CD8⁺ T Cells Regulate Bone Tumor Burden Independent of Osteoclast Resorption

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Abstract

Blockade of osteoclast (OC) activity efficiently decreases tumor burden as well as associated bone erosion in immune-compromised animals bearing human osteolytic cancers. In this study, we showed that modulation of antitumor T-cell responses alters tumor growth in bone, regardless of OC status, by using genetic and pharmacologic models. PLCγ2−/− mice, with dysfunctional OCs and impaired dendritic cell (DC)–mediated T-cell activation, had increased bone tumor burden despite protection from bone loss. In contrast, Lyn−/− mice, with more numerous OCs and a hyperactive myeloid population leading to increased T-cell responses, had reduced tumor growth in bone despite enhanced osteolysis. The unexpected tumor/bone phenotype observed in PLCγ2−/− and Lyn−/− mice was transplantable, suggesting the involvement of an immune component. Consistent with this hypothesis, T-cell activation diminished skeletal metastasis whereas T-cell depletion enhanced it, even in the presence of zoledronic acid, a potent antiresorptive agent. Importantly, injection of antigen-specific wild-type cytotoxic CD8⁺ T cells in PLCγ2−/− mice or CD8⁺ T-cell depletion in Lyn−/− mice normalized tumor growth in bone. Our findings show the important contribution of CD8⁺ T cells in the regulation of bone metastases regardless of OC status, thus including T cells as critical regulators of tumor growth in bone. Cancer Res; 71(14); 4799–808. ©2011 AACR.

Introduction

Bone metastases represent a serious complication of many cancers, including breast, prostate, and lung tumors, as well as multiple myeloma. In the bone marrow (BM), there is a mutually positive interaction between osteoclasts (OC) and cancer cells that is known as the vicious cycle (1). Growth factors secreted by the tumor cells, such as receptor activator of NF-κB–ligand (RANKL), stimulate the bone resorptive activity of OCs. In turn, bone-stored factors including TGF-β are released and enhance tumor growth (1). Experimental evidence suggests that OCs play a central role in modulating the tumor/bone vicious cycle. Antagonism of RANKL, by using recombinant osteoprotegerin (Fc-OPG) or soluble anti-RANKL antibody (Ab), reduces OC number and significantly decreases tumor burden in murine models of breast cancer bone metastasis and multiple myeloma (2). Similarly, treatment with zoledronic acid (ZOL), an N-bisphosphonate compound highly effective in inducing OC apoptosis and suppressing bone resorption, protects mice injected with human breast cancer cells from developing tumor growth in bone (3). Based on these findings, the OC is now the principal therapeutic target for bone metastases (4). However, not all patients with bone metastases respond well to antiresorptive therapy, and one third develops further skeletal-related events within 2 years of initiating these therapies (5). Thus, the results from clinical studies suggest that other bone marrow–residing cells, in addition to OCs, could be regulating tumor growth in bone.

