Tumor Necrosis Factor-α (TNF) Stimulates RANKL-induced Osteoclastogenesis via Coupling of TNF Type 1 Receptor and RANK Signaling Pathways*

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Joint destruction because of matrix degradation and excessive bone loss characterizes inflammatory bone diseases such as osteolysis, osteoarthritis, and rheumatoid arthritis (1–4). Accumulation of inflammatory cells and their secreted products at the inflammation site attracts osteoclasts and their precursor cells, leading to further deterioration of the bone component (5–7). Tumor necrosis factor-α (TNF), interleukin-1 (IL-1), and receptor activator of NF-κB ligand (RANKL, also known as OPGL and ODF), are abundant in sites of inflammation and are known to promote osteoclast recruitment, differentiation, and activation (8–12). Osteoclast differentiation per se requires activation of the RANK/RANKL pathway (13, 14). Recent evidence points out that RANKL is also secreted by T helper 1 lymphocytes, the cells responsible for secretion of the pro-inflammatory cytokines TNF and IL-1 (12). More importantly, a direct role of T cell secreted RANKL in promoting joint inflammation, bone, and cartilage destruction has been established (12). Thus, RANKL and TNF may orchestrate bone and tissue dissolution in inflammatory bone diseases.

In general, TNF receptor family members when activated recruit TNF receptor-associated factor (TRAF) proteins to their cytoplasmic tail. Acting as adaptor proteins, TRAFs bind to and activate several downstream tyrosine and serine/threonine kinases, including c-Src, Akt/PKB, and MEKK-1. These in turn activate several downstream tyrosine and serine/threonine kinases, including c-Src, Akt/PKB, and MEKK-1. These in turn prompt activation, primarily via phosphorylation events, of a signalsome-residing molecules, such as (a) activation of IκB kinase (IKK) pathway, (b) MEKK1 >> ERK, and (c) MEKK1 >> JNK >> c-Jun/AP-1. RANKL transmits its signal via a member of the TNF receptor family, RANK (15). The complete repertoire of RANK intracellular signaling is unclear; however, similar to other TNF receptors, it recruits members of the TRAF adapter proteins and activates AP-1 and NF-κB (16, 17). Activation of these transcription factors entails phosphorylation of inhibitory proteins followed by release and nuclear translocation of the transcription factors. Thus, a considerable overlap exists in the signal transduction pathways transmitted by RANKL and TNF receptors.

TNF (18, 19) recognizes two receptors: TNF-receptor 1 (TNFr1) and TNF-receptor 2 (TNFr2). We have previously shown that TNF promotes osteoclastogenesis (21, 22), whereas TNF-receptor 2 is inhibitory. Among the important events following TNF and RANKL occupancy of their respective receptors, as already mentioned, are mobilization of NF-κB and c-Jun/AP-1 (16, 17, 23). The pivotal role that NF-κB plays in osteoclast recruitment is established by the fact that mice lacking both the p50 and p52 NF-κB subunits develop a form of osteopetrosis in which the animal is completely devoid of osteoclasts (24, 25). Likewise, ablation of the c-fos component of

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AP-1 resulted in a similar osteopetrotic bone phenotype (26). Finally, dominant-negative blockade of ERK signaling dampens osteoclastogenesis (27). We and others have shown that inflammatory osteolysis reflects TNF-induced recruitment of osteoclasts that resorb alveolar bone leading to edentulism (21). Abundant RANKL in sites of bone erosion further suggests that local differentiation and activation of osteoclasts by pro-inflammatory cytokine is a likely occurring event. Because TNF is also abundant in inflamed bone sites and plays a major role in progression of the disease, understanding the molecular mechanisms by which this inflammatory cytokine accelerates RANK-induced osteoclastogenesis will provide the basis for prevention of inflammatory bone osteolysis.

In this study, we show that TNF closely regulates RANK/RANKL-induced osteoclastogenesis. TNF markedly accelerates basal osteoclastogenesis induced by RANKL via its TNFR1. We also find that RANKL and TNF ultimately induce expression and recruitment of TRAF2, TRAF6, MEKK-1, and c-Src. These events lead to TNFR1-dependent activation of NF-κB, ERK, and c-Jun/AP-1, induction of RANK, and osteoclastogenesis.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All antibodies were purchased from Santa Cruz (Santa Cruz, CA). Recombinant murine TNF and M-CSF were purchased from R & D Systems Inc. (Minneapolis, MN). RANKL was produced in Escherichia coli by fusion of the region from lysine 158 to the C terminus of the mouse cDNA to thioredoxin and will be described in detail elsewhere. ECL kit was obtained from Pierce. All other chemicals were obtained from Sigma.

