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Receptor Activator of Nuclear Factor-κB Ligand Expression by Human Myeloma Cells Mediates Osteoclast Formation *in Vitro* and Correlates with Bone Destruction *in Vivo*[1]

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ABSTRACT

Multiple myeloma (MM) is an incurable B-cell malignancy able to mediate massive destruction of the axial skeleton. The aim of this study was to examine the involvement of the tumor necrosis factor-ligand family member, receptor activator of nuclear factor-κB ligand (RANKL), and its naturally occurring antagonist, osteoprotegerin (OPG), in MM biology. Using flow cytometry and two independent anti-RANKL antibodies, we demonstrate RANKL expression in $CD38^{+++}CD45^{+}$ and $CD38^{+++}CD45^{-}$ myeloma plasma cell (MPC) subpopulations derived from patients with osteolytic MM. In addition, highly purified subpopulations of MPC express mRNA for both transmembrane and soluble RANKL isoforms but lack expression of OPG mRNA and protein. We also show that RANKL expressed by MPC is functional as *in vitro* coculture of $CD38^{+++}CD45^{+}$ and $CD38^{+++}CD45^{-}$ MPC subpopulations with peripheral blood mononuclear cells resulted in the formation of multinucleate, tartrate-resistant acid phosphatase-positive osteoclasts-like cells capable of forming typical resorption pits. Furthermore, high expression of membrane-associated RANKL by $CD38^{+++}$ MPC correlated with the presence of multiple radiological bone lesions in individuals with MM. Together, our data strongly suggest that RANKL expression by MPC confers on them the ability to participate directly in the formation of osteoclast *in vivo* and extends our knowledge of the involvement of RANKL and OPG in the osteolysis characteristic of this disease.

INTRODUCTION

MM[4] is characterized by the presence of a monoclonal population of terminally differentiated MPC that home to the BM (1, 2). A predominant clinical feature of MM patients is bone destruction, which accounts for much of the morbidity and mortality associated with MM. Although some patients exhibit diffuse osteopenia, most present with multiple discrete lytic lesions adjacent to nests of MPC (3–5). The precise molecular mechanisms responsible for osteolysis are unknown. However, it is clear from histomorphometric studies that osteolysis is caused by an uncoupling of the normal process of bone remodeling, leading to an increase in OC activity and bone resorption (6, 7). Several known OAFs are produced locally by MPC or by stromal cells in response to MPC (5, 7, 8). These include lymphotoxin (9, 10), IL-1 (11–13), TNF-α (13), IL-6 (14, 15), parathyroid hormone-releasing protein (16, 17), and MIP-1α (18). Although previous studies have shown that MPCs produce all of these OAFs, their contribution to the osteolytic disease is unclear.

The TNF-ligand family member RANKL has been shown to play a critical role in OC development and activation (19, 20). A secreted form of RANKL has also been identified (21). The activity of RANKL is antagonized by its decoy receptor, OPG (22), a soluble TNF-receptor family member that acts by binding to RANKL and preventing its ligation with RANK (22). OPG-knockout mice develop extensive osteoporosis because of an increased number of mature OCs, indicating that OPG plays a significant role in the regulation of osteoclastogenesis (23). Thus, the RANKL:OPG ratio is a critical determinant of OC formation (24), and a number of reports now demonstrate that this ratio is significantly altered in malignancy-associated osteolysis by factors produced by tumor cells (24, 25).

The role of RANKL in MM has been controversial. Clearly, OAFs produced by MPC are capable of up-regulating RANKL expression by stromal cells (26–28). However, a recent study has demonstrated that human MM cell lines or primary MPC obtained from MM patients do not express RANKL on their cell surface (28). Moreover, Roux *et al.* (29) demonstrated RANKL expression by immunohistochemistry in MM patient biopsy material in BM stromal cells but not in MPC. Contrary to these findings, Sezer *et al.* (30, 31) recently showed RANKL expression immunohistochemically in MPC in patient biopsy material.

In the present study, we demonstrate by RT-PCR and flow cytometry that $CD38^{+++}$ human MPCs express RANKL but lack detectable expression of OPG at both the transcriptional and protein level. Furthermore, when cultured *in vitro* with peripheral blood-derived OC precursors, these cells were capable of supporting OC formation and subsequent bone resorption in a RANKL-dependent manner, in the complete absence of a stromal osteoblast layer. Our studies also demonstrate that high expression of membrane-associated RANKL by MPC was directly correlated with the presence of osteolytic bone lesions in patients with myeloma. These studies therefore suggest that the accumulation of OCs and formation of osteolytic lesions adjacent to nests of MPC is attributable, at least in part, to MPC-derived RANKL.

MATERIALS AND METHODS

Cell Culture. Human PBMCs were obtained from normal volunteers and isolated on separation gradients (Lymphoprep, Nycomed Pharma, Oslo, Norway). After centrifugation at 400 × *g* for 30 min at 4°C, the buffy layer was removed with a transfer pipette and washed three times in HBSS (Life Technologies, Inc., Gaithersburg, MD), containing 5% FCS (CSL Limited, Victoria, Australia). Isolated PBMCs were resuspended at a concentration of

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[4] The abbreviations used are: MM, multiple myeloma; v/v, volume for volume; MPC, myeloma plasma cell; TNF, tumor necrosis factor; IL, interleukin; OAF, OC-activating factor; OC, osteoclast; RANKL, receptor activator of nuclear factor-κB ligand; MIP, macrophage inflammatory protein; BMMC, bone marrow mononuclear cell; RT-PCR, reverse transcription-PCR; OPG, osteoprotegerin; sRANKL, soluble receptor activator of nuclear factor-κB ligand; BM, bone marrow; TM-RANKL, transmembrane receptor activator of nuclear factor-κB ligand; RANK, receptor activator of nuclear factor-κB; FACS, fluorescence-activated cell sorter; PBMC, peripheral blood mononuclear cell; TACE, TNF-alpha converting enzyme; TRAP, tartrate-resistant acid phosphatase; M-CSF, macrophage colony-stimulating factor.

