed status.

Attainment of macrophage characteristics upon JNK inhibition

Because the TRAP$^+$ mononuclear pOCs derived from BMMs returned to TRAP$^-$ status following JNK blockade, we next inquired whether this transition is also accompanied by an increase in macrophage characteristics. To determine this probability, the macrophage cell-surface markers CD11b, CD68, CD14 and F4/80 were assessed by flow cytometry. As expected, BMMs expressed these markers (Fig. 3). CD11b-positive cell counts remained high in both JNK-inhibitor (SP600215)-treated and untreated cells (Fig. 3A). The number of CD68-, F4/80- and CD14-positive cells decreased as BMMs became pOCs (Fig. 3B-D). Upon SP600125 treatment of pOCs, the number of cells positive for either CD68, F4/80 or CD14 reached a level that was comparable to or higher than that found in BMMs (Fig. 3B-D). The mean fluorescence intensity (MFI) of the stained cells also showed a similar pattern of changes (Fig. 3E). Thus, SP600215 treatment of pOCs led to



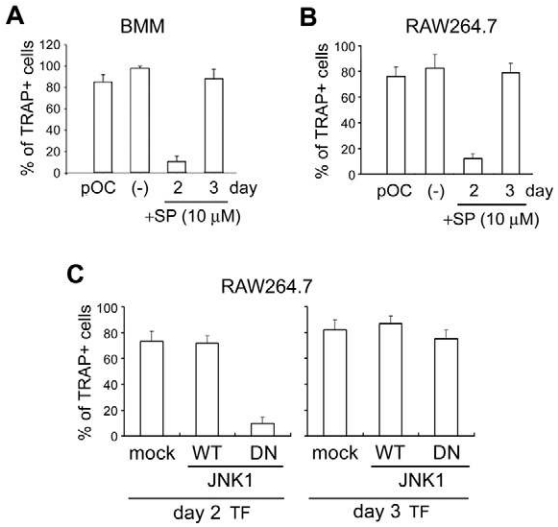


Fig. 2. Stage-dependent induction of phenotypic reversion by JNK inhibition. (A) BMMs were cultured with M-CSF (30 ng/ml) plus RANKL (100 ng/ml). After 2 or 3 days, SP600125 (+SP) or vehicle (–) was added and cells were further incubated for 24 hours before TRAP staining. pOCs were also stained for comparison. (B) RAW264.7 cells were cultured with RANKL (100 ng/ml). Cells were treated with SP600215 at the indicated day or vehicle at day 2 for 24 hours and were then stained for TRAP. (C) RAW264.7 cells cultured with RANKL (100 ng/ml) for 2 (left) or 3 (right) days were transfected with wild type (WT) or dominant negative (DN) JNK. Cells were further incubated with RANKL for 24 hours and the percentage of TRAP$^+$ cells was determined. Data are the mean ± s.d. and are representative of three independent experiments.

Fig. 1. Reversion of committed pOCs by blocking JNK activity. (A) Experimental scheme of osteoclastogenesis from mouse BMMs. Non-adherent bone marrow cells (BMCs) were cultured with M-CSF (30 ng/ml) for 3 days to generate BMMs. BMMs were cultured with M-CSF (30 ng/ml) plus RANKL (100 ng/ml) for 2 days to generate pOCs. (A-C) pOCs were incubated for 48 hours with the NF-κB inhibitor SN50 (SN, 20 μM), JNK inhibitor SP600125 (SP, 10 μM), MEK inhibitor PD98059 (PD, 10 μM), p38 inhibitor SB203580 (SB, 10 μM) or the vehicle (Vh, 0.1% DMSO) in the presence of M-CSF (30 ng/ml) and the indicated combination of RANKL (R, 100 ng/ml), TNFα (T, 20 ng/ml) and IL1α (I, 10 ng/ml). Cells were stained and the number of TRAP$^+$ MNCs was counted. (A,D-F) pOCs were incubated for 24 hours with the inhibitor as above in the presence of M-CSF and RANKL (+MR). Cells were either stained for TRAP (D,E) or incubated with CCK-8 (F) to assess the percentage of TRAP$^+$ cells and the viability of cells, respectively. (A,G) pOCs were treated for 24 hours with SP600125 at the indicated concentration (G) together with M-CSF and RANKL. (A,H) pOCs were incubated for 24 hours with or without SP600125 (10 μM) in the presence of M-CSF and RANKL, and mRNA levels of *TRAP*, calcitonin receptor (CTR) and MMP9 were analyzed by real-time PCR. (C,E-H) Error bars represent s.d. from triplicate samples. Similar results were obtained in two other experiments. txt, treatment.

