

CD47 Regulates Bone Mass and Tumor Metastasis to Bone

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Abstract

CD47, also called integrin-associated protein, plays a critical role in the innate immune response and is an atypical member of the immunoglobulin superfamily that interacts with and activates $\beta 3$ integrins. $\beta 3$ integrin^{-/-} mice have defective platelet and osteoclast function and are protected from bone metastasis. The role of CD47 in skeletal homeostasis and bone metastasis has not been described. CD47^{-/-} mice had increased bone mass and defective osteoclast function *in vivo*. Although the number of functional osteoclasts formed by differentiating CD47^{-/-} bone marrow macrophages was decreased, high doses of RANKL rescued differentiation and function of CD47^{-/-} osteoclasts *ex vivo* and rescued the osteoclast defect in CD47^{-/-} mice. Inhibition of nitric oxide (NO) synthase, which is expressed at higher levels in CD47^{-/-} osteoclasts, also rescued the osteoclast defect in CD47^{-/-} cells. We then examined the consequences of this osteoclast defect in bone metastasis. In a model of tumor metastasis to bone, bone tumor burden was decreased in the CD47^{-/-} mice compared with wild-type (WT) controls, with no decrease in s.c. tumor growth in CD47^{-/-} mice. There was decreased tumor-associated bone destruction in the CD47^{-/-} mice compared with WT controls, consistent with a defect in osteoclast function that was not rescued by the presence of tumor. Our data show that CD47 regulates osteoclastogenesis, in part, via regulation of NO production, and its disruption leads to a decrease in tumor bone metastasis. CD47 is a novel therapeutic target to strengthen bone mass and diminish metastatic tumor growth in bone. [Cancer Res 2009;69(7):OF1-9]

Introduction

Bone metastases cause hypercalcemia, bone loss, fractures, and pain and are thus a significant cause of morbidity and mortality in cancer patients (1). Several tumor cell types (e.g., breast and prostate carcinomas and melanomas) metastasize to bone and lead to bone degradation via activation of bone-resorbing osteoclasts (1–4). Osteoclasts are formed from the fusion of monocytes/macrophages and are characterized by their large size, the presence of multiple nuclei, and positive staining for tartrate-resistant acid phosphatase (TRAP). Two cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL), are necessary for the survival and

differentiation of macrophages into osteoclasts *in vitro* and *in vivo* (5). The osteoclast attaches to bone via the α v β 3 integrin through formation of the cytoskeletal organizations called actin rings. The resorbing osteoclast has a ruffled membrane and expresses cathepsin K, which is necessary for degrading the organic matrix of bone, and vacuolar proton pumps (H⁺-ATPases), which secrete acid and thus result in bone resorption by dissolving the inorganic matrix (5).

The presence of tumor cells in the bone microenvironment results in osteoclast and osteoblast recruitment and activation. This activation stimulates the release of growth factors from stromal cells as well as from the bone matrix, which further promote tumor growth in bone (1–4, 6). This is known as the vicious cycle of bone metastasis. We have previously shown that hyperfunctional osteoclasts are permissive for the growth of tumor cells that home to bone (7, 8). In addition, mice globally deficient in the $\beta 3$ integrin, which show a profound defect in platelet and osteoclast function, are protected from bone metastasis and tumor-associated bone destruction (9–11).

CD47 (also called integrin-associated protein) was first isolated through its interaction with the α v β 3 integrin, which is prominently expressed on osteoclasts (12–15). Expressed on all vascular cells, CD47 is an atypical member of the immunoglobulin superfamily (16) with a single IgV domain, five transmembrane segments, and a cytoplasmic tail (17). CD47 associates *in cis* in the plane of the membrane with α v β 3 and other integrins (12–15) and can augment integrin function by this association (18). It has recently been discovered that CD47, when ligated by thrombospondin-1 (TSP1), inhibits nitric oxide (NO) signaling in vascular cells (19). In platelets, TSP1-CD47 signaling opposes the NO/cyclic guanosine 3',5'-monophosphate (cGMP) inhibition of integrin activation (20), thus facilitating platelet aggregation. CD47 inhibition of NO signaling has also been shown in several other *in vivo* models, suggesting that limiting NO signaling is a primary function of the TSP1-CD47 system (19–26). Recent data indicate that the differentiation of bone marrow macrophages into osteoclast *in vitro* is sensitive to NO concentration, such that low levels of NO stimulate osteoclast formation, whereas increased NO inhibits formation of osteoclast (27). Therefore, CD47 may modulate NO signaling in osteoclast.

Although CD47 associates with α v β 3 and augments its function, $\beta 3$ ^{-/-} and CD47^{-/-} mice have rather different phenotypes. $\beta 3$ ^{-/-} mice are a model of Glanzmann's thrombasthenia, displaying profound defects in platelet aggregation and clot retraction (10). In contrast, CD47^{-/-} mice have normal bleeding times and a modest decrease in platelet numbers (28). These differences can be accounted by the fact that CD47 augments integrin signaling but is not essential for integrin-mediated adhesion (19). CD47 also has integrin-independent functions. For example, CD47-SIRP1 α interactions are important in innate immune reactions, negatively regulating phagocytosis *in vivo*. The presence of CD47 on hematopoietic

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cells provides them with a "self-signal" that inhibits their phagocytosis (16, 29). The SIRP1 α -CD47 interaction has also been postulated to be necessary for macrophage fusion, which is essential for the formation of osteoclasts (30, 31). Recently, Lundberg and colleagues (31) reported a defect in formation of osteoclasts from macrophages in the presence of blocking antibodies to CD47.

Because CD47 is proposed to function in macrophage fusion, interacts with and activates α v β 3, and modulates NO signaling, we hypothesized that CD47^{-/-} mice would have altered osteoclast differentiation and function. Here, we show that CD47^{-/-} mice have a modest increase in bone volume due to defective osteoclast function *in vivo*. Furthermore, CD47^{-/-} macrophages are defective in osteoclast formation *in vitro*. The osteoclast defect is rescued by increased administration of RANKL both *in vitro* and *in vivo*, and inhibition of NO synthase (NOS) rescues CD47^{-/-} osteoclast differentiation *in vitro*. To test the role of CD47 in a pathophysiologic context in which osteoclast function is central to disease pathogenesis, we evaluated bone metastasis and tumor-associated bone destruction in CD47^{-/-} mice.

Materials and Methods

Cells. The B16-F10 C57BL/6 murine melanoma cell line was purchased from the American Type Culture Collection and modified to express firefly luciferase (B16-FL; ref. 32).