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1×10^7 cells/ml in α-MEM medium containing 10% (α-MEM-10) and used for OC coculture experiments. Cryopreserved BMMC samples from MM patients were collected by the Transfusion Laboratory, Institute of Medical and Veterinary Science, South Australia. The use of all normal donor and patient-derived material was used after informed donor consent and approved by the Ethics committees of the Institute of Medical and Veterinary Science and the Royal Adelaide Hospital.

Isolation of Total RNA. Total RNA was extracted from patient-derived BMMC using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD), as recommended by the manufacturer. Patient BMMCs were sorted on the basis of their expression of CD38, and 2.5–5 $\times 10^5$ cells were collected for RNA extraction.

RT-PCR Analysis. First-strand cDNA was synthesized from 1–2 μg of total RNA using a reverse transcription kit, Superscript II (Invitrogen Life Technologies, Inc., Carlsbad, CA). cDNA was then used as a template for amplification by semiquantitative PCR using AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT), as described previously (25). DNA was amplified under the following cycling conditions: denaturation 94°C/1 min, annealing at 58–62°C/1 min, and extension at 72°C/1 min for 22 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 30–40 cycles for other primer pairs, such that all products could be assayed in the log-linear phase. This was followed by a final extension step at 72°C for 10 min. Most of the primer sequences used and their expected product sizes are as described previously (25). Primer sequences used to amplify and detect sRANKL and TM-RANKL isoforms are shown in Table 1. PCR amplification products were resolved by electrophoresis on a 2% w/v agarose gel and visualized by SYBR Gold (Molecular Probes, Eugene, OR) staining at 570 nm. The relative amounts of PCR products were determined by quantitating the intensity of the bands using a Fluorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA), as described previously (25). Negative controls, which had no cDNA added to the reaction mixture, were performed with all PCR reactions.

Immunofluorescence Staining of Surface Antigens. Expression of RANKL was determined by flow cytometry. BMMCs were blocked in blocking buffer comprised of HBSS supplemented with 20 mM HEPES (pH 7.35) and 5% (v/v) FCS, 2% (v/v) normal human serum, and 0.4% (v/v) BSA and stained with a mouse anti-RANKL antibody (TRANCE; MAB626, R&D Systems, Inc., Minneapolis, MN) for 1 h on ice. After washing twice in HBSS supplemented with 5% (v/v) FCS (HHF), cells were incubated with a 1:50 dilution of biotinylated goat antimouse IgG antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) for 45 min on ice. Cells were washed again and incubated on ice for 30 min with a 1:100 dilution of streptavidin antibody conjugated to FITC (Caltag Laboratories, Burlingame, CA). Purified mouse $IgG2_b$ (1A6.11; a kind gift from Prof. L. K. Ashman, University of Newcastle, Australia) was used as an isotype-matched negative control for the TRANCE antibody. In two-color stains, anti-CD38 directly conjugated to phycoerythrin (BD PharMingen, San Diego, CA) was added in the final incubation step with streptavidin-FITC. Purified mouse IgG1 (BD PharMingen) was used as the isotype-matched control for CD38. All samples were fixed in "FACS Fix" (1% v/v formaldehyde, 2% w/v D-glucose, and 0.02% w/v sodium azide in PBS) and analyzed on an Epics-XL-MCL flow cytometer (Beckman Coulter, Hialeah, FL), on the basis of their forward and side light scatter properties and their FITC and phycoerythrin fluorescence.

Cell Permeabilization for Immunofluorescence Staining. Cells were blocked in blocking buffer, washed twice in PBS, and fixed for 20 min in 1% paraformaldehyde at room temperature. Cells were then washed twice in HHF and 0.1% saponin (Sigma Diagnostics, Inc., St. Louis, MO) to permeabilize the membrane. Cells were immunostained as described above, with all washes and antibody dilutions in HHF and 0.1% saponin. Intracellular RANKL expression was detected with a rabbit anti-RANKL polyclonal antibody (Sc-6073; Santa Cruz Biotechnology, Santa Cruz, CA). The isotype-matched negative control was purified rabbit immunoglobulin with no human reactivity. The rabbit anti-RANKL antibody was used to examine the expression of cytoplasmic RANKL protein, because the epitope identified by the monoclonal anti-TRANCE antibody was paraformaldehyde-fixation sensitive.

Osteoclast Coculture Assay. Because primary MPCs do not have the capacity to divide or survive for extended periods *in vitro,* and the temporal requirements for the exposure of developing OC precursors to RANKL (32), we derived a method for generating OC in coculture with MPC. First, normal PBMCs were plated onto slices of sperm whale dentine (1×10^6 cells/slice) in α-MEM-10 medium in 96-well plates. Cells were allowed to adhere for 1 h at 37°C, and nonadherent cells were removed by washing three times in HHF. Adherent PBMCs were cultured on the dentine slices in 0.2 ml of α-MEM-10 supplemented with 25 ng/ml recombinant human M-CSF (Genetics Institute, Cambridge, MA) for 1 week, and the media were replenished every 3 days. On day 7 of the culture, MM patient BMMCs were stained for CD38 and CD45 antigens and sorted on the basis of surface expression. Each of the sorted populations ($CD38^-$, $CD38^+$, $CD38^{+++}45^+$, and $CD38^{+++}45^-$) were added to wells at a concentration of 2×10^4 cells/well. For each population, there were four conditions (each condition was performed in triplicate): (*a*) no exogenous treatment; (*b*) soluble, recombinant human RANKL/IL-1β (each 2 ng/ml; R&D Systems, Inc.) added on day 16 of culture to activate and prolong the survival of any OC formed, because in our experience, the MPCs in these populations would be poorly viable at this time and therefore be suboptimal to "support" OC development; (*c*) rhOPG (50 ng/ml) added on day 7; and (*d*) rhOPG (50 ng/ml) and RANKL/IL-1β (each 2 ng/ml) added on days 7 and 16, respectively. As a negative control, PBMCs were cultured in the absence of sorted cells and in conditions (*a*) to (*d*). This was to provide a baseline level of spontaneous OC formation attributed to PBMCs alone. As a positive control, PBMCs were treated with 50 ng/ml soluble RANKL in medium containing 25 ng/ml recombinant human M-CSF. For each condition, the medium was changed every 3 days for the duration of 21 days, and the dentine slices and wells were stained for the presence of TRAP-positive cells, and the dentine slices were examined for the presence of resorption lacunae. The numbers of $TRAP^+$ cells were scored by light microscopic analysis, and $TRAP^+$ cells with three or more nuclei were scored as positive. Results were enumerated in triplicate wells $\pm$ SE, and the significant differences between treatments were determined using Student's *t* tests (two tailed, paired).

