

# HCO<sub>3</sub><sup>-</sup>/Cl<sup>-</sup> anion exchanger SLC4A2 is required for proper osteoclast differentiation and function

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Contributed by Laurie H. Glimcher, September 9, 2008 (sent for review August 1, 2008)

As the only cell capable of bone resorption, the osteoclast is a central mediator of skeletal homeostasis and disease. To efficiently degrade mineralized tissue, these multinucleated giant cells secrete acid into a resorption lacuna formed between their apical membrane and the bone surface. For each proton pumped into this extracellular compartment, one bicarbonate ion remains in the cytoplasm. To prevent alkalization of the cytoplasm, a basolateral bicarbonate/chloride exchanger provides egress for intracellular bicarbonate. However, the identity of this exchanger is unknown. Here, we report that the bicarbonate/chloride exchanger, solute carrier family 4, anion exchanger, member 2 (SLC4A2), is up-regulated during osteoclast differentiation. Suppression of *Slc4a2* expression by RNA interference inhibits the ability of RAW cells, a mouse macrophage cell line, to differentiate into osteoclasts and resorb mineralized matrix *in vitro*. Accordingly, *Slc4a2*-deficient mice fail to remodel the primary, cartilaginous skeletal anlagen. Abnormal multinucleated giant cells are present in the bone marrow of *Slc4a2*-deficient mice. Though these cells express the osteoclast markers CD68, cathepsin K, and NFATc1, compared with their wild-type (WT) counterparts they are larger, fail to express tartrate-resistant acid phosphatase (TRAP) activity, and display a propensity to undergo apoptosis. *In vitro* *Slc4a2*-deficient osteoclasts are unable to resorb mineralized tissue and cannot form an acidified, extracellular resorption compartment. These data highlight SLC4A2 as a critical mediator of osteoclast differentiation and function *in vitro* and *in vivo*.

apoptosis | NFATc1 | osteopetrosis | acidification

Bone is a remarkable biomaterial composed of organic and inorganic molecules that remodels to preserve structural integrity and adapt to stress. Two cells execute this process: the osteoblast and osteoclast, which synthesize and catabolize bone respectively. An imbalance between osteoclast and osteoblast activity perturbs bone quality, leading to fractures or skeletal deformities (1, 2).

The osteoclast is a multinucleated giant cell that differentiates from myeloid precursors under the influence of the osteoblast-derived cytokines, macrophage-colony stimulating factor (M-CSF) and receptor activator for nuclear factor- $\kappa$ B ligand (RANKL). This process is controlled by the transcription factor, nuclear factor of activated T cells c1 (NFATc1), which is induced by RANKL and governs the expression of genes necessary for osteoclast formation and function (3). After differentiation, the osteoclast polarizes, forming a resorption lacuna between its apical membrane and the mineralized bone surface. Within this space, the osteoclast secretes enzymes, as well as acid, via an apical, H<sup>+</sup>-vacuolar ATPase. The low pH of the resorption lacuna activates these proteolytic enzymes and promotes dissolution of crystalline calcium phosphate. To maintain electroneutrality, a parallel chloride/proton antiporter releases a chloride ion with each proton (4). In humans and mice, mutations in either the proton pump (*TCIRG1*) or the chloride/proton antiporter (*CLCN7*) lead to osteopetrosis, a condition characterized by high bone mass due to an inability of osteoclasts to remodel bone (5).

