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Inhibitory Immunoglobulin-Like Receptors LILRB and PIR-B Negatively Regulate Osteoclast Development

Yu Mori,*‡ Sukenao Tsuji,* Masanori Inui,* Yuzuru Sakamoto,* Shota Endo,* Yumi Ito,* Shion Fujimura,* Takako Koga,‡ Akira Nakamura,* Hiroshi Takayanagi,‡ Eiji Itoi,† and Toshiyuki Takai†*‡

Osteoclasts, multinucleated cells of myeloid-monocytic origin, are responsible for bone resorption, which is crucial for maintenance of bone homeostasis in concert with bone-forming osteoblasts of nonhematopoietic, mesenchymal origin. Receptor activator of NF-κB ligand (RANKL) and M-CSF, expressed on the surface of and secreted by osteoblasts, respectively, are essential factors that facilitate osteoclast formation. In contrast to the activation processes for osteoclast formation, inhibitory mechanisms for it are poorly understood. Herein we demonstrate that inhibitory Ig-like receptors recruiting Src homology 2 domain-containing tyrosine phosphatase 1 (SHP-1) are expressed on osteoclast precursor cells like other myeloid cells, and that they play a regulatory role in the development of osteoclasts. We detected cell-surface expression of paired Ig-like receptor (PIR)-B and four isoforms of leukocyte Ig-like receptor (LILR)B on cultured osteoclast precursor cells of mouse and human origin, respectively, and showed that all of these ITIM-harboring inhibitory receptors constitutively recruit SHP-1 in the presence of RANKL and M-CSF, and that some of them can suppress osteoclast development in vitro. Fluorescence energy transfer analyses have suggested that the constitutive binding of either murine PIR-B or its human ortholog LILRB1 to MHC class I molecules on the same cell surface comprises one of the mechanisms for developmental regulation. These results constitute the first evidence of the regulation of osteoclast formation by cell-surface, ITIM-harboring Ig-like receptors. Modulation of these regulatory receptors may be a novel way to control various skeletal system disorders and inflammatory arthritis.


B one is continuously remodeled through bone formation by osteoblasts and bone resorption by osteoclasts, both of which innovate the bone structure and sustain its structural and functional integrity. Osteoclasts are multinucleated cells derived from bone marrow hematopoietic precursor cells of myeloid-macrophage lineage, whereas osteoblasts are of nonhematopoietic, mesenchymal origin. It is of particular importance to elucidate the molecular mechanisms for bone homeostasis mediated by osteoclasts and osteoblasts for the development of novel therapeutic strategies for various skeletal system disorders, such as excessive bone formation (osteopetrosis), bone reduction (osteoporosis), and inflammatory arthritic diseases, including rheumatoid arthritis.

Osteoblasts and other bone marrow stromal cells express two essential factors for osteoclast differentiation (osteoclastogenesis), namely receptor activator of NF-κB ligand (RANKL), a member of the TNF family, and M-CSF (1, 2). RANKL is considered to be a crucial cytokine that drives the development of monocyte-macrophage-lineage cells into osteoclasts (3), while M-CSF is indispensable for the proliferation and survival of osteoclast precursor cells (4). RANKL stimulation of osteoclast precursor cells leads to recruitment of TNFR-associated factor (TRAF)6 to RANK, and activates key nuclear factors c-Fos and NF-κB, which then induce the expression of genes for osteoclastogenesis governed by a key transcription factor, NFATc1 (5). Mice deficient in RANKL, RANK, TRAF6, NF-κB, or c-Fos show impaired osteoclast differentiation and osteopetrosis (1, 2, 6). In addition to this pivotal molecular sequence involving a cytokine, its receptor, intracellular signal mediators, and transcription factors, we (7, 8) and others (9, 10) recently identified a novel and indispensable signal cascade initiated by two membrane-associated, homodimeric adaptor molecules, namely FCγR and DAP12, both of which harbor an ITAM that, upon tyrosine phosphorylation initiated by RANKL, conveys Syk-mediated calcium signaling pivotal for the induction of NFATc1. Several cell-surface receptors in the immune system

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Abbreviations used in this paper: RANKL, receptor activator of NF-κB ligand; β1m, β2-microglobulin; BMM, bone marrow monocyte/macrophage-lineage cells; FCγR, Fc receptor common γ subunit; FRET, fluorescence resonance energy transfer; LILR, leukocyte Ig-like receptor; MNC, multinucleated cells; MOC, multinucleated mature osteoclast; PIR, paired Ig-like receptor; POC, prefusion osteoclast; SHP-1, SIRP, signal regulatory receptor; TRAP, tartrate-resistant acid phosphatase.