TRAP Staining. The appearance of large, multinucleated cells staining positive for TRAP was indicative of the formation of OCs in coculture. Cells were fixed in 4% (w/v) gluteraldehyde in PBS and stained using a commercially available Leukocyte Acid Phosphatase kit (Sigma Chemical Co., St. Louis, MO).

Identification of Resorption Pit Formation. To assess bone resorption by OCs after coculture, dentine slices were treated with 6 M ammonium hydroxide for 2 h and ultrasonicated for 30 min to remove cell debris, washed in 70% ethanol, and dried overnight. The dentine slices were then mounted on stubs, carbon coated, and visualized on a Philips XL-20 scanning electron microscope, as described previously (33). The number of identifiable contiguous resorption pits (resorption sites) were determined for each dentine slice. The area of each resorption site was quantitated from SE images, using QUANTIMET image analysis software (Leica, Cambridge, United Kingdom) as described previously (34).

Serum Calcium Measurement. Total serum calcium measurements were performed as part of routine pathological assessment of MM patients, using the arsenazo III dye method (35), and analyzed on an Olympus 5400 general chemistry analyzer.

Radiographic Identification of Skeletal Lesions. Femora, tibiae, skull, lumbar vertebrae, and pelvis were radiographed using a Faxitron X-ray system

Table 1 *RT-PCR primers and conditions for the amplification of human mRNAs*

Isoform		Primer sequence (5′–3′) (sense/antisense)	Annealing temp. (°C)[a]	Cycle no.[a]	Product size (bp)[a]
Both	S	AACAGGCCTTTCAAGGAGCTG	60	35	538
TM-RANKL	S	GCAGCGTCGCCCTGTTCTTCTA	60	35	712
sRANKL	S	ATCTTCAGAGTTTCGACTTTATCAAC	60	40	732
Both	AS	TAAGGAGGGGTTGGAGACCTCG	60		
Both	Probe	GCCATCCACCATCGCTTTCTCTGC	42		

[a] Annealing temperature, cycle number, and expected product size refer to the product generated by PCR amplification with the particular sense (S) primer with the antisense (AS) primer.

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(Hewlett Packard, McMinnville, OR), as part of routine diagnostic skeletal survey. Patients were scored as either "+" or "−" depending on the presence or absence of osteolytic lesions, respectively. In addition, using a modification of the Durie and Salmon method for skeletal involvement (36), patients were scored according to the following system: 0 = no osteolytic lesions; 1 = one or more osteolytic lesion in one site; or 2 = multiple lesions in multiple sites. Significant differences between groups were determined using the Mann-Whitney *U* test.

Data Analysis. For semiquantitative RT-PCR analysis, experiments were performed in triplicate, and the data are represented as mean ± SE. For comparisons between molecular and radiographic parameters, the Mann-Whitney *U* test and Student *t* test were used to determine the statistical difference between two nonparametric and parametric populations, respectively.

RESULTS

Expression of RANKL by Patient-derived MPCs. RANKL expression in normal and myeloma BM was determined by dual color immunofluorescence staining, using CD38 as a marker of MPC. CD38 staining of myeloma BM revealed three characteristic subpopulations: (*a*) mature myeloma (and normal) plasma cells express high levels of CD38 and are designated here as $CD38^{+++}$; (*b*) monocytoid cells express intermediate levels of CD38 and are designated here as $CD38^{+}$; and (*c*) $CD38^{-}$ cells include a variety of other hematopoietic lineages, including non-B lymphoid cells present in the BM. As seen in Fig. 1, membrane-bound RANKL was highly expressed by $CD38^{+++}$ MPCs in the myeloma BM. The majority of the MPCs were found to express RANKL, although the staining was heterogeneous. Normal BM, on the other hand, contains few plasma cells (<2%), as indicated in Fig. 1, where there is a corresponding lack of the $CD38^{+++}$ population in the normal BM. However, in normal marrow, there was a notable shift in both the $CD38^{+}$ and $CD38^{-}$ populations when intracellular RANKL expression was examined in cells isolated from five normal donors (data not shown). These staining patterns were confirmed by substituting the CD38 antibody with an antibody directed against CD138 (syndecan-1), an additional, independent marker of MPC (data not shown). Fig. 2 shows MPC cells from 15 patients, each with varying degrees of RANKL expression, determined by gating on $CD38^{+++}$ cells. In 9 of 15 patients tested (UPN 1, 3, 4, 11, 12, 13, 14, 16, and 17), RANKL was strongly expressed on the surface of MPC. In the remaining 6 patients, membrane RANKL was weakly expressed. Similar results were observed when intracellular RANKL expression was examined (data not shown).

