MCP-1-induced Human Osteoclast-like Cells Are Tartrate-resistant Acid Phosphatase, NFATc1, and Calcitonin Receptor-positive but Require Receptor Activator of NFκB Ligand for Bone Resorption*

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MCP-1 (monocyte chemotactic protein-1) is a CC chemokine that is induced by receptor activator of NFκB ligand (RANKL) in human osteoclasts. In the absence of RANKL, treatment of human peripheral blood mononuclear cells with macrophage colony-stimulating factor and MCP-1 resulted in tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells that are positive for calcitonin receptor (CTR) and a number of other osteoclast markers, including nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (NFATc1). Although NFATc1 was strongly induced by MCP-1 and was observed in the nucleus, MCP-1 did not permit the formation of bone-resorbing osteoclasts, although these cells had the typical TRAP+/CTR+ multinuclear phenotype of osteoclasts. Despite a similar appearance to osteoclasts, RANKL treatment was required in order for TRAP+/CTR+ multinuclear cells to develop bone resorption activity. The lack of bone resorption was correlated with a deficiency in expression of certain genes related to bone resorption, such as cathepsin K and MMP9. Furthermore, calcitonin blocked the MCP-1-induced formation of TRAP+/CTR+ multinuclear cells as well as blocking osteoclast bone resorption activity, indicating that calcitonin acts at two stages of osteoclast differentiation. Ablation of NFATc1 in mature osteoclasts did not prevent bone resorption activity, suggesting NFATc1 is involved in cell fusion events and not bone resorption. We propose that the MCP-1-induced TRAP+/CTR+ multinuclear cells represent an arrested stage in osteoclast differentiation, after NFATc1 induction and cellular fusion but prior to the development of bone resorption activity.

Osteoclasts are large, tartrate-resistant acid phosphatase (TRAP)+-positive multinuclear cells that resorb bone (1). In addition to many unique functions and characteristic microscopic features in particular, osteoclasts are also positive for the G protein-coupled calcitonin receptor (CTR) (2), the serine protease cathepsin K (CTSK) (3), and a subcellular structure known as the F-actin ring that is associated with bone resorption activity (4, 5). Osteoclasts differentiate from hematopoietic precursors of monocyte/macrophage lineages (1), through the effects of receptor activator of NFκB ligand (RANKL) present on the surface of stromal cells and osteoblasts (6, 7). Osteoclast differentiation can be promoted in vitro by using soluble recombinant RANKL in the presence of macrophage colony-stimulating factor (M-CSF). M-CSF is necessary for human osteoclast differentiation by supporting cell viability. In the absence of RANKL, M-CSF-treated cells differentiate into macrophage-like cells. Calcitonin is secreted from the thyroid gland in response to serum calcium and inhibits the bone resorbing activity of mature osteoclasts by binding the calcitonin receptor. Indeed, the presence of the calcitonin receptor is thought to be a later marker of osteoclast differentiation, and its presence is often taken as marker of a mature bone-resorbing osteoclast (8).

MCP-1 (monocyte chemotactic protein 1) is a member of the CC chemokine superfamily that plays a critical role in the recruitment and activation of leukocytes during acute inflammation (9). In addition, MCP-1 is found at the site of tooth eruption, a feature consistent with a role in osteoclast biology (10). The cellular effect of MCP-1 is mediated by the CC chemokine receptor 2, a G protein-coupled receptor that is induced by RANKL (11) and stimulates the phosphoinositide 3-kinase signaling pathways (12). We have shown previously that MCP-1 is induced by RANKL and promotes osteoclast fusion into multinuclear cells (13). Furthermore, we have also demonstrated that cells treated with MCP-1 without RANKL can also form TRAP+ multinuclear cells that closely resemble osteoclasts but are unable to resorb bone (13). GM-CSF overrides the osteoclast differentiation pathway, leading to dendritic cells even in the presence of RANKL. In this process, GM-CSF suppresses the RANKL-mediated induction of MCP-1. However, adding back MCP-1 results in the reversal of GM-CSF repression of osteoclast differentiation, suggesting that MCP-1 has a number of different potent effects on osteoclast differentiation (13).

NFATc1 is a transcription factor involved in T cell maturation that has been reported recently as the master switch regulator of osteoclast formation (14). In mouse cells, transfectant constitutive NFATc1 appears sufficient for induction of bone-resorbing osteoclasts (15, 16). In mouse, NFATc1 has also been shown to regulate expression of major osteoclast markers, including CTSK (17) and TRAP (18). NFATc1 is activated by de-phosphorylation by calcineurin through the calcmodulin/calcineurin cascade, which involves calcium signaling (19).

RANKL potently induces chemokines during osteoclast differentiation (11, 13). We previously presented a model of the actions of chemokines in general and MCP-1 in particular on osteoclast differentiation

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5 The abbreviations used are: TRAP, tartrate-resistant acid phosphatase; RANK, receptor activator of NFκB; RANKL, RANK ligand; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; CTR, calcitonin receptor; NFATc1, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1; CTSK, cathepsin K; TBP-2, thio-redoxin binding protein-2; PBMC, peripheral blood mononuclear cells; siRNA, small interfering RNA; TXN, thioredoxin; MAPK, mitogen-activated protein kinase; GFP, green fluorescent protein.

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coated culture plates (BD Biosciences). After 14 days, mature osteoclasts were then removed by using dissociation buffer (Invitrogen), suspended in medium, and then layered onto a two-stage step gradient of 40% serum with a 70% serum cushion as described by Collin-Osdoby et al. (21). Cells were centrifuged at 100 × g for 3 min. The lower fraction, enriched for multinuclear cells (70–80% of cells after this procedure were multinuclear), was then plated at 1.5 × 10^3 cells per well of a 96-well plate containing dentine slices and was incubated for various times. Mononuclear control cells were plated on dentine at 5 × 10^3 cells per well in order to provide sufficient cells for RANKL-mediated cell fusion. Cells on dentine slices were stained for TRAP activity and for nuclei content and photographed under light and fluorescent microscopy. Dentine slices were then cleaned in chloroform, sputter coated with gold, and observed by scanning electron microscopy.