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have also been suggested to be associated with Fc\(\gamma\) or DAP12 on osteoclast precursor cells (7, 11).

On the other hand, osteoprotegerin (12), SHIP (13), and SH2 domain-containing tyrosine phosphatase 1 (SHIP-1) (14) are suppressive elements for osteoclastogenesis (6), because targeted disruption or naturally occurring loss-of-function mutation of the genes for the secretory protein or one of the intracellular phosphatases resulted in increased osteoclastogenesis leading to osteoporosis. Osteoprotegerin is a secreted TNF receptor-related protein that acts as a decoy receptor that blocks RANKL binding to RANK on osteoclasts (15), while SHIP in osteoclasts inhibits PI3K, an inducer of cell survival, cytoskeletal rearrangement, and motility, and thus regulates the activation of osteoclasts (6, 16). Additionally, viable motheaten (me/ me\(^e\)) mice, in which tyrosine phosphatase activity of cytosolic enzyme SHP-1 is partially lost (residual activity, 10–20%) (14), show severe defects in hematopoiesis and exhibit bone reduction due to an increased number of osteoclasts and the enhanced bone resorptive activity (14). It is suggested that SHP-1 plays a role in signals downstream of RANKL (17) as well as in a pathway involving LPS stimulation (18). These observations indicate the presence of intracellular regulatory processes involving SHP-1 that inhibit relatively early stages of osteoclast development (6). However, little is known about the molecular network for this inhibition, compared with the wealth of evidence of the cell-surface receptors and intracellular activation cascades facilitating osteoclastogenesis (7, 11).

In the immune system, SHP-1 activation takes place downstream of various inhibitory receptors, such as human leukocyte Ig-like receptor LILR-B (19–21), signal regulatory receptor (SIRP)\(\alpha\) (22), murine paired Ig-like receptor (PIR)-B (23, 24), and gp49B (25, 26). SHP-1 is recruited, via its SH2 domain, to the phosphotyrosine residues of the ITIM present in the cytoplasmic isoforms of human LILRB, namely LILRB1, B2, B3, and B4 (also known as ILT72/LIR-1/CD85j, ILT4/LIR-2/CD85d, ILT5/LIR-3/CD85a, and ILT3/LIR-5/CD85k, respectively), and murine PIR-B in osteoclasts derived from human peripheral blood and mouse bone marrow, respectively. Our findings strongly suggest that the LILRB- and PIR-B-associated constitutive signaling mediated by SHP-1 regulates the differentiation of osteoclast precursors in concert with RANKL and M-CSF. Fluorescence resonance energy transfer (FRET) examination revealed that the constitutive cis-interaction of LILRB1 with its ligands, MHC class I molecules, on the surface of the same osteoclast precursor cells contributes to developmental regulation.

**Materials and Methods**

**Cell culture**

Human PBMCs were obtained individually by separating peripheral blood from healthy volunteers by density gradient centrifugation with Histopaque 1077 (Sigma-Aldrich). according to the guidelines of the Ethical Committee of Tohoku University Graduate School of Medicine. CD14\(^+\) monocytes were then purified by positive selection with MACS (Miltenyi Biotec). To generate multinucleated mature osteoclasts (MOCs), CD14\(^+\) monocytes were cultured at 5 \(\times\) 10\(^5\) cells/ml in \(\alpha\)-MEM, supplemented with 10% FCS and penicillin-streptomycin, for 7 days in the presence of 100 ng/ml RANKL and 20 ng/ml M-CSF (PeproTech). Tartrate-resistant acid phosphatase (TRAP\(^+\)) multinucleated cells (MNCs, \(\geq\)3 nuclei) were counted. Proliferation of osteoclasts (POCs) were obtained at 72 h in culture in the same medium as that for MOCs. Macrophages were obtained by 7 days of culture of CD14\(^+\) monocytes in the medium containing M-CSF but not RANKL.

C57BL/6 (B6) mice were purchased from Charles River Japan. The generation of PIR-B-deficient (\(Pirb^{–/–}\)) mice was described previously (31). Mice were maintained and bred in the animal unit of the Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). According to guidelines for experimental animals defined by The Institute of Development, Aging, and Cancer Animal Studies Committee. The induction of murine osteoclasts in vitro was performed as described previously (7). Briefly, bone marrow cells were cultured at 5 \(\times\) 10\(^5\) cells/ml in \(\alpha\)-MEM with 10% FCS containing 10 ng/ml M-CSF. After 2 days, adherent cells were used as bone marrow monocyte/macrophage lineage cells (BMMs). The BMMs were further cultured for 3 days in the presence of 100 ng/ml RANKL and 10 ng/ml M-CSF to generate MOCs. TRAP\(^+\) MNCs (\(\geq\)3 nuclei) were counted. POCs were obtained by a 48-h culture in the same medium as that for MOCs. Murine osteoblasts were prepared as described previously (7).