Animals. CD47^{-/-} mice on a pure C57BL/6 background were housed under pathogen-free conditions according to the guidelines of the Division of Comparative Medicine, Washington University School of Medicine. The animal ethics committee approved all experiments. For generation of CD47^{-/-} mice, CD47^{+/-} females were crossed to CD47^{+/-} males and the females were allowed to have two litters.

Micro-computed tomography. Tibias and femurs were suspended in agarose and the right proximal tibial and femoral metaphyses were scanned by micro-computed tomography (μ CT; μ CT-40; Scanco Medical) as described previously (33, 34). For image acquisition, the tibias were placed in a 17-mm holder and scanned. The image consisted of 50 slices. The trabecular region was selected using contours inside the cortical shell on each two-dimensional image. The growth plate was used as a marker to determine a consistent location to start analysis. A three-dimensional cubical voxel model of bone was built, and the calculations were made as follows: relative bone volume over total bone volume (BV/TV), trabecular number, and thickness. A threshold of 300 (of 1,000) was used to differentiate trabecular bone from nonbone.

Histology, bone histomorphometry, and longitudinal growth measurements. Mouse tibias were fixed in formalin and decalcified in 14% EDTA. Paraffin-embedded sections were stained with H&E and separately for TRAP. Trabecular bone volume and tumor area were measured according to a standard protocol using Bioquant Osteo (Bioquant Image Analysis Corp.). Bone sections were blinded before analysis. Methylmethacrylate-embedded lumbar vertebral bodies of wild-type (WT) and CD47^{-/-} mice were stained for von Kossa on a counterstain of 0.5% basic fuchsin. Longitudinal growth was measured by use of calipers on whole body as well as isolated femurs.

Serum CTX assay. CTX was measured from WT or CD47^{-/-} mouse fasting serum by using a CTX ELISA system (Nordic Bioscience Diagnostics).

Flow cytometry (fluorescence-activated cell sorting). Whole bone marrow was isolated from WT and CD47^{-/-} mice and incubated in blocking medium (2.4G2 hybridoma with 4 mL of mouse IgG) and then incubated with FITC-conjugated anti-mouse F4/80 or anti-mouse CD47 (miap301) with an anti-rat FITC secondary antibody on ice for 20 min. The cells were then washed twice and analyzed on a FACScan flow cytometer (BD Biosciences).

***In vitro* osteoclast assays.** Whole bone marrow was isolated from WT C57BL/6 mice and plated in M-CSF containing CMG-14-12 cell culture

supernatant (1:10, v/v) in α -MEM containing 10% fetal bovine serum in Petri dishes for 3 d to generate primary bone marrow macrophages. Bone marrow macrophages were lifted and equal numbers were plated in 48-well dishes in osteoclast medium, α -MEM containing 10% FBS, CMG-14-12 supernatant (1:20, v/v), and glutathione *S*-transferase-RANKL (50 or 100 ng/mL), and incubated at 37°C for 5 d to generate osteoclasts. TRAP staining was performed according to the manufacturer's instructions (Sigma-Aldrich). In Fig. 4, 0, 3, 10, 30, and 100 μ g/mL of *N*-nitro-*L*-arginine methyl ester (L-NAME) were added to cultures at the same time with M-CSF and RANKL, and medium was changed every day. Osteoclast cultures were fixed after 4 d in culture and stained for TRAP.

Reverse transcription and quantitative PCR. Reverse transcription and quantitative PCR (qPCR) methods were carried out as described previously (8). The following qPCR primers were used: CD47, GGCGCAAAG-CACCGAAGAAATGTT (forward) and CCATGGCATCGCGCTTATCCATTT (reverse); inducible NOS (iNOS), GGCAGCTGTGAGACCTTTG (forward) and GCATTGGAAGTGAAGCGTTTC (reverse); and glyceraldehyde-3-phosphate dehydrogenase, TCAACAGCAACTCCCCTCTTCCA (forward) and ACCCTGTGCTGTAGCCGTATTCA (reverse).

Actin ring formation and bone resorption assays. Actin ring formation and bone resorption assays were performed as described (35). Briefly, the cells plated on bovine bone slices were fixed with 3% paraformaldehyde in PBS for 20 min. Filamentous actin was stained with FITC-labeled phalloidin at 0.3 μ g/mL in PBS. For staining of the resorption lacunae (pits), the cells were brushed off the bone with a toothbrush. The slices were incubated with 20 μ g/mL of peroxidase-conjugated wheat germ agglutinin for 1 h. After washing in PBS, 0.52 mg/mL of 3,3'-diaminobenzidine with 0.1% H₂O₂ was added onto the bone slices for 15 min. Pit area was determined from five \times 4 fields by using Osteo software (Bioquant) blinded to genotype.

***In vivo* RANKL injections.** RANKL (100 μ g) in a volume of 40 μ L was injected subperiosteally in the midline calvaria in 8-wk-old mice daily for 5 consecutive days. On the 6th day, serum was collected, mice were sacrificed, and the calvarial bone was isolated. TRAP staining was performed on fixed, decalcified, and paraffin-embedded calvarial bone.

Tumor and bone metastasis models. For intracardiac injections, the operator was blinded to genotype. Mice were anesthetized and inoculated intracardially via the left ventricular chamber with 10⁵ B16-FL cells in 50 μ L PBS as previously described (7). Bioluminescence imaging was performed on days 7, 10, and 12 after B16-FL cell inoculation. Mice were sacrificed and underwent blinded necropsy on day 12 after tumor cell injection. Mice were discarded from the final analysis if the animal died before day 12 or if necropsy showed a large mediastinal tumor indicative of injection of tumor cells into the chest cavity, not the left ventricle.

For intratibial injections, mice were anesthetized, and 1 \times 10⁴ B16-FL cells in 50 μ L PBS were injected into the right tibia. PBS (50 μ L) was injected into the left tibia as an internal control. Animals were radiographed in two dimensions using an X-ray system to confirm intratibial placement of the needle (Faxitron Corp.). Bioluminescence imaging was performed on days 7 and 9 after B16-FL cell inoculation. Mice were sacrificed and underwent necropsy on day 9 after B16-FL inoculation. Mice with i.m. locations of tumors were discarded from the analysis. For s.c. injections, mice were anesthetized, and 5 \times 10⁵ B16-FL cells in 100 μ L PBS were injected s.c. on the dorsal surface of the mouse at two sites. Tumor growth was monitored over the 14-d period after B16-FL injections, and bioluminescence imaging was performed 5, 7, 10, and 14 d after B16-FL inoculation. The experiment was terminated due to the presence of large necrotic tumors.