The bone microenvironment is a reservoir of several immune cell types. Memory T cells have been found in the bone marrow of patients with breast cancer, implicating them in cancer immune surveillance (6). Interestingly, some of the therapies aimed at disrupting the mutual interaction between cancer cells and OCs also have immunomodulatory effects. For example, TGF-β, which is released into the bone marrow microenvironment by the resorptive OC, inhibits T-cell proliferation, natural killer (NK) cell function, and antigen presentation (7). Thus, blockade of TGF-β at sites of metastases may locally activate T-cell function, initiating an antitumor immune response. ZOL, in addition to its antiresorptive effect, can activate cytotoxic γ/δ-T cells (8) and inhibit certain populations of myeloid-derived cells with T-cell–suppressor abilities. Unfortunately, to date, the contribution of T cells in modulating the tumor/bone vicious cycle has not been evaluated because most models of bone metastases use human breast cancer cells injected into immunocompromised mice.
The present study aimed to examine the relative contribution of immune cells and OCs in the tumor/bone vicious cycle by using a syngeneic mouse model. We turned to the B16 melanoma model of bone metastases because these cells: (i) grow in C57BL/6 immunocompetent mice, and (ii) metastasize to bone following intracardiac injection. B16 is a relatively poorly immunogenic cell line, although it can induce a modest, but specific, T-cell response (9). Thus, by using B16 cells we can take advantage of genetically manipulated mice with specific immune phenotypes. Phospholipase C gamma (PLCγ) 2 is an enzyme converting phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3) leading to activation of protein kinase C (PKC) and calcium pathways. PLCγ2−/− mice have broadly compromised immune responses due to impaired B-cell development, NK-cell cytotoxic activity, and dendritic cell (DC)-mediated antigen presentation leading to defective T-cell activation (10–13). Furthermore, PLCγ2−/− mice are osteopetrotic due to reduced OC number and functionality (14, 15). Lyn is a Src family member that is mainly involved in down-modulation of several intracellular pathways, including PLCγ2 activation (16). Lyn−/− mice have increased B-cell-mediated immune responses, expanded macrophages, mast cells, and DCs (17, 18). Due to a hyperactive myeloid population, T-cell responses are also enhanced, and Lyn−/− mice develop autoimmunity with age (19). Furthermore, Lyn−/− mice have decreased bone mass and more numerous OCs due to enhanced RANKL signaling and PLCγ phosphorylation (16, 20).

Because PLCγ2 deficiency impacts OC formation and function, we expected that PLCγ2−/− mice would have decreased bone tumor burden following B16 tumor inoculation. Furthermore, we would have anticipated increased tumor growth in bone in Lyn−/− mice on the basis of their hyperactive OC phenotype. On the contrary, we found that PLCγ2−/− mice are more susceptible to tumor growth in bone, despite their OC defect, whereas Lyn−/− mice display significant inhibition of tumor growth in bone, in the face of more numerous OCs. Despite the lack of PLCγ2 and Lyn expression in T cells, aberrant myeloid-mediated T-cell activation is responsible for these unexpected findings. Our data show that CD8+ T cells modulate tumor growth in bone regardless of genetic or pharmacologic OC inhibition, thus expanding the current tumor/bone vicious cycle model to include an immune component in addition to the OC.

Materials and Methods

Tumor cells and animal models of bone metastases

B16 mouse melanoma cells were obtained from Dr. David Fisher (Harvard Medical School). These cells have been characterized by expression arrays and Western blot analysis for melanocyte markers such as melanin, tyrosinase, c-kit, and microphthalmia transcription factor. Pigmented tumor formation in mice further confirmed that B16 are melanoma cells. This assay is routinely used in the laboratory for their characterization and it was conducted within the last few weeks of the experiment.

Firefly-conjugated B16 cells (B16-FL) were injected intrathecally (IT), intracardiac (LV), or subcutaneously (s.c.) in 6-week-old, female mice (ref. 21; Supplementary Data). For CD4+ or CD8+ T-cell depletion we injected 100 μg/100 μL YTS191L.12 or YTS169.4.2.1 mAb, respectively, starting 1 day before tumor inoculation and continuing every other day for the duration of the experiment. ZOL (0.75 μg/mouse) was administered s.c. 10 and 4 days before tumor inoculation, and mice were sacrificed 2 weeks later. This dosing of ZOL was designed to produce drug levels similar to those achieved with the clinical dosing regimen of 4 mg Zometa for the treatment of bone metastases (22).

Bone histomorphometry

Mouse tibias were decalcified and processed for bone histomorphometry as described previously (21). Because PLCγ2−/− and Lyn−/− mice have different basal bone mass compared with wild-type (WT) mice, we calculated bone loss as percentage of bone volume (BV)/total bone volume (TV) relative to their tumor-free controls (arbitrarily set at 100%) using Bioquant Osteo.

Bone marrow transplantation

Female, 5-week-old C57BL/6 mice were lethally irradiated using a 137Cs source with 900 rads to generate recipient mice. Bone marrow was harvested from 6-week-old, female PLCγ2−/− and Lyn−/− mice or WT littermates, suspended in PBS, and 200 μL containing a concentration of 107 cells was injected into the lateral tail vein of recipient mice to generate PLCγ2−/− and Lyn−/− radiation chimeras and WT controls.

