Chapter 4
Inhibitory regulation of osteoclast bone resorption by SIRPα

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Abstract

Osteoclasts mediate bone resorption, which is critical for bone development, maintenance and repair. Proper control of osteoclast development and function is important and deregulation of these processes may lead to bone disease, such as osteoporosis. Previous studies have shown that the cytosolic protein tyrosine phosphatase SHP-1 acts as a suppressor of osteoclast differentiation and function, but putative inhibitory receptors that mediate recruitment and activation of SHP-1 in osteoclasts have remained unknown. In the present study, we identify the SHP-1-recruiting inhibitory immunoreceptor SIRPα as a negative regulator of osteoclast activity. SIRPα is expressed by osteoclasts, and osteoclasts from mice lacking the SIRPα cytoplasmic tail and signaling capacity display enhanced bone resorption in vitro. Consequently, SIRPα-mutant mice have a significantly reduced cortical bone mass. Furthermore, osteoclasts from SIRPα-mutant mice show an enhanced formation of actin rings, known to be instrumental in bone resorption. SIRPα mutation did not significantly affect osteoclast formation, implying that the role of SIRPα was limited to the regulation of mature osteoclast function. This identifies SIRPα as a bona fide inhibitory receptor that regulates the bone resorption activity and supports a concept in which osteoclast function is balanced by the signaling activities of activating and inhibitory immunoreceptors.
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Introduction

Osteoclasts are multinucleated cells from the macrophage-lineage that are highly specialized in bone resorption and as such play a pivotal role in normal bone development, turnover and repair. Deregulation of osteoclast differentiation and function may lead to pathological conditions such as osteoporosis or osteopetrosis and therefore both the formation and the activity of osteoclasts need to be tightly controlled. The development of osteoclasts involves the differentiation and fusion of myeloid precursor cells into mature bone-resorbing osteoclasts, and this is driven by the action of receptor activator of NF-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF)\(^1^\).  

The immunoreceptor tyrosine-based activation motif (ITAM) is a highly conserved region in the cytoplasmic domain of signaling chains of adapter proteins and receptors and is a critical mediator of intracellular signals. ITAM signaling is required for the differentiation and function of B and T cells in adaptive immunity and regulates the function of innate immune cells, including natural killer cells, and myeloid cells such as macrophages, neutrophils and dendritic cells. Recent studies have demonstrated that ITAM adapter proteins are also involved in the formation and function of osteoclasts. Mice deficient in both of the ITAM adapter proteins DNAX-activating protein (DAP)\(^1\) and Fc receptor γ-chain (FcR\(γ\)) are osteopetrotic owing to impaired osteoclast formation and bone resorption\(^4^\). Consistent with this, mice deficient in the tyrosine kinases Syk and c-Src, acting downstream of DAP12 and FcR\(γ\), develop a severe osteopetrosis, which is apparently due to a diminished bone resorption\(^6\). Furthermore, triggering of DAP12 and FcR\(γ\) associated activating immunoreceptors, such as TREM-2 (triggering receptors expressed on myeloid cells-2), PIR-A (paired immunoglobulin-like receptor-A), OSCAR (osteoclast-associated receptor) and SIRP\(β\)\(^1\) (signal regulatory protein beta 1) enhanced osteoclastogenesis and promoted bone resorption, suggesting that activating immunoreceptor signaling plays a regulatory role in osteoclast formation and function\(^5;9\). 

In the immune system, ITAM receptor signaling is generally counterbalanced by inhibitory receptors that recruit cytosolic Src homology-2-containing protein tyrosine phosphatases (SH2-PTPases), such as SHP-1 and/or SHP-2, via cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Interestingly, \textit{motheaten} mice with a deficiency or loss-of-function mutation in SHP-1, which constitutes the major cytosolic SH2-PTPase in hematopoietic cells, develop a severe osteoporosis, which is associated with an increase of osteoclast differentiation and bone resorption, indicating that SHP-1 controls osteoclast formation and activity\(^10^\). It is therefore anticipated that osteoclasts express inhibitory receptors that, through recruitment and activation of SHP-1, inhibits osteoclast formation and/or activation. However, until now the relevant inhibitory receptors on osteoclasts have not been defined. 