***In vivo* bioluminescence imaging.** Mice were injected i.p. with 150 mg/kg D-luciferin (Biosynthesis) in PBS 10 min before imaging. Imaging was performed using a charge-coupled device camera (IVIS 100; exposure time of 1 or 5 min, binning of 8, field of view of 15 cm, f/stop of 1, and no filter) in collaboration with the Molecular Imaging Center Reporter Core. Mice were anesthetized by isoflurane (2% vaporized in O₂) and C57BL/6 mice were shaved to minimize attenuation of light by pigmented hair. For analysis, total photon flux (photons per second) was measured from a fixed region of interest in the tibia/femur, the mandible, or the local s.c. tumor using Living Image 2.50 and IgorPro software (Wavemetrics).

Results

CD47^{-/-} mice have increased bone volume due to dysfunctional osteoclasts. CD47 interacts with and activates $\beta 3$ integrins. $\beta 3$ ^{-/-} mice display osteopetrosis due to osteoclast dysfunction. Therefore, we hypothesized that CD47^{-/-} mice might have an

osteoclast defect. To test this hypothesis, μ CT analysis on tibias from 8-week-old WT and CD47^{-/-} littermates was performed. We observed an increase in trabecular BV/TV in CD47^{-/-} mice compared with WT mice (Fig. 1A and B). There was no change in trabecular number, but the trabecular thickness was increased in

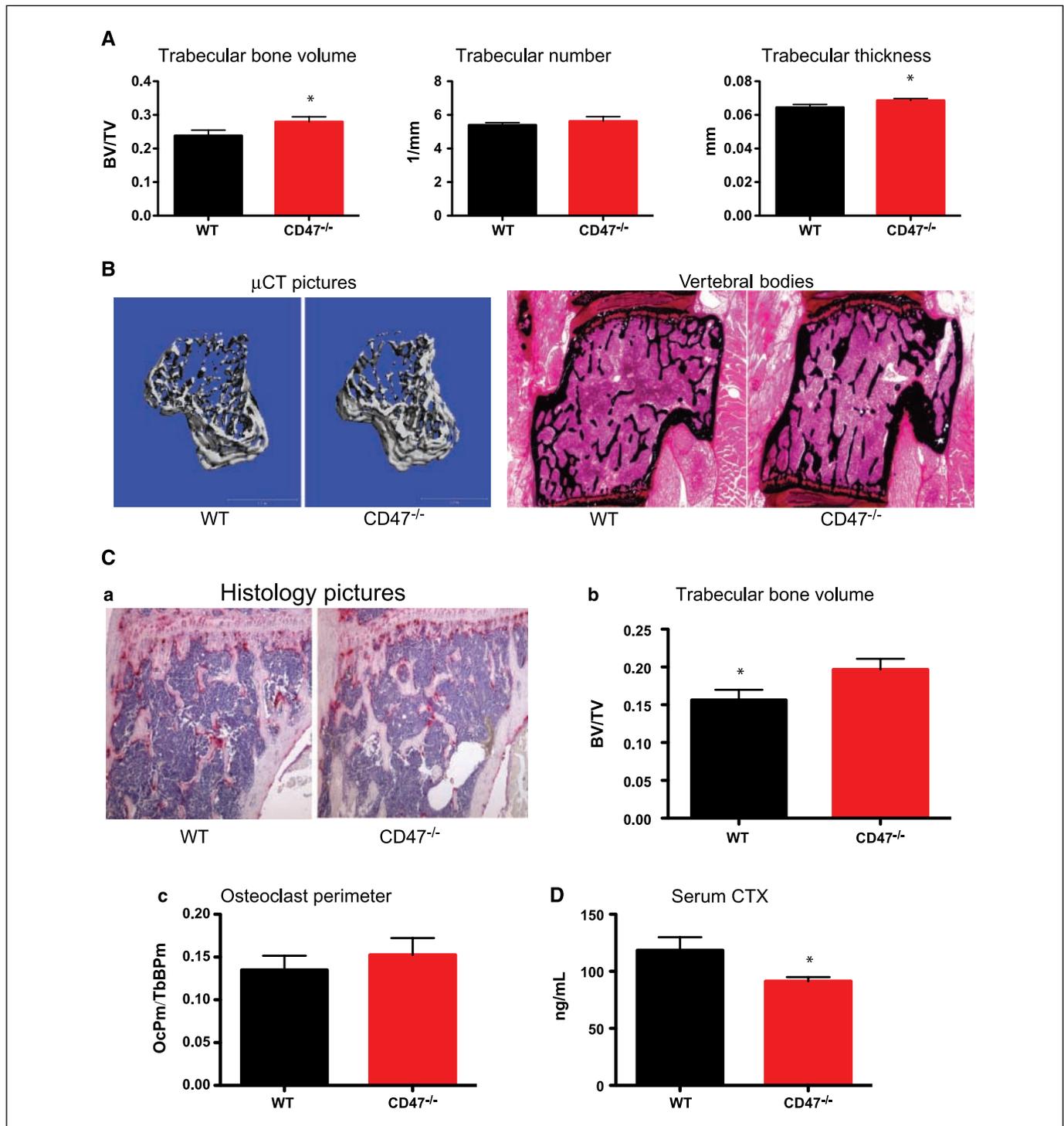


Figure 1. CD47^{-/-} mice have increased bone volume due to dysfunctional osteoclasts. **A**, WT and CD47^{-/-} tibias were subjected to μ CT analysis of bone parameters (WT, $n = 5$; CD47^{-/-}, $n = 5$). Trabecular BV/TV by μ CT ($P = 0.04$); trabecular number by μ CT ($P = 0.22$); trabecular thickness by μ CT ($P = 0.04$). **B**, representative images of three-dimensional μ CT reconstruction of WT and CD47^{-/-} tibias (left) and lumbar vertebral bodies of WT and CD47^{-/-} mice stained with von Kossa (right). **C**, a, representative histologic TRAP-stained tibial sections. b, BV/TV in tibias by histomorphometry ($P = 0.02$). c, osteoclast perimeter/trabecular bone perimeter ($P = 0.23$). **D**, collagen breakdown products (CTX) were measured in the serum of starved WT and CD47^{-/-} mice ($P = 0.03$).

