Myeloid-Derived Suppressor Cells Function as Novel Osteoclast Progenitors Enhancing Bone Loss in Breast Cancer

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Abstract

Enhanced bone destruction is a hallmark of various carcinomas such as breast cancer, where osteolytic bone metastasis is associated with increased morbidity and mortality. Immune cells contribute to osteolysis in cancer growth, but the factors contributing to aggressive bone destruction are not well understood. In this study, we show the importance of myeloid-derived suppressor cells (MDSC) in this process at bone metastatic sites. Because MDSC originate from the same myeloid lineage as macrophages, which are osteoclast precursors, we hypothesized that MDSC may undergo osteoclast differentiation and contribute to enhanced bone destruction and tumor growth. Using an immunocompetent mouse model of breast cancer bone metastasis, we confirmed that MDSC isolated from the tumor-bone microenvironment differentiated into functional osteoclasts both in vitro and in vivo. Mechanistic investigations revealed that nitric oxide signaling was critical for differentiation of MDSC into osteoclasts. Remarkably, osteoclast differentiation did not occur in MDSC isolated from control or tumor-bearing mice that lacked bone metastasis, signifying the essential cross-talk between tumor cells and myeloid progenitors in the bone microenvironment as a requirement for osteoclast differentiation of MDSC. Overall, our results identify a wholly new facet to the multifunctionality of MDSC in driving tumor progression, in this case as a novel osteoclast progenitor that specifically drives bone metastasis during cancer progression.

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Introduction

Myeloid-derived suppressor cells (MDSC) play a pivotal role in cancer progression by suppressing both innate as well as adaptive immunity (1, 2). Accumulation of MDSC has been reported in almost all cancers, both in preclinical models and human patients (3–5). Tumor progression is associated with gradual accumulation of MDSC in the blood, lymph nodes, and spleen. MDSC accumulate in the primary tumor as well as at the metastatic tumor sites. Recent studies have substantiated that MDSC inhibit the antitumor immunity and promote tumor expansion and metastasis at distant sites, including the bone (6, 7). An increase in the infiltration of MDSC in the bone marrow has also been reported in tumor-bearing mice. Further, elimination or reduction in MDSC numbers significantly delays and limits tumor growth in the bone (8).

Bone is 1 of the major metastatic sites for carcinomas of the breast, prostate, and lung as well as multiple myeloma (9). Approximately 65% to 80% of patients with disseminated breast disease show skeletal metastasis (10, 11). In order for cancer to establish in the bone, tumor cells secrete a variety of growth factors and cytokines that induce differentiation and activation of osteoclasts, which degrade bone, facilitating tumor growth. During normal bone remodeling, macrophages and monocytes remain the major precursors of osteoclasts (12). Stimulation of these cells in vitro with macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) induces their differentiation into multinucleated osteoclasts.

MDSC are a heterogeneous population comprising of immature myeloid cells (IMC). Under normal conditions, the IMC differentiate into mature macrophages, dendritic cells, and granulocytes. However, in pathologic conditions including cancer, IMC differentiation is inhibited resulting in the accumulation of immunosuppressive MDSC (13). Because MDSC are progenitors of macrophages, which differentiate into osteoclasts, and MDSC numbers are elevated in breast cancer patients, we sought to determine if MDSC in the tumor microenvironment within the bone undergo osteoclast differentiation and contribute to enhanced bone destruction and tumor growth in an immunocompetent mouse model of breast cancer.

Results of the studies clearly showed that MDSC from tumor-bearing mice with bone metastasis differentiate into...
functional osteoclasts. Further, investigation of the underlying molecular mechanisms of MDSC differentiation into osteoclasts indicates nitric oxide (NO) signaling as the key pathway regulating the differentiation. Collectively, the present study reports a novel role for MDSC as osteoclast-forming cells, contributing to enhanced osteolysis during breast cancer bone dissemination. As MDSC are elevated in other osteolytic cancers, it remains possible that such osteolytic potential of MDSC may play a vital role in increased bone destruction and growth of tumors in the bone microenvironment, and targeting MDSC can be an effective strategy to reduce skeletal morbidity in osteolytic cancers.