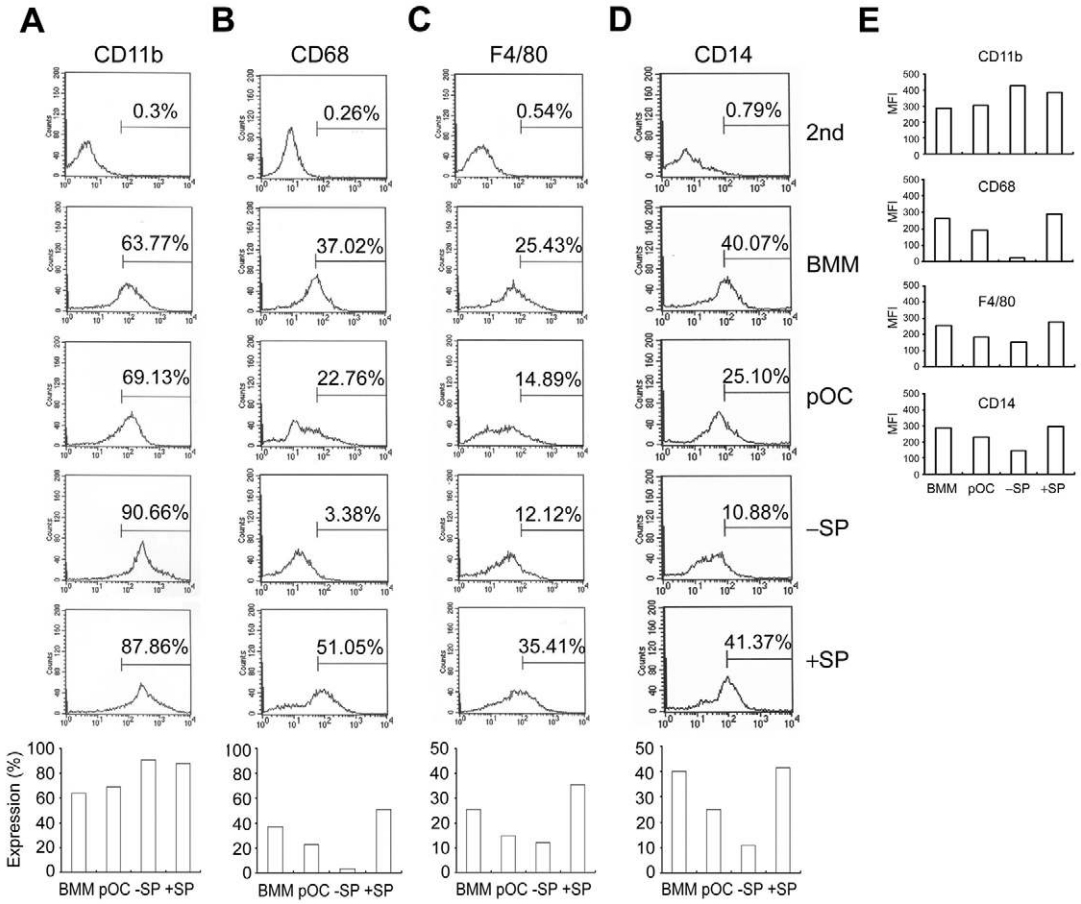


Fig. 3. Expression changes of macrophage surface markers by JNK-inhibitor treatment. BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 2 days to generate pOCs. pOCs were further incubated with M-CSF and RANKL for 24 hours in the absence (–SP) or presence (+SP) of SP600125. (A-D) Cells were stained with fluorescein-conjugated anti-CD11b (A), -CD68 (B), -F4/80 (C) or CD14 (D) as surface markers of macrophages and/or monocytes, and were then subjected to flow-cytometry analyses. The *x*-axes represent fluorescence intensity for FITC-zymosan-positive cells. Bar graphs show the percentage of cells positive for each surface marker. (E) Histograms represent the MFI of the stained cells. Similar results were observed in another experiment.

an increase in cell population that express the macrophage markers, suggesting that the cells have reversed to macrophages from pOCs.

To further characterize the nature of the cells that returned from pOCs to a TRAP$^-$ status in response to JNK blockade, we tested the phagocytic activity of the cells. Cells were incubated with FITC-labeled zymosan microspheres, vigorously washed to remove free microspheres and stained with propidium iodide to visualize each cell in fluorescence microscopic fields. The phagocytosed microspheres showed as yellow in the merged images (Fig. 4A). It has been reported that phagocytic activity decreases when BMMs differentiate into osteoclasts (Mochizuki et al., 2006). In agreement with previous reports, BMMs showed a higher percentage of phagocytic cells and higher amounts of phagocytosed microspheres per cell than pOCs in our study (Fig. 4A). Analysis after 24 hours of incubation of pOCs with RANKL in the absence of SP600125 found the number of phagocytic cells and the extent of phagocytic activity per cell to be further decreased (Fig. 4A). This decrease in phagocytic-cell number and activity level was blocked by the presence of SP600125 during the incubation period (Fig. 4A). The phagocytic activity was also evaluated by flow cytometric analysis. The percentage of phagocytic pOCs decreased to 49.16% from the 80.97% of BMMs (Fig. 4B,C). The culture of pOCs for another day in the absence of SP600125 further decreased the number of cells displaying phagocytic activity to 19.24%, whereas incubation of pOCs with SP600125 increased the number of phagocytic cells to 67.64% (Fig. 4B,C).

Macrophages have the potential to differentiate to dendritic cells in the presence of GM-CSF and interleukin-4 (IL4) (Alnaeeli et al., 2006). We examined the response of pOCs treated with SP600125 in comparison with BMMs and vehicle-untreated pOCs when the cells were cultured in the presence of GM-CSF plus IL4. SP600125-treated cells behaved like BMMs, showing generation of CD11c$^+$ F4/80$^-$ dendritic cells (Fig. 4D,E). Collectively, the results shown in Figs 3 and 4 suggest that the cells converted to TRAP$^-$ from pOCs upon SP600125 treatment became macrophage-like.

Effects of JNK inhibition on pOCs derived from human precursor cells

We next investigated whether the conversion of committed TRAP$^+$ pOCs to TRAP$^-$ cells by JNK blockade could also be induced in human cells. Human peripheral blood monocytes (PBMCs) were cultured in osteoclastogenic medium until the percentage of TRAP$^+$ cells reached ~80% (Fig. 5A). The addition of SP600125 to the TRAP$^+$ pOCs for 24 hours reduced the TRAP$^+$ cell percentage to ~30% (Fig. 5B). As was the case with BMM-derived