Signal regulatory protein alpha (SIRP\(α\)) is an inhibitory immunoreceptor that contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which upon ligand binding become phosphorylated, and mediate the recruitment and activation of the cytosolic tyrosine phosphatases (PTPases) SHP-1 and SHP-2\(^13\). The extracellular
region of SIRPα is composed of immunoglobulin (Ig)-like domains, which share a particularly close structural similarity to that of TcR and BcR (B cell receptor) chains\textsuperscript{17,18}. SIRPα is predominantly expressed by myeloid and neuronal cells\textsuperscript{19-21}. The broadly expressed CD47 surface receptor acts as a cellular ligand for SIRPα and has been proposed to act as a signal of ‘self’\textsuperscript{20,22-24}, that by interacting with SIRPα controls cellular effector functions, including growth, differentiation, adhesion transendothelial migration of monocytes and granulocytes and migration of Langerhans cells\textsuperscript{25,26}. A role for SIRPα in osteoclast differentiation and/or function has not been established. Of relevance, \textit{in vitro} studies have suggested that CD47-SIRPα interactions may play a role in the fusion of macrophage leading to multinucleated cell formation, as occurs also during osteoclastogenesis\textsuperscript{27-29}.

In the present study, we have investigated the involvement of SIRPα signaling in osteoclast differentiation and activation, employing mice that have a defect in SIRPα signaling\textsuperscript{30}. Our results show that SIRPα-mutant mice have significantly decreased cortical bone mass, in spite of an apparently normal osteoclast development \textit{in vivo} and \textit{in vitro}. Furthermore, osteoclasts generated from these mice show an increased bone resorption capacity \textit{in vitro}. This is associated with an enhanced formation of actin rings, which are known to be important for proper bone degradation. This identifies SIRPα as an inhibitory receptor that selectively controls osteoclast function.

\textbf{Materials and methods}

\textit{Mice}

C57BL/6 mice with a targeted deletion of the SIRPα cytoplasmic region have been described previously\textsuperscript{30}. The mice that were originally generated onto the 129/Sv background had been backcrossed onto the C57BL/6 mice for ten generations. Wild type C57BL/6 mice of the same genetic background were maintained together with the SIRPα-mutant mice in the breeding facility of the Vrije Universiteit Medical Center. Mice were kept in specifically pathogen free conditions according to FELASA recommendations and used at 6 weeks of age. Permission for animal experiments described here was obtained from the Animal Welfare committee of the Vrije Universiteit.

\textit{Bone marrow isolation}

Bone marrow cells were isolated as described before\textsuperscript{31}. The cells were resuspended through a 21-gauge needle and filtered over a 100 µm pore size Cell Strainer filter (Falcon/Becton Dickinson, Franklin Lakes, NJ) and kept on ice in culture medium [\(\alpha\)-Minimal Essential Medium (Gibco BRL, Paisley, Scotland)] supplemented with 5% fetal calf serum (HyClone, Logan UT), 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (antibiotic antmyotic solution Sigma, St. Louis, MO) until further use.
**Bone marrow cell populations**

Various hematopoietic cell populations in the bone marrow, including granulocytes, monocytes, immature myeloid cells, lymphoid cells, erythroid cells and early blasts were identified as described by double staining with biotinylated ER-MP12 mAb (against CD31) and FITC-labeled ER-MP20 mAb (against Ly-6C) (generously provided by Dr P. Leenen, Department of Immunology, Erasmus University Medical Center, Rotterdam, The Netherlands). After 1 hour incubation at 4°C, cells were washed three times with PBS-0.1% BSA. ER-MP12 binding was detected using Alexa 633-streptavidin conjugate (Molecular Probes, Eugen, OR) for 30 min at 4°C. After three washes with PBS-0.1%BSA, cells were analyzed on a FACS Calibur (BD Biosciences, San Jose, CA) using Cell Quest™ software.

**Osteoclastogenesis**

Bone marrow cells were plated in 96-well flat bottom tissue culture treated plates (Costar, Cambridge, MA) at a density of 1.0x10^5 cells per well in 150 µl culture medium containing 30 ng/ml recombinant murine M-CSF (R&D systems, Minneapolis, MI) and 20 ng/ml recombinant murine RANKL (RANKL-TEC, R&D systems, Minneapolis, MI). Cells were cultured on 650 µm thick bovine cortical bone slices or on plastic. Culture media were replaced every 3 days. After either 5 days, in case of TRACP staining, or 6 days for the analysis of bone resorption, wells were washed with PBS and either fixed in 4% PBS buffered formaldehyde and stored at 4°C (for TRACP staining) or in water (for bone resorption) respectively. TRACP staining was performed according to manufacturer's instruction (Sigma Aldrich, St Louis, MO).

**RNA analysis and real-time quantitative PCR**

RNA isolation and real-time quantitative PCR was performed as described in detail. Real time PCR primers were designed using the Primer Express software, version 2.0 (Applied Biosystems, Foster City, Ca, USA) (Table 1). To avoid amplification of genomic DNA, each amplicon spanned at least one intron. To test for PCR efficiencies, one of the osteoclast samples was used to generate a standard curve for all the genes studied. The PCR reactions of the different amplicons had equal efficiencies. Porphobilinogen deaminase (PBGD) was used as the housekeeping gene. Expression of this gene was not affected by any of the experimental conditions. Samples were normalized for the expression of PBGD by calculating the ΔCt (Ct of gene of interest – Ct of PBGD) and expression of the different genes is expressed as 2^(-ΔCt).

