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 demonstrate the importance of myeloid-derived suppressor cells (MDSC) in this process at bone metastatic sites. Since 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.

Edited Precis: Seminal findings define a novel, crucial role for myeloid-derived suppressor cells in driving bone metastasis, with implications for how to block this deadly aspect of many cancers including breast cancer.
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 anti-tumor immunity and promote tumor expansion and metastasis at distant sites, including the bone (6). 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 one of the major metastatic sites for carcinomas of the breast, prostate, lung as well as multiple myeloma (9). About 65-80% 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 multi-nucleated 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. But in the pathological conditions including cancer, IMC differentiation is inhibited resulting in the accumulation of immunosuppressive MDSC (13). Since MDSC are
MDSC are novel osteoclast progenitors of macrophages, which differentiate into osteoclasts and that MDSC numbers are elevated in breast cancer patients, we sought to determine if MDSC in the tumor microenvironment within the bone undergoes osteoclast differentiation and contribute to enhanced bone destruction and tumor growth in an immunocompetent mouse model of breast cancer.

Results of the studies clearly demonstrated 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 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 intra-cardiac route. After 10-12 days, when bone metastases were observed by non-invasive luciferase imaging, mice were sacrificed and bone marrow cells were collected. RBC were lysed using ACK RBC lysis buffer. Cells were incubated with Fc block for 15 min at 4°C. For sorting of total MDSC population (MDSC$^{(+\;\text{bone mets})}$), cells were stained with APC-conjugated anti-CD11b antibody and PE-Cy7-conjugated Gr-1 antibody (eBioscience, San Diego, CA) for 30 min at 4°C. After washing with sterile PBS, CD11b$^+$Gr-1$^+$ MDSC were sorted using BD FACS ARIA III (BD Biosciences, San Jose, CA). CD11b$^+$Gr-1$^+$ MDSC were further stained for additional markers including CD115 PE, F4/80 PE Cy5, CD80 FITC, Ly6C Per CP Cy 5.5 and Ly6G PE antibodies. MDSC were isolated from inguinal, axillary, branchial and thymus lymph nodes (MDSC$^{(\text{Lymph nodes})}$), lungs(MDSC$^{(\text{Lungs})}$), blood (MDSC$^{(\text{Blood})}$) and spleen (MDSC$^{(\text{Spleen})}$) of tumor-challenged mice showing bone metastasis. MDSC were also isolated from tumor-bearing mice but without visible bone metastasis (MDSC$^{(-\;\text{bone mets})}$) and from age-matched control mice (MDSC$^{(\text{control})}$). Expression of arginase and iNOS were detected by permeabilization of cells and staining with iNOS-PE antibody and arginase antibody followed with Alexa 488 conjugated secondary antibody (eBioscience).

In vitro osteoclastogenesis assay. $10^5$ MDSC were seeded in 200 µl α-MEM medium in 48-well plate (Corning Inc., Corning, NY) in the presence of 44 ng/ml M-CSF and 100 ng/ml RANKL (kind gifts from Dr. Xu Feng, UAB) (14). For some experiments, 25 µM NG-Monomethyl-L-arginine, monoacetate salt (L-NMMA) was added to the MDSC cultures (a kind gift from Dr. J. Zmijewski, UAB) (15). Media was changed every 2 days. On days 8-9, 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 M acetate buffer for 20 min at RT. At the end of incubation, cells were stained in 0.2M acetate buffer containing 0.5mg/ml naphtol AS-MX phosphate and 1.1mg/ml fast red TR salt (Sigma-Aldrich, St. Louis, MO) for 30-45 min at 37°C till color change was noted. Nuclei were stained using hematoxylin for 30 sec. 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 performed in triplicate.

**In vitro bone resorption assay.** MDSC and BMM (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 M ammonium hydroxide and mechanical agitation. Bone slices were analyzed using a Olympus FluorView 300 Laser Scanning Confocal Microscope. A quantitative analysis of osteolysis was performed by measuring the percentage of the resorbed areas as compared to the entire bone surface using Adobe Photoshop Software.

