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TNF Receptor Type 1 Regulates RANK Ligand Expression by Stromal Cells and Modulates Osteoclastogenesis

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Abstract TNFα is a major osteoclastogenic cytokine and a primary mediator of inflammatory osteoclastogenesis. We have previously shown that this cytokine directly targets osteoclasts and their precursors and that deletion of its type-1 receptor (TNFr1) lessens osteoclastogenesis and impacts RANK signaling molecules. Osteoclastogenesis is primarily a RANK/RANKL-dependent event and occurs in an environment governed by both hematopoietic and mesenchymal compartments. Thus, we reasoned that TNF/TNFr1 may regulate RANKL and possibly RANK expression by stromal cells and osteoclast precursors (OCPs), respectively. RT-PCR experiments reveal that levels of RANKL mRNA in WT stromal cells are increased following treatment with 1,25-VD_3 compared to low levels in TNFr1-null cells. Expression levels of OPG, the RANKL decoy protein, were largely unchanged, thus supporting a RANKL/OPG positive ratio favoring WT cells. RANK protein expression by OCPs was lower in TNFr1-null cells despite only subtle differences in mRNA expression in both cell types. Mix and match experiments of different cell populations from the two mice phenotypes show that WT stromal cells significantly, but not entirely, restore osteoclastogenesis by TNFr1-null OCPs. Similar results were obtained when the latter cells were cultured in the presence of exogenous RANKL. Altogether, these findings indicate that in the absence of TNFr1 both cell compartments are impaired. This was further confirmed by gain of function experiments using TNFr1- null cultures of both cell types at which exogenous TNFr1 cDNA was virally expressed. Thus, restoration of TNFr1 expression in OCPs and stromal cells was sufficient to reinstate osteoclastogenesis and provides direct evidence that TNFr1 integrity is required for optimal RANK-mediated osteoclastogenesis. J. Cell. Biochem. 93: 980–989, 2004.

Key words: osteoclast; stromal cells; TNF receptor; RANKL

Homeostasis of bone metabolism is a lifelong process required for protection against bone loss diseases. The fate of bone remodeling is controlled by precise machinery that includes functional coupling between osteoclasts (bone resorbing cells) and osteoblasts (bone forming cells) and their secreted products [Teitelbaum et al., 1995; Teitelbaum, 2000]. Osteoclasts arise from marrow monocytes and macrophages which, under precisely defined conditions, differentiate into multi-nucleated bone resorbing cells [Teitelbaum et al., 1995; Teitelbaum, 2000]. Previous studies have shown that steroid induction of co-cultures consisting of marrow macrophages and stromal cells resulted in the formation of bona fide osteoclasts [Suda et al., 1992; Teitelbaum et al., 1997]. Recent studies established that the osteoblast-secreted factors M-CSF and RANKL are required for osteoclastic differentiation [Takahashi et al., 1991; Tanaka et al., 1993; Lacey et al., 1998; Kong et al., 1999]. RANKL, a member of the TNF family, is a transmembrane protein produced by stromal and activated T cells [Khosla, 2001; Jones et al., 2002]. The extracellular portion of this factor is cleaved and is also considered a potent inducer of osteoclast differentiation [Lacey et al., 1998]. RANKL exerts its biological function through binding to its receptor RANK that is expressed by monocytes/macrophages [Li et al., 2000]. A decoy receptor, termed osteoprotegerin (OPG), also secreted by stromal

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cells, prevents RANKL binding to RANK by binding the cytokine and neutralizing its activity [Aubin and Bonneleye, 2000; Simonet et al., 2001; Udagawa et al., 2003].

The osteoclastogenic role of other TNF family members, particularly TNFα, has been described. We and others have shown that TNFα exacerbates osteoclastogenesis and leads to bone erosion in various inflammatory bone diseases [Lam et al., 2000; Schwarz et al., 2000; Zhang et al., 2000; Clohisy et al., 2003]. The most likely scenario is that TNFα propagates osteoclastic formation and activity by RANKL-primed cells. In this regard, our previous studies indicate that TNFα acts through its type 1 receptor to synergistically increase osteoclastogenesis by RANKL-induced marrow macrophages [Abu-Amer et al., 1997, 2001; Zhang et al., 2000]. Supporting this notion are findings by Lam et al. [2000] indicating that suboptimal concentrations of RANKL are sufficient and required to support TNF stimulation of osteoclastogenesis. We also have reported that deletion of TNFr1, the receptor that signals TNF-induced osteoclastogenesis, results in reduced overall RANKL signaling and osteoclastogenesis in particular [Zhang et al., 2000].

