Supplementary Information

Materials and Methods

Reagents

Recombinant mouse RANK ligand and M-CSF were purchased from PeproTech EC (London, UK). Anti-β-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO). Anti-Pim-2 antibody was obtained from Abcam (Cambridge, MA). Anti-c-fos antibody was obtained from Cell Signaling (Danvers, MA). Anti-NFATc1 (7A6) and anti-cathepsin K antibodies, and normal mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SMI-16a and SN50 were purchased from Calbiochem (Darmstadt, Germany). IMG-2001 was purchased from Imgenex (San Diego, CA).

Differentiation of osteoclasts

To generate osteoclasts, RAW264.7 cells were cultured in 96-well plates (750 cells per well in 96 well plates) in α-MEM containing 10% FBS for 4 days in the presence of RANKL (25 ng/ml). The cells were stained for TRAP after the 3-day culture and TRAP-positive multinucleated cells (MNCs) containing three or more nuclei were counted.

Bone resorption assay

The bone resorption assay was performed as previously described(Shinohara, *et al* 2015). RAW264.7 cells were cultured in Corning Osteo-Assay Surface 96-well plates (Corning, Lowell, MA) in α-MEM containing 10% FBS and RANKL (25 ng/ml) in the presence or absence of SMI-16a (10 μM). After 5 days of culture, the attached cells were removed from the slides using 6% sodium hypochlorite. The areas of dentin resorption were determined using image-analysis software (NIH Image J System).

Co-culture of primary mouse bone marrow cells with myeloma cell lines

Primary mouse bone marrow cells were isolated from the tibiae or femurs of 5-week-old male C57BL/6J mice. The cells (10^6 cells per well in 24-well plates) were pretreated with RANKL (25 ng/ml) and M-CSF (10 ng/ml) for 1 day, and then RPMI8226, KMS11, U266 or INA-6 cells (10^3/well) were co-cultured in RPMI1640 plus 10% FBS for 5 days. The cells were stained for TRAP after the 3-day cultivation and TRAP-positive multinucleated cells (MNCs) containing three or more nuclei were counted.

Western blotting

The cultured cells were collected and lysed in RIPA lysis buffer (Santa Cruz). The lysates containing equal amounts of proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore). After blocking with 5% non-fat skim milk, the membranes were probed with optimally diluted primary antibodies overnight at 4°C followed by washing and incubation with peroxidase-conjugated secondary antibodies for 45 min at ambient temperature. Immunoreactive bands were visualized using the Immobilon Western chemo luminescent system (Millipore). After stripping off the bound antibodies, the membranes were re-probed with another antibody.

Small interfering RNA (siRNA) transfection

For transient silencing of Pim-2, Pim-2 siRNA (50-CAGGAGGCAAGGAUCGGGCAGCUUU-30 and 50-AAAGCUGCCCGAUCCUUGCCUCCUG-30) (Invitrogen) was transfected into RAW264.7 cells using a Lipofectamine RNAi Max Reagent (Invitrogen, Carlsbad, CA, USA). Stealth RNAi siRNA Negative Control High GC (Invitrogen) was transfected as a control.

Luciferase assay

NF-κB luciferase reporter was obtained from Stratagene (La Jolla, CA, USA). RAW264.7 cells were transfected with NF-κB reporter vector with the aid of Lipofectamine LTX^{TM} reagent (Invitrogen). GL3-basic vector (Promega, Madison, WI, USA) was used as the empty-vector control. The cells were treated with RANK ligand in the presence or absence of SMI-16a (20 μM) for 24 h. The efficiency of the transfection was standardized by co-transfection with pTK-Renilla (Promega). Total cell lysates were prepared with the Dual-Glo® Luciferase Assay System (Promega) and assessed for luciferase activity.

[Ca^{2+}]i oscillation

Intracellular Ca^{2+} ([Ca^{2+}]i) oscillation was performed as described previously(Shinohara, *et al* 2015). RAW264.7 cells (1×10^5 cells per 35 mm dish) were incubated with RANKL (25 ng/ml) for 24 hours in the presence or absence of SMI-16a (10 μM). For [Ca^{2+}]i measurement, cells were incubated with 5 μM fluo-4 AM and 0.05% pluronic F127 for 30 minutes in serum-free α-MEM, and washed twice with Hanks' balanced salt solution

(HBSS). At an excitation wavelength of 488 nm and emission at 520 nm for fluo-4, the cells were analyzed simultaneously at 5 second intervals using a fluorescence microscope (Nikon). To estimate time courses of intracellular Ca^{2+} levels in a single cell, fluorescence intensity of fluo-4 AM was measured, and the results were expressed as percent of a maximum increase obtained by addition of 10 μM ionomycin (Wako) at the end of experiments. Fluorescence intensity was analyzed by NIH Image J.

Animal model and histological analyses

All animal experiments were conducted under the regulation and permission of the Animal Care and Use Committee of the University of Tokushima, Tokushima, Japan (toku-dobutsu 13094). MM mouse models were also prepared by the intra-tibial inoculation of mouse 5TGM1 MM cells to ICR nu/nu mice (CLEA Japan) at 4–6 weeks old as described previously. Mice were treated with SMI-16a intraperitoneally at 20 mg/kg or saline every other day for 2 weeks after confirming MM cell growth on day 5. After the treatment, the tibiaes were taken out, and fixed for 2 days in 10% PFA/PBS, decalcified in 10% EDTA for 1 week at 4°C, and embedded in paraffin. Five μm-thick serial sections were prepared. After deparaffinization, the sections were incubated with primary antibodies overnight at 4°C. A horseradish peroxidase-streptavidin detection system (Dako, Carpinteria, CA), or Alexa Fluor 594-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-mouse IgG antibodies (Life technologies, Grand Island, NY) were used to detect the immunoreactivity. For hematoxylin and eosin staining, the sections were incubated with hematoxylin solution for 1 minute, and washed with water, followed by eosin staining for 2 minutes.

Statistical analysis

Statistical analysis was performed using Student's *t*-test or one-way analysis of variance (ANOVA). $P \leq 0.05$ was considered as a significant difference. All statistics were performed using the Statistical Package for Social Sciences (SPSS 13.0 for Windows; Chicago, IL).

Reference

Shinohara, H., Teramachi, J., Okamura, H., Yang, D., Nagata, T. & Haneji, T. (2015) Double Stranded RNA-Dependent Protein Kinase is Necessary for TNF-alpha-Induced Osteoclast Formation In Vitro and In Vivo. *J Cell Biochem,* **116,** 1957-1967.

Supplementary Figure legend

Supplementary Figure 1

Mice with intra-tibial inoculation of 5TGM1 cells were treated with SMI-16a at 20 mg/kg or saline intraperitoneally every other day from day 6 to day 21. The tibiae were taken out at day 21. The tibiae were also taken out from control mice without 5TGM1 cell inoculation (far left). Cathepsin K (CTK) immunoreactivity (upper panel) and background staining with control IgG (middle panel) were analyzed on 5-μm thick paraffin-embedded serial sections. The serial sections were also stained with hematoxylin and eosin (H&E) (lower panel). The scale bar indicates 100 μm.

Supplementary Figure 1