**In vivo MDSC depletion.** To deplete MDSC in vivo, mice were injected intra-peritoneally with 1.5 mg gemcitabine (Sigma-Aldrich, St. Louis, MO) 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 BM of gemcitabine treated
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mice and were differentiated into osteoclasts as described above. MDSC from non-gemcitabine
treated mice were included as control.

**In vivo MDSC transfer assay.** MDSC were isolated from the bone of tumor-bearing mice with
bone metastasis (MDSC\textsuperscript{(+ bone mets)}), as described above. 2.5 x 10\textsuperscript{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, prior to injection of MDSC\textsuperscript{(+ bone mets)}
in vivo, mice were injected with 1400W (Cayman Chemical Company, Ann Arbor, MI)
(10mg/kg body weight) intra-peritoneally, 2 days before MDSC\textsuperscript{(+ bone mets)} 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, Wayne, PA). The formalin-fixed bones
were then decalcified in 2.5% EDTA, pH 8.0, for 2 weeks. Five µm paraffin-embedded sections
were used for histology.

To demonstrate that transferred MDSC differentiated into osteoclasts in vivo, MDSC\textsuperscript{(+
bone mets)} from tumor-challenged BALB/c mice (CD45.2 genotype) were injected into long bones
of congeneric, non-tumor challenged CD45.1\textsuperscript{+} female BALB/c mice as described above. After 8
days, mice were sacrificed and bone marrow cells were collected. Cells were stained with
CD45.2-PE antibody to detect presence of adoptively-transferred MDSC. Cells were also stained
with antibody to cathepsin-k (osteoclast marker) followed by Alexa 488 conjugated secondary
antibody. MDSC, which stained positive for CD45.2 and cathepsin-k were sorted and were
differentiated in vitro into osteoclasts as described above.
**In vitro suppression assay.** Following sacrifice of tumor-challenged mice with bone metastasis, MDSC were sorted from the BM (MDSC\(^{(+ \text{ bone mets})}\)) and CD4\(^{+}\) T cells were sorted from the spleen. CD4\(^{+}\) T cells were then labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE) as per the manufacturer’s instructions (Molecular Probes, Eugene, OR). Following labeling, CFSE-CD4\(^{+}\) T cells were cultured with MDSC in 1:1 ratio in media containing 0.75 \(\mu\)g/ml anti-CD3 and 4 \(\mu\)g/ml anti-CD28 antibodies along with 50 \(\mu\)M \(\beta\)-mercaptoethanol for 72 hrs. As a control CD4\(^{+}\) T cells were cultured in the absence of MDSC. After 72 hrs, cells were harvested and 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 tartarate-resistant acid phosphatase (TRAP) staining as described previously (16). All the microscopic images were obtained using 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 is exactly 250 \(\mu\)m 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 performed by using Bioquant Image Analysis software (R&M Biometrics, Nashville, TN) (19). Numbers of osteoclasts per bone surface were calculated.

**Semi-quantitative Reverse Transcription (RT)-PCR.** Total RNA was isolated from MDSC and BMM using TRIzol reagent (Invitrogen). One \(\mu\)g of total RNA was reversed-transcribed to cDNA with iScript cDNA synthesis kit (Bio-Rad). PCR amplification was performed using primers specific for MMP9, TRAP, carbonic anhydrase II (Car2), cathepsin K (Ctsk) and GAPDH using the following condition: preheating at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension 72°C for 30 s in a 30 cycle reaction, followed by final
extension 72°C for 5 min. PCR was performed with Dream Taq Green 2x PCR mix from Fermentas (Glen Burnie, MD) in a 50-μl reaction volume. PCR primer sequences are