**Antibodies**

The mAbs used for immunophenotypic and functional assays were as follows: purified and FITC-conjugated anti-LILRB1 (clone GH17/75; BD Pharmingen), PE-conjugated and purified anti-LILRB2 (clone 42D1, rat IgG2a, Beckman Coulter), purified and FITC-labeled anti-LILRB3 (clone 222821; R&D Systems), purified and PC-5 conjugated anti-LILRB4 (clone ZM3.8; Beckman Coulter), anti-PIR-A/B (6C1; a generous gift from Drs Hiromi Kubagawa and Max D. Cooper, University of Alabama at Birmingham), anti-SIRP\(\alpha\) (clone P84; BD Pharmingen), anti-HLA-A/B/C (clone G46-2.6; BD Pharmingen), anti-human \(\beta_2\)-microglobulin (\(\beta_2\)-m) (clone TU99; BD Pharmingen), anti-H2K^D^ (clone 28-8-6; BD Pharmingen); and anti-mouse \(\beta_2\)-m (clone S19.8; BD Pharmingen). The Abs used for immunoblot analysis were as follows: biotinylated anti-ILT3 (anti-LILRB4, R&D Systems), and anti-phosphotyrosine (4G10) mAbs and anti-SHP-1 Abs (Upstate Biotechnology).

**Microarray analysis**

Microarray analysis was performed on Affymetrix Human Genome U133 Plus 2.0 arrays using mRNAs prepared from purified monocytes, cultured macrophages, POCs, and MOCs as described above.

**Flow cytometric analysis**

Cell-surface expression of LILRs or PIR-B was assessed by flow cytometric analysis (FACS LSR, BD Biosciences) using the specific mAbs described above. For the detection of LILRB3 expression, the secondary reagent was PE-labeled rat anti-mouse Abs (BD Pharmingen). For the detection of PIR-B expression, anti-PIR-A/B (6C1) labeled with Alexa Fluor 647 (Invitrogen) was used.

**Assessment of osteoclastogenesis after Ab stimulation**

Human CD14\(^+\) monocytes were obtained as described above. For crosslinking experiment, the anti-LILRB1 (GH17/75), LILRB3 (222821), and LILRB4 (ZM3.8) mAbs and isotype control Abs were converted to F(ab')\(_2\) using an ImmunoPure IgG1 Fab and F(ab')\(_2\) preparation kit (Pierce). Twenty-four-well flat-bottom plates were coated overnight at 4°C with F(ab')\(_2\), at the concentration of 20 \(\mu\)g/ml in PBS. CD14\(^+\) monocytes were plated at 5 \(\times\) 10\(^5\) per well and then cultured with 100 ng/ml RANKL and 20 ng/ml M-CSF for 7 days. Osteoclastogenesis was assessed by counting TRAP\(^+\) multinucleated cells.

**Murine bone histology**

Murine computed tomography was performed using \(\mu\)CT40 (Scanco Medical). Murine skeletal tissues were fixed in paraformaldehyde, decalcified in EDTA, dehydrated in ethanol, and then embedded in paraffin. Sections were stained with H&E or for TRAP activity using a TRAP staining kit (TaKaRa Biomedicals).
**Pit formation assay**

For assessment of the bone resorption activity of bone marrow cell-derived osteoclasts, bone marrow cells from B6 and Pirb/H11002 mice were cultured on calcium phosphate matrices for 8 days in the presence of various concentrations of RANKL and 20 ng/ml M-CSF (PeproTech). After induction of the osteoclasts, the area of bone resorption was determined with image analysis software (Adobe Photoshop 7.0).