DC generation

DCs were generated from bone marrow progenitor cells from 5- to 8-week-old C57BL/6 mice, as previously described (23; Supplementary Data). DCs were matured with 1 μg/mL lipopolysaccharide (LPS) overnight, and upregulation of mature DC markers was confirmed by fluorescence-activated cell sorting (FACS).

T-cell adoptive transfer

CD8+ T cell were magnetically isolated from WT mice vaccinated with GP10025-33 (10 μg/mL; Bio-Synthesis Inc.) pulsed DCs by using CD8+ T Cell Isolation Kit (Miltenyi Biotec). Reactivity of GP100-specific CD8+ T cells was confirmed in vitro by IFN-γ release following peptide stimulation. A sample of 1 × 106 CD8+ T cells plus 200 μL of interleukin (IL)-2 (2,000 U/mL; Chemicon) were adoptively transferred into tumor-bearing (IT-injected) PLCγ2−/− mice via tail vein. Subsequently, GP10025-33 peptide-pulsed (4 hours), LPS-matured DCs were s.c. administered on the day of the adoptive transfer to further activate GP100-specific CD8+ T cells. Tumor-bearing WT and PLCγ2−/− mice receiving IL-2 alone were used as controls. Tumor growth was monitored on days 8, 10, and 12 by bioluminescence imaging (BLI).

Detection of GP10025-33-specific CD8+ T cell

Spleens were isolated from B16-FL s.c. injected mice. Spleenocytes were cultured with 10 μg/mL GP10025-33 peptide, in
the presence of Golgistop (Becton Dickinson), to inhibit cytokine secretion. After 8 hours, samples were stained with α-CD8-fluorescein isothiocyanate (FITC) Ab (Becton Dickinson), fixed, permeabilized using Cytofix/Cytoperm Kit (Becton Dickinson), co-stained with R-phycoerythrin (PE)-conjugated α-IFN-γ Ab, and analyzed by FACS.

Cytometric bead array
A sample of 5 × 10^6 splenocytes from B16-FL s.c.-injected mice was plated in a 96-well/plate with 200 µL of RPMI-1640 and 10% mouse serum. Cells were restimulated with irradiated B16-FL (2,500 rads) for 3 days. Supernatants were harvested and IFN-γ production was assessed by FACS analysis using the Tγ1/γ2 Assay Kit (Becton Dickinson) according to the manufacturer’s instructions.

Statistical analysis
For each in vivo experiment, 4 to 6 mice per group were used. Experiments were done in triplicate and analyzed using Student’s t-test. In calculating 2-tailed significance levels for equality of means, equal variances were assumed for the 2 populations. Results were considered significant at P < 0.05, and are indicated with an asterisk (*).

Results
PLCy2^−/− mice have increased bone tumor burden despite defective OC function
Deletion of PLCγ2 in mice results in an osteopetrotic phenotype due to OC defects (14, 15). Based on the tumor/bone vicious cycle model in which OC activity is required to sustain tumor growth in bone, we predicted that PLCγ2^−/− mice would be protected from bone metastases. To test this hypothesis, we injected luciferase-labeled B16 cells (B16-FL) IT, to test tumor growth in bone, or into the left cardiac ventricle (LV), to test tumor tropism to bone, of 6-week-old PLCγ2^−/− and WT littermates. Presence of bone tumors was measured by BLI 8 and 10 days after tumor inoculation, and by histologic examination on day 12. Unexpectedly, despite the OC defect, PLCγ2^−/− mice showed significantly increased tumor burden in bone (Fig. 1A). Histologic analysis confirmed the increase in tumor area in PLCγ2^−/− bones (Fig. 1B and C).