MMP-9 Forward 5′-CTTCTTCTCTGGACGTCAAATG-3′
Reverse 5′-CATTTTGGAACCTCACACGCT-3′

Car2 Forward 5′-AGAGAACTGGGACAAGGACTT-3′
Reverse 5′-CCTCCTTTCAGCCTGATTGT-3′

Ctsk Forward 5′-GATGCTTACCCATATGTGGGC-3′
Reverse 5′-CATATCCTTTGTTTCCCCAGC-3′

TRAP Forward 5′-GCCAAGATGGATTATCATGGTG-3′
Reverse 5′-CAGAGACATGATGAAAGTCAGCG-3′

GAPDH Forward 5′-ACATCATCCCTGGCATCCACTG-3′
Reverse 5′-TCATTGAGAGCAATGCCAGC-3′

Thirty micro-liters of PCR mixture was separated on 2% agarose gel for electrophoretic analysis. All semi-quantitative RT-PCR assays were independently performed at least three times.

**Western blot analysis.** MDSC\(^{(+}\)bone mets\), MDSC\(^{(-}\)bone mets\), MDSC\(^{(-}\)Lymph nodes\), MDSC\(^{(-}\)Lungs\), MDSC\(^{(-}\)Blood\) and MDSC\(^{(-}\)Spleen\) were sorted from tumor-bearing mice as described above. Whole cell lysates were prepared using RIPA buffer containing protease and phosphatase inhibitors as per the manufacturer’s instructions (Thermo Scientific, Rockford, IL). Protein concentrations were measured using 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-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
MDSC are novel osteoclast progenitors transferred to nitrocellulose membranes (Millipore, Bedford, MA) followed by blocking with 2% non-fat milk and incubation with primary antibodies, overnight at 4°C. Beta-actin antibody was used as a loading control. After washing the primary antibody with 1× tris-buffered saline with Tween-20 (TBST) (3×10min), suitable secondary antibodies, conjugated to horseradish peroxidase, were applied for 1hr at room temperature, then washed with TBST (3×10min) and blots were then incubated with enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Piscataway, NJ) according to manufacturer's directions and developed on a Fuji LAS-3000 chemiluminescence developer. All the primary antibodies were obtained from Cell Signaling, Boston, MA 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, Piscataway, NJ.

**Measurement of nitric oxide.** Levels of nitric oxide (NO) were detected by using 4-amino-5-methylamino- 2’, 7’-difluorofluorescein diacetate (DAF-FMDA) (Molecular Probes, Eugene, OR) reagent and the Griess Reagent (Promega, Madison, WI), as per the manufacturer’s instructions. Briefly, MDSC (10⁴ cells/well) were cultured in the presence of RANKL and M-CSF as mentioned before for 3 days. As controls, MDSC grown in LPS along and MDSC grown in LPS along with RANKL and M-CSF were included (20). For detecting NO levels by Griess reagent, culture supernatants were collected from MDSC differentiating into osteoclasts at different time points under conditions mentioned above. The assay was carried out as per manufacturer’s instructions and data was normalized to standard curve (with known concentrations of nitrite) and expressed as final nitrite concentrations in media (21). Nitric oxide
levels were also measured for MDSC cultures differentiating into osteoclasts in the presence of LNMMA, a NO inhibitor. The assay was repeated at least three times.

**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 along with RANKL and M-CSF. Detection of urea was carried out using QuantiChrom Urea Assay kit (BioAssay Systems, Hayward, CA) (20). The results are reported as urea concentrations in media. Results are derived from three different samples tested in triplicate.

**Measurement of superoxide.** Superoxide levels in MDSC differentiating into osteoclasts were detected by flow cytometry by incubating for 20 min at RT with dihydroxyethidium (DHE, 10 μM; Molecular Probes, Eugene, OR) following manufacturer’s recommendations. Cells were then washed twice in PBS and the percent positive cells were determined by flow cytometry (20). As controls, MDSC grown in LPS and in LPS along with RANKL and M-CSF were included. The assay was performed at least three times.