Materials and Methods

Isolation of MDSC

Female BALB/c mice were injected with $10^5$ 4T1(fLuc) cells, a kind gift from Dr. Xiaoyuan Chen (Stanford University), via the intracardiac route. After 10 to 12 days, when bone metastases were observed by noninvasive luciferase imaging, mice were sacrificed and bone marrow cells were collected. RBCs were lysed using the ACK RBC lysis buffer. Cells were incubated with Fc block for 15 minutes at 4°C. For sorting of total MDSC population (MDSC<sub>bone</sub>), cells were stained with APC-conjugated anti-CD11b antibody and PE-Cy7-conjugated Gr-1 antibody (eBioscience) for 30 minutes at 4°C. After washing with sterile PBS, CD11b<sup>−</sup>Gr-1<sup>−</sup> MDSC were sorted using BD FACS ARIA III (BD Biosciences). CD11b<sup>−</sup>Gr-1<sup>−</sup> MDSC were further stained for additional markers including CD115 PE, F4/80 PE Cy5, CD80 FITC, Ly6C Per CP Cy5.5, and Ly6G PE antibodies. MDSC were isolated from inguinal, axillary, brachial, and thymus lymph nodes (MDSC<sub>lymph nodes</sub>), lungs (MDSC<sub>lung</sub>), blood (MDSC<sub>blood</sub>), and spleen (MDSC<sub>spleen</sub>) of tumor-challenged mice showing bone metastasis. MDSC were also isolated from tumor-bearing mice but without visible bone metastasis (MDSC<sub>non bone</sub> control) and from age-matched control mice (MDSC<sub>control</sub>). Expression of arginase and iNOS was detected by flow cytometry. Cells were stained with antibody to iNOS-PE antibody and arginase antibody followed with Alexa 488-conjugated secondary antibody (eBioscience).

In vitro osteoclastogenesis assay

For the assay, $10^5$ MDSC were seeded in 200 μL α-MEM medium in a 48-well plate (Corning Inc.) in the presence of 44 ng/mL M-CSF and 100 ng/mL RANKL (kind gifts from Dr. Xu Feng, The University of Alabama at Birmingham, Birmingham, AL; ref. 14). For some experiments, 25 μmol/L NG-monomethyl-L-arginine, monooacetate salt (L-NMMA) was added to the MDSC cultures (a kind gift from Dr. J. Zmijewski, The University of Alabama at Birmingham, Birmingham, AL; ref. 15). Media was changed every 2 days. On days 8 to 9, the presence of osteoclasts was detected by tartarate-resistant acid phosphatase (TRAP) staining. Briefly, media was removed carefully and cells were washed once in PBS before fixing in 0.2 mol/L acetate buffer for 20 minutes at room temperature (RT). At the end of incubation, cells were stained in 0.2 mmol/L acetate buffer containing 0.5 mg/mL naphthol AS-MX phosphate and 1.1 mg/mL fast-red TR salt (Sigma-Aldrich) for 30 to 45 minutes at 37°C till color change was noted. Nuclei were stained using hematoxylin for 30 seconds. Cells were washed twice in PBS and suspended in PBS (16). Cells showing 3 or more nuclei were considered as osteoclast. As a positive control, bone marrow-derived macrophages (BMM) from tumor-bearing mice were cultured under identical conditions. All assays were conducted in triplicate.

In vitro bone resorption assay

MDSC and BMMs ($10^5$ cells/well) were seeded on bovine cortical bone slices plated in 24-well culture plates and cultured under conditions indicated in individual experiments to promote osteoclast formation and bone resorption. The bone slices were then harvested, and the cells were subsequently removed with 0.25 mol/L ammonium hydroxide and mechanical agitation. Bone slices were analyzed using an Olympus Fluorview 300 Laser Scanning Confocal Microscope. A quantitative analysis of osteolysis was conducted by measuring the percentage of the resorbed areas as compared with the entire bone surface using Adobe Photoshop Software.

In vivo MDSC depletion

To deplete MDSC in vivo, mice were injected intraperitoneally with 1.5 mg gemcitabine (Sigma-Aldrich) twice in the first week and once per week thereafter (17, 18). Treatment was started on day 10 post-4T1(fLuc) challenge, when tumor was established and metastasis to the bone was confirmed by luciferase imaging. Upon sacrifice of mice on day 17 post-tumor challenge, MDSC were sorted from the bone marrow of gemcitabine-treated mice and were differentiated into osteoclasts as described earlier. MDSC from non-gemcitabine-treated mice were included as controls.

In vivo MDSC transfer assay

MDSC were isolated from the bone of tumor-bearing mice with bone metastasis (MDSC<sub>bone</sub>), as described earlier. A total of $2.5 \times 10^5$ MDSC in 50 μL PBS were injected in the long bones of BALB/c mice and was followed by a second injection of MDSC after 4 days. As a control, PBS was injected. Alternatively, before injection of MDSC<sub>bone</sub> in vivo, mice were injected with 1400W (10 mg/kg body weight; Cayman Chemical Company) intraperitoneally, 2 days before the MDSC<sub>bone</sub> injection. Injections were given every 2 days till the end of the experiment. On day 10, mice were sacrificed and femur and tibia were collected and fixed in 4% buffered-formalin for 2 days and were subjected to micro-CT analysis (Micro-CT40; SCANCO Medical). The formalin-fixed bones were then decalcified in 2.5% EDTA, at pH 8.0, for 2 weeks. Thereafter, 5-μm paraffin-embedded sections were used for histology.