Figure 1. PLCγ2^−/− mice display increased tumor burden in bone, but not tumor-associated bone loss. B16-FL cells were injected LV or IT into 6-week-old WT and PLCγ2^−/− littermates. A, BLI at days 8 and 10 showed higher tumor burden in PLCγ2^−/− mice (LV: *P < 0.001 vs. WT mice; IT: *P < 0.05 vs. WT mice, n = 4). B, tartrate-resistant acid phosphatase (TRAP)-stained sections from WT and PLCγ2^−/− tibiae showed OCs (red cells in 2.5× magnification insert) and tumor cells (T). C, histologic analysis of tumor area/total bone area showed a significant increase in tumor growth in PLCγ2^−/− mice (*P < 0.05 vs. WT) despite decreased OC number/bone perimeter (D: *P < 0.05 vs. WT). E, bone loss was calculated as the percentage of BV/TV relative to tumor-free controls (CTR). Although WT mice underwent 50% bone loss after tumor injection, only less than 20% bone loss occurred in PLCγ2^−/− mice (*P < 0.05 vs. WT). F, increased bone loss in WT mice was further confirmed by higher CTX levels. Data are expressed as fold induction from baseline, arbitrarily set as 1, and significant lower CTX levels in PLCγ2^−/− mice are indicated (*P < 0.05 vs. WT; n = 5 for WT and n = 4 for PLCγ2^−/−).
These results were also replicated using non-labeled parental B16 cells (data not shown), indicating that the presence of the luciferase was not responsible for the altered tumor growth.

Interestingly, consistent with impaired basal osteoclastogenesis, tumor-bearing PLCγ2−/− animals continued to display a 3-fold decrease in OC number compared with equally treated WT littermates (Fig. 1D). Despite the increase in tumor burden, PLCγ2−/− mice were still protected from tumor-associated bone destruction as determined by more than 80% of remaining trabecular BV/TV relative to their tumor-free controls. In contrast, tumor-bearing WT mice displayed approximately 50% reduction in BV/TV (Fig. 1E). Decreased tumor-associated bone erosion in PLCγ2−/− mice was further confirmed by low serum levels of Collagen type I fragment (CTX), a marker of tumor-associated bone erosion in approximately 50% reduction in BV/TV (Fig. 1E). Decreased free controls. In contrast, tumor-bearing WT mice displayed approximately 50% reduction in BV/TV (Fig. 1E). Decreased tumor-associated bone erosion in PLCγ2−/− mice was further confirmed by low serum levels of Collagen type I fragment (CTX), a marker of tumor-associated bone erosion in approximately 50% reduction in BV/TV (Fig. 1E).

Increased bone tumor burden in PLCγ2−/− mice is transplantable

Because PLCγ2−/− mice have broadly compromised myeloid and NK cell functions (10–13), we explored whether the increased bone tumor burden in PLCγ2−/− mice was mediated by cells of the hematopoietic compartment. We transplanted bone marrow cells from WT or PLCγ2−/− donors into lethally irradiated 5-week-old C57BL/6 WT mice. Three weeks after transplantation, B16-FL cells were inoculated IV. Similar to PLCγ2−/− mice, tumor growth in bone was increased in WT mice carrying PLCγ2−/− marrow cells (PLCγ2−/− > WT), as determined by BLI (Fig. 2A) and histologic analysis (Fig. 2B and C). PLCγ2−/− > WT-transplanted mice were protected from tumor-induced bone loss, and the number of OCs per bone perimeter was approximately 4-fold less compared with WT > WT transplants (Fig. 2D and E). Based on the dysfunctional PLCγ2−/− OC phenotype, these findings indicate that cells of hematopoietic origin, other than OCs, contributed to enhance tumor growth in the bone of PLCγ2−/− mice.

*Lyn−/− mice are protected from bone tumor growth despite increased OC responsiveness*

To determine whether an aberrant immune condition might contribute to regulation of tumor growth in bone regardless of the OC status, we turned to Lyn−/− mice, characterized by enhanced myeloid cell and OC functionality.

