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Biochemical and Biophysical Research Communications 321 (2004) 7–12
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GM-CSF cannot substitute for M-CSF in human osteoclastogenesis

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Received 6 April 2004
Available online 3 July 2004

Abstract

Osteopetrotic mice lacking functional M-CSF recover with ageing, suggesting alternate osteoclastogenesis pathways exist. One alternative is GM-CSF, treatment with which improves the osteopetrosis. Our objective was to determine whether GM-CSF could replace M-CSF in human osteoclastogenesis in vitro. Human CFU-GM precursors cultured with RANKL differentiate into osteoclasts without added M-CSF, indicating constitutive production of M-CSF. Addition of M-CSF antibody completely inhibited differentiation, demonstrating M-CSF-dependence in vitro. Co-treatment with low concentrations (0.01 ng/mL) of GM-CSF for 14 days or higher concentrations (10 ng/mL) for the first 1–2 days enhanced osteoclastogenesis but this effect was blocked with M-CSF antibody. Treatment with GM-CSF transiently increased M-CSF mRNA expression at 3 h but suppressed expression at 7–14 days. Neither FLT3-ligand nor VEGF supported osteoclastogenesis in the absence of M-CSF. Thus, in vitro human osteo clastogenesis is dependent on M-CSF and the stimulatory effects of GM-CSF are mediated by M-CSF. Rescue by GM-CSF in M-CSF-deficiency is unlikely to be directly mediated by FLT3-ligand or VEGF.

Keywords: Osteoclasts; Granulocyte–macrophage colony-stimulating factor; Macrophage-colony-stimulating factor; Colony forming unit-granulocyte–macrophage

Osteoclasts (OC) are specialised cells responsible for bone resorption that differentiate from hematopoietic precursors [1–3]. Macrophage-colony-stimulating factor (M-CSF) and RANKL are essential for osteoclastogenesis in vitro [4–8]. The critical role of M-CSF was demonstrated in studies of osteopetrotic mice with a *Csfm*^{op}/*Csfm*^{op} (*op*/*op*) gene mutation that lack functional M-CSF [9–12]. These animals display defects in macrophage and OC formation but the severe osteopetrosis corrects with age, suggesting the existence of alternative molecular mechanisms [13–15]. Granulocyte–macrophage colony-stimulating factor (GM-CSF) is one potential candidate because it can directly stimulate OC precursor proliferation [16–19], although treatment with high-dose GM-CSF does not correct osteopetrosis in these mice [20], and *op*/*op* mice that are also GM-CSF deficient recover with ageing [21]. However, Myint et al. [22] showed that administration of low doses of GM-CSF and/or IL-3 corrects osteopetrosis in young *op*/*op* mice and noted that GM-CSF-deficient *op*/*op* mice show a marked delay in recovery from osteopetrosis compared to single mutant *op*/*op* mice. Other growth factors that have been shown to compensate for the absence of functional M-CSF include the angiogenic cytokine, vascular endothelial growth factor (VEGF), and Flt-3 ligand [23–25].

Although previous studies have aided in understanding the role that GM-CSF plays in osteoclastogenesis, the majority of this knowledge derives from animal models, with limited data available from human systems. The aim of this study was to determine, in an in vitro human model employing CFU-GM precursors, whether GM-CSF could replace M-CSF in osteoclastogenesis.

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.06.097

We report that human osteoclast differentiation is dependent on M-CSF and although GM-CSF treatment stimulates osteoclastogenesis, this effect is mediated by endogenous M-CSF production.

Materials and methods

Materials. Eagle's minimum essential media (MEM) and penicillin/streptomycin were purchased from Sigma. One hundred times non-essential amino acids and fetal bovine serum (FBS) were purchased from CSL Biosciences (Parkville, Australia). Ficoll–Paque was purchased from Pharmacia Biotech. rhGM-CSF, rhVEGF, rhFLT3-L, and polyclonal antibody anti-M-CSF were purchased from Chemicon. rhM-CSF was generously provided by Genetics Institute. Soluble hRANKL coupled to GST fusion protein (sRANKL) was generously provided by Drs. Matthew Gillespie and Julian Quinn, St. Vincent's Institute of Medical Research, Melbourne, Australia.