**Confocal microscopy and FRET analysis**

Confocal laser scanning microscopy of human PBMC-derived POCs was performed with Alexa Fluor 546-conjugated anti-LILRB1, LILRB2, LILRB3, LILRB4, and β2m mAbs, and Alexa Fluor 647-conjugated anti-PIR-A/B, SIRP/H9251, and SIRP/H9252 mAbs, and Alexa Fluor 647-conjugated H-2Kb/Db mAb. All of these mAbs were directly labeled with an Alexa Fluor 546 or 647 monoclonal labeling kit (Invitrogen). POCs were visualized by FluoView FV1000 confocal microscopy (Olympus), which was controlled with FV-10ASW software. Alexa Fluor 546/647 FRET imaging was performed with excitation at 543 nm and detection of donor Alexa Fluor 546 and FRET emission, followed by 633 nm excitation and acceptor Alexa Fluor 647 detection. After image acquisition, FRET efficiencies were calculated using FV-10ASW software. FRET efficiency (E) was obtained as follows: $E = 1 - P_D/P_T \times 100$ where $P_D$ indicates the fluorescence intensity of prebleach donor cells and $P_T$ the fluorescence intensity of postbleach donor cells. $E$ of >0.05 was defined as the threshold level for substantial transfer efficiency.

**Immunoblot analysis**

For immunoprecipitation analysis, cell lysates were sequentially incubated with anti-LILRB1, anti-LILRB2, anti-LILRB3, anti-LILRB4, anti-PIR-A/B, and protein-G conjugated Sepharose beads. Immunoprecipitates were separated by SDS-PAGE using 15% gels, followed by transfer to polyvinylidene difluoride (Millipore) membranes. Each membrane was probed with HRP-conjugated anti-phosphotyrosine mAb and anti-SHP-1 mAb. The probed membranes were stripped with strip buffer and then reprobed with anti-LILRB1, anti-LILRB2, anti-LILRB3, anti-LILRB4, and anti-PIR-A/B.

**Statistical analysis**

Statistical significance was assessed by means of Student’s t test, with $p < 0.05$ being considered significant.

**Results**

Four isoforms of LILRB are expressed on human osteoclasts in vitro

In the immune system, the ITAM-mediated activation signal is often coupled with counter-regulation via ITIM-harboring receptors that recruit SHP-1 (27, 32, 33). In the present study, we first attempted to determine whether the expression of inhibitory isoforms of human LILR, LILRBs, could be detected on POCs or MOCs in vitro. To this end, we first examined the gross profiles of mRNA expression of LILRB molecules in human peripheral blood monocytes, in vitro-cultured macrophages, POCs, and MOCs by means of microarray analysis. As shown in Fig. 1A, the mRNA expression profiles of the individual LILRBs exhibited several characteristics for each cell lineage. As a common observation, high mRNA expression of LILRB1–B4 was observed for every lineage. In peripheral blood monocytes, LILRB1, A2, A5, and A6 mRNA was distinctive. In macrophages, LILRB1 and LILRB5 expression was reduced compared with that in monocytes, while the expression of LILRB2 and LILRB6 was sustained. In POCs, the expression levels of LILRBs, LILRA5 in particular, were decreased when compared with those in monocytes. In MOCs, the expression profiles of LILRA1–A6 were roughly the same as those in POCs. B. Flow cytometric analysis of cell-surface expression on myeloid-lineage cells stained with mAbs against LILRB1 (GHI/75), LILRB2 (42D1), LILRB3 (22281), and LILRB4 (ZM3.8). Representative data are shown from three separate experiments with similar results, each of which employed the set of myeloid-lineage cells prepared individually from three donors.
LILRB1–B4 was distinctive. In macrophages prepared by culturing monocytes with M-CSF for 6 days, LILRB4 and LILRB5 expression was markedly enhanced, while LILRB1–B3 expression was not remarkably altered. In POCs after 4 days of culture of monocytes with RANKL and M-CSF, a positive control RANK mRNA was significantly induced, while the LILRB1–B4 mRNA level was relatively unchanged. In MOCs after 6 days of culture of monocytes with RANKL and M-CSF, the expression profile of LILRB1–B5 was roughly the same as those in the case of POCs. Thus, through this microarray analysis, we verified the expression of significant amounts of LILRB1–B4 mRNAs during the development of osteoclasts, suggesting the possible involvement of these inhibitory receptors in human osteoclastogenesis. Although we monitored in parallel the profiles of mRNA expression of the activating receptors, LILRAs, finding, for example, that the expression of LILRA2 and LILRA6 was significant in both POCs and MOCs, we hereafter focus on the analysis on LILRBs in this study.

Next we attempted to detect the cell-surface expression of LILRB1–B4 on immature osteoclasts, POCs, by flow cytometry (Fig. 1B). Fully matured MOCs in culture were not suitable for this study because they were too large for the instrument. The expression levels of LILRB1, LILRB2, and LILRB3 on POCs were comparable to those on monocytes and macrophages. It is interesting that the LILRB4 levels on POCs were higher than those on monocytes and macrophages. Collectively, the flow cytometric analyses revealed that LILRB1, B2, B3, and B4 were distinctly expressed on the surface of POCs, suggesting the inhibitory roles of these receptors.

