NF-κB p50 and p52 Regulate Receptor Activator of NF-κB Ligand (RANKL) and Tumor Necrosis Factor-induced Osteoclast Precursor Differentiation by Activating c-Fos and NFATc1*

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Postmenopausal osteoporosis and rheumatoid joint destruction result from increased osteoclast formation and bone resorption induced by receptor activator of NF-κB ligand (RANKL) and tumor necrosis factor (TNF). Osteoclast formation induced by these cytokines requires NF-κB p50 and p52, c-Fos, and NFATc1 expression in osteoclast precursors. c-Fos induces NFATc1, but the relationship between NF-κB and these other transcription factors in osteostegenesis remains poorly understood. We report that RANKL and TNF can induce osteoclast formation directly from NF-κB p50/p52 double knockout (dKO) osteoclast precursors when either c-Fos or NFATc1 is expressed. RANKL- or TNF-induced c-Fos up-regulation and activation are abolished in dKO cells and in wild-type cells treated with an NF-κB inhibitor. c-Fos expression requires concomitant RANKL or TNF treatment to induce NFATc1 activation in the dKO cells. Furthermore, c-Fos expression increases the number and resorptive capacity of wild-type osteoclasts induced by TNF in vitro. We conclude that NF-κB controls early osteoclast differentiation from precursors induced directly by RANKL and TNF, leading to activation of c-Fos followed by NFATc1. Inhibition of NF-κB should prevent RANKL- and TNF-induced bone resorption.

Osteoclasts are specialized bone-resorbing cells derived from multipotent myeloid progenitor cells. They play a crucial homeostatic role in skeletal modeling and remodeling and destroy bone in many pathologic conditions (1, 2). Understanding of the regulation of osteoclast formation, activation, and survival has increased dramatically in recent years following the identification of osteoprotegerin and of receptor activator of NF-κB (RANK) and its ligand, RANKL (3–5). RANKL is a member of the TNF superfamily (6, 7). It is expressed by a variety of cell types, particularly osteoblast/stromal cells, and its expression by these cells increases in response to a variety of factors, including cytokines, growth factors, and hormones, to induce osteoclast formation, activation, and survival in normal and disease states (2, 8, 9). Osteoprotegerin is a decoy receptor for RANKL that negatively regulates bone resorption by binding to RANKL, and thus, preventing it binding to RANK on osteoclasts or their precursors. Thus, in many circumstances, osteoclast formation is regulated indirectly by accessory cells.

In addition to passively responding to RANKL, osteoclasts also actively regulate their own formation, activation, and survival, both positively and negatively. For example, they produce both ligands (10, 11) and receptors (12–14) for positive regulatory cytokines, such as TNF and interleukin-1, as well as for interferon-β, which negatively regulates their formation (15, 16). TNF has been implicated in postmenopausal and inflammation-associated bone loss mainly by inducing expression of RANKL (8, 17) and macrophage colony-stimulating factor (M-CSF) (18, 19) by accessory cells. By this mechanism, these cytokines indirectly increase osteoclastogenesis. In addition, TNF increases expression of RANK and c-Fms on the surface of osteoclast precursors to amplify RANKL and M-CSF signaling (20, 21). TNF also induces activation of the transcription factors NF-κB, AP-1, and nuclear factor of activated T cells c1 (NFATc1, also known as NFAT2) in osteoclast precursors (reviewed in Refs. 1 and 22, 23), thereby directly controlling the differentiation of these precursors to osteoclasts (24–27).

NZF-κB, AP-1 and NFATc1 are essential for RANKL-induced osteoclastogenesis (28–31) and are activated downstream in the RANKL/RANK signaling pathway to induce a variety of responses in osteoclast precursors (29, 32). c-Fos, a component of the dimeric transcription factor, AP-1 (reviewed by Karsenty

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and Wagner (23)), mediates RANKL stimulation of osteoclast formation by transcriptionally inducing NFATc1 (29, 33). NFATc1 is a member of the NFAT transcription factor family of five proteins that regulate the expression of cytokines and immunoregulatory genes in several cell types (34–36). NFATc1 rescues osteoclastogenesis in cells lacking c-Fos (29, 33, 37–39), indicating that c-Fos is upstream of NFATc1.

