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Abstract

Introduction: Breast cancer remains the second leading cause of cancer related deaths for women in the United States. Metastasis is regulated not only by intrinsic genetic changes in malignant cells, but also by the microenvironment, especially those associated with chronic inflammation. We have reported that mice that suffer from autoimmune arthritis have significantly increased incidence of bone and lung metastasis and decreased survival associated with breast cancer. In this study, we evaluated the mechanism underlying the increased metastasis.

Methods We used two mouse models; one that develops spontaneous autoimmune arthritis (SKG mice) injected with metastatic breast cancer cells (4T1), and second, that develops spontaneous breast cancer (MMTV-PyV MT mice) injected with type II collagen to induce AA. Mast cell levels and metastasis were monitored.

Results: Our studies show that the metastatic niches (bone and lung) in the arthritic mice with breast cancer express significantly higher numbers of mast cells as compared to their non-arthritic counterparts. We report that to begin with, the non-tumor bearing mice with arthritis have significant upregulation in mast cell population and the population of mast cells increases three folds with breast cancer because the tumor cells express stem cell factor SCF which is a chemotactic growth factor for mast cells and this interaction helps in the differentiation, migration, maturation, proliferation and survival of mast cells, thus remodeling the microenvironment by increasing the population of mast cells and further contributing to increased metastasis. We report that the differentiation of mast cells from bone marrow derived stem cells was significantly higher in the arthritic versus the non-arthritic tumor-bearing mice. We report that the major underlying cause of increased metastasis in arthritic milieu is triggered by the tumor derived SCF/cKit signaling (SCF binds to the cKit receptor on mast cells). Thus, targeting the SCF/cKit interaction reduced the differentiation of mast cells and consequently reduced breast cancer-associated metastasis.

Conclusion: This is the first report to show that mast cells play a critical role in not only remodeling the tumor microenvironment but also the metastatic niche to facilitate efficient metastasis through SCF/cKit interaction in **breast cancer with arthritis**.

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INTRODUCTION: Breast Cancer (BC) remains the second leading cause of cancer-related death in women. In 2011, an estimated 1,596,670 new cases of breast cancer are expected to be diagnosed in women and about 571,950 are expected to die from the disease due to metastasis [1]. The most common site of metastasis is the bone and bone disseminated breast cancer is incurable [2]. The reason/s for this site-specificity is not well understood. Several studies have demonstrated that sites of chronic inflammation are associated with the establishment and growth of tumor cells [2]. One such common inflammatory condition in humans is autoimmune arthritis (AA), which causes inflammation and deformity of the joints as well as increased cellular infiltration and inflammation of the lungs [3]. Although AA and BC are different diseases, some of the underlying molecular processes that characterize AA also affect cancer progression and metastasis. Indeed, the bones and lungs are not only the most common sites of chronic inflammation linked to AA, but are also frequent sites of BC metastasis. In addition, there are epidemiologic studies indicating that BC patients with rheumatoid arthritis (RA) have poor prognosis and high mortality in comparison to BC patients without RA [4]. Despite this knowledge, it has never been questioned whether or not these inflamed sites are an ideal milieu for BC cells to home and grow [5]. Thus, an understanding of the molecular mechanisms and factors that facilitate BC-associated metastasis in arthritic conditions is desperately needed and highly significant. We believe that this understanding will aid in the development of improved and novel therapeutic options for patients with metastatic BC.

BODY: For the past 5 years, I have been exploring this idea and have reported that the inflammatory microenvironment caused by AA serves as a chemo attractant for recruitment, retention, and proliferation of BC cells in the bones and lungs [6, 7]. We have shown this in two relevant transgenic models that mimic the human disease: one that develops spontaneous AA (SKG mice) and is induced to develop mammary gland tumors [6] and the second that develops spontaneous mammary gland tumors (PyV MT mice) and is induced to develop AA [7]. Metastasis is regulated not only by intrinsic genetic changes in malignant cells, but also by the microenvironment. The preference of breast cancer cells to grow in the bone and lung is underscored by the fact that 65-75% of patients with advanced disease develop metastases in these organs. There are no methods to predict the increased risk of breast cancer-associated metastasis influenced with arthritis and current treatments have notable limitations. Hence, in this study, we propose to analyze the in-depth mechanism contributing to the increased breast cancer associated metastasis in an arthritic environment. Our published data [6, 7] suggest that several cytokines such as IL-17, VEGF, PGE₂, IL-6, M-CSF, and TNF- α were significantly up regulated in the circulation as well as in the bone and lung microenvironment of the arthritic mice. Several of these proinflammatory factors (known to influence metastasis) are produced by Mast cells (MCs) which are activated by tumor-derived stem cell factor (SCF) and its receptor c-Kit [8].

HYPOTHESIS: Increase in MCs triggered by SCF/c-Kit signaling within the arthritic bone or lung may be the underlying factor for increased metastasis in the arthritic mice and that targeting the SCF-cKit interaction and/or the downstream factors associated with this signaling may prevent metastasis

Specific Aim 1: To test whether MCs are activated by SCF via c-Kit signaling in the arthritic versus non-arthritic mice

Specific Aim 2: To test the same parameters as Aim 1 in the MMTV-PyV MT mice (a model of spontaneous breast cancer) which are induced to develop arthritis by administration of type II collagen at 14 weeks of age.

Specific Aim 3: To test the efficacy of inhibiting the activation of MCs by anti-SCF or anti-c-Kit in preventing metastasis in both models.

Results:

Statement of work:

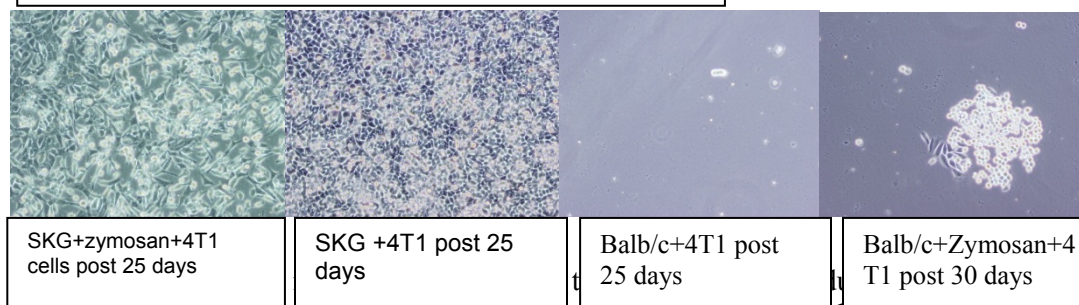
Specific Aim 1: To test whether MCs are activated by breast tumor-derived SCF via c-Kit signaling in the arthritic versus non-arthritic mice.

Method: We used the established mouse model of spontaneous arthritis (known as the SKG mice) that was injected with syngeneic metastatic breast cancer cells (4T1 cells) in the mammary fat pad to develop unifocal mammary gland tumors. SKG mice without tumor and normal Balb/c mice with and without tumor were used as controls. The SKG mice is a mutant on the Balb/c background which spontaneously develop T cell-mediated chronic autoimmune arthritis as a consequence of a mutation of the gene encoding an SH2 domain of ZAP-70, a key signal transduction molecule in T cells. This mutation impairs positive and negative selection of T cells in the thymus, leading to thymic production of arthritogenic autoimmune CD4⁺ T cells. The mice succumb to symmetrical joint swelling. Genetic deficiency of IL-6, IL-1, or TNF- α inhibit development of arthritis in SKG mice, similar to the effects of anticytokine therapy in human arthritis. These clinical and immunopathological characteristics of arthritis in these mice make the strain a suitable model for testing our hypothesis. Although SKG mice have the mutation, they remain healthy under the specific pathogen free (SPF) condition and therefore are designated as pro-arthritic. They develop severe arthritis when given one intraperitoneal (ip) injection of 2 mgs/100ul of zymosan A. Thus we have models of arthritis, pro-arthritis and no arthritis to test our hypothesis. Two month old mice were injected with zymosan A and joint swelling macroscopically examined starting at 14-days post zymosan. Thirty days post zymosan A, 100% of the mice developed polyarthritis. At this point, mice were challenged with syngeneic 4T1 breast cancer cells (1×10^5 cells per mouse in 100ul PBS) in the mammary fat pad. Mice were sacrificed 4 weeks post tumor challenge.