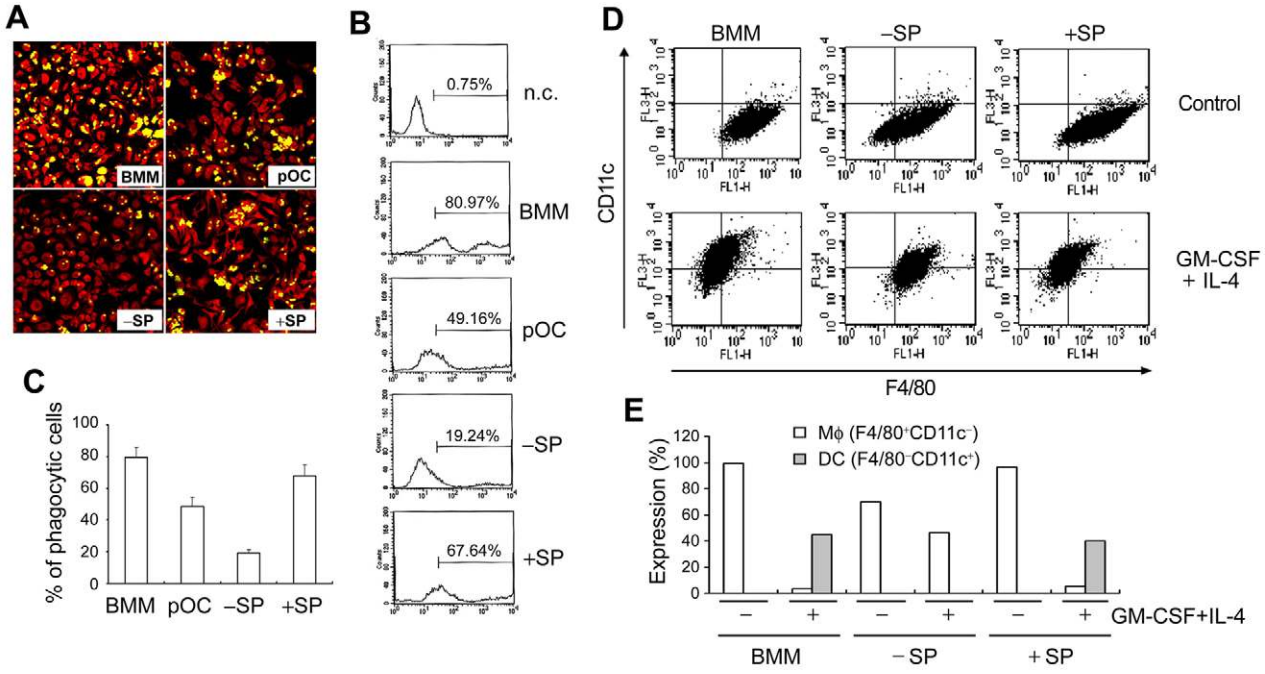


Fig. 4. Determination of phagocytic activity and dendritic differentiation potential. (A-C) BMMs, pOCs and pOCs treated with (+SP) or without (–SP) SP for 24 hours were incubated with FITC-labeled zymosan for 45 minutes as described in the Materials and Methods. After washing, cells were permeabilized and incubated with propidium iodide. (A) Cells that have taken up zymosan appear yellow because of overlap of green (FITC-zymosan) and red (propidium iodide) fluorescences in confocal microscopy when images were merged. (B) The cells were also analyzed for FITC fluorescence by flow cytometry. n.c., negative control cells incubated without FITC-zymosan and stained with FITC-Ig. The *x*-axes represent fluorescence intensity for FITC-zymosan-positive cells. (C) Histograms show the percentage of phagocytic cells determined by flow cytometry. Error bars represent s.d. from triplicate samples. Results are representative of three independent experiments. (D,E) BMMs, and –SP and +SP cells were stained for CD11c and F4/80 before and after culture in dendritic-differentiation medium as described in the Materials and Methods. The percentage of dendritic cells (DCs) ($F4/80^{-}CD11c^{+}$) and macrophage (MΦ) population ($F4/80^{+}CD11c^{-}$) was determined by flow cytometry.

pOCs (Fig. 4), the number of PBMC-derived pOCs with phagocytic activity decreased (Fig. 5C,D). SP600125 treatment of human pOCs for 24 hours caused an increase in the phagocytic cell population from 24.31% to 35.29% (Fig. 5C,D). These data demonstrate that the phenotypic reversion of pOCs by JNK inhibition is conserved between mouse and human osteoclast-differentiation programs.

Involvement of NFATc1 in pOC reversion by JNK blockade

Several transcription factors are crucial for osteoclast differentiation. Among them, NFATc1 has been suggested to play a master-switch role in osteoclast differentiation (Takayanagi et al., 2002a). A previous report showing that cooperative activity between Jun-Fos complexes and NFATc2 is necessary for NFATc1 expression (Ikeda et al., 2004) suggests possible JNK involvement in NFATc1 regulation. We found that SP600125 treatment blocked the RANKL-induced transcriptional activity of NFATc1 in pOCs (Fig. 6A). In line with the reduced activity, both the mRNA and protein levels of NFATc1 were downregulated by SP600125 treatment of pOCs (Fig. 6B,C). These data suggest that NFATc1 is a key downstream player in the phenotypic reversion by JNK blockade. To support this notion, we knocked down the NFATc1 expression level in pOCs by using retrovirus for *NFATc1* siRNA and assessed the percentage of $TRAP^{+}$ cells. When NFATc1 expression was reduced by siRNA (Fig. 6D), $TRAP^{+}$ cell percentage was substantially decreased (Fig. 6E,F). Furthermore, the retroviral infection of active *NFATc1* prevented the decrease in $TRAP^{+}$ cells by SP600125 (Fig. 6G,H). Therefore, the expression of NFATc1 controlled by JNK activity at the pOC stage is required for maintaining the $TRAP^{+}$ phenotype for further differentiation into mature osteoclasts.

Role of CaMK in the JNK-mediated maintenance of osteoclastic commitment

We next analyzed the gene-expression-profile changes in pOCs caused by JNK inhibition by using Affymetrix human GeneChip. pOCs were generated from three different human PBMC preparations in osteoclastogenic medium and further cultured in the presence (SP+ cells) or absence (SP– cells) of SP600125 for 24 hours. The gene-expression levels in the SP+ and SP– cells were compared together and with those in PBMCs (SP0 cells). The genes for which the expression level increased or decreased in SP– cells compared with that in SP0 cells with the change being attenuated by SP treatment by more than 1.5-fold in all three different samples were collected (see Table S1 and S2 in the supplementary material). Of special interest, *CamKIV* (*CamK4*) mRNA decreased by 1.73-fold in response to SP600125 treatment in pOCs (Fig. 7A). We then sought to delineate the relationship between CaMK and the JNK-blockade-induced reversion of pOCs. The expression of CaMK-family kinases was confirmed by real-time PCR in BMMs, pOCs and pOCs further cultured in the presence or absence of SP600125. The mRNA expression of *CamKIIα* (*CamK2a*), *CamKIIδ* (*CamK2d*) and *CamKIV* increased in pOCs compared with the level in BMMs (Fig. 7B). Treatment of pOCs with SP600125 decreased *CamKIIδ* and *CamKIV* mRNA, whereas *CamKIIα* was not affected (Fig. 7B). Thus, we hypothesized that CaMKIIδ and CaMKIV are responsible for JNK-mediated maintenance of osteoclastic