**Tyrosine phosphorylation of LILRB during osteoclastogenesis**

To further characterize the LILRB protein expression in osteoclasts, we examined their tyrosine phosphorylation profile during osteoclastogenesis by means of immunoblot analysis (Fig. 2A). Cellular lysates of human monocytes, macrophages, POCs, and MOCs were subjected to immunoprecipitation (IP) with LILRB mAbs. The immunoprecipitates were separated by SDS-PAGE and immunoblotted (IB) with anti-phosphotyrosine (PY) (4G10), anti-SHP-1, and anti-LILR mAbs. Representative data are shown from three separate experiments with similar results, each of which employed the set of myeloid-lineage cells prepared individually from three donors. B and C, Down-regulation of osteoclastogenesis by LILRB stimulation. Human CD14+ monocytes were plated on dishes coated with F(ab')2 of LILRB1, LILRB3, LILRB4, or the isotype control murine IgG at the concentration of 20 μg/ml overnight at 4°C. After cell culture with RANKL and M-CSF for 7 days, TRAP staining was performed (B) and TRAP-MNCs (n > 3) were counted (C). Representative data are shown from three separate experiments with similar results, each of which employed the set of myeloid-lineage cells prepared individually from three donors.

**FIGURE 2.** LILRBs show constitutive tyrosine phosphorylation and association with SHP-1 and down-regulate osteoclast differentiation. A, Cellular lysates of human monocytes (Mono), macrophages (Mφ), POCs, and MOCs were subjected to immunoprecipitation (IP) with LILRB mAbs. The immunoprecipitates were separated by SDS-PAGE and immunoblotted (IB) with anti-phosphotyrosine (PY) (4G10), anti-SHP-1, and anti-LILR mAbs. Representative data are shown from three separate experiments with similar results, each of which employed the set of myeloid-lineage cells prepared individually from three donors. B and C, Down-regulation of osteoclastogenesis by LILRB stimulation. Human CD14+ monocytes were plated on dishes coated with F(ab')2 of LILRB1, LILRB3, LILRB4, or the isotype control murine IgG at the concentration of 20 μg/ml overnight at 4°C. After cell culture with RANKL and M-CSF for 7 days, TRAP staining was performed (B) and TRAP-MNCs (n > 3) were counted (C). Representative data are shown from three separate experiments with similar results, each of which employed the set of myeloid-lineage cells prepared individually from three donors.
receptor phosphotyrosylation. We failed to detect any recruitment of SHP-2 or SHIP to these LILRB proteins in each lineage of cells (data not shown). Thus, we concluded that LILRB1, B2, B3, and B4 in POCs and MOCs were constitutively tyrosine phosphorylated and were constitutively associated with SHP-1.

**FIGURE 3.** cis-Association between MHC class I molecules and LILRB1 or LILRB2 on POCs. A, Colocalization of either LILRB1 or LILRB2 and HLA-A/B/C, as seen on FRET analysis. From left to right, excited donor (Alexa Fluor 546-conjugated mAbs to LILRB1 (GHI/75), LILRB2 (42D1), LILRB3 (222821), LILRB4 (ZM3.8), and β2m (TU99)) images (green), excited acceptor (Alexa Fluor 647-conjugated HLA-A/B/C mAb (G46-2.6)) images (red), FRET signal images (pseudocolor), and differential interference contrast (DIC) images are shown. FRET images indicate the cis-association between LILRB1 or β2m and HLA-A/B/C. Red to blue color indicators represent strong to weak FRET. B, Energy transfer between different pairs was detected as the increase in donor fluorescence after acceptor photobleaching. FRET efficiency (%) = (1 − fluorescence intensity of prebleach donor cells/fluorescence intensity of postbleach donor cells) × 100. FRET efficiency >5% was defined as the threshold level for substantial transfer efficiency. Data are the means ± SEM for at least 25 POCs prepared from one donor. *: FRET effect considered to be significant.