Animal Numbers for Aim 1: (A) SKG + 4T1 = 10 mice; SKG + Zymosan + 4T1 = 10 mice; SKG only = 4 mice; SKG + Zymosan = 4 mice; (B) Balb/c + 4T1 = 10 mice; Balb/c + Zymosan + 4T1 = 10 mice; Balb/c only = 4 mice; Balb/c + Zymosan = 4 mice. Total mice: 56 mice

Results: We evaluated the tumor burden coupled with bone and lung metastasis in the arthritic SKG mice versus non-arthritic Balb/c. We report: **1)** Significantly higher tumor burden in arthritic SKG+4T1, SKG+Zymosan+4T1 versus Balb/c+4T1, Balb/c+Zymosan+4T1. **2)** The lung metastasis was almost three times higher in arthritic (SKG \pm zymosan+breast cancer) versus non-arthritic Balb/C mice with breast cancer. **3)** The bone marrow cells were stained with pancytokeratin to look for the metastatic epithelial cells through flow cytometry. We found significantly higher pancytokeratin positive staining in the bone marrow of arthritic SKG with \pm zymosan and challenged with 4T1 cells versus non-arthritic Balb/c \pm zymosan+4T1 cells. **4)** The x-ray imaging confirmed the metastatic lesions in the arthritic SKG mice injected with 4T1 cells as compared to the non-arthritic Balb/c. **5)** The blood was collected from the animals and looked for the circulating tumor cells. Seven out of 10 SKG mice \pm zymosan with breast cancer showed circulating tumor cells in the blood culture post 23-35 days from the endpoint. On the contrary, three out of 10 Balb/c mice with breast cancer showed tumor cells in blood culture post 30-35 days.

Figure 1: Tumor cells in Blood culture: Representative



metastasis is significantly higher, we evaluated if mast cells are playing a pivotal role.

6) We cultured the bone marrow cells. After flushing the bone marrow and processing it, we added IL-3 and SCF to the bone marrow cells. Addition of IL-3 and SCF will differentiate the bone marrow cells to immature and mature mast cells. Every three days, we collected the supernatant and added fresh IL-3 and SCF to it. It takes one month to generate mast cells from the bone marrow progenitors.

Below is the total number of mast cells generated. We counted the mast cells from each plate per group. A) SKG + 4T1: Approximately on an average 150×10^4 mast cells were counted. B) SKG+zymosan+4T1: Approximately on an average 100×10^4 mast cells counted. C) Balb/c +4T1: Approximately on an average 35×10^4 cells counted; D) Balb/c + zymosan+4T1: Approximately on an average 30×10^4 cells counted. To confirm that the cells counted are mast cells, we stained the cells with CD117 antibody, also called KIT or C-Kit receptor expressed on the surface of mast cells (Figure 2). We also confirmed that the cells we saw under the microscope are mast cells (Figure 3) by staining the cells with toluidine staining (Figure 4). Both ways, it was confirmed and we got positive CD117 staining with flow cytometry as well as positive toluidine staining.

Figure2: *In vitro* mast cell culture post 30 days: CD117 population

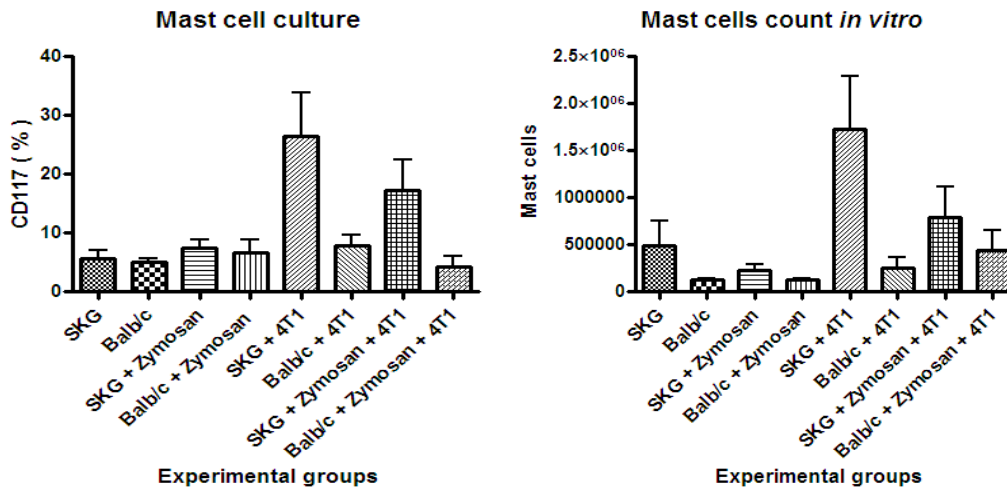
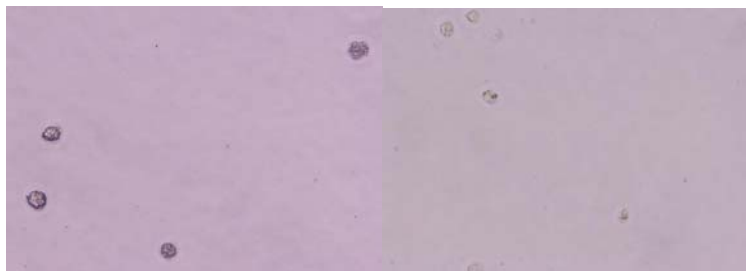
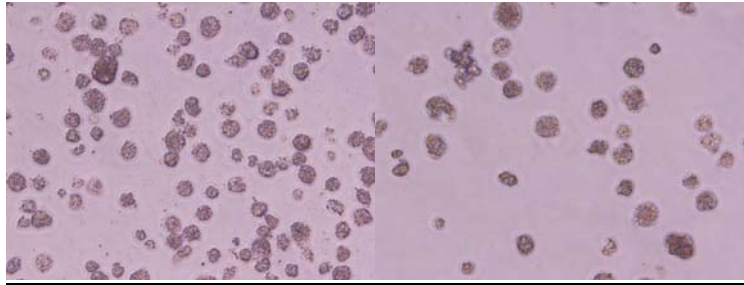


Fig 3 : Representative mast cell population under the microscope.



Balb/c injected with 4T1 cells

Balb/c + zymosan+ 4T1 cells



SKG injected with 4T1 cells

SKG + zymosan+ 4T1 cells

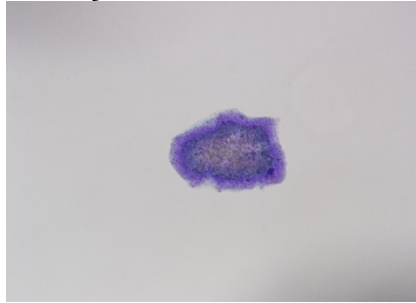


Fig 4: Representative image of mast cell staining to confirm that the cells seen under the microscope are mast cells: Toluidine staining stains mast cells as purplish blue

From the above data , we confirmed that the bone marrow cells differentiating into mast cells by addition of IL-3 and SCF is significantly higher in arthritic SKG mice versus non-arthritic Balb/c mice when induced with breast cancer

7) We evaluated the mast cell population in the lungs of these mice. The lungs were processed and stained with CD117 FITC and SCF PE to understand the SCF- C-Kit interaction. We found significantly higher population of positive mast cell staining and SCF staining in the lungs of arthritic SKG induced with breast cancer versus non-arthritic Balb/c induced with breast cancer.

8) The processed bone marrow cells were also stained with CD117 and SCF and higher population of CD117 positive and SCF positive cells were seen in the bone marrow of arthritic versus non-arthritic mice induced with breast cancer.

9) The tumors were also processed to determine the population of mast cells and SCF. We found significantly high population of CD117 positive mast cells and its ligand SCF in the tumor microenvironment of arthritic SKG mice with breast cancer as compared to non-arthritic Balb/c mice with breast cancer.

From these above data we confirmed that the SCF/c-Kit signaling is significantly up regulated in the arthritic microenvironment, hence up regulating the population of mast cells through C-kit receptor expressed on the surface of mast cells. Hence, we can assume that Mast cells are activated by breast tumor-derived SCF via c-Kit signaling in the arthritic versus non-arthritic mice.

Specific Aim 2: To test the same parameters as Aim 1 in the MMTV-PyV MT mice (a model of spontaneous breast cancer) which are induced to develop arthritis by administration of type II collagen at 14 weeks of age. The mouse mammary gland tumor virus MMTV driven polyoma middle T antigen (PyV MT) transgenic mice were induced to develop autoimmune arthritis by administration of type II collagen. The PyV MT mice develop spontaneous palpable mammary gland tumors by 12 weeks of age and progress to large adenocarcinomas by 24 weeks. 50-60% of these mice also develop lung metastasis between the ages of 20-24 weeks. By 26-28 weeks, the primary tumors are 10% of the body weight and the mice have to be sacrificed. Mice were injected with 50ul of 2mg/ml Type II collagen (CII) in complete Freund's adjuvant (CFA) intradermally ~1.5cms distal from base of the tail at 14 weeks of age. This is the most commonly used model of AA in a C57BL/6 background. All the parameters studied in Aim 1 were also done in Aim 2.

Animal numbers: PyV MT mice injected with CII at 14 weeks: 6 mice; PyV MT mice with no arthritis: 6 mice; C57BL/6 mice injected with CII : 6 mice; C57BL/6 mice with no arthritis: 6 mice. Total mice: 24 mice. *Please note:* We repeated the experiments from Aim 2 with large n numbers (n=10 from each group) and once again confirmed our results.