Osteoclastogenesis cultures. Human umbilical cord blood was obtained from healthy donors under a protocol approved by Barwon Health Research and Ethics Advisory Committee. The isolation of mononuclear cells from cord blood, preparation of CFU-GM colonies, generation of osteoclasts from CFU-GM, and the quantitation of formation and resorption have been previously described [16]. In all cultures, sRANKL was added at 125 ng/mL. Dentine slices were included in all osteoclast formation/resorption experiments.

Real-time PCR analysis of M-CSF mRNA expression. Pooled CFU-GM colonies were incubated at 9×10^5 cells in 12-well plates in the presence of sRANKL with or without GM-CSF (10 ng/mL). Total RNA was isolated at 3 h, 1, 2, 4, 7, and 14 days by lysing cells in RNAzolB and reverse transcribed to cDNA as previously described [26]. Quantitation of M-CSF mRNA was assessed using real-time PCR (Assays-on-Demand Gene Expression product, part number 4331182; Hs00174164_m1, SIG1020000, 1020100000, Applied Biosystems) with an ABI PRISM 7700 Sequence Detection Instrument. The method of comparison was based on reference to GAPDH mRNA as an internal control for RNA (delta-delta method). GAPDH primers were from Proligo and were designed using Primer Express software (Applied Biosystems)—sequences: GAPDH for 5′-CCACATCGCTCAGACACCAT-3′; GAPDH rev 5′-CCAGGCGCCCAATACG-3′.

For each treatment/time point mRNA was extracted separately from 3 wells of a 12-well plate and the real-time PCR was performed in duplicate.

Statistical analysis. Data are expressed as means ± SEM where applicable. Differences between groups were determined using one-way analysis of variance followed by Fisher's multiple comparison test or two-way analysis of variance followed by Tukey's multiple comparison test. Statistical significance was set at $p<0.05$.

Results

Endogenous production of M-CSF by CFU-GM cultures

In cultures treated with sRANKL alone, the number of OC formed was comparable to that seen in the presence of sRANKL plus exogenous M-CSF (25 ng/mL), although the OC were significantly smaller (30% of control) and resorbed less (30% of control) (Fig. 1). Addition of a neutralising M-CSF antibody caused a concentration-dependent inhibition of formation, size, and dentine resorption, with complete abolition of resorption at 2 μg/mL (Fig. 1).