In the current study, we examined the mechanism(s) by which TNF/TNFr1 modulates osteoclastogenesis. We find that TNFr1 integrity is required for basal and regulated expression of RANKL by stromal cells. We also report that TNFr1 is required for normal signaling of RANK on marrow macrophages but to a lesser degree compared with stromal cells. "Mix and match" experiments, show that wild type stromal cells significantly, but not completely, restore osteoclastogenesis by TNFr1-null bone marrow macrophages otherwise known as osteoclast precursors (OCPs). In addition re-introduction of TNFr1 expression in TNFr1-null stromal cells and OCPs using retroviral infections restores osteoclastogenesis. Our findings point out that endogenous expression of TNFr1 is required for optimal RANK/RANKL-induced osteoclastogenesis. These studies further set the stage for deletion analysis of the TNFr1 domain-mediated osteoclast differentiation.

MATERIALS AND METHODS

Reagents

M-CSF was purchased from R&D Systems, Inc. (Minneapolis, MN). RANKL was purchased from Peprotech (Rocky Hill, NJ). 1,25-dihydroxyvitamin-D_3 (1,25-VD_3) was purchased from BIOMOL (Plymouth Meeting, PA) RT-PCR kit was from Promega (Madison, WI).

Animals

TNFr1 knockout mice and their wild type controls were purchased from Jackson Labs (Bar Harbor, ME).

Cell Culture

Bone marrow macrophages, referred to as OCPs were isolated from whole bone marrow of 4–6 week old mice and incubated in tissue culture plates, at 37°C in 5% CO_2, in the presence of 10 ng/ml M-CSF [Clohisy et al., 1989]. After 24 h in culture, the non-adherent cells were collected and layered on a Ficoll–Hypaque gradient. Cells at the gradient interface were collected and plated in α-MEM, supplemented with 10% heat-inactivated fetal bovine serum, at 37°C in 5% CO_2 in the presence of 10 ng/ml M-CSF, and plated according to experimental conditions.

Primary Stromal Cells

Stromal cells were obtained from 4 to 6-week-old mice. Whole marrow was flushed from long bones and plated in α-MEM supplemented with 10% heat-inactivated FBS for 4 days. Adherent cells were then lifted with trypsin/EDTA and re-plated for an additional 4 days. This cell population, which was monocytes-depleted (using non-specific esterase staining), was stromal cell-enriched and was used for further experimentation.

Osteoclast Generation

Three methods were used to generate osteoclasts in vitro: (1) purified marrow OCPs were cultured at 1×10^6 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. (2) OCPs and primary stromal cells were purified as described earlier and co-cultured at 10:1 ratio, respectively, in the presence of 10 nM 1,25-VD_3. Cultures were supplemented with fresh media and 1,25-VD_3 every 3 days. Bona fide osteoclasts fully formed on days 7–8 of culture. (3) Whole bone marrow was cultured at 3×10^6 cells/ml/cm^2 in the presence of 1,25-VD_3. Medium and steroid were refreshed every 3 days. Multinucleated osteoclasts were formed on days 7–8.

Retrovirus Vector Construction and Preparation

The ΔU3 retroviral vector was used to express TNFr1 in murine primary OCPs and stromal cells. The TNFr1 cDNA was inserted in the ΔU3 vector using XbaI and BamH1 cloning sites to form the ΔU3-TNFr1.

293GPG packaging cells were cultured in DMEM with 10% heat-inactivated FBS supplemented with puromycin, G418, and tetracycline as previously described [Ory et al., 1996]. ΔU3-TNFr1 was purified by $CsCl_2$ gradient centrifugation. ΔU3-TNFr1 was then cotransfected with a plasmid encoding hygromycin into 293GPG cells using LipofectAmine 2000 (Invitrogen, Carlsbad, CA). Hygromycin-resistant stably transfected clones were selected in the presence of 100 μg/ml Hygromycin B (Sigma Chemical Co., St. Louis, Missouri). The clones producing the highest titer of virus, as determined by percent transduction of OCPs, were expanded. Supernatant containing the virus-TNFr1 was harvested after withdrawal of antibiotics from the 293GPG culture conditions at 48, 72, and 96 h post transfections.

Infection of Stromal Cells and OCPs

Purified stromal cells were lifted with trypsin/EDTA and maintained in suspention in Teflon beakers for 24 h. Likewise, gradient purified OCPs were placed in Teflon beakers in the presence of M-CSF for 24 h. Cells were then infected with the ΔU3-TNFr1 virus (collected supernatant) in the presence of 8 μg/ml polybrene (Sigma Chemical Co.), for 24 h without antibiotic selection. Cells were then incubated in fresh media in suspension for an additional 2 days after which they were plated according to experimental conditions.

Reverse Transcriptase-PCR

Total mRNA was extracted from control and treated stromal cells and OCPs using Trizol reagent (Invitrogen). RT-PCR was carried out with a kit (Invitrogen) using the following primers: RANKL: sense-5′ttctatttcagagcgcagat3′, antisense-5′agtcatgttggagatcttgg3′ (expected size 399 bp), OPG: sense-5′accactactacacagacagc3′, antisense-5′aggagaccaaagacactgca3′ (expected size 316 bp), RANK: sense-5′cctgattgcaactgcctgta3′, antisense-5′tgcttcctgggtcaaaaatc3′ (expected size 402 bp), Actin: sense-5′ gggtcagaaggactcctat3′, antisense-5′ gtaacaatgccatgttcaat3′.