**Bone resorption**

Bone resorption was visualized and quantified as previously described with minor adaptations. Cells were removed with 0.25 M NH₄OH for 30 minutes. The slices were washed in distilled water, incubated in a saturated alum (KAl(SO₄)₂·12H₂O) solution for 10 minutes, washed in distilled water and stained with Coomassie Brilliant blue. The surface areas of individual resorption pits were measured using Image-Pro Plus software (MediaCybernetics, Silver Spring, MD).
**Immunoprecipitation and western blot analysis**

For immunoprecipitation of SIRPα, bone marrow cells were plated in 48-well flat bottom tissue culture treated plates (Costar, Cambridge, MA) at a density of 2.5x10^5 cells per well in 400 µl culture medium containing 30 ng/ml recombinant murine M-CSF (R&D systems) and 20 ng/ml recombinant murine RANKL (RANKL-TEC, R&D systems). Culture media were replaced every 3 days. After 6 days 1 mM sodium orthovanadate (Na_3VO_4, New England Biolabs, Leusden, The Netherlands) was added to culture medium where indicated. After incubation for 30 minutes at 37°C, 5% CO_2 cells were washed twice with ice-cold PBS supplemented with 1 mM Na_3VO_4. Subsequently, cells were lysed with Igepal lysis buffer (Sigma) containing 1 mM Na_3VO_4 and protease inhibitor cocktail (Roche) for 1 hour at 4°C. The lysates were clarified by centrifugation and precleared by incubation with protein G-Sepharose beads (Amersham Biosciences, Uppsala, Sweden) for 1 hour. SIRPα was immunoprecipitated by addition of rat anti-mouse SIRPα (clone P84, IgG1, BD Pharmingen, San Diego, CA) bound to protein G-Sepharose beads for 1 hour at 4°C. Beads were washed with Igepal lysis buffer containing 1 mM Na_3VO_4 and protease inhibitor cocktail and resuspended in SDS sample buffer. Western blot analysis was performed with anti-SHP-1 (SH-PTP1, rabbit polyclonal antibody, Santa Cruz Biotechnology, Heidelberg, Germany) and anti-SIRPα (Ab8120, Rabbit polyclonal, Abcam, Cambridge, United Kingdom) antibodies, followed by HRP-linked secondary antibodies. Proteins were detected with Supersignal West Dura (Pierce, Rockford, IL, USA) and a Gelimager (Epi Chemi II Darkroom combined with a 12-bit SensiCam charged-coupled device camera driven by Labworks 4.0 (UVP, Inc., Upland, CA, USA).
**MicroCT imaging**
Three-dimensional reconstructions of trabecular and cortical bone of the tibiae were generated by employing a high-resolution microCT system (μCT 40; Scanco Medical AG, Brüttisellen, Switzerland). Tibiae of 6 male wild type and 6 male SIRPα-mutant mice, all of which were 42 days old, were mounted in a cylindrical specimen holder (polyetherimide; 20.5 mm outer diameter; wall thickness, 0.7 mm) to be captured in a single scan. They were secured with synthetic foam and were completely submerged in deionized water. Scans with an isotropic resolution of 10 μm were made employing a 70 kV peak-voltage X-ray beam. The computed linear attenuation coefficient of the X-ray beam in each volume element (voxel) was stored in an attenuation map and represented by a grey value in the reconstruction. Specific volumes of interest (VOIs) were selected. To analyze trabecular bone a region of 5% of the tibiae length above and under the metaphysis was evaluated. Cortical bone analysis was performed in the region between 45% until 55% along the length of the tibiae. To discriminate between bone and background, the reconstructions were segmented using an adaptive threshold determination procedure as previously described. All bone regions were segmented using the same threshold for comparison purposes. Multiple cortical and trabecular bone parameters were determined using morphometric software supplied by the manufacturer. For trabecular bone: BV/TV: bone volume fraction, Tb.N: trabecular number, Tb.Th: trabecular thickness, Tb.Sp: trabecular separation, and Conn.D: connectivity density. For cortical bone, cortical thickness (Ct.Th) and second moment of inertia (MOI) were determined.

**Histology**
Tibiae of 42 days old wild type and SIRPα-mutant mice were fixed for 24 h at room temperature in 4% formaldehyde and 1% glutaraldehyde, buffered in 0.1M sodium cacodylate buffer (pH 7.4). Next, tibiae were decalcified for two weeks in a solution containing 10% EDTA, 1% formaldehyde and 1% glutaraldehyde, which was replaced twice a week. Fixed specimens were dehydrated through a series of ethanol and embedded in Epon 812 (TAAB laboratories, Aldermaston, United Kingdom). Semi-thin sections (1-2 μm) were cut and stained with methylene blue. The number of osteoclasts per mm trabecular surface area was determined using QWin-software (Leica, Wetzlar, Germany).