To show that transferred MDSC differentiated into osteoclasts in vivo, MDSC<sub>bone</sub> from tumor-challenged BALB/c mice (CD45.2 genotype) were injected into long bones of congenic, non–tumor-challenged CD45.1<sup>−</sup> female BALB/c mice as described earlier. After 8 days, mice were sacrificed and bone marrow cells were collected. Cells were stained with CD45.2-PE antibody to detect the presence of adoptively transferred MDSC. Cells were also stained with antibody to...
The presence of osteoclasts within the bone sections was detected by TRAP staining as described previously (16). All the microscopic images were obtained using a Leica DMI4000B microscope, attached to a Leica DFC500 digital camera. The microscopic images were obtained using a Leica DMI4000B

Sawant et al. (2013) conducted an in vitro suppression assay following sacrifice of tumor-challenged mice with bone metastasis. MDSC were sorted from the bone marrow (MDSC(M+/bone mets)) and CD4⁺ T cells were sorted from the spleen. CD4⁺ T cells were then labeled with carboxyfluorescein diacetate and succinimidyl ester (CFSE) according to the manufacturer’s instructions (Molecular Probes). Following labeling, CFSE-CD4⁺ T cells were cultured with MDSC in 1:1 ratio in media containing 0.75 μg/mL anti-CD3 and 4 μg/mL anti-CD28 antibodies together with 50 μmol/L β-mercaptoethanol for 72 hours. As a control, CD4⁺ T cells were cultured in the absence of MDSC. After 72 hours, cells were harvested and the presence of CD4⁺ T cells labeled with CFSE was detected by flow cytometry.

Immunohistochemistry

The presence of osteoclasts within the bone sections was detected by TRAP staining as described previously (16). All the microscopic images were obtained using a Leica DMi4000B microscope, attached to a Leica DFC500 digital camera. The LASv3.6.0 software was used to optimize picture quality. A region of interest was selected that was exactly 250μm distal to the growth plate, and extending 1 mm downward (thereby avoiding the primary spongiosa) through the metaphysis of the femur and tibia. Standard bone histomorphometry was carried out by using Bioquant Image Analysis software (R&M Bio-metrics; ref. 19). The number of osteoclasts per bone surface was calculated.

Semiquantitative reverse transcription PCR

Total RNA was isolated from MDSC and BMMs using TRIzol reagent (Invitrogen). Then, 1 μg of total RNA was reversed-transcribed to cDNA with an iScript cDNA synthesis kit (Bio-Rad). PCR amplification was carried out using primers specific for MMP9, TRAP, carbonic anhydrase II (Car2), cathepsin K (Ctsk), and GAPDH using the following conditions: preheating at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds in a 30-cycle reaction, followed by final extension at 72°C for 5 minutes. PCR was carried out with the Dream Taq Green 2× PCR mix from Fermentas in a 50-μL reaction volume. The PCR primer sequences used are

MMP-9
- Forward 5’-CTTCTTCTCTGAGCTAAATG-3’
- Reverse 5’-CATTCTGGAACACTACGCGC-3’
- Car2
- Forward 5’-AGAGAAGTGGAACAAGACTT-3’
- Reverse 5’-CTTCTTCTTGAGCTACGAGC-3’
- Ctsk
- Forward 5’-GATGGTCACTTAGGGGAC-3’
- Reverse 5’-CATATCTCTTTGTTCCCGAC-3’

TRAP
- Forward 5’-GCAAGATGAGTTTATGGGTC-3’
- Reverse 5’-CAGAGAGATGGAAGTGCACAGC-3’
- GAPDH
- Forward 5’-ACATCTACTCTGTACCTCAGTG-3’
- Reverse 5’-TCATTTGAGAAGTCCGAC-3’

Thirty microliters of PCR mixture was separated on 2% agarose gel for electrophoretic analysis. All semiquantitative reverse transcription PCR (RT-PCR) assays were independently conducted at least 3 times.