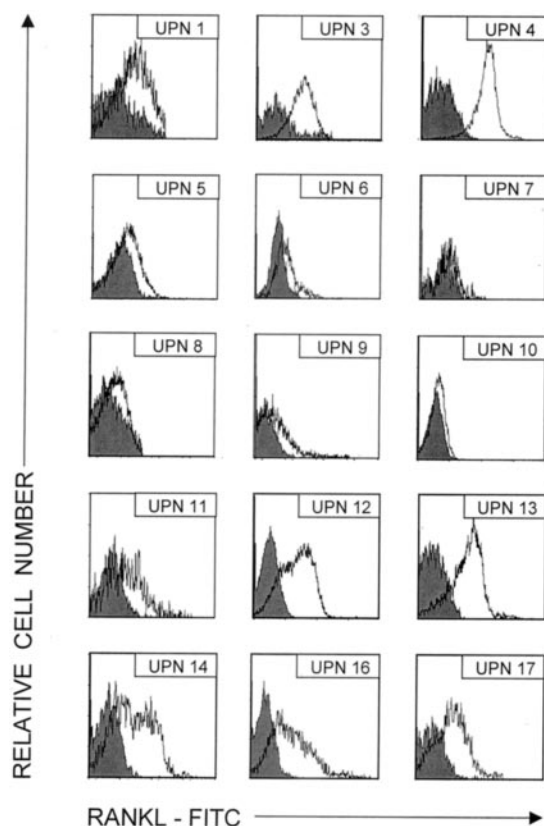

Fig. 2. Cell surface RANKL expression in $CD38^{+++}$ cells from 15 MM patients. Total BM cells from 15 different MM patients were stained with anti-RANKL antibody, and the level of RANKL expression by MPC was determined by gating on $CD38^{+++}$ cells alone. White histogram represents RANKL staining, and black histogram shows staining with the isotype-matched negative control antibody.

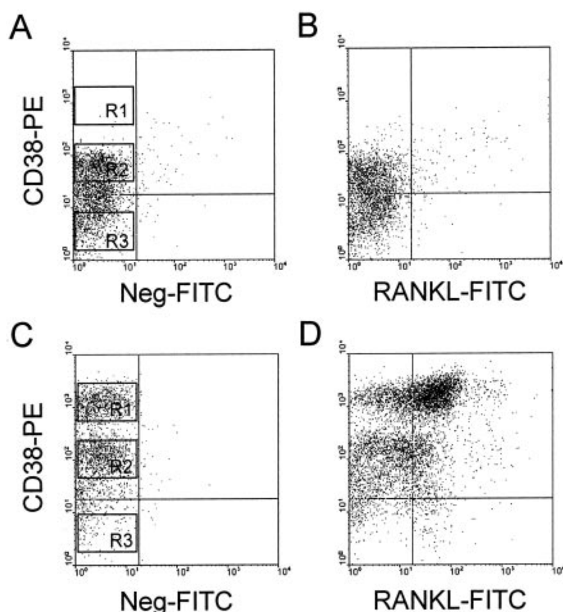

Fig. 1. Two-color flow cytometric analysis of BMMC stained with CD38 and TRANCE (anti-RANKL) antibodies. BMMC from normal donors (*A* and *B*) or MM patient (*C* and *D*) were stained with CD38, TRANCE (anti-RANKL), or the corresponding negative control monoclonal antibodies, as indicated and analyzed by two-color flow cytometry. R1 defines $CD38^{+++}$ plasma cells, R2 defines $CD38^{+}$ monocytoid cells, and R3 represents $CD38^{-}$ lymphoid cells present in BM. *A*, in normal marrow, few $CD38^{+++}$ cells are present as expected, with correspondingly few cells positive for RANKL (*B*), whereas in MM BM (*C*), many $CD38^{+++}$ MPC are evident, and these were found to express cell surface RANKL (*D*). These staining patterns are typical of cells obtained from five donors of each type.

Gene Expression of RANKL and Other TNF Family Members by Patient MPC. To determine the gene expression profile of various cell populations in MM patients, RT-PCR was performed on patient-derived FACS-sorted cells. Cells were sorted on the basis of their CD38 expression giving rise to lymphoid ($CD38^{-}$), monocytoid ($CD38^{+}$), and MPC ($CD38^{+++}$) populations (Fig. 3*A*). RANKL mRNA expression was consistently detected in the $CD38^{+}$ and $CD38^{+++}$ populations (Fig. 3*B*). When primers were used to specifically amplify either the sRANKL or TM-RANKL isoforms, the $CD38^{+++}$ MPCs were found to express a message for both isoforms. Conversely, $CD38^{+}$ cells were found to express predominantly the TM-RANKL isoform. OPG mRNA expression was either very weak or not found in $CD38^{+++}$ MPC (Fig. 3*B*). Consistent with data for normal B-lineage cells (37), RANK mRNA was also expressed in $CD38^{+++}$ MPC (Fig. 3*B*). As expected, RANK mRNA was also found in monocytoid cells ($CD38^{+}$ $CD14^{+}$), which are likely to include OC precursors in these BM aspirates (data not shown).

MPCs Are Capable of Supporting Osteoclastogenesis. To test whether the RANKL expressed by MPC was functional and of likely

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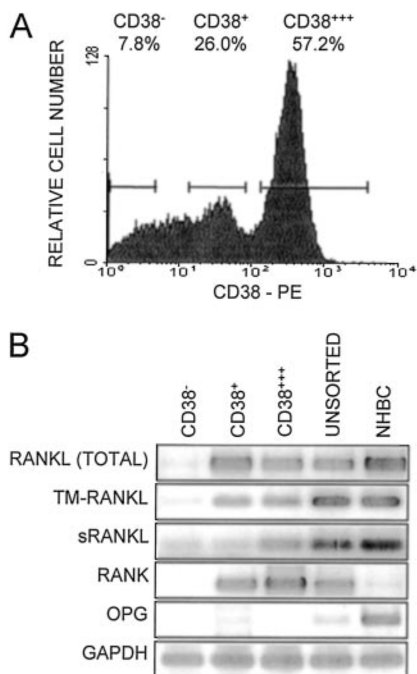