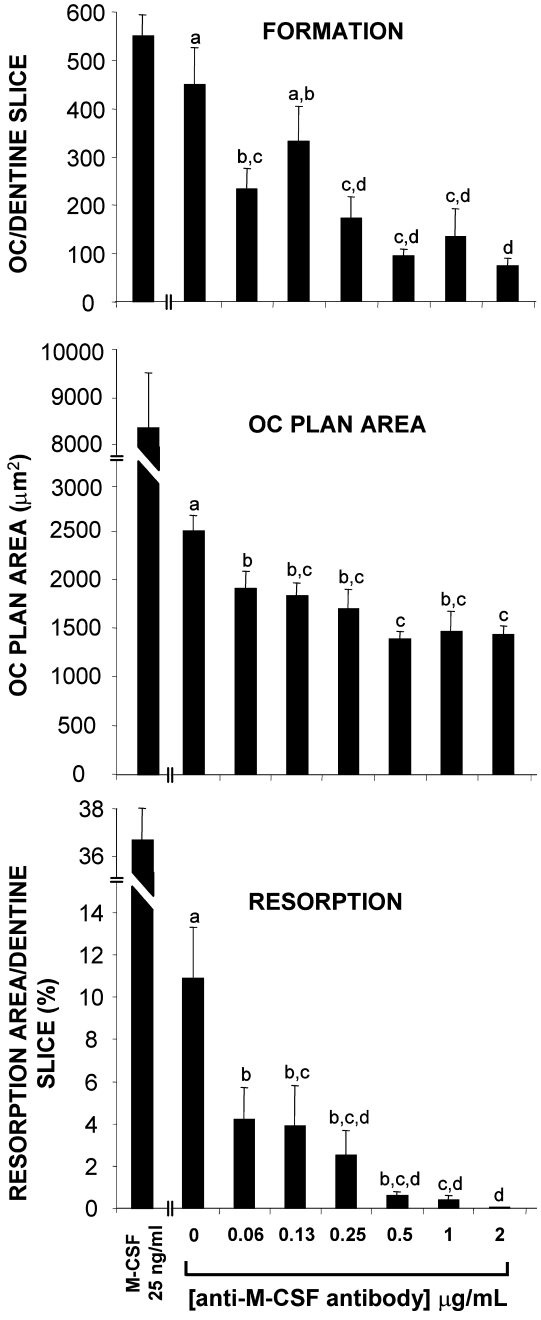

Fig. 1. Human osteoclastogenesis is dependent on M-CSF. Osteoclastogenesis cultures treated with sRANKL (125 ng/mL) without added M-CSF were co-treated with increasing concentrations of anti-M-CSF antibody for 14 days. Results are expressed as means ± SEM ($n=6$ dentine slices/group). Groups with different superscripts are significantly different; $p<0.0001$; one-way ANOVA; Fisher's multiple comparison test.

Promotion of osteoclast size and resorption by GM-CSF in the absence of exogenous M-CSF

When sRANKL-treated CFU-GM cultures were co-treated for 14 days with increasing concentrations of GM-CSF there was a biphasic response in resorption

