

CHARACTERIZATION OF THE BONE MARROW MICROENVIRONMENT DURING THE
DEVELOPMENT OF TRIPLE NEGATIVE BREAST CANCER METASTASES

by

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ABSTRACT

Triple negative breast cancer (TNBC) is a subtype that lacks expression of progesterone and estrogen receptors, and does not over-express human epidermal growth factor receptor 2 (HER2). TNBC is not responsive to standard therapies that target these receptors and is associated with poor prognosis, a high rate of recurrence, and aggressive metastasis. The **purpose** of this study was to characterize the bone marrow microenvironment during the process of primary tumor growth and metastasis in murine TNBC models. We **hypothesize** that the expression of the inflammatory chemokines KC/CXCL1 and MCP-1/CCL2 in the bone marrow microenvironment promotes the development of breast cancer metastasis. The 2225LM murine tumor has gene expression patterns comparable to human tumors of the basal-like TNBC phenotype and the T11 murine tumor mirrors the claudin-low TNBC phenotype. In our studies, tumors (1-2 mm) are implanted in either the subcutaneous flank or the mammary fat pad of syngeneic, female mice and are excised ~2 weeks later to reliably generate growth of seeded metastases. Previous data from our laboratory found significantly increased levels of KC/CXCL1 and MCP-1/CCL2 in primary tumors of both tumor lines compared to tissue from naïve mice. In the present study, bone marrow is flushed from femurs and tibias of tumor-implanted mice, separated into cellular and soluble components and assayed by ELISA. MCP-1 is significantly elevated in the extracellular component of bone marrow of T11-implanted mice. KC/CXCL1 is not significantly elevated in either the extracellular or intracellular bone marrow of T11-implanted mice. KC/CXCL1 is expressed by inflammatory cells and osteoblasts and is a chemoattractant for inflammatory cells and certain tumor cells. MCP-1/CCL2 is expressed by a variety of inflammatory cells and myeloid progenitor cells and is a chemoattractant for

monocytes/macrophages. Our results are consistent with the premise that an increase in MCP-1/CCL2 in the bone marrow promotes the development of claudin-low breast cancer lung metastasis.

INTRODUCTION

Metastasis requires that a tumor cell is able to dissociate from the primary tumor, enter circulation or the lymphatics, invade another tissue, and grow in a new environment (Joyce, 2008). Investigations into the patterns of metastases have resulted in the discovery of cellular and molecular changes in tissues prior to the infiltration of a tumor cell. Research in the last decade has deemed these changes in the molecular and cellular composition of tissues, the formation of a “pre-metastatic niche”. A pre-metastatic niche is the tissue microenvironment that supports tumor cell attraction, retention, and growth (Kaplan, 2006). The microenvironment attracts and supports the tumor cell(s) by the changes in soluble mediators, molecules involved in the communication between cells of the immune system, and altering the presence of cell populations involved in immune response.

Research studies suggest that cells involved in the alteration of tissue into a pro-tumor microenvironment are cells of the immune system, which normally function to defend the body from pathogens and maintain homeostasis. Cells of the immune system are produced in the bone marrow by the process of hematopoiesis. Hematopoiesis is the process in which hematopoietic stem cells in the bone marrow differentiate and mature into lymphocytes and myeloid cells based on chemical signals. These myeloid cells have been shown to play key roles in cancer-related inflammation and are the focus of this project. Inflammation is thought to be

a major contributor to the tumor microenvironment, as it has the ability to promote tumor cell insurgence and sustain tumor growth (Caronni, 2014).

Bone marrow-derived myeloid cells that have been shown to play a significant role in this inflammatory pre-metastatic niche include monocytes, macrophages and neutrophils (Caronni, 2014). Monocytes, macrophages, and neutrophils are derived from the same bone marrow precursor cell. The differentiation and maturation of these cells depend on chemical signals present in the bone marrow at the time of production.