**FIGURE 4.** PIR-B in osteoclasts shows constitutive tyrosine phosphorylation and association with SHP-1. A, Expression of PIR-B on osteoclasts but not on osteoblasts. Flow cytometric analysis of cell-surface PIR-A and PIR-B expression on macrophages (Mφ), POCs, and cultured osteoblasts from B6 (black lines) and Pirb−/− (gray lines) mice, stained with anti-PIR-A/B mAb (6C1). Gray lines indicate PIR-A expression. The mean fluorescence intensity for each peak for macrophages and POCs is shown in the panels. In osteoblasts, PIR-A/B expression was not detected. B and C, Constitutive tyrosine phosphorylation of, and SHP-1 association with, PIR-B in osteoclasts. Cellular lysates of murine macrophages (Mφ), POCs, and MOCs were subjected to immunoprecipitation (IP) with anti-PIR-A/B mAb. The immunoprecipitates were separated by SDS-PAGE and immunoblotted (IB) with anti-phosphotyrosine (PY) mAb (4G10) followed by anti-PIR-A/B blotting (B) or anti-SHP-1 blot (C). PIR-B (~130 kDa) is constitutively tyrosine phosphorylated and is recruiting SHP-1 in POCs and MOCs.
Down-modulation of osteoclast differentiation by LILRB aggregation

Constitutive phosphotyrosylation of LILRB1, B2, B3, and B4, and their association with SHP-1 during osteoclastogenesis, prompted us to examine whether osteoclast development in vitro might be suppressed by forced stimulation of LILRB molecules on monocytes. We coated culture dishes with F(ab’)_2 of each LILRB mAb and then plated CD14/monocytes and cultured them in the presence of RANKL and M-CSF. As shown in Fig. 2, stimulation of LILRB1, LILRB3, and LILRB4 significantly inhibited the development of MOCs compared with that with the isotype control mAbs. LILRB4 aggregation caused the most significant inhibition of the formation of TRAP/MOCs (Fig. 2, B and C). However, this LILRB stimulation did not cause any impairment of the cytoskeletal structure when examined by F-actin staining (data not shown). Thus, it was suggested that LILRB1, B3, and B4 could have the potential to inhibit osteoclastogenesis upon interaction with their cognate ligands in vitro. Thus far, our attempts to demonstrate a possible inhibitory role of LILRB2 in osteoclastogenesis were not successful, possibly due mainly to a limited variety of LILRB2-specific mAbs (data not shown).

cis-Binding between MHC class I molecules and LILRB1 and LILRB2 on the POC cell surface

The possible LILRB-mediated regulation of osteoclastogenesis suggests a crucial role of the interaction with their cognate ligands. LILRB1 and LILRB2, as well as their murine counterpart PIR-B, are known to recognize a broad range of classical and nonclassical MHC class I molecules (35, 36), while neither LILRB3 nor LILRB4 binds MHC class I molecules (37). Recently, we verified that human LILRB2 can bind HLA-A/B/C on the surface, namely cis-binding, of the same human basophilic leukemia cells, KU812, on confocal microscopy and FRET (38). Likewise, we also demonstrated that murine PIR-B recognizes H-2Kb/Db in cis on the same mast cell surface (38). These preceding observations led us to examine whether cis-interaction between LILRB1 or LILRB2 and MHC class I molecules could be demonstrated on osteoclast precursor cells. To obtain any evidence for cis-binding between LILRB1 or LILRB2 and MHC class I molecules, we performed FRET analysis with each Alexa 546-conjugated anti-LILRB mAb as an energy donor and Alexa 647-conjugated anti-HLA-A/B/C mAb as an acceptor, since the efficiency of the FRET from donor to acceptor represents a very sensitive indicator of the molecular colocalization on a scale of 2–10 nm (39). Fig. 3 summarizes the donor-acceptor FRET effect observed on the confocal laser microscopy. When POCs were doubly stained with Alexa 546-conjugated anti-LILRB mAb and then excited with a 532-nm laser, we detected strong FRET from the excited LILRB1 and LILRB2 mAb signal to the HLA-A/B/C mAb signal (7.5 ± 0.6 and 12.5 ± 0.7%, respectively). As a positive control, β2m showed strong energy transfer with HLA-A/B/C (12.0 ± 1.2%). In contrast, negative control

FIGURE 5. PIR-B-deficient osteoclasts exhibit up-regulated differentiation and enhanced bone resorption in vitro. A, Bone marrow-derived monocyte/macrophage progenitors (BMMs) derived from B6 and Pirb⁻/⁻ mice were cultured in the presence of 0, 10, or 100 ng/ml RANKL and 20 ng/ml M-CSF for 7 days followed by TRAP staining. B, Number of TRAP⁺ multinuclear cells developed from BMMs of B6 and Pirb⁻/⁻ mice. After BMM culture, TRAP⁺ multinucleated (n > 3) cells were counted. Data are the means ± SD of triplicate determinations and are representative of three independent experiments with similar results. C, BMMs derived from B6 and Pirb⁻/⁻ mice were cultured on calcium phosphate matrices in the presence of 0, 10, or 100 ng/ml RANKL and 20 ng/ml M-CSF. The percentage resorption pit area was measured as described in Materials and Methods. Data are the means ± SD of triplicate determinations and are representative of three independent experiments with similar results.
LILRB3 and LILRB4 did not show a significant energy transfer with HLA-A/B/C on POCs (2.3 ± 0.4% and 3.5 ± 0.4%, respectively). These findings strongly suggest that LILRB1 and LILRB2, but not LILRB3 or LILRB4, can associate with MHC class I molecules in cis on the POC surface, as was shown for LILRB2 binding to MHC class I molecules on a basophilic leukemia cell line (38).

