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Ly49Q, an ITIM-bearing NK receptor, positively regulates osteoclast differentiation

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ABSTRACT

Osteoclasts, multinucleated cells that resorb bone, play a key role in bone remodeling. Although immunoreceptor tyrosine-based activation motif (ITAM)-mediated signaling is critical for osteoclast differentiation, the significance of immunoreceptor tyrosine-based inhibitory motif (ITIM) has not been well understood. Here we report the function of Ly49Q, an Ly49 family member possessing an ITIM motif, in osteoclastogenesis. Ly49Q is selectively induced by receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) stimulation in bone marrow-derived monocyte/macrophage precursor cells (BMMs) among the Ly49 family of NK receptors. The knockdown of Ly49Q resulted in a significant reduction in the RANKL-induced formation of tartrate-resistance acid phosphatase (TRAP)-positive multinucleated cells, accompanied by a decreased expression of osteoclast-specific genes such as *Nfatc1*, *Tm7sf4*, *Oscar*, *Ctsk*, and *Acp5*. Osteoclastogenesis was also significantly impaired in Ly49Q-deficient cells *in vitro*. The inhibitory effect of Ly49Q-deficiency may be explained by the finding that Ly49Q competed for the association of Src-homology domain-2 phosphatase-1 (SHP-1) with paired immunoglobulin-like receptor-B (PIR-B), an ITIM-bearing receptor which negatively regulates osteoclast differentiation. Unexpectedly, Ly49Q deficiency did not lead to impaired osteoclast formation *in vivo*, suggesting the existence of a compensatory mechanism. This study provides an example in which an ITIM-bearing receptor functions as a positive regulator of osteoclast differentiation.

Introduction

Bone homeostasis is controlled by the coordinated balance maintained between bone formation by osteoblasts and bone resorption by osteoclasts [1]. Osteoclasts, multinucleated cells that uniquely have the ability to resorb bone, play a central role in calcium homeostasis as well as bone remodeling [2]. Increased osteoclast differentiation and function have been implicated in the pathogenesis of various osteopenic conditions, including postmenopausal osteoporosis and bone loss in inflammatory arthritis [3,4]. Therefore, understanding the regulatory mechanisms of osteoclast differentiation and function is important for the development of novel therapeutic strategies for these disorders.

Osteoclasts differentiate from monocyte/macrophage lineage cells in the presence of macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) [1,2]. This process is tightly regulated by mesenchymal lineage cells such as osteoblasts and bone marrow stromal cells, which provide M-CSF and RANKL [1,2]. M-CSF signaling through its receptor, c-Fms, is required for the survival and proliferation of osteoclast precursor cells [5]. RANKL binding to its receptor, RANK, results in the activation of tumor necrosis factor receptor-associated factor 6 (TRAF6), c-Fos, and calcium signaling pathways, each of which is essential for the induction and activation of nuclear factor of activated T cells (NFAT) c1, a critical transcription factor for osteoclastogenesis [1,6]. In fact, mice deficient in RANKL, RANK, TRAF6, c-Fos, and NFATc1 exhibit severe osteopetrosis due to impaired osteoclastogenesis [1,3,7].

In addition to RANKL and M-CSF, costimulatory signals mediated by immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptors, DNAX-activating protein 12 (DAP12), and Fc

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receptor common γ subunit (FcRγ), are indispensable for osteoclastogenesis [8,9]. The immunoglobulin-like receptors, such as triggering receptors expressed on myeloid cells-2 (TREM-2), paired immunoglobulin-like receptor (PIR)-A, osteoclast-associated receptor (OSCAR), and signal-regulatory protein (SIRP) β1, which associate with DAP12 or FcRγ in osteoclast precursor cells, play a key role in the costimulatory signals required for osteoclastogenesis [1,10], although the ligands of these receptors in the skeletal system have not yet been identified.

