The Metastatic Breast Cancer Microenvironment in Bone Exhibits Unique BMP Signaling

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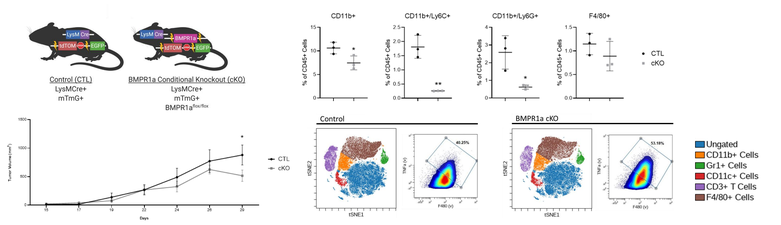
Abstract

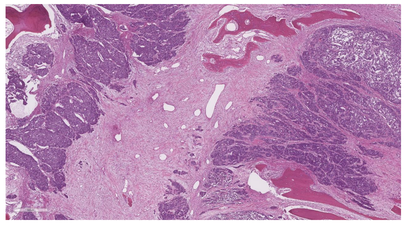
Breast cancer (BC) patient prognosis has improved for localized BC, yet metastatic BC continues to cause high mortality with a 5 year survival rate of only 27%. Approximately 70% of BC metastases occur in the bone, with a tumor microenvironment (TME) composed primarily of BC cells, immune cells, and bone cells. In these cellular compartments, bone morphogenetic protein (BMP) signaling exhibits unique TME dependent tumor promoter and suppressor effects. Previous studies in our lab have found BMPs promote myeloid progenitors, polarization of M2 macrophages, and tumor progression in a BMPR1a LysMCre conditional knockout mouse model. Yet to establish BMPs as a viable target for the treatment of metastatic bone, the mechanisms behind BMPs promoting BC bone metastases must be investigated. To further investigate BMP dependent myeloid heterogeneity in cancer, we have utilized a cohort of non-treatment naïve BC patient bone biopsies to unveil the distinct myeloid populations in the TME of BC bone metastases. Differential gene expression analysis revealed a subset of patient samples with a high myeloid gene signature, corresponding with increased chemokine, cytokine and JAK/STAT signaling gene pathways in addition to enhanced BMP signaling. Digital Spatial Profiling via the NanoString GeoMx platform and multiplexed immunohistochemistry staining via the AKOYA Vectra Polaris platform were used to analyze the spatial context of the TME in our cohort of patient bone. We found enhanced myeloid cell infiltration, myeloid heterogeneity and M2 macrophage polarization in the TME of high myeloid gene signature patient samples. This precision oncology analysis into the unique landscape of the metastatic bone TME revealed BMP driven phenotypes in distinct TME cellular components. To then determine if the TME features observed in the metastatic bone samples was reflective of altered innate trained immunity regulated by BMPs, we investigated the requirement for BMP signaling in myeloid inflammatory responses both in vitro and in a mouse model of BC bone metastasis. We found BMPs alter the ability for macrophages to undergo innate trained immunity functions and BC bone metastases alter myeloid inflammatory responses in mice. Investigating the heterogeneity and functions of myeloid cells in the TME of bone metastatic BC will help advance therapeutic target development for BC patients with bone lesions. Expanding therapeutic options for metastatic BC patients will improve patient quality of life and reduce deaths caused by metastasis.

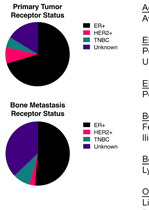
BMPs Support Myeloid Heterogeneity & Tumors



BMPs Required To Maintain Myeloid Progenitors and Promote Tumor Progression. Previous work in the Owens lab utilized a transgenic mouse model with LysMCre restricted deletion of BMPR1a to investigate the requirement of BMP signaling in myeloid cells. Reduced myeloid populations were found in the bone marrow and spleen of BMPR1a cKO mice. MyC-CaP prostate cancer flank tumors in BMPR1s cKO mice exhibited restricted tumor progression and enhanced macrophage infiltration. CyTOF analysis identified an increase in M1 macrophages from BMPR1a cKO tumors.