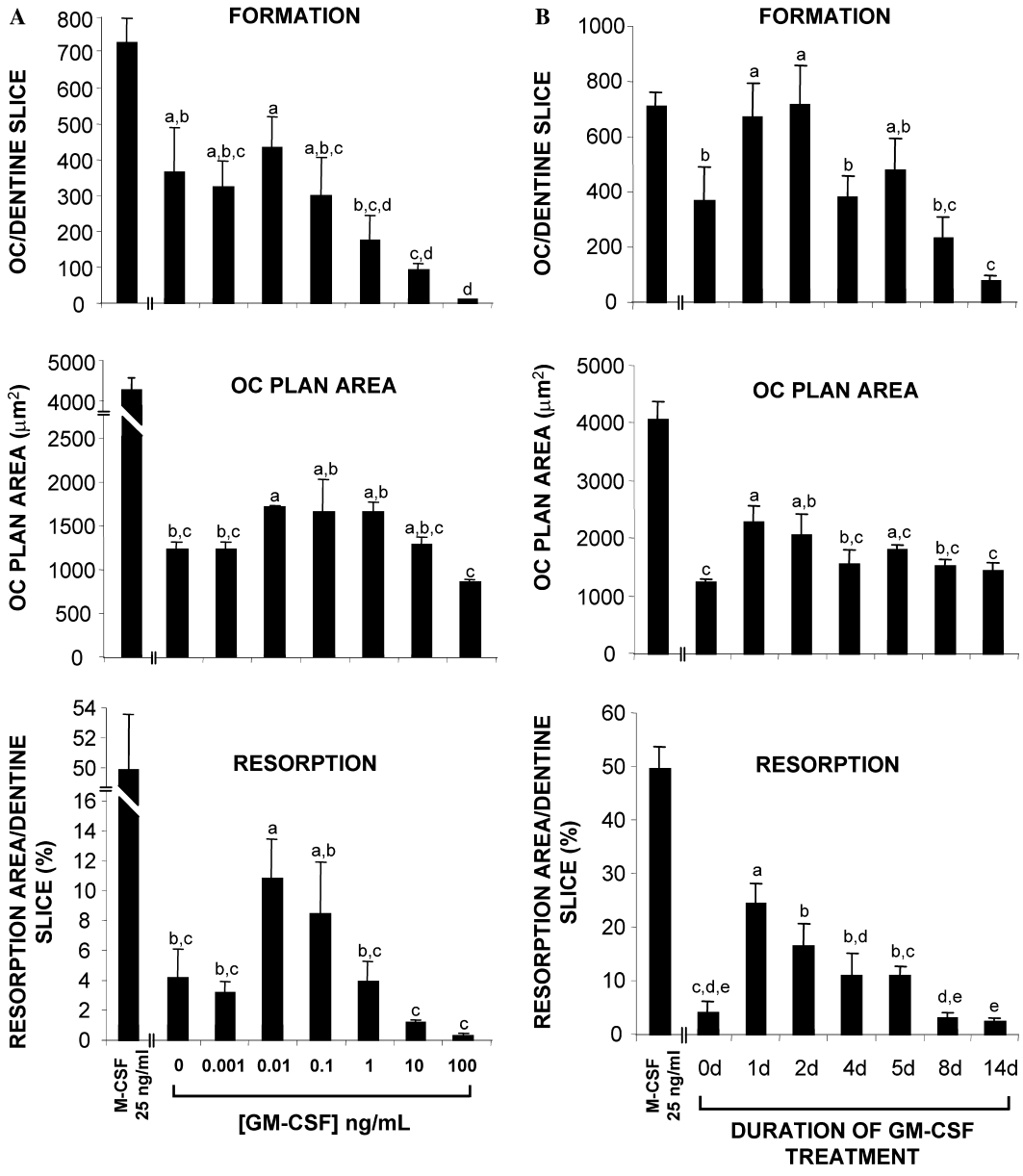

Fig. 2. GM-CSF stimulates osteoclastogenesis in the absence of exogenous M-CSF. (A) Osteoclastogenesis cultures treated with sRANKL without added M-CSF were co-treated with increasing concentrations of GM-CSF for 14 days. (B) GM-CSF (10 ng/mL) was added at the commencement of culture and removed at times indicated. Results are expressed as means ± SEM ($n = 6$ dentine slices/group). Groups with different superscripts are significantly different ($p < 0.02$); one-way ANOVA; Fisher's multiple comparison test.

(Fig. 2A). A concentration-dependent increase in the percentage area of dentine resorbed was observed with a peak response between 0.01 and 0.1 ng/mL. A small increase in the size of the OC also occurred but there was no significant effect on formation. Concentrations of GM-CSF of 1 ng/mL or greater were associated with progressive inhibition of formation, cell size, and resorption. GM-CSF (100 ng/mL) completely inhibited OC formation and resorption with generation of dendritic cell clusters (DC) evident in these cultures.

Progressive removal of GM-CSF (10 ng/mL) from the commencement of culture demonstrated that short exposure to GM-CSF for the first 24–48 h of culture increased OC number, plan area, and resorption (Fig. 2B). Continued exposure to GM-CSF was associated with progressive inhibition of all these parameters and promotion of DC formation (Fig. 2B).

Osteoclastogenesis promoted by GM-CSF is indirectly mediated via endogenous M-CSF production

In these experiments CFU-GM cultures were treated with sRANKL alone or together with GM-CSF (10 ng/mL) for the first 24 h. Cultures treated with GM-CSF

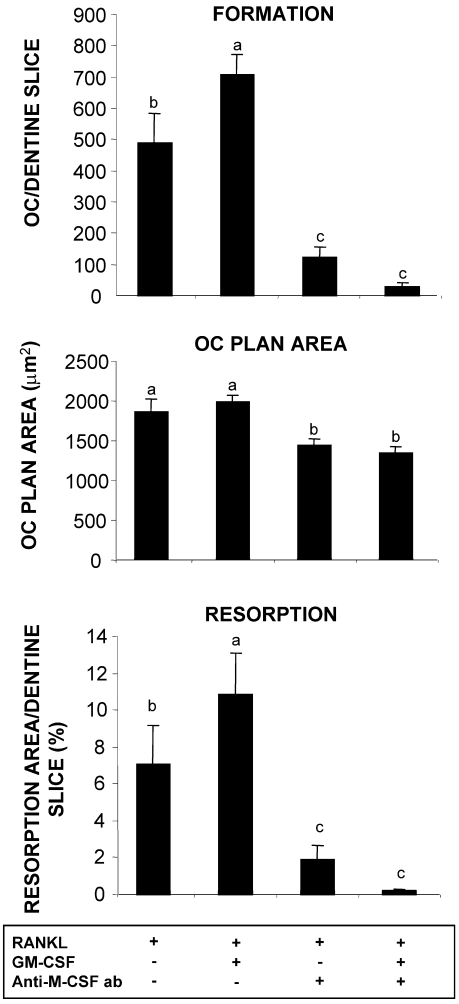

Fig. 3. GM-CSF-induced osteoclastogenesis was blocked by co-treatment with anti-M-CSF polyclonal antibody. Osteoclastogenesis cultures treated with sRANKL without added M-CSF were co-treated with anti-M-CSF antibody (1 μg/mL) for 14 days. GM-CSF (10 ng/mL) was added from the commencement of culture and removed after 24 h. Results are expressed as means ± SEM ($n = 6$ dentine slices/group). Groups with different superscripts are significantly different; $p < 0.0001$; one-way ANOVA; Fisher's multiple comparison test.