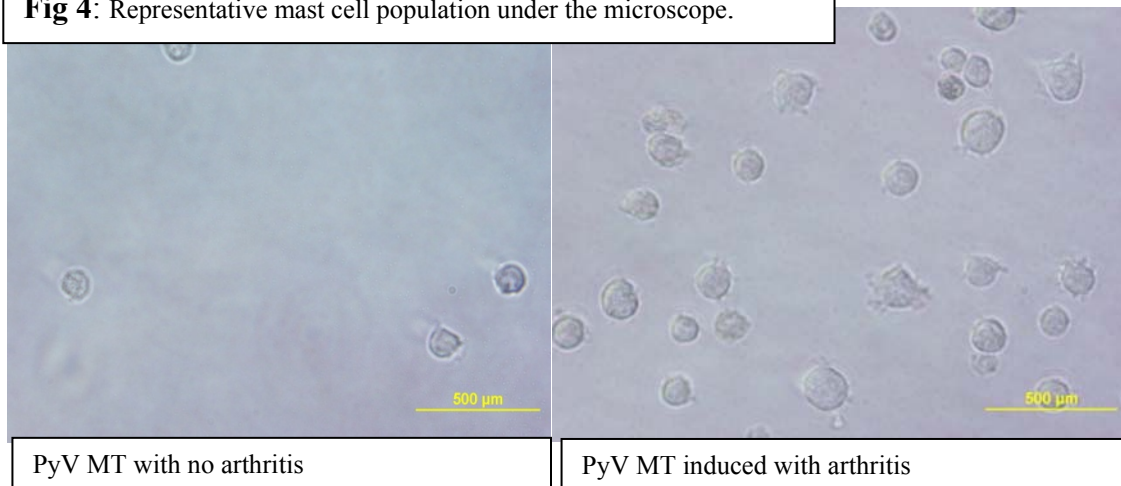
Results: We evaluated the tumor burden coupled with bone and lung metastasis in the arthritic PyV MT mice versus non-arthritic PyV MT. We report: **1)** Significantly higher tumor burden in PyV MT mice induced with arthritis as compared to non-arthritic PyV MT **2)** The lung metastasis was significantly higher in arthritic versus non-arthritic PyV MT. **3)** The bone marrow cells were stained with pancytokeratin to look for the metastatic epithelial cells through flow cytometry. We found significantly higher pancytokeratin positive staining in the bone marrow of arthritic PyV MT mice versus non-arthritic PyV MT. **4)** The x-ray imaging confirmed the metastatic lesions in the bones of arthritic PyV MT versus non-arthritic PyV MT

After confirming that metastasis along with tumor burden is significantly higher in the arthritic versus non-arthritic PyV MT, we tried to evaluate the contribution of mast cells in the enhanced metastasis.

5) We cultured the bone marrow cells. After flushing the bone marrow and processing it, we added IL-3 and SCF to the bone marrow cells. Addition of IL-3 and SCF will differentiate the bone marrow cells to immature and mature mast cells. Every three days, we collected the supernatant and added fresh IL-3 and SCF to it. It takes one month to generate mast cells from the bone marrow progenitors.

Below is the total number of mast cells generated. We counted the mast cells from each plate per group. A) PyV MT + CII : Approximately on an average 100×10^4 mast cells were counted. B) PyV MT only with no arthritis : Approximately on an average 40×10^4 mast cells counted. To confirm that the cells counted are mast cells, we stained the cells with CD117 antibody. We also confirmed that the cells we saw under the microscope and counted are mast cells by staining the cells with toluidine staining .Both ways, it was confirmed and we got positive CD117 staining with flow cytometry as well as positive toluidine staining. Fig 4 shows the representative mast cell population under the microscope with significantly high number of mast cells in arthritic PyV MT versus non-arthritic PyV MT

Fig 4: Representative mast cell population under the microscope.



We evaluated the mast cell population in the lungs of these mice. The lungs were processed and stained with CD117 FITC and SCF PE to understand the SCF- C-Kit interaction. We found higher population of positive mast cell staining and SCF staining in the lungs of arthritic PyV MT versus non-arthritic PyV MT.

8) The processed bone marrow cells were also stained with CD117 and SCF and higher population of CD117 positive and SCF positive cells were seen in the bone marrow of PyV MT mice induced with arthritis as compared to non-arthritic Py V MT mice.

9) The tumors were also processed to determine the population of mast cells and SCF. We found significantly high population of CD117 positive mast cells and its ligand SCF in the tumor microenvironment of arthritic PyV MT mice versus non-arthritic PyV MT.

Specific Aim 1 & 2:

We repeated the endpoints of Specific 1 and 2 again. In summary, From Aim 1, we did not see any difference between the metastasis as well as expression of mast cells and expression of bone marrow differentiated mast cells between SKG ± zymosan with breast cancer. Therefore in the figures below, we are showing representation of SKG mice challenged with breast cancer. It is to be noted that we saw the same results with SKG mice with zymosan challenged with breast cancer (data not shown). We saw exactly the same trend in both arthritic SKG mice ± zymosan.

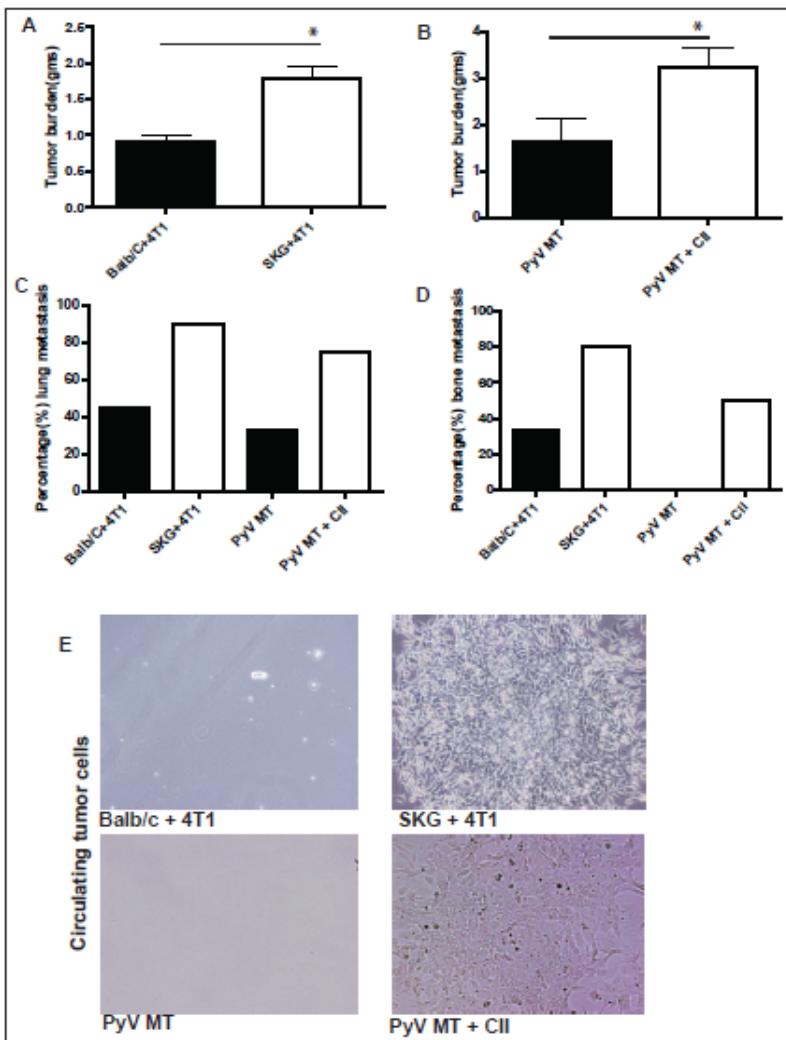


Figure 5: Higher tumor burden coupled with higher metastasis in arthritic versus non-arthritic BC bearing mice

A and B: Significant increase in tumor burden in SKG versus Balb/C (*P<0.05) and arthritic PyV MT versus PyV MT (*P<0.05) mice. **C and D:** Percentage of lung and bone metastasis respectively. **E:** Light microscopic images of circulating tumor cells cultured from peripheral blood of arthritic versus non-arthritic tumor bearing mice. (A-E: N=10 mice). **Significant increase in primary tumor burden and lung and bone metastasis in arthritic versus non-arthritic mice with BC**