Fig. 3. Gene expression of RANKL and OPG in sorted populations of MM BM. In *A,* total BM from MM patients were stained and sorted on the basis of CD38 expression. RNA was extracted from 2.5–5 × 10^5 sorted cells or total unsorted BM. *B,* RT-PCR analysis of mRNA expression of total RANKL, and TM- and sRANKL isoforms, RANK and OPG in sorted cells from a representative MM patient and from normal human bone cells (*NHBC*) as a comparative control. Total RANKL was expressed in $CD38^{+++}$ MPC, $CD38^{+}$ (monocytoid), and $CD38^{-}$ (lymphoid) populations. Soluble RANKL was more restricted in expression to the $CD38^{+++}$ population. The results are representative of those obtained from three separate MM patients' cells.

in vivo significance, OC-forming cocultures were performed. A limitation of this type of study is the poor survival and viability of primary MPC when these are cultured *in vitro*. To address this problem, MM patient-derived BMMCs were stained for both CD38, a marker for MPC, and CD45, a marker for immature MPC (Fig. 4*A*). $CD38^{+++}45^{+}$ MPCs would be expected to have increased survival capability and thus would be most likely to support OC formation in an *in vitro* setting. Furthermore, the requirements of OC precursors for exposure to RANKL have been demonstrated to be temporal, such that exposure between days 7 and 14 of a 21-day culture period has been shown to be necessary and adequate for human OC formation from PBMC precursors grown in the presence of M-CSF (32). Therefore, various subpopulations of BMMCs were sorted by FACS and cocultured with PBMCs pretreated with M-CSF for 7 days, as described in "Materials and Methods." To demonstrate the formation of morphologically identifiable OCs, OCs were defined as $TRAP^{+}$ cells with at least three nuclei. PBMCs cultured in M-CSF in the absence of MM cell subpopulations gave rise to few, if any, morphologically identifiable OCs (Fig. 4*B*). However, $CD38^{+++}45^{+}$ MPCs were observed to support the formation of a significant number of $TRAP^{+}$ OCs with three or more nuclei ($P < 0.03$; Fig. 4*B*). We reasoned that the coculture conditions were still suboptimal because of the relatively poor survival of even the $CD38^{+++}45^{+}$ MPCs. Therefore, to prolong the survival and activity of any OCs formed in this assay system, recombinant RANKL and IL-1β were added to replicate wells at day 16 of the coculture at concentrations which promote the survival and the activity of existing OCs but not *de novo* OC formation. Indeed, the addition of this cytokine cocktail to PBMCs cultured alone did not result in the formation of any additional OCs (Fig. 4*B*). However, on the addition of RANKL and IL-1β to the coculture with $CD38^{+++}45^{+}$ MPCs, significantly more large, multinucleated (5–30 nuclei) $TRAP^{+}$ OCs were formed ($P < 0.03$; Fig. 4*B*). To demonstrate that OC formation in this system occurred through the RANKL/RANK signaling pathway, recombinant OPG was added to cultures on day 7, at the time of MPC addition. In all instances, OPG abolished the formation of identifiable OCs ($P < 0.005$; Fig. 4*B*), suggesting that the process by which MPCs support osteoclastogenesis is mediated through RANKL.

To test the function of the newly formed OCs, cocultures were also performed where the cells were cultured on dentine slices, and the level of resorption was assessed by scanning electron microscopy after 3 weeks. Consistent with the patterns of TRAP staining, cultures of PBMC alone, in the presence or absence of recombinant RANKL and IL-1β added at day 16 of the culture, gave rise to few recognizable resorption lacunae (Fig. 5*B*). In contrast, coculture of PBMCs with $CD38^{+++}45^{+}$ MPCs resulted in the formation of TRAP+ OCs [Fig. 5*A* (*a*)], which gave rise to small but clearly defined resorption lacunae [Fig. 5*A* (*b*) and Fig. 5*B*]. However, with the addition of RANKL and IL-1β on day 16 of culture, much more extensive resorption was observed, with pits of ~10–15 μm in diameter, indicating that the OCs formed were activated or survived longer and were better able to resorb bone [Fig. 5*A* (*d*)]. The increased size and degree of multinucleation of the formed OC in the presence of added