Clinical Breast Cancer Bone Metastases





Clinical Characteristics of Study Cohort

Age At Primary Surgery
Average 52.7 (29-80)

Age At Bone Surgery
Average 55.7 (36-80)

ER Status At Primary Tumor
Positive 6/7
Unknown 1/7

ER Status At Bone Metastasis
Positive 7/7

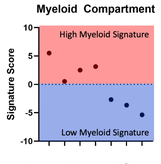
Bone Metastasis Site
Femur 6/7
Iliac 1/7

Bone Pathology
Lytic 7/7

Other Metastatic Sites
Liver 3/7
Brain 1/7
Bladder 1/7
Bone 2/7
None 1/7

Investigating the Tumor Microenvironment in Breast Cancer Patient Bone Metastases. A cohort of 47 metastatic bone archival FFPE samples from non-treatment naïve breast cancer patients at the University of Colorado Cancer Center was generated, with the majority of cases exhibiting ER+ primary tumors and ER+ bone metastases. From this cohort, 7 metastatic breast cancer patient bone samples were selected which had ER+ bone metastases and lytic bone pathology. Hematoxylin and eosin (H&E) staining in representative patient bone sample demonstrates distinct features of lytic bone pathology, stroma, tumor cells and immune cells within the TME.

Gene Expression of Bone Metastases

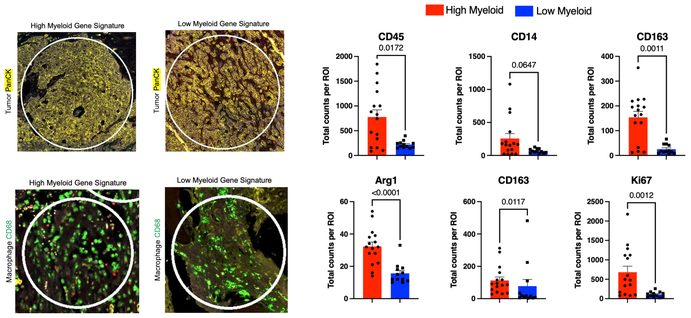


Undirected Differential Expression in Myeloid High	
Cytokine and Chemokine Signaling	2.225
Cytotoxicity	2.128
Immune Cell Adhesion and Migration	2.115
JAK-STAT Signaling	2.039
Interferon Signaling	2.013
TGF-beta Signaling	1.654

Gene	Log2 Fold Change	P-value
CXCL12	4.26	0.000871
S100A8	6.65	0.00171
JAK2	1.52	0.00336
NKG7	4.54	0.00351
CSF3R	3.45	0.00398
CXCR4	1.34	0.00506

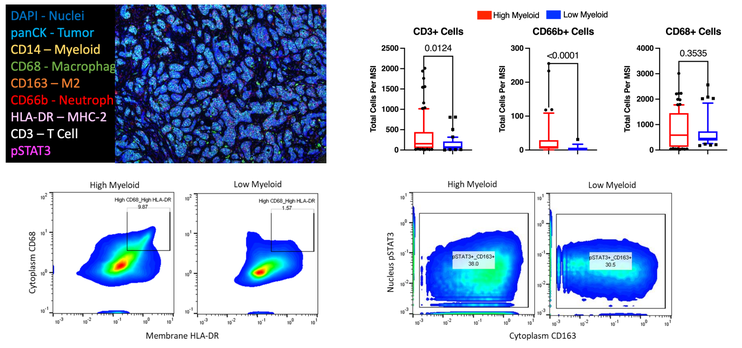
Inflammatory Gene Signatures In Myeloid Enriched Bone Metastases. Gene expression was assessed in 7 archival FFPE bone biopsies with the NanoString human Immune Oncology 360 gene expression panel. Distinct separation of bone samples based on high (n=4) and low (n=3) myeloid gene expression signatures was found. Differential gene expression analysis between high vs low myeloid gene signature patient samples revealed enriched inflammatory genes in the high myeloid patient samples.