Western blot analysis

MDSC(M+/bone mets), MDSC(M+/bone mets), MDSC(Lymph nodes), MDSC(Lungs), MDSC(Blood), and MDSC(Spleen) were sorted from tumor-bearing mice as described earlier. Whole-cell lysates were prepared using RIPA buffer containing protease and phosphatase inhibitors according to the manufacturer’s instructions (Thermo Scientific). Protein concentrations were measured using a commercially available BCA protein assay kit (Thermo Scientific). For each sample, 100 μg of protein was used to detect the levels of HIF-1α, Phospho-ERK, Phospho-phosphoinositide 3 (PI3) kinase, Phospho-Akt, Total ERK, Total-Pi3 kinase, Total-Akt, and β-actin by Western blot analysis. Following denaturation, the samples were separated on a 10% polyacrylamide gel and transferred to nitrocellulose membranes (Millipore) followed by blocking with 2% non-fat milk and incubation with primary antibodies, overnight at 4°C. The β-actin antibody was used as a loading control. After washing, the primary antibody with 1× tris-buffered saline with Tween-20 (TBST; 3 × 10 minutes) and suitable secondary antibodies, conjugated to horseradish peroxidase, were applied for 1 hour at room temperature, then washed with TBST (3 × 10 minutes) and blots were then incubated with enhanced chemiluminescence reagent (GE Healthcare Life Sciences) according to the manufacturer’s instructions and data was developed on a Fuji LAS-3000 chemiluminescence developer. All the primary antibodies were obtained from Cell Signaling and were used at the recommended dilutions. A donkey anti-rabbit secondary antibody was used for all the proteins except for HIF-1α, for which a sheep anti-mouse secondary antibody was used. Both the secondary antibodies were purchased from GE Healthcare Life Sciences.

Measurement of nitric oxide

Levels of NO were detected by using 4-amino-5-methylamino-2, 7-difluorofluorescein diacetate (DAF-FM DA; Molecular Probes) reagent and the Griess Reagent (Promega) according to the manufacturer’s instructions. Briefly, MDSC (10⁴ cells/well) were cultured in the presence of RANKL and M-CSF for 3 days as described earlier. As controls, MDSC grown in LPS alone and MDSC grown in LPS together with RANKL and M-CSF were included (20). For detecting NO levels by using the Griess reagent, culture supernatants were collected from MDSC differentiating into osteoclasts at different time points under conditions mentioned earlier. The assay was conducted according to the manufacturer’s instructions and data was normalized to a standard curve (with known concentrations of
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MDSC isolated from the bone marrow of tumor-bearing mice have potential to differentiate into osteoclasts establishing that these are indeed immunosuppressive cells.

Measurement of arginase activity

The concentration of urea, an end product of the arginase pathway, was used as an estimate of the arginase activity in the culture supernatants of MDSC differentiating into osteoclasts. Controls were MDSC cultured in LPS alone and together with RANKL and M-CSF. Detection of urea was carried out using the Quantichrom Urea Assay kit (BioAssay Systems; ref. 20). The results are reported as urea concentrations in media. Results are derived from 3 different samples tested in triplicate.

Measurement of superoxide

Superoxide levels in MDSC differentiating into osteoclasts were detected by flow cytometry by incubating for 20 minutes at room temperature with dihydroxyethidium (DHE, 10 μmol/L; Molecular Probes) according to the manufacturer’s recommendations. Cells were then washed twice in PBS and the percentage of positive cells were determined by flow cytometry (20). As controls, MDSC grown in LPS alone and in LPS together with RANKL and M-CSF were included. The assay was conducted at least 3 times.

Statistical analysis

Data were analyzed by 1-way ANOVA. A Tukey test was also applied for multiple comparisons wherever applicable. Values provided are the mean ± SE, and the differences were considered significant if P was less than 0.05.

Results

Isolation and characterization of MDSC from the breast cancer bone metastasis model

For identifying a possible role of MDSC as osteoclast progenitors, an osteolytic breast cancer cell line constitutively expressing firefly luciferase 4T1(fLuc), syngeneic in BALB/c mice, was injected via the intracardiac route into syngeneic female BALB/c mice. Bone metastasis was confirmed in the tumor-bearing mice after 10 to 12 days post-injection by noninvasive imaging. MDSC populations in the bone marrow were characterized using specific cell surface markers. The CD11b and Gr-1 MDSC phenotype was further confirmed as the population that was CD80<sup>+</sup>, CD115<sup>+</sup>, and F4/80<sup>-</sup> (Supplementary Fig. S1). The absence of F4/80 indicated that these cells were not already committed to differentiate into macrophages. The MDSC were a mixed population of granulocytic Ly6C<sup>+</sup>Ly6G<sup>-</sup> cells and monocytic Ly6C<sup>-</sup>Ly6G<sup>+</sup> MDSC. MDSC were isolated from both bone marrow and lung using the same phenotype. Further, the isolated MDSC actively suppressed proliferation of splenic CD4<sup>+</sup> T cells; thus establishing that these are indeed immunosuppressive cells (Supplementary Fig. S1).