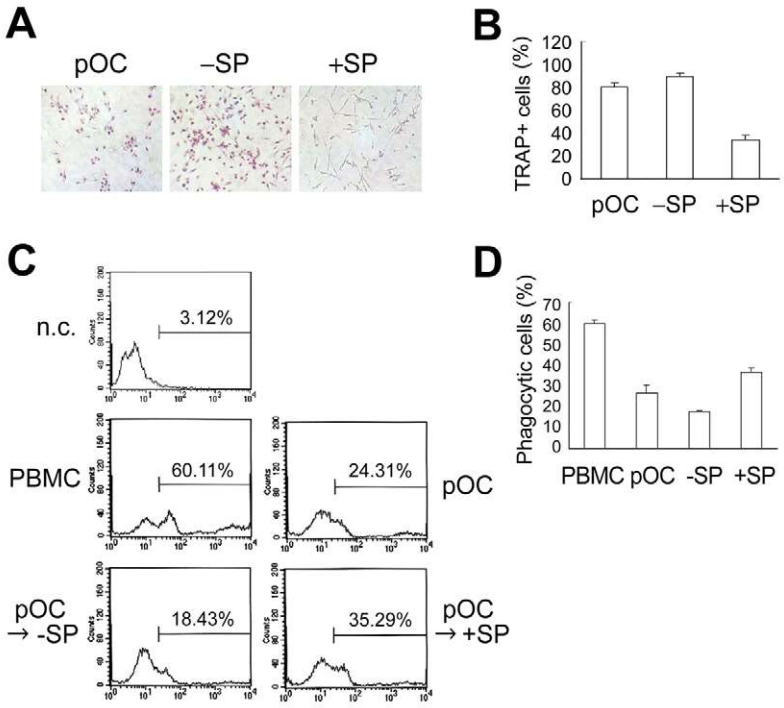


Fig. 5. Reversion of human PBMC-derived pOCs by JNK blockade. (A,B) PBMCs were cultured with M-CSF (100 ng/ml) and RANKL (100 ng/ml) for 3 days to the pOC stage. pOCs were treated with (+SP) or without (–SP) SP600125 (10 μM) for 24 hours and TRAP$^+$ cells were counted. (C,D) PBMCs, PBMC-derived pOCs and pOCs treated with (+SP) or without (–SP) SP for 24 hours were incubated with FITC-zymosan and subjected to flow cytometry as described in the Materials and Methods. (C) n.c., negative control cells incubated without FITC-zymosan and stained with FITC-Ig. The *x*-axes represent fluorescence intensity for FITC-zymosan-positive cells. (B,D) Error bars represent s.d. from triplicate samples. Similar results were obtained in three independent experiments.

commitment. To gain evidence for this notion, we knocked-down CaMKII or CaMKIV expression in pOCs using siRNA. The respective *CamKIIδ* or *CamKIV* siRNAs were effective and specific (Fig. 7C). After CaMKII or CaMKIV knockdown, the percentage of TRAP$^+$ cells was substantially reduced (Fig. 7D). In addition, a significantly higher percentage of pOCs that were transduced with *CamKIIδ* and *CamKIV* siRNA showed phagocytic activity compared with the cells transduced with control siRNA (Fig. 7E).

Because NFATc1 also mediated the JNK-blockade-dependent reversion of pOCs (Fig. 6), we inquired whether CaMK activity would be linked to NFATc1 regulation. First, the effect on NFATc1 expression of CaMK downregulation by siRNA was assessed. Introduction of *CamKIIδ* and *CamKIV* siRNA in pOCs resulted in a decrease in the NFATc1 level (Fig. 8A). Next, the effect of CaMK-activity inhibition with the pharmacological reagent KN93 was examined. KN93 treatment also reduced NFATc1 expression (Fig. 8B), suggesting that the catalytic activity of CaMK is required for NFATc1 expression in pOCs. Consistently, KN93 treatment of pOCs decreased the percentage of TRAP$^+$ cells (Fig. 8C) and increased phagocytic activity (Fig. 8D). These data suggest that JNK modulates the expression of CaMK and subsequently that of NFATc1 to maintain the commitment status for successful completion of osteoclastic differentiation.

Discussion

Both lineage commitment from HSCs and subsequent differentiation in the bone microenvironment are essential steps for osteoclast generation. However, the osteoclastic-lineage commitment has been ill-defined because of lack of markers specific for cells committed to osteoclastogenesis. In this regard, it was reported that mouse bone-marrow monocytic cells rendered to become RANK$^+$ by treatment with M-CSF, which were designated as late-stage precursors, could still differentiate into macrophage cells (Arai et al., 1999). In that study, it was proposed that the stage of osteoclastic commitment is late in the differentiation process (Arai et al., 1999). In another study, mouse BMMs exposed to RANKL together with M-CSF and TGFβ for 24 hours, under culture conditions in which full osteoclastic differentiation is completed by 72 hours, were considered as cells committed to osteoclastogenesis (Mochizuki et al., 2006). These 'committed' cells exhibited moderate levels of RANK and NFATc1 and a low level of TRAP, and could not differentiate to dendritic cells in the presence of GM-CSF (Mochizuki et al., 2006). The TRAP$^+$ pOCs used in our study are probably at the state comparable to the 'committed' cells in the latter study (Mochizuki et al., 2006) or at a point a little further progressed in the osteoclastic differentiation process. Regardless of the precise differentiation status of pOCs, it is clear that JNK inhibition caused loss of the TRAP$^+$ phenotype of pOCs even in the presence of RANKL in our study. However, the cells already fused did not lose their TRAP$^+$ characteristics. Therefore, JNK activity is required until the fusion stage during osteoclastogenesis to maintain the status of osteoclastic commitment. Our study is distinct from previous genetic and pharmacological studies (David et al., 2002; Ikeda et al., 2004) that investigated the effects of a JNK blockade that was present from the beginning of the differentiation, which can not distinguish the role of JNK for the initiation of commitment itself from the role of JNK after the commitment has been established.

The cells converted to TRAP$^-$ from TRAP$^+$ pOCs by JNK inhibition appeared macrophage-like. Several lines of data support the macrophage-likeness of the converted cells. First, FACS analyses showed that CD68, CD14 and F4/80 expression became lower in pOCs and further decreased in untreated pOCs compared with BMMs, whereas it was comparable between the SP600125-treated pOCs and BMMs (Fig. 3). Second, the phagocytic activity of cells gradually decreased as the BMM or PBMC osteoclast precursor cells differentiated to osteoclasts (Figs 4, 5). Treatment with the JNK inhibitor SP600125 at the pOC stage reversed the direction of phagocytic activity change, i.e. untreated cells showed lower phagocytic activity than pOCs, whereas SP600125-treated cells had higher activity than pOCs (Figs 4, 5). Furthermore, pOCs treated with SP600125 showed a behavior more like BMMs in conditions in which dendritic differentiation was driven by GM-CSF and IL4 (Fig. 4D,E). In addition, removal of SP600125 at the pOC stage after 24 hours of treatment allowed normal osteoclast formation (data not shown), suggesting that treated cells retained osteoclastogenic potential. Thus, JNK blockade in pOCs seems to return the committed cells to the precursor-like state, reversing the differentiation program.