In the immune system, the balance between the activating signals mediated by the ITAM and the inhibitory signals mediated by the immunoreceptor tyrosine-based inhibitory motif (ITIM) determine the level of the immune response [11,12]. The activating signals are mediated by the tyrosine kinases, including the Src and spleen tyrosine kinase (Syk) families, whereas the inhibitory signals are mediated by protein tyrosine phosphatases and lipid phosphatases which are recruited to ITIM [12]. Interestingly, viable motheaten mice (me^v/me^v), which have a catalytically defective Src-homology domain-2 phosphatase-1 (SHP-1), exhibit severe osteoporosis caused by enhanced osteoclastic bone resorption, suggesting that SHP-1 controls osteoclast differentiation and function [13,14]. Although recent studies have reported that certain ITIM-bearing receptors, including PIR-B, SIRPα, platelet endothelial cell adhesion molecule-1 (PECAM-1) and CMRF-35-like molecule-1 (CLM-1), inhibit osteoclastogenesis through SHP-1 activation [15–18], the function of the ITIM-bearing receptors remains to be elucidated further.

The murine Ly49 receptor family is comprised of both activating and inhibitory molecules in target cell recognition by natural killer (NK) cells [19]. Ly49Q is a type II transmembrane protein which contains the ITIM motif at the N-terminus of the cytoplasmic region and associates with MHC class I *in cis* [20]. Whereas most of the Ly49 receptors are expressed on NK cells, Ly49Q is expressed on plasmacytoid dendritic cells (pDCs), macrophages, and neutrophils, but not NK cells [20–22]. Several Ly49 receptors inhibit NK cell functions, including cytokine production and cytolytic activity. Treatment of activated macrophages with an anti-Ly49Q antibody induces rapid spreading and the formation of cell polarity through the reorganization of the actin cytoskeleton [20]. Following inhibitory Ly49 receptor engagement with MHC class I ligand, the phosphorylation of a tyrosine residue within the ITIM present in the cytoplasmic region facilitates the binding and activation of the SHP-1 and SHP-2 phosphatases, and the attenuation of intracellular signals [23]. Although Ly49Q is able to associate with SHP-1 and SHP-2 via its tyrosine-phosphorylated ITIM [20], toll-like receptor 9 (TLR9)-dependent antiviral responses were diminished *in vivo*, and the production of cytokines such as interferon-α (IFN-α) and interleukin (IL)-12 from pDCs in response to TLR9 was impaired *in vitro*, suggesting that Ly49Q is necessary for the activation of the innate immune response [24]. Similarly, the TLR9-mediated production of inflammatory cytokines such as IL-6 and tumor necrosis factor-α (TNF-α) was impaired in macrophages derived from Ly49Q-deficient mice [22].

In this study, we report that RANKL stimulation caused a selective induction of Ly49Q during osteoclast differentiation, which effect was diminished by FK506-mediated inhibition of calcineurin–NFAT activation. The knockdown or genetic ablation of Ly49Q in osteoclast precursor cells resulted in the significant suppression of osteoclastogenesis *in vitro*. Thus, these findings suggest that Ly49Q positively regulates osteoclast differentiation, and led us to investigate the mechanisms underlying this positive regulation and the bone phenotype in Ly49Q-deficient mice.

Materials and methods

Mice and analysis of the bone phenotype. The generation of Ly49Q-deficient mice was described previously [24]. All mice were maintained under specific-pathogen free conditions. Ly49Q-deficient mice were maintained and bred in the International Medical Center of Japan. All animal experiments were approved by the Animal Study Committee of Tokyo Medical and Dental University or the International Medical Center of Japan. Three-dimensional microcomputed tomography (microCT) scanning was performed using a ScanXmate-A100S Scanner (Comscan Techno, Kanagawa, Japan). Three-dimensional microCT images were reconstructed and the structural indices calculated using a three-dimensional image analysis system (TRI/3D-BON; RATOC System Engineering, Tokyo, Japan). Analyses of bone histology were performed as described [8,25,26].

In vitro osteoclastogenesis. In vitro osteoclastogenesis was performed as described previously [6,8,25,26]. Briefly, bone marrow cells were cultured with 10 ng/ml M-CSF (R&D Systems, Minneapolis, MN) to obtain bone marrow-derived monocyte/macrophage precursor cells (BMMs). These cells were cultured with 5 or 50 ng/ml RANKL (PeproTech, Rocky Hill, NJ) and M-CSF for 3 days. Osteoclastogenesis was evaluated by tartrate-resistance acid phosphatase (TRAP) staining.