Monocytes are cells that circulate in the bone marrow, blood, and spleen and have the ability to produce inflammatory chemical signals and phagocytose cells and toxic molecules. Macrophages are phagocytic cells that reside in tissues and are known to play a role in tissue homeostasis. They are able to recognize pathogens and induce production of inflammatory cytokines (Geissman, 2010).

Neutrophils are cells that are typically the first leukocytes to localize to a site of inflammation. They are able to eliminate pathogens via both intra and extracellular mechanisms including phagocytosis and the formation of extracellular traps. Distinctive features of neutrophils include a polymorphic nucleus and the ability to release granules that use anti-microbial molecules and proteases to kill pathogens. Neutrophils are released from the bone marrow in a mature state whereas macrophages are released from the bone marrow as monocytes and mature into macrophages once they begin to reside in certain tissues (Kolaczowska, 2013). However, not all monocytes released from the bone marrow mature into macrophages. Monocytes that continue in the body's circulation avoid maturation into

macrophages. Evidence suggests that once an immune cell arrives in tissue, tumor cells can modify inflammatory cell types to alter them into tumor promoting rather than tumor suppressive (Joyce, 2008). For macrophages, investigations have indicated that these cells can switch from a tumor suppressive M1 phenotype to a pro-tumor M2 phenotype. Neutrophils follow the same trend; N1 neutrophils are thought to be anti-tumor and N2 neutrophils are thought to be pro-tumor.

Osteoblasts are cells that originate from mesenchymal stem cells in the bone marrow and are responsible for bone formation and remodeling. Recent studies suggest that osteoblasts increase expression of inflammatory cytokines in the presence of cancer cells (Bussard, 2010). Cancer cells in the bone marrow have also been shown to promote osteoclastogenesis and bone resorption (Chen, 2010).

Inflammation that is produced by the immune system because of wound healing is usually self-limiting. However, when the inflammation is not self-limiting, it creates a prime environment for cell proliferation. Observing an increase in the production of these macrophage and neutrophil cell populations in the bone marrow can indicate that an immune response is taking place. Enhanced myeloid cell production from the bone marrow during an infection is expected; however, during certain inflammatory conditions, hematopoietic stem progenitor cells are thought to respond to stimuli and proliferate to replenish immune cells (Baldrige, 2011). An enhanced amount of signals that stimulate myeloid cell production in the bone marrow may indicate infection or inflammation elsewhere. The same chemical signals

that stimulate the bone marrow during inflammation are expected during the formation of an inflammatory pre-metastatic niche.

Chemical signals of interest in the bone marrow that stimulate the production of myeloid cells include MCP-1 and KC. Monocyte Chemoattractant Protein MCP-1/CCL2 is produced by a variety of cell types including macrophages and fibroblasts. This chemokine has been shown to promote migration of inflammatory monocytes from the bone marrow into the circulation (Yadav, 2010). Keratinocyte chemoattractant, or KC/CXCL1 is expressed by inflammatory cells and osteoblasts and is a chemoattractant for inflammatory cells and certain tumor cells. Previous data from our laboratory found increased levels of KC/CXCL1 and MCP-1/CCL2 in primary tumors and in lungs with visible metastases of both tumor lines, compared to tissue from naïve mice.