CD47^{-/-} tibias (Fig. 1A and B). Von Kossa staining of the lumbar vertebral bodies, a marker for mineralized matrix, confirmed an increase in bone volume and trabecular thickness in CD47^{-/-} mice (Fig. 1B). The increase in trabecular bone volume (BV/TV) was also confirmed by histomorphometry on histologic sections (Fig. 1C, a and b). We did not observe a difference in longitudinal growth of CD47^{-/-} mice as measured by femur length and whole body length (Supplementary Fig. S1). Interestingly, we also observed that osteoclast perimeter/total trabecular bone perimeter was not changed in CD47^{-/-} mice *in vivo* (Fig. 1C, c). To confirm that this increase in BV/TV was due to dysfunctional osteoclasts, we measured serum CTX, the COOH-terminal telopeptide of collagen type I that is cleaved on bone resorption by osteoclasts. We observed a decrease in CTX activity (Fig. 1D), which along with an increase in trabecular bone volume suggests that the osteoclast activity was decreased in CD47^{-/-} mice *in vivo* (Fig. 1E).

CD47^{-/-} osteoclast defect can be rescued by high doses of RANKL *in vitro*. To investigate if the osteoclast defect is cell autonomous in CD47^{-/-} mice, we first determined if macrophages or osteoclast precursors were decreased in CD47^{-/-} bone marrow. We stained WT and CD47^{-/-} whole bone marrow with CD47-FITC

as control and F4/80 as a macrophage marker and carried out flow cytometry. We did not observe a significant difference in F4/80⁺ cells in CD47^{-/-} bone marrow compared with WT controls (Fig. 2A). To test the differentiation of macrophages to osteoclasts, we cultured whole bone marrow in M-CSF alone for 3 days to enrich for macrophages. An equal number of macrophages were then cultured in the presence of M-CSF and RANKL to promote differentiation into osteoclasts for 7 days. At a dose of 50 ng/mL of RANKL, we observed induction of CD47 transcript levels over the course of osteoclast differentiation in WT but not in CD47^{-/-} cells (Fig. 2B). We observed that WT macrophages produced large, multinucleated, TRAP-positive osteoclasts by day 5, but only a few osteoclasts formed from CD47^{-/-} macrophages. When WT and CD47^{-/-} macrophages were plated onto bovine bone slices, there was a decrease in the number of multinucleated CD47^{-/-} osteoclasts with multiple actin rings visualized by phalloidin staining (Fig. 2C). However, when the dose of RANKL was increased 2-fold to 100 ng/mL, the osteoclast differentiation defect was largely rescued in CD47^{-/-} cells. This was reflected in equivalent numbers of osteoclasts with multiple actin rings formed on bovine bone from macrophages of both genotypes (Fig. 2C). To determine

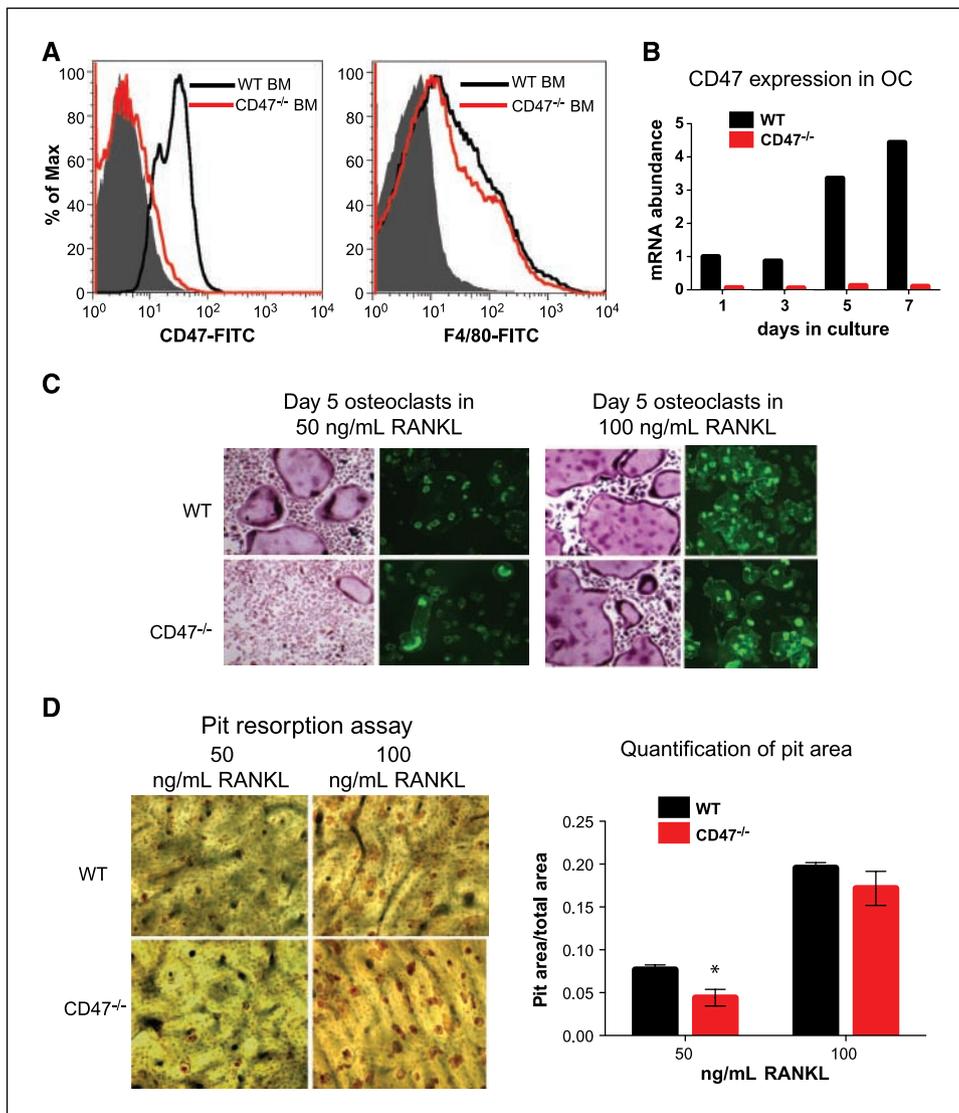


Figure 2. CD47^{-/-} osteoclast defect can be rescued by high doses of RANKL *in vitro*. **A**, fluorescence-activated cell sorting analysis on WT and CD47^{-/-} total bone marrow (BM). WT and CD47^{-/-} total bone marrow was stained for CD47 and F4/80. **B**, qPCR graph showing induction of CD47 transcript over the 7-d course of osteoclast differentiation. **C**, representative images of day 5 osteoclasts cultured on bone and stained for TRAP (left) and cultured on bovine bone and stained for actin rings (right) in the presence of 50 ng/mL M-CSF and 50 or 100 ng/mL of RANKL. **D**, representative images of day 5 osteoclasts cultured on bone and stained for resorption lacunae in the presence of 50 ng/mL M-CSF and either 50 or 100 ng/mL of RANKL. The pit (resorption lacunae) area was measured by histomorphometry. RANKL: 50 ng/mL ($P = 0.04$) and 100 ng/mL ($P = 0.18$).