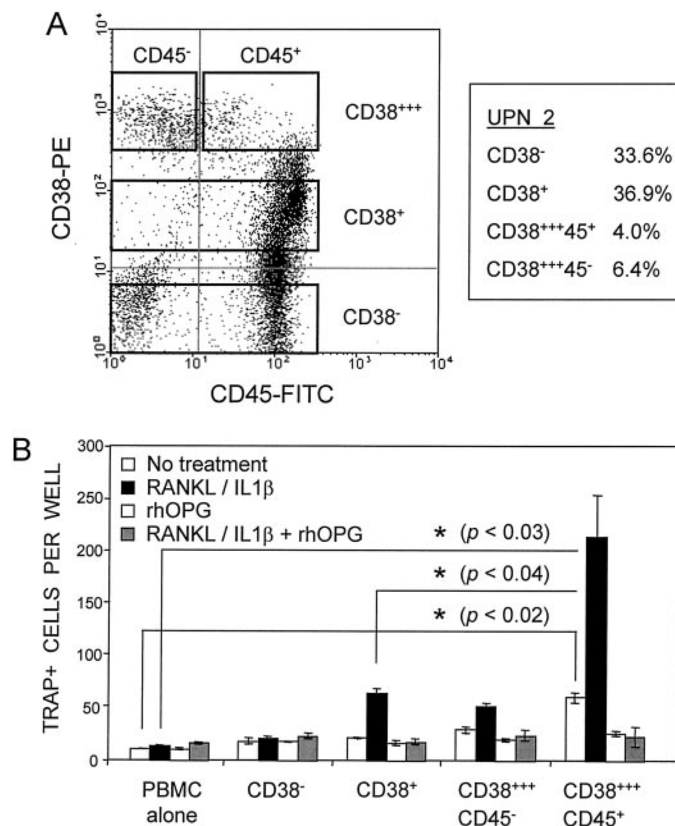

Fig. 4. $CD38^{+++}$ MPC are capable of supporting the formation of multinucleated $TRAP^{+}$ cells in an *in vitro* coculture assay. In *A,* patient-derived BM was stained with CD38 and CD45 monoclonal antibodies, and sort gates were drawn, as indicated, to generate $CD38^{-}$, $CD38^{+}$ (both independent of CD45 expression), $CD38^{+++}45^{-}$, and $CD38^{+++}45^{+}$ populations, which were included in coculture assays with normal human PBMCs, as described in "Materials and Methods." Percentages of cells in each subpopulation are indicated. In *B,* MM BM subpopulations were cocultured as described, with PBMCs that had been pretreated for 7 days with M-CSF (25 ng/ml). Cultures received either no additional treatment, recombinant RANKL and IL-1β (each 2 ng/ml) at day 16 of the culture, recombinant human OPG at day 7, or OPG (50 ng/ml) at day 7 followed by RANKL and IL-1β (each 2 ng/ml) at day 16. The numbers of $TRAP^{+}$ cells were scored by light microscopic analysis of stained wells after 21 days of culture. $TRAP^{+}$ cells with three or more nuclei were scored as positive. Results shown are means of triplicate wells ± SE. Significant differences between treatments are indicated and were determined using Student's *t* test (two tailed, paired).

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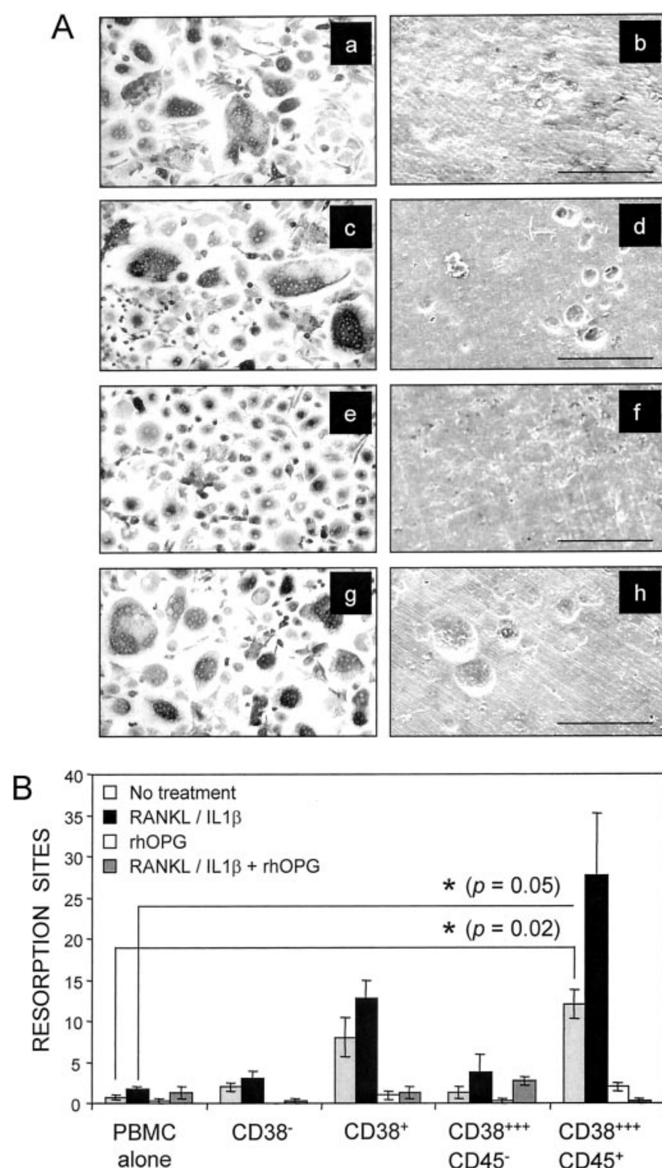

Fig. 5. $CD38^{+++}$ MPC are capable of supporting osteoclast formation and bone resorption. *A,* scanning electron micrographs showing the bone resorption mediated by multinucleated $TRAP^+$ OCs. With no additional treatment, the $CD38^{+++}45^+$ MPC cells were capable of supporting the formation of TRAP+ OC-like cells (*a*) capable of resorption (*b*). Notably, the numbers of resorption lacunae corresponded closely with the numbers of TRAP+ multinucleated cells. With the addition of RANKL/IL-1β on day 16 of culture, multinucleation was increased (*c*), and these cells were activated and able to resorb bone more effectively (*d*). Supplementation of media with recombinant OPG at the time of addition of sorted MPC abolished the formation of large TRAP+ cells (*e*) and resorption lacunae (*f*). Recombinant RANKL (50 ng/ml) added to PBMC alone was used as a positive control, which resulted in phenotypically identical OC to the MPC-PBMC coculture (*g*), although these had a greater resorption capability (*h*). Bars represent 100 μm. In *B,* resorption sites, defined as regions of contiguous resorption pits, were counted after visualization by SE. The data are plotted as the mean number of resorption sites, per dentine slice +/− the SE, for four bone slices per treatment. The experiments were repeated two times with different myeloma patient cells, with essentially similar results.