RESULTS

Osteoclast Formation Is Reduced in RANKL-Induced OCPs, Whole Marrow Cultures, and Co-Cultures of Stromal Cells With OCP Cells From TNFr1−/− Mice

We have shown in the past that cultures from TNFr1-null mice generate significantly less osteoclasts compared to their wild-type counterparts. To further elucidate the osteoclastogenic potential of TNFr1-derived cells, we conducted a comprehensive experiment in which we compared the data from three different osteoclast formation systems. First, OCPs from wild type and TNFr1-null mice were cultured in the presence of M-CSF and RANKL, cytokines required for direct induction of osteoclastogenesis. Second, whole marrow cells from wild type or TNFr1-null mice were cultured in the presence of 1,25-VD_3, a steroid required for inducing osteoclastogenesis. Third, osteoclast precursor cells from either wild type or TNFr1-null mice were co-cultured with an ST2 cell line that supports osteoclastogenesis in the presence of 1,25-VD_3. The results of these experiments are summarized in Figure 1, and indicate that TNFr1 plays a critical role in direct and indirect induction of osteoclastogenesis. Further examination of the data indicates that osteoclastogenesis established by direct induction with RANKL of TNFr1-null OCPs is less than that seen in wild type (Fig. 1A), and was approximately 74% of WT (Fig. 1B). In contrast, whole bone marrow cultures from TNFr1 knockout mice generate far less osteoclasts (33%) compared to wild type cultures. Lower osteoclast counts (57%), compared with RANKL-treated TNFr1-null OCPs, were obtained from co-cultures of TNFr1-null OCPs with ST2 cells (Fig. 1B).

These in vitro data indicate that OCPs from TNFr1-null mice do not respond fully to osteoclastogenic factors (26% less osteoclasts compared with wild type counterparts). Furthermore, induction of TNFr1-null OCP osteoclastogenesis using 1,25-VD_3-stimulated ST2 cells, presumably through induction of endogenous RANKL expression, also failed to restore osteoclastogenesis. More notably, primary stromal cells in the whole marrow culture have a severely diminished capacity of supporting osteoclastogenesis, indicating that expression of TNFr1 by both OCPs and stromal cells might be essential for optimal osteoclastogenesis.

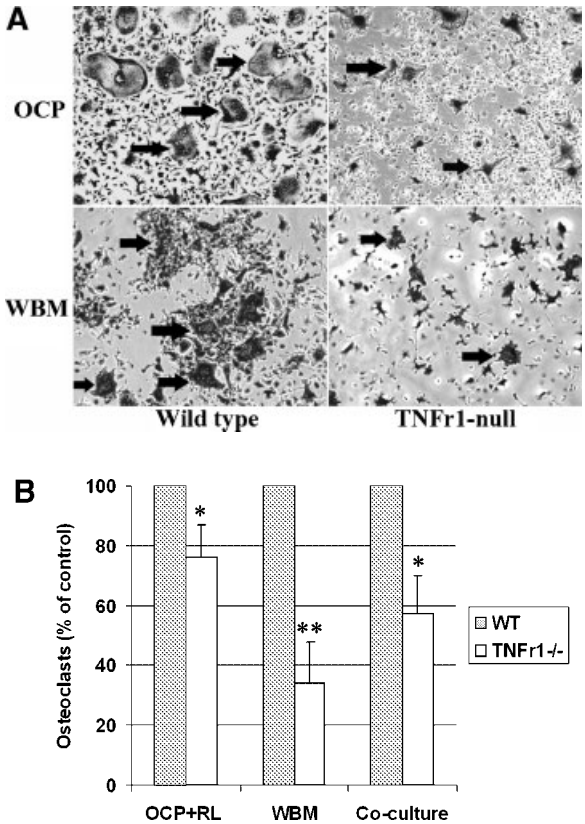

Fig. 1. Retarded osteoclastogenesis by TNFr1-null cell cultures. Osteoclast precursors (OCPs) or whole bone marrow (WBM) cells were cultured under osteoclastogenic conditions. In brief, cells were isolated as described under ''Materials and Methods.'' OCPs were plated at 1×10^6 cells/ml in the presence of 10 ng/ml M-CSF and 20 ng/ml RANKL for 4 days. WBM cultures were plated at 3×10^6 cells/ml/cm^2 with 10 nM 1,25-VD_3 for 8 days. **A**: Cultures were then fixed and TRAP-stained. Osteoclasts are indicated with arrows. Results from OCPs and ST2 co-cultures (treated with 1,25-VD_3) were similar to OCPs + RANKL (not shown). **B**: TRAP positive cells with >3 nuclei were counted in quadruplicate wells from three different experiments (*$P < 0.05$; **$P < 0.005$).