In our study, the molecular mechanism underlying the JNK-inhibition-induced reversal of osteoclastic commitment was

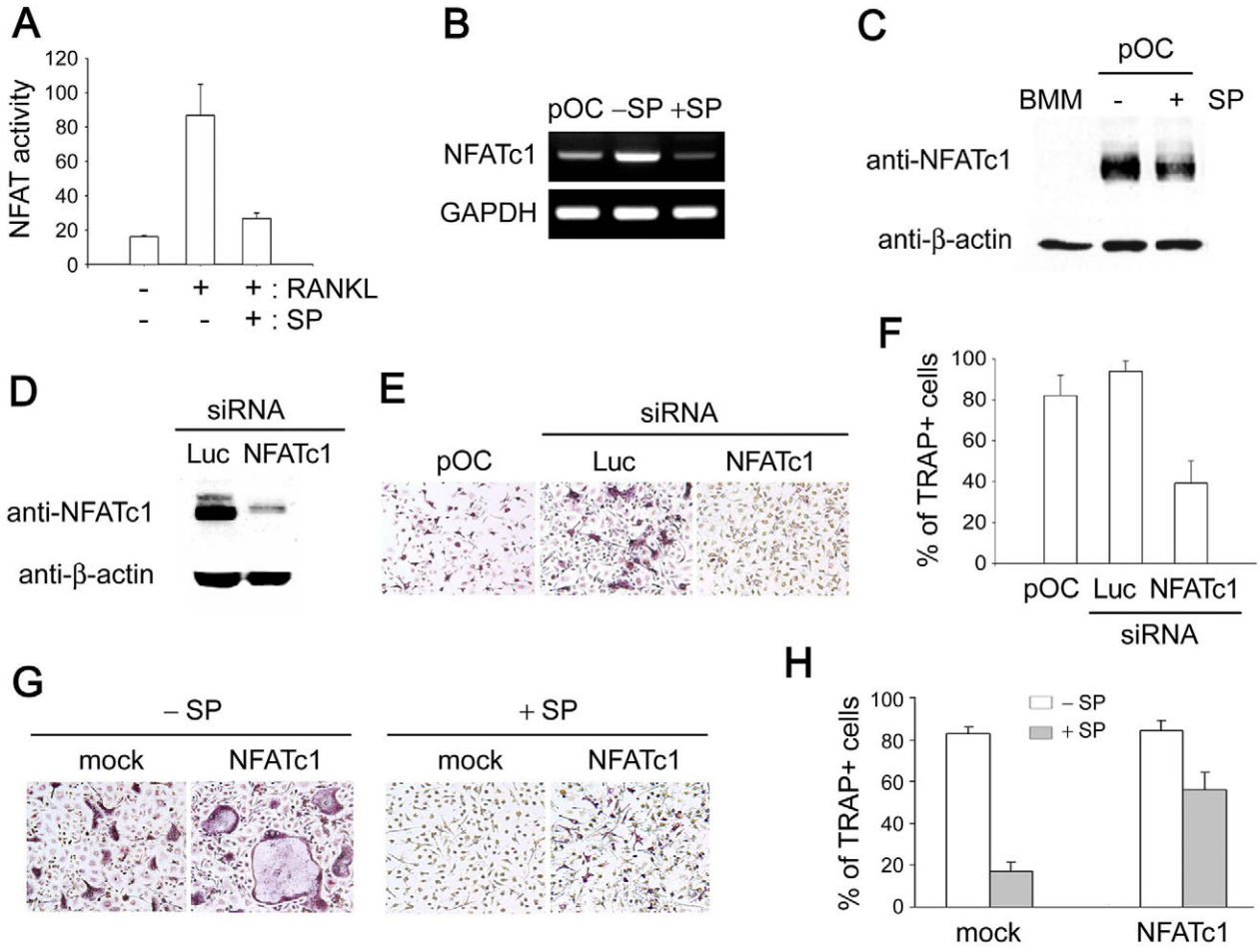


Fig. 6. Involvement of NFATc1 in the reversion of differentiation by JNK inhibition. (A) RAW264.7 cells were transfected with NFAT-reporter luciferase plasmid and stimulated with RANKL for 24 hours in the presence or absence of SP600125 (SP, 10 μM). Luciferase activity was assessed with cell lysates. (B,C) BMM-derived pOCs were treated with or without SP600125 (10 μM) for 24 hours. The mRNA (B) and protein (C) levels of NFATc1 were determined by reverse transcriptase (RT)-PCR and western blotting analyses, respectively. (D-F) pOCs were infected with *NFATc1* siRNA or control siRNA (Luc) retroviruses. (D) The reduced level of NFATc1 was determined by western blotting. (E) Infected cells were cultured with RANKL (100 ng/ml) and M-CSF (30 ng/ml) for 24 hours before TRAP-staining. (F) The percentage of $TRAP^+$ cells was assessed. (G,H) pOCs were infected with active NFATc1 or control retroviruses and incubated with or without SP600125 (10 μM) in the presence of RANKL and M-CSF for 24 hours. The percentage of $TRAP^+$ cells was determined. (A,F,H) Data are mean ± s.d. of triplicate samples. All results are representative of three independent experiments.

attributable to CaMK and NFATc1. JNK inhibition reduced the expression of both NFATc1 and CaMK (Figs 6, 7). NFATc1 is a key transcription factor for the expression of *TRAP* and of other osteoclastogenesis-associated genes (Ikeda et al., 2004; Kim et al., 2005; Matsumoto et al., 2004; Sharma et al., 2007). The upregulation of NFATc1 by RANKL requires an auto-amplification process in which active NFATc1 binds to its own promoter and enhances transcription (Asagiri et al., 2005). JNK inhibition also decreased specifically the expression level of CaMKIIδ and CaMKIV in pOCs (Fig. 7A,B). This downregulation of CaMK by JNK inhibition appeared to involve AP-1, the complex of Fos and Jun family members, because SP600125 reduced AP-1 activity in promoter reporter assays (data not shown). It is likely that Jun phosphorylation that otherwise enhances the transcription activity of AP-1 was blocked by SP600125. In support of this notion, promoter-sequence analyses revealed two and one AP-1 binding sites in CaMKIIδ and CaMKIV, respectively (data not shown). Despite several documentations describing the involvement of CaMK in the regulation of JNK activity, there has been no report showing CaMK regulation by JNK in any cell system. To our knowledge, our study is the first to show JNK-mediated regulation of CaMK. Interestingly, although the overexpression of NFATc1 in SP600125-treated pOCs resulted in the restoration of the $TRAP^+$ population, it could not facilitate the generation of mature, multinuclear osteoclasts (Fig. 6G,H). Thus, it is likely that the JNK-CaMK-NFATc1 pathway described in the present study is not sufficient to promote the differentiation of osteoclasts from pOCs. These results not only suggest that NFATc1 downstream of JNK is important for the maintenance of osteoclastic commitment, but also indicate that other JNK-dependent signaling pathways are required to fully support osteoclast differentiation.