We first demonstrate that the 4T1 tumor burden is significantly higher in the arthritic SKG versus the non-arthritic Balb/C mice (Fig 5A). Second, we substantiate this finding in the PyV MT mice with higher tumor burden in the arthritic PyV MT versus the non-arthritic PyV MT mice (Fig 5B). Third, metastasis to the lungs

and bones were compared. We report that 9 out of 10 SKG mice show lung metastasis while only 4 out of 10 Balb/C mice show metastasis (Fig 5C). Similarly, 7 out of 10 arthritic PyVMT mice show lung metastasis while only 3 out of 10 non-arthritic PyVMT mice show the same (Fig 5C). Similar trends were observed with bone metastasis with 8 out of 10 SKG mice developing bone metastasis while only 3 out of 10 Balb/c developing the same (Fig 5D). Likewise, 5 out of 10 arthritic PyVMT mice developed bone metastasis while none of the non-arthritic PyVMT developed bone metastasis (Fig 5D). We detected

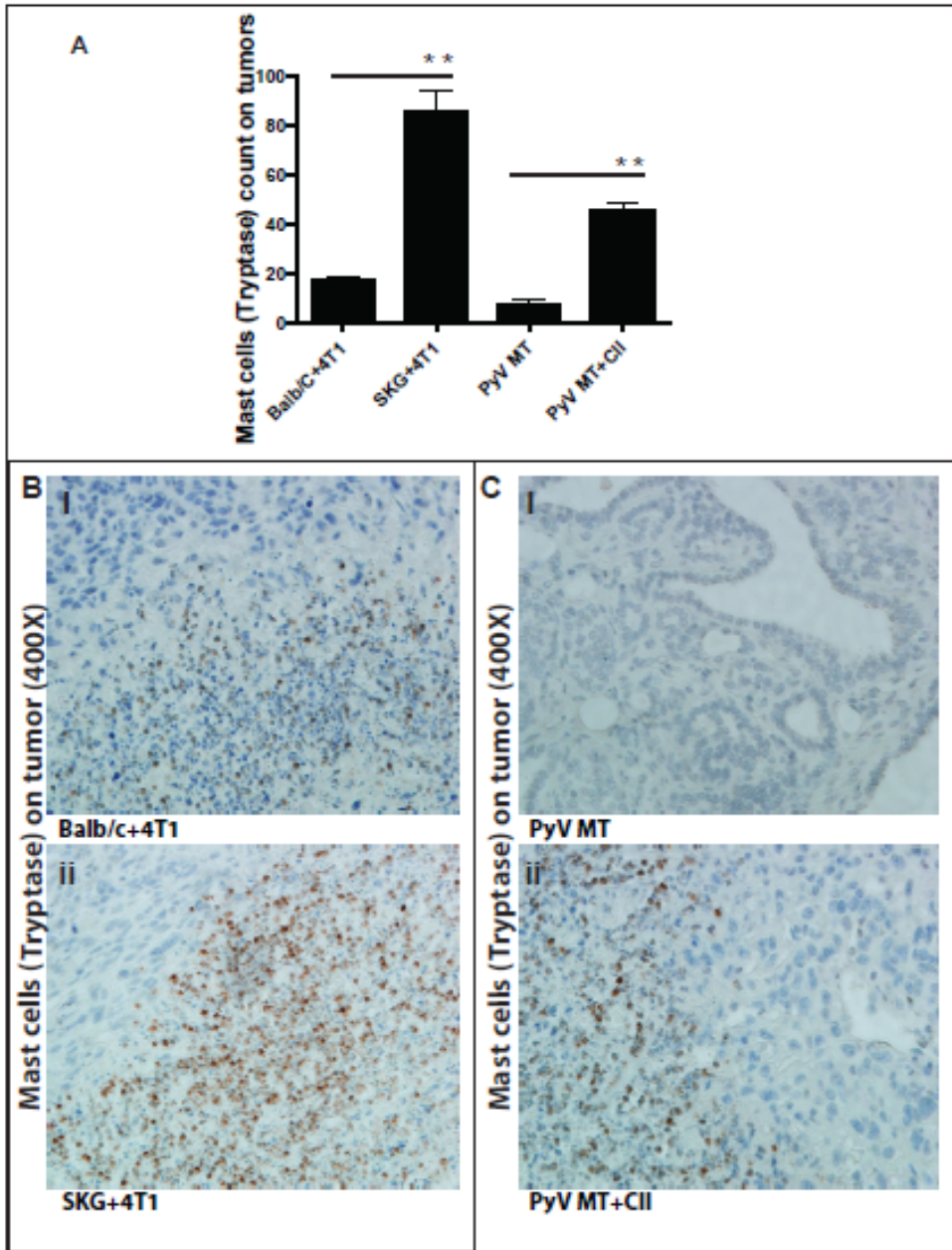
higher levels of CTCs (that formed colonies within 3-weeks of culture) in the blood of the tumor-bearing arthritic mice compared to the blood from the tumor-bearing non-arthritic mice (Fig 5E).

Figure 6: Expression of MC tryptase staining on tumors

A: Significant increase in MCs tryptase count in the tumors of arthritic SKG mice challenged with 4T1 cells versus non-arthritic Balb/c mice injected with 4T1 cells (**P<0.01) and in arthritic PyV MT mice induced with CII versus non-arthritic PyV MT mice (**P<0.01). Representative images of tryptase staining in **B** and **C:** tumors taken at 400x magnification. (N=6 mice per experimental group and 10 fields per section). Brown staining represents MCs tryptase expression.

Mast cell (MC) population in the tumor microenvironment and metastatic niche

We assessed the expression of Mast cells (MCs) in the tumor and metastatic niches (bone and lung). Tryptase is the most abundant secretory granule-derived serine contained in MCs that has been used as a marker for mast cell activation. In addition, tryptase has been shown to be a sensitive and specific marker for the



localization of mast cells in tissues. By doing MC tryptase staining by IHC, we detected significantly higher number of MCs expression in the tumors of arthritic versus non-arthritic mice with BC (Figure 6A). We detected huge infiltration of MCs in the tumors of SKG versus Balb/c mice with BC (Figure 6B) and in the PyV MT mice induced with arthritis versus PyV MT mice with no arthritis (Figure 6C). We further identified significantly higher levels of MCs in the sites of metastasis: lungs (Figure 7) and bones (Figure 8) of arthritic versus non-arthritic mice in both (SKG and PyVMT) models. This suggested that the arthritic milieu may directly influence MC recruitment and activation within the tumor and the metastatic microenvironment. Thus, data suggest that there is an increase in MC migration and activation within the tumor as well as in the metastatic niches as indicated by the increased MC expression. However, to further confirm the *in-vivo* infiltration of MCs, toluidine staining was also conducted on

tissue sections. MCs contain granules (metachromatic) composed of heparin and histamine. Toluidine blue stains MC blue-purple (metachromatic staining) and the background blue (orthochromatic staining). Clearly, in both the arthritic PyVMT and SKG mice, we observe a significantly increased infiltration of MCs within the tumor (Fig 9A-B), lung (Fig 9C-D) and bone (Fig 9E-F). Shown in the Figures 6-9 are representative MC staining in the tissues from n=1 mouse per experimental group. Also shown in Fig 5B is a magnified image of MC infiltration in the 4T1 tumor section of an arthritic SKG mouse. To quantitate the results (Figures 6-9), MCs were counted under the microscope (n=6 mice per experimental group and 10 fields per section per organ) and the mean numbers reflected in the graph. Interestingly, in the non-tumor bearing SKG and CII-injected C57BL/6 mice, the MC population was upregulated compared to tumor-bearing Balb/c or PyVMT mice (Figures 6-9) indicating that the arthritic milieu by itself can increase the MC population in the bones and lungs creating a conducive niche that attracts the SCF expressing tumor cells. SCF binds to the c-Kit receptor on MCs and helps in the differentiation, migration, maturation, and survival of MCs, remodeling the microenvironment by increasing the population of MCs. These findings provide a new insight into the role of MCs in tumors and the relation among inflammation, immunosuppression, and tumor progression. Thus, we believe that the higher tumor burden and metastasis seen in the arthritic mice (Fig 5) may be due to the increase in MC infiltration and propagation in the tumors and metastatic niches. The MC expression in the lungs and bones of non-arthritic control mice (Balb/c and C57BL/6) with no tumor was found to be almost negligible (data not shown).