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**Figure 2.** Increased bone tumor burden in PLCγ2−/− mice is transplantable. Three weeks after transplantation, B16-FL were injected IV into WT mice transplanted with WT (WT > WT) or with PLCγ2−/− (PLCγ2−/− > WT) bone marrow cells. A and B, BLI and TRAP-stained long bone sections showed increased tumor growth in bone of PLCγ2−/− > WT (**, *P < 0.01 vs. WT > WT mice; n = 6; OCs are visualized in insert 2.5 × magnification). Histomorphometry in PLCγ2−/− > WT transplanted animals revealed (C) increased tumor area/total bone area (**, *P < 0.05 vs. WT > WT mice), (D) reduced OC number (**, *P < 0.05 vs. WT > WT mice), and (E) reduced tumor-induced bone loss (**, *P < 0.05 vs. WT > WT mice).
In contrast to PLCγ2−/− animals, Lyn−/− mice displayed significant inhibition of tumor growth in bone following either intratibial or intracardiac tumor inoculation (Fig. 3A and B). Analysis of tumor area/total bone area confirmed a 4-fold decrease in tumor burden in Lyn−/− mice (Fig. 3C). However, despite the significantly reduced bone tumor burden, Lyn−/− mice had increased OC numbers as WT mice (Fig. 3D and E). Consistent with this finding, Lyn−/− mice had elevated serum CTX levels (Fig. 3F). These data indicated that increased OC responsiveness is not sufficient to enhance tumor growth in bone of Lyn−/− mice.

Reduction in bone tumor burden in Lyn−/− is transplanted

To determine whether cells of the hematopoietic compartment were responsible for reduced bone tumor burden in Lyn−/− mice, we carried out bone marrow transplants, in which WT recipient mice carried WT (WT > WT) or Lyn−/− (Lyn−/− > WT) marrow cells. We found that Lyn−/− > WT transplanted animals were protected from B16-FL tumor growth in bone, as determined by BLI (Fig. 4A) and histomorphometric analysis (Fig. 4B). Similar to Lyn−/− mice, Lyn−/− > WT transplants displayed disproportionate bone loss for their tumor burden, showing similar OC number and percentage of BV/TV as WT > WT transplants despite the 4-fold decrease in bone tumor burden (Fig. 4C–E). Due to the hyperactive Lyn−/− OC phenotype, these data indicate that cells of hematopoietic origin, other than OCs, contribute to reduced tumor growth in bone of Lyn−/− mice.

Modulation of T-cell activation alters tumor burden in bone independent of OC activity

T cells are known to be modulators of antitumor immune responses (24). To determine whether T cells might be regulating tumor growth in bone, we injected WT mice with the α-CTLA4 Ab, which enhances T-cell proliferation and/or function (25), following intracardiac inoculation of B16-FL. α-CTLA4 Ab significantly suppressed tumor growth in bone, as shown by BLI and histomorphometric analysis (Fig. 5A and B). Conversely, Nude mice, which lack all T-cell subsets, had enhanced tumor growth in bone compared with control animals (Fig. 5C). Histologic analysis confirmed an abundance of tumor cells in the bones of Nude mice compared with their controls (Fig. 5D). To determine which T-cell subset might be regulating tumor growth in bone, we turned to MHCI−/− mice, which do not have CD8+ T cells, and MHCI−/− mice, which lack CD4+ T cells. Absence of either CD4+ or CD8+ T cells significantly increased B16-FL tumor growth in bone following intracardiac inoculation. MHCI−/− mice had >4-fold increase and MHCI−/−/− mice had >2.5-fold increase in bone tumor burden by day 10 as compared with WT mice (Fig. 5E and F).

Because the tumor/bone vicious cycle model considers OC activity to be central to tumor proliferation in bone, we sought to determine the relative contribution of OCs and T cells in this process. We tested the efficacy of ZOL antiresorptive
treatment in providing protection from tumor growth in bone in mice depleted of CD4+ or CD8+ T cells. WT mice were treated with ZOL 10 and 4 days before B16-FL intratibial inoculation, and α-CD4+ or α-CD8+ T-cell–depleting Abs were administered 1 day before and every 2 days after injection of cancer cells. Efficiency of T-cell depletion was confirmed at the end of the experiment by FACS analysis. Despite blockade of tumor-associated bone loss (Supplementary Data 1), we found that the antitumor effect of ZOL was significantly reduced in CD8−/− mice, we isolated spleens from T-cell abnormalities are responsible for tumor growth in bone of PLCγ2−/− and Lyn−/− mice