In our study, both gene knockdown of *CamKIIδ* and *CamKIV*, and treatment of cells with the CaMK inhibitor KN93 elicited the reversion of pOCs to $TRAP^-$ cells while elevating the phagocytic activity (Figs 7, 8). During the progression of our study, Sato et al. showed that KN93 blocked Fos induction by RANKL in BMMs and suggested that CaMKIV regulates Fos transcription through

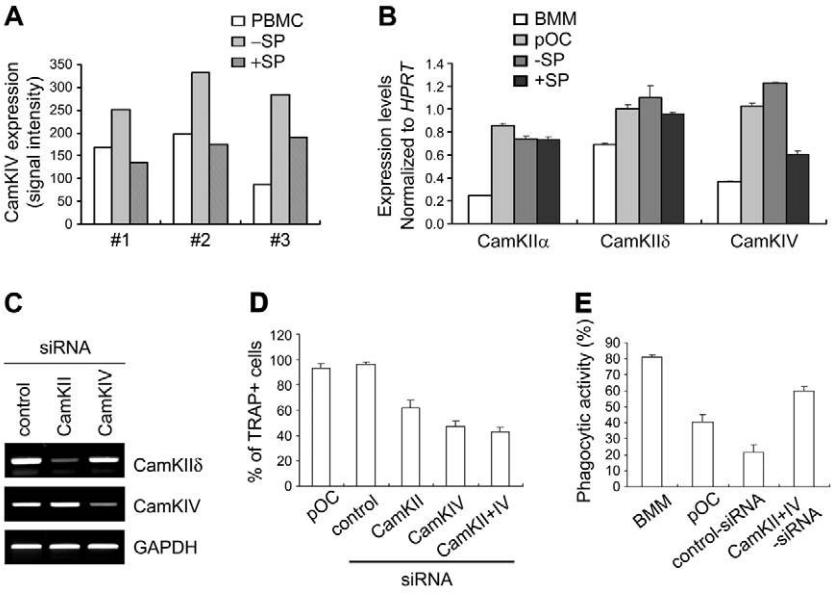


Fig. 7. Role of CaMK in the JNK-dependent maintenance of the committed state. (A) Relative signal intensity of CaMKIV from the DNA microarray analysis of three different human samples (1-3) described in the Materials and Methods is presented. (B) Mouse BMM-derived pOCs were treated with (+SP) or without (–SP) SP600125 (10 μM) for 24 hours in the presence of RANKL (100 ng/ml) and M-CSF (30 ng/ml). Expression of CaMKIIα, CaMKIIδ and CaMKIV was examined by real-time PCR. (C-E) pOCs were transfected with oligonucleotides for *CamKIIδ* and/or *CamKIV* siRNA. (C) Knockdown of CaMK expression was confirmed by reverse transcriptase (RT)-PCR. (D) Cells were TRAP-stained and the percentage of $TRAP^+$ cells was determined. (E) Cells were also evaluated for phagocytic activity with FITC-labeled zymosan. (B,D,E) Error bars represent s.d. from triplicate samples. Results are representative of three independent experiments.

CREB phosphorylation (Sato et al., 2006). In pOCs, gene knockdown of either *CamKIIδ* or *CamKIV* led to a reduction in Fos level (data not shown). Fos, in turn, is one of the key factors required for NFATc1 induction by RANKL (Matsuo et al., 2004; Takayanagi et al., 2002a). Consistently, the addition of both CaMK siRNA and KN93 to pOCs decreased NFATc1 levels (Fig. 8A,B). One interesting observation was that the addition of *Fos* siRNA reduced CaMKIIδ and CaMKIV levels (data not shown). Because the promoters of those CaMK proteins contain AP-1-binding sites (see above), it is plausible that Fos upregulation by CaMK feeds back to elevation of CaMKIIδ and CaMKIV expression. The NFATc1 auto-amplification and CaMK-Fos positive feedback might reinforce the profound and sustained expression of NFATc1 in the late phase of osteoclastogenesis. In our present study, SP600125 decreased NFATc1 levels in pOCs even in the continuous presence of RANKL, and introduction of *NFATc1* siRNA converted $TRAP^+$ cells to $TRAP^-$ (Fig. 6). Therefore, NFATc1 is the ultimate point to which the JNK signaling reaches through CaMK for the maintenance of osteoclastic-commitment status (Fig. 9). For activation of both CaMK and NFATc1, Ca^{2+} is required. pOCs manifest an oscillation response in the intracellular Ca^{2+} level (Takayanagi et al., 2002a). Therefore, in addition to the expression induction of CaMK and NFATc1, RANKL signaling might stimulate their activity by regulating intracellular Ca^{2+} in pOCs to sustain the osteoclastic-commitment state.

In summary, we showed here for the first time that JNK is specifically involved in maintaining the commitment state during osteoclastogenesis by RANKL. We also provided evidence that this function of JNK is mediated by regulation of CaMK, which is crucial for maintaining high NFATc1 levels in committed cells. The JNK-CaMK pathway might be an efficient therapeutic target for blocking cells already committed for osteoclast formation.

Materials and Methods

Reagents and antibodies

Recombinant soluble human RANKL (310-01), murine RANKL (315-11), human M-CSF (300-25), murine M-CSF (315-02) and IL1α (211-11A) were purchased from Peprotech EC (London, UK). Recombinant murine TNFα (416-MT-50) was obtained from R&D Systems (Minneapolis, MN). Polyclonal antibodies (Abs) against NFATc1 (sc7294) and CD14 (sc-9150) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin monoclonal Ab (mAb) was purchased from Sigma-Aldrich (St Louis, MO). Rat FITC-conjugated anti-mouse CD11b mAb (557396), hamster PE-Cy7-labeled anti-CD11c mAb (558079), rat IgG2b-PE and mouse IgM-FITC were obtained from BD Pharmingen (San Jose, CA). Rat FITC-labeled anti-mouse F4/80 mAb (Cl:A3-1) was purchased from Serotec (Raleigh, NC). FITC-zymosan (Z2841) was purchased from Molecular Probe (Carlsbad, CA). SN50 (NF-κB inhibitor) (481480), SP600125 (JNK inhibitor) (420119), PD98059 (MEK inhibitor) (513000) and SB203580 (p38 inhibitor) (559389) were obtained from Calbiochem (San Diego, CA). Acid Phosphatase kit (387A) was obtained from Sigma-Aldrich. Cell-counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan).