NF-κB comprises a family of five transcription factors, and expression of both p50 and p52 is required for osteoclast formation (30, 31). NF-κB p50/p52 double knock-out (dKO) mice do not form osteoclasts, whereas osteoclast formation in p50 or p52 single knock-out mice is normal. The dKO mice have increased numbers of CD11b+/RANK+ osteoclast precursors in their spleens (40), indicating that p50/p52 expression is required for progression of these cells along the osteoclast differentiation pathway. The defect cannot be rescued by treatment of dKO splenocytes with RANKL or TNF (40), each of which activates NF-κB in wild-type (WT) osteoclasts (25, 26).

This is important because TNF can induce osteoclast formation in vitro from WT, RANKL−/−, or RANK−/− osteoclast precursors (41–43), indicating that p50 and p52 expression is required for this in vitro effect of TNF. Despite these observations, the mechanism whereby TNF induces osteoclast formation in vitro in the absence of RANKL/RANK signaling remains unknown.

Although it is established that RANKL activates c-Fos and NFATc1 in osteoclasts or their precursors (29, 33), there are conflicting data on its effects on NF-κB activation (44). Recent studies have indicated that NF-κB p65 and to a lesser extent p50 proteins are recruited along with NFATc2 to the NFATc1 promoter within 1 h of treatment of osteoclast precursors with RANKL (45). c-Fos is not recruited to the NFATc1 promoter until much later (at 24 h), by which time NFATc2 and NF-κB p65 and p50 are no longer detectable. Interestingly, by this time, NFATc1 has been recruited to its own promoter. These investigators suggested that RANKL induces cooperation of NFATc2 pre-existing in precursors with other transcription factors, such as NF-κB to activate initial induction of NFATc1, followed by a later auto-amplification phase of NFATc1 to induce osteoclast formation. Despite these demonstrations of transient NF-κB p65 and p50 association with NFATc2, but not NFATc1, on the NFATc1 promoter, it is still not clear what the relationship is among NF-κB, c-Fos, and NFATc1 during the early events that mediate RANKL or TNF-induced osteoclast formation. This is exemplified by the pathways illustrated in recent review papers of signaling downstream from RANK (46, 47). Thus, it is still not clear whether the essential role of NF-κB p50 and p52 in osteoclast formation is upstream or downstream of c-Fos given that expression of both is required for osteoclastogenesis in vitro and in vivo, nor is it clear whether it is necessary for NF-κB p50 and p52 to interact with NFATc1 in osteoclast precursors for their differentiation into osteoclasts.

Here, we report that RANKL or TNF can induce osteoclast formation from NF-κB dKO osteoclast precursors when c-Fos is expressed, indicating that NF-κB is upstream of c-Fos. RANKL or TNF treatment and c-Fos expression in dKO cells also induces NFATc1 expression, and retroviral expression of NFATc1 plus treatment with these cytokines rescue the defect in osteoclast formation. These findings indicate that interaction between NFATc1 and NF-κB p50 or p52 is not required for NFATc1 to execute its osteoclastogenic effect.

**MATERIALS AND METHODS**

**Animals**—Generation of NF-κB p50/p52 dKO mice (C57BL/6 × 129) was described previously (30), and mice were used when they were 3–6 weeks old. Littermates of dKO mice that have normal teeth eruption and skeletal development were used as WT controls. The Institutional Animal Care and Use Committee approved all animal studies.

**Reagents**—Recombinant human M-CSF, murine RANKL, interleukin-1β, and TNFα were purchased from R&D Systems, Inc. (Minneapolis, MN). Polybrene and puromycin were obtained from Sigma. NF-κB activation inhibitor was purchased from Calbiochem.