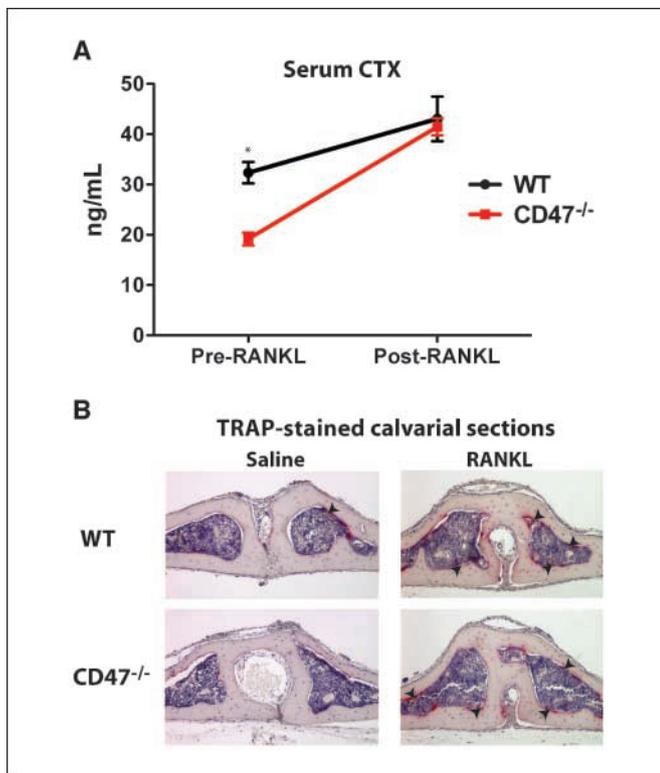


Figure 3. The osteoclast dysfunction in CD47^{-/-} mice is rescued by *in vivo* RANKL injections. **A**, 100 μ g RANKL was delivered subperiosteally onto the midline calvaria of WT ($n = 5$) and CD47^{-/-} ($n = 5$) mice. Serum CTX was measured before and after RANKL injections. WT versus CD47^{-/-} pre-RANKL, $P < 0.01$; WT versus CD47^{-/-} post-RANKL, $P = 0.74$; WT pre-RANKL versus post-RANKL, $P = 0.03$; CD47^{-/-} pre-RANKL versus post-RANKL, $P < 0.01$. **B**, representative images of TRAP-stained calvarial sections. Arrows, recruited osteoclasts.

the functional capacity of CD47^{-/-} osteoclasts to resorb bone, we stained the bones with wheat germ agglutinin to measure the areas of resorption lacunae after 5 days on bovine bone. There was a significant decrease in bone resorption by CD47^{-/-} osteoclasts in

50 ng/mL RANKL; however, the resorption capacity of CD47^{-/-} osteoclasts was similar to WT at a high dose of RANKL (Fig. 2D).

The osteoclast dysfunction in CD47^{-/-} mice was rescued by *in vivo* RANKL injections. To determine if RANKL could rescue CD47^{-/-} osteoclast function *in vivo* as was observed *in vitro*, we injected 100 μ g RANKL subperiosteally into the midline calvaria of 8-week-old WT and CD47^{-/-} mice. We measured serum CTX before and after RANKL injections in these mice. Before RANKL injection, CD47^{-/-} mice had lower serum CTX as we have shown before (Fig. 3A). However, after RANKL administration, osteoclast activation was not significantly different between WT and CD47^{-/-} mice as measured by serum CTX (Fig. 3A). Furthermore, direct visualization of osteoclast recruitment by TRAP staining on histologic sections of calvarial bone in WT and CD47^{-/-} mice confirmed the CTX results (Fig. 3B). We conclude that a high dose of RANKL rescued the cell-autonomous osteoclast defect in CD47^{-/-} mice.

NOS inhibition restores osteoclast differentiation in CD47^{-/-} cells. Several tissues from CD47^{-/-} mice have increased NO levels (36). We differentiated WT and CD47^{-/-} macrophages into osteoclasts in the presence of M-CSF and RANKL. We observed that iNOS expression levels were higher in CD47^{-/-} osteoclast cultures compared with WT controls (Fig. 4A). It has been previously shown that increased levels of NO lead to a block in osteoclast differentiation (27). We hypothesized that this increase in NO levels is responsible for the inhibition of osteoclast differentiation in CD47^{-/-} cells. We differentiated WT and CD47^{-/-} macrophages into osteoclasts in the presence of L-NAME, a pan-NOS inhibitor. In CD47^{-/-} cells, we observed a dose-dependent rescue of osteoclast differentiation with L-NAME administration. We observed a biphasic effect of L-NAME on WT cells, where, at a low dose of L-NAME, there was modest enhancement of osteoclast formation and, at a high dose of L-NAME, there was inhibition of osteoclast differentiation (Fig. 4B). The osteoclast inhibitory dose of L-NAME on WT cells was enhancing in CD47^{-/-} cells. Taken together, these data support that the increased levels of iNOS in CD47^{-/-} cells negatively affect osteoclast formation.

CD47^{-/-} mice have decreased tumor burden and bone loss in an intracardiac metastasis model. The data above indicate

