Reagents

Recombinant mouse CD97 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). Recombinant human M-CSF and RANKL were gifts from Dr. Y. Choi (University of Pennsylvania, Philadelphia, PA). Recombinant mouse TNFα was purchased from BioLegend (San Diego, CA, USA). The antibodies used for flow cytometric analysis were the following: anti-mouse CD3, anti-mouse CD45R (B220), anti-mouse CD11b (Mac-1), anti-mouse CD117 (c-kit), anti-mouse CD115 (c-fms). All antibodies were obtained directly conjugated to fluorochromes or biotinylated from BD Pharmingen (San Jose, CA, USA) and eBioscience (San Diego, CA, USA). Antibodies against NFATc1 and c-fos were purchased from BD Pharmingen or Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and antibodies against IκBα, phospho-c-jun, and β-actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Media and reagents for cell culture were purchased from Gibco, Inc. (Carlsbad, CA, USA) and Hyclone, Inc. (Logan, UT, USA).

Cell cultures and osteoclast measurement

Bone marrow cells were isolated from femurs and tibia of WT or CD97 KO mice. Isolated cells were washed with α modified essential medium (α-MEM), and cultured (5×10^4 cells/well in 96 well plate) with complete media (10 % HIFBS, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin) in the presence of recombinant human M-CSF (30 ng/ml), and RANKL (10 or 30 ng/ml). M-CSF and RANKL were replenished with each medium change. Bone marrow macrophage/monocyte (BMM) cells were prepared by incubating total bone marrow cells overnight in complete α-MEM. Non-adherent cells were collected and mononuclear cells were prepared using Ficoll-Hypaque (GE Healthcare, Piscataway, NJ, USA) density gradient centrifugation. Buffy coat cells were collected and used for BMM cultures (1×10^4 cells/well in 96 well plate).

To determine osteoclast formation, cultured cells were fixed with 2.5 % glutaraldehyde in PBS for 15 min at room temperature. Cells were then stained for tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO, USA). For evaluating the number of OCLs, TRAP-positive and cells containing more than 3 nuclei were

counted. The OCLs area was measured under a magnification of 10x using the CellSens image analyzing software.

Transfection of CD97 specific siRNA

BMM cells were isolated and cultured with complete α-MEM in the presence of M-CSF (30 ng/ml) for 3 days. BMM cells were then transfected with 100 nM of siRNA against CD97 for knockdown or negative control siRNA (Qiagen, Valencia, CA, USA) using HiPerFect transfection reagent (Qiagen, Valencia, CA, USA) for 6 hours. CD97 specific siRNA was purchased from Qiagen, which contain siRNA from 4 different sites. At the end of transfection medium was replaced with fresh complete α-MEM and cultured for additional 3 days. Three days after siRNA transfection, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) with TRAP staining kit, RNA extraction, or cell lysates were collected for western blot analysis.

Cell proliferation assay and migration assay

Cell proliferation assay was performed using WST-1 reagent according to the manufacturer's recommendation (Roche, Mannheim, Germany). Briefly, bone marrow cells (5×10^4 cells) were plated in a 96 well plate and treated with M-CSF (30 ng/ml) and/or RANKL (30 ng/ml) for up to 6 days prior to the assay. Cells are incubated with the WST-1 for 1 hour. After incubation, the amount of formazan dye formed is quantitated with to measure the number of viable cells spectrophotometer at 450 nm.

Cell migration assay was performed using modified Boyden chamber assay according to the manufacturer's recommendation (Cell Biolabs, Inc., San Diego, CA). Briefly, bone marrow cells (1×10^6 cells) from WT and CD97 KO mice were placed in the upper chamber, which was separated from lower chamber by the polycarbonate membrane (8 μm pore size) in a 96-well plate. Cells were incubated in the presence or absence of HIFBS and/or the combination of M-CSF and RANKL for 12 hrs. Migratory cells passed through the membrane and attached to the bottom side of membrane. These cells were dissociated, lysed, and quantified using fluorescent dye.