**siRNA Studies**—siRNA for NFATc1 was designed using primers spanning 493 bp and containing the T7 RNA polymerase promoter, 5′-TAA TAC GAC TCA CTA TAG GGG AAC. ACT ATG GCT ATG CAT CC-3′ and 5′-TAA TAC GAC TCA CTA TAG GGA CTG CCA TGG CGA CTG CTG-3′. NFM1c1 and green fluorescent protein (GFP) siRNA were prepared identically using dicer siRNA generation kit (Genlanitis, San Diego, CA) according to the manufacturer’s protocol. Osteoclasts were grown on collagen-coated plates and then removed using dissociation buffer and centrifugation through 40–70% serum to separate the multinuclear cells (21), which were then plated onto dentine slices in minimal essential medium supplemented with 10% fetal calf serum and 25 ng/ml M-CSF and 20 ng/ml RANKL. siRNA (final concentration 12.5 nM) was transfected by using 3 μl of FuGENE 6 (Roche Applied Science) per 0.5 ml of medium as described (22).

**RNA Studies**—Cultures were lysed and RNAs were isolated as described previously (13). Total RNA was converted into cDNA, and quantitative PCRs were performed and analyzed using SYBR Green I Supermix (Bio-Rad) in a Bio-Rad i-Cycler as described previously (13, 20). Standard curves were generated for all PCR assays. Primers and conditions for quantitative PCR assays were as described previously (20) except for the following primer sets: c-Fos, 5′-AGCCAAATGCCCGCAACCGGA-3′ and 5′-GCAACCTTGGCAAATCTCCTGCTC-3′; CD14, 5′-CGTTCGACCCTGTCGGTG-3′ and 5′-AGCTTTTCCTTATTGGCTCCA-3′; CSF1R, 5′-GGAACGCCTAGACGACGAA-3′ and 5′-CTCTACACAGATGCTGATC-3′; STAT1, 5′-GGTTGGCTTGAAGG-3′ and 5′-GATAATGTCACCCTGTTGCT-3′; GPX1, 5′-GTTGGCTTGAAGG-3′ and 5′-GATAATGTCACCCTGTTGCT-3′; TXN, 5′-GGAACGCCTAGACGACGAA-3′ and 5′-CTCTACACAGATGCTGATC-3′; MMP9, 5′-GGTTGGCTTGAAGG-3′ and 5′-GATAATGTCACCCTGTTGCT-3′.

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**RESULTS**

**Characterization of MCP-1-treated Cells**—The phenotype of cells treated with MCP-1 in the presence of M-CSF was compared with authentic osteoclasts differentiated with RANKL (Fig. 1). The cells treated with MCP-1 are TRAP⁺ and multinuclear as are osteoclasts (Fig. 1A) but do not appear as spread as osteoclasts. The MCP-1-treated cells

**EXPERIMENTAL PROCEDURES**

**Preparation and Culture of Human Monocytes**—Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (Amersham Biosciences) density gradient centrifugation, as described previously (20). PBMCs were plated at 10⁶ cells/cm², and nonadherent cells were removed by washing in normal saline. Cells were cultured in a 5% CO₂ atmosphere in minimal essential media supplemented with 10% fetal calf serum, 1% penicillin/streptomycin (all from Invitrogen). All media were supplemented with 25 ng/ml of M-CSF in all experiments. Human PBMC grown in the above medium containing M-CSF proliferate over 21 days and develop into macrophage-like mononuclear cells. Soluble RANKL (20 ng/ml) was added to induce osteoclast formation. TRAP⁺ multinuclear cells were similarly differentiated by using 25 ng/ml of MCP-1 instead of RANKL. M-CSF, MCP-1, and RANKL were purchased from PeproTech (Rocky Hill, NJ). SB203580 and U0126 were purchased from Calbiochem and were used at 20 and 10 μM, respectively. Human calcitonin was used at 50 ng/ml (Sigma). All data are based on a minimum of three replicate experiments performed independently on different occasions using different donor bloods (from the authors M. S. K., C. J. D., and N. A. M.). Where stated in the text, some experiments had more than three independent replicates. All cultures were performed for 21 days, unless stated otherwise.

After 21 days, PBMC cultures were fixed in acetone, citrate, and formaldehyde solution and stained for TRAP using the leukocyte acid phosphatase staining kit (Sigma). TRAP⁺ cells that had three or more nuclei were considered multinuclear. Hoechst stain (Cellomics, Pittsburgh, PA) was used to visualize nuclei. Rhodamine-conjugated phalloidin was used to stain for F-actin (Invitrogen). NFAT activation HitKit HCS reagent kit (Cellomics, Pittsburgh, PA) was used to visualize activation of NFAT transcription factor proteins.

**Bone Resorption Assays**—Two types of culture procedure were used. In a continuous exposure bone resorption assay, cells were cultured in standard medium as above and were differentiated directly on a 16-mm² sperm whale dentine slice in the well of a 96-well plate. This form of assay represents exposure of the dentine slice to cells for 21 days. The second form of assay involves incubation of mature osteoclasts on dentine slices for limited periods of time. Osteoclasts were prepared by culturing PBMCs in RANKL containing medium using type I collagen-
FIGURE 1. Analysis of cellular and molecular phenotypes of multinuclear cells. A, light microscopy of cellular appearance and electron microscopy of bone resorption phenotype of cells treated with either MCP-1 or RANKL. Both MCP-1- and RANKL-treated cells are TRAP+ and multinucleated (upper panel), but MCP-1-treated cells are negative for bone resorption (lower panel). Bar represents 100 μm. B, confocal imagery of cellular phenotype after treatment with M-CSF and MCP-1 or M-CSF and RANKL. Hoechst stain for nuclei (blue); both treatment regimes show multiple nuclei in the cells. Rhodamine conjugate phalloidin stain for actin (red); M-CSF and MCP-1 treatments are negative for the F-actin ring (upper panel). C, expression of MMP9, integrin αv, NFATc1, CTR, and Cals1 after treatment with M-CSF+MCP-1 or M-CSF+RANKL. D, expression of STAT3, STAT1, FBP, KCO21, c-fos, and c-jun after treatment with M-CSF+RANKL or M-CSF+MCP-1. E, expression of CSF1R, RANK, CD44, CSF2RA, and CD14 after treatment with M-CSF+RANKL or M-CSF+MCP-1. F, expression of TXK, QPXL, and TBP2 after treatment with M-CSF+RANKL or M-CSF+MCP-1.
are also negative for pit formation on dentine, whereas the authentic RANKL-treated osteoclasts form multiple deep pits on dentine slices (Fig. 1A). Confocal imagery using Hoechst stain confirmed multinuclearity of MCP-1-treated cells (Fig. 1B). In contrast to well defined F-actin rings in osteoclasts, TRAP+ multinuclear cells differentiated with MCP-1 did not show F-actin rings. Both cell types demonstrated similar nuclear localization of the crucial osteoclast transcription factor NFAT (Fig. 1B).