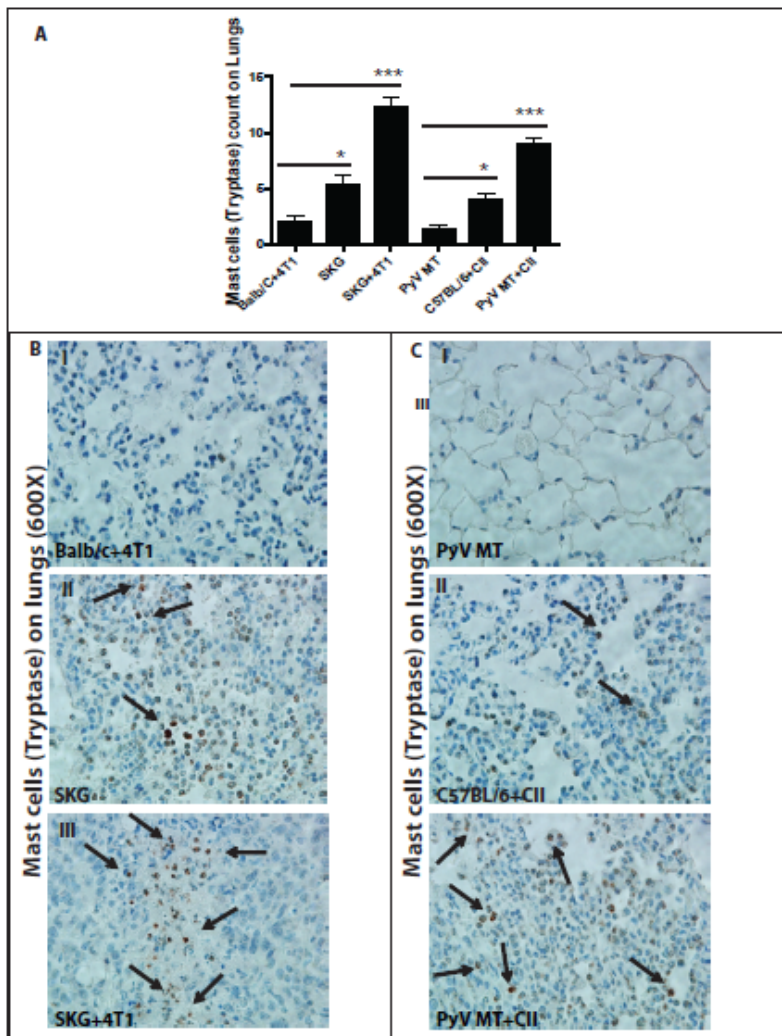


Figure 7: Expression of MCs tryptase staining on lungs

A: Significant increase in MCs tryptase count in the lungs of arthritic SKG mice challenged with 4T1 cells versus non-arthritic Balb/c mice injected with 4T1 cells (** $P < 0.001$) and in arthritic PyV MT mice induced with CII versus non-arthritic PyV MT mice (** $P < 0.001$). Significant increase in MCs tryptase is also observed in the lungs of arthritic SKG mice with no tumor versus Balb/c mice with BC (* $P < 0.05$). Similarly, we see the same trend in arthritic C57BL/6 mice induced with CII versus spontaneous BC model of PyV MT mice with no arthritis (* $P < 0.05$). Representative images of tryptase staining in **B** and **C**: lungs taken at 600x magnification. (N=6 mice per experimental group and 10 fields per section). Brown staining represents MCs tryptase expression

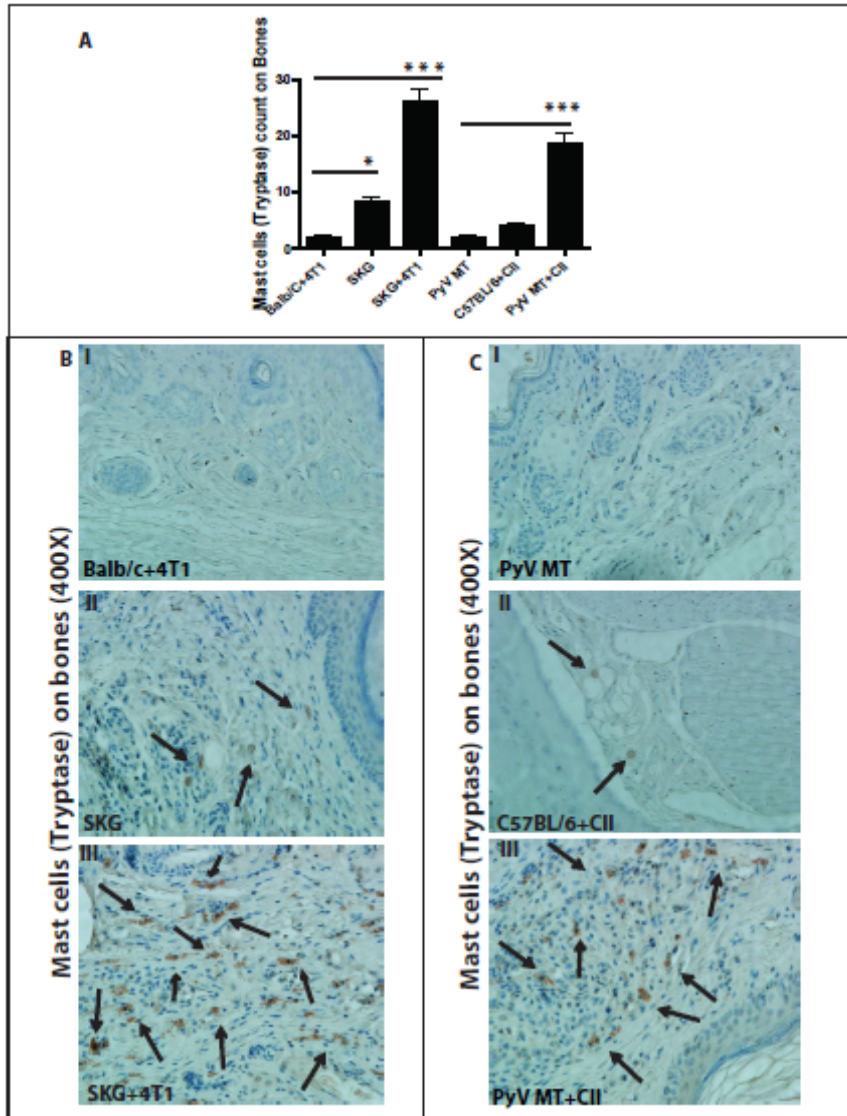


Figure 8: Expression of MCs tryptase staining on bones

A: Significant increase in MCs tryptase count in the bones of arthritic SKG mice challenged with 4T1 cells versus non-arthritic Balb/c mice injected with 4T1 cells (** $P < 0.001$) and in arthritic PyV MT mice induced with CII versus non-arthritic PyV MT mice (** $P < 0.001$). Significant increase in MCs tryptase is also observed in the bones of arthritic SKG mice with no tumor versus Balb/c mice with BC ($P < 0.05$). Similarly, though not significant but we see the same trend in arthritic C57BL/6 mice induced with CII versus spontaneous BC model of PyV MT mice with no arthritis. Representative images of tryptase staining in **B and C:** bones taken at 400x magnification. (N=6 mice per experimental group and 10 fields per section). Brown staining represents MCs tryptase expression.

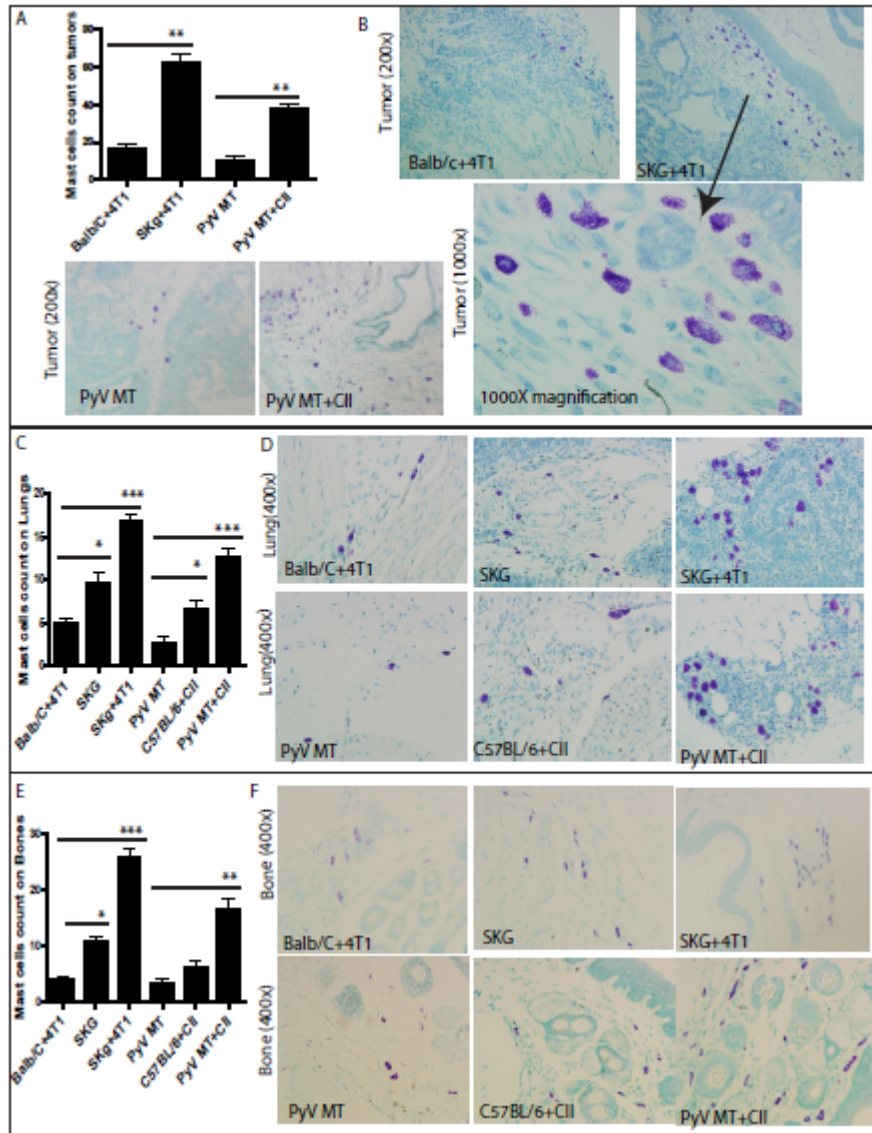


Figure 9: Toluidine staining to confirm MC expression

Significant increase in MCs toluidine count in arthritic mice with BC (SKG+4T1 and PyV MT+CII) versus non-arthritic (Balb/c+4T1 and PyV MT) BC models in **A**) tumors (**P<0.01), **C**) Lungs (***) and **E**) bones

(***P<0.001,**P<0.01).