**Confocal microscopy**
Bone marrow cells were seeded on cortical bone slices and allowed to mature for 5.5 days in the presence of M-CSF and RANKL as described above. Slices were washed in PBS and fixed in acetone at -20°C for 4 minutes, and subsequently dried at room temperature. Non-specific binding to cells was blocked for 30 minutes with 10% normal goat serum followed by an overnight incubation at 4°C with rat anti-mouse SIRPα (clone P84, IgG1, BD Pharmingen, San Diego, CA)⁴⁷. After three washes with PBS the slides were incubated with an Alexa 488-conjugated goat anti-rat IgG antibody
(Molecular Probes). Rat anti-mouse CD25 (clone PC61.5.3, IgG1)\(^{39}\) served as an isotype-matched negative control. F-actin was stained according to a method described previously using Alexa 488-phalloidin (Molecular Probes)\(^{40}\). Nuclei were stained with propidium iodide (Sigma). Image stacks were generated using confocal laser scanning microscopy (Leica, Wetzlar, Germany) using an Argon laser (Alexa 488 and propidium iodide).

**Results**

*Osteoclastogenesis is independent of osteoclast SIRP\(\alpha\) signaling*

The inhibitory receptor-associating PTPase SHP-1 has been demonstrated to play a non-redundant negative role in osteoclast differentiation and activation\(^{10,12}\), but the putative inhibitory immune receptors that mediate SHP-1 recruitment and activation and induce down-stream signaling events in osteoclasts have not been identified. Confocal microscopy was used to study the cellular expression and localization of SIRP\(\alpha\) in osteoclasts that were generated from murine bone marrow cells and cultured on cortical bone slices in the presence of M-CSF and RANKL. Consistent with the generalized expression of SIRP\(\alpha\) among cells of the myeloid lineage, a plasma membrane and diffuse cytoplasmic staining was observed for SIRP\(\alpha\) in both multinucleated osteoclasts as well as in mononuclear osteoclast precursors (Figure 1A). The SIRP\(\alpha\) cytoplasmic tail contains ITIM motifs that are responsible for the recruitment of the cytosolic tyrosine phosphatase SHP-1\(^{15}\). In order to evaluate the binding of SHP-1 to SIRP\(\alpha\) in osteoclasts we performed an immunoprecipitation experiment. As shown by SIRP\(\alpha\) immunoprecipitation and Western blotting with a SHP-1-specific antibody (Figure 1B), SHP-1 associates constitutively with SIRP\(\alpha\) in osteoclasts at least under the *in vitro* conditions employed here.

To investigate a direct role for SIRP\(\alpha\) in osteoclast differentiation and activity, we performed studies in SIRP\(\alpha\)-mutant mice. These mice, which were described previously\(^{30}\), lack most of the cytoplasmic region of SIRP\(\alpha\) including all of the ITIMs and the proline-rich domains implicated in signaling.