Despite a lack of PLCγ2 and Lyn expression in T cells, myeloid cell abnormalities [impaired DC function in PLCγ2−/− (23), myeloid hyperactivity in Lyn−/− mice (19)] could induce aberrant T-cell–mediated antitumor responses. To determine the presence of activated T cells in tumor-bearing mice, we isolated spleens from PLCγ2−/− and Lyn−/− mice 2 weeks after B16-FL s.c inoculation. We examined IFN-γ release by activated T cells, restimulated in vitro for 3 days with irradiated B16-FL cells. This assay showed impaired IFN-γ release in PLCγ2−/− and hyper-production in Lyn−/− cultures compared with WT cultures (Fig. 6A). Furthermore, IFN-γ release was observed in response to parental B16 cells (data not shown). To further determine whether the antitumor T-cell response was specific to the B16 cell line, splenocytes isolated from tumor-bearing mice were stimulated for 8 hours with tumor antigen GP100, expressed by B16 cells. FACS analysis showed that the percentage of CD8+ IFN-γ producing PLCγ2−/− T cells responding to GP100 was significantly less than those in WT mice, whereas in Lyn−/− mice it was 2-fold more than that in WT mice (Fig. 6B), supporting the conclusion that PLCγ2−/− and Lyn−/− mice have altered T-cell responses to tumor.

To further determine whether T-cell abnormalities were responsible for increased bone tumor burden in PLCγ2−/− mice, we adoptively transferred tumor-specific cytotoxic CD8+ T cells into PLCγ2−/− mice. Because B16 melanoma cells express the antigen GP100, we generated GP100-reactive CD8+ T cells by injecting twice GP10025–35 peptide-pulsed...
mature DCs into WT mice. Two weeks later, CD8+ T cells were isolated from spleens and their functionality was confirmed in vitro in response to the antigen (Supplementary Data 2). GP100-reactive CD8+ T cells were then adoptively transferred into PLCy2−/− mice IT injected with B16-FL cells, along with GP100-pulsed WT DCs and IL-2. Mice injected with IL-2 alone were used as controls. IL-2 itself did not affect tumor growth in this model (Supplementary Data 3). Adoptive transfer of GP100-reactive CD8+ T cells reduced tumor burden in PLCy2−/− mice compared with null mice receiving IL-2 alone (Fig. 6C and D). Conversely, Ab-mediated CD8+ T-cell depletion in tumor-bearing Lyn−/− mice enhanced tumor burden in bone by BLI, although CD4+ depletion did not cause increased tumor burden (Fig. 6E). To simultaneously measure tumor burden and confirm CD4/CD8 depletion, we conducted FACS analysis on tumor-bearing bones and found that CD8+ depletion led to more tumor growth in bone marrow, as determined by TRP-1+ staining, a surface marker of B16 cells (Fig. 6F).
Thus, CD8+ T cells contributed to the regulation of tumor growth in bone in PLCγ2−/− and Lyn−/− mice regardless of OC functionality.

Discussion

Animal studies using human breast cancer cells injected in immunocompromised mice strongly support a tumor/bone vicious cycle model in which blockade of OC function can reduce tumor growth in bone and prevent associated bone loss (26, 27). ZOL, a potent inhibitor of OC activity, is widely used in the clinic to treat patients with bone metastases. ZOL confers protection from tumor-induced bone loss and reduces skeletal complications such as bone pain, pathologic fractures, bone surgery, and hypercalcemia. Unfortunately, one third of patients with bone metastases who respond to ZOL treatment develop further skeletal-related events within 2 years of initiating the antiresorptive therapy (5). Therefore, the tumor/bone vicious cycle model must be revisited to include other bone marrow–derived cells, in addition to the OCs that can participate in restraining or encouraging the expansion of tumor in the bone microenvironment, thus altering the efficacy of antiresorptive therapies. By using immunocompetent mice, we showed that alterations in T-cell functions can bypass the requirement for OCs in tumor growth within bone and reduce the efficiency of ZOL treatment.