The failure of bone resorption in the TRAP+ multinuclear cells from MCP-1 treatment was attributed previously to insufficient induction of CTSK, although levels of TRAP mRNA were essentially identical in the two cell types (13). The failure to resorb bone may be because of a lack of expression of key osteoclast genes, despite the similarity in appearance of these MCP-1-treated cells to osteoclasts. To understand further the phenotype of MCP-1-induced TRAP+ multinuclear cells, the expression of genes related to osteoclast function was compared with that in authentic osteoclasts differentiated with RANKL (Fig. 1, C–F). Matrix metalloproteinase 9 (MMP9) is a proteinase involved in bone resorption. MMP9 expression was similar in character to that of CTSK (13), in that expression was lower in the non-bone-resorbing TRAP+ multinuclear cells induced with MCP-1 (Fig. 1C). Integrin αV (a subunit of the αV–β3 integrin) had a similar pattern of expression with lower levels in MCP-1-induced TRAP+ multinuclear cells (Fig. 1C). These data provide further evidence that that MCP-1-mediated TRAP+ multinuclear cells lack essential genes required for the bone resorption function of an osteoclast (Fig. 1A).

Higher mRNA levels were observed for NFATc1 in MCP-1 treated cells compared with RANKL-treated cells in all of six independent experiments with an average difference of 16-fold (n = 6, p = 4.8 × 10−5). Similarly, CTR (6.3-fold, n = 5, p = 2.0 × 10−5) and calmodulin 1 (3.4-fold, p = 1.2 × 10−6) were also higher in TRAP+ multinuclear cells induced by MCP-1 compared with osteoclasts. Most surprisingly, the mRNAs for these three genes were more abundant in the MCP-1-induced multinuclear cells than in osteoclasts differentiated with RANKL.

A series of transcription factors was examined (Fig. 1D), and all were more abundant in MCP-1-treated cells than in RANKL-treated cells. In particular, c-Fos and c-Jun were 26-fold (p = 4.2 × 10−5) and 2.7-fold (p = 2.5 × 10−5), respectively, more abundant in MCP-1-treated cells compared with RANKL-treated cells. These data suggest that the failure of MCP-1-treated osteoclast-like cells to resorb bone cannot be attributed to insufficient NFATc1, c-Fos, or c-Jun, transcription factors that are considered essential for osteoclast differentiation. Significantly greater levels of STAT1 (p = 3.8 × 10−6), STAT3 (p = 4.4 × 10−7), and far upstream element-binding protein (p = 1.5 × 10−7) were observed in the MCP-1-treated cells compared with RANKL treatment. Kox31 is a transcription factor of the zinc finger family that we showed was significantly repressed by RANKL during human osteoclast differentiation (20). Kox31 mRNA is significantly higher in MCP-1-treated cells compared with authentic osteoclasts (p = 0.01) but still substantially lower than the level found in macrophage-like cells treated with M-CSF alone (50 copies per ng of total RNA; data not shown).

A series of cell surface proteins were investigated: c-Fms, RANK, CD44, CSF2RA, and CD14. Of these proteins, c-Fms, RANK, CD44, and CSF2RA are induced by RANKL and M-CSF treatment relative to macrophage-like cells treated with M-CSF alone (20). The cell surface marker CD14 is higher in cells treated with M-CSF alone and is repressed by RANKL and M-CSF treatment (values are 265 ± 33 versus 22.7 ± 1.5 copies per ng of total RNA, respectively, with p = 1.7 × 10−5). MCP-1 represses CD14 in a manner similar to RANKL treatment relative to M-CSF alone (Fig. 1F). Low CD14 is considered a characteristic of osteoclasts; MCP-1-treated cells have significantly lower CD14 mRNA compared with authentic RANKL-derived osteoclasts (p = 0.005). In other respects, the TRAP+ multinuclear cells from MCP-1 treatment had significantly more mRNA for c-Fms (n = 4, p = 2.3 × 10−3), RANK (n = 4, p = 2.3 × 10−3), CD44 (p = 5.5 × 10−4), and CSF2RA (p = 0.009) than in authentic osteoclasts differentiated with RANKL.

Finally, genes related to redox regulation were investigated. Thioredoxin (TXN), glutathione peroxidase-1 (GPX1), and thioredoxin-binding protein-2 (TBPI) were all significantly different in MCP-1-treated cells compared with authentic osteoclasts. Notably, we showed previously that addition of exogenous MCP-1 to RANKL-treated cells enhanced osteoclast formation (13). If MCP-1 and RANKL, either separately or together, activate the same molecular pathways leading to TRAP+ multinuclear cells, a similar pattern of response to inhibitors would be expected. On the other hand, a differential pattern of response would be expected if MCP-1 and RANKL treatment utilized different mechanisms of cell fusion.

To investigate the signaling pathway of multinucleation, p38 and MEK1/2 MAPK inhibitors (SB203580 and U0126, respectively) were utilized to inhibit cell fusion. These inhibitors were tested in cultures treated with MCP-1, RANKL, and MCP-1 plus RANKL combined treatment. The following three parameters were measured to reflect the extent of cell fusion in different cultures: the total number of TRAP+ cells with three or more nuclei, the number of nuclei per cell, and the size of multinuclear cells measured on the longest axis.