Firstly, we studied osteoclast formation *in vitro* by culturing bone marrow cells from wild type and SIRP\(\alpha\)-mutant mice in the presence of M-CSF and RANKL. Notably, no difference in bone marrow composition was observed with respect to the relative numbers of granulocytes, monocytes, immature myeloid cells, lymphoid cells, erythroid cells and early blasts, that were identified on the basis of CD31 (ER-MP12) and Ly-6C (ER-MP20) expression\(^{32}\) (Figure 1C and D). After differentiation for 6 days in the presence of M-CSF and RANKL, multinucleated osteoclasts were identified after staining for TRACP (tartrate-resistant acid phosphatase) activity and DAPI. No significant differences in the formation and differentiation of mature osteoclasts were found between the cultures from SIRP\(\alpha\)-mutant and wild type mice, regardless whether the cells had been cultured on cortical bone slices (Figure 1) or on plastic (Figure 2). In particular, there were no differences in the average numbers of nuclei in multinucleated osteoclasts (Figure 1E and F, and figure 2A and B), indicating that SIRP\(\alpha\) signaling is
Figure 1. SIRPα signaling is not required for osteoclastogenesis. (A) Osteoclasts generated from bone marrow cells of wild type mice by culturing for 5.5 days in the presence of M-CSF and RANKL and seeded on cortical bone slides express SIRPα. Abundant SIRPα was seen both in mononuclear (arrow heads) and multinucleated (arrows) cells. No staining was observed with the isotype-matched negative control (rat anti-mouse CD25). The cells were fixed and stained with the anti-SIRPα monoclonal antibody (clone p84, rat IgG) or rat anti-mouse CD25 (clone PC61.5.3, IgG1) and Alexa 488 (green) conjugated goat anti-rat IgG antibody and evaluated by confocal microscopy. Nuclei were visualized by propidium iodide (red) staining. Bar = 100 μm. (B) Osteoclasts derived from bone marrow cells of the wild type mice and cultured on plastic in the presence of M-CSF and RANKL. After 30 minutes incubation with culture medium supplemented with Na3VO4, cells were lysed and cellular lysate was subjected to immunoprecipitation (IP) with anti-SIRPα (clone P84, rat IgG) coupled to G-Sepharose beads. Western blot analysis was performed with antibodies against SHP-1 (SHPTP1, rabbit polyclonal antibody) and anti-SIRPα (Ab8120, Rabbit polyclonal). The result
clearly shows association of SIRPα with SHP-1. (C) Bone marrow cells of 6 weeks old male control and SIRPα-mutant mice were subjected to flow-cytometric analysis with antibodies against CD31 (ER-MP12) and Ly-6C (ER-MP20). Flow cytometric dot-plot of ER-MP12/20 labeled bone marrow cells. (D) Six different cell populations, including granulocytes, monocytes, immature myeloid cells, lymphoid cells, erythroid cells and early blasts could be distinguished. No difference was found in bone marrow composition between wild type and SIRPα-mutant mice based on the percentage of the variant subpopulations. Data are presented as mean ± SEM. (E) Osteoclasts, generated from bone marrow cells of wild type and SIRPα-mutant mice and cultured on cortical bone slides in the presence of M-CSF and RANKL for 5 days, were stained for TRACP, a specific enzyme marker of osteoclasts. Nuclei were visualized with DAPI. Note that mononuclear, binuclear and multinucleated cells (arrow) are present in both cultures. Bar = 100 µm. (F) Number of multinucleated (> 3 nuclei) cells per cm² per 10⁵ bone marrow cells originally seeded. Data are expressed as mean ± SEM of two independent experiments (both wild type and SIRPα-mutant mice n=5). No differences were seen in the number of osteoclasts, generated from the bone marrow of both wild type and SIRPα-mutant mice. (G) The cultured osteoclasts were analyzed by qPCR for the osteoclast markers DC-STAMP, calcitonin receptor, cathepsin K and TRACP. The abundance of mRNA relative to that of PBGD is shown. No differences were found between wild type and SIRPα-mutant mice. Data are presented as mean ± SEM.

Figure 2. Similar osteoclast formation from wild type and SIRPα-mutant bone marrow on plastic. (A) Osteoclasts, generated from bone marrow cells of wild type and SIRPα-mutant mice and cultured in the presence of M-CSF and RANKL for 5 days, were stained for TRACP. Nuclei were visualized with DAPI. Note that mononuclear, binuclear and multinucleated cells (arrow) are present in both cultures. Bar = 100 µm. (B) Number of multinucleated (> 3 nuclei) cells per cm² per 10⁵ bone marrow originally seeded. Data are presented as mean ± SEM of two independent experiments (both wild type and SIRPα-mutant mice n=5). No differences were found in the number of osteoclasts, generated from the two types of bone marrow. (C) The osteoclasts cultured on plastic were analyzed by qPCR for the osteoclast markers DC-STAMP, calcitonin receptor, cathepsin K and TRACP. The abundance of mRNA relative to that of PBGD is shown. Levels of osteoclast marker genes were similar between wild type and SIRPα-mutant. Data are expressed as mean ± SEM. No differences were found between wild type and SIRPα-mutant mice.
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dispensable for cellular fusion during osteoclastogenesis. This is perhaps somewhat surprising in light of previous studies in which a role for CD47-SIRPα interactions in macrophage fusion was suggested\textsuperscript{27-29}. Furthermore, the expression levels of the osteoclast markers DC-STAMP (dendritic cell-specific transmembrane protein), calcitonin receptor, cathepsin K and TRACP were determined by qPCR (real-time quantitative PCR) and these were unaltered in osteoclasts from SIRPα-mutant mice as compared to the wild type cells (Figure 1G and figure 2C), strongly suggesting a similar degree of osteoclast differentiation. Taken together, these data show that SIRPα is present on osteoclasts, but its signaling does not play a significant role in osteoclast formation and differentiation.