TNFr1−/− Stromal Cells Express low Levels of RANKL mRNA

To further investigate the possible mechanisms for retarded osteoclastogenesis by TNFr1-null cultures, we examined mRNA expression levels of RANK and RANKL in OCPs and stromal cells, respectively, under naive and osteoclastogenic conditions. Consonant with its role in osteoclastogenesis, RT-PCR experiments reveal that expression levels of RANKL message in wild type stromal cells are markedly elevated following 1,25-VD_3 treatment (Fig. 2). In contrast, message expression levels of the cytokine in TNFr1-null stromal cells are only moderately augmented following exposure to

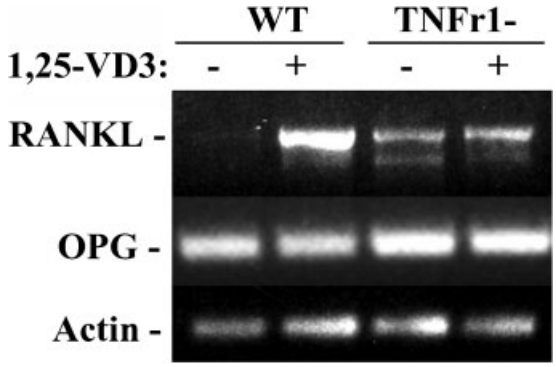

Fig. 2. Lower expression of RANKL by 1,25-VD_3-treated TNFr1-null stromal cells. Stromal cells were isolated from marrow of wild type and TNFr1-null mice. mRNA expression of RANKL and OPG following treatment with1,25-VD_3 was measured by RT-PCR.

1,25-VD_3 (2 fold). Because OPG regulates RANKL availability and positive ratio of RANKL/OPG is required to support osteoclastogenesis, we also examined expression of OPG mRNA under the same conditions. Our results show little but not significant change in OPG mRNA expression levels in naïve or treated wild type and TNFr1-null stromal cells (Fig. 2). The positive ratio of RANKL/OPG was overwhelmingly in favor of WT rather than TNFr1-null cultures and exceeded 20 folds in favor of WT cells. Altogether, these data show impaired expression of RANKL message by 1,25-VD_3-stimulated TNFr1-null stromal cells.

Wild Type Stromal Cells Significantly Rescue Osteoclastogenesis by TNFr1−/− OCPs

Having established that TNFr1-null stromal cells poorly express RANKL in response to the osteotropic hormone 1,25-VD_3, we reasoned that wild type stromal cells might restore osteoclastogenesis by TNFr1-null OCPs. To address this issue, wild type stromal cells were co-cultured with TNFr1-null OCPs in the presence of 1,25-VD_3. Control groups included co-cultures of stromal cells with OCPs from either wild type or TNFr1-null animals. The results indicate that wild type stromal cells induce osteoclastogenesis by TNFr1-null OCPs up to 80% compared with wild type OCPs, 200% compared with TNFr1-null co-cultures (Fig. 3), and resemble the effect of exogenous RANKL treated OCPs seen in Figure 1B.

To confirm that RANKL message expression by wild type stromal cells is indeed the factor directly responsible for this increase, we

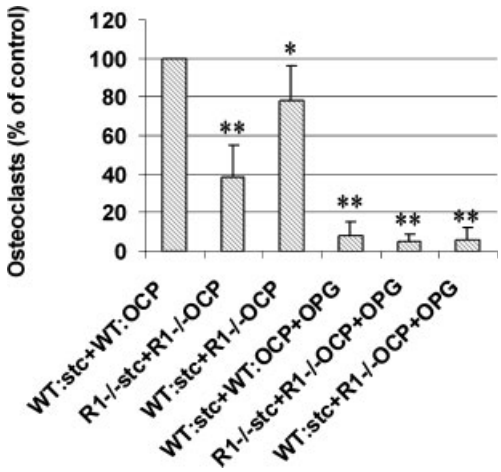

Fig. 3. Wild type stromal cells partially rescue osteoclastogenesis by TNFr1-null OCPs. Wild type (WT:stc) or TNFr1-null (R1−/−stc) stromal cells were isolated and co-cultured with wild type or TNFr1-null OCPs (R1−/−OCP) as shown in the presence of 1,25-VD_3. Parallel cultures were treated with 100 ng/ml OPG for the duration of the experiments. Cultures were fixed on day 7 and TRAP-stained. Results represent average of three independent experiments with quadruplicate wells in each condition (*$P < 0.05$; **$P < 0.005$).

included in parallel cultures increasing doses of OPG. The decoy receptor (100 ng/ml) was sufficient to block osteoclastogenesis by these cultures (Fig. 3). Taken together, the data presented in this figure indicate that subdued RANKL message expression by TNFr1-null stromal cells significantly contributes to plummeted osteoclastogenesis. The data also validate our previous findings that TNFr1 expression by OCPs is also required for optimal osteoclastogenesis [Zhang et al., 2000].