**Constructs and Transfection**—The coding regions of genes were amplified by PCR from cDNAs and cloned into the pMX-puro retroviral vector (33, 48). Each 5′ primer contains a Kozak sequence following the start codon. c-Fos was murine, and NF-κB p50 and p52 were of human origin. The pMSCV-ncNFATc1 construct was a gift from Dr. N. Clipstone (Northwestern University, Chicago, IL) (49). The pMX-GFP-puro vector was used as a control for infection efficiency. These retrovirus vectors were transiently transected into the Plat-E retroviral packaging cell line (50), and viral supernatant was collected 48 h later.

**Osteoclastogenesis and Viral Infection**—Splenocytes were extracted from spleens through a fine wire mesh and cultured with conditioned medium from a M-CSF-producing cell line (51) (1:20 dilution) for 3 days in α-modified essential medium with 10% fetal calf serum (Hyclone Laboratories, Logan, UT) to enrich for osteoclast precursors, which we named M-CSF-dependent splenocytes (MDS). Then the cells were infected with the retroviral supernatants in the presence of M-CSF (10 ng/ml) and Polybrene (8 μg/ml). On day 2 after infection, puromycin (2 μg/ml) was added to the cultures to select for gene-integrated cells. ~50–80% of MDS were GFP-positive under fluorescence microscopy 2 days after infection, and this increased to >90% following puromycin treatment. The cells were cultured with M-CSF (10 ng/ml) for 3–7 days. RANKL (10 ng/ml) and TNF (20 ng/ml) (these doses for RANKL and TNF effectively induce osteoclast formation from WT spleen cells) were added every other day. The experiments were stopped during this time based on a visual assessment of multinucleated cell formation using an inverted microscope. Optimal osteoclast formation occurs in WT cell preparations 1–2 days after those from the dKO mice. The cells were fixed, stained for tartrate-resistant acid phosphatase (TRAP) activity to identify osteoclasts as TRAP+ cells containing ≥3 nuclei, and counted, as described previously (52). For functional studies, infected cells were cultured on bone slices for 10 days under the same conditions as described above. Osteoclasts were then removed, resorption pits were visualized after 0.1% toluidine blue staining, and the mean pit area was measured, as described previously (53).

**Quantitative Real-time RT-PCR**—RNA from MDS or infected cells was extracted using the RNeasy kit and the
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**RESULTS**

**NF-κB Is Upstream from c-Fos in RANKL and TNF-induced Osteoclast Formation**—c-Fos, like NF-κB, is activated by RANKL and TNF to induce osteoclast formation (15), but it is not known whether c-Fos can substitute for NF-κB expression in osteoclast precursors. To examine this question, we overexpressed c-Fos in NF-κB p50/p52 dKO MDS. MDS were used as osteoclast precursors in our study because NF-κB dKO mice have severe osteopetrosis, which prevents harvesting of bone marrow cells. We first examined whether NF-κB p50, p52, and p50+p52 retroviral expression would rescue the defect in osteoclast formation in dKO MDS when the cells were treated with RANKL. Overexpression of p50+p52 in dKO cells rescued osteoclast formation, inducing ~150 osteoclasts/96 wells (Fig. 1A). In comparison, RANKL-treated GFP-infected WT cells typically formed ~300 TRAP⁺ osteoclasts (data not shown), indicating that the maximal rescue efficiency of our system is ~50% of that of WT cells. c-Fos expression alone, like p50, p52, or p50+p52, did not induce osteoclasts in the absence of RANKL (Fig. 1, A and B). However, when RANKL was added to c-Fos-expressing dKO cells, the combination rescued the osteoclast formation defect, inducing ~150 osteoclasts (Fig. 1, B and C). The TRAP⁺ cells that formed on plastic dishes and bone slices from dKO cells overexpressing c-Fos appeared similar to those of WT MDS treated with M-CSF and RANKL, and they formed numerous resorption pits on bone slices, typical of mature WT osteoclasts (Fig. 1D). These data indicate that c-Fos activated by RANKL can efficiently substitute for the lack of p50 and p52 in dKO cells in these culture conditions and that the resulting c-Fos-overexpressing osteoclasts have a bone resorptive capacity similar to that of WT cells.