Quantitative RT-PCR and GeneChip analysis. Real-time quantitative RT-PCR analysis was performed as described [26]. Briefly, total RNA was extracted by ISOGEN (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 0.5 μg of total RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative RT-PCR analysis was performed with the LightCycler apparatus (Roche Applied Science, Indianapolis, IN) using SYBR Green Real time PCR Master Mix (TOYOBO, Osaka, Japan). All primer sequences are available upon request. GeneChip analysis was performed as described previously [6,27].

Flow cytometric analysis. BMMs were removed from culture plates by treatment with Trypsin–EDTA (Invitrogen). Cell surface expression of Ly49Q was confirmed by staining with biotinylated anti-Ly49Q antibody [20] followed by Streptavidin–allophycocyanin conjugates (eBioscience, San Diego, CA). Stained cells were analyzed by FACSCantoII using Diva software (BD Biosciences, San Jose, CA).

Knockdown analysis. The short hairpin RNA (shRNA) duplexes were constructed based on the sequences obtained from the shRNA library of the RNAi Consortium. RNA targeting regions with a hairpin sequence (shLy49Q-1 sense: 5′-gatccGTAAACAGATATGTGA GCATTCTCGAGAATGCTCACATATCTGTTTACTTTTTg-3′; shLy49Q-1 antisense: 5′- aattcAAAAAGTAAACAGATATGTGAGCATTCTCGAGAA TGCTCACATATCTGTTTACg-3′; shLy49Q-2 sense: 5′-gatccGAACA TGTCTACCCATGTATTCTCGAGAATACATGGGTAGACATGTTCTTTTTg-3′; shLy49Q-2 antisense: 5′-aattcAAAAAGAACATGTCTACC CATGTATTCTCGAGAATACATGGGTAGACATGTTCg-3′; shNFATc1 sense: 5′-gatccGCCGCAGAACACTACAGT TATCTCGAGATAACTG-TAGTGTTCTGCGGCTTTTTg-3′; shNFATc1 antisense: 5′- aattcAAAAAGCCGCAGAACACTACAGTTATCTCGAGATAACTGTAGTGTTC TGCGGCg-3′) were ligated into the RNAi-Ready pSIREN-RetroQ-ZsGreen Vector (Clontech, Mountain View, CA) at the BamHI and EcoRI sites. pSIREN-RetroQ-ZsGreen-shLy49Qs, pSIREN-RetroQ-ZsGreen-shNFATc1 or a control pSIREN-RetroQ-ZsGreen-shRNA that specifically targets Luciferase was used to transfect BMMs. The retrovirus supernatants were obtained by transfecting the retroviral vectors into the Plat-E packaging cell line using Fugene6 (Roche Applied Science). BMMs were infected with retroviruses for 12 h before RANKL stimulation.

Immunoblot analysis. Cell lysates were subjected to immunoblot analysis using specific antibodies for NFATc1 (Santa Cruz Biotechnology, Santa Cruz, CA), c-Fos (Calbiochem, La Jolla, CA), and β-actin (Sigma–Aldrich, St. Louis, MO). For immunoprecipitation analysis, cells were solubilized in lysis buffer (1% Nonidet P-40 in 50 mM NaCl, 50 mM Tris–HCl, 5 mM EDTA, 1 mM NaF, 2 mM