**Statistical analysis.** Data were analyzed by one-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 \( p < 0.05 \).
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RESULTS

Isolation and characterization of MDSC from the breast cancer bone metastasis model. For identifying a possible role of MDSC as osteoclast progenitors, osteolytic breast cancer cell line constitutively expressing firefly luciferase 4T1(fLuc), syngeneic in BALB/c mice, were injected via intra-cardiac route into syngeneic and immunocompetent BALB/c mice. Bone metastasis was confirmed in the tumor-bearing mice after 10-12 days post challenge by non-invasive imaging. MDSC population in the bone marrow were characterized using specific cell surface markers. CD11b and Gr-1 MDSC phenotype was further confirmed as the population that was CD80\textsuperscript{Lo}, CD115\textsuperscript{+} and F4/80\textsuperscript{-} (Supplemental Figure S1). 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\textsuperscript{+}Ly6G\textsuperscript{+} cells and monocytic Ly6C\textsuperscript{-}Ly6G\textsuperscript{-} MDSC. MDSC were isolated from both bone marrow and lung using same phenotype. Furthermore, the isolated MDSC actively suppressed proliferation of splenic CD4\textsuperscript{+} T cells; thus establishing that these are indeed immunosuppressive cells (Supplemental Figure S1).

MDSC have potential to differentiate into osteoclasts. To determine if MDSC differentiated into osteoclasts, MDSC, isolated from bone marrow of tumor-bearing mice with bone metastasis (MDSC\textsuperscript{(+bone mets)}) were cultured in medium containing M-CSF and RANKL. MDSC from the lungs of tumor-bearing mice with metastasized tumor (MDSC\textsuperscript{(+lung mets)}) and from bone marrow of tumor-bearing mice but without bone metastasis (MDSC\textsuperscript{(-bone mets)}) were also included in the study. Bone-marrow-derived macrophages were used as a positive control. The cells were fixed and stained by tartarate-resistant acid phosphatase (TRAP) after 10 days. Results of this indicated that MDSC\textsuperscript{(+bone mets)} stained positively for TRAP as evidence for osteoclast differentiation (Figure 1A). However, MDSC\textsuperscript{(+lung mets)} and MDSC\textsuperscript{(-bone mets)} did not undergo...
osteoclast differentiation. MDSC were also isolated from the lymph nodes (MDSC\textsubscript{(Lymph Nodes)}), spleen (MDSC\textsubscript{(Spleen)}) and blood (MDSC\textsubscript{(Blood)}) of tumor bearing mice showing bone metastasis. Phenotypically, such MDSC were similar to MDSC\textsuperscript{(+bone mets)} but failed to differentiate into osteoclasts suggesting that the bone microenvironment is critical for osteoclast differentiation of MDSC (Supplementary Figure S2).

Further, MDSC\textsuperscript{(+bone mets)} expressed other osteoclast specific markers, including cathepsin-K, carbonic anhydrase-2, and MMP-9, starting at day 4 of osteoclast differentiation (Figure 1B). However, MDSC\textsuperscript{(-bone mets)} did not express any of the osteoclast specific markers. As MDSC\textsuperscript{(+bone mets)} differentiated into osteoclasts, expression of F4/80, which is a macrophage specific marker, was detected by flow cytometry. As shown in Supplementary Figure S3A, MDSC\textsuperscript{(+bone mets)} did not express F4/80 during osteoclast differentiation which demonstrated that MDSC\textsuperscript{(+bone mets)} 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 \textit{in vitro} and \textit{in vivo} (22, 23). To determine if MDSC-derived osteoclasts were capable of degrading bone, a bone resorption assay was performed. As shown in Figure 2, osteoclasts differentiated from MDSC\textsuperscript{(+bone mets)} were functional as they degraded bone, indicated by the presence of numerous resorption pits (Figure 2). As expected, MDSC from control mice (MDSC\textsuperscript{(control)}) and MDSC\textsuperscript{(-bone mets)} failed to resorb bone.