PLCγ2 plays a critical role in bone homeostasis and immune function (12, 13, 15, 23, 28–30). Previous studies using a tumor-rejection assay, in which MHCI−/− deficient susceptible target cells were injected intraperitoneally in WT or PLCγ2−/− mice, suggested compromised tumor elimination in PLCγ2−/− mice (31). Consistent with this finding, we observed increased s.c. tumor growth and elevated metastatic dissemination following intravenous injection of B16-FL cells, showing broadly impaired antitumor immune responses (Supplementary Data 4). However, in bone, the OC resorptive activity is thought to be central in regulating metastatic dissemination and tumor burden (1). By measuring direct tumor growth in bone or tropism of tumor cells to bone, we show that PLCγ2−/− OC defect is not sufficient to prevent bone metastases, thus challenging the current tumor/bone vicious cycle model.

Mirroring the PLCγ2−/− phenotype, deletion of Lyn, a negative regulator of PLCγ activity, leads to increased immune activation (18, 19, 32, 33). In agreement with a hyperactive
immune condition, Lyn−/− mice are protected from s.c. tumor growth and metastatic dissemination. Lyn−/− mice are also more prone to bone loss due to increased OC number (20). Although more numerous OCs are expected to create a highly favorable environment for tumor growth in bone, our data show that increased immune activation is sufficient to counteract the OC-mediated pro-tumor effect, thus protecting Lyn−/− mice from bone metastases.

Immune cells have emerged as significant regulators of primary cancer development as well as metastasis into ectopic tissues. Tumor antigens are presented to naïve T cells, leading to activation of CD4+ and CD8+ T cells. Subsequently, tumor-specific T cells home to tumor sites, where they kill antigen-positive tumor cells (34). However, tumor cells can also escape the immune system, developing the ability to grow in an immunologically intact host. Several factors released by tumor cells can directly or indirectly induce T-cell immune suppression. TGF-β, released by OCs (35), has positive proliferative effects on tumor cells but also very potent anti-inflammatory activity (7). Thus, the antitumor effects of OC blockade and/or inhibition of TGF-β pathway could, at least in part, depend on interference with T-cell–mediated antitumor responses. Furthermore, we showed that global T-cell deficiency increases, whereas T-cell activation protects from, growth of B16-FL cells in bone. CD8+, and to a lesser extent CD4+, T cells have antitumor capabilities. However, T-cell manipulations do not seem to affect the capacity of the cancer cells to reach bone, presumably because T-cell activation occurs after the cells have arrived in the bone microenvironment.

Importantly, we found that tumor growth in bone in CD8+ T-cell–depleted mice occurs independently of ZOL-mediated OC blockade. This finding is in apparent contradiction with previous reports showing that ZOL reduces bone tumor burden in immunocompromised animals. However, a direct comparison between the antitumor effect of ZOL in immunocompetent and immunodeficient mice has never been reported. ZOL is effective in decreasing tumor burden in T-cell–deficient mice compared with untreated immunodeficient controls. However, the antitumor effect of ZOL in CD8+ T-cell–depleted mice is significantly reduced compared with immunocompetent WT animals treated with the antiresorptive agent. This observation has important clinical relevance because ZOL is the current treatment of choice for patients with bone metastases. Although ZOL has been shown to successfully reduce incidence of bone metastases, not all patients respond well (5). Considering that cancer patients often have immune imbalances, our data suggest that reduced T-cell antitumor immune responses could be responsible for tumor growth in bone despite ZOL-mediated OC blockade.