Macrophage Characterization by Digital Spatial Profiling



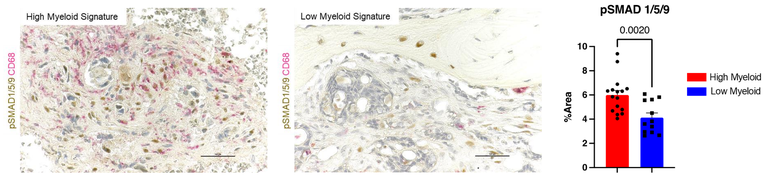
Digital Spatial Profiling of Breast Cancer Bone Metastases Reveals Macrophage Phenotypes. NanoString Digital Spatial Profiling (DSP) was used to determine the distinct proteomic characteristics of the tumor, macrophage, and T cell regions of tumor induced bone disease from metastatic patients. Regions of interest (ROIs) were selected in 7 patient biopsies, with 4 tumor, macrophage, and T cell ROIs selected for each patient. Protein expression was analyzed by antibody counts in ROIs for each antigen.

Macrophage Heterogeneity in Bone Metastases



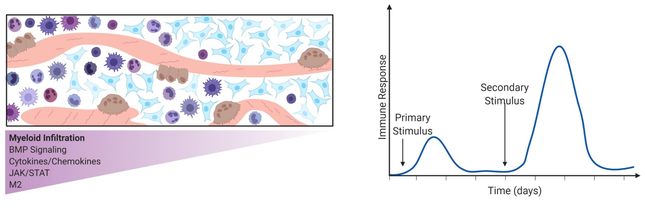
Multiplexed Immunohistochemistry Analysis of Macrophage Heterogeneity. Vectra Polaris multiplexed immunohistochemistry staining was used to analyze the singe cell protein expression of 7 breast cancer bone metastasis patient samples. Polaris analysis revealed co-expression of markers on individual cells to aid in cell phenotyping and signaling. InForm phenotype training allowed for identification of cell phenotypes based on marker expression. Cell segmentation data from InForm was then exported as FCS files and concatenated into High Myeloid and Low Myeloid groups and analyzed in FlowJo to investigate expression heterogeneity.

BMP Signaling in Bone Metastases



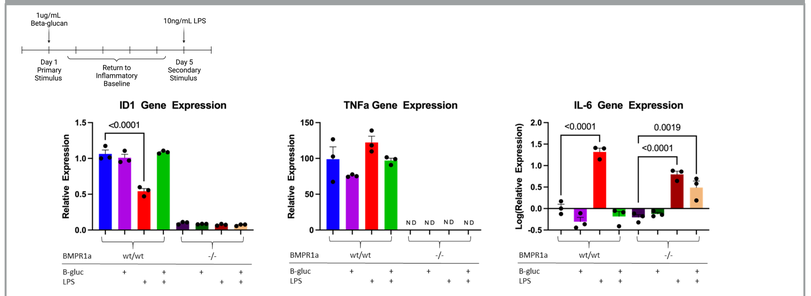
BMP Signaling Enriched In Breast Cancer Patient Bone Metastases with High Myeloid Gene Signature. Immunohistochemistry staining for pSMAD1/5/9 (brown) identifies BMP signaling in patient bone samples, shown both in tumor cells and the surrounding tumor microenvironment including CD68 macrophages (pink). Quantitation of pSMAD1/5/9 staining in ImageJ shows enriched BMP signaling in High Myeloid patients.