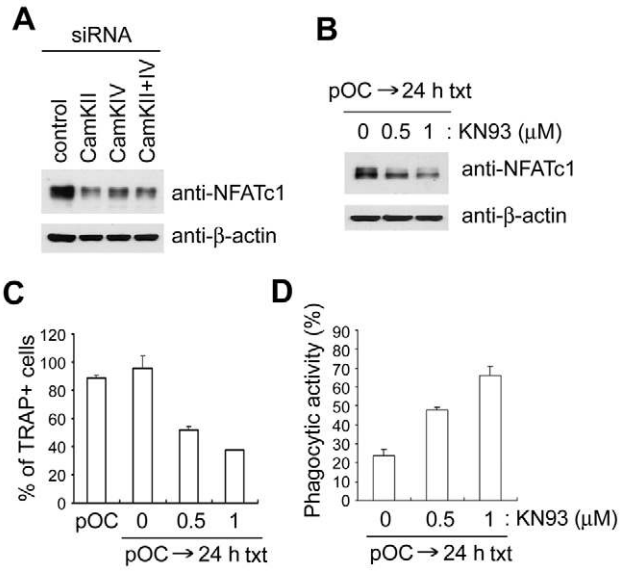


Fig. 8. Modulation of NFATc1 level by CaMK in pOCs. (A) Mouse BMM-derived pOCs were transfected with oligonucleotides for *CamKIIδ* and/or *CamKIV* siRNA, and the protein level of NFATc1 was analyzed by western blotting. (B-D) pOCs were treated for 24 hours with or without KN93 at the indicated concentrations in the presence of RANKL and M-CSF. NFATc1 protein level (B), the percentage of $TRAP^+$ cells (C) and phagocytic activity (D) were determined. (C,D) Error bars represent s.d. from triplicate samples. Results are representative of three independent experiments. txt, treatment.

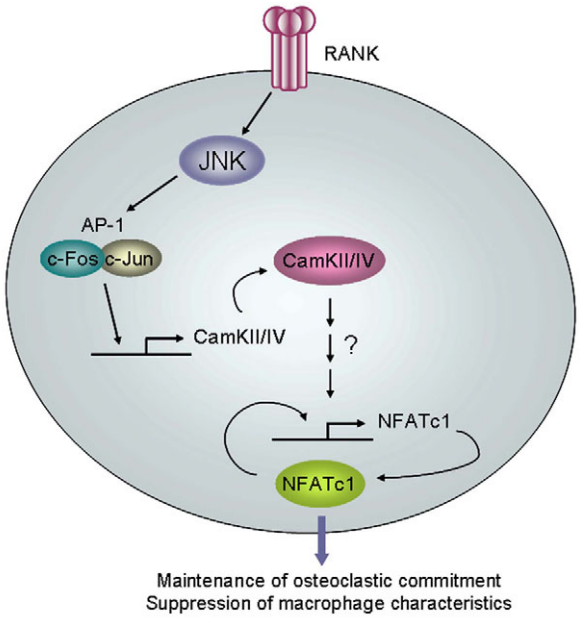


Fig. 9. A diagram illustrating the role of JNK, CaMK and NFATc1 in maintaining the committed state during osteoclastogenesis by RANKL. JNK stimulates AP-1 transcription-factor activity and upregulates CaMKII and CaMKIV in committed pOCs. The elevated CaMK level in pOCs leads, through a mechanism yet to be elucidated, to a sustained increase in NFATc1 level, which is required to keep *TRAP* gene expression 'on' in committed pOCs.

Osteoclastogenic culture of mouse bone-marrow-derived macrophages

For osteoclastogenesis from mouse bone-marrow-derived precursor cells, 6- to 8-week-old ICR mice were used (Charles River Laboratories, Wilmington, MA). Animal experimental protocols were approved by the committee on the care and use of animals in research at Seoul National University. Mouse bone-marrow space of the tibiae and femora was flushed with α-minimal essential medium (MEM) containing 100 U/ml penicillin and 100 μg/ml streptomycin. After removing erythrocytes by lysing in ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.2), the bone-marrow cells were plated on 100-mm culture dishes and cultured in α-MEM supplemented with 10% fetal bovine serum (FBS) for 16-24 hours in 5% CO_2 at 37°C. Non-adherent cells were collected, plated on 100-mm bacterial dishes and cultured for 3 days in the presence of 30 ng/ml M-CSF. The adherent cells were considered to be BMMs and were used as osteoclast precursor cells. BMMs were seeded on 48-well plates at 5×10^4 cells/well and cultured with 100 ng/ml RANKL plus 30 ng/ml M-CSF. Cells were stained for TRAP using the Acid Phosphatase kit as per the manufacturer's instruction (Sigma-Aldrich). The percentage of $TRAP^+$ cells among the total cells and the number of MNCs containing more than three nuclei were scored. After 2 days of BMM culture in the osteoclastogenic medium, more than 80% of total cells were $TRAP^+$ and most of the cells were pOCs, with a few small MNCs. The maximum level of formation of fully mature osteoclasts was achieved after 4 days.

Cell-viability assay

Cell viability was assessed using CCK-8 according to the manufacturer's protocol (Dojindo Laboratories). Cells were incubated with the CCK-8 reagent (100 nM) for 45 minutes and optical density was determined at 450 nm.

Osteoclastogenic culture of RAW264.7 cells

For osteoclastogenesis from RAW264.7 cells, cells were seeded in 48-well plates at 1×10^4 cells/well in α-MEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in the presence of 100 ng/ml RANKL without M-CSF addition. RAW264.7-derived pOCs were obtained after 2 days and mature osteoclasts were observed after ~3.5-4 days.

In some experiments, RAW264.7 cells were cultured with 100 ng/ml RANKL for 2 or 3 days. Cells were transfected for 3 hours with the wild-type or dominant-negative JNK plasmid DNA using Lipofectamine 2000 reagent (Invitrogen) in α-MEM/10% FBS. At 16 hours after transfection, RANKL was added to 100 ng/ml and cells were further incubated for 24 hours before TRAP staining.

