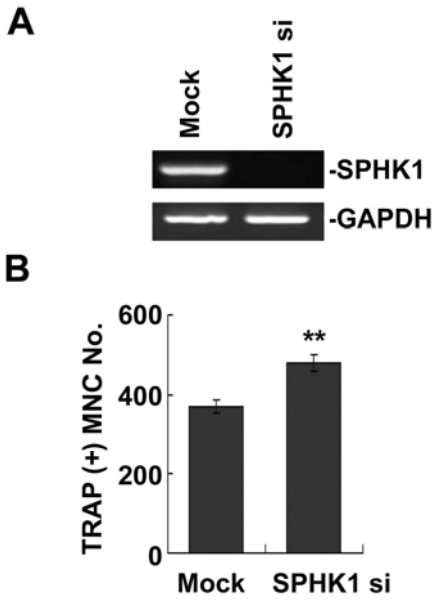
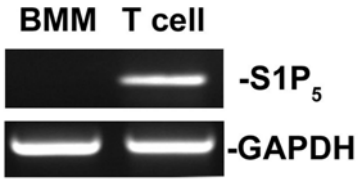
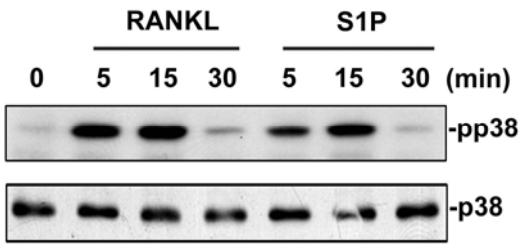
Supplementary data



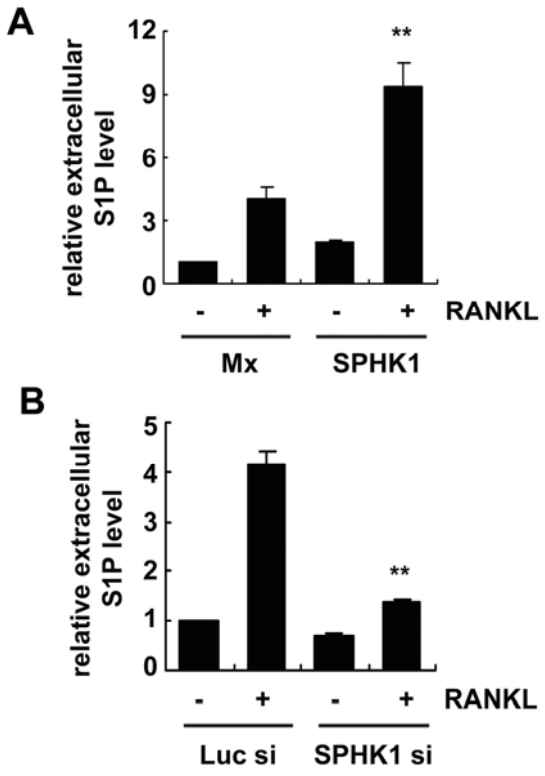
Supplementary Figure 1 SPHK1 siRNA increases RANKL-induced osteoclastogenesis in RAW264.7 cell culture. (**A**) RAW264.7 cells were transfected with oligocassettes containing SPHK1 siRNA target sequences or mock DNA. The expression levels of SPHK1 mRNA were evaluated by RT-PCR. (**B**) RAW264.7 cells transfected with SPHK1 siRNA or mock DNA were cultured for 4 days in the presence of RANKL (100 ng/ml). Cells were then TRAP-stained and the number of $TRAP^+$ multinuclear cells (MNC) was counted. ** $p < 0.005$ vs. control.



Supplementary Figure 2 $S1P_5$ receptor is detected in T cells with the primers used for BMM analyses. RNA were extracted from BMM and T cells and analyzed for $S1P_5$ mRNA levels by RT-PCR.

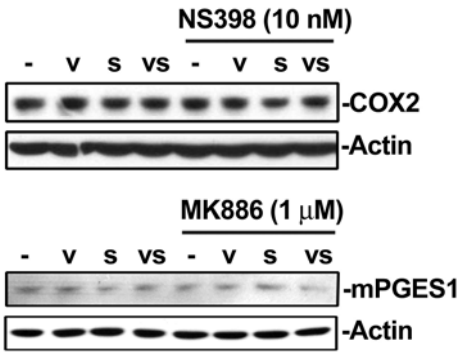


Supplementary Figure 3 Extracellular S1P induces p38 activation in BMMs. Cells were serum-starved for 3 h and stimulated with RANKL (1 μg/ml) or S1P (1 μM) for indicated time. The phosphorylated and total levels of p38 were examined by Western blotting.