Osteoclast bone resorption is inhibited by SIRPα signaling

To determine whether SIRPα is a critical regulator of osteoclast function, we compared the bone resorption activity of \textit{in vitro} generated bone marrow-derived osteoclasts from wild type and SIRPα-mutant mice cultured on bovine cortical bone slices. Osteoclasts from the SIRPα-mutant mice proved to resorb considerably more bone as compared to those from wild type mice (1.0±0.3% and 3.2±0.5% respectively) (Figure 3A and B). This difference in osteoclast activity was not only reflected in the total amount of bone resorbed, but also by a difference in the average pit size (1.0±0.1mm\textsuperscript{2} and 1.9±0.2mm\textsuperscript{2}, respectively), suggesting that the bone resorption capacity of individual osteoclasts was increased (Figure 3C). These data show that SIRPα-derived signals play a regulatory role in osteoclast function.

\begin{figure}
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\caption{Increased bone resorption in osteoclasts from the SIRPα-mutant mice. Bone marrow cells were cultured on cortical bone slices in the presence of M-CSF and RANKL for 6 days. (A) The slices were stained with Coomassie Brilliant blue. Bar = 100 µm. (B,C) The bone resorption area and pit size were determined using Image-Pro Plus software. Note that both the total resorption area and pit size, reflecting the bone resorbing capacity of individual cells, is enhanced in the SIRPα-mutant mice. Data are expressed as mean ± SEM of two independent experiments (both wild type and SIRPα-mutant mice n=10). (*) p< 0.01}
\end{figure}
Figure 4. Reduced cortical bone mass in SIRPα-mutant mice. Tibiae from 42 days old male wild type and SIRPα-mutant mice were examined by three-dimensional microCT. Multiple cortical and trabecular bone parameters were compared. (A) The cortical thickness at the midshaft of the tibiae was significantly reduced in the SIRPα-mutant mice. Data are expressed as mean ± SEM. (*) p<0.05 (B) Three-dimensional reconstruction of the cortex at the midshaft of the tibiae of wild type and SIRPα-mutant mice. (C) Note that the tibial length is similar for both genotypes. Data are expressed as mean ± SEM. (*) p<0.05 (D) Comparison of the body weight of wild type and SIRPα-mutant mice shows a small (i.e. 13%) but significant reduction in the body weight of the SIRPα-mutant mice. Data are presented as mean ± SEM. (E) Histological staining with methylene blue of the wild type and SIRPα-mutant mice tibiae. Micrograph of osteoclasts along trabecular bone. Bar = 10 µm. (F,G) Note that there are no differences in osteoclasts features, such as the number of osteoclasts per mm trabecular bone and number of nuclei per osteoclast. Data are expressed as mean ± SEM. Figure shown is representative of n=6.
Reduced cortical bone mass in SIRPα-mutant mice
To examine whether the enhanced bone resorption capacity of osteoclasts generated by cells isolated from SIRPα-mutant mice is reflected by a reduced bone mass in vivo, the tibiae of SIRPα-mutant mice and wild type mice were analyzed for a variety of bone parameters using micro-computed tomography (microCT). This demonstrated that the cortical thickness (Ct.Th) of the bone, measured at the midshaft of the tibiae, was significantly less in the SIRPα-mutant mice (0.21±0.016mm and 0.16±0.005mm, respectively) (Figure 4A and B). This reduction occurred in spite of a normal length of the tibiae (Figure 4C). Other parameters for trabecular and cortical bone, including bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular spreading (Tb.Sp), connectivity density (Conn.D) and second moment of inertia (MOI), did not differ significantly between the wild type and SIRPα-mutant mice. Furthermore, the SIRPα-mutant mice displayed a small (i.e. 13%) but significant reduction in their body weight (Figure 4D), a finding that was also reported previously, which may in fact be explained by the reduction in volume of cortical bone, that represents about 75% of the total bone in the body.

To confirm in vivo that the reduced cortical thickness observed in the SIRPα-mutant mice was a result of an enhanced bone resorption and was not caused by a difference in osteoclast number, the tibiae of the wild type and SIRPα-mutant mice were analyzed by histology for the presence and number of osteoclasts (Figure 4E). Apparently, the defect in SIRPα signaling of the mutant mice did not affect the number of osteoclasts per mm of trabecular bone surface (Figure 4F). Also, there was no difference in the average number and distribution of nuclei per osteoclast between the two genotypes (Figure 4G). Finally, there was no significant difference in osteoblast activity and the amount of osteoid as determined at the shaft of the tibiae (Figure 5), suggesting a similar bone formation capacity in SIRPα-mutant and wild type animals.

Collectively, these in vivo results demonstrate that SIRPα-mutant mice have significantly reduced cortical bone mass, in spite of apparently normal osteoclast numbers and normal osteoblast function. This provides further support for the idea that SIRPα signaling plays inhibitory role in osteoclast activity.