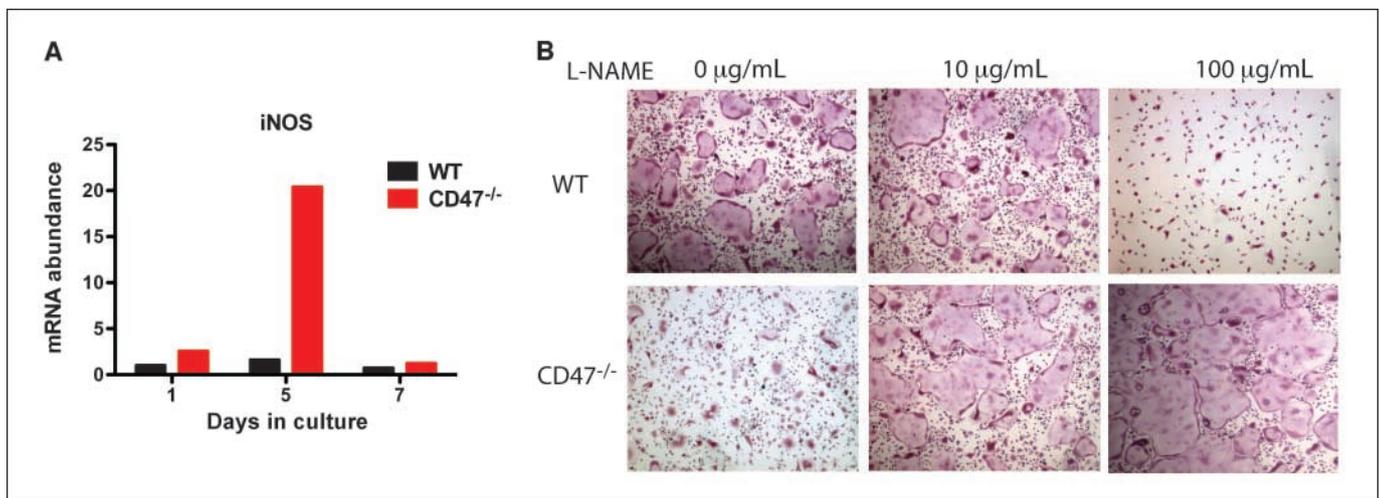


Figure 4. NOS inhibition restores osteoclast differentiation in CD47^{-/-} cells. **A**, WT and CD47^{-/-} macrophages were differentiated into osteoclast in the presence of 50 ng/mL M-CSF and 50 ng/mL RANKL. RNA was isolated at days 1, 5, and 7, and cDNA was made. qPCR analysis was carried out with specific primers to iNOS. **B**, WT and CD47^{-/-} macrophages were differentiated into osteoclasts in the presence of M-CSF, RANKL, and L-NAME, a pan-inhibitor of NOS, for 5 d.

that, at high doses of RANKL, CD47 was not necessary for osteoclast differentiation and function. RANKL is produced from T cells and osteoblasts during inflammatory conditions such as arthritis and bone metastasis. RANKL is not expressed by B16-FL cells (data not shown). We thus examined bone metastasis and osteolysis in CD47^{-/-} mice to determine if this local increase in RANKL is able to rescue the CD47^{-/-} osteoclasts in this pathophysiologic context. We evaluated bone metastasis in WT and CD47^{-/-} mice using murine melanoma B16-F10 cells engineered to express firefly luciferase (B16-FL). We measured bone tumor burden by real-time bioluminescence on days 7, 10,

and 12 after intracardiac B16-FL injection, a route of administration that allows for bone metastasis rather than lung infiltration of injected cells (9, 32). There was a significant decrease in tumor burden in the femoral/tibial bones and the mandible of CD47^{-/-} mice compared with WT mice (Fig. 5B and C) as measured by bioluminescence and confirmed by histomorphometric measurement of tumor volume in histologic sections at day 12 after B16-FL inoculation (Supplementary Fig. S2A; Fig. 5B and C). Tumor cells are known to secrete factors that activate osteoclasts to degrade bone and induce osteolysis, which is a significant outcome of bone metastases (1–4, 6). Trabecular bone volume was measured in