TNF plays important roles in osteoclast formation, activation, and survival in a number of pathologic conditions (9, 56) by stimulating RANKL expression in stromal and other cell types. To determine whether TNF, like RANKL, could induce osteoclast formation directly when c-Fos was overexpressed in dKO MDS, we treated c-Fos-expressing dKO MDS with TNF. We observed numerous TRAP⁺ osteoclasts. However, the number of osteoclasts formed (~80/well) was less than in the c-Fos/RANKL-treated cultures (~150/well; Fig. 1B).

To determine whether there is impaired RANKL or TNF-induced c-Fos up-regulation and AP-1 binding activity in the absence of NF-κB p50 and p52, we compared c-Fos mRNA expression and activation in dKO and WT MDS. RANKL or TNF stimulated c-Fos mRNA expression in WT cells but not in dKO cells (Fig. 2A). Accordingly, RANKL or TNF induced binding of c-Fos protein to an AP-1 oligonucleotide probe in an EMSA using nuclear extracts from WT MDS but not using dKO cells. In contrast, binding to the SP-1 control probe was similar between dKO and WT nuclear extracts, indicating equal loading in the EMSA (Fig. 2B). To further determine whether NF-κB is upstream from c-Fos induced by RANKL or TNF, we treated WT cells with a newly developed NF-κB inhibitor (57) and demonstrated that NF-κB inhibitor treatment prevented RANKL or TNF-induced c-Fos expression (Fig. 2C) and activation (data not shown). Finally, we infected c-Fos-deficient cells with NF-κB p50+p52 retroviral constructs and treated

QiaShredder from Qiagen (Valencia, CA). cDNA synthesis was performed as described previously (40). Quantitative PCR amplification was performed with gene-specific primers using an iCycler real-time PCR machine using iQ SYBR Green supermix (both from Bio-Rad Laboratories) according to the manufacturer’s instructions. The primer sequences are as follows: NFATc1, forward, 5'-CACATTCTGGTCCATACGA-3', and reverse, 5'-CGTGTAGCTGCAATAAGG-3'; c-fos, forward, 5'-CTGTCAACACAAGGACCTTT-3', and reverse, 5'-AGAGATAGCTGCTCTACITGG-3'; β-actin, forward, 5'-ACCCA-GATCATGTGTGAGAC-3', and reverse, 5'-GTCAGGATCT-TCACTAGGTGT-3'. The relative standard curve method was used to calculate the amplification difference for each primer set (54). The standard curve was made from four points corresponding to 10-fold cDNA serial dilution for each gene. For each sample, the relative amount was calculated from its respective standard curve. The quantity of c-fos or NFATc1 mRNA was then obtained by division of each value by the actin value. Standards and samples were run in triplicate.

**Western Blot Analysis**—Infected cells were lysed with radioimmunoprecipitation buffer (50 mM HEPES at pH 7, 1% Triton X-100, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 150 mM NaCl with protease inhibitors, and sodium orthovanadate). The lysates (20 μg of protein) were resolved by 10% SDS-PAGE and immunoblotted with a rabbit anti-c-Fos, mouse anti-NFATc1 (both from Santa Cruz Biotechnology, Santa Cruz, CA), or a mouse anti-actin (Sigma) antibody.