The protons released by the vacuolar proton pump are supplied by carbonic anhydrase II, which catalyzes the formation of

carbonic acid from water and carbon dioxide. For each proton ejected by the pump, an equimolar amount of base in the form of bicarbonate (HCO<sub>3</sub><sup>-</sup>) is retained in the cytoplasm. A HCO<sub>3</sub><sup>-</sup>/Cl<sup>-</sup> anion exchanger in the basolateral membrane prevents cytoplasmic alkalization by providing egress for excess base. Accordingly, anion exchange inhibitors repress the bone resorbing activity of osteoclasts, and incubation of active osteoclasts in Cl<sup>-</sup>-free media results in an increase in cytoplasmic pH (4, 6–8). Despite these data, the genetic identity of the basolateral HCO<sub>3</sub><sup>-</sup>/Cl<sup>-</sup> anion exchanger in osteoclasts, and its role in bone remodeling *in vivo*, has not been described. Here, we report that the HCO<sub>3</sub><sup>-</sup>/Cl<sup>-</sup> anion exchanger SLC4A2 (also known as AE2) is up-regulated during osteoclastogenesis through an NFATc1-dependent pathway and is a critical mediator of osteoclast development and function *in vivo* and *in vitro*.

## Results

***Slc4a2* Is Up-Regulated during Osteoclastogenesis in an NFATc1-Dependent Manner.** NFATc1 is induced by RANKL and orchestrates the osteoclast differentiation program (3). We recently generated a conditional knockout allele of *Nfatc1* (*Nfatc1<sup>fl/fl</sup>*), and deletion in the postnatal period using *Mx1-Cre* (*Nfatc1<sup>ΔΔ</sup>*) resulted in severe osteopetrosis with a marked attenuation of osteoclast differentiation (9). Because NFATc1 is required for expression of many genes important for osteoclast function, we reasoned that novel osteoclast regulators exist within the universe of NFATc1-dependent transcripts in these cells. A comparative DNA microarray analysis of FACS-sorted, bone marrow osteoclast precursors (BmOcpPs) incubated with MCSF and RANKL was performed (9). This analysis revealed that the bicarbonate/chloride anion exchanger, *Slc4a2*, was expressed 5-fold higher in NFATc1-sufficient cells (Fig. 1A). The use of alternative promoters leads to multiple *Slc4a2* isoforms designated *Slc4a2a*, *Slc4a2b1*, *Slc4a2b2*, *Slc4a2c1*, and *Slc4a2c2* (10, 11). Though the *Slc4a2a* and *Slc4a2b* isoforms are ubiquitously expressed, the *Slc4a2c1* isoform is stomach restricted (10). In response to RANKL, the *Slc4a2a* isoform was up-regulated almost 10-fold in BmOcpPs (Fig. 1B), whereas expression of *Slc4a2b1*, *Slc4a2b2*, *Slc4a2c1*, and *Slc4a2c2* was not discernable (Fig. 1B). Consistent with our microarray results (Fig. 1A), *Slc4a2a* was not induced in NFATc1-deficient BMOcpPs treated with RANKL, and cyclosporine A (CsA), an inhibitor of the NFATc1-activating phosphatase calcineurin, attenuated *Slc4a2a*

Author contributions: J.W., L.H.G., and A.O.A. designed research; J.W. and A.O.A. performed research; J.W., L.H.G., and A.O.A. analyzed data; and J.W., L.H.G., and A.O.A. wrote the paper.

This work was supported by a sponsored research grant from Merck & Co., Inc. (to L.H.G.) and an unrestricted Abbott Scholar Award in Rheumatology Research (to A.O.A.). L.H.G. has equity in and is on the corporate board of directors of the Bristol-Myers Squibb Company.