Deletion of PIR-B leads to accelerated osteoclastogenesis in vitro

To circumvent the lack of a suitable model system for verifying any physiological role of LILRB in bone remodeling, we utilized gene-targeted mice lacking PIR-B (31), the murine counterpart of any physiological role of LILRB in bone remodeling, we utilized To circumvent the lack of a suitable model system for verifying in vitro

Deletion of PIR-B leads to accelerated osteoclastogenesis in vitro

Next, samples of PIR-B-sufficient or -deficient macrophages, POCs, and MOCs were immunoblotted with Abs against PIR, phosphotyrosine, and SHP-1 (Fig. 4A). As was the case for human LILRBs (Fig. 2A), PIR-B was constitutively tyrosine phosphorylated in POCs and MOCs, as was the case for murine B cells and other myeloid cells (36, 24, 40). We detected constitutive SHP-1 association with PIR-B in POCs and MOCs (Fig. 4C).

We then cultured bone marrow cells from PIR-B-deficient or -sufficient mice in the presence of M-CSF and RANKL and compared the osteoclastogenesis. As shown in Fig. 5, A and B, MOC development was significantly accelerated in PIR-B-deficient cells in the presence of either a lower (10 ng/ml) or higher (100 ng/ml) concentration of RANKL. The resorption pit area produced on calcium phosphate matrices was also larger for the culture of PIR-B-deficient MOCs than for PIR-B-sufficient B6 mice (Fig. 5, C and D), indicating that PIR-B deletion may lead to accelerated bone resorption primarily due to increased osteoclasts. These analyses indicate that murine osteoclastogenesis in the presence of RANKL and M-CSF in vitro is also negatively regulated by PIR-B, as was the case for human LILRB1, B3, and B4 described above.

cis-Association between MHC class I molecules and PIR-B on the murine POC cell surface

The finding of cis-binding of LILRB1 to MHC class I molecules on the same POC cell surface shown in Fig. 3 led us to examine whether the murine LILRB1/B2 counterpart PIR-B could also bind MHC class I molecules on the same POC surface in cis. Again, we performed FRET analysis with Alexa 546-conjugated anti-PIR-A/B mAb as an energy donor and Alexa 647-conjugated anti-H-2Kb/Db mAb as an acceptor. Fig. 6A shows that a FRET effect was clearly detected for PIR-B and H-2Kb/Db as well as for PIR-A/B and H-2Kb/Db. A negative control, SIRPα, a receptor for CD47, did not show any significant FRET effect with H-2Kb/Db, while a positive control, β2m, yielded distinct FRET with H-2Kb/Db, confirming that the FRET analysis was valid. Fig. 6B summarizes the FRET effect for each donor-acceptor combination. These findings strongly suggest that PIR-B can bind to MHC class I molecules in cis on the POC surface, as was the case for LILRB1 and LILRB2 on POCs (Fig. 3) and for PIR-B on the mast cell surface (38).

PIR-B-deficient mice are not osteoporotic

Finally, we compared bone histology and bone morphometric parameters of the tibia of B6 and Pirb−/− mice at various ages. We
anticipated a decreased bone mass, namely osteoporosis, in PIR-B deficiency, as was observed for SHP-1-mutant viable motheaten (me+/me+) mice (14); unexpectedly, however, we failed to demonstrate any osteoporosis on either bone histology at 1.5 mo of age (Fig. 7A) or micro CT analyses at 3 mo of age (Fig. 7B), a significant decrease in bone volume, or an increase in osteoclast number or their surface area, or in bone resorption rate in Pirb−/− mice at any age examined (Fig. 7C). Osteoblastic parameters of the tibia of Pirb−/− mice also showed no significant alterations in osteoblast function in terms of osteoblast surface area, bone formation rate, osteoid thickness, or mineral apposition rate (Fig. 7D). These results indicate that the deletion of PIR-B does not significantly alter either the osteoclast or osteoblast function in vivo, at least up to 6 mo of age.