**NFATc1 Nuclear Translocation**—c-Fos or GFP virus-infected MDS were cultured with M-CSF and RANKL in 96-well culture plates to generate osteoclasts. After mature osteoclasts were observed, cells were fixed with 10% neutral buffered formalin and permeabilized using 0.1% Triton X-100. Immunofluorescent staining was performed using mouse anti-NFATc1 antibody followed by Cy3-conjugated anti-murine immunoglobulin (Jackson ImmunoResearch, West Grove, PA). Subcellular localization of Cy3-labeled NFATc1 was observed using fluorescence microscopy.

**Electrophoretic Mobility Shift Assay (EMSA)**—To assess c-Fos activation, EMSA was performed as described previously (55). Briefly, 5 μg of nuclear extracts prepared from untreated MDS or RANKL- or TNF-treated MDS were incubated with 32P-end-labeled 21-mer double-stranded oligonucleotide containing the consensus AP-1 site (5'-CGTGTAGCTGCAACTCAGC GGAAA-3') (Santa Cruz Biotechnology) for 30 min at room temperature. The DNA-protein complex formed was then separated from free oligonucleotide on 5% native polyacrylamide gels. Binding specificity was examined by competition with 30-fold excess unlabeled AP-1 oligonucleotide. SP-1 binding sequence 5'-CGACGCGGCCGCCCACATC-3' (Invitrogen) was used as a loading control.

**Statistics**—All experiments were performed more than once with similar results. Results are given as mean ± S.E. Comparisons were made by analysis of variance and Mann-Whitney’s U test for unpaired data. P values <0.05 were considered statistically significant.
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A

No, TRAP+ Ocs/well

\[ \begin{array}{cccc}
\text{p50/p52} & \text{GFP} & \text{p50/p52} & \text{p50} \\
\text{RANKL} & 0 & 0 & 0 \\
\end{array} \]

FIGURE 1. c-Fos is downstream of NF-κB in RANKL-induced osteoclastogenesis. A and B, NF-κB dKO splenocytes were cultured with M-CSF for 3 days to generate osteoclast precursors, called MDS (as described above). MDS were infected with retroviral supernatant containing NF-κB p50+p52, p50, or p52 (A) or c-Fos (B) with M-CSF for 2 days, selected in medium containing puromycin (2 μg/ml) plus M-CSF (10 ng/ml) for 2 days, and cultured with M-CSF and RANKL (10 ng/ml) or TNF (20 ng/ml) for 5 days (n = 4 wells/group), and TRAP+ osteoclasts were counted. C, c-Fos mRNA was assessed by real-time RT PCR using total RNA from c-Fos- or GFP-infected MDS after culture for 4 days. Values (means of triplicate loadings plus S.E.) are the -fold changes of c-fos/actin in c-Fos- over GFP-infected samples. D, TRAP-stained c-Fos- or GFP-infected dKO cells cultured with RANKL on plastic dishes (panels a and d) or bone slices (panels b and e). Resorption pits (panels f, arrows) produced by these cells (panels c and f) stained with toluidine blue; osteoclasts and resorption pits from WT cells were induced by c-Fos/RANKL (panels g–i), *p < 0.05 versus GFP-treated groups.

them with RANKL. As expected, overexpression of NF-κB in these cells did not rescue their defect in osteoclast formation (data not shown). These findings indicate that c-Fos is downstream of NF-κB in RANKL and TNF-induced osteoclast formation.