The MEK1/2 inhibitor, U0126, had a profound repressive effect on the formation of multinuclear cells in all treatment regimes (Fig. 2A). In contrast, the addition of the p38 MAPK inhibitor, SB203580, did not have a significant effect on the number of TRAP+ multinuclear cells formed in MCP-1-treated cultures (139 ± 5 versus 126 ± 3, p = 0.21; Fig. 2A). These data suggest that MCP-1-mediated multinuclear cell formation is more sensitive to MEK1/2 blockade than p38 MAPK blockade. The situation in authentic osteoclasts differentiated with RANKL, or in cultures treated with MCP-1 plus RANKL, was similar but with subtle differences in comparison to cultures treated with MCP-1 alone.
FIGURE 2. Multinucleation and NFATc1 induction are dependent on MEK1/2 signaling. 

A, the effect of inhibitors on multinuclear cell count. Cultures were treated with various combinations of M-CSF, MCP-1, and RANKL as indicated, with or without SB203580 or U0126. Addition of SB203580 had a minor effect on the number of TRAP^+ multinuclear cells in all treatment regimes. Addition of U0126 suppressed TRAP^+ multinuclear cells in all treatment regimes. *, significant differences, where \( p < 0.01 \). 

B, the effect of inhibitors on the average number of nuclei per cell. Cultures were treated as indicated. The addition of either SB203580 or U0126 significantly reduced the average number of nuclei per cell. Cultures were treated as indicated. The addition of either SB203580 or U0126 significantly reduced the average number of nuclei per cell in all treatment regimes. * and **, significant differences, where \( p < 0.01 \).

C, M-CSF, M-CSF+MCP-1, M-CSF+RANKL, M-CSF+RANKL+MCP-1, SB203580, U0126.

D, the effect of inhibitors on the expression of NFATc1. Cultures were treated with various combinations of M-CSF, RANKL, SB203580, and U0126 as indicated. The addition of U0126 significantly reduced the expression of NFATc1. * and **, significant differences, where \( p < 0.01 \).
The addition of SB203580 to RANKL-treated cultures resulted in a 26% reduction in the number of TRAP\(^{+}\) multinuclear cells (from 217 ± 3 to 160 ± 7, \(p = 1.5 \times 10^{-3}\); Fig. 2A), a reduction in the number of nuclei per cell (from 7.2 ± 0.7 to 3.2 ± 0.6, \(p = 6.3 \times 10^{-7}\); Fig. 2B), and a reduction in the size of cells (from 136 ± 6 to 49 ± 5 \(\mu m\), average longest axis, \(p = 9.2 \times 10^{-11}\); Fig. 2C) when compared with control RANKL treatment.

SB203580 had a similar effect on cultures treated with MCP-1 plus RANKL. Repression of multinuclear cell count, nuclear content, and size was observed as for RANKL-treated cells, although MCP-1 plus RANKL cultures started from a higher level of all parameters (Fig. 2, A and B). U0126 potently suppressed the number of TRAP\(^{+}\) multinuclear cells in all cultures, whether treated with either MCP-1, RANKL, or MCP-1 plus RANKL (\(p = 1.1 \times 10^{-5}, 1.2 \times 10^{-6},\) and 8.9 \(\times 10^{-5}\), respectively; Fig. 2A). Taken together, these data suggest that the effects of SB203580 and U0126 are consistent in the three treatment groups, with SB203580 treatment resulting in significant but quantitative effects on parameters of multinuclear cells, whereas U0126 virtually eliminated multinuclear cells in all treatment regimes. Moreover, U0126-treated cells remained mononuclear (Fig. 2, B and C), suggesting that U0126 inhibited multinucleation by blocking cell fusion.

We reported previously that GM-CSF and cyclosporin A can suppress RANKL-mediated osteoclast formation, leading to mononuclear cells, and that an addition of exogenous MCP-1 reverses the effect of these inhibitors, resulting in TRAP\(^{+}\) multinuclear cells (13). We tested the capacity of MCP-1 to reverse the inhibition of SB203580 and U0126. Cultures treated with RANKL and SB203580 had an average of 3.2 ± 0.6 nuclei per cell and were on average 49 ± 5 \(\mu m\) on the longest axis. Addition of MCP-1 to cells treated with RANKL and SB203580 resulted in an increase in nuclear count (6.6 ± 0.5 nuclei per cell) comparable with authentic RANKL-induced osteoclasts (7.2 ± 0.7 nuclei per cell; Fig. 2B) and an increase in average cell size (71 ± 9 \(\mu m\); Fig. 2C). Although these cells were smaller than osteoclasts derived from RANKL treatment (136 ± 6 \(\mu m\)), the increase in nuclear content and increase in cell size suggests that exogenous MCP-1 can partially overcome SB203580 inhibition. In marked contrast, exogenous MCP-1 failed to improve any feature of the potent inhibition of osteoclast formation mediated by U0126 (Fig. 2, A–C). These data suggest that multinuclear cell formation in general requires MEK1/2 signaling.

High NFATc1 expression was present in both RANKL- and MCP-1-induced TRAP\(^{+}\) multinuclear cells (Fig. 1C). We measured the expression of NFATc1 in cultures exposed to SB203580 and U0126 (Fig. 2D). The potent RANKL-mediated induction of NFATc1 was unaffected by SB203580 but was totally abolished by U0126 (Fig. 2D). Taken together, these data are consistent with the hypothesis that NFATc1 expression is correlated with multinuclear cell formation and that the induction of NFATc1 by RANKL flows through MEK1/2.

Addition of Exogenous Calcitonin Inhibits Fusion Induced by MCP-1—Calcitonin receptor was highly up-regulated in MCP-1-treated cells compared with RANKL-treated cells. This leads to the hypothesis that calcitonin may act to prevent fusion of multinuclear cells induced by MCP-1, as well as its recognized role in inhibiting fusion of osteoclasts differentiated with RANKL.