Hypothesis: Myeloid Cell BMP Signaling in Bone TME



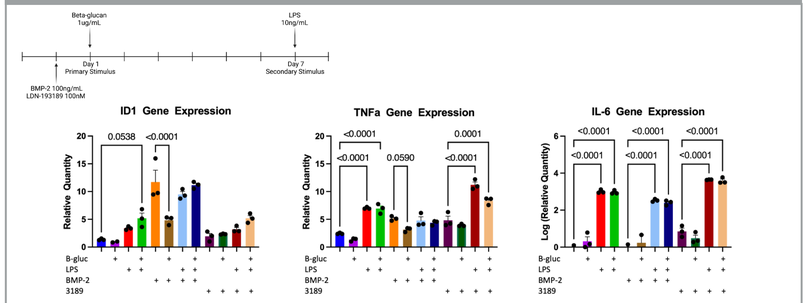
The myeloid enrichment observed in the metastatic bone from breast cancer patients may be reflective of a maladaptive trained immunity phenotype driven by BMP dependent myeloid memory. Trained innate immunity results from epigenetic and metabolic reprogramming of myeloid cells, allowing for enhanced responses of innate immune cells to later inflammatory stimulation.

Myeloid Memory in BMPR1a$^{-/-}$ Macrophages



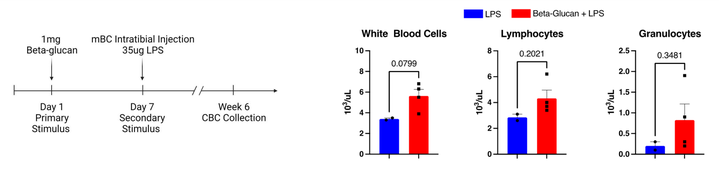
Loss of BMP Signaling Alters Myeloid Memory. Bone marrow macrophage cell lines derived from wild type and LysMCre BMPR1a conditional knockout mice underwent innate immune entrainment. Cells were treated with Beta-glucan for 24hrs followed by 5 days of culturing then LPS for 24hrs followed by RNA collection. Gene expression analysis measured BMP signaling through ID1 and inflammatory response through IL-6 and TNFalpha.

Modulated BMP Signaling in Myeloid Memory



Macrophage Treatment with BMP-2 or BMP Inhibitor Alters Myeloid Memory. Raw264.7 macrophages were treated with BMP-2 or BMP inhibitor LDN-193189 24hrs before undergoing innate immune entrainment, for a total of 48hrs of treatment with BMP stimulus or inhibitor. Cells were subsequently treated with Beta-glucan for 24hrs followed by 6 days of culturing then LPS for 24hrs followed by RNA collection. Gene expression analysis measured BMP signaling through ID1 and inflammatory response through IL-6 and TNFalpha.

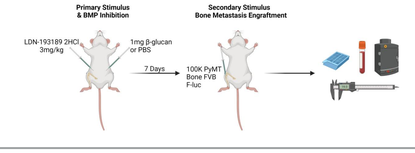
Trained Immunity in Mouse Bone Metastases



Preliminary Evidence of Trained Immunity Modeled in Mouse Breast Cancer Bone Metastases. To investigate how trained immunity functions in mice with metastatic bone lesions, FVB mice were treated with Beta-glucan 7 days prior to receiving LPS and intratibial injections of the syngeneic MMTV-PyMT metastatic bone cell line to form bone metastases. Analysis of complete blood counts after 6 weeks of metastatic tumor growth revealed preliminary evidence of trained immunity with trending increases of immune cells.

Summary & Next Steps

- Myeloid gene signature enriched patient bone samples have enhanced inflammatory signaling (JAK/STAT & chemokine/cytokine), myeloid infiltration, M2-like polarization, & BMP signaling
- Myeloid memory is modulated by BMP signaling *in vitro*
  - Trained immunity increases ID1 gene expression
  - LDN-193189 treatment increases macrophage inflammatory response
- Trained immunity can be modeled in mice with breast cancer bone metastases
- Next Step: Investigate if BMP signaling is required for trained immunity in a mouse model of breast cancer bone metastases



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