**Animals**—C3H/HeN males were purchased from Harlan Industries (Indianapolis, IN). Knockout mice for TNF receptors p55 and p75 and their wild type controls were provided by Drs. Warner Lesslauer (Hoffman-LaRoche) and Mark Moore (Genentech Inc., South San Francisco, CA), respectively.

**Cell Culture**—Bone marrow macrophages were isolated from whole bone marrow of 4–6-week-old mice and incubated in tissue culture plates, at 37°C in 5% CO₂, in the presence of 10 ng/ml M-CSF (28). After 24 h in culture, the nonadherent cells were collected and layered on a Ficoll-Hypaque gradient. Cells at the gradient interface were collected and plated in α-minimum essential medium, supplemented with 0.5% heat-inactivated fetal bovine serum, at 37°C in 5% CO₂ in the presence of 10 ng/ml M-CSF, and plated according to each experimental condition.

**Osteoclast Generation**—Purified marrow macrophages were cultured at 1 × 10⁶ cells/ml in the presence of 10 ng/ml M-CSF and 20 ng/ml RANKL for 4 days. Cultures were supplemented with M-CSF and RANKL on day 2 of culture.

**Immunoblotting**—Total cell lysates were boiled in the presence of 2× SDS-sample buffer (0.5 M Tris- HCl, pH 6.8, 10% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromphenol blue, distilled water) for 30 s and subjected to electrophoresis on 8–12% SDS-polyacrylamide gel electrophoresis (29). Proteins were transferred to nitrocellulose membranes using asemi-dry blotter (Bio-Rad) and incubated in blocking solution (10% skim milk prepared in phosphate-buffered saline containing 0.05% Tween 20) to reduce nonspecific binding. Membranes were washed with phosphate-buffered saline/Tween buffer and exposed to primary antibodies (1 h at room temperature up to overnight at 4°C), washed again four times, and incubated with the respective secondary horseradish peroxidase-conjugated antibodies (1 h, room temperature). Membranes were washed extensively (5 × 15 min), and an ECL detection assay was performed following the manufacturer’s directions.

**Electrophoretic Mobility Shift Assay**—Nuclear fractions were prepared as described previously (30, 31). In brief, monolayers of bone marrow macrophages grown in 100-mm² tissue culture dish were washed twice with ice-cold phosphate-buffered saline. Cells were lifted from the dish by treating with 5 mM EDTA and 5 mM EGTA in phosphate-buffered saline. Cells were then resuspended in hypotonic lysis buffer A (10 mM HEPES, pH 7.8, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 5 μg/ml leupeptin) and incubated on ice for 5 min, and Nonidet P-40 was added to a final concentration of 0.64%. Nuclei were pelleted, and the cytosolic fraction was carefully removed. The nuclei were then resuspended in nuclear extraction buffer B (20 mM HEPES, pH 7.8, 420 mM NaCl, 1.2 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin), vortexed for 30 s and rotated for 30 min in 4°C. The samples were then centrifuged, the nuclear proteins in the supernatant were transferred to fresh tubes, and protein content was measured using standard BCA kit (Pierce). Nuclear extracts (10 μg) were incubated with an end-labeled double stranded oligonucleotide probe containing the sequence 5′-AAAA CAG GGG GCT TTC CCT CCT C-3′ (32) derived from the xB3 site of the TNF promoter or with commercial cJun/AP-1 oligonucleotide (Santa Cruz). The reaction was performed in a total of 20 μl of binding buffer (20 mM HEPES, pH 7.8, 100 mM NaCl, 0.5 mM dithiothreitol, 1 μg of poly(dI-dC), and 10% glycerol) for 30 min at room temperature. Samples were then fractionated on a 4% polyacrylamide gel and visualized by exposing dried gel to film.