**MDSC induce bone destruction \textit{in vivo.}** To corroborate the \textit{in vitro} finding that MDSC\textsuperscript{(+bone mets)} form functional osteoclasts, these MDSC were injected in the tibia of female BALB/c mice. Ten days later, femur and tibia were analyzed by micro-CT and histochemical staining for
MDSC are novel osteoclast progenitors detecting bone destruction. Mice injected with MDSC\(^{(+\text{bone mets})}\) showed significantly more bone destruction compared to the PBS control by micro-CT (Figure 3A and Supplementary Figure S3B). Histochemical analysis clearly showed increased osteoclast numbers by the TRAP assay (Figure 3B&C, Supplementary Figure S3B).

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

**Nitric oxide 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 nitric oxide (NO) production (2, 24, 25). 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 (Figure 4A-B, Supplemental Figures S4 & S5). Further, the NO levels were greatly elevated only in MDSC\(^{(+\text{bone mets})}\) and not in MDSC\(^{\text{control}}\), MDSC\(^{(-\text{bone mets})}\), MDSC\(^{\text{(Lymph Nodes)}}\), MDSC\(^{\text{(Lungs)}}\), MDSC\(^{\text{(Blood)}}\), and MDSC\(^{\text{(Spleen)}}\) as these MDSC differentiated into osteoclasts (Figures 4C-D, 5A, Supplemental Figure S4 & S5), thus demonstrating a possible role for NO in inducing osteoclasts differentiation of MDSC\(^{(+\text{bone mets})}\).

**Nitric oxide is essential for differentiation of MDSC into osteoclasts.** Next, to determine that NO is essential for osteoclast differentiation of MDSC\(^{(+\text{bone mets})}\), these cells were cultured in
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RANKL and M-CSF in the presence of L-NMMA, which is a specific inhibitor of inducible nitric oxide synthase (iNOS) (15). Results clearly showed that MDSC^{(+bone mets)} failed to differentiate into osteoclasts in the presence of 25 µM L-NMMA, demonstrating that NO production is crucial for the differentiation of MDSC^{(+bone mets)} into the osteoclasts (Figure 5B&C).

Furthermore, to delineate a pivotal role of NO in the differentiation of MDSC^{(+bone mets)} into osteoclasts, prior to \textit{in vivo} transfer of MDSC^{(+bone mets)}, mice were injected with 1400W, a specific iNOS inhibitor, intra-peritoneally. 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 along with MDSC^{(+bone mets)}, signifying the importance of NO in MDSC-mediated bone damage \textit{in vivo} (Supplemental Figure S6).

\textbf{Nitric oxide elevation is accompanied with increased activation of PI3 kinase, ERK and HIF-1α.} Since 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-1 (HIF-1α) is known to be up-regulated 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. Besides, NO levels are elevated in MDSC under hypoxia (26). As shown in Figure 6A, HIF-1α levels were decreased upon treatment of MDSC^{(+bone mets)} with L-NMMA. HIF-1α levels were higher in MDSC^{(+bone mets)} compared to MDSC^{(-bone mets)} (Figure 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 (Figure 6C). As expected, low levels of phosphorylated PI3
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kinase and ERK were detected in MDSC(\textsuperscript{(Lymph Nodes)}), MDSC(\textsuperscript{(Lymph Nodes)}), MDSC(\textsuperscript{(Lungs)}), MDSC(\textsuperscript{(Blood)}), and MDSC(\textsuperscript{(Spleen)}) (Supplemental Figure S7).

Taken together, this study clearly demonstrates that MDSC, in the bone micro-environment with disseminated tumor, are novel osteoclast progenitors which contribute to osteolysis of breast cancer. Further, studies delineate a NO-dependent mechanism which drives MDSC differentiation into osteoclasts in the bone micro-environment, via 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 micro-environment 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 osteoclastogenesis. 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 nearly 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, upon 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 osteoclastogenesis (28). As described previously, HIF-1α stimulates as well as regulates osteoclasts (29, 30). In line with these reports, the present study clearly demonstrates 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\textsuperscript{(+bone mets)} only. Studies have demonstrated that NO induces HIF-1α activation via MAPK and PI3kinase signaling pathway (27, 31) and further analysis of bone derived MDSC following cancer dissemination revealed that PI3 kinase levels are elevated during osteoclast differentiation of MDSC. L-NMMA treatment of MDSC\textsuperscript{(+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\textsuperscript{(+bone mets)}.