showed a significant increase in formation and resorption that was completely blocked by co-treatment with M-CSF antibody for 14 days (Fig. 3). Time course studies of precursors treated with sRANKL alone revealed that M-CSF mRNA expression progressively increased at 7 and 14 days (Fig. 4). Co-treatment of these cultures with GM-CSF (10 ng/mL) produced a biphasic effect on M-CSF mRNA expression with a transient 2.1-fold increase at 3 h but significant inhibition at 7 and 14 days (Fig. 4).

FLT3-L or VEGF cannot substitute for M-CSF in human osteoclastogenesis

Co-treatment of CFU-GM cultures with either FLT3-L (100 ng/mL) or VEGF (100 ng/mL) and

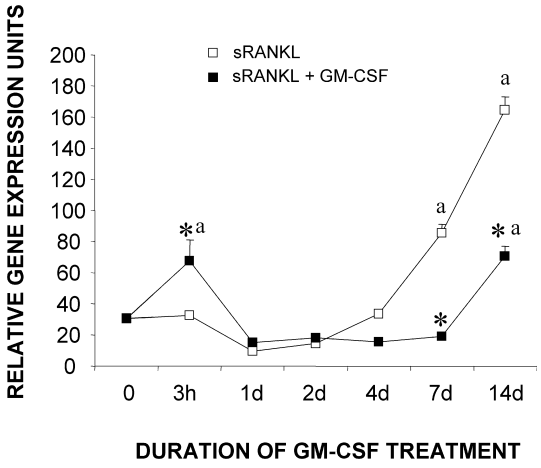

Fig. 4. Regulation of M-CSF mRNA expression in osteoclastogenesis cultures by GM-CSF. OC precursors were cultured in 12-well plates with sRANKL in the absence of added M-CSF. GM-CSF (10 ng/mL) was added from the commencement of culture and total RNA was harvested for M-CSF mRNA quantitation by real-time PCR at times indicated. Results are expressed as means ± SEM ($n = 3$ wells/group). [a]$p = 0.0001$ versus the zero time-point; *$p = 0.0001$ versus RANKL treatment alone; two-way ANOVA; and Tukey's multiple comparison test.

M-CSF antibody did not result in osteoclastogenesis. In addition, FLT3-L or VEGF did not further contribute to osteoclastogenesis above that was elicited by M-CSF and sRANKL (Fig. 5).

Discussion

We have shown that human CFU-GM cultures produce sufficient endogenous M-CSF to support OC differentiation in the absence of added M-CSF. Furthermore, we have shown that GM-CSF treatment enhances osteoclastogenesis in these cultures by increasing their endogenous M-CSF production. We previously reported that GM-CSF has a biphasic effect on human osteoclastogenesis; short-term treatment potentiated OC differentiation by proliferating precursors whereas persistent exposure favoured DC formation [16]. These findings resulted from a culture system in which exogenous M-CSF was supplied at levels that stimulated maximal osteoclastogenesis.

In the current study, we assessed the capacity of GM-CSF to directly support human osteoclastogenesis in the absence of M-CSF. Osteoclast formation and resorption occurred with RANKL treatment in the absence of exogenous M-CSF. The efficiency of generation was only 10–50% of that achieved with optimum levels of M-CSF (25 ng/mL) and was due to the endogenous production of M-CSF, confirmed by expression of M-CSF mRNA throughout the culture period and the inhibitory effect of M-CSF antibody. Very low concentrations of GM-CSF present for the entire culture period or higher