Re-Introduction of TNFr1 to Stromal Cells Lacking This Receptor Induces RANKL and Osteoclastogenesis

The data presented thus far suggest but do not prove that TNFr1 regulates the osteoclastogenic potential of stromal cells, especially RANKL expression by these cells. To further investigate this proposition, we examined the direct effect of TNFr1 by re-introducing the receptor into TNFr1-null stromal cells. Contrary to hypo-regulated RANKL mRNA expression by TNFr1-null stromal cells, we find that viral re-introduction of TNFr1 to these cells enhances responsiveness of RANKL mRNA to osteoclastogenic conditions (Fig. 4). Thus, TNFr1 directly impacts RANKL mRNA regulation.

Having established successful introduction of TNFr1, we turned to assess the osteoclastogenic

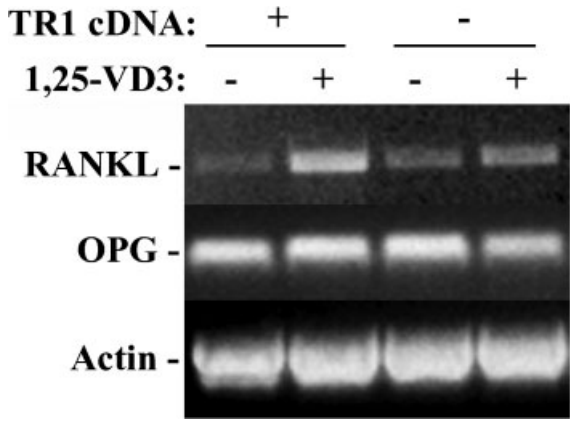

Fig. 4. Re-introduction of TNFr1 (TR1) cDNA in TNFr1-null stromal cells restores RANKL expression. Stromal cells were isolated from TNFr1-null mice. Cells were infected with mock or with TNFr1 cDNA for 2 days. Cells were then incubated with or without 1,25-VD_3 and RT-PCR was performed for RANKL and OPG transcripts.

recovery in presence of this receptor. Attesting to our hypotheses, osteoclast formation by precursor wild type cells (namely, OCPs) resumes in a normal pattern (92%+/−16 of control) when co-cultured with TNFr1-restored knockout stromal cells (Fig. 5). Osteoclast levels in co-cultured wild type OCPs with TNFr1−/− stromal cells infected with exogenous TNFr1 cDNA exceeds those obtained from co-cultured with TNFr1-deficient stromal cells (Fig. 5). The seemingly incomplete gain of osteoclastogenesis

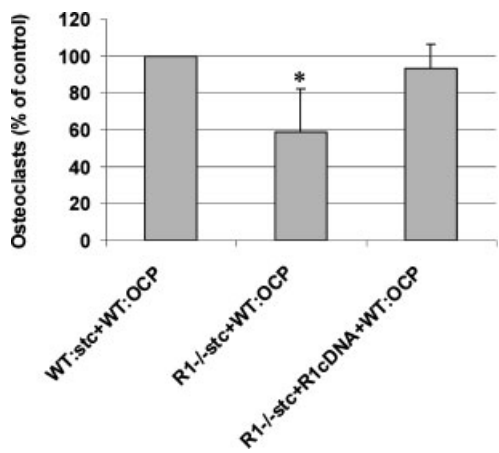

Fig. 5. TNFr1 expression by stromal cells is required for optimal osteoclastogenesis. Wild type OCPs were co-cultured with wild type stromal cells (WT:stc), TNFr1-null stromal cells (R1-/-stc), or with TNFr1-null stromal cells infected with exogenous TNFr1 cDNA (R1-/-stc+R1cDNA). Mature cultures were fixed and TRAP-stained as described elsewhere. Data are presented as osteoclast counts from quadruplicate wells of three independent experiments (*$P < 0.05$).

in these experiments may be simply due to suboptimal re-introduction of the TNF receptor.

TNFr1 Re-Introduction to TNFr1-Null OCPs Supports Osteoclastogenesis

Our findings indicate that reduced levels of RANKL exhibited by TNFr1-null stromal cells can be restored only partially (74%) by exogenous addition of the cytokine (Fig. 1B). Therefore, RANKL bioavailability alone cannot account for reduced osteoclastogenesis by OCPs stimulated with RANKL and M-CSF. Thus, it is reasonable to speculate that TNFr1 integrity and/or related signaling events are required for resumption of normal osteoclastogenesis. To test this possibility, we re-introduced TNFr1 into TNFr1-null OCPs using a retroviral delivery system. Control experiments with LacZ and immunoblots for TNFr1 show successful expression of the receptor in TNFr1-null OCPs (Fig. 6A,B). Functionality of the newly introduced TNFr1 receptor was confirmed by NF-κB activation with TNF induction (not shown). Similar studies with these cells were previously published [Abu-Amer et al., 2001].