**RESULTS**

**TNF Accelerates RANKL-mediated Osteoclastogenesis by Bone Marrow Macrophages via Its Type 1 Receptor**—We have shown that TNF stimulates osteoclastogenesis in whole marrow cultures and in co-culture of osteoclast precursors with stromal cells (21, 22). Basal osteoclastogenesis in these two systems was induced by 1,25-dihydroxyvitamin D₃. Given the presence of both stromal and osteoclast precursors in those cultures, it is not clear which cell type is targeted by TNF. To address this issue, a pure population of osteoclast precursors, in the form of monocytes/macrophages, was cultured in the presence of RANKL and M-CSF for 4 days. Cells were then treated with carrier or TNF for 24 h, after which cultures were fixed and stained for tartrate-resistant acid phosphatase, a hallmark of osteoclasts (21). We found that TNF dramatically enhances basal osteoclastogenesis by RANKL-induced bone marrow macrophages (Fig. 1). A 4–5-fold increase in the number of multi-nucleated osteoclasts and a 4–12-fold increase in cell size were observed. The average number of osteoclasts in RANKL-treated wild type cultures (quadruplicate wells ± S.D., n = 3) was 134 ± 21 compared with 732 ± 115 in RANKL + TNF-

**FIG. 1.** TNF accelerates RANKL-induced osteoclastogenesis via its type 1 receptor. Osteoclast precursor cells were isolated from the bone marrow of 4–6-week-old mice (wild type, TNFR1−/−, and TNFR2−/−) as described under “Experimental Procedures.” Pure marrow macrophages (>90%) were plated in 48-well plates at 1 × 10⁶ cells/ml using α-minimum essential medium supplemented with 10% heat-inactivated fetal calf serum and 10 ng/ml M-CSF. Cultures were placed at 37°C with 5% CO₂, and were treated with 20 ng/ml soluble RANKL for 3–4 days. TNF (10 ng/ml) was then added to one half of the cultures for an additional 24 h. Developing osteoclasts were then fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity following the manufacturer’s directions. Tartrate-resistant acid phosphatase-positive (purple) mono and multi-nucleated large cells are osteoclasts and their committed precursors. Results represent three independent experiments. Images represent 20× magnification taken by light microscope.
TNF transmits its biological signals via two receptors, TNFR1 and TNFR2, and we have shown previously that TNFR1 is the pro-osteoclastic moiety (21, 22). Using cells derived from mice lacking individual TNF receptor, we found that cells deleted of TNFR1 generate far less osteoclasts in response to RANKL than their wild type counterparts (52 ± 14 versus 134 ± 21, respectively; p < 0.005) (Fig. 1). More importantly, addition of TNF to wild type cells while generating exuberant osteoclast response overnight (top right panel), it failed to impact osteoclastogenesis by TNFR1-null cells (middle right panel). Number of osteoclasts in RANKL-treated wild type cells was 52 ± 14, which was statistically not different from the number of osteoclasts from TNFR1 treated with RANKL and TNF (61 ± 21 osteoclasts). Furthermore, size and multi-nucleation of osteoclasts in TNFR1 knockout cells were not affected by TNF treatment.

In contrast, osteoclastogenesis by cells lacking TNFR2 yet expressing TNFR1 resumed in a similar manner seen in wild type cells (bottom panels). Consistent with our previous observations (22), we found an increased number of osteoclasts (189 ± 17) from TNFR2 mice in basal conditions (+ RANKL), which was dramatically increased with TNF addition (847 ± 79, p < 0.001). Increased basal and TNF-induced osteoclastogenesis by TNFR2-null cells is consistent with the role of this receptor as an osteoclast suppressor (22) and further enforces the requirement of TNFR1 for normal osteoclastogenesis.

**TNFR1 Regulates Expression of TRAF2, TRAF6, c-Src, and MEKK1 in Osteoclasts**—We next turned to investigate the molecular pathway(s) by which TNF accelerates RANKL-induced osteoclastogenesis. Signaling of TNF and RANKL requires recruitment of TRAFs and activation of subsequent kinases. Thus, we examined the expression of these proteins in osteoclasts differentiated in vitro compared with their precursors. We found that osteoclasts express elevated levels of TRAF2, TRAF6, c-Src, and MEKK1, whereas expression of these proteins by untreated precursor cells was negligible (Fig. 2A). Contrary to that, we found that expression of these proteins in TNFR1-null osteoclasts is reduced. These data are consistent with the pro-osteoclastic role of TNFR1.

Having established the expression levels of TRAF2, TRAF6, and MEKK1 in osteoclasts, we examined expression of these proteins in precursor cells in response to RANKL, TNF, and other pro-osteoclastic agents following 24 h of treatment. We found that expression of TRAF2, TRAF6, and MEKK1 is highly induced in wild type cells by RANKL, TNF, and pro-osteoclastic agents, such as lipopolysaccharide and IL-1 (Fig. 2B). These observations point out that expression of these proteins is likely essential for osteoclast differentiation evident by their reduced expression in TNFR1-null osteoclasts, which were poorly generated with RANKL (Fig. 2A). These findings further support the possibility that TNFR1 is essential for TNF-enhanced activation of the RANK/RANKL pathway.