C-Fos Up-regulates NFATc1 Expression in NF-κB dKO Osteoclast Precursors, and NFATc1 Overexpression Rescues the Defect in Osteoclast Formation in NF-κB dKO Cells—NFATc1 functions downstream of c-Fos during osteoclastogenesis in WT cells (29). If this is also the case in dKO cells, we should not observe increased NFATc1 expression in RANKL-treated dKO cells, and NFATc1 expression should be rescued by c-Fos overexpression. We found that RANKL treatment of WT, but not dKO MDS, increased NFATc1 mRNA 5-fold. In contrast, c-Fos overexpression plus RANKL induced NFATc1 mRNA in both WT and dKO cells, as assessed by real-time RT-PCR (Fig. 3A). Consistent with this, c-Fos/RANKL-induced c-Fos osteoclasts had increased NFATc1 protein expression (Fig. 3B), but RANKL alone did not induce endogenous c-Fos or NFATc1 expression in dKO MDS in the absence of c-Fos retroviral infection (Fig. 3B). NFATc1 activation by RANKL in both WT and dKO osteoclasts was examined by immunofluorescence staining using an anti-NFATc1 antibody. In WT or dKO osteoclast precursors cultured in the absence of RANKL, NFATc1 is detectable only in the cytoplasm regardless of c-Fos overexpression (Fig. 3C, panels a–d). In mature WT osteoclasts, NFATc1 translocated to nuclei in response to RANKL plus either GFP or c-Fos infection (Fig. 3C, panels e and f). By contrast, in dKO MDS only infected with c-Fos, no mature osteoclasts formed, and therefore, no NFATc1 translocation was observed (Fig. 3C, panel g). However, NFATc1 was observed in
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FIGURE 3. c-Fos expression and RANKL induce NFATc1 expression and activation in NF-κB dKO osteoclast precursors during osteoclast differentiation. A, WT or NF-κB dKO MDS were generated, infected with c-Fos or GFP, and treated with M-CSF ± RANKL, and NFATc1 expression was assessed as described in the legend for Fig. 1. *, p < 0.05 versus WT cells. B, c-Fos and NFATc1 protein expression levels in c-Fos and GFP-infected dKO cultures treated with and without RANKL for 5 days were assessed by Western blot analysis. C, mature osteoclasts were generated from WT or NF-κB dKO MDS that were infected with c-Fos or GFP retroviral supernatants in the presence of M-CSF ± RANKL or PBS. When multinucleated cells with the morphologic features of osteoclasts were observed under an inverted microscope, the cells were stained with anti-NFATc1 antibody to identify nuclear translocation (×10; insets are ×40).

FIGURE 4. NFATc1 expression and RANKL or TNF treatment induce osteoclast formation from NF-κB dKO osteoclast precursors. A, NF-κB dKO MDS were generated, infected with NFATc1, c-Fos or GFP, and treated with M-CSF ± RANKL or PBS, and TRAP+ osteoclast formation was assessed as described in the legend for Fig. 1. B, TRAP+ osteoclasts (Ocls, arrows) formed in the NFATc1-infected cultures treated with PBS, but their numbers were much smaller than in the cultures treated with RANKL. C, NF-κB dKO MDS were generated, infected with NFATc1 or GFP, and treated with M-CSF ± TNF or PBS, and TRAP+ osteoclast formation was assessed, as described in the legend for Fig. 1. D, NFATc1 mRNA was assessed by real-time RT PCR using total RNA from c-Fos- or GFP-infected MDS. *, p < 0.05 versus GFP-infected group.
peaked 1 day later (Fig. 5, A–C), associated with lower levels of NFATc1 expression and fewer osteoclasts. This suggests that the weaker osteoclastogenic effect of TNF in comparison with RANKL in these cultures may be due to lower induced NFATc1 expression.

c-Fos Expression Increases the Resorptive Activity of Osteoclasts Induced from WT MDS by TNF—Having demonstrated that c-Fos can substitute for NF-κB in osteoclast formation, we next examined whether c-Fos could increase the bone-resorbing capacity of mature osteoclasts induced by TNF. The ability of TNF to induce osteoclast formation and bone resorption in vitro has been controversial (13). However, we found that TNF-induced osteoclasts do resorb bone (Fig. 6), although the pits were smaller and shallower than those formed in response to RANKL (data not shown). c-Fos also significantly increased the TNF-induced pit area (Fig. 6).