RANKL and IL-1β [Fig. 5*A* (*c*)] are consistent with reports that these cytokines are also involved in the late stages of OC fusion and subsequent function (38). The addition of recombinant OPG at day 7 (at the time of MPC addition) inhibited the formation of TRAP+ cells [Fig. 5*A* (*e*)] and the resorption of dentine by these OCs [Fig. 5*A* (*f*)]. Cocultures with $CD38^{+++}45^-$ mature MPCs, which have a very poor survival potential *in vitro,* gave rise to few resorption lacunae, even on the addition of RANKL and IL-1β on day 16 (Fig. 5*B*). This strongly implies that the critical stages of OC formation in this culture system are supported by viable ($CD38^{+++}45^+$) MPC and not simply the exogenous recombinant RANKL and IL-1β. Consistent with our previous findings (34), only 15–20% of the TRAP+ cells were capable of creating a resorption site on the dentine slice. However, the number of resorption sites corresponded closely with the relative number of TRAP+ cells in each condition examined (compare Fig. 4*B* with Fig. 5*B*). As a positive control in this system, a high concentration of recombinant RANKL (50 ng/ml) was added from day 7 of the culture of PBMC alone. This gave rise to OC phenotypically identical to those derived from the coculture of PBMCs with $CD38^{+++}45^+$ MPCs when 25-fold less RANKL was added at day 16 of the culture [Fig. 5*A* (*g*)]. The resorption pits in the positive control were generally larger (638.2 +/− 74.48 μm^2; mean +/− SE, n = 76), when compared with those generated by coculture with $CD38^{+++}45^+$ MPCs [193.87 +/− 16.13 μm^2, mean +/− SE, n = 78; compare Fig. 5*A* (*d*) with Fig. 5*A* (*h*)]. This difference in pit characteristics between the positive control and pits formed in coculture likely reflects the poor viability of primary MPC *in vitro,* even under the pro-survival and activation conditions used here, but could also be caused by suboptimal relative cell densities or other aspects of the assay. Nevertheless, this is the first demonstration that purified MPCs have been shown to participate in *de novo* OC formation, in the absence of any other stromal cell layer.

Elevated RANKL Expression by Myeloma Cells Correlates with Radiologically Detectable Osteolysis. To further examine the likely *in vivo* relevance of RANKL expression by MPCs, we conducted a blind study to correlate expression with parameters of bone loss in MM patients. Radiographs of multiple bones (femora, tibiae, skull, lumbar vertebrae, and pelvis) were assessed for the presence of radiologically detectable bone lesions as part of routine diagnostic skeletal survey. In initial studies, patients were scored as either positive or negative with regard to the presence or absence of radiologically detectable lytic lesions, respectively. When these data were plotted with reference to the expression of TM-RANKL (Fig. 6*A*), it was evident that the expression of high levels of TM-RANKL by $CD38^{+++}$ MPCs was associated with the presence of osteolytic bone lesions (P = 0.01). Using a modification of the Durie and Salmon system (36), patients were further subdivided and scored according to those who possessed no radiographic lesions (score = 0), those who possessed one or more lesions in one site (score = 1), and those who possessed more than one lesion in multiple sites (score = 2). Correlations were then sought between osteolytic score and expression of RANKL by MPCs derived from the same patients as described above (Fig. 6*B*). High levels of TM-RANKL expression by $CD38^{+++}$ MPCs were associated with the presence of multiple osteolytic bone lesions (P = 0.01; Fig. 6*B*). Together with the above *in vitro* data, this strongly implies that the expression of RANKL by MPCs has a functional role in the osteolysis observed in MM. No correlation was observed when the level of TM-RANKL expression was compared with the level of total serum calcium on the day of BM acquisition (data not shown). Furthermore, there was no difference between the levels of serum calcium in patients with osteolytic lesions and those without (data not shown), consistent with the findings of Durie *et al.* (39).

DISCUSSION

MM is a B-cell malignancy that has profound effects on the skeleton. Bone destruction is mediated by OAFs, which are produced locally by MPCs, or by osteoblasts/BM stromal cells in response to MPCs. These factors stimulate OC formation and survival and ultimately increase overall OC activity. A number of MPC-derived OAFs, including IL-6, IL-1β, TNFα, MIP-1α, and parathyroid

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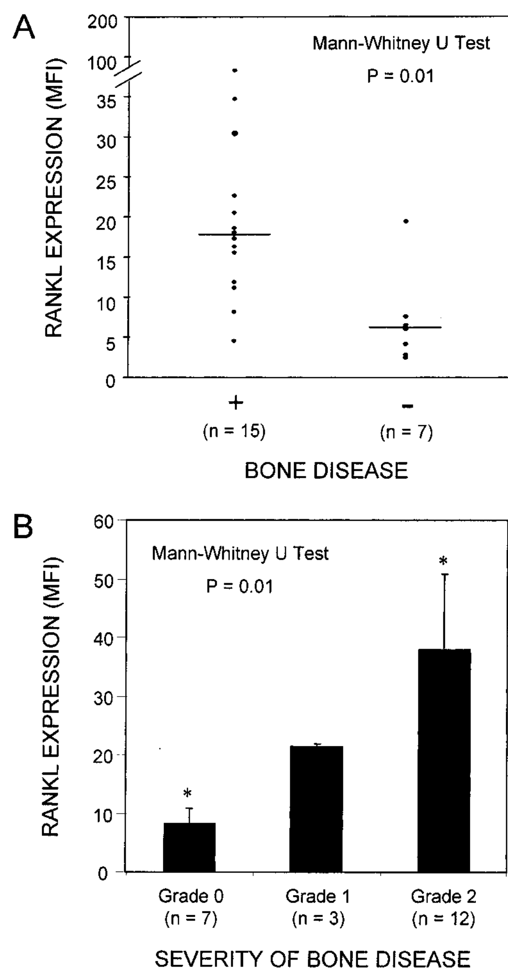

Fig. 6. Elevated levels of membrane RANKL expression is associated with the presence of radiographically detectable osteolytic lesions. In *A,* in a blind survey, multiple radiographs were assessed for the presence or absence of osteolytic lesions and MM patients and accordingly scored as either positive or negative. These data were plotted with respect to the mean fluorescence intensity (*MFI*) of TM-RANKL expression on $CD38^{+++}$ patient's MPC assessed as described in Fig. 2. Statistical significance was determined using a Mann-Whitney *U* test for nonparametric data. $P < 0.01$ was considered significant as indicated. In *B,* patients were further graded according to a modified Durie and Salmon classification system and plotted with reference to TM-RANKL expression. Statistical significance of the difference between grades 1 and 2 with grade 0 was determined using a Mann-Whitney *U* test for nonparametric data. $P < 0.01$ was considered significant as indicated.

hormone-releasing protein, have now been shown to act by up-regulating the expression of RANKL and/or down-regulating the expression of OPG in osteoblastic stromal cells (27, 28). Although RANKL has been implicated in the pathogenesis of MM in an indirect fashion (27, 28), the expression of RANKL by human MPC themselves has been the subject of considerable controversy (29, 30, 40). Our study demonstrates unequivocally the direct expression of RANKL by MPCs.