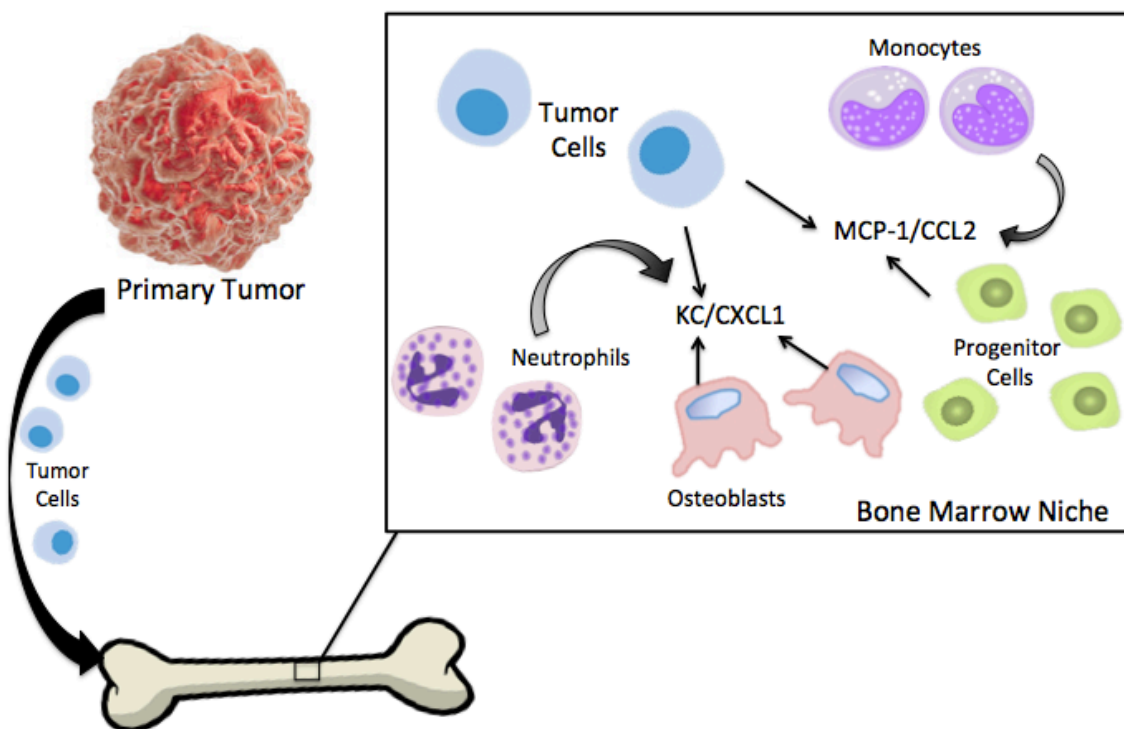


FIGURE 1: Bone Marrow Microenvironment. KC/CXCL1 and MCP-1/CCL2 may influence the bone marrow to promote bone remodeling and cancer cell colonization.

Despite a common tissue of origin, subtypes of breast cancers are quite diverse. Triple negative breast cancer (TNBC) is a subtype unique in that its tumors lack the expression of progesterone and estrogen receptors, and do not over-express human epidermal growth factor receptor 2 (HER2) (Foulkes, 2010). Because of this, treatment of Triple Negative Breast Cancer is not responsive to standard therapies, which target these receptors. This subtype of breast cancer accounts for 15-20% of all breast cancers and is associated with poor clinical prognosis, high rate of recurrence, and aggressive metastasis (Chiorean, 2013).

The purpose of this study was to characterize the bone marrow microenvironment during the process of primary tumor growth and metastasis in murine TNBC models. We hypothesize that the expression of the inflammatory chemokines KC/CXCL1 and MCP-1/CCL2 at metastatic sites promotes the development of breast cancer metastasis.

MATERIALS AND METHODS

Mice: Syngenic Balb/c female mice were obtained from Charles River Laboratories and were housed and handled in accordance with IACUC regulations.

Tumor Implantation: T11 and 2225LM tumors were obtained from Dr. Jason Herschkowitz, University at Albany, SUNY. Tumor sections (1-2mm) were implanted in either the subcutaneous flank or the mammary fat pad of mice. The tumors were then excised approximately 14 days after implantation. The mice were euthanized approximately 30 days post tumor resection.