Involvement of NO in osteoclast differentiation of MDSC\textsuperscript{(+bone mets)} was further confirmed by using a specific iNOS inhibitor. Nitric oxide 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). Bone marrow-derived macrophages from mice lacking iNOS showed reduced osteoclast formation and bone resorption (33, 34). Inhibition of NO levels in WT mice using iNOS inhibitors also showed reduced osteoclast potential of bone marrow derived macrophages. These observations further corroborate the present findings.

MDSC from tumor-bearing mice with bone metastasis also induced osteolysis \textit{in vivo} in syngeneic mice. This further indicates that these cells are primed to be osteoclast progenitors and the bone microenvironment \textit{in vivo} triggers their differentiation into functional osteoclasts. Increased osteolysis in MDSC\textsuperscript{(+bone mets)} injected mice was result of increased osteoclast numbers. It was interesting that 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...
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migration (35, 36). Our data indicated that as MDSC\(^{(+\text{bone mets})}\) differentiate into osteoclasts, they express MMP-9 and this may contribute to their migration \textit{in vivo}. By congeneric transfer of MDSC\(^{(+\text{bone mets})}\) 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\(^{(+\text{bone mets})}\)-secreted growth factors such as IL-1, IL-6, M-CSF, which, may further stimulate endogenous macrophages in the bone microenvironment to differentiate into osteoclasts. Based on 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 between 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. Nearly 83% of breast tumors metastasized to bone express osteopontin (OPN), which contributes to osteolysis by inducing expression of cathepsin K and MMP-9 which 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 like 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\(^{(+\text{bone mets})}\) (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). So, presence of such pro-osteolytic factors may induce differentiation of MDSC\(^{(+\text{bone mets})}\) 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 the carcinomas with 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 anti-tumorigenic drug, but also for reducing bone destruction. Indeed, our \textit{in vivo} study showed that gemcitabine treated mice, not only had less MDSC, but the tumor growth in the bone was also reduced (Supplementary Figure S8). Furthermore, 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, and contribute to aggressive osteolysis. For long, this population of myeloid cells was thought of as an immunosuppressive
MDSC are novel osteoclast progenitors population. Evidence from this study further adds an intriguing multi-faceted role for MDSC in cancer bone pathology.
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REFERENCES


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FIGURE LEGENDS

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]. 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 semi-quantitative PCR. All the experiments were repeated 3 times independently.

Figure 2: MDSC(+bone mets)-derived osteoclasts are functional in bone resorption. (A) MDSC(control), MDSC(+bone mets) and MDSC(-bone mets) were seeded on sterile porcine cortical bone slices (10^5 cells/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) Percent 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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Figure 3: Syngeneic transplantation of MDSC from bone metastasis induce increased bone destruction in recipient mice in vivo. (A) 2.5 x 10^5 MDSC from mice bearing bone metastasis were injected in the tibia of syngeneic, normal female mice on days 1 and 5. As a control, mice were injected with PBS. On day 10, mice were sacrificed and MDSC-injected tibia and adjacent femur were processed for micro-CT analysis to determine the extent of bone destruction. A representative image for each experimental group is shown [N=3]. Paraffin sections of the above femur and tibia were stained by TRAP to detect the presence of osteoclasts. Representative images of 20x (B) and 40x (C) magnifications are shown. Images were taken using Leica DMI4000B microscope, attached to a Leica DFC500 digital camera. The LASv3.6.0 software was used to optimize picture quality. (D) MDSC(+bone mets) from CD45.2 genotype BALB/c mice were injected into tibia of non-tumor challenged CD45.1 congeneic BALB/c mice as described in Materials and Methods. On day 8, mice were sacrificed and bone marrow cells were collected from the MDSC-injected tibia and adjacent femur. Cells were stained with antibodies to CD45.2 and cathepsin k. Presence of CD45.2^+ cells, which also stained for cathepsin k were sorted. Sorted cells were differentiated into osteoclasts as mentioned previously. A representative data set showing presence of transferred MDSC(+bone mets) and differentiation into osteoclast is presented [N=3].