Using more sensitive techniques than have been used previously (28, 29), we found that RANKL was expressed at both the mRNA and protein level in patient-derived MPCs. We have shown that RANKL was detected by flow cytometry at varying levels in all patient-derived MPCs. In some patients, RANKL was highly expressed on the MPC cell surface, whereas others had very little membrane-associated RANKL but had higher levels of cytoplasmic RANKL. This difference in expression is potentially caused by the cleavage of membrane-bound RANKL by TACE, a recently identified metalloproteinase, which is known to cleave RANKL and other TNF family members from the cell surface (41). By RT-PCR, we found that TACE is expressed by MPC,[5] raising the possibility that in MM, TACE may cleave membrane-bound RANKL from the surface of MPC. TACE-like activity may account in part for the lack of immunostaining for RANKL observed in MPC observed by others (40). We have also shown using isoform-specific RT-PCR primers that MPCs express the secreted isoform of RANKL, as well as the transmembrane isoform. Functionally, there is no specific difference between the various RANKL isoforms (41, 42); however, it may be speculated that differences might be observed at the microenvironmental level, with respect to the focal or disseminated nature of the resulting lytic lesions. Consistent with our study, Sezer *et al.* (30) recently reported the expression of RANKL in cells morphologically identified as MPCs in patient biopsy material using immunohistochemical methods. Our findings using two independent anti-RANKL antibodies and RT-PCR analysis of highly purified FACS-sorted subsets confirm and extend these earlier studies.

Importantly, we have shown that primary MPCs, in addition to expressing RANKL, are capable of directly supporting osteoclastogenesis *in vitro*. Immature MPCs ($CD38^{+++}45^{+}$), when cocultured with PBMCs, were able to support $TRAP^{+}$, multinucleated OC formation. The addition of recombinant OPG to cocultures simultaneously with the addition of MPCs completely blocked the formation and activity of OC in these cocultures, showing that OC formation was RANKL mediated. A problem with using patient-derived cells of this type is their limited survival and proliferative capacity *in vitro*. This was overcome by adding the MPC to the PBMC on day 7 of culture. By day 16, the majority of MPC had undergone apoptosis, because the culture conditions described here were only able to promote their survival for 48–96 h (data not shown). However, this brief window of exposure of OC precursors to MPC-expressed RANKL and the other factors important in the early stages of OC differentiation proved to be adequate for triggering OC maturation, consistent with findings using recombinant RANKL to generate OC from PBMCs (32). To complete the process of differentiation and further activate developed OC (38), low concentrations of recombinant RANKL and IL-1β were added to some cocultures on day 16, concentrations which were inadequate to support OC differentiation in the absence of stromal cells. This resulted in a significant increase in the number of fully functional multinucleated $TRAP^{+}$ OC, which were able to effectively resorb bone. This profound increase in the number of $TRAP^{+}$ cells was unique to the $CD38^{+++}45^{+}$ MPCs cocultured with PBMCs. The $CD38^{+++}45^{-}$ MPCs have a more mature phenotype and less proliferative potential (43), undergo rapid apoptosis when cultured *ex vivo,* and may, in part, explain why these cells are unable to support osteoclastogenesis. Therefore, in addition to merely supporting the survival of the osteoclast precursors, our studies indicate that MPCs are also able to promote the entire process of OC formation in a RANKL-dependent manner.

OPG is a key regulator of OC formation by functioning as a decoy receptor for RANKL (22). We have shown by RT-PCR and ELISA that OPG is not expressed by patient-derived MPCs. This is consistent with studies by Shipman and Croucher (44), who also demonstrate that patient-derived cells lack OPG expression and, moreover, cause a down-regulation of stromal cell-derived OPG. This is a significant finding, because *in vivo,* the relative ratio of RANKL to OPG has been shown to be an important parameter of both physiological bone remodeling (45) and an important determinant for the progression of bone disease in MM (27, 28). Therefore, the localized expression of membrane-bound and soluble RANKL by MPC, without the compen-

[5] A. N. Farrugia *et al.,* unpublished observations.

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satory expression of OPG, will contribute to an overall shift in the ratio of RANKL to OPG, in favor of OC formation.

The expression of RANKL by MPC is likely to be a highly significant finding from a clinical perspective. In the cohort of 22 MM patients in this study, the level of expression of RANKL by MPC was significantly higher in cells from patients with multiple identifiable osteolytic lesions than those from patients without osteolytic lesions. Serum calcium levels on the other hand were not informative with respect to RANKL expression, consistent with the findings of Durie *et al.* (39), who reasoned that calcaemia in MM may fluctuate for a number of reasons distinct from pathological bone loss, including calcium absorption and its excretion rate (39). Together, our data strongly suggest that in addition to other known OAFs, the expression of RANKL by MPC may play a significant role in the focal formation of OCs. This is supported by the *in vitro* OC formation data presented here and points to a possible direct pathophysiological role for MPC-derived RANKL in MM. Our data are consistent with the recent findings of others (30, 31, 46), who also show that RANKL expression correlates closely with osteolytic bone disease in patients with MM.

Taken together with existing data, our results imply that MM is a disease in which the tumor cell, the MPC, in addition to producing autocrine growth factors, deregulates the normal process of bone remodeling, in favor of OC formation, by a number of means: (*a*) by producing and secreting RANKL themselves; (*b*) by inducing the expression of RANKL and down-regulation of OPG expression by osteoblasts and stromal cells via the production of other OAFs; and (*c*) by replacing the marrow space normally occupied by OPG-expressing lineages of cells with MPCs that do not express OPG. Consistent with a number of recent studies (47–50), our study suggests the possibility of blocking RANKL-mediated osteoclastogenesis by using recombinant OPG as a treatment for MM-induced osteolysis, which may significantly reduce the morbidity associated with this disease.

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