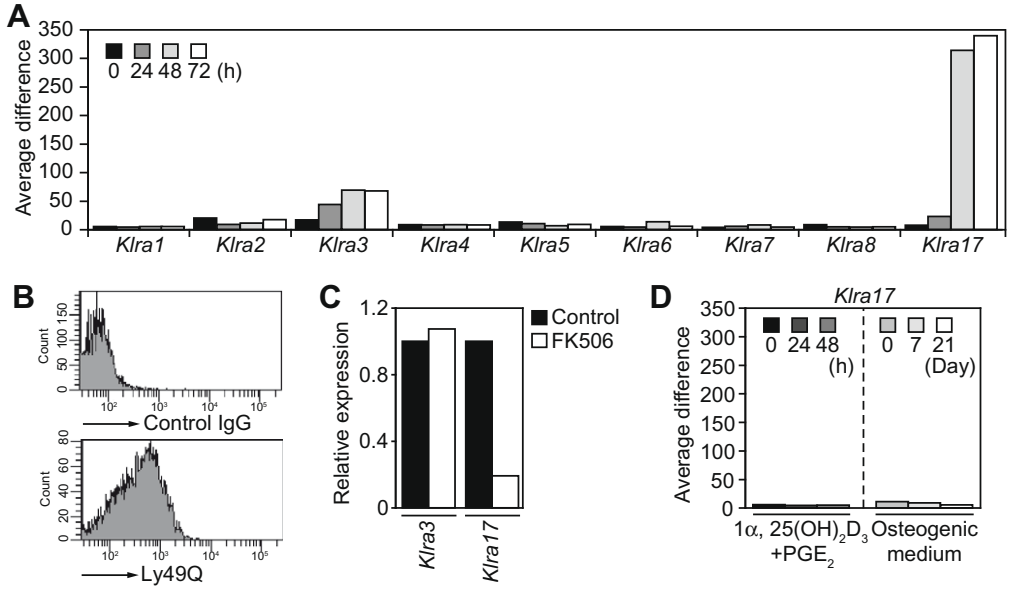

Fig. 1. Expression of Ly49 receptors in osteoclasts and osteoblasts. (A) GeneChip analysis of the mRNA expression of Ly49 receptors during osteoclast differentiation after RANKL stimulation. (B) Flow cytometric analysis of Ly49Q expression on BMMs treated with RANKL for 2 days. (C) Effect of FK506 treatment on the mRNA expression of *Klra3* and *Klra17* in osteoclasts. (D) GeneChip analysis of the mRNA expression of *Klra17* in calvarial osteoblasts treated with 10 nM 1α, 25$(OH)_2D_3$ and 1 μM PGE_2 or osteogenic medium (50 μM ascorbic acid, 10 nM dexamethasone and 10 mM β-glycerophosphate).

PMSF), supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Science). Immunoprecipitation was carried out by incubation with the anti-SHP-1 antibody (Cell Signaling Technology, Beverly, MA) followed by the addition of protein G–Sepharose (GE Healthcare, Buckinghamshire, UK). Immune complexes were separated by electrophoresis followed by blotting with anti-PIR-B (R&D Systems), anti-PECAM-1(Santa Cruz Biotechnology), and anti-SHP-1 antibodies.

Statistical analysis. Statistical analysis was performed using the unpaired two-tailed Student's *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$;