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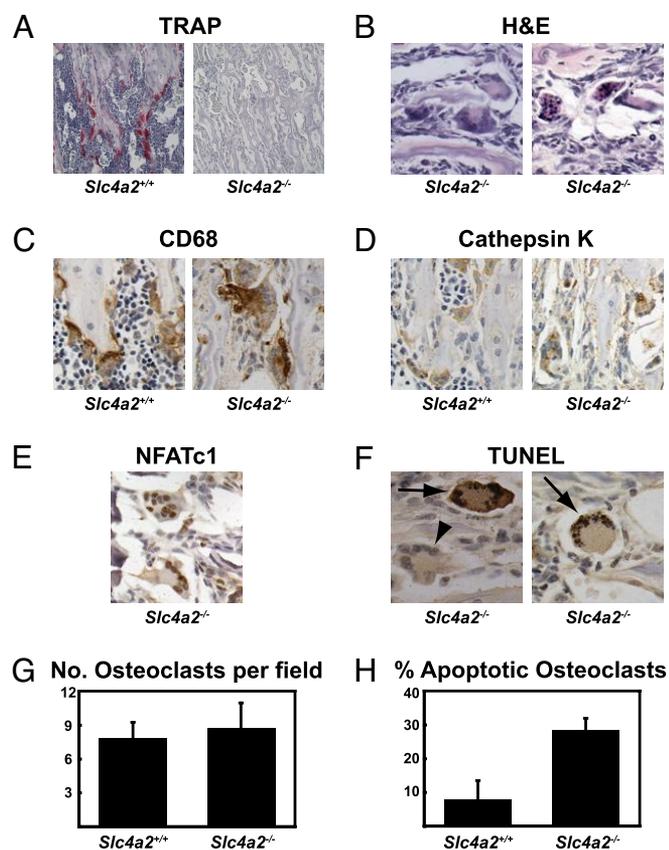
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**Fig. 4.** Abnormal osteoclast development and increased osteoclast apoptosis in *Slc4a2*-deficient mice. (A) TRAP stain of the tibial metaphysis (40 $\times$  objective). (B) H&E stain showing multinucleated giant cells without (Left) and with (Right) apoptotic features in *Slc4a2<sup>-/-</sup>* mice. (C–E) Immunohistochemistry for (C) CD68, (D) cathepsin K, and (E) NFATc1. (F) TUNEL staining at the femoral metaphysis of *Slc4a2<sup>-/-</sup>* mice. Note brown staining in the condensed nuclei of apoptotic *Slc4a2<sup>-/-</sup>* osteoclasts (arrows) compared with a nonapoptotic one (arrowhead). (G) Number of cathepsin K-positive, multinucleated cells per microscopic field (40 $\times$  objective) in the distal femoral metaphysis ( $P > 0.5$ , *Slc4a2<sup>+/+</sup>* vs. *Slc4a2<sup>-/-</sup>*) (H) Percentage of cathepsin K-positive, multinucleated cells with cytologic features of apoptosis in the distal femoral metaphysis ( $P < 0.01$ , *Slc4a2<sup>+/+</sup>* vs. *Slc4a2<sup>-/-</sup>*). The images in (B)–(F) were cropped from pictures obtained in the femoral metaphysis with 40 $\times$  or 60 $\times$  ([F] only) objectives. Images are representative of at least three littermate-matched, 3-week-old mice analyzed per genotype. Data in (G) and (H) are the average plus 5D of three mice per genotype.

differentiation (Fig. 5B–D). Moreover, *Slc4a2<sup>-/-</sup>* osteoclasts were completely unable to resorb dentin (Fig. 5E). Because SLC4A2 promotes gastric acid secretion (13, 15) and osteoclasts secrete protons to resorb bone, the ability of *Slc4a2<sup>-/-</sup>* cells to form an acidic, extracellular compartment was tested. Whereas WT osteoclasts formed large, acridine orange-positive discs, indicating acidic extracellular resorption pits, *Slc4a2<sup>-/-</sup>* cells did not (Fig. 5F). Taken together, these data show that SLC4A2 facilitates optimal osteoclast differentiation and is required for acid secretion.