TNFr1 knockout OCPs with the newly introduced TNFr1 were then cultured in the presence of RANKL or co-cultured with wild type stromal cells and stimulated with 1,25-VD_3. The data depicted in Figure 7 show a near complete recovery of osteoclasts by TNFr1 null cells that harbor the exogenously introduced receptor compared with wild type cultures and in contrast with baseline osteoclastogenesis by TNFr1-null OCPs. This increase was nearly 95% of wild type cultures. Thus, expression of intact TNFr1 on OCPs is required for RANKL-induction of osteoclastogenesis and sufficient for nearly complete rescue of the phenotype. Similarly, inclusion of 1,25-VD_3-stimulated WT stromal cells in a coculture with TNFr1-restored OCPs results in osteoclastogenic rate indistinguishable from control cultures (not shown). Thus, TNFr1 regulates both RANKL and RANK signaling of osteoclastogenesis by stromal and OCP cells, respectively.

DISCUSSION

Excessive osteoclastic activity and bone erosion in inflammatory osteolysis and rheumatoid arthritis are significant clinical problems, and if left untreated, may lead to disability. Pro-inflammatory cytokines, such as TNFα, play a major role in the progression and severity of inflammatory bone loss diseases [Maini et al., 1993; Feldmann et al., 1995; Schwarz et al., 2000; Abu-Amer, 2003]. The ability of TNFα to enhance osteoclast differentiation and activity has been widely investigated [Lam et al., 2000; Zhang et al., 2000; Clohisy et al., 2002]. The cytokine markedly stimulates pre-osteoclasts into mature bone resorbing osteoclasts. Our studies in this matter have established that the cytokine transmits its osteoclastogenic signals through its type-1 receptor, namely TNFr1 [Abu-Amer et al., 1997, 2001; Zhang et al., 2000]. In fact, deletion of this receptor hinders osteoclastogenesis by marrow derived from these animals. Discovery of the prime osteo-

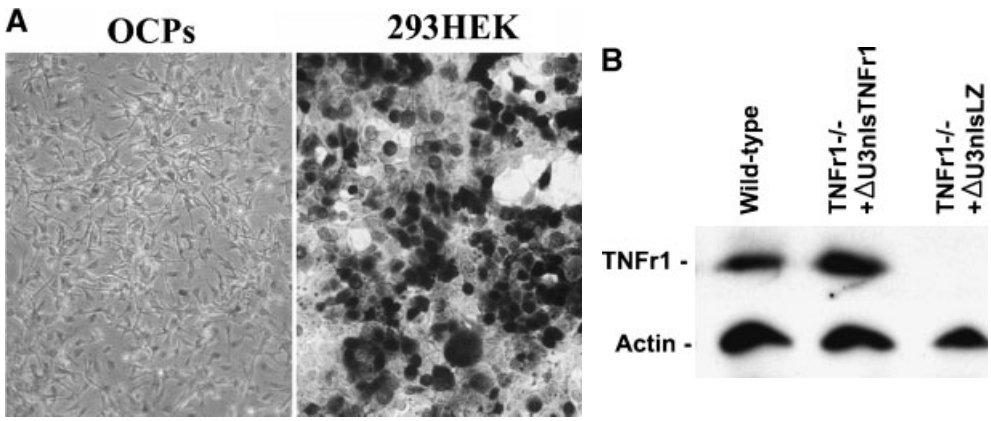

Fig. 6. Re-introduction of hTNFr1 into OCPs using retroviral vector ΔU3nlsTNFr1 [Ory et al., 1996]. Lac Z fragment was displaced with hTNFr1 using PCR techniques. 293GPG cells were transfected with control (Lac Z) or vector containing hTNFr1 cDNA. Viral supernatants were collected 24 up to 96 h post infection. OCPs from TNFr1- null mice were then infected with control or TNFr1-bearing virus in the presence of M-CSF1. Three days-old cells in culture were fixed and stained with X-gal (**A**) or lysed for detection of TNFr1 expression by immunoblots (**B**).