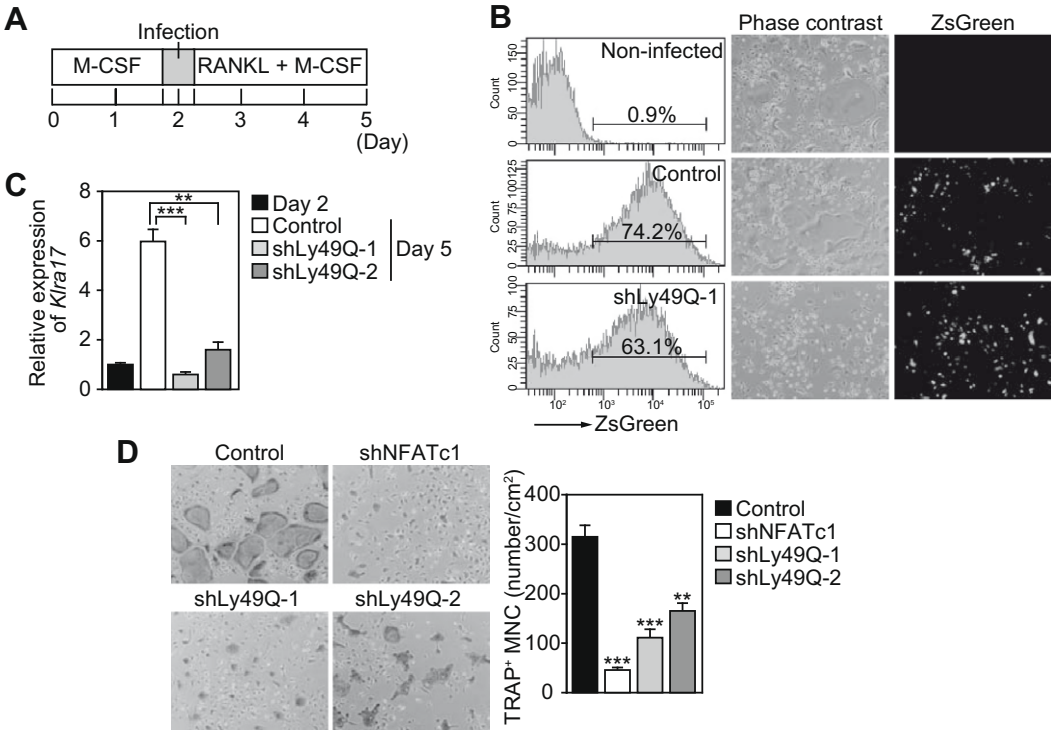

Fig. 2. Contribution of Ly49Q to RANKL-induced osteoclastogenesis. (A) A schematic of the *in vitro* osteoclast culture system and the retroviral transfer. The infected cells were cultured for 3 days in the presence of RANKL and M-CSF. (B) Infection efficiency of retroviruses. After 2 days of RANKL stimulation, the efficiency of infection was determined by assaying ZsGreen expression with flow cytometry (left panel) or fluorescence microscopy (right panel). (C) The mRNA expression of *Klra17* (encoding Ly49Q) in osteoclast precursor cells transduced with retroviral vectors expressing shLy49Q (quantitative RT-PCR). Total RNA was isolated on the indicated days. The level of mRNA expression was normalized with *Gapdh* expression. (D) The effect of the retrovirus-mediated introduction of the shRNA for Ly49Q on RANKL-induced osteoclastogenesis.

***$P < 0.001$; n.s., not significant, throughout the paper). All data are expressed as means ± SEM.

Results

Selective expression of Ly49Q during osteoclast differentiation

The expression of Ly49 family receptors during osteoclast differentiation induced by RANKL and M-CSF was analyzed by GeneChip analysis (Fig. 1A). Among the Ly49 family receptors, *Klra17* (encoding Ly49Q) was highly and selectively induced during osteoclastogenesis. The protein expression of Ly49Q in BMMs was confirmed by flow cytometric analysis (Fig. 1B). In addition, the expression of *Klra17*, but not *Klra3* (encoding Ly49C), was markedly reduced by FK506, an inhibitor of calcineurin, suggesting that the induction of Ly49Q is dependent on the calcineurin–NFAT pathway (Fig. 1C). On the other hand, Ly49Q was not induced in calvarial osteoblasts treated with 1α, $25(OH)_2D_3$, and prostaglandin E_2 (PGE_2), which support osteoclast differentiation, or when cultured in an osteogenic medium which induces osteoblast differentiation (Fig. 1D). These results prompted us to examine the role of Ly49Q in osteoclastogenesis.

Inhibition of osteoclast differentiation by RNA interference (RNAi)-mediated knockdown of Ly49Q

To assess the role of Ly49Q during osteoclastogenesis, we performed RNAi-mediated knockdown of Ly49Q in the osteoclastogenesis induced by RANKL and M-CSF, using the retroviral short hairpin RNA (shRNA) delivery system (Fig. 2A). The infection efficiency was determined by analyzing the expression of ZsGreen, a green fluorescent protein encoded by the retrovirus vector, using flow cytometry. BMMs were transduced with retroviral vectors expressing shRNA targeting two different regions of Ly49Q (shLy49Q-1 and shLy49Q-2). As shown in Fig. 2B, more than 60% of the cells were positive for ZsGreen, indicating that shRNA-expressing retroviral vectors were efficiently transduced into BMMs. The mRNA expression of *Klra17* in osteoclast precursor cells was analyzed by quantitative RT-PCR, and was found to be significantly reduced by shLy49Q-1 as well as shLy49Q-2 (Fig. 2C). When the expression of NFATc1, the key transcription factor for osteoclastogenesis, was suppressed by shRNA targeting NFATc1 (shNFATc1), osteoclast differentiation was markedly inhibited (Fig. 2D). Based on previous studies [15–18], the ITIM-containing receptors negatively regulate osteoclastogenesis, but contrary to our expectation, the knockdown of Ly49Q significantly suppressed, rather than enhanced, the formation of the TRAP-positive multinucleated cells (MNCs) induced by RANKL and M-CSF (Fig. 2D). The difference in the inhibitory effects of shLy49Q-1 and shLy49Q-2 may result from the difference in the knockdown efficiency (see Fig. 2C). These results suggest that Ly49Q is a positive regulator of osteoclastogenesis, even though Ly49Q contains an ITIM motif.