## Discussion

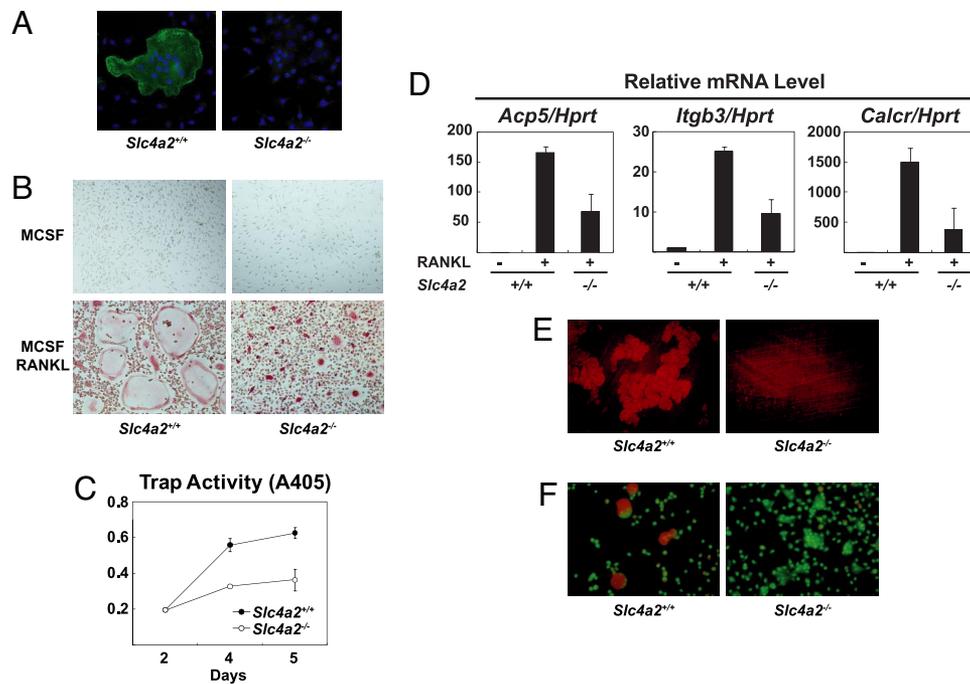
Bone resorption begins with the differentiation of macrophage precursors into osteoclasts, which adhere to the bone surface and polarize to form an extracellular hemivacuole into which acid is secreted. The secretion of protons into the resorption lacuna generates an intracellular acid deficit. A basolateral  $\text{HCO}_3^-/\text{Cl}^-$  anion exchanger regulates intracellular pH through the efflux of excess bicarbonate (4, 8). Until this study, the genetic identity of this exchanger was unknown. Here, we define *Slc4a2* as an

NFATc1-regulated transcript in osteoclasts, whose mutation blocks skeletal remodeling.

The SLC4 family of anion transporters share a common cytoplasmic N-terminal domain, followed by a polytopic transmembrane domain responsible for anion transport (16). The use of alternative, tissue-specific promoters leads to the transcription of variant 5' mRNAs that encode truncated N-terminal isoforms. Osteoclasts up-regulate the *Slc4a2a* isoform, but not the *Slc4a2b* and *Slc4a2c* isoforms (Fig. 1B). Whether NFATc1 directly regulates *Slc4a2a* expression in osteoclasts is currently under investigation. Two different strains of *Slc4a2* knockout mice have been described. The first (*Slc4a2a,b<sup>-/-</sup>*) targeted the *Slc4a2a,b* isoforms (15, 17), leaving expression of *Slc4a2c* intact, a stomach-specific isoform. The second strain (*Slc4a2<sup>-/-</sup>*), used here, deleted all five isoforms (13). Though both strains display perinatal lethality, *Slc4a2<sup>-/-</sup>* mice are growth retarded and edentulous, whereas *Slc4a2a,b<sup>-/-</sup>* mice appear grossly normal (13, 15, 17). Here, we show that *Slc4a2<sup>-/-</sup>* mice fail to remodel the primary cartilaginous anlagen leading to the absence of cortical bone and a discernable BM cavity (Fig. 3A–E). Although the skeletal phenotype of *Slc4a2a,b<sup>-/-</sup>* has not been reported, the lack of growth retardation in this strain, a common feature of severe osteopetrosis, suggests that SLC4A2c may compensate for SLC4A2a in osteoclasts. Further studies are needed to resolve this important issue.