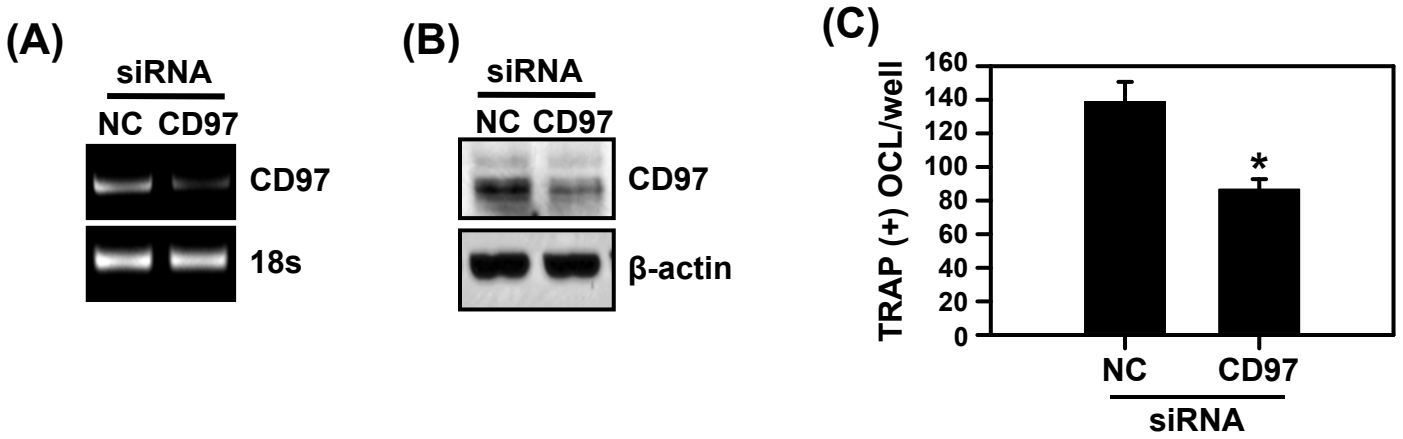
Micro-CT and Histomorphometric analyses

Trabecular and cortical morphometry within the metaphyseal region of the distal femur was quantified using micro-CT (μCT40, Scanco Medical AG, Bassersdorf, Switzerland). Three-dimensional images were reconstructed using standard convolution back-projection algorithms with Shepp and Logan filtering, and rendered at a discrete density of 578, 704 voxels/mm^3 (isometric 12-μm voxels). Threshold segmentation of bone from marrow and soft tissue was performed in conjunction with a constrained Gaussian filter to reduce noise. Volumetric regions for trabecular analysis were selected within the endosteal borders to include secondary spongiosa of femoral metaphyses located 960 μm (6 % of length) from the growth plate. Trabecular morphometry was characterized by measuring the bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular spacing (Tb.Sp). Cortical morphometry was analyzed within a 600 μm long section at mid-diaphysis of the femur and included measurements of average thickness and cross-sectional area.
For histomorphometric analysis, all measurements were confined to the secondary spongiosa and restricted to an area between 400 and 2000 μm distal to the growth plate-metaphyseal junction of the distal femur.

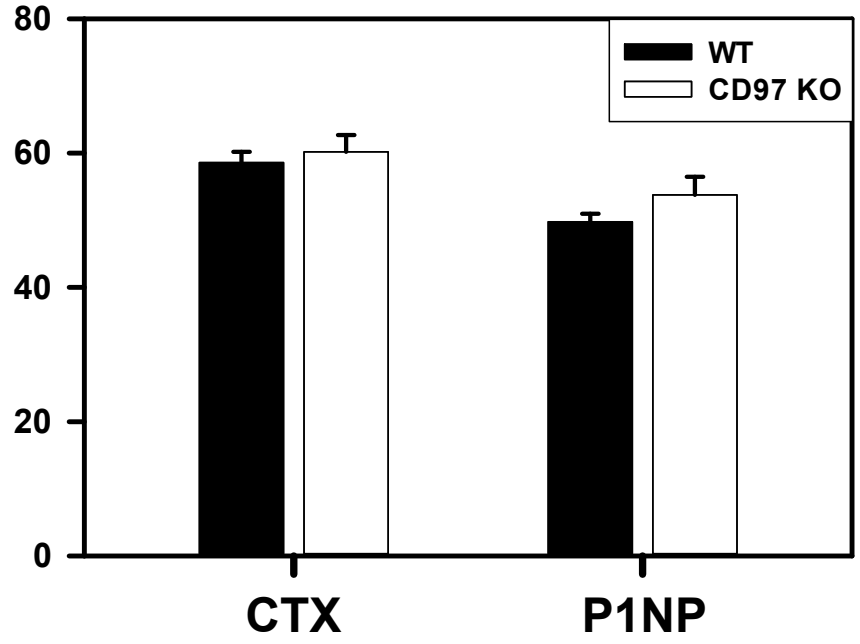
Reverse transcription and real time PCR

Total RNA was isolated from the cells or tissues using TRI-reagent (Molecular Research Center, Cincinnati, OH, USA) followed by reverse transcription. PCR amplification was performed using gene-specific PCR primers and Taq polymerase. Specific primer set was used as follows: murine CD97 (Forward: 5'-AGA ATC TGC TCC GAG ACT TC-3'; reverse: 5'-CCG ACC AGG AAT ATG ATT G-3'), murine 18s (Forward: 5'-CAT GTG GTG TTG AGG AAA G-3'; reverse: 5'-GCC CAG AGA CTC ATT TCT T-3'). The amplified products were run in a 1.5 % agarose gel, stained with ethidium bromide (EtBr) and photographed under UV illumination. Real time PCR was performed using gene-specific primers and gene-specific Taqman probes from ABI (Applied Biosystems). Specific Taqman primer was used as follows: murine CD97 (Mm00516248), murine RANKL (Mm00441908), murine OPG (Mm01205928), murine IL-1α (Mm00439620), murine CCL2 (Mm00441242), murine CCL3 (Mm00441258), murine IL-6