**RANKL Activation of Erk and c-Jun Is Diminished in the Absence of TNFR1**—Recruitment of TRAF2, TRAF6, and MEKK1 to the cytoplasmic tail of TNF receptor family members facilitates binding and activation of multiple downstream kinases. These in turn, lead to activation of (a) MEKK1 > JNK > c-Jun/AP-1, (b) MEKK1 > MEK > ERK, and (c) TRAFs > IkB kinases > IkB > NF-κB signaling pathways, all of which are essential for osteoclast differentiation and survival (24, 26, 27). Thus, we asked whether, similar to the impaired TRAFs and MEKK1 expression observed in TNFR1-null cells, one or more of these downstream pathways are also impaired.

To this end we first examined expression and phosphorylation of MEK1 and ERK1/2 in RANKL-treated wild type and TNFR1 knockout cells. Activation of MEK1 is manifested by its phosphorylation, at least in part by MEKK1. MEKK1 then phosphorylates ERK1/2, leading to their dimerization and nuclear translocation. Reflecting reduced expression of MEKK1 in RANKL-generated TNFR1-null osteoclasts, we found that MEKK1 phosphorylation induced by RANKL is significantly reduced in TNFR1-null compared with wild type cells (Fig. 3A). Hypophosphorylation of the mitogen-activated protein kinase is specific because MEKK1 protein was equally expressed in all conditions. Similarly, we found that ERK1/2 proteins are equally expressed in both cell types (Fig. 3B), and their expression is unaffected by the addition of RANKL. Interestingly, however, and despite the equal expression of the protein in both cell types, phosphorylation of the mitogen-activated protein kinases resembled that observed with the upstream kinase MEKK1. Namely, although ERK1/2 undergo rapid phosphorylation within 10 min, which is sustained up to 4 h after exposure to RANKL, little or no phosphorylation was observed in RANKL-treated TNFR1-null cells. Residual and transient phosphorylation of ERK1/2 in TNFR1 knockout cells was detected only after 40 min of exposure to RANKL. This finding correlates well with reduced expression of MEKK1 in TNFR1-null osteoclasts (generated with RANKL) (Fig. 2A). Moreover, given the established role of ERK activation as essential for osteoclast activation and survival (27), these findings support the notion that TNFR1 is required for normal osteoclastogenesis.

MEKK-1, via JNK, also leads to c-Jun phosphorylation and nuclear translocation. Using electrophoretic mobility shift assay, we provide evidence that c-Jun is activated in osteoclast precursors induced by RANKL (Fig. 4). More importantly, we
TNFr1 Regulates RANKL-mediated Osteoclastogenesis

Fig. 3. RANKL induces phosphorylation of MEK1 as well as ERK1 and 2 in a TNFr1-dependent manner. Osteoclast precursors (macrophages) from wild type (WT) and TNFr1 knockout mice were treated with RANKL for the time points indicated. Cell lysates were then subjected to western immunoblots using anti-MEK1 and phosphoMEK1 antibodies (A) and anti-ERK1/2 and phosphoERK1/2 antibodies (B).

Fig. 4. Activation of cJun/AP-1 transcription factor by RANKL and TNF is severely reduced in TNFr1 knockout cells. Wild type and TNFr1- cells were treated with RANKL or TNF for the time points indicated. Nuclear extracts were then prepared and subjected to electrophoretic mobility shift assay using a cJun/AP-1 labeled oligonucleotide.

We found that activation of c-Jun/AP-1 by RANKL is severely reduced in TNFr1-null compared with wild type cells. Mirroring its pro-osteoclastic effect (Fig. 1), TNF alone, although activating the transcription factor in wild type cells as expected (Fig. 4, lanes 6 and 7), fails to trigger such response in TNFr1-null cells (Fig. 4, lanes 12–14). Lack of transcription factor activation in TNFr1-null cells is in keeping with its principal role in induction of RANKL-mediated osteoclastogenesis.