Bone Marrow Isolation: The tibias and femurs of the mice were harvested. The epiphyses were separated from the diaphyses of the bones and 0.5-1.0 mL media was used to flush the diaphyses. The epiphyses were manually chopped with scissors and added to the flushed media. The flush was filtered through a 70-micron filter. The flush was centrifuged for 10 minutes at 10 °C at 1000 rpm. The supernatant was then extracted. The erythrocytes in the cell pellet were lysed using a Terry Fox lysis buffer and then resuspended in Iscove's Modified Dulbecco's Medium (Gibco, Catalog number: 21056023). A cell count by hemocytometer was performed using 10% Acetic Acid and Trypan Blue.

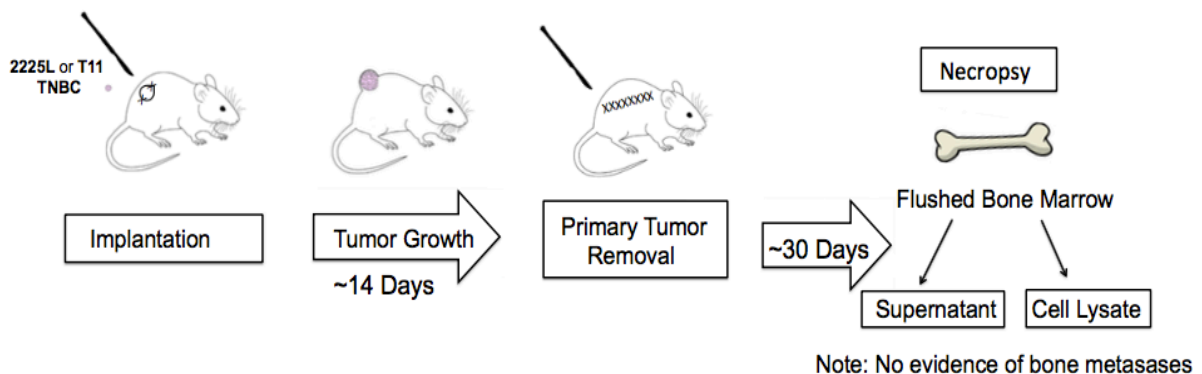


FIGURE 2: Experimental Model

Chemokine Analyses: Chemokines were quantified in both the supernatant and the cell lysate using MCP-1/CCL2 and KC/CXCL1 sandwich ELISAs (R&D Systems) following R&D Systems recommended protocols. Chemokine levels were analyzed from three groups of mice, naïve (n=8), T11-implanted (n=16), and 2225LM-implanted (n=16).

Statistical Analysis: For comparison of two groups, we used an unpaired, nonparametric, two-tailed Mann-Whitney t-test with a confidence interval of 95 percent.

RESULTS

The chemokine levels in each group of mice were compared using a Mann-Whitney T-test. MCP-1/CCL2 levels in the extracellular component of bone marrow of T11-implanted mice were significantly elevated ($p=0.004$) compared to the MCP-1/CCL2 levels in the naïve group (Figure 3A). However, KC/CXCL1 was not significantly elevated in the extracellular component of the bone marrow of T11 or 2225LM-implanted mice compared to the naïve group (Figure 3B). We also analyzed the KC/CXCL1 levels in the intracellular component of the bone marrow (cell lysate) and found that, although KC/CXCL1 was elevated in the T11-implanted mice compared to the naïve mice, the increase was not statistically significant. (Figure 5). Chemokine levels were not significantly affected by the location of tumor implantation (subcutaneous heteropic vs. orthotopic in the mammary fat pad, data not shown). Chemokine levels were also compared to the incidence of visible lung metastases. The data did not show a significant association between MCP-1/CCL2 and the incidence lung macrometastases (Figure 4A). There was also no significant association between KC/CXCL1 levels and the incidence of lung macrometastases (Figure 4B).