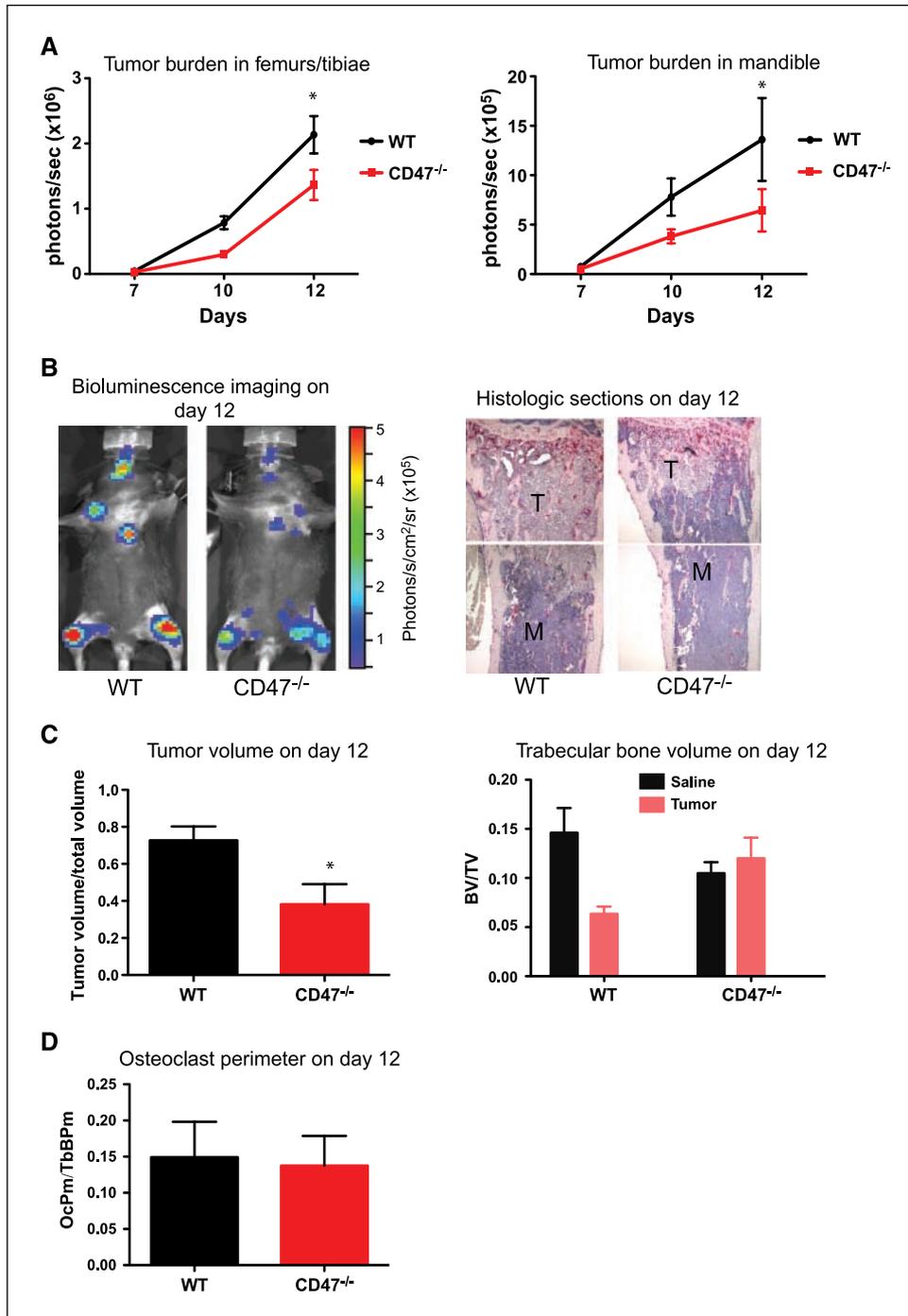


Figure 5. CD47^{-/-} mice have decreased tumor burden and bone loss in an intracardiac metastasis model. **A**, B16-FL cells were injected into the left ventricular chamber of WT ($n = 3$) and CD47^{-/-} ($n = 3$) mice. Tumor burden in the femur/tibia ($P = 0.003$) and the mandible ($P = 0.05$) as measured by bioluminescence imaging 7, 10, and 12 d after tumor cell injection. **B**, representative images of bioluminescence and of tibial histologic bone sections. **M**, marrow; **T**, tumor. **C**, tumor volume/total volume in the tibiae of WT ($n = 3$) and CD47^{-/-} ($n = 3$) mice at day 12 was measured by histomorphometric analysis ($P = 0.02$). Trabecular bone volume in the tibiae of WT ($n = 3$) and CD47^{-/-} ($n = 3$) mice at day 12 was measured by histomorphometric analysis. WT saline versus tumor, $P < 0.01$; CD47^{-/-} saline versus tumor, $P = 0.51$. **D**, osteoclast perimeter in the tibiae of WT ($n = 3$) and CD47^{-/-} ($n = 3$) mice at day 12 was measured by histomorphometric analysis. Three independent experiments showed similar results.

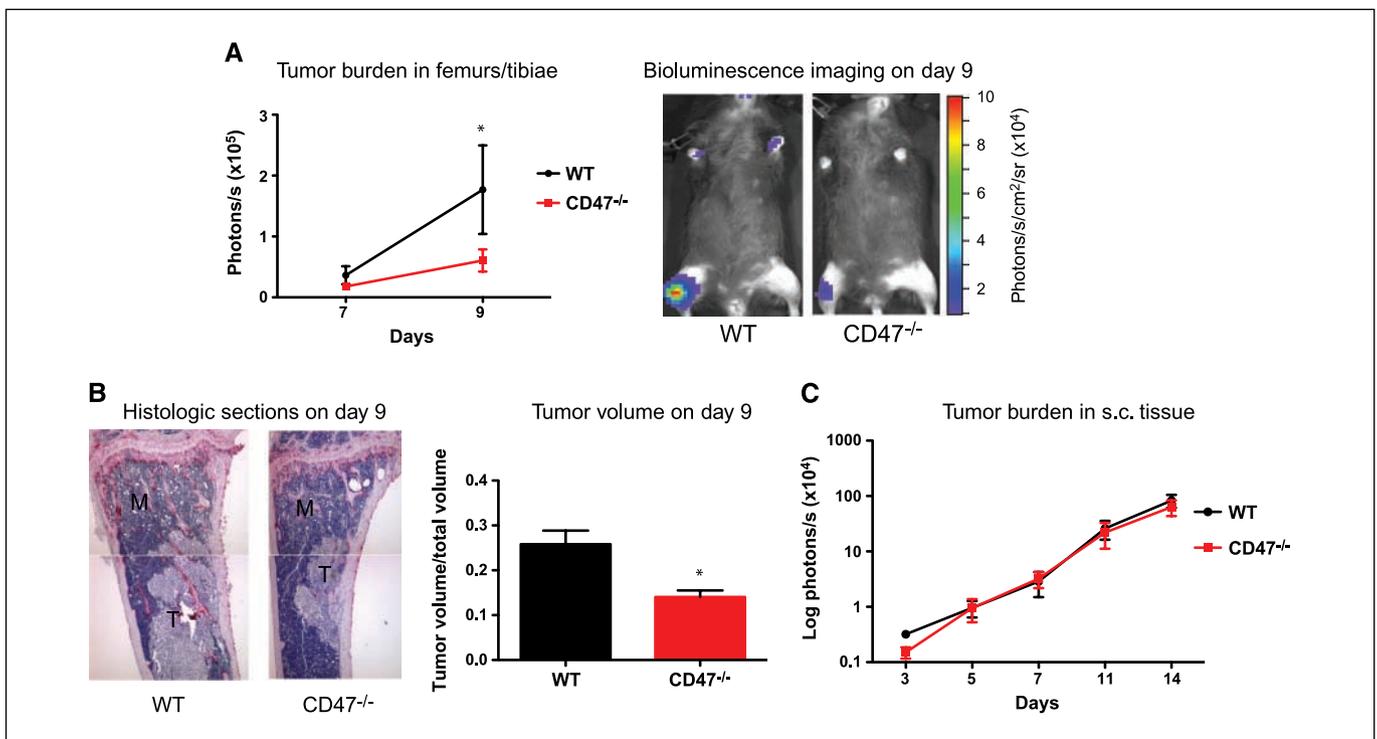


Figure 6. CD47^{-/-} mice have decreased tumor burden and bone loss in an intratibial metastasis model but not in a s.c. model. **A**, B16-FL cells were injected directly into the tibia of WT ($n = 6$) and CD47^{-/-} ($n = 5$) mice. Tumor burden in the tibiae was measured by bioluminescence imaging at days 7 and 9 after B16-FL injection ($P = 0.05$). Representative bioluminescence images are shown. **B**, representative images of tibial histologic bone sections at day 9. Tumor volume/total volume in the tibiae of WT ($n = 6$) and CD47^{-/-} ($n = 5$) mice at day 12 was measured by histomorphometric analysis ($P = 0.03$). Three independent experiments showed similar results. **C**, B16-FL cells were injected s.c. into the hind flank of mice, and tumor burden was measured by bioluminescence imaging at days 5, 7, 10, and 14 after B16-FL injection.

WT and CD47^{-/-} mice injected with B16-FL or saline. Whereas there was significant bone loss in B16-FL in WT mice, there was no bone loss in CD47^{-/-} bones injected with B16-FL, consistent with the osteoclast dysfunction in CD47^{-/-} mice (Fig. 5B and C). We did not observe a difference in osteoclast perimeter/trabecular bone perimeter in tumor-bearing CD47^{-/-} bones (Fig. 5D). Thus, we conclude that CD47^{-/-} mice had decreased bone tumor burden and tumor-associated bone destruction, and the local increase in RANKL levels *in vivo* was not sufficient to rescue the osteoclast defect in CD47^{-/-} mice.