Supplementary Figure 4 S1P secretion is modulated by SPHK1 overexpression and siRNA in BMMs. (**A**) BMMs infected with HA-SPHK1 or the control retroviruses were labeled with ^{32}Pi and treated with RANKL. The culture supernatants were collected and S1P levels were determined. ** $p < 0.005$ vs. control virus-infected cells. (**B**) BMMs infected with SPHK1 siRNA or luciferase siRNA retroviruses were labeled with ^{32}Pi and treated with RANKL. The culture supernatants were collected and S1P levels were

determined. ** $p < 0.005$ vs. control virus-infected cells.



Supplementary Figure 5 COX2 and mPGES1 protein levels are not affected by short time treatment with VtD_3 and S1P. Osteoblasts were stimulated with S1P (1 μM) and/or VtD_3 (10^{-8} M) for 10 min after treatment with NS398 or MK886 for 30 min. The COX2 and mPGES1 protein levels were determined by Western blotting.

Supplementary Table 1 The nucleotide sequences used for plasmid construction

type of experiments	nucleotide sequences
human SPHK1 expression vector	5’-GAATTCATGGATCCAGCGGGCGGCCCCCGG-3’ 5’-GTCGACTCATAAGGGCTCTTCTGGCGGTGG-3’
mouse SPHK1 siRNA vector	5’-GATCCCCTATGGAACTTGACTGTCCATTCAAG AGATGGACAGTCAAGTT CCATATTTTTA-3’ 5’-AGCTTAAAAATATGGAACTTGACTGTCCATCTC TTGAATGGACAGTCAAGTTCCATAGGG-3’
luciferase siRNA vector	5’-GATCTGTATAATACACCGCGCTACTTGATATCCG GTAGCGCGGTGTATTATACTTTTTTCCAAA-3’ 5’-AGCTTTTGGAAAAAAGTATAATACACCGCGCT ACCGGATATCAAGTAGCGCGGTGTATTATACA-3’

Supplementary Table 2 Sequences of PCR primer pairs

genes	primer sequences
mouse SPHK1	5’-ACAGACCATCCAAAGGTAGTTT-3’ (forward) 5’-CTCTATTCTGTGCTCAGTCTGTC-3’ (reverse)
mouse SPHK2	5’-GTACTCATGTTGGGCATCTT-3’ (forward) 5’-CATACTCCACTAACTCCCCA-3’ (reverse)
mouse $S1P_1$	5’-TCCATGTAAACTGGGTCAAG-3’ (forward) 5’- AAAGGTGCTGTAGGGGTTAG-3’ (reverse)
mouse $S1P_2$	5’-TTTTAAAATTGGGACAGGGT-3’ (forward) 5’-TTCTCCACAGGATTTAGCAA-3’ (reverse)
mouse $S1P_3$	5’-ATGGCATTTGCTCTTGTTTA-3’ (forward) 5’-TATTTTTCCCTTAACCCAGC-3’ (reverse)
mouse $S1P_4$	5’-AACTGTGGGTATGACTCTGG-3’ (forward) 5’-ATACAGTTGGAACAGTTGGG-3’ (reverse)
mouse $S1P_5$	5’-CTAGGTCTGGAAATTTGGCT-3’ (forward) 5’-AACTGAAGTTGCCAGAATCA-3’ (reverse)
mouse RANKL	5’- AGCAGGGAAGGGTTGGACA-3’ (forward) 5’- CAGGTTTGCAGGACTCGAC-3’ (reverse)

mouse OPG	5'-CCACTCTTATACGGACAGCT-3' (forward) 5'-TCTCGGCATTCACTTTGGTC -3' (reverse)
mouse GAPDH	5'-ACCACAGTCCATGCCATCAC-3' (forward) 5'-TCCACCACCCTGTTGCTGTA-3' (reverse)

Reagents

Sphingosine 1-phosphate was purchased from BIOMOL (Plymouth Meeting, PA). Anti-SPHK1 antibody has been described (Terada *et al,* 2004). Anti-SPHK1 and anti-SPHK2 were purchased from Exalpha Biologicals (Maynard, MA, USA). Antibodies against phospho-p38, p38, phospho-ERK, and ERK were obtained from Cell Signaling. Anti-hemagglutinin (HA) antibody was purchased from COVANCE (Berkeley, CA, USA) and anti-TRAF6 (cat. no. AAP426) was purchased from Stressgen (Ann Arbor, MI, USA). Anti-COX2 antibody was from BD Transduction Laboratories (Lexington, KY, USA) and anti-mPGES1 was purchased from Cayman (Ann Arbor, MI). [γ-^{32}P]ATP (3000 Ci/mmol) and [^{32}P] orthophosphate were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Terada N, Banno Y, Ohno N, Fujii Y, Murate T, Sarna JR, Hawkes R, Zea Z, Baba T, Ohno S. (2004) Compartmentation of the mouse cerebellar cortex by sphingosine kinase. *J Comp Neurol* **469:**119-127.