Ly49Q as a positive regulator of osteoclast differentiation

Given our observation that the knockdown of Ly49Q resulted in the inhibition of RANKL-induced osteoclastogenesis, we investigated the expression of genes implicated in osteoclast differentiation and function by quantitative RT-PCR (Fig. 3A). Although the mRNA expression of *Tnfrsf11a*, *Csf1r*, or *Fos* was not changed by the Ly49Q knockdown, the expression levels of *Ctsk*, *Acp5*, *Oscar*, and *Tm7sf4* (encoding dendritic cell-specific transmembrane protein, DC-STAMP) were significantly decreased as compared to control. In addition, *Nfatc1* expression was potently suppressed by Ly49Q knockdown. Similar results were obtained with immunoblot analysis (Fig. 3B).

It is well documented that ITAM-mediated calcium signaling is crucial for the robust induction of NFATc1 during osteoclastogenesis [1,8]. Thus, it is likely that Ly49Q contributes to the activation of the calcium signaling, but how does an ITIM-bearing receptor stimulate calcium signaling? In the regulation of paired immunoglobulin-like receptors, negative regulation usually occurs in the presence of the activatory receptor recognizing the common ligands [11]. We therefore hypothesized that Ly49Q does not func-

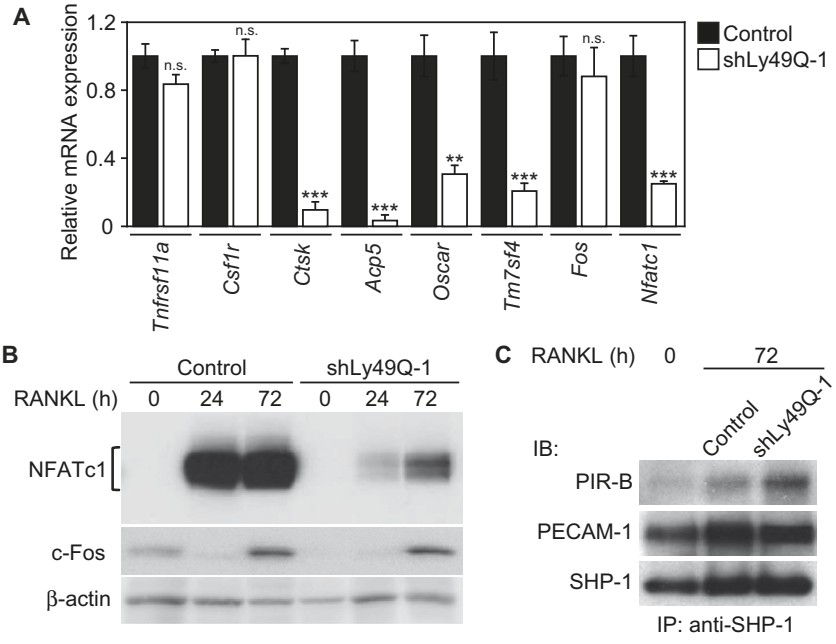

Fig. 3. Role of Ly49Q in RANKL-induced osteoclast differentiation. (A) Effect of Ly49Q knockdown on osteoclast-related gene expression (quantitative RT-PCR analysis). BMMs infected with retroviral vectors expressing control shRNA or shLy49Q-1 were cultured for 3 days with RANKL and M-CSF. (B) Effect of the Ly49Q knockdown on protein expression of NFATc1 and c-Fos (immunoblot analysis). (C) Effect of the Ly49Q knockdown on the association of SHP-1 with PIR-B or PECAM-1 in osteoclasts.

tion as a negative regulator, due to the lack of a corresponding activating receptor in the osteoclast lineage, and that Ly49Q efficiently associates with SHP-1 to deprive other ITIM-bearing inhibitory receptors of SHP-1, thus disabling the negative regulation. In fact, the association of SHP-1 with PIR-B, but not PECAM-1, was enhanced by the knockdown of Ly49Q (Fig. 3C). This result suggests that Ly49Q competes with PIR-B for the association of SHP-1, thereby functioning as a suppressor of PIR-B-mediated inhibitory effect on osteoclastogenesis.