DISCUSSION

Expression of NF-κB, c-Fos, and NFATc1 is required for osteoclast precursors to differentiate into osteoclasts in response to RANKL. Here, we show that c-Fos and NFATc1 induction is downstream of NF-κB p50 and p52 activation in RANKL-mediated signaling in osteoclast precursor differentiation because either c-Fos or NFATc1 can efficiently substitute for NF-κB p50 and p52 in RANKL-treated NF-κB p50/p52 dKO cells to induce osteoclast formation and bone resorption. Furthermore unexpectedly, c-Fos or NFATc1 can substitute for NF-κB in TNF-stimulated osteoclast formation from NF-κB dKO cells in vitro. NFATc1 has been described as the master switch for regulating terminal differentiation of osteoclasts downstream from RANKL, and its expression is regulated by c-Fos (29, 33). Our findings indicate that NF-κB p50 and p52 play an essential role regulating the early differentiation of osteoclast precursors upstream from c-Fos and NFATc1 in response to RANKL and TNF, both of which can induce osteoclast formation directly from osteoclast precursors in vitro.

The importance of these findings is that expression of RANKL and TNF is increased in a variety of common bone diseases associated with increased bone resorption, including postmenopausal osteoporosis, rheumatoid arthritis, and periodontal disease (2, 58, 59). These two cytokines can increase the expression of each other by accessory cells and of TNF by osteoclasts and their precursors (60, 61). By this latter mechanism, TNF could amplify its osteoclastogenic effects in an autocrine auto-amplifying manner, setting up what we propose is an auto-amplifying cycle of increasing osteoclastogenesis whereby TNF induces more osteoclasts and their precursors, which produce more TNF to induce formation of more osteoclasts, and so on. Excessive production of TNF in joints of patients with rheumatoid arthritis could account in large part for the aggressive bone resorption seen in affected joints of these patients. The
efficacy of anti-TNF therapy in many rheumatic patients supports this assertion (62).

Overexpression of NF-κB p50 and p52 or c-Fos alone in dKO MDS did not induce osteoclast formation in our cultures. Although NFATc1 overexpression alone did induce small numbers of osteoclasts, as with NF-κB p50 and p52 or c-Fos, concomitant treatment with cytokines was required to rescue the osteoclast formation defect efficiently. Currently, we do not know why cytokines are required for this efficient rescue of the osteoclast formation defect. When c-Fos or NFATc1 was overexpressed alone in c-fos−/− cells, this, too, did not rescue the osteoclastogenesis defect and needed the addition of RANKL for efficient rescue (33). We therefore speculate that this retroviral rescue system has an intrinsic deficiency that prevents full recovery of osteoclastogenesis or that to initiate the osteoclast formation program, these virus-encoded transcription factors need to be activated through RANKL signaling and interact with a yet to be identified RANKL-induced molecule. However, it is also possible that other mechanisms are involved in RANKL-induced osteoclast formation. Further studies will be required to address this particular issue.

To date, there is no evidence that NF-κB activates c-Fos directly, and there are no consensus NF-κB binding sites on the c-Fos promoter. Indirect activation of c-Fos by NF-κB through serum-response factors has been described in embryonic fibroblasts (63). Recently, we have found that NF-κB binding activity was significantly higher in c-fos−/− MDS when compared with WT cells, supporting the argument that NF-κB is upstream rather than downstream of c-Fos (64). The report that NF-κB p65 and p50 transiently interact with NFATc2, but not with NFATc1, on the NFATc2 promoter is intriguing and warrants further study to determine exactly what the role of NF-κB p50 and p52 and NFAT2 proteins is in the early stages of osteoclastogenesis.