Exogenous calcitonin (up to 50 ng/ml) was added to cultures treated with MCP-1 to generate TRAP\(^{+}\) multinuclear cells. Similarly, calcitonin was added to cells treated with RANKL to produce authentic osteoclasts. Calcitonin resulted in a similar dose response of reduction in the number of multinuclear cells in both treatments (Figs. 3, A and B), confirming the hypothesis that the calcitonin receptor is active in MCP-1-generated TRAP\(^{+}\) multinuclear cells. Calcitonin (at 50 ng/ml) significantly reduced the number of TRAP\(^{+}\) multinuclear cells in both MCP-1 treatment (133 ± 29 versus 21 ± 6, \(p = 8 \times 10^{-5}\)) and RANKL-treated cells (240 ± 11 versus 89 ± 12, \(p = 2 \times 10^{-4}\)) (Fig. 3C). Calcitonin had no significant effect on cells treated with M-CSF alone (Fig. 3C). The visual appearance of RANKL- and MCP-1-treated cultures was remarkably similar after treatment with calcitonin (Fig. 3D). These TRAP\(^{+}\) multinuclear cells that remained after calcitonin treatment were generally of smaller size in both MCP-1- and RANKL-treated cultures (Fig. 3D), indicating that calcitonin reduces the number and the size of multinuclear cells. Exogenous calcitonin reduced the size of RANKL-treated cells by ∼3-fold (\(p = 2.2 \times 10^{-5}\)). Because the RANKL-treated osteoclasts are larger to begin with than TRAP\(^{+}\) multinuclear cells from MCP-1 treatment, calcitonin has a proportionately greater effect on the RANKL-treated cells compared with MCP-1-treated cells. Calcitonin at 50 ng/ml completely inhibited bone resorption by osteoclasts (Fig. 3D). Confocal imagery showed NFAT was still residing in the nuclei of the cells treated with calcitonin, regardless of whether they were from the MCP-1 or the RANKL treatments (Fig. 3E). Most interestingly, the F-actin ring, clearly evident in RANKL-treated osteoclasts, was absent after calcitonin treatment (Fig. 3E). These data suggested that calcitonin was able to prevent the MCP-1-mediated fusion of monocytes into TRAP\(^{+}\) multinuclear cells.

**MCP-1-treated Cells Are Able to Differentiate into Osteoclasts and Become Positive for Bone Resorption Activity after RANKL Exposure**—The presence of RANKL mRNA in TRAP\(^{+}\) multinuclear cells differentiated with MCP-1 suggested the hypothesis that such cells could become proficient for bone resorption if provided with RANKL. The gene expression profile of such cells suggested also that several osteoclast-related genes are at least as abundant in MCP-1-induced TRAP\(^{+}\) multinuclear cells as in osteoclasts, and therefore may indicate an increased facility to differentiate. We tested this hypothesis by exposing TRAP\(^{+}\) multinuclear cells from MCP-1 treatment to RANKL on dentine slices.

Initially, the time course of bone resorption of RANKL-treated human PBMCs grown directly on dentine slices was established (Fig. 4A). PBMCs grown on dentine slices and exposed to RANKL were negative for bone resorption activity at 7 days of culture, whereas at 14 days bone resorption was evident on 7 ± 2% of the surface of the dentine slice. By 21 days of continuous RANKL treatment, the amount of resorption significantly increased to 47 ± 8% (\(p = 3.4 \times 10^{-3}\)). Thus, the percentage of resorption increased exponentially, as more mature osteoclasts were formed (Fig. 4A). However, cultures exposed to continuous treatment with either M-CSF alone or M-CSF and MCP-1 were negative for bone resorption (Fig. 4, B and D).

Multinuclear cells induced by MCP-1 were grown on collagen-coated plates for 14 days and then purified over serum step gradients and plated
FIGURE 3. Exogenous calcitonin inhibits the multinuclear phenotype. A, cultures were treated continuously with calcitonin at the concentration indicated (ng/ml) in the presence of M-CSF and RANKL. B, cultures were treated continuously with calcitonin at the concentration indicated (ng/ml) in the presence of M-CSF and MCP-1. C, addition of exogenous calcitonin (50 ng/ml) TRAP\(^+\) multinuclear cells were significantly suppressed in both M-CSF- and MCP-1-treated cells and M-CSF- and RANKL-treated cells. *, significant differences compared with M-CSF and MCP-1 or M-CSF and RANKL treatment and exogenous calcitonin-added cells, where p < 0.01. D, simultaneous treatment with calcitonin and either MCP-1 or M-CSF and RANKL treatment and TRAP\(^+\) multinuclear cells.
onto dentine slices. Cells were then cultured on dentine for 7 days in the presence of RANKL. Controls were PBMC-cultured on collagen-coated plates for 14 days in the presence of RANKL, purified similarly over serum step gradients, plated onto dentine slices, and then exposed to RANKL for 7 days. A further control included PBMC grown on collagen-coated plates in the presence of M-CSF alone and then plated onto dentine slices and exposed to RANKL for 7 days.

Mononuclear cells treated with M-CSF for 14 days and then exposed to RANKL for 7 days showed a small amount of bone resorption (0.76 ± 0.54%). This indicated that 14 days of incubation with M-CSF did not eliminate the capacity to differentiate into an osteoclast. Incidentally, this represented more bone resorption than that observed in PBMCs exposed for 7 days continuously to RANKL (Fig. 4A), perhaps suggesting that osteoclast progenitors are able to proliferate during 14 days of incubation with M-CSF.