Analysis of Ly49Q-deficient mice

We analyzed *in vitro* osteoclast differentiation in BMMs derived from Ly49Q-deficient mice. The formation of the TRAP-positive MNCs induced by RANKL and M-CSF was significantly reduced in the Ly49Q-deficient cells as compared with the wild-type (WT) cells (Fig. 4A). To evaluate the number of osteoblast progenitors in bone marrow, we performed a colony formation assay. The numbers of alkaline phosphatase-positive colony-forming unit-fibroblast (CFU-F) and von Kossa-positive CFU-osteoblast (CFU-OB) colonies in the culture of bone marrow cells were comparable in the WT and Ly49Q-deficient mice (data not shown). These results suggest that Ly49Q positively regulates osteoclast differentiation, without affecting osteoblastic bone formation.

Finally, to elucidate the role of Ly49Q *in vivo*, we analyzed the bone phenotype of Ly49Q-deficient mice. Unexpectedly, microCT analysis indicated no apparent difference between Ly49Q-deficient mice and WT littermates (Fig. 4B). For example, there was no difference in trabecular bone volume between the Ly49Q-deficient mice and WT littermates (Fig. 4D). Bone morphometric analysis also indicated no significant difference in osteoclast surface or osteoclast number in the metaphyseal region of the tibia (Fig. 4C and D). Similarly, the eroded surface was unchanged, suggesting that the osteoclastic bone resorption in Ly49Q-deficient mice was normal (Fig. 4D). Osteoblastic parameters, including osteoblast surface, osteoid surface, and osteoid volume, were comparable (data not shown). These results demonstrate that Ly49Q deficiency does not significantly influence osteoclast differentiation and function *in vivo* under physiological conditions.

Discussion

The crucial role of ITAM-bearing adaptors that associate with immunoglobulin-like receptors in osteoclast differentiation has