Enhanced actin ring formation in the SIRPα-mutant mice
To gain further insight into the mechanism underlying the increased bone resorption by the SIRPα-mutant mice, we focused on actin ring formation in osteoclasts. In bone resorbing osteoclasts these actin rings are an intrinsic part of the adhesive structure, the so-called sealing zone, that isolates the resorptive microenvironment from the general extracellular space and is a prerequisite for efficient bone resorption. Of relevance, signaling by activating immunoreceptors, which is mediated via c-Src and Syk, promotes actin ring formation. We therefore anticipated that SIRPα signaling might affect actin ring formation. Osteoclasts were generated from bone marrow cells on cortical bone slices and stained with fluorescent labeled phalloidin to visualize filamentous actin. In osteoclasts of both mouse strains, actin rings were identified...
flattened shape, no visible Golgi and more condensed nuclear chromatin in contrast to the osteoblasts with low activity. Data are expressed as mean ± SEM. (C) The amount of osteoid at the shaft of the tibiae was not significantly different between wild type and SIRPα-mutant mice. Data are presented as mean ± SEM. Figure shows data obtained from 6 mice of 42 days old.

(Figure 6A), but not all osteoclasts displayed actin rings. In the SIRPα-mutant mice the proportion of osteoclasts with actin rings was significantly higher (Figure 6B). Nearly all mutant osteoclasts contained at least one actin ring, whereas this was 75% of wild type osteoclasts. Moreover, the average number of actin rings per osteoclast was higher in mutant compared to the wild type cells (Figure 6B). Again, in these experiments the average number of nuclei of the osteoclasts analyzed did not differ between the two mouse strains (data not shown). Taken together, these findings indicate that SIRPα inhibits actin ring formation, and this may explain, at least in part, the elevated bone resorption activity of SIRPα-mutant osteoclast.

Discussion

In the present study, we have investigated the involvement of SIRPα, as a prototypic inhibitory receptor, in osteoclast formation and bone resorption by using SIRPα-mutant mice that lack the cytoplasmic domain of the receptor and therefore carry an intrinsic defect in signaling. Our results demonstrate that SIRPα is expressed by osteoclasts and that SIRPα signaling plays a non-redundant regulatory role in the bone resorption activity of osteoclasts. This conclusion is based on the following in vitro and in vivo observations. In vitro generated osteoclasts from the SIRPα-mutant mice showed a strongly enhanced bone resorption activity in comparison to the cells generated from wild type mice. In vivo this enhancement of osteoclast activity was also reflected in a decrease in cortical thickness of the tibiae. These findings are in agreement with studies performed in motheaten mice that lack the cytosolic tyrosine phosphatase SHP-1, which is considered to be a major cytosolic PTPase in hematopoietic cells, and is
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Figure 6. Enhanced actin ring formation in SIRPα-mutant mice. Osteoclasts derived from bone marrow cells of the wild type and SIRPα-mutant mice were cultured on cortical slides and stained with Alexa fluor 488 phalloidin. (A) Images were obtained by confocal laser scanning microscopy. Actin rings are in green, nuclei (PI-staining) are in red. Bar = 100 µm. (B) The number of actin rings per osteoclast was determined. In the SIRPα-mutant mice almost all osteoclasts contain an actin ring, and the percentage of osteoclasts with more than one actin ring is increased. On average 35±5.4 (mean ± SEM) osteoclasts per mouse (n=5 per genotype) were analyzed. (*) p<0.05

Known to transduce at least some of the intracellular signals generated by SIRPα in macrophages,10,12 in fact, to our knowledge SIRPα, which does indeed associate with SHP-1 in osteoclasts as shown in the present study, represents the first reported example of a bona fide ITIM-containing inhibitory immunoreceptor that inhibits osteoclast activity.

In contrast to the reported observations in SHP-1-deficient mice, which display both enhanced osteoclast formation and bone resorption activity, we did not observe any detectable differences in the formation and differentiation of osteoclasts, evaluated either in vitro or in vivo, between wild type and SIRPα-mutant mice. This indicates that the SIRPα-mutant reproduces only the part of the SHP-1 phenotype that relates to the
regulation of bone resorption, and suggests that other inhibitory immune receptors that signal via SHP-1 may also be relevant, particularly with respect to osteoclast formation. To our knowledge, only one candidate SHP-1-binding inhibitory receptor, i.e. the CMRF-35-like molecule 1 (CML-1), with such an activity has been reported. However, while overexpression of CML-1 inhibits osteoclastogenesis in an in vitro model employing RAW264.7 cells, CML-1 is actually downregulated during normal osteoclastogenesis. Although this strongly suggests that inhibitory receptors can indeed have inhibitory activity with respect to osteoclastogenesis, the relevant inhibitory receptor(s) controlling osteoclast formation in vivo remain to be identified.