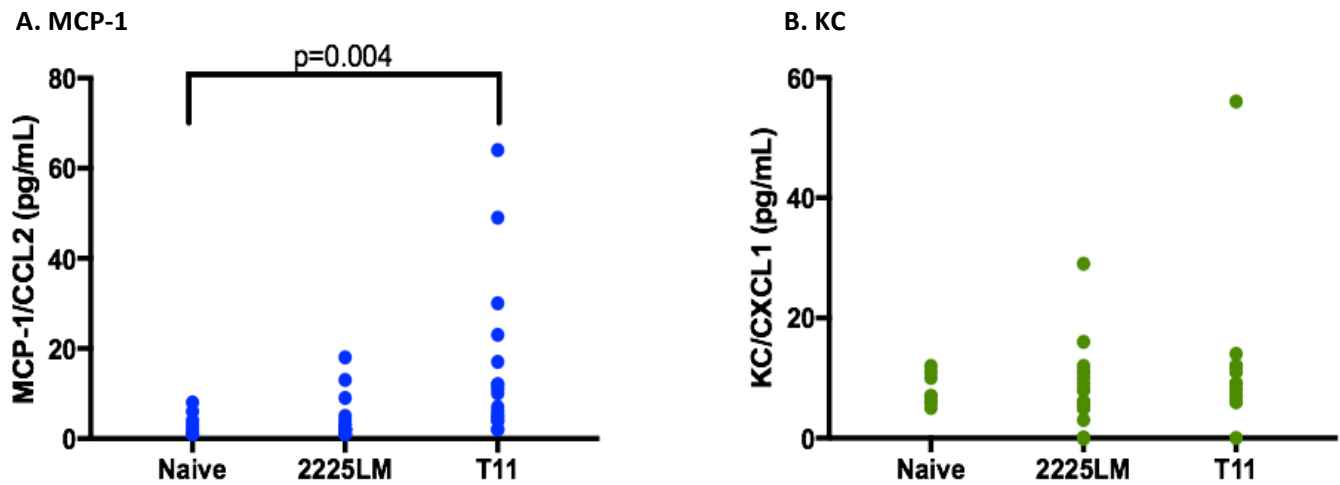


FIGURE 3: Levels of Extracellular Chemokines in Bone Marrow A. MCP-1/CCL2 was significantly elevated in the T-11 implanted mice compared to the naïve mice ($p=0.004$). B. KC/CXCL1 was not significantly different in the T11 and 2225LM-implanted mice compared to the naïve mice.