Significant increase in MCs toluidine staining was also observed in arthritic control (SKG and C57BL/6+CII) mice with no tumor as compared to non-arthritic (Balb/c mice with BC and spontaneous BC PyV MT mice) in **C**) lungs (*P<0.05) and **E**) bones (*P<0.05) We observed the same trend of MC expression with toluidine staining in tumors, lungs and bones of arthritic versus non-arthritic BC models as in MCs tryptase staining (Figures 2-4). Representative images of toluidine staining in **B**) tumors (200x), **D**) Lungs (400x) and **F**) Bones (400x) magnification. (N=6 mice

per experimental group and 10 fields per section per organ). Purple-blue staining represents MCs toluidine expression.

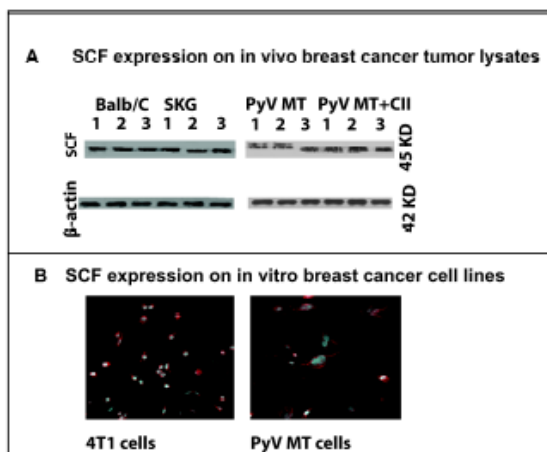


Figure 10: SCF expression on the tumors

Mast cell migration to the tumor site and the following activation may be the prerequisite for their promoting effect on tumors. In this regard, SCF is possibly involved. SCF is a potent mast cell attractant that stimulates directional motility of MCs. SCF is a chemotactic factor as well as a growth factor and that the ckit receptor on MCs can transduce signals leading to both cell proliferation and increased directional cell motility. In our study, to confirm that MC activation and proliferation in the tumors and sites of metastasis is mediated by tumor derived SCF, we assessed the

expression of SCF on 4T1 and PyV MT tumors *in vivo* by western blotting. We observed that SCF was highly expressed by the 4T1 and PyV MT tumors *in vivo* (Figure 10A). But, there was no difference in SCF protein expression levels between tumors derived from arthritic versus non-arthritic mice indicating that the arthritic milieu does not influence SCF levels. SCF expression was further assessed in the 4T1 and PyVMT tumor cell lines *in vitro* by IF. We observed the expression of SCF in both the cell lines (Figure 10)

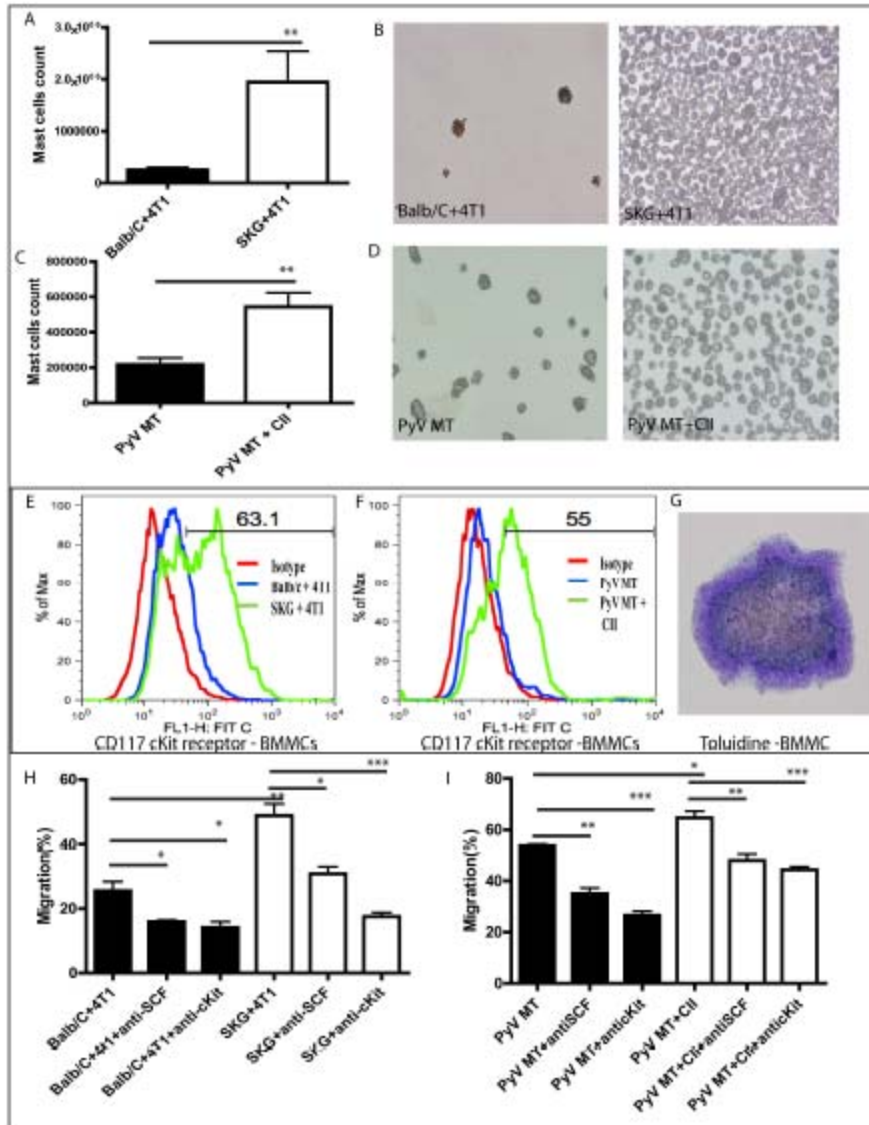


Figure 11: Generation of BM derived MCs

Significant increase in MCs count in tumor bearing **A)** SKG (**P<001) and **C)** arthritic PyV MT (**P<001) mice (N=10). **B and D)** : Representative images of MCs at 400x magnification. **E and F)** : Flow cytometric analysis of percent cells expressing cKit receptor on MCs **G)** : Representative image of toluidine staining for MC (1000x magnification). **H and I)** : Significant increase in migration of 4T1 and PyV MT cells towards the MCs from tumor bearing arthritic mice (BMMCs) (**P<001 and *P<0.05). Pretreatment of 4T1 and PyV MT cells with anti-SCF antibody or adding anti-c-Kit antibody to the MCs in the lower chamber significantly decreased the migration of the tumor cells towards the MCs (*P<0.05, **P<0.01, ***P<0.001).

Increased differentiation of MCs from BM derived hematopoietic precursors in arthritic versus non-

arthritic mice with BC

We cultured 2×10^6 BM cells in the presence of IL-3 and SCF for 30 days to induce the precursor cells to differentiate into MCs. Numbers of MCs from SKG and PyVMT mice are represented in Fig 11A and C respectively. Light microscopic images of differentiated MCs from SKG and PyVMT mice are shown in Fig 11B and D respectively. Significant increase in MC differentiation was observed in tumor-bearing SKG and arthritic PyVMT mice as compared to tumor-bearing non-arthritic Balb/c mice and non-arthritic PyVMT mice (Fig 11A - D). We also observed significant increase in the bone marrow derived mast cells

(BMMCs) in non-tumor bearing arthritic SKG and C57BL/6+CII mice versus tumor bearing non-arthritic Balb/c+4T1 and PyV MT mice (data not shown). Figure 11 B and D shows the light microscopic view of MC morphology. Cells were stained for (c-Kit receptor) CD117 expression and analyzed by flow cytometry. Representative histogram is shown in Fig 11E and F. In addition, to confirm that the cells were indeed MCs, toluidine staining was conducted and representative staining is shown in Fig 11G. Data suggest that the BM-derived precursor cells that are pre-destined to differentiate into MCs are significantly higher in the arthritic BM milieu. It can therefore be speculated that AA is affecting hematopoiesis and inducing the production of MCs- thus creating a microenvironment appropriate for tumor cells to reside and form metastasis.