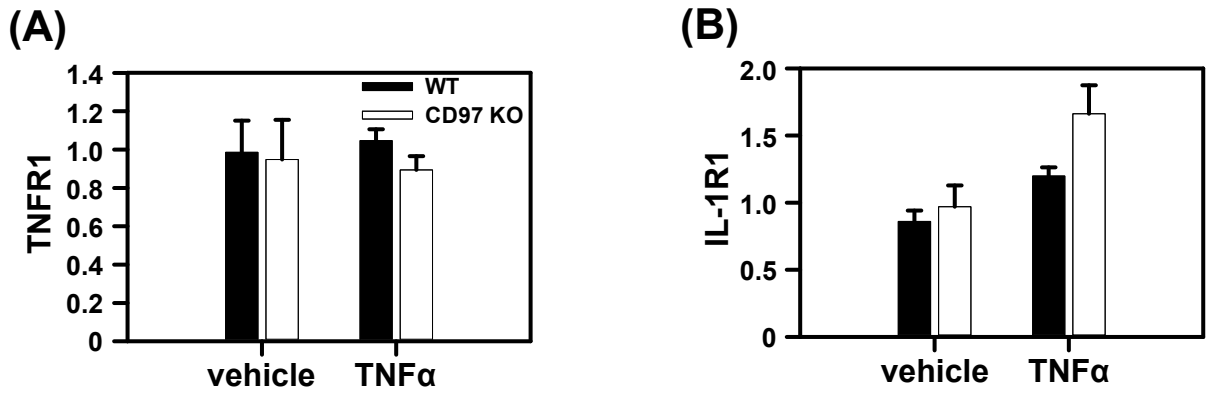
(Mm00446190), murine TNFR1 (Mm00441875), murine IL-1R1 (Mm00434237), and GAPDH (Mm99999915). PCR was run in an ABI 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA). The relative quantification of target gene expression was normalized to a GAPDH expression for each sample.



Supplemental Figure 1. CD97 regulates osteoclast formation. (A-C) BMM cells were transfected with CD97 siRNAs or negative control (NC) siRNA and cultured for 4 days with M-CSF and RANKL (30 ng/ml). (A) RT-PCR and (B) Western blot analyses were performed to detect CD97 mRNA and protein expression, respectively. (C) Cultured BMM cells were fixed and stained for TRAP. The number of TRAP(+) osteoclasts with 3 or more nuclei were counted and presented as per well. Experiments were performed at least 3 times and the representative data are shown. Values represent mean±SEM (N=5-6). *, Significant effect of CD97 siRNAs treatment compared to NC.



Supplemental Figure 2. Serum levels of CTX and P1NP (pg/ml) were measured by ELISA. N=9-11. Serum samples from female mice were collected after fasting overnight at the time of sacrifice. Values represent mean±SEM.



Supplemental Figure 3. TNFα treatment did not alter TNFR1 (A) and IL-1R1 (B) mRNA expression in calvaria from both WT and CD97 KO mice. Total RNA from calvaria of WT and CD97 KO mice was extracted after 4 days of TNFα treatment supracalvarially.

Table 1. Cytokine and chemokine levels in serum from WT and CD97 KO mice that were treated with TNFα (0.75 µg) daily for 4 days over calvaria. Mice were fasted overnight and serum was collected for 32 multiplex analysis, http://www.emdmillipore.com/US/en/product/MILLIPLEX-MAP-Mouse-CytokineChemokine-Magnetic-Bead-Panel---Premixed-32-Plex---Immunology-Multiplex-Assay,MM_NF-MCYTMAG-70K-PX32?bd=1#. Data are presented TNFα treatment/vehicle treatment (T/C) and cytokine levels in the serum WT and CD97 KO mice were compared. Value represent mean±SEM. *, Significant effect of CD97 KO in response to TNFα treatment.

T/C ratio	IL-1α	IL-1β	IL-6	CCL2	CCL3
WT	0.55±0.05	0.87±0.12	1.46±0.55	1±0.05	1.11±0.14
CD97 KO	0.6±0.07	1.66±0.22*	19.27±7.01*	1.2±0.13	1.14±0.09