Mature human osteoclasts from RANKL-treated cells cultured on collagen-coated plates survive serum gradient purification and accumulate many resorption pits and trails on dentine over the following 7 days of RANKL exposure. As expected, mature osteoclasts had significantly greater bone resorption activity (35 ± 7.0% n = 3, p = 8.4 × 10^-3), compared with M-CSF alone-treated cells (Fig. 4, C–E). Multinuclear cells purified from MCP-1-treated cultures and plated onto dentine in the presence of RANKL were positive for bone resorption activity, degrading 8.0 ± 1.5% of the dentine surface after 7 days of exposure to RANKL (Fig. 4, C–E). This amount of bone resorption was significantly greater than that compared with M-CSF alone-treated cells (p = 0.012) but was significantly less than that from cells treated continuously with RANKL, whether replated from collagen or not. In order to put these data into perspective, the bone resorption activity of multinuclear cells produced by MCP-1 treatment (purified and then exposed to dentine slices with RANKL) was compared with the time course of bone resorption from continuous exposure to RANKL (Fig. 4A). Cultures with prior MCP-1 treatment followed by RANKL treatment had a similar amount of bone resorption activity after 7 days on dentine as did cultures from 14 days of continuous RANKL exposure on dentine (Pearson correlation, R^2 = 1). These data provided evidence that MCP-1-treated cells can differentiate efficiently into authentic bone-resorbing osteoclasts and indicated that the induction of RANK by MCP-1 (Fig. 1E) may precondition such cells to differentiation in the presence of RANKL.

**NFATc1 Regulates Calcitonin Receptor—**NFATc1 expression was high in MCP-1-induced TRAP^+^ multinuclear cells, as was CTR expression. We tested the hypothesis that NFATc1 regulates CTR in mature osteoclasts by ablating NFATc1 expression using siRNA and then measuring the expression of a number of genes suspected to be NFATc1 targets. Mature osteoclasts were differentiated in continuous exposure to RANKL for 14 days on collagen-coated plates. Osteoclasts were dissociated, purified over serum step gradients, and replated prior to transfection with siRNA. Medium was changed after 6 h, and cells were harvested at 72 h post-exposure to siRNA. RANKL and M-CSF were present throughout the transfection. Total RNA was harvested, and real time quantitative PCR was used to measure mRNA content relative to 18 S rRNA. Exposure to anti-NFATc1 siRNA resulted in potent suppression (knock down by 88 ± 12%, p = 3.6 × 10^-3) of NFATc1 in mature osteoclasts (Fig. 5A). An equally potent flow on knock down of calcitonin receptor (84 ± 13% reduction, p = 6.5 × 10^-3) by NFATc1 siRNA was observed (Fig. 5A). In contrast, control siRNA against an unrelated protein, GFP, did not influence gene expression (Fig. 5A). These data are consistent with NFATc1 regulating calcitonin receptor.

The high calcitonin receptor mRNA in MCP-1-treated cells may be explained by the high content of NFATc1 mRNA in these cells. In complete contrast, other genes related to bone resorption were not affected significantly by NFATc1 siRNA, including the hydrogen ATPase (ATP6vC), integrin αv, CT SK, TRAP, and MMP9.

**siRNA Knock Down of NFATc1 in Mature Osteoclasts—**Multinuclear cells induced by MCP-1 have the visual appearance of human osteoclasts, being TRAP^+^/CTR^−^ responsive to calcitonin and with high nuclear NFATc1 content. These data suggest that NFATc1 expression was not sufficient for bone resorption activity. If NFATc1 is the master regulator of human osteoclast function, knock down of NFATc1 should decrease bone resorption activity. In contrast, if NFATc1 is involved primarily in cell fusion events, and not bone resorption, then siRNA against NFATc1 should have no effect on the bone resorption phenotype of mature human osteoclasts.

Mature osteoclasts were differentiated on collagen-coated plates in the presence of RANKL for 14 days, purified over serum step gradients, and then the culture was split into identical parts and replated onto dentine slices in the presence of RANKL. The replicate cultures were made for parallel transfection with siRNA against NFATc1 and control GFP. Cells were exposed to siRNA for 6 h, and then the medium was changed, and the bone resorption area was assayed by electron microscopy at 96 h post-transfection. Osteoclasts transfected with control siRNA against GFP yielded 8.5 ± 2% resorption after 96 h (Fig. 5, B and C). We have established previously that osteoclasts transfected with siRNA to GFP do not differ in bone resorption from identical nontransfected cultures, indicating that under the optimized conditions of Selinger et al. (22), bone resorption is not influenced nonspecifically by siRNA. In marked contrast, NFATc1 siRNA-transfected osteoclasts had a significantly greater area of bone resorption activity (19 ± 2% n = 6, p = 1.2 × 10^-3) after 96 h (Fig. 5, B and C). This outcome was in complete contrast to the expectation of decreased bone resorption with ablation of NFATc1 by siRNA, based on the proposition of NFATc1 as a master regulator. The experiment was repeated on six independent occasions; all six replicate experiments had similar outcomes (Fig. 5D).

**DISCUSSION**

We demonstrated previously that MCP-1 is induced by RANKL in human osteoclast differentiation. MCP-1 stimulates the formation of osteoclasts in the presence of RANKL and, in the absence of RANKL, stimulates the formation of TRAP^+^ multinuclear cells that have the appearance of osteoclasts. We also showed that the receptors for MCP-1 (CCR2 and CCR4) are induced by RANKL (11), providing evidence for an autocrine loop for MCP-1 in human osteoclasts. In this study, we further investigate the phenotype of MCP-1-induced TRAP^+^ multinuclear cells and test several hypotheses derived from the data.