RANKL-induced Phosphorylation of IxB and Activation of NF-xB Is TNFr1-dependent—NF-xB is essential for osteoclast formation. Activation of this transcription factor is a consequence of IxB phosphorylation and dissociation of the IxB/NF-xB complex followed by nuclear translocation of NF-xB and binding to DNA (23). We have documented that expression of the adaptor proteins TRAF2 and TRAF6 is impaired in RANKL-generated TNFr1 knockout osteoclasts (Fig. 2), a phenomenon consistent with reduced osteoclastogenesis by the same cells (Fig. 1). Thus, we asked whether reduced osteoclastogenesis in RANKL-treated TNFr1-null cells also reflect impaired NF-xB activation, a process mediated through TRAF protein recruitment (23). First, we document that RANKL induces rapid phosphorylation of IxB manifested by the indicated slow migrating band (Fig. 5A, IxB-p). This phosphorylation persists for 2 h and diminishes after 3 h. Reflecting IxB phosphorylation, our data show that similar to TNF, RANKL activates NF-xB in wild type cells, evidenced by increased DNA binding activity with time (Fig. 5B, lanes 2–4). In contrast and enforcing the role of TNFr1 in RANKL-induced osteoclastogenesis, we found that IxB is hypophosphorylated (Fig. 5A) and that NF-xB nuclear translocation and DNA binding activity are severely reduced in cells lacking TNFr1 (Fig. 5B, lanes 8–14). Interestingly, in TNFr1-null cells, activation of NF-xB was severely reduced in the presence of RANKL and absent following TNF treatment. These data, once again, highlight the essential role of TNFr1 as an activator of NF-xB, a transcription factor critical for osteoclast formation.

TNFr1 Regulates RANK—Thus far, our data indicate that signaling of osteoclastogenesis by TNFr1 and RANK is seemingly overlapping, and activation of TNFr1-dependent events accelerates RANKL-induced osteoclastogenesis. More specifically, deletion of TNFr1 dampens RANKL-induced expression, phosphorylation, and activation of key proteins required for normal osteoclastogenesis, such as TRAFs, MEKK1, c-Src, IxB, NF-xB, AP-1/c-Jun, and ERK proteins. Thus, it is reasonable to hypothesize that endogenous TNFr1 signaling regulates RANK expression/function. To address this issue, we examined RANK expression by TNFr1-null cells. To this end, marrow macrophages from wild type and TNFr1-null cells were cultured for 4 days in the absence or presence of RANKL, and levels of RANK expression were assessed by immunoblots. We found that expression of RANK is reduced in the absence of TNFr1 (Fig. 6, lanes 1–4) and remain low in the presence of RANKL and/or TNF (Fig. 6, lanes 2 and 3). In contrast, levels of RANK were elevated in wild type cells (Fig. 6, lane 5) and were synergistically increased when treated with RANKL and TNF (lane 8). These observations suggest that signals transmitted by the TNFr1, likely to be NF-xB and/or AP-1-dependent genes, regulate RANK expression.

DISCUSSION

Osteoclastogenesis is mediated via ligation of RANKL to its transmembranal receptor, RANK (13, 14). Furthermore, osteoclast recruitment and activation is markedly induced in states of inflammatory bone diseases (5–7). In this regard, we and
TNFr1- Wild type

TNF: - - - - - - +
RANKL: - - - - - - +
RANK- 1 2 3 4 5 6 7 8

Fig. 6. TNF, acting through its type 1 receptor, induces RANK expression synergistically with RANKL. Wild type and TNFr1 knockout cells were treated with vehicle, TNF, RANKL, or both cytokines for 24 h. Cells were then lysed, and RANK expression was measured by immunoblotting of equal amounts of total cell proteins. Equal protein loading was further confirmed by incubating nitrocellulose membrane in Ponceau S solution.

others have documented a primary role for TNF in stimulating osteoclastogenesis in vitro and in vivo (21, 22, 33, 34). The recent discovery of RANK signaling pathway in osteoclasts and their precursors enables us to investigate the direct effect, if any, of pro- and anti-inflammatory factors on RANK-induced osteoclastogenesis.