To explore the function of SLC4A2 in osteoclasts, an RNAi approach was used using RAW clone 6 cells (Fig. 2B). Two shRNAs against *Slc4a2* blocked osteoclast differentiation (Fig. 2C and D). In contrast, multinucleated giant cells expressing osteoclast lineage markers could be identified in the BM of *Slc4a2<sup>-/-</sup>* mice (Fig. 4B–E). Moreover, primary *Slc4a2*-deficient macrophages formed multinucleated giant cells in response to RANKL (Fig. 5B). However, similar to the knockdown cells, *Slc4a2*-deficient osteoclasts expressed less TRAP activity (Figs. 4A and 5C). In addition, these cells expressed lower levels of osteoclast differentiation markers (Fig. 5D). However, though the block in differentiation appears to be partial, the osteoclasts that form in *Slc4a2<sup>-/-</sup>* cultures were completely defective in the resorption of dentin (Fig. 5E) and vacuole acidification (Fig. 5F). Our histologic data indirectly support a role for SLC4A2 in osteoclast development. In other models, where genes are mutated that purely affect osteoclast function, like *Src<sup>-/-</sup>* and *Igfb3<sup>-/-</sup>* mice, increased osteoclast numbers are observed (18, 19). In contrast, we found similar numbers of cathepsin K-positive multinucleated cells in *Slc4a2<sup>+/+</sup>* and *Slc4a2<sup>-/-</sup>* mice (Fig. 4G). Certainly, increased numbers of osteoclasts in *Slc4a2<sup>-/-</sup>* mice could be offset by increased apoptosis (Fig. 4H), in addition to a defect in differentiation.

It is possible that *Slc4a2*-deficient osteoclasts do not resorb mineralized matrix because they fail to express molecules necessary to efficiently resorb bone, in addition to an inability to form an acidified vacuole (Fig. 5F). For example,  $\beta_3$  integrin is necessary for attachment and spreading of osteoclasts (18), and this molecule is not optimally expressed in *Slc4a2*-deficient cells (Fig. 5D). Consistent with this, *Slc4a2*-deficient osteoclasts do not spread normally (Fig. 5B). How an anion exchanger could affect differentiation and gene expression is not apparent. Certainly, perturbations to cytoplasmic pH, generated even before activation of the proton pump, may affect signaling pathways. Furthermore, SLC4A2 may have functions in the osteoclast independent of its anion exchange activity. For example, in red blood cells, the N-terminal domain of SLC4A1, a homolog of SLC4A2, anchors the membrane to the cytoskeleton (16). Mutations in *SLC4A1* cause hereditary spherocytosis, a genetic disease characterized by anemia due to erythrocyte fragility. Clearly, further experimentation is needed to resolve the independent contributions of SLC4A2 to osteoclast differentiation and function.



**Fig. 5.** SLC4A2 is required for bone resorption and extracellular acidification by osteoclasts *in vitro*. (A) Immunostaining for SLC4A2 on MCSF-primed BM cells cultured with MCSF and RANKL. (B) TRAP stain and (C) supernatant TRAP assay of spleen cells cultured with MCSF (B) only, or MCSF and RANKL. (D) qRT-PCR analysis of spleen cells cultured with MCSF, or MCSF and RANKL. (E) Lectin-TRITC stain of dentin slices cultured with spleen cells and MCSF and RANKL. (F) Acridine orange stain of spleen cells cultured on dentin slices with MCSF and RANKL.