SCF-cKit signaling is the underlying mechanism of increased metastasis in arthritic mice with BC

To determine that the chemotactic migration of tumor cells into metastatic sites is indeed SCF-MCs mediated, we conducted migration assays. SCF expressing 4T1 and PyV MT cells (Figure 10B) were placed in the upper chamber and BMMCs (Figure 11A-G) of arthritic and non-arthritic mice in the lower chamber. We found significant increase in the migration of 4T1 and PyVMT cells towards the MCs that were derived from tumor-bearing arthritic SKG or PyVMT mice (Fig 11H and I respectively). We further showed that pre-treatment of 4T1 and PyVMT cells with anti-SCF antibody or adding anti-cKit antibody to the MCs in the lower chamber significantly decreased the migration of the tumor cells towards the MCs (Fig 11H and I). The data clearly suggest that the SCF/c-Kit signaling is one of the major drivers of BC-associated bone and lung metastasis. This is the first study that undoubtedly establishes the mechanism behind the homing of the circulating BC cells to the lungs and bones of arthritic mice.

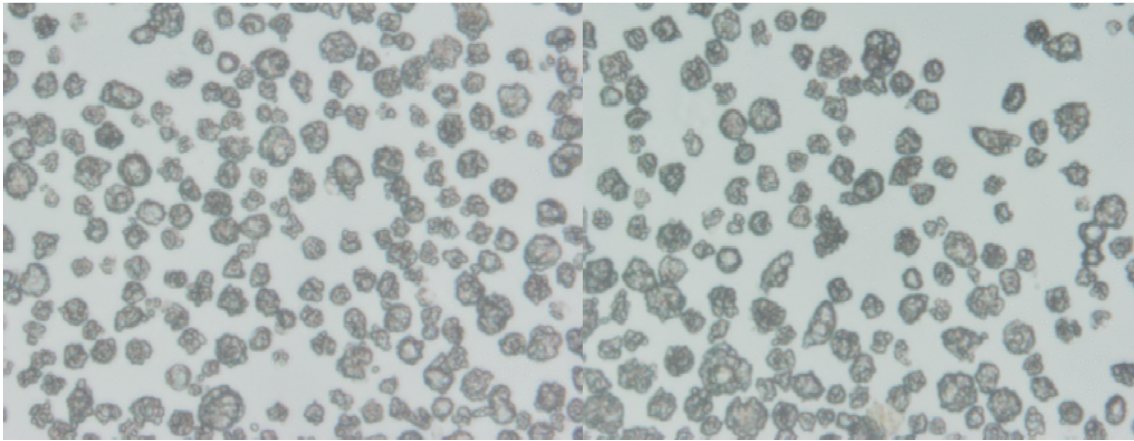
Specific Aim 3: To test the efficacy of inhibiting the activation of MCs by anti-SCF or anti-c-Kit in preventing metastasis in both models. We also used celecoxib, a known anti-arthritic drug alone or in combination with the anti-SCF or anti-c-Kit. In the future, other critical cytokines identified from aims 1 and 2 will be targeted. At 2 months of age, 50 SKG mice were given one ip dose of zymosan A (2 mg/100µl of saline). One month post zymosan, mice were challenged with syngeneic 4T1 breast cancer cells in mammary fat pad. Tumors were allowed to grow for 2 weeks. When tumors were ~5% of the mouse body weight, 10 mice were treated with anti-SCF+celecoxib and 10 mice were treated with anti-C kit receptor + celecoxib(50ug/mouse, EBiosciences). One ip injection of 10µg/ml of purified anti-SCF antibody (R&D Systems,Minneapolis,MN) and one ip injection of 50 ug of anti-C Kit receptor only administered. Celecoxib (20mg/kg in 100 uls of 10%DMSO from Pfizer), a known anti-arthritic and anti-tumor agent was gavaged starting at the same time as the anti-SCF/anti C kit receptor antibody but was given daily until sacrifice except for the weekends. Ten mice were treated with celecoxib alone and another 10 mice were treated with anti-SCF/anti C Kit receptor alone. Ten control mice received 5µg/ml (100µl volume) of control Rat IgG/ goat IgG antibody in saline (BD biosciences) + 100uls of 10%DMSO. Ten mice were left untreated. Mice were sacrificed two weeks post anti-SCF/anti C kit receptor treatment.

Animal numbers for Aim 3. (A) SKG + zymosan A + 4T1 challenge:10 mice: no treatment; 10 mice: celecoxib (20mg/kg in 10% DMSO in 100uls, gavage); 10 mice:anti-SCF (10ug/ml in 100 uls of saline); 10 mice:anti-SCF +celecoxib; 10 mice: anti-C kit receptor +celecoxib; 10 mice: anti-C kit receptor; 10 mice: control rat IgG antibody + 10% DMSO; 10 mice: control goat IgG antibody. **Total mice for aim 3 with SKG model: 50 mice X 2 (for anti-c-Kit) = 120 mice.**

Results

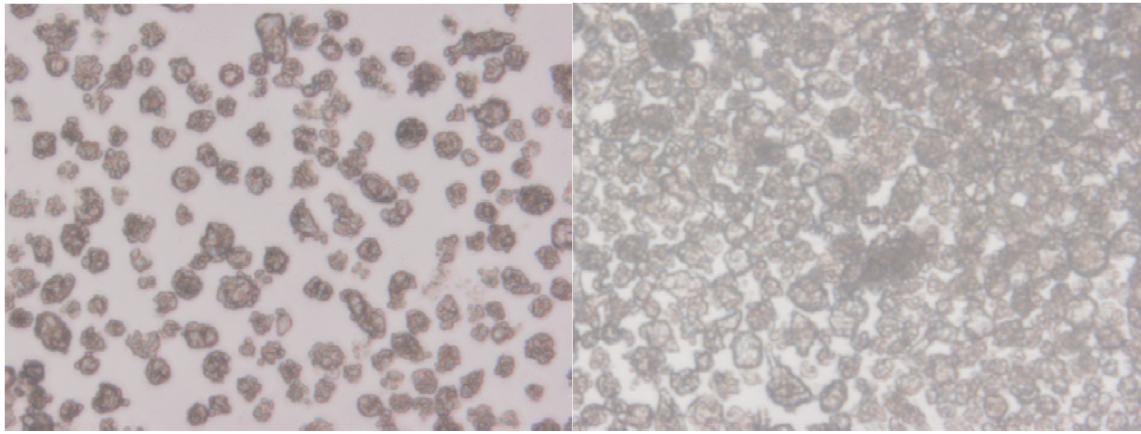
The bone marrow cells were processed from the mice and cultured in vitro for 45 days with IL-3 and SCF. The mast cells were counted and it was observed that the differentiation of bone marrow cells to mast cells was significantly lower in the SKG mice injected with 4T1 cells and then treated with anti- C Kit receptor expressed on the mast cells. The population of mast cells in Balb/c mice did not show significant difference since the mast cells in Balb/c non-arthritic mice was significantly low to begin with as seen in Figures 2 and 3.

Figure 12: Representation of the mast cells from SKG mice challenged with 4T1 cells followed by treatment.