MDSC have potential to differentiate into osteoclasts

To determine if MDSC differentiated into osteoclasts, MDSC isolated from the bone marrow of tumor-bearing mice with bone metastasis (MDSC<sup>+</sup>bone mets<sup>-</sup>) were cultured in medium containing M-CSF and RANKL. MDSC from the lungs of tumor-bearing mice with metastasized tumor (MDSC<sup>+</sup>bone mets<sup>-</sup>) and from bone marrow of tumor-bearing mice but without bone metastasis (MDSC<sup>-</sup>bone mets<sup>-</sup>) were also included in the study. BMs were used as a positive control. The cells were fixed and stained by TRAP after 10 days. Results of this staining indicated that MDSC<sup>+</sup>bone mets<sup>-</sup> stained positively for TRAP as evidence for osteoclast differentiation (Fig. 1A). However, MDSC<sup>+</sup>bone mets<sup>-</sup> and MDSC<sup>-</sup>bone mets<sup>-</sup> did not undergo osteoclast differentiation. MDSC were also isolated from the lymph nodes (MDSC<sup>+</sup>lymph Node<sup>-</sup>), spleen (MDSC<sup>+</sup>spleen), and blood (MDSC<sup>+</sup>blood) of tumor-bearing mice showing bone metastasis. Phenotypically, such MDSC were similar to MDSC<sup>+</sup>bone mets<sup>-</sup> but failed to differentiate into osteoclasts, suggesting that the bone microenvironment is critical for osteoclast differentiation of MDSC (Supplementary Fig. S2).

Further, MDSC<sup>+</sup>bone mets<sup>-</sup> expressed other osteoclast-specific markers, including cathepsin-K, carbonic anhydrase-2, and MMP-9, starting at day 4 of osteoclast differentiation (Fig. 1B). However, MDSC<sup>-</sup>bone mets<sup>-</sup> did not express any of the osteoclast-specific markers. Because MDSC<sup>+</sup>bone mets<sup>-</sup> differentiated into osteoclasts, expression of F4/80, which is a macrophage-specific marker, was detected by flow cytometry. As shown in Supplementary Fig. S3A, MDSC<sup>+</sup>bone mets<sup>-</sup> did not express F4/80 during osteoclast differentiation, which showed that MDSC<sup>+</sup>bone mets<sup>-</sup> did not differentiate into macrophages and, thus, were a true novel population of osteoclast progenitor.

MDSC-derived osteoclasts are functional and capable of bone resorption

Next, we sought to determine if MDSC-derived osteoclasts from bone metastasis are functional. A hallmark of functional osteoclasts is their ability to degrade bone in vitro and in vivo (22, 23). To determine if MDSC-derived osteoclasts were capable of degrading bone, a bone resorption assay was conducted. As shown in Fig. 2, osteoclasts differentiated from MDSC<sup>+</sup>bone mets<sup>-</sup> were functional as they degraded bone, indicated by the presence of numerous resorption pits (Fig. 2). As expected, MDSC from control mice (MDSC<sup>+</sup>control) and MDSC<sup>-</sup>bone mets<sup>-</sup> failed to resorb bone.

MDSC induce bone destruction in vivo

To corroborate the in vitro finding that MDSC<sup>+</sup>bone mets<sup>-</sup> form functional osteoclasts, these MDSC were injected into the tibia of female BALB/c mice. Ten days later, femur and tibia were analyzed by micro-CT and histochemical staining for detecting bone destruction. Mice injected with MDSC<sup>+</sup>bone mets<sup>-</sup> showed significantly more bone destruction compared with the PBS control on micro-CT imaging (Fig. 3A; Supplementary Fig. S3B). Histochemical analysis clearly showed increased osteoclast numbers by the TRAP assay (Fig. 3B&C; Supplementary Fig. S3B).

To confirm that injected MDSC differentiated into osteoclasts in vivo and caused bone destruction, a congenic bone destruction was carried out wherein MDSC<sup>+</sup>bone mets<sup>-</sup> from CD45.2<sup>-</sup> genotype mice were transferred into the tibia of non-
tumor-challenged congenic CD45.1\(^+\) mice. After 8 days, the presence of MDSC\(^{-}\)bone mets\(^+\) was detected in injected tibia by CD45.2 labeling and these MDSC also expressed cathepsin K, an osteoclast marker, and differentiated into osteoclasts in vivo (Fig. 3D).