In this study, we established that TNF acts directly on osteoclast precursors, thus providing a clear target to prevent TNF signaling in states of bone inflammation. Similar to TNF, RANKL signaling involves recruitment and activation of key proteins essential for osteoclastogenesis, some of which include TRAFs, MEKK1, c-Src, IκB kinases, ERKs, c-Jun, and NF-κB (16, 17). The obvious overlap between TNF and RANKL stimulation of their respective pathways, their abundance in sites of bone inflammation, and their documented pro-osteoclastic role prompted us to investigate possible cooperative signaling between TNF receptors and RANK with regard to events leading to osteoclastogenesis. We found that levels of TRAF2, TRAF6, and MEKK1 are increased in response to treatment of precursors with osteoclastogenic agents, such as RANKL, TNF, IL-1, and lipopolysaccharide, and parallel their high level of expression in differentiated osteoclasts. Interestingly, and in support of its essential role in osteoclastogenesis (35, 36), we found expression of TRAF6 is significantly higher than TRAF2. In any case, expression of TRAF2 and TRAF6 by RANKL or TNF-treated macrophages and high expression of these factors in fully differentiated osteoclasts is in keeping with their essential role for c-Jun and NF-κB activation, respectively (37, 38). Similar to our previous findings that TNF mobilizes and activates c-Src (21, 30), we show in this report that expression of the tyrosine kinase is elevated in RANKL-generated osteoclasts.

We also report that RANKL induces expression of the upstream mitogen-activated protein kinase, MEKK-1. MEKK-1 normally binds to and is activated by TRAF2 (37). In its activated form, MEKK-1 induces and activates, among other functions, the JNK and MEK1, leading to c-Jun and ERK activation, respectively (37). Based on these observations, it is reasonable to propose that RANKL-activated pathways converge at the level of TRAF adapter proteins. According to this scenario and consistent with previous observations (36, 37), TRAF6-c-Src complex leads to NF-κB activation, whereas TRAF2-MEKK1 primarily activates the ERK and c-Jun pathways. These pathways are not entirely distinct because of cross-talk at different stages (39, 40).

By analogy to RANKL and using similar mechanisms, TNF is capable of inducing NF-κB and c-Jun/AP-1 (20, 23). TNF exerts its effects via two receptors, TNFr1 and TNFr2. Both receptors share functional properties, but it is the TNFr1 that has been implicated as pro-osteoclastic (21, 22). Although its direct RANKL-independent osteoclastogenic potential remains disputed (41, 42), our data indicate that TNF exerts robust osteoclastogenesis by RANKL primed pre-osteoclasts. Supporting this observation is our finding that deletion of the TNFr1 attenuates endogenous and in vitro RANKL-mediated osteoclastogenesis subsequent to reduced NF-κB, ERK, and c-Jun/AP-1 activation. Reduced activation of NF-κB, ERKs, and c-Jun/AP-1 in TNFr1 knockout cells is most likely secondary to hypophosphorylation of upstream regulatory proteins, such as IκB, MEK, and JNK, two of which are documented in this study. These impaired signals appear to be TNFr1-dependent, because basal osteoclastogenesis resumes in TNFr1-expressing TNFr2-null cells in a manner greater than that seen by their wild type counterparts.

The most challenging of our findings is the endogenous effect of TNF on RANKL-mediated osteoclastogenesis. Although equal amounts of total protein were examined, expression levels of RANK were very little in TNFr1-null cells compared with wild type controls. Interestingly, administration of either TNF or RANKL has little or no impact on RANK expression in both species. In contrast, combined addition of TNF and RANKL, while eliciting no effect on TNFr1-null cells, synergistically induce RANK expression within 24 h. Thus, regulation of RANK is under the aegis of TNFr1 and requires preactivation with RANKL.

First, our findings point out that basal osteoclast formation by TNFr1 is reduced in the absence of TNFr1. Thus, endogenous expression of this receptor is essential to maintain higher level of osteoclastic pool. Further treatment with TNF of cells derived from the TNFr1-null mice fails to augment osteoclastogenesis. Second, examination of NF-κB and AP-1 transcription factors in TNFr1-null nuclear extracts points to reduced DNA binding of both factors compared with wild type-derived cells. This finding mirrors reduced osteoclastogenesis by TNFr1-null cells. These observations are in keeping with the essential role of NF-κB and AP-1 transcription factors in osteoclast differentiation and activation (16, 17, 26, 43). Third, the finding that TNFr1 transmits signals that up-regulate RANK expression and support osteoclastogenesis favorably argues that RANK gene expression may be regulated by NF-κB and/or AP-1 transcriptional machinery.

In conclusion, our findings suggest that overlapping signaling mechanisms of RANKL and TNF result in exuberant osteoclastogenesis and may mediate severe bone resorption. TNFr1 is required for both basal RANK expression/signaling and for the cytokine-enhanced phase of osteoclastogenesis.

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TNF\(_{\text{R}1}\) Regulates RANKL-mediated Osteoclastogenesis

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