Figure 4: Nitric oxide levels are elevated in MDSC(+bone mets) during osteoclast differentiation. MDSC, derived from the bone marrow of tumor-bearing mice were cultured in the presence of M-CSF and RANKL with or without LPS. On days 2, 5 and 8 of differentiation, culture supernatants were collected to assay arginase levels and NO levels. Quantitative differences in arginase level as measured by urea production are provided (A). Levels of ROS
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were detected by flow cytometry by incubating MDSC\(^{(+\text{bone mets})}\) for 20 min at RT with dihydroxyethidium after 5 days of culture in osteoclast differentiation medium (B). Culture supernatants were also used to detect NO levels by Griess assay (C). MDSC\(^{(\text{control})}\), MDSC\(^{(+\text{bone mets})}\) and MDSC\(^{(-\text{bone mets})}\) were differentiated into osteoclasts in M-CSF and RANKL containing medium with or without LPS. Five days post differentiation, levels of NO were detected by addition of DAF-FMDSA. Flow cytometry was used to quantify NO levels. A representative histogram is shown along with percentage of cells with increased NO content. All the experiments were repeated 5 times independently [\(*p<0.05, **p<0.001\)].

**Figure 5:** Nitric oxide is essential for differentiation of MDSC\(^{(+\text{bone mets})}\) into osteoclasts. (A) Nitric oxide levels were detected on day 8 in MDSC\(^{(\text{control})}\), MDSC\(^{(+\text{bone mets})}\) and MDSC\(^{(-\text{bone mets})}\) as they differentiated into osteoclasts. A representative data from 3 independent experiments is presented [\(N=3, *p<0.05\)]. (B) MDSC\(^{(+\text{bone mets})}\) were differentiated into osteoclasts in the presence of L-NMMA. Griess assay was carried out to detect NO levels. Data is representative of 3 different experiments [\(N=3, **p<0.001\)]. (C) MDSC\(^{(+\text{bone mets})}\) were differentiated into osteoclasts in the presence of L-NMMA and presence of osteoclasts was detected by TRAP assay [\(N=3\)].

**Figure 6:** MDSC\(^{(+\text{bone mets})}\)–derived osteoclasts have elevated HIF-1\(\alpha\), ERK and PI3 kinase pathways. MDSC\(^{(+\text{bone mets})}\) and MDSC\(^{(-\text{bone mets})}\) were isolated from tumor-bearing mice and cultured in the presence or absence of L-NMMA for 4 days following which cell lysates were prepared. Presence of HIF-1\(\alpha\) was detected by Western blot as mentioned in Materials and Methods (A). MDSC\(^{(+\text{bone mets})}\) and MDSC\(^{(-\text{bone mets})}\) were isolated from tumor-bearing mice and
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cell lysates were prepared. Lysates containing equal amounts of total protein were separated on SDS-PAGE and transferred onto nitrocellulose membranes. Detection of HIF-1α (B), ERK, PI3 kinase and β-actin (C) were performed as described in Materials and Methods [N=3].
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

(A) Graph showing the concentration of NO in medium (µM) with different treatments:

- LPS
  - +
  - -
- RANKL
  - +
  - -
- M-CSF
  - +
  - -

(B) Bar graph showing the concentration of NO in medium (µM) with different treatments:

- M-CSF + RANKL
  - +
  - +
- L-NMMA
  - -
  - +

(C) Images comparing the effects of M-CSF + RANKL and L-NMMA treatments.
Figure 6
Myeloid-derived suppressor cells function as novel osteoclast progenitors enhancing bone loss in breast cancer

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