BMM and osteoclast cultures

Bone marrow cells were obtained by flushing the marrow space of tibiae and femora from 5-6 week-old ICR mice with α-minimal essential medium (MEM) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were suspended in α-MEM supplemented with 10% fetal bovine serum (FBS), plated on 100-mm bacterial dishes, and cultured for 16-24 h with M-CSF (5 ng/ml) in 5% CO_2 at 37℃. Nonadherent cells were completely removed and remaining cells further cultured for 3 days in the presence of 30 ng/ml M-CSF. The adherent cells were considered bone marrow-derived macrophages (BMMs) and used as osteoclast precursor cells. To achieve osteoclast differentiation, BMMs seeded in 96-well plates at 2 x 10^4 cells/well or in 6-well plates at 1.5 x 10^5 cells/well were cultured for 4-5 days with 30 ng/ml M-CSF and 100 ng/ml RANKL. Retrovirus-infected cells were cultured for 5-6 days. Osteoclasts were identified by staining for tartrate-resistant acid phosphatase (TRAP) using Leukocyte Acid Phosphatase Assay kit (Sigma). TRAP-positive cells containing more than 3 nuclei

were counted as osteoclasts.

Generation of retroviruses and BMM infection

Generated recombinant pMX-HA-SPHK1 and pSuper-retro-siRNA plasmids and their parental vectors were transfected into Plat-E cells. 2 x 10^6 Plat-E cells were transfected for 6 h with 4 μg of plasmids and 10 μl Lipofectamine 2000 (Invitrogen) in DMEM. The medium was replaced with DMEM/10%FBS and cells were further incubated. Next day the medium was changed with 4 ml α-MEM/10%FBS and culture was continued for 48 h. Cell culture medium containing viral particles were collected and centrifuged for 10 min at 1500 x *g*. The supernatants were stored at -70℃ until use. For infection with retroviruses, BMMs plated in 6-well plates (1 x 10^6 cells/well) were incubated with the virus-containing supernatant (2 ml/well), polybrene (10 μg/ml) and M-CSF (30 ng/ml) for one day. Equal volume of α-MEM/10% FBS containing M-CSF (30 ng/ml) was added and the incubation was continued for another 24 h.

Co-immunoprecipitation

For immunoprecipitation from pOc, the cells were washed twice with PBS and disrupted with a lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM NaF, 2 mM NaVO4, 1% Triton X-100, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mM PMSF, 20 mM Tris-Cl, pH 7.4). 500 μg of cell lysates were incubated with 4 μg anti-TRAF6 antibody (Stressgen, cat. no. AAP426, rabbit polyclonal) or 4 μg control rabbit Ig (Sigma) for 4 h at 4℃ with constant rocking. After adding 50 μl of protein A bead slurry, the mixtures were incubated for 2 h at 4℃ with constant rocking. The beads were precipitated by centrifugation and washed 4 times with the lysis buffer. The precipitates were separated by 10 % SDS-PAGE and transfer to PVDF membrane. After blocking with 5% skim milk for 1 h at 25℃, the membrane was probed with anti-SPHK1 antibody (Exalpha; 1:400 dilution) for overnight at 4℃. Then the membrane was washed twice with TBS-T, and incubated with a light chain specific anti-rabbit Ig conjugated to horseradish peroxidase (Jackson Laboratory, cat. no. 211-032-171; 1:10000 dilution) for 1 h at room temperature. The immune complexes were detected by using an enhanced chemiluminescence system (Pierce, Rockford, IL, USA). The membrane was stripped and reprobed with of anti-TRAF6 antibody (Stressgen, cat. no. AAP426; 1:1000 dilution). For HEK cells, 500 μg of lysates were pre-cleared with 50 μl of protein A bead slurry for 2 h at 4℃ before immunoprecipitation. In the control lysate blots, 20 μg of cell lysates were used.