**NO levels are elevated as MDSC differentiate into osteoclasts**

The mechanisms by which MDSC promote immunosuppression are by increased arginase activity, reactive oxygen species (ROS), and/or NO production (2, 24, 25).

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**Figure 1.** MDSC from bone metastasis are primed for osteoclast differentiation. A, MDSC\(^{-}\)bone mets\(^+\), MDSC\(^{-}\)bone mets\(^-\), and MDSC\(^+\)lung mets\(^-\) were cultured in the presence of M-CSF and RANKL for osteoclast differentiation. Bone marrow-derived macrophages were used as a positive control. Presence of osteoclasts was detected by TRAP staining after 10 days. A representative image for each treatment group is shown (n = 5). A Leica DMI4000B microscope, attached to a Leica DFC500 digital camera, was used for obtaining images. B, bone marrow macrophages, MDSC\(^{-}\)bone mets\(^+\), and MDSC\(^{-}\)bone mets\(^-\) were differentiated into osteoclasts as mentioned in Materials and Methods. On days 2, 4, and 7 of differentiation, cells were collected and RNA was isolated. cDNA was synthesized and used to detect expression of TRAP, MMP-9, cathepsin K, and carbonic anhydrase-2 by semiquantitative PCR. All the experiments were repeated 3 times independently.

**Figure 2.** MDSC\(^+\)bone mets\(^-\) differentiated osteoclasts are functional in bone resorption. A, MDSC\(^+\)bone mets\(^-\), MDSC\(^-\)bone mets\(^+\), and MDSC\(^-\)bone mets\(^-\) were seeded on sterile porcine cortical bone slices (10\(^5\) cells per slice). After 13 days of culture, presence of pits (resorption areas) was detected by using a Olympus FluorView 300 laser scanning confocal microscope. A representative image for each experimental group is presented. Experiments were repeated 3 times independently. B, percentage of resorption was calculated from the laser scanning confocal microscope images using Adobe Photoshop software. Results are representative of 3 independent experiments (\(*\), P < 0.001).
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To examine whether any of these mechanisms were involved in differentiation of MDSC into osteoclasts, arginase activity, ROS, and NO levels were measured at various stages of osteoclast differentiation of MDSC. Arginase activity and ROS levels remained unchanged (Fig. 4A–B; Supplementary Figs. S4 and S5). Further, the NO levels were greatly elevated only in MDSC(+/bone mets) and not in MDSC(control), MDSC(−/bone mets), MDSC(Lungs), MDSC(Blood), and MDSC(Spleen) as these MDSC differentiated into osteoclasts (Figs. 4C and D, 5A; Supplementary Figs. S4 and S5), thus showing a possible role for NO in inducing osteoclast differentiation of MDSC(+/bone mets).

**NO is essential for differentiation of MDSC into osteoclasts**

Next, to determine that NO is essential for osteoclast differentiation of MDSC(+/bone mets), these cells were cultured in RANKL and M-CSF in the presence of l-NNMA, which is a specific inhibitor of inducible nitric oxide synthase (iNOS; ref. 15). Results clearly showed that MDSC(+/bone mets) failed to differentiate into osteoclasts in the presence of 25 μmol/L l-NNMA, showing that NO production is crucial for the differentiation of MDSC(+/bone mets) into the osteoclasts (Fig. 5B and C).

Further, to delineate a pivotal role of NO in the differentiation of MDSC(+/bone mets) into osteoclasts, before in vivo transfer of MDSC(+/bone mets) mice were injected with 1400W, a specific iNOS inhibitor, intraperitoneally. Treatment with 1400W was continued until the end of the experiment, at which point mice were sacrificed to collect MDSC-injected long-bones for micro-CT analysis. Data clearly showed reduced bone damage in the bone of mice injected with 1400W together with MDSC(+/bone mets), signifying the importance of NO in MDSC-mediated bone damage in vivo (Supplementary Fig. S6).

**NO elevation is accompanied with increased activation of PI3 kinase, ERK, and hypoxia-inducible factor-1α**

Because elevation of NO was specific to MDSC(+/bone mets), which differentiated into osteoclasts, we then investigated the pathways that might contribute to high NO production in MDSC(+/bone mets). Hypoxia-inducible factor-1α (HIF-1α) is known to be upregulated in MDSC in the tumor microenvironment (26). Considering the hypoxic tumor microenvironment of the bone, we hypothesized that these MDSC may have elevated HIF-1α levels. In addition, NO levels are elevated in...
MDSC under hypoxia (26). As shown in Fig. 6A, HIF-1α levels decreased on treatment of MDSC(−bone mets) with L-NMMA. HIF-1α levels were higher in MDSC(+bone mets) compared with MDSC(−bone mets) (Fig. 6B). NO can further induce HIF-1α via signaling through PI3 kinase or ERK or Akt (27). MDSC(+bone mets) also showed elevated levels of phosphorylated PI3 kinase and ERK (Fig. 6C). As expected, low levels of phosphorylated PI3 kinase and ERK were detected in MDSC(Lymph Nodes), MDSC(Lungs), MDSC(Blood), and MDSC(Spleen) (Supplementary Fig. S7).