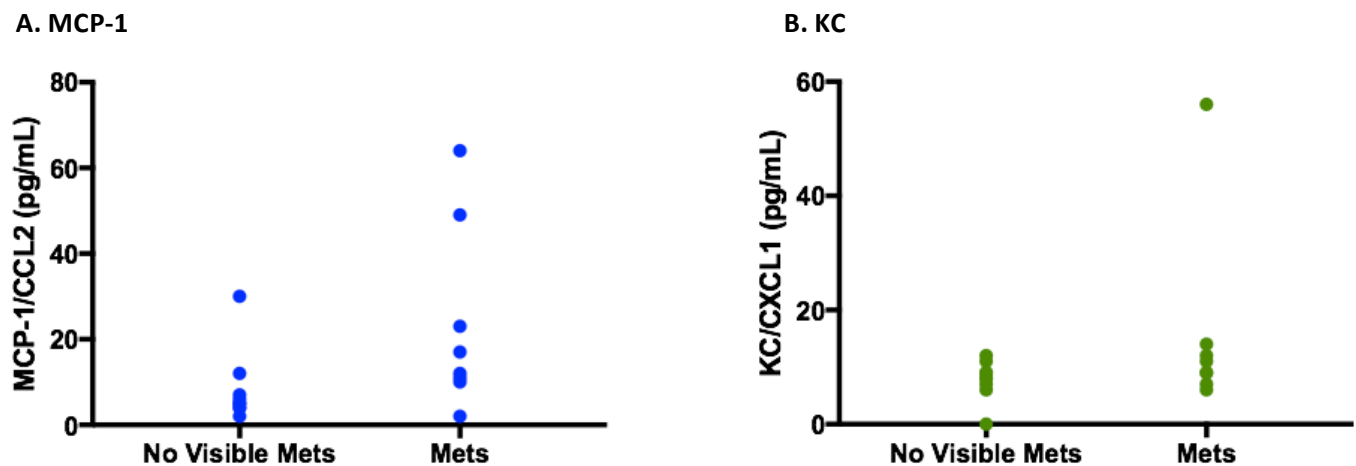


FIGURE 4: Bone Marrow Extracellular Chemokine Levels and Incidence of Lung Metastases A. MCP-1/CCL2 was elevated in T11-implanted mice with visible lung metastases ($p=0.06$). B. KC/CXCL1 was not significantly changed in T11-implanted mice with visible lung metastases.

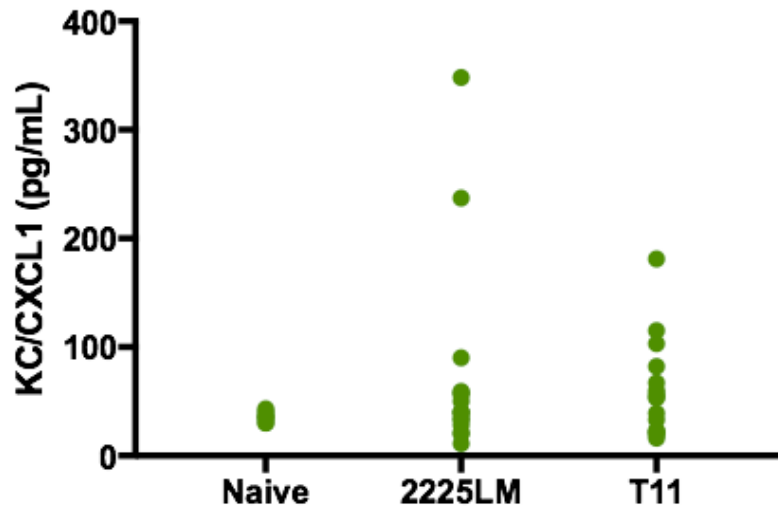


FIGURE 5: Levels of intracellular KC in Bone Marrow. KC/CXCL1 was not significantly elevated in the T11-implanted mice compared to the naïve mice ($p=0.08$).

DISCUSSION

During this study we sought to characterize the chemokine levels of the bone marrow during primary tumor growth and metastasis. Because of their role in metastasis to the lungs, we chose to investigate MCP-1 and KC in both the intracellular and extracellular components of the bone marrow. These molecules may influence the bone marrow to promote bone remodeling and cancer cell colonization. We expected levels of these chemokines in the bone marrow microenvironment to be elevated in tumor-implanted mice compared to naïve mice. However, 2225LM-implanted mice did not show significantly elevated levels of either chemokine. We have shown that secretory MCP-1/CCL2 is elevated in T11-implanted mice. This finding supports the idea of chemokine changes in the bone marrow microenvironment during the processes of primary tumor growth and metastasis. Similar increases in bone marrow levels of MCP-1/CCL2 have been reported via cytokine analysis of bone culture supernatants (Sosnoski, 2012).

Future studies will inhibit MCP-1 to investigate its role in metastatic progression and further characterize the cytokine profile in the bone marrow. Possible cytokines of interest may include G-CSF and CXCL12, which have been shown to play a role in neutrophil migration from the bone marrow (Christopher, 2009). We will also investigate levels of intracellular MCP-1 as well as the source of MCP-1 in the bone marrow of T11 implanted mice. Possible sources of MCP-1 include T11 tumor cells and myeloid progenitor cells in the bone marrow. An increase in the production of myeloid progenitor cells in the bone marrow may indicate an inflammatory response such as the development of a pre-metastatic niche.

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