It is currently unclear why *Slc4a2*<sup>-/-</sup> osteoclasts do not display histochemical TRAP activity *in vivo*, despite the expression of other osteoclast markers (Fig. 4 A and C–E). Osteoclast TRAP is translated as a monomer with low phosphatase activity. Proteolytic cleavage excises an internal, repressive peptide resulting in a more active heterodimer (20). The lack of histochemical TRAP activity in *Slc4a2*<sup>-/-</sup> mice may be related to inefficient processing of the TRAP monomer due to improper vacuole acidification. Reports showing that cathepsins process monomeric TRAP (20), and that cathepsins are more stable and active at low pH (21), support this hypothesis. However, osteoclasts from *Atp6i*<sup>-/-</sup> and *Cln7*<sup>-/-</sup> mice, which also display acidification defects, express TRAP activity (22, 23). Given the importance of TRAP to osteoclast function (24), further investigation is needed to explain this observation.

The most intriguing finding in this study was the dramatic increase in osteoclast apoptosis observed in *Slc4a2*<sup>-/-</sup> mice (Fig. 4 F and H). Compared with the large body of knowledge about osteoclast differentiation, relatively little is known about osteoclast apoptosis *in vivo*. The reversal phase of bone resorption is associated with cell death of osteoclasts, most dramatically observed at the termination of lactation (25). Two drugs, estrogen and bisphosphonates, currently used to treat osteoporosis, induce osteoclast apoptosis (26, 27). Although the mechanism by which *Slc4a2* deficiency results in osteoclast apoptosis is unresolved, it may be a direct consequence of cytoplasmic alkalization in the absence of HCO<sub>3</sub><sup>-</sup>/Cl<sup>-</sup> transport activity. In multiple systems a rise in intracellular pH triggers apoptosis (28). Alternatively, increased cell death may not be intrinsic to *Slc4a2*<sup>-/-</sup> osteoclasts, but rather a consequence of proapoptotic signals generated in these mice. For example, sex hormones, glucocorticoids, and TGFβ all promote osteoclast apoptosis (29) and could be deregulated in *Slc4a2*<sup>-/-</sup> mice. *Slc4a2*<sup>-/-</sup> osteoclasts may also die more readily because of decreased matrix or stromal cell-derived prosurvival signals.

Taken together, our data suggest therapeutics targeting SLC4A2 may block bone degradation by at least three mecha-

nisms: inhibiting acidification of the resorption lacuna, reducing TRAP activity, and initiating osteoclast apoptosis. The induction of cell death is a particularly attractive mechanism of action because intermittent inhibition of SLC4A2 could have long-lasting effects on osteoclast numbers and thus obviate the need for daily therapy.

## Materials and Methods

**Mice.** The generation of the *Nfatc1* conditional knockout strain, and the use of *Mx1-cre* to delete *Nfatc1* in osteoclast precursors, is described elsewhere (9). *Slc4a2*<sup>-/-</sup> mice were obtained from G.E. Shull (University of Cincinnati, Cincinnati, OH) (13). Experimental protocols were approved by the Standing Committee on Animals at the Harvard Medical School and were designed with institutional and National Institutes of Health guidelines for the humane use of animals.

**Cell Culture.** All cells were maintained in Minimum Essential Medium-Alpha (Cellgro) containing 10% FBS and 1% penicillin/streptomycin. RANKL was a gift from Y. Choi (University of Pennsylvania, Philadelphia). Osteoclast differentiation in RAW clone 6 cells (12), a gift of K.P. McHugh (Harvard Medical School, Boston), was induced by adding 200 ng/ml RANKL for 4 days. The isolation of CD11b<sup>low</sup>-CD3e<sup>-</sup>B220<sup>-</sup>c-kit<sup>+</sup>fms<sup>+</sup> BMOcPs, used for the experiments in Fig. 1 A–C, is described elsewhere (9, 30). For immunofluorescence studies, MCSF-primed BM cells were used as osteoclast precursors (18). For mouse spleen cell cultures, a single cell suspension of splenocytes was cultured for 3 days with 30 ng/ml MCSF (R&D Systems) and differentiation was induced by adding 500 ng/ml RANKL for 5 days. Biochemical assays for TRAP were performed as described (31, 32) or with a commercial kit (Sigma).