Taken together, this study clearly shows that MDSC, in the bone microenvironment with disseminated tumor, are novel osteoclast progenitors which contribute to osteolysis of breast cancer. Further, studies delineate a NO-dependent mechanism that drives MDSC differentiation into osteoclasts in the bone microenvironment via the HIF-1α signaling pathway. Thus, targeting MDSC in breast cancer patients will not only reduce tumor growth but also lower the growth of breast cancer in the bone.

Discussion

The present study elucidates a novel role for MDSC as osteoclast progenitors in breast cancer. Importantly, the finding that only MDSC from the bone microenvironment with disseminated breast cancer were capable of undergoing osteoclast differentiation suggests the importance of interaction of MDSC with other cells, including cancer cells, and the reactive stroma that might induce appropriate stimuli for osteolysis. Although results of the current study delineating the role of MDSC as osteoclast progenitor are from using a breast cancer model, these findings can be also extended to other osteolytic carcinomas such as lung and multiple myeloma that predominantly metastasize to bone.

MDSC constitute approximately 1–30% of cells in the bone marrow of normal mice (13). However, following bone metastasis of breast cancer, MDSC numbers are elevated not only at the primary tumor site, but also at metastatic sites including lung, liver, and bone. It is very interesting from the results of the present study that only resident MDSC isolated from the bone microenvironment following cancer dissemination can become osteoclasts. Studies to understand possible mechanisms that might have triggered the differentiation of bone-derived MDSC into osteoclasts indicated the significance of NO signaling.

It is likely that increased hypoxia in the bone, on tumor growth, triggers osteoclast differentiation of MDSC. Hypoxia and HIF-1α expression have been known to enhance osteolytic bone metastases of breast cancer by promoting...
dramatically increased specific activation via MAPK and PI3 kinase signaling pathway HIF-1α, and PI3 kinase pathways. MDSC(+) were differentiated into osteoclasts. Representative data from 3 independent experiments is presented (n = 3; *, P < 0.05). B, MDSC (+)-bone mets) were differentiated into osteoclasts in the presence of L-NMMA. Griess assay was carried out to detect NO levels. Data are representative of 3 different experiments (n = 3; **, P < 0.001). C, MDSC(+)-bone mets) were differentiated into osteoclasts in the presence of L-NMMA and presence of osteoclasts was detected by TRAP assay (n = 3).

osteoclastogenesis (28). As described previously, HIF-1α stimulates and regulates osteoclasts (29, 30). In line with these reports, the present study clearly shows that HIF-1α levels are dramatically increased specifically only in MDSC(+)-bone mets) and this is the only MDSC population that differentiated into osteoclasts. Interestingly, HIF-1α also induces NO production via iNOS (26), which again was found to be elevated in MDSC(+)-bone mets) only. Studies have shown that NO induces HIF-1α activation via MAPK and PI3 kinase signaling pathway (27, 31) and further analysis of bone-derived MDSC following cancer dissemination revealed that P3 kinase levels are elevated during osteoclast differentiation of MDSC. i-NMMA treatment of MDSC (+)-bone mets) not only reduced the NO levels in these MDSC but also drastically reduced the HIF-1α levels. Thus, it is likely that increased HIF-1α levels, combined with elevated NO levels in MDSC, promote osteoclast differentiation of MDSC(+)-bone mets).

Involvement of NO in osteoclast differentiation of MDSC(+)-bone mets) was further confirmed by using a specific iNOS inhibitor. NO is known to induce osteoclast differentiation of macrophages (32, 33). In the present study, very high levels of NO were observed as macrophages differentiated into osteoclasts (data not shown). BMMs from mice lacking iNOS showed reduced osteoclast formation and bone resorption (33, 34). Inhibition of NO levels in wild-type (WT) mice using iNOS inhibitors also showed reduced osteoclast potential of BMMs. These observations further corroborate the findings in the present study.