TNF, like RANKL, can activate a variety of signaling pathways in osteoclasts and their precursors (reviewed in Refs. 8, 58, and 65) that induce osteoclast formation and activation. Our observation of a sequence of induction by RANKL and TNF beginning with NF-κB followed by c-Fos and NFATc1 suggests that these cytokines may share a common pathway to induce osteoclast precursor differentiation in addition to activating c-Fos. However, RANKL and TNF recruit different adapter proteins, TNF receptor-associated factor (TRAF) 6 (66) and TRAF2 (67), respectively, to their receptors to mediate downstream signaling, suggesting that they should activate different pathways. RANKL has been reported to regulate stimulated, but not basal, osteoclast formation by a pathway involving NF-κB-inducing kinase and NF-κB p100 processing to p52 in the NF-κB alternative pathway (68). However, these authors did not observe this effect with short term TNF treatment, again suggesting that RANKL and TNF may use different pathways. Further studies will be required to determine what these are.

In the later stages of our cultures after 24–48 h when osteoclasts begin to form, RANKL and TNF induced expression of NFATc1 in c-Fos-infected dKO MDS. That NFATc1 is essential for osteoclastogenesis and works downstream of c-Fos has been established by genetic studies (29, 33). For example, overexpression of NFATc1 retrovirus plus RANKL treatment rescued the osteoclastogenesis defect in Fos−/− osteoclast precursors (33). The recent studies by Asagiri et al. (45) reporting that NF-κB p65 and p50 bind transiently to the promoter region of NFATc1 along with NFATc2 within 1 h of treatment with RANKL highlight the complexity of the signaling downstream from RANKL/RANK interaction and our limited understanding of how RANKL mediates osteoclast formation.

The current paradigm on osteoclastogenesis posits that most cytokines, hormones, and growth factors induce osteoclast formation and bone resorption predominantly by an indirect mechanism whereby they promote RANKL and M-CSF expression in osteoblast/stromal cells, rather than by directly acting on osteoclast precursors to mediate the differentiation of RANK-expressing precursors into mature osteoclasts. Indeed, it has been proposed recently that stromal cells expressing M-CSF make a greater contribution to TNF-mediated osteoclast formation than osteoclast precursors (18) and that osteoclast formation in vitro requires prior priming of these precursors by RANKL (69). However, the claim in this latter study has now been disproved (42, 43). Thus, it is now clear that TNF can induce osteoclast formation both directly and indirectly at least in vitro, although the relative contributions of these two mechanisms in vivo remain uncertain.

Our findings suggest that osteoclastogenesis is controlled by a hierarchical transcriptional program in which NF-κB activation appears to be the first event and is followed by c-Fos and NFATc1 activation. NF-κB is not essential for the commitment of hematopoietic stem cells to the osteoclast progenitor cells because these cells can give rise to functional osteoclasts if the current paradigm on osteoclastogenesis posits that most cytokines, hormones, and growth factors induce osteoclast formation and bone resorption predominantly by an indirect mechanism whereby they promote RANKL and M-CSF expression in osteoblast/stromal cells, rather than by directly acting on osteoclast precursors to mediate the differentiation of RANK-expressing precursors into mature osteoclasts. Indeed, it has been proposed recently that stromal cells expressing M-CSF make a greater contribution to TNF-mediated osteoclast formation than osteoclast precursors (18) and that osteoclast formation in vitro requires prior priming of these precursors by RANKL (69). However, the claim in this latter study has now been disproved (42, 43). Thus, it is now clear that TNF can induce osteoclast formation both directly and indirectly by RANKL or TNF in the early stages of osteoclast precursor differentiation, rather than during the later stages where an NFATc1 inhibitor would be predicted to work, based on published data (70, 71) and on our data. Blockade of NF-κB signaling using such an approach may be an effective therapeutic strategy to prevent bone loss in a variety of common bone diseases. This overall beneficial effect of NF-κB inhibition in preclinical studies contrasts with the disappointing results with the calcineurin inhibitor, cyclosporin A, which induces osteoporosis in humans (72), presumably because it inhibits not only NFATc1 activation in osteoclasts but also in osteoblasts by down-regulating expression of the NFATc1 target gene, ostein, in osteoblasts, and thus, reducing bone formation directly (73).

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