**RNA Extraction and Quantitative Real-Time PCR.** Total RNA was extracted using an RNeasy kit (Qiagen). cDNA was synthesized using AffinityScript cDNA Synthesis Kit (Stratagene). qRT-PCR reactions were performed using the primers listed in supporting information (SI) Table S1 and the Brilliant II SYBR Green QPCR Master Mix (Stratagene) in an Mx3005P QPCR System (Stratagene). Relative amounts of mRNA were calculated by the ΔΔCt method using *Hprt1* as an internal control.

**SLC4A2 Western Blot and Immunofluorescence.** An affinity purified rabbit antibody to mouse SLC4A2 was provided by S.L. Alper (Harvard Medical School, Boston) (33). For immunofluorescence, osteoclast precursors were cultured with

MCSF and RANKL for 5 days, fixed, permeabilized, and stained with anti-SLC4A2 and goat anti-rabbit Alexa488 (Invitrogen). Hoechst (Sigma) was used as a nuclear counterstain.

**shRNA-Mediated Knockdown of *Slc4a2* mRNA.** The RNAi Consortium at the Broad Institute (34) provided lentivirus-based shRNA constructs targeting *Slc4a2* (Table S2), or control sequences, which were used to infect RAW clone 6 cells. Assays were performed on a stable pool of transduced cells.

**Histology and Immunohistochemistry.** Tissues were fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin. TRAP staining was performed as described (31). The following monoclonal antibodies were used for immunohistochemistry: anti-CD68 (FA-11; Abcam), anti-cathepsin K (182-12G5; Calbiochem), and anti-NFATc1 (7A6; PharMingen). The appropriate HRP conjugated secondary antibodies and 3,3'-diaminobenzidine revealed binding of primary antibodies to their respective antigens. TUNEL was performed using the In Situ Cell Death Detection Kit (Roche).

**Quantification of Osteoclasts and Apoptosis *in Vivo*.** To quantify osteoclasts and apoptotic osteoclasts, three nonoverlapping microscopic fields, obtained with a 40 $\times$  objective, were analyzed per mouse from femur sections stained for cathepsin K. Osteoclasts were defined as cathepsin K-positive cells with two or more nuclei. Cells with chromatin condensation or nuclear fragmentation were considered apoptotic (29).

**Osteologic Matrix Resorption and Dentin Resorption Assay.** RAW clone 6 cells were plated on Osteologic slices (BD Biosciences), treated with RANKL for 6 days, and analyzed following manufacturer's instructions. For dentin assays, spleen cells were plated on dentin slices (provided by P. Hauschka, Harvard Medical School, Boston) and differentiated with MCSF and RANKL for 6 days. Resorption pits were revealed using wheat germ lectin-TRITC (Sigma) after sonication in 0.5 M NH<sub>4</sub>OH.

**Evaluation of Extracellular Acidification Using Acridine Orange.** Acridine orange staining was performed as described (35). Briefly, spleen cells were plated on dentin slices, differentiated with MCSF and RANKL for 6 days, and incubated with 5  $\mu$ g/ml acridine orange (Sigma) for 15 min. The cells were observed under a fluorescence microscope with a 490 nm excitation filter and a 525 nm arrest filter.

**Statistical Analysis.** Unpaired, two-tailed Student's *t* tests were used for statistical comparisons. A *P* value of <0.05 was considered significant.

**ACKNOWLEDGMENTS.** The authors thank Dr. Nir Hacohen and the Immune Circuits Group, and the RNAi consortium at the Broad Institute, for advice and the lentiviral shRNA vectors. We acknowledge Dorothy Zhang for expert histology preparation. This work was supported by National Institutes of Health Grant AI31541 (to L.H.G.) and Merck & Co., Inc. (L.H.G.). A.O.A. acknowledges the Abbott Scholar Award in Rheumatology Research and the 2007 American Society for Clinical Investigation Young Investigator Award.

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