MDSC from tumor-bearing mice with bone metastasis also induced osteolysis in vivo in syngeneic mice. This further indicates that these cells are primed to be osteoclast progenitors and the bone microenvironment in vivo triggers their differentiation into functional osteoclasts. Increased osteolysis in MDSC(+)-bone mets)-injected mice was the result of increased osteoclast numbers. It was interesting that the femur, adjacent to the MDSC-injected tibia, also showed significant amount of bone destruction. One of the possibilities is that the MDSC-generated osteoclasts could migrate to the neighboring femur and induce osteolysis. MMP-9 is critical for osteoclast migration (35, 36). Our data indicated that as MDSC(+)-bone mets) differentiate into osteoclasts, they express MMP-9, and this may contribute to their migration in vivo. By congenic transfer
of MDSC(\textsuperscript{+}bone mets) it was clear that transferred MDSC remained confined in injected tibia and did not migrate to the adjacent femur (data not shown). Therefore, there is a strong possibility that the observed bone destruction in the adjacent femur may be due to MDSC(\textsuperscript{+}bone mets)-secreted growth factors such as interleukin 1 (IL-1), IL-6, M-CSF, which may further stimulate endogenous macrophages in the bone microenvironment to differentiate into osteoclasts. On the basis of these observations, it may be anticipated that in the bone carrying metastasized tumor, infiltration of MDSC would function in a dual capacity; first, MDSC can directly contribute to osteolysis by differentiating into osteoclasts and, secondly, MDSC-produced cytokines can induce endogenous osteoclast progenitors to induce bone damage.

It is clear from the current study that a cross-talk among MDSC, tumor cells, and the bone microenvironment is necessary for MDSC differentiation into osteoclasts. It remains possible that soluble factors secreted by tumor cells in the bone ‘prime’ these MDSC as osteoclast progenitors. Approximately 83% of breast tumors metastasized to bone express osteopontin (OPN), which contributes to osteolysis by inducing expression of cathepsin K and MMP-9 that are essential for osteoclast function (37). 4T1 cells used in this study have been known to express OPN (38). In addition, breast cancer cells metastasized to the bone also secrete various chemokines such as MCP-1 and RANTES, which are known to enhance osteoclastogenesis (39). Interestingly, MCP-1 can induce NO secretion, a molecular mediator that is essential for osteoclast differentiation of MDSC(\textsuperscript{+}bone mets) (40). MDSC express CCR-2, which is a receptor for MCP-1, and thus are responsive to this chemokine (41). Elevated levels of both MCP-1 and RANTES were observed in the 4T1 breast cancer model as cancer metastasizes to bone, which corroborated with published reports (data not shown). Therefore, the presence of such pro-osteolytic factors may induce differentiation of MDSC(\textsuperscript{+}bone mets) into osteoclasts.

Noting that MDSC are novel osteoclast progenitors, it will be interesting to investigate further the potential of MDSC from the breast cancer patients to induce osteolysis. Ongoing studies are focused on obtaining peripheral MDSC from breast cancer patients with bone metastasis, with further studies planned with MDSC from the bone marrow aspirates of these patients.

Overall, the present study gives a new impetus to the role of MDSC in tumor progression, especially for carcinomas with a propensity to metastasize to the bone. It will also allow designing better treatment regimen for patients with breast cancer bone pathology. For example, gemcitabine, a commonly used chemotherapy agent for breast and lung carcinomas (42–44) is also known to specifically inhibit MDSC (18, 45). Thus, gemcitabine may be used not only as an antitumorigenic drug, but also for reducing bone destruction. Indeed, our in vivo study showed that gemcitabine-treated mice not only had lesser MDSC, but also the tumor growth in the bone was reduced (Supplementary Fig S8). Further, bisphosphonates are commonly used for breast cancer patients with bone metastasis (46, 47). Interestingly, bisphosphonates also inhibit MDSC (8). In the context with the present study, it is relevant to speculate that bisphosphonates, besides inhibiting tumor angiogenesis and inducing apoptosis, can also directly reduce osteolysis by inhibiting MDSC, which are novel osteoclast progenitors.

In summary, the findings presented here provide a novel role for MDSC as cells capable of differentiating into functional, bone-resorbing osteoclasts that contribute to aggressive osteolysis. For long, this population of myeloid cells was thought of as being an immunosuppressive population. Evidence from this study further adds an intriguing multifaceted role for MDSC in cancer bone pathology.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Sawant, S. Ponnazhagan
Development of methodology: A. Sawant, J. Jules, C. Lee, S. Ponnazhagan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Sawant, X. Feng, S. Ponnazhagan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Sawant, J. Deshane, S. Ponnazhagan
Writing, review, and/or revision of the manuscript: A. Sawant, J. Jules, B. Harris, S. Ponnazhagan
Study supervision: S. Ponnazhagan

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References


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