PBMC culture

Experiments with human blood samples were approved by the Institutional Review Board for Medical Research Ethics. Peripheral blood of healthy donors was separated by centrifugation on Ficoll-Histopaque (25-072-CV, Cellgro), washed with PBS and then resuspended in α-MEM supplemented with 10% FBS. For osteoclast formation, PBMCs were cultured in α-MEM/10% FBS in the presence of 100 ng/ml M-CSF and 100 ng/ml RANKL as previously described (Chang et al., 2007). After 3 days of culture, pOCs were formed. Mature osteoclasts were generated after 7 days.

Retroviral transduction

Recombinant retroviruses were prepared as previously described (Huang et al., 2000). pOCs derived from BMMs were infected with the prepared retroviruses for ~12-24 hours.

Phagocytosis assay

Cultured cells (2×10^5/ml) were incubated with FITC-zymosan for 45 minutes. After washing three times with 1% FBS/PBS to remove free FITC-zymosan, cells were fixed, permeabilized and stained with propidium iodide. The fluorescence of cells was detected by confocal microscopy (OLYMPUS-FV300) and FACS analyses (FACSCalibur, BD).

Dendritic-cell differentiation

Cells (5×10^5 cells/ml) were cultured in modified RPMI 1640 medium (BioWhittaker) in the presence of 10 ng/ml IL4 (Peprotech) and 10 ng/ml GM-CSF (Immunex/Amgen) for 3 days based on the method as described previously (Steptoe et al., 2002). To determine the phenotype of differentiated dendritic cells, cells were stained by FITC-labeled anti-F4/80 and PE-Cy7-labeled anti-CD11c, and were subjected to cytometric analyses (Alnaeeli et al., 2006).

Flow-cytometry analysis

Antibodies used for FACS analysis are described above. For surface-marker staining, cells were incubated with mAbs for 20 minutes on ice and washed three times with PBS. Flow-cytometric analysis was performed, using a FACSCalibur flow cytometer. CellQuest software (Becton Dickinson) was used for data acquisition and analysis.

Real-time PCR and conventional reverse transcriptase (RT)-PCR analysis

For real-time PCR analysis, total RNA was isolated with the RNeasy total RNA isolation kit (Qiagen, Hilden, Germany) and reverse transcribed using RT Superscript II (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. PCR reactions were performed using ABI Prism 7500 Sequence Detection System with SYBRGreen PCR master mix (Applied Biosystems, Warrington, UK) for 40 cycles of 15-second denaturation at 95°C and 60-second amplification at 60°C. Quantitative PCR primer sequences were as follows: 5′-AGATTTGTGGCTGTGGGCGA-3′ (TRAP forward); 5′-AAGTCAGCGCCCATCGTCTG-3′ (TRAP reverse); 5′-GCAACCGAACCTGGTCCAACTAT-3′ (CTR forward); 5′-AAGCAGCAATCGACAAGGAGTGA-3′ (CTR reverse); 5′-GACGGCACGCCTTGGTGTAG-3′ (MMP9 forward); 5′-GGCCCTCAAAGATGAACGGG-3′ (MMP9 reverse); 5′-TCAGCCTGCATCGCCTATATCC-3′ (CaMKIIα forward); 5′-AAGTGGACGATCTGCCATTTGC-3′ (CaMKIIα reverse); 5′-CTGCCTTTGAACCTGAAGCATTG-3′ (CaMKIIδ forward); 5′-TGAACGTGTGGGTTGAGGATGAT-3′ CaMKIIδ reverse); 5′-GGAGGAGACCTCCAGTATGGTGC-3′ (CaMKIV forward); 5′-CTCCTCAGTCATGGGGTCCATTT-3′ (CaMKIV reverse). Dissociation curves were analyzed with SDS software (Applied Biosystems) and data were presented as relative expression normalized by the expression of HPRT. The RT-PCR analyses were performed as described (Huang et al., 2006). The sequences of primers used are as follows: 5′-GGTGGAAGACGTACTTCCTAGCTG-3′ (NFATc1 forward); 5′-CTTCCAGGCTGGGCAGGT-3′ (NFATc1 reverse); 5′-CTGGCACACCTGGGTATCTT-3′ (CaMKIIδ forward); 5′-ATTCTGGTGACGGAAAATCG-3′ (CaMKIIδ reverse); 5′-AGCTGGTCACAGGAGGAGAA-3′ (CaMKIV forward); 5′-AATTTTGAGGGGTGCATCAG-3′ (CaMKIV reverse); 5′-ACCACAGTCCATGCCATCAC-3′ (GAPDH forward); and 5′-TCCACCACCCTGTTGCTGTA-3′ (GAPDH reverse).

Western blotting analysis

Cells were disrupted in a lysis buffer containing 1% NP-40 and the cell lysates were subjected to western blotting as previously described (Ryu et al., 2006).

Gene knockdown

The siRNA target sense sequence for NFATc1 was 5′-AGACGTACTTCCTAGCTGCAA-3′ and the loop sequence was 5′-TTGATATCCG-3′. The siRNA-insert DNA was cloned into pSuper-retro vector (Oligoengine, Seattle, WA) using a *Bam*HI and *Hind*III site. For knockdown of CaMK genes, RNA-duplex oligonucleotides (Invitrogen, Carlsbad, CA) were used. The sequences were 5′-UUCUGGAGAAAGAUACCCAGGUGUG-3′ for *CamKII* and 5′-UCCACAAUCCUGUCAAACAGUUCUC-3′ for *CamKIV*.

Gene-expression profiling

Human PBMCs and PBMC-derived pOCs treated with (SP+) or without (SP–) the JNK inhibitor SP600125 for 24 hours were used for the microarray analysis. Total RNA was extracted using the RNeasy total RNA isolation kit (Qiagen). RNA (10 μg) from each sample was transcribed to double-stranded cDNA using SuperScript II RT (Invitrogen) with oligo-dT primer containing the T7 RNA polymerase site on the 5′ end. The cDNA serves as the template for an in vitro transcription reaction in the presence of biotin-modified ribonucleotides to produce single-stranded RNA. The biotin-labeled RNA was fragmented and hybridized with the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA) as per the manufacturer's instruction. The array chips were scanned with the GeneArray scanner (Affymetrix). Data analysis was performed using Microarray Suite (MAS) 5.0. (Affymetrix).

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