Altered T-cell activation is also central to the tumor/bone phenotype observed in PLCγ2−/− and Lyn−/− mice. Although B16 is a poorly immunogenic cell line, it has been reported that it can induce a modest antitumor T-cell response (9). We further confirmed this observation by identifying CD8+ T cells that can respond to the endogenous B16 antigen, GP100. Specifically, we observed enhanced GP100-specific CD8+ T cells in tumor-bearing Lyn−/− mice but a blunted response in PLCγ2−/− animals. Release of IFN-γ by these tumor-specific CD8+ T cells was also enhanced in Lyn−/− mice, although this is barely detectable in PLCγ2−/− animals. Importantly, depletion of CD8+ T cells restores tumor growth in bone in Lyn−/− mice, whereas adoptive transfer of tumor-specific WT CD8+ T cells into PLCγ2−/− mice reduces bone tumor burden.

Despite not being expressed in T cells, both PLCγ2 and Lyn indirectly govern T-cell activation by modulating myeloid cell functions. Defective DC-mediated antigen presentation (13, 23) probably underlies the reduced number of tumor-specific CD8+ T cell in PLCγ2−/− mice. Because of impaired DC functions, PLCγ2−/− mice are protected from antigen-induced arthritis, an inflammatory condition strongly dependent on T-cell activation (23). It is very likely that DC defects impair T-cell activation in PLCγ2−/− tumor-bearing mice, thus enhancing bone tumor burden even in the absence of functional OCs. An additional explanation for the tumor phenotype in PLCγ2−/− mice could be defective NK-cell cytotoxicity (12). It is unlikely that NK-cell abnormalities are primarily responsible for increased tumor growth in these mice, because NK-cell depletion in WT mice can only slightly increase bone tumor burden without reaching the levels observed in PLCγ2−/− animals (Supplementary Data 5). Conversely, Lyn−/− mice have expanded macrophage and DC populations (18, 36) as well as hyperactive myeloid cells that augment T-cell responses and IFN-γ production (19). Indeed, we found that tumor-bearing Lyn−/− mice display increased tumor-antigen–specific CD8+ T cells in spleen compared with WT. Therefore, similar to PLCγ2 deficiency, absence of Lyn in the myeloid population is likely to be responsible for enhanced T-cell antitumor responses.

To the best of our knowledge, this report is the first to analyze the relative contribution of bone and immune cells in development of bone metastases. We now provide compelling evidence that a condition of immune deficiency can interfere with the antitumor effects of OC blockade. We used both genetic models of immune and OC modulation (Lyn and PLCγ2), as well as pharmacologic inhibition of OCs and T cells. These findings suggest that the immune status can affect the efficacy of antiresorptive treatments in reducing bone metastases and emphasize the need to further explore the beneficial effects of combined T-cell stimulation and antiresorptive therapies in patients with bone metastases.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We gratefully thank Tonia Thompson for research administration and Karon Hertlein for secretarial support (Department of Orthopaedics, Washington University). This work was supported by NIH to R. Faccio (grant no. ROI AR53628) and ARRA to R. Faccio (grant no. AR052705 and EB007568), A.C. Hirbe (grant no. T32HL007088), and K. Weilbaecher (grant no. R01 52152) and the Barnes-Jewish Foundation to D.V. Novack (grant AR53628) and ARRA to R. Faccio (grant no. 63181), NIH to D.V. Novack (grant R01 52152). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 28, 2010; revised April 6, 2011; accepted May 12, 2011; published OnlineFirst May 20, 2011.
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Correction: CD8⁺ T Cells Regulate Bone Tumor Burden Independent of Osteoclast Resorption

In this article (Cancer Res 2011;71:4799–808), which was published in the July 15, 2011, issue of Cancer Research (1), the author listing was incomplete. Specifically, the listing was missing an institution. In addition, a funding source and grant number were missing from the Acknowledgments section.

The correct author listing and missing acknowledgments are provided. The authors regret these errors.

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Acknowledgments
This work was also supported by NIH to David Piwnica-Worms (grant no. P50CA94056). The histologic and mCT analysis was supported by The Center for Musculoskeletal Biology and Medicine, Award Number P30AR057235 from the National Institute of Arthritis, Musculoskeletal and Skin Diseases.

Reference

Published online January 17, 2012.
doi: 10.1158/0008-5472.CAN-11-3840
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Cancer Res 2011;71:4799-4808. Published OnlineFirst May 20, 2011.

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