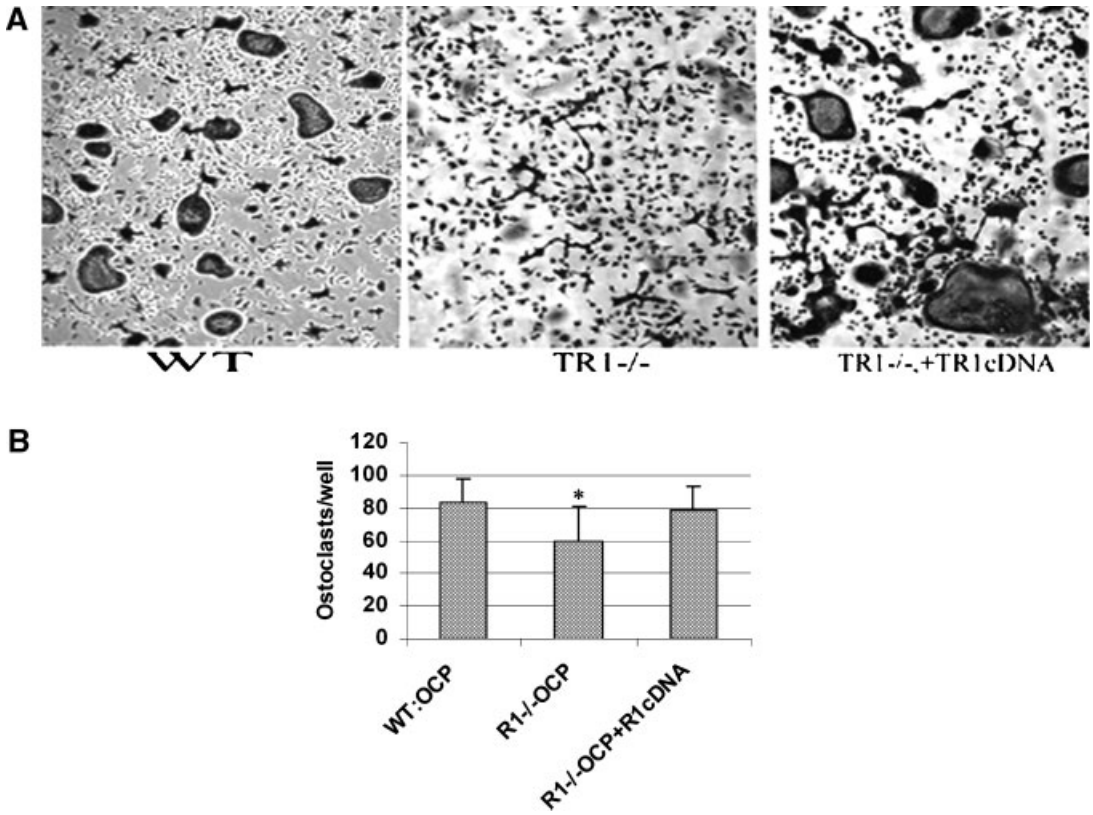

Fig. 7. Re-introduction of TNFr1 (TR1) into TNFr1-null cells, rescues osteoclastogenesis. **A**: TNFr1-null marrow macrophages, pre-treated with RANKL, were infected with ΔU3nlsTNFr1 (TR1cDNA), and cultured with M-CSF and RANKL for 4 days. Cultures were then fixed and TRAP-stained. **B**: Multi-nucleated (>3 nuclei/cell) TRAP-stained osteoclasts were counted in quadruplicate wells from three independent experiments ($*P < 0.05$).

clastogenic factor, RANKL, permits direct examination of the mechanism by which TNF transmits osteoclastogenesis.

Despite conflicting reports regarding the mode of action of TNF on osteoclastogenesis [Kobayashi et al., 2000; Lam et al., 2000; Pettit et al., 2001], it is widely accepted that TNF works in harmony with RANKL to exacerbate osteolytic activity by mature osteoclasts and RANKL-primed precursors [Lam et al., 2000; Zhang et al., 2000]. Our previous findings indicate that TNFr1 mediates the TNF-osteoclastogenic effect and that in vitro cultures obtained from TNFr1-null mice exhibit poor osteoclastogenic potential. We also reported that RANK downstream signaling pathways, including TRAF6, IκB/NF-κB, ERK, and JNK MAP kinases, are diminished in TNFr1-null OCPs [Zhang et al., 2000]. Altogether, these observations prompted us to speculate that endogenous integrity of TNFr1 in OCPs may be required for optimal osteoclastogenesis.

Similar to OCPs, stromal cells also express TNF receptors, respond to the cytokine, and produce osteoclastogenic cytokines in response to various stimuli [Kaplan et al., 1996]. Our finding that TNFr1-null marrow cultures, containing both OCP and stromal cells lacking TNFr1, generate less osteoclasts compared with co-cultures in which only OCPs are TNFr1-deficient, hints that TNFr1 is also essential for optimal osteoclastogenic potential by stromal cells. Therefore, we have taken a comprehensive approach to assess the contribution of the TNF/TNFr1 axis to osteoclastogenesis by OCP and stromal cells. First, we used whole bone marrow cultures in vitro from wild type and TNFr1-null mice. The results from these experiments show a significant reduction of osteoclast numbers by TNFr1-null cultures compared with their wild type counterparts. Thus, it is reasonable to suggest that a cellular defect in expression or secretion of osteoclastogenic factor(s) is responsible for reduced osteoclasto-