MCP-1-induced TRAP^+^ multinuclear cells that are negative for bone resorption are indeed capable of differentiating into bone-resorbing osteoclasts when provided with RANKL. Such TRAP^+^ multinuclear cells stimulated by MCP-1 may be an intermediate stage of osteoclast differentiation. We had proposed previously a model in which these cells represent a stage in the process of recruitment and activation of human osteoclast precursors that are attracted to the site of RANK-RANKL signaling due to the production of chemokines (13). The pres-
FIGURE 4. MCP-1-induced TRAP$^+$ multinuclear cells are positive for bone resorption after treatment with RANKL. A, graph shows percent of resorption area per dentine slice after continuous M-CSF and RANKL treatment for 7, 14, and 21 days. Asterisk indicates the significant difference compared with M-CSF plus RANKL treatment at 21 days, where $p < 0.01$. B, graph shows percent of resorption area per dentine slice after 21 days of continuous treatment with M-CSF alone, M-CSF plus MCP-1, or M-CSF plus RANKL. M-CSF- and RANKL-treated cells are positive for bone resorption, whereas others are negative for bone resorption. C, cells were grown on collagen-coated plates for 14 days with either M-CSF alone, M-CSF plus MCP-1, or M-CSF plus RANKL. All mature cells were then plated on dentine and treated with M-CSF plus RANKL for 7 days. Graph shows percent of resorption area per dentine slice. M-CSF plus MCP-1-treated cells have significantly enhanced resorption activity compared with M-CSF-treated cells. *, significant difference compared with M-CSF plus MCP-1 treatment, where $p < 0.05$. #, significant difference compared with M-CSF plus RANKL treatment, where $p < 0.05$. D, light and electron microscopy of cells and bone resorption on dentine slices. Cells were grown continuously on dentine slices as in B. Treatment with M-CSF alone (upper left panel) results in mononuclear macrophage-like cells that are negative for bone resorption (lower left panel). Treatment with MCP-1 (in the presence of M-CSF) results in TRAP$^+$ multinuclear cells (upper center panel) that are negative for bone resorption (lower center panel). Treatment with RANKL (in the presence of M-CSF) results in TRAP$^+$ osteoclasts (upper right panel) that have substantial bone resorption activity (lower right panel). Extensive resorption pits on dentine can be seen in both light and electron microscopy (upper and lower panels on right). E, cellular appearance and bone resorption activity of cells cultured on collagen-coated plates for 14 days prior to exposure to RANKL for 7 days on dentine slices. Cells with prior M-CSF treatment (upper left panel) were able to produce a low number of osteoclasts, evidenced by bone resorption (lower left panel). TRAP$^+$ multinuclear cells from MCP-1 treatment became positive for bone resorption after exposure to RANKL (upper and lower center panels), with visible osteoclast trails. Osteoclasts differentiated with RANKL for 14 days prior to plating on dentine showed substantial bone resorption with pits and trails (upper and lower right panel). Upper panels are all light microscopy with TRAP stain, and lower panels are electron microscopy. Bar is 100 μm.
Knockdown of NFATc1 in mature human osteoclasts alters calcitonin receptor expression and increases bone resorption. A, graph shows efficiency of knockdown of NFATc1 gene using siRNA against NFATc1 compared with siRNA against GFP. *, significant difference compared with control GFP siRNA transfection, where \( p < 0.01 \). Assays were for NFATc1, CTR, vacuolar proton-ATPase (ATP6C), CTSK, integrin \( \alpha V \) subunit, MMP9, and TRAP.

B, transfection of NFATc1 siRNA increases bone resorption activity of mature osteoclasts. Cells were cultured with M-CSF and RANKL over 14 days on collagen-coated plates. Mature osteoclasts were then plated on dentine and transfected with siRNA for 96 h. Graph shows percent of resorption area per dentine slice in each treatment group. Data are the mean of six independent experiments. *, significant difference compared with control siGFP transfection, where \( p < 0.01 \).

C, light microscopy of siRNA-transfected cells shows TRAP \( ^{+} \) multinuclear cells with resorption pits and trails. GFP siRNA-transfected cells are shown in the upper panel, and NFATc1 siRNA-transfected cells are shown in the lower panel. Bar is 100 \( \mu m \).

D, electron micrographs of representative areas of dentine slices. Upper and lower panels are matched pairs from all six independent experiments. Bone resorption activities were positive for both GFP and NFAT siRNA-transfected cells; however, more persistent trails appear to be a feature of the cells treated with siRNA against NFATc1 (lower panels). Bar is 200 \( \mu m \).
MCP-1-mediated TRAP⁺ Multinuclear Cells

ent data are consistent with the model. MCP-1-treated cells have many features in common with osteoclasts and are able to differentiate into authentic bone-resorbing osteoclasts in the presence of RANKL. The further differentiation into osteoclasts in the presence of RANKL may be facilitated by the expression of RANK, which is at a higher level in MCP-1-treated cells than that found in authentic osteoclasts. MCP-1-derived TRAP⁺ multinuclear cells had higher expression of osteoclast-related nuclear factors and cell surface receptors, showing a similar but different phenotype to RANKL-mediated osteoclasts. MCP-1-treated cells lacked expression of genes required for bone resorption activity, including proteases MMP9 and cell adhesion molecules, such as the αv subunit of integrin αvβ3, which is required for formation of the F-actin ring (4, 5, 24). In keeping with the lack of expression of the αv subunit, MCP-1-mediated multinuclear cells lacked the F-actin ring, whereas RANKL-mediated multinuclear cells clearly showed the F-actin ring structure.

NFATc1 is induced by RANKL in mouse (15, 16, 18) and human (20) osteoclast differentiation and is claimed to be the master regulator of mouse osteoclast formation and function (14). Most interestingly, MCP-1-mediated TRAP⁺ multinuclear cells had abundant expression of NFATc1, calmodulin 1, and CTR. The presence of NFATc1 in MCP-1-treated cells was detected by quantitative PCR of mRNA and verified by nuclear staining for NFAT and confocal imagery. The induction of NFATc1 by RANKL was dependent on MEK1/2 signaling but not p38 MAPK signaling. The MEK1/2 inhibitor U0126 suppressed NFATc1 induction by RANKL and also suppressed osteoclast formation. Similarly, U0126 potently inhibited MCP-1-mediated multinuclear cell formation. In contrast, inhibition of the p38 MAPK pathway by SB203580 did not inhibit the RANKL-mediated induction of NFATc1 and permitted multinuclear cell formation in both RANKL- and MCP-1-treated cultures, although these cells were smaller and had reduced nuclear content. These data suggest MEK1/2 signaling flowing through ERK1/2 is necessary for NFATc1 induction by RANKL and for multinuclear cell formation by RANKL and MCP-1.