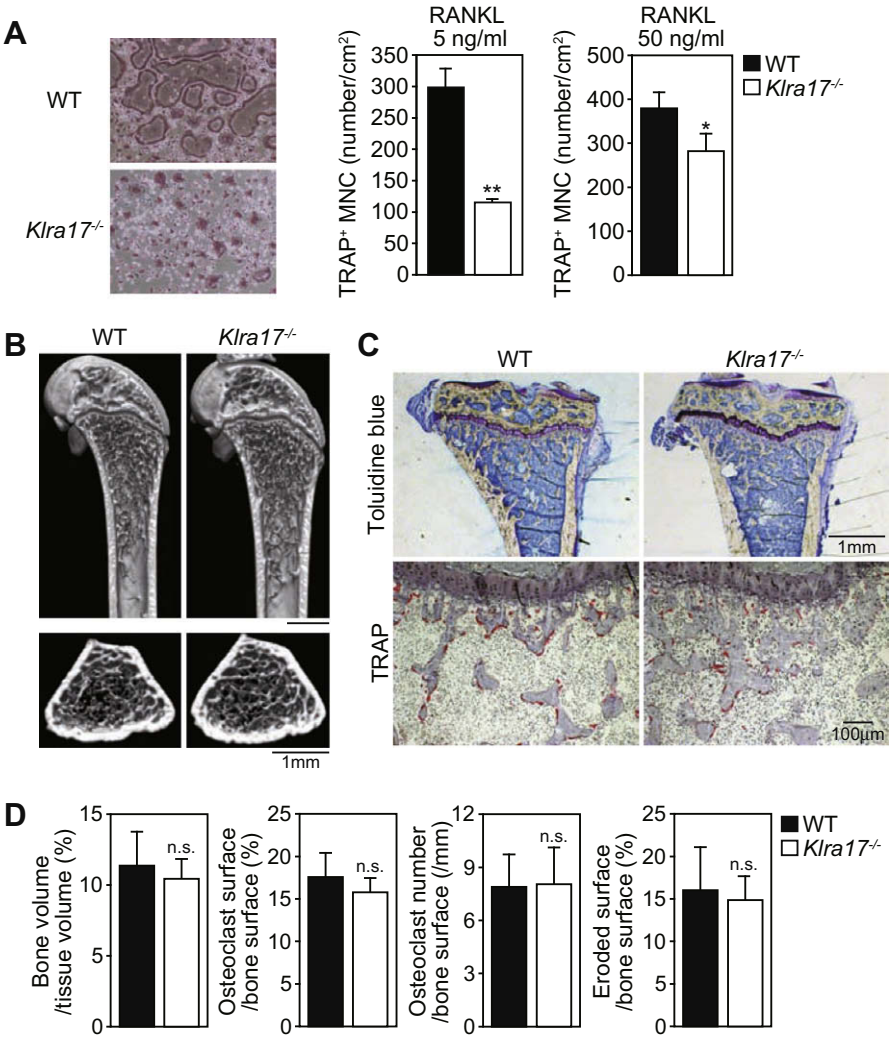

Fig. 4. Bone phenotype of Ly49Q-deficient (*Klra17*$^{-/-}$) mice. (A) Osteoclast differentiation in WT and Ly49Q-deficient BMMs stimulated with 5 and 50 ng/ml of RANKL in the presence of M-CSF. (B) MicroCT analysis of the femora of WT and Ly49Q-deficient mice (upper photograph: longitudinal view; lower photograph: axial view of the metaphyseal region). (C) Histological analysis of the proximal tibiae of WT and Ly49Q-deficient mice (upper photograph: toluidine blue staining; lower photograph: TRAP staining). (D) Bone volume determined by microCT analysis and osteoclastic parameters measured by bone morphometric analysis of WT and Ly49Q-deficient mice at the age of 9–10 weeks ($n = 4$).

been established [1,10]. However, the function of ITIM-bearing receptors has not been well understood. In the present report, we investigated the role of the ITIM-bearing NK receptor Ly49Q in osteoclastogenesis, because Ly49Q is specifically induced by RANKL stimulation in BMMs. Among NFAT family members, NFATc1 functions as the essential regulator for osteoclastogenesis [1,6] by inducing a number of genes which are involved in osteoclast differentiation and function [1,7]. We showed the RANKL-induced expression of Ly49Q, is dependent on the calcineurin–NFAT pathway, suggesting that Ly49Q induction is closely connected with the gene induction program activated during osteoclastogenesis. Interestingly, Ly49Q positively regulates *in vitro* osteoclastogenesis, unlike other ITIM-bearing receptors [15–18].

We explored the mechanisms underlying the positive regulation of osteoclast formation by an ITIM-bearing receptor. Since the expression level of SHP-1 is not changed by RANKL stimulation, it is likely that Ly49Q competes with other ITIM-bearing receptors for the recruitment of the limited amount of SHP-1 protein available during osteoclastogenesis. As shown in Fig. 3C, the recruitment of SHP-1 to PIR-B increased in the absence of Ly49Q. It is thus possible that the positive regulation by Ly49Q is based on the competition between Ly49Q and PIR-B, the latter of which is known to inhibit osteoclastogenesis through SHP-1. In the reported examples of the paired receptor system, the inhibitory receptor usually exerts its negative function in the presence of a corresponding activatory receptor and common ligands. Although the detailed mechanisms remain to be identified, Ly49Q may not be functional, possibly due to the lack of a corresponding activatory receptor in osteoclast precursor cells. Recent reports demonstrated that other ITIM-bearing receptors convey positive signals. For example, TREM-like transcript-1 (TLT-1), an ITIM-bearing receptor expressed on platelets, enhances calcium signaling by facilitating the recruitment of SHP-2 to the ITIM motif [28]. More recently, we demonstrated that Ly49Q promotes rapid polarization of neutrophils and tissue inflammation in the presence of inflammatory stimuli, although Ly49Q inhibits the adhesion and spreading of neutrophils at steady state [29]. These dual function of Ly49Q in neutrophils appeared to be mediated by changing the effecter phosphatase from SHP-1 to SHP-2 [29]. However, the role of SHP-2 in osteoclasts is still unknown and an important subject for future research.

In contrast to the *in vitro* observation, however, these results show a lack of an overt bone phenotype in Ly49Q-deficient mice. The role of Ly49Q may thus be compensated by other molecules *in vivo*. Indeed, mice lacking ITIM-bearing receptors, including PIR-B-deficient and SIRPα-mutant mice, also displayed a normal or subtly altered bone phenotype [15,16], suggesting that the ITIM-bearing receptor system is redundant. However, it has been shown that the expression of the MHC class I molecule, the ligand for Ly49Q, is induced by inflammatory cytokines such as TNF-α [30]. Therefore, it is possible that Ly49Q has a role in the enhanced osteoclastogenesis which occurs under various pathological conditions, including rheumatoid arthritis. This study provides an interesting example in which an ITIM-bearing receptor functions as a positive regulator of osteoclast differentiation and suggests a greater functional complexity of paired receptor systems than previously appreciated.

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