**Discussion**

LILRB1 and LILRB2 are human orthologs of murine PIR-B because of the similarity in their chromosomal localization, overall
structure, and ligand-binding specificities (36, 41). Murine homologs or orthologs of human LILRB3 and LILRBB4 have not been identified. In this study, we demonstrated that human LILRB1, B2, B3, and B4 are expressed on cultured osteoclast precursor cells derived from peripheral blood monocytes, and that LILRB1/B3/B4 could be inhibitory for osteoclast development in the presence of RANKL and M-CSF. We also showed that one of the inhibitory mechanisms could comprise the constitutive cis-binding of LILRB1 to MHC class I molecules on the same osteoclast precursor cells, leading to the constitutive recruitment of SHP-1. A role of LILRB3 and LILRB4 was also shown to be down-modulation of osteoclast development through their association with SHP-1, as in the case of LILRB1, but we could not analyze possible mechanisms further due to the lack of information on their ligands.

In contrast to the distinct expression of LILRB1, B2, B3, and B4 on the osteoclast surface, expression of the activating isoforms, LILRAs, was not prominent, at least in their mRNA levels. On microarray analysis, the tendency was observed that the expression of inhibiting LILRBs dominated that of activating LILRAs concomitant with differentiation into osteoclasts as well as into macrophages from monocyctic cells. The dominant expression of LILRAs over LILRBs may be required for proper regulation of osteoclast development. Additionally, these observations may indicate a putative role of activating LILRAs in the enhancement of osteoclast development, as demonstrated by the accelerated osteoclastogenesis upon stimulation of murine receptors, including OSCAR (osteoclast-associated receptor), PIR-A, TREM-2 (triggering receptor expressed on myeloid cells 2), and SIRPβ1, with specific Abs (7), albeit that the physiological ligands have not been identified for LILRAs other than LILRA1, whose binding to HLA-B27 has been observed (42).

The inhibitory role of LILRB1 in osteoclastogenesis was also shown by the murine cell system in which osteoclast development was accelerated among cells from mice deficient in LILRB1/B2 ortholog PIR-B. However, bone tissues from Pirb−/− mice were normal; namely, there was no apparent modulation in osteoclast number or function, at least up to 6 mo of age, as assessed by bone morphometric parameters. It is possible that we can identify the direct effect of PIR-B deletion on osteoclastogenesis in vitro, while in an in vivo setting we might fail to detect enhanced osteoclast development, possibly due to the involvement of other physiological factors such as other inhibitory receptors not identified yet. In this context, we have already noted the mRNA expression of a SHIP-utilizing inhibitory Ig-like receptor, FcγRIIB, in osteoclast precursor cells and mature osteoclasts (7). Likewise, a family of cell-surface Ig-like proteins termed CMRF-35-like molecules (CLM) includes an ITIM-harboring inhibitory isoform, CLM-1 (43). It is noteworthy that forced expression of CLM-1 on osteoclastogenic cell line RAW264.3 prevents its development into osteoclasts and activation, although CLM-1 did not show substantial expression on osteoclasts (44). These and other regulatory Ig-like receptors on myeloid-lineage cells might also be critical for osteoclastogenesis by regulating intracellular signals upon their interaction with cognate ligands expressed on the osteoclast precursor cells themselves or surrounding stromal cells including osteoblasts. In the immune system, it has already been shown that these inhibitory Ig-like receptors recruit SHIP or SHP-1 upon tyrosine phosphorylation of their ITIM in the intracellular portion. These regulatory receptors could compensate for the effect of PIR-B deficiency in vivo.

In clinical settings, LILRB1 was found to be highly polymorphic and associated with susceptibility to rheumatoid arthritis in HLA-DRB1-shared epitope-negative subjects (45). On the other hand, LILRB4 polymorphisms have been screened for their possible association with atopy, but no apparent association was detected (46). The association of either LILRB3 or LILRB4 with bone disorders, such as rheumatoid arthritis or bone metabolic diseases, including osteoporosis and osteoporosis, has not been reported so far. Our present study immediately raises the question of whether any polymorphisms in LILRB1, B2, B3, or B4 are linked with any bone disorders. Examination of these polymorphisms and their association with immune and osseous disorders will increase our understanding of the physiological roles of LILRBs. In particular, LILRB4 could be a novel therapeutic target for inflammatory bone diseases and osteoclastogenic disorders because of its relatively preferential expression on osteoclast precursor cells as well as its significant inhibitory role in differentiation into mature osteoclasts.

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Disclosures
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References