In mouse studies, transfected constitutive NFATc1 has been considered sufficient for osteoclast formation (14). In the literature, many papers define osteoclasts without reference to the bone resorption phenotype. Cells are commonly considered osteoclasts if they are positive for the calcitonin receptor (considered as a late differentiation marker), TRAP⁺, and are multinuclear giant cells. If this less exacting definition of an osteoclast is accepted, then the high NFATc1 in MCP-1-treated cells appears to be correlated with the appropriate TRAP⁺/CTR⁺ and multinuclear phenotype. However, the lack of bone resorption in the presence of high NFATc1 runs counter to the idea that NFATc1 alone is sufficient for osteoclast formation and function, at least in human cells. In contrast, the data presented in this paper support a role for NFATc1 in the formation of multinuclear cells through cell fusion. At least in the human, induction of NFATc1 by MCP-1 does not induce osteoclast function in the absence of RANKL, although it produces TRAP⁺/CTR⁺ multinuclear cells. Furthermore, if NFATc1 was the master regulator of human osteoclast formation and function, knock down of NFATc1 should inhibit bone resorption. We showed previously that bone resorption in mature osteoclasts transfected with siRNA against cathepsin K was inhibited significantly, indicating that transfected siRNA can be used effectively with mature osteoclasts (22). Most surprisingly, siRNA to NFATc1 did not inhibit bone resorption, but rather, bone resorption was significantly increased compared with control transfections with anti-GFP siRNA. CTR was also potently knocked down in mature osteoclasts by siRNA to NFATc1, so we cannot rule out that the increased bone resorption in these experiments was because of reduction in CTR or some other unknown gene that is regulated by NFATc1. These data suggest a complex role for NFATc1 in regulating bone resorption in mature human osteoclasts and reinforce the connection between NFATc1 expression and cellular fusion events.

The correlation of high NFATc1 with high CTR in MCP-1-treated cells and the results of siRNA knock down of NFATc1 suggest a direct regulatory function of NFATc1 to induce the CTR gene. Previous data have suggested that CTR is regulated by NFATc1 (15), but CTR is generally considered as a late stage marker (8). Addition of exogenous calcitonin significantly reduced the number of MCP-1-mediated TRAP⁺ multinuclear cells, blocking the fusion process. Calcitonin significantly reduced the number of RANKL-mediated TRAP⁺ multinuclear osteoclasts and also suppressed bone resorption, agreeing with previous observations (25). Our data show that calcitonin can inhibit chemokine-stimulated cell fusion, presumably through the MCP-1-induced CTR. These data suggest that CTR may be expressed under chemokine signaling and therefore may represent both the intermediate and late stages of osteoclast differentiation. In the presence of abundant calcitonin, one may expect the formation of osteoclasts through cell fusion to be inhibited. The existence of feedback regulation of osteoclast formation by physiological calcium levels through calcitonin seems a logical proposition. This idea also predicts that calcitonin may have benefits in inflammatory bone loss, where high chemokine production is observed, by inhibiting the fusion of pre-osteoclasts stimulated by chemokines in the absence of RANKL.

Relative sensitivities of different genes to the repressive effects of cyclosporin on RANKL-mediated induction also supports CTR as an NFATc1 target in human osteoclasts. Cyclosporin A is an inhibitor of calcineurin-mediated activation of NFATc1. We previously have shown that cyclosporin A potently inhibits the formation of human osteoclasts by RANKL. CTR was highly sensitive to cyclosporin A inhibition, with a maximal repression of RANKL-mediated induction at 250 ng/ml (20). In contrast, induction of cathepsin K and TRAP by RANKL was unaffected at cyclosporin concentrations up to 1 μg/ml (20). The present data suggest that CTR is regulated by NFATc1 and also show that, at least in mature human osteoclasts, NFATc1 ablation does not lead to a reduction in expression of CTSK nor TRAP, consistent with our previous observations using cyclosporin A. These data are in contrast to published studies in mouse systems, where evidence is convincing that CTSK and TRAP are NFATc1 targets, although cyclosporin is used at higher concentrations in mouse studies (10 μg/ml) that may interfere with multiple targets (26). The differences between human and mouse systems await further clarification.

In a similar manner to the high levels of NFATc1 in MCP-1-treated cells, the other critical osteoclast-related transcription factors, c-Jun and c-Fos, were also more abundant in MCP-1-treated cells than in RANKL-treated cells. c-Fos was particularly more abundant (27-fold) in MCP-1-treated cells. c-Fos (as a component of AP1) has been suggested as an activator of NFATc1 (18); our data are compatible with that interpretation, as 27-fold induced c-Fos is positively correlated with 16-fold induced NFATc1. Despite this possible relationship between c-Fos and NFATc1, the lack of bone resorption activity in MCP-1-induced TRAP⁺/CTR⁺ multinuclear cells indicates that an unknown RANKL signaling pathway is required for activation of bone resorption in human osteoclasts even in the presence of nuclear NFAT.

Thioredoxin and glutathione peroxidase-1 were lower in MCP-1-treated cells than in RANKL-treated cells (2.3- and 4.9-fold, respectively). TBP2 (thioredoxin-binding protein-2) is repressed during osteoclast differentiation (23) but was up-regulated in MCP-1-treated cells (3.7-fold). TBP2 negatively regulates thioredoxin and other redox-reg-
oclast biology and the kind gift of dentine slices. We also thank Dr. Ghafar redox-regulated activation of AP1, NFκB, and NFAT in osteoclasts (23, 27, 28). NFATc1 and AP1 components (c-Fos and c-Jun) are more abundant in MCP-1-treated cells, compared with RANKL-treated cells. Because MCP-1-treated TRAP$^+$ multinuclear cells are capable of differentiating into osteoclasts on treatment with RANKL, we assume that these redox regulators, such as TBp2, do not permanently block further differentiation and that a redox status permissive for osteoclast differentiation is acquired through RANKL. We have not yet investigated the effect of altering redox states in the TRAP$^+$ osteoclast-like cells that are negative for bone resorption, possibly because of insufficient integrin αV, MMP9, cathepsin K, absent F-actin ring, and an inappropriate redox status. MCP-1-induced multinuclear cells are a valid intermediate in human osteoclast differentiation because they become positive for bone resorption when presented with RANKL, in keeping with our previous model (13). Calcitonin inhibited MCP-1-mediated cell fusion, suggesting that calcitonin can repress early cell fusion events as well as mature osteoclast function. Therefore, an important calcitonin regulatory checkpoint exists at an intermediate stage of osteoclast differentiation, interacting with chemokine signaling.

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