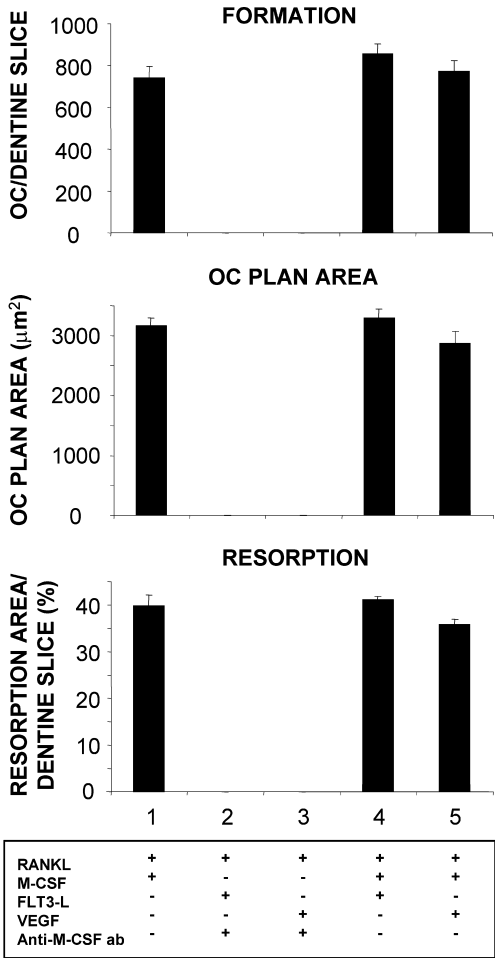

Fig. 5. FLT3-L and VEGF cannot substitute for M-CSF in human osteoclastogenesis in vitro. Osteoclastogenesis cultures treated with sRANKL for 14 days were co-treated with 100 ng/mL FLT3-L or VEGF in the presence of either 1 μg/mL anti-hM-CSF antibody (2 and 3) or 25 ng/mL M-CSF (4 and 5). Treatment 1 is the control (M-CSF and sRANKL). All treatments were added from the commencement of culture. Results expressed as means ± SEM ($n = 6$ dentine slices/group).

concentrations present for only the first 24–48 h of the culture promoted OC differentiation. This was not a direct effect, however, as GM-CSF treatment produced a transient increase in M-CSF mRNA and osteoclastogenesis was completely repressed by co-treatment with a neutralising antibody to M-CSF. A similar stimulation of endogenous M-CSF by GM-CSF was reported in a human monocyte/UMR 106 co-culture system [27]. Prolonged exposure of OC precursors to high concentration GM-CSF (10 ng/mL) for >48 h inhibited M-CSF mRNA expression, which may be due to differentiation to a DC phenotype.

Direct stimulation of OC precursor proliferation by GM-CSF has been reported [16–19], whereas a direct action on precursor differentiation has not been reported in vitro. A recent study demonstrated that in *op/op* mice estrogen deficiency increases OC formation that is blocked by antagonists to vascular endothelial growth factor (VEGF) [28]. These in vivo data reinforce previous in vitro data that VEGF, acting through Flt-1, replaced M-CSF in support of OC differentiation [23] and enhanced bone resorption and survival of mature OC through VEGF receptors, KDR/Flk-1 and/or Flt-1, expressed on mature OC [24]. Similarly, Flt-3 ligand is able to support osteoclastogenesis from non-adherent murine bone marrow cells in the absence of exogenous M-CSF [25]. Recovery from osteopetrosis in *op/op* mice by treatment with low-dose GM-CSF may be mediated via these alternate mechanisms. However, it would appear from our in vitro data that VEGF and Flt-3 ligand do not influence osteoclastogenesis in humans.

In conclusion, we have shown that in vitro human osteoclastogenesis is absolutely dependent on M-CSF and that GM-CSF, Flt-3 ligand, and VEGF cannot substitute for M-CSF in this regard. We have also shown that GM-CSF, at low concentrations or with short, early exposure, promotes osteoclastogenesis by increasing endogenous M-CSF expression. The work highlights the complex interactions occurring in osteoclast differentiation systems, even in vitro, and the importance of determining the molecular basis of responses induced by treatments.

Acknowledgments

The authors thank Drs. Matthew Gillespie and Julian Quinn, St. Vincent's Institute of Medical Research, Melbourne, Australia, and Genetics Institute for generously providing sRANKL and recombinant human M-CSF, respectively. We also thank Dr. Margaret Henry for assistance with statistical analysis.

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