The observation that SIRPα-mutant mice do not show any detectable defects in osteoclast formation in general, and osteoclast multinucleation in vitro (Figure 1E) and in vivo (Figure 4G), may be somewhat unexpected in light of the previously suggested role of CD47-SIRPα interactions in macrophage fusion in vitro. One explanation for this apparent discrepancy could be that osteoclast fusion is indeed mediated by CD47-SIRPα interactions, but does not involve concomitant SIRPα signaling. However, an alternative explanation is that CD47-SIRPα interactions, while relevant for fusion of some of the tested macrophage populations, do not play a role during osteoclast fusion at all. It was recently reported by Lundberg et al. that osteoclasts differentiated from CD47-deficient bone marrow cells in the presence of M-CSF and RANKL do not differ in size or number of nuclei per osteoclast suggesting that normal fusion occurs. Interestingly, while the results of the latter study strongly argue against a role for CD47-SIRPα interactions in osteoclast fusion, they did provide evidence for a role of CD47-SIRPα interactions in osteoclast formation, as reduced numbers of TRACP-positive osteoclasts were observed in the absence of CD47 in vitro as well as in vivo, and also by adding blocking antibodies against CD47 or SIRPα in vitro. Unfortunately, neither the bone resorption activity of CD47-deficient osteoclasts nor the bone mass were evaluated by Lundberg et al. and it therefore remains undecided whether the inhibition of osteoclast function by SIRPα signaling depends on CD47-SIRPα interactions or involves SIRPα signaling that is independent from CD47. Taken together it seems that: (i) osteoclast formation is supported by CD47-SIRPα interactions, but it is independent of SIRPα signaling, (ii) cell fusion of osteoclast precursors is independent of both CD47-SIRPα interactions and SIRPα signaling, and (iii) osteoclast function is inhibited by SIRPα signaling.

Although our results provide evidence for an inhibitory role of SIRPα signaling in osteoclast bone resorption, it seems possible that not all osteoclasts are equally dependent on SIRPα regulation. For instance, the decrease in cortical thickness observed in SIRPα-mutant mice in vivo was not paralleled by a detectable effect on trabecular bone mass, suggesting that SIRPα primarily regulates the activity of osteoclasts located at the shaft of long bones. Previously, we and others have demonstrated functional heterogeneity between osteoclasts at distinct anatomical locations, including those from cortical and trabecular bone. Zenger et. al. showed that the intracellular localization of monomeric TRACP was altered in distal...
Inhibitory regulation of osteoclast bone resorption by SIRPα

Figure 7. Hypothetical mechanism of the regulation of osteoclast function by activating and inhibitory receptors. The ITAM bearing proteins FcRγ and DAP12 that associated with the activating immunoreceptors such as TREM2 becomes phosphorylated by Scr upon ligand binding. This leads to recruitment of Syk, which in turn induces actin ring formation and thereby activates bone resorption. To control the resorption activity, osteoclasts also expressed inhibitory receptors like SIRPα which recruit the tyrosine phosphatases SHP-1 upon ligand binding. SHP-1 in turn inhibits actin ring formation and thereby the resorption activity of osteoclasts.

Our results also provide insight into the mechanism of regulation by SIRPα of osteoclastic bone resorption. In particular, we observed that the enhanced bone resorption by SIRPα-mutant osteoclasts was associated with an increased actin ring formation. The formation of actin rings, which form the foundation of the so-called...
sealing zone, is a prerequisite for osteoclastic bone resorption, essentially because it allows the generation of an isolated acidic compartment in which bone matrix is degraded by e.g. proteases. Actin ring formation is controlled by $\alpha_v\beta_3$ integrins, which upon ligand binding trigger autophosphorylation and activation of the tyrosine kinase c-Src. Activated c-Src phosphorylates ITAM motifs on activating immunoreceptor-associated adaptor proteins, in particular DAP12 or FcR$\gamma$, which in turn leads to the recruitment and activation of Syk$^{8,47,48}$. Syk phosphorylates a variety of substrates, including vinculin and paxillin. These proteins promote actin polymerization and the resultant formation of the osteoclast sealing zone. Of relevance in this context, mice deficient in c-Src or Syk develop osteopetrosis, which is due to a decreased osteoclast activity$^{8,49,50}$. Osteopetrosis is also observed in DAP12 and FcR$\gamma$ double knock-out mice$^6$. We propose that SIRP$\alpha$, and perhaps other myeloid inhibitory receptors as well, function as the inhibitory counterparts of activating immunoreceptors in this pathway. Figure 7 presents a hypothetical mechanism of the regulation of osteoclast function by activating and inhibitory immune receptors. It will be interesting to establish at which level(s) the proposed SIRP$\alpha$-SHP-1 signal integrates with the $\alpha_v\beta_3$ integrin/c-Src/activating immunoreceptor/adaptor/Syk/actin pathway.

In conclusion, we demonstrate that SIRP$\alpha$ signaling controls osteoclast bone resorption. In particular, SIRP$\alpha$ inhibits the generation of actin rings and the formation of the osteoclast sealing zone. More knowledge on the regulation of osteoclast activity by SIRP$\alpha$ may lead to novel methods can lead to treat of osteoporosis and other bone remodeling diseases.
Reference List


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