CD47^{-/-} mice have decreased tumor burden and bone loss in an intratibial metastasis model but not in a s.c. model. We have previously shown that platelets are critical for the homing of tumor cells to bone (9). CD47^{-/-} mice have been shown to have a mild decrease in platelet numbers (28). To further confirm that this decrease in tumor burden in CD47^{-/-} mice after intracardiac injections of B16-FL cells was not due to compromised homing of tumor cells to bone, we turned to a more direct model of late-stage bone metastasis that eliminates initial tumor-homing steps of the metastatic process. We injected equal numbers of B16-FL cells directly into the tibiae of WT and CD47^{-/-} littermates and performed bioluminescence on days 7 and 9 after B16-FL inoculation. Bioluminescence imaging showed decreased tumor burden over time in the CD47^{-/-} mice compared with WT mice (Fig. 6A). Histomorphometric measurement of tumor volume in histologic sections at day 9 after B16-FL inoculation confirmed this decrease (Supplementary Fig. S2B; Fig. 6B).

To confirm that the decrease in bone tumor burden in CD47^{-/-} mice was specific to CD47 function in the bone microenvironment and not a result of intervention by the immune system or tumor-associated angiogenesis in the CD47^{-/-} mice, we measured local tumor burden in s.c. injected B16-FL cells. We observed no change in s.c. tumor burden as measured by bioluminescence on days 5, 7, 10, and 14, at which point the experiment was terminated due to necrotic tumors (Fig. 6C). We conclude that the decrease in tumor burden in CD47^{-/-} mice was specific to the bone compartment and not due to compromised immunity or platelet interactions.

Discussion

We show here that CD47^{-/-} mice exhibited mild osteopetrosis and decreased osteoclast function *in vivo* and *in vitro*. *In vitro*, CD47^{-/-} macrophages did not form as many multinucleated spread osteoclasts as WT cells. Bone resorption as measured by pit staining was decreased in CD47^{-/-} cells *in vitro*. Interestingly, this osteoclast defect was rescued by delivery of high doses of RANKL both *in vitro* and *in vivo*, suggesting that CD47 may cooperate with RANKL during osteoclast development/function. We observed that iNOS levels were increased in CD47^{-/-} osteoclasts and inhibition of NOS rescued the osteoclast differentiation defect in CD47^{-/-} cells. We also observed decreased tumor burden and osteolysis in CD47^{-/-} bone after intracardiac or intratibial delivery of tumor cells.

The first role known for CD47 was based on its physical and functional interaction with $\alpha v\beta 3$ (37). It is well known that the

integrin $\alpha\beta3$ plays a critical role not only in osteoclast function (11) but also in the differentiation of macrophages into osteoclast (38). CD47 has been shown to augment $\alpha\beta3$ function by physical association, leading to increased integrin clustering and therefore increases in avidity. Furthermore, CD47 signals through G α i to activate $\alpha\beta3$ via an "inside-out" signaling pathway analogous to that used by G protein-coupled receptors on platelets to activate $\alpha\text{IIb}\beta3$ via activation of the small G protein Rap1 (20). In platelets, NO-stimulated cGMP levels oppose Rap1b activation. CD47 acts to prevent this inhibitory effect of NO, thus allowing more GTP loading of Rap1b, resulting in $\alpha\text{IIb}\beta3$ activation (20). It is likely that CD47 in osteoclasts can activate $\alpha\beta3$ in a similar fashion.

Although CD47 could be playing a role in osteoclast differentiation and function by augmenting $\alpha\beta3$ signaling, it could also exert its effects on osteoclasts in a $\beta3$ -independent manner. The defect in osteoclast differentiation can be overcome by addition of high doses of M-CSF to cultures by increasing expression of c-fms (39). In contrast, we observed that exogenous RANKL administration could rescue the osteoclast defect in CD47^{-/-} mice *in vivo* and *in vitro*, suggesting that CD47 might play a role in osteoclastogenesis by enhancing RANKL signaling. It is possible that CD47 and RANKL might stimulate osteoclasts in parallel pathways that merge at a common signaling molecule. Our data suggest that the NO/cGMP pathway might be the link between CD47 and RANKL signaling.

There is a growing literature on the role of NO in osteoclast differentiation and function. Often, the proposed "Goldilocks effect" comes into play in NO regulation. NO has been reported to both promote and inhibit osteoclast differentiation/survival (27, 40, 41) and osteoclast function (40, 42, 43). Low signaling levels of NO derived from eNOS (NOSIII) and/or nNOS (NOSI) are most likely needed to support osteoclast differentiation, whereas higher levels, such as those produced by iNOS, become inhibitory. We found that iNOS was significantly induced in CD47^{-/-} osteoclast and inhibition with a NOS inhibitor, L-NAME, rescued CD47^{-/-} osteoclast differentiation (Fig. 4B). Many signaling effects of NO are mediated via stimulation of soluble guanylate cyclase, production of cGMP, and activation of protein kinase G. However, there are effects of NO that are accomplished by its direct interaction with protein thiols (S-nitrosylation) to modify their enzymatic activity (44, 45). Src, a kinase downstream of RANK signaling known to have a critical role in osteoclast differentiation and function (46), is a target of inhibitory S-nitrosylation (47). This modification of Src by NO in osteoclasts might have a significant effect on osteoclast function. Such a finely balanced regulatory mechanism

is suggested by our finding that only a 2-fold increase in RANKL concentration *in vitro* permits formation of CD47^{-/-} osteoclasts that are as functional as WT osteoclasts.

The second major finding of our study is that the role of CD47 in osteoclast differentiation/function has a significant effect on the interaction of tumor cells within the bone microenvironment. The decreased osteoclast function in CD47^{-/-} mice leads to a decrease in bone tumor burden and osteolysis, consistent with the model of the vicious cycle of bone metastasis (1–4). Notably, the increase in bone volume in CD47^{-/-} was only ~4% compared with WT mice, not as profound a change as observed in the osteopetrotic $\beta3$ ^{-/-} mice (11). It is interesting that the relatively modest, rescuable decrease in osteoclast function in CD47^{-/-} mice *in vivo* could result in a more pronounced decrease in tumor burden and osteolysis. These results suggest that the relationship between the osteoclast and the tumor cell with regard to the vicious cycle of bone metastasis is not one of direct proportionality. The complex signaling pathways involved in the relationship between tumor cells and bone cells no doubt involve amplification steps. Both Src kinase-initiated pathways and the NO/cGMP pathways implicated here are classic examples of signal amplifiers.

One of the major complications of currently available therapies for bone metastasis, such as bisphosphonates, is their harsh effect on bone, leading to conditions such as suppressed bone turnover or osteonecrosis of the jaw (48, 49). We observed that CD47^{-/-} mice displayed a mild increase in bone volume and a decrease in tumor burden in bone. Our results suggest the potential for manipulating CD47 signaling as a novel approach to treatment of osteoporosis in the general population and to treatment of bone metastasis in cancer patients without causing severe complications to the skeleton.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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