genesis by TNFr1-null cultures. Next, we attempted to identify the factor or cell type responsible for the osteoclastogenic deficiency. Recognizing that stromal cell secreted-RANKL is sufficient to induce osteoclastogenesis, we turned to conduct mix and match co-cultures of stromal and OCP cells from WT and TNFr1-null mice. Our data clearly indicate that mRNA expression levels of RANKL by TNFr1-null stromal cells in response to 1,25-VD_3 stimulation, are significantly reduced. As a result, the capacity of these cells to support osteoclastogenesis is limited. To validate this assumption, wild type stromal cells were used to support osteoclastogenesis by TNFr1-null OCPs. These experiments revealed that wild type stromal cells only partially supported osteoclastogenesis and was approximately 20% below the levels seen in wild type control cultures. These findings were also similar to experiments utilizing direct stimulation of osteoclast formation by RANKL-induction of TNFr1-null OCPs. Outcome of these experiments indicates that levels of osteoclasts generated from TNFr1-null OCPs with RANKL are very similar to those generated with wild type stromal cells (74 and 79% of control, respectively). Once again these levels fell significantly below levels of osteoclasts in wild type cultures.

Altogether, the findings thus far indicate that TNFr1 deficiency affects both hematopoietic and mesenchymal compartments relevant to osteoclastogenesis. This conclusion was evident by gain of function experiments entailing reintroduction of TNFr1 cDNA in stromal cells or OCPs lacking the endogenous gene. In this respect, RANKL gene expression is regulated by 1,25-VD_3 in stromal cells bearing the exogenous TNFr1. These stromal cells efficiently support osteoclastogenesis when co-cultured with wild type OCPs or with TNFr1-null OCPs harboring the newly introduced exogenous TNFr1 cDNA. The level of osteoclastogenesis by these cultures (both stromal cells and OCPs with restored expression of TNFr1) was very similar to that seen with wild type cultures.

Reduction of osteoclastogenesis by TNFr1-null OCPs stimulated by RANKL may be due to functional overlapping of RANK and TNFr1 signaling molecules which is compromised in the absence of TNFr1. In support of this notion, we have published ample data establishing that, (1) TNFr1 is the primary TNF receptor supporting osteoclastogenesis [Abu-Amer et al., 2001], (2) TNFα and its type 1 receptor mediate endotoxin-induced osteoclastogenesis and bone resorption [Abu-Amer et al., 1997], (3) neutralizing TNFα activity with soluble decoy molecules and blocking its down stream NF-κB signaling alleviates inflammation and bone erosion [Clohisy et al., 2002], (4) TNFr1 is essential for induction of normal osteoclastogenesis by RANKL [Zhang et al., 2000], and (5) levels of TRAF2, TRAF6, MEKK1, NF-κB, and c-Jun/AP-1 are reduced in TNFr1-null OCPs [Zhang et al., 2000], and absence of TNFr1 significantly dampens RANKL signal transduction pathways, such as NF-κB and AP-1 activation. More importantly, the osteoclastogenic factor interleukin-1, which signals through a distinct pathway, although including TRAF6, induces osteoclastogenesis by TNFr1-null cultures to levels similar to those of WT cultures (unpublished observations).

Thus, our data provide evidence that intact TNFr1 is required for optimal RANKL expression and RANKL-induced osteoclastogenesis. However, domain specificity of TNFr1 related to this function remains unclear. In this regard, it is not clear whether ligation of TNFr1 to basal levels of circulating TNF sets the threshold of RANKL-induced osteoclastogenesis. This does not appear to be the case since cultures obtained from TNF knockout mice (cytokine not receptor) generate osteoclasts at levels indistinguishable from wild type cultures (unpublished observations). Thus, it is reasonable to speculate that deletion of TNFr1 interrupts intracellular protein complexes shared by TNF receptor family members, such as RANK, an event that hampers optimal signaling by these molecules. In this regard, several deletion mutants of the TNFr1 intracellular domain were constructed to test their contribution to optimal osteoclastogenesis. When completed, these studies should identify specific TNFr1 regulatory region(s) that impact basal and most likely inflammatory osteoclastogenesis.

Regulation of RANKL expression by TNFr1 in stromal cells was also noted. The interplay between TNFr1 and RANKL in stromal cells is not known, however, our observations suggest that 1,25-VD_3 induction of RANKL may require intact TNFr1 machinery. We find support for this finding from earlier studies showing that cytokines such as IL-1β, IL-6, IL-11, IL-17, and TNFα, increase the expression of RANKL with decrease of OPG expression in osteoblasts/

stromal cells [Nakashima et al., 2000]. Furthermore, it was shown by Gerstenfeld et al. [2001] that in a model of bone repair, healing is delayed in the TNFr1/2 deficient mice. Likewise, in a marrow ablation model, recruitment of osteoblasts, and mRNA expression of type-I collagen and osteocalcin are impeded in TNFr-null compared to wild type mice [Gerstenfeld et al., 2001]. Although the exact mechanism of such phenomenon is unclear, our studies offer clues for future directions. Altogether, it is likely that RANKL gene expression is regulated by other TNF/TNFr family members.

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