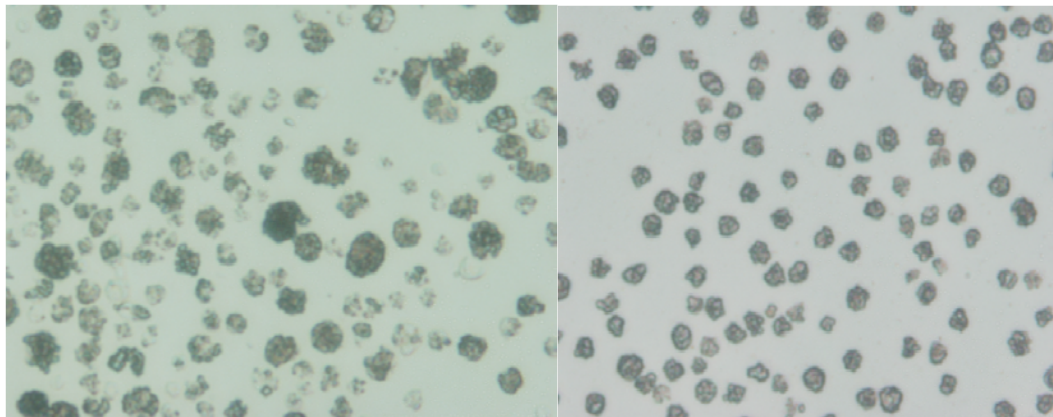
SKG + 4T1 + untreated

SKG + Zymosan + 4T1 + Untreated



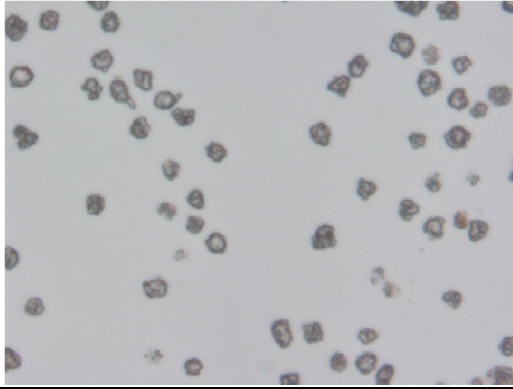
SKG + 4T1 + zymosan+Goat IgG isotype

SKG + 4T1 + Goat IgG isotype

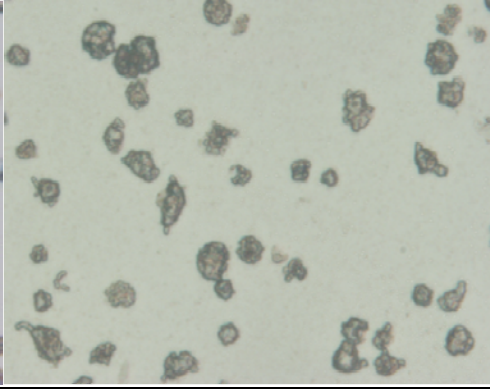


SKG + 4T1 + Rat IgG isotype

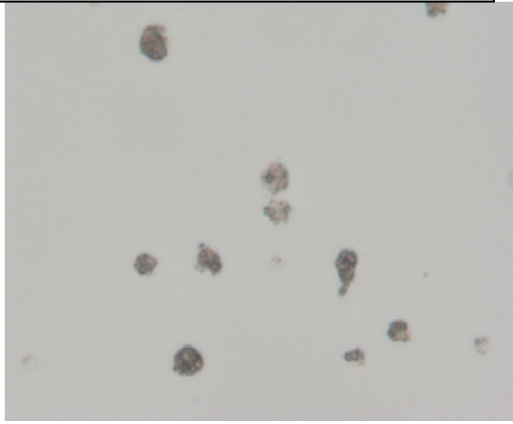
SKG + Zymosan + 4T1 + Rat IgG isotype



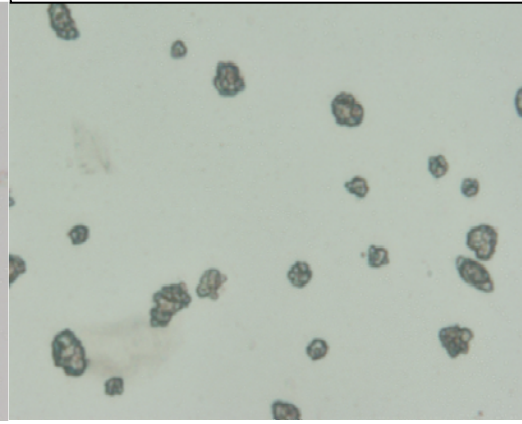
SKG + 4T1 + Celecoxib



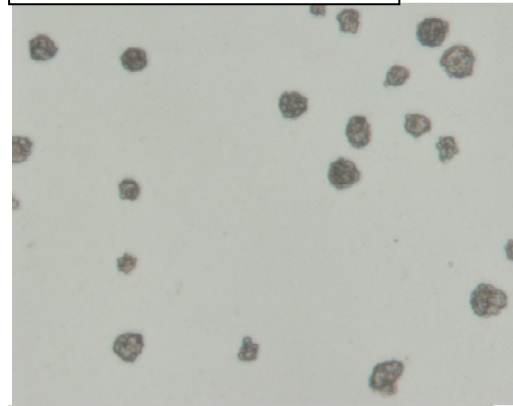
SKG + Zymosan + 4T1 + Celecoxib



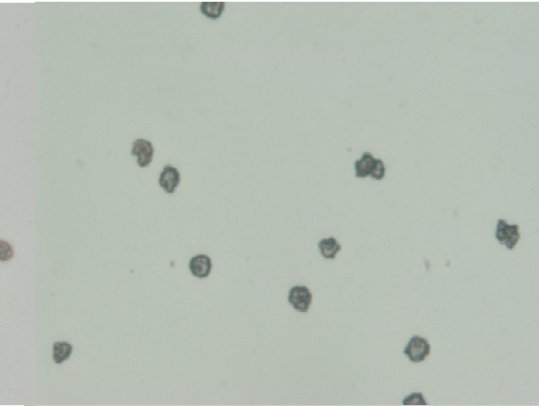
SKG + 4T1 + anti- SCF



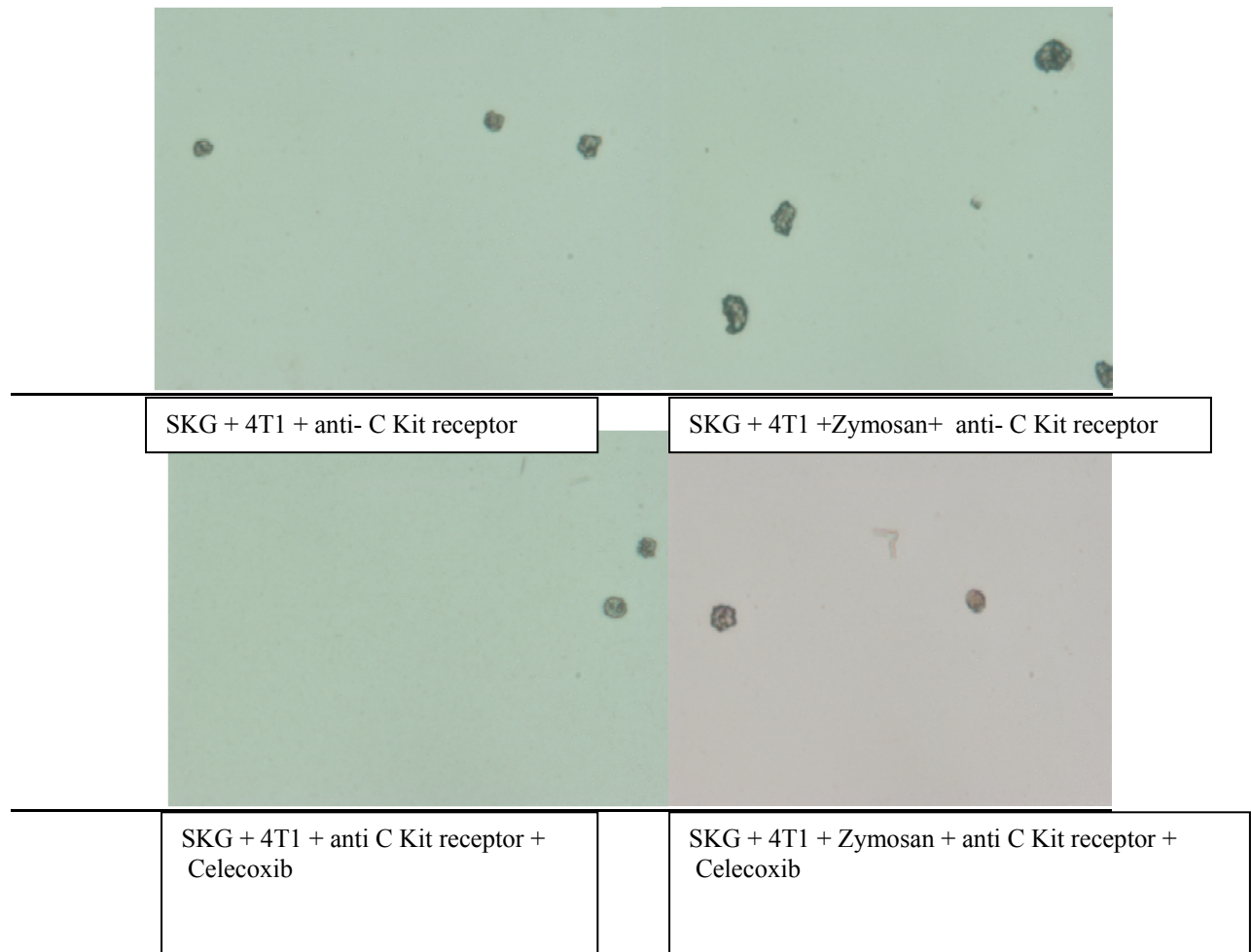
SKG + Zymosan + 4T1 + anti- SCF



SKG + 4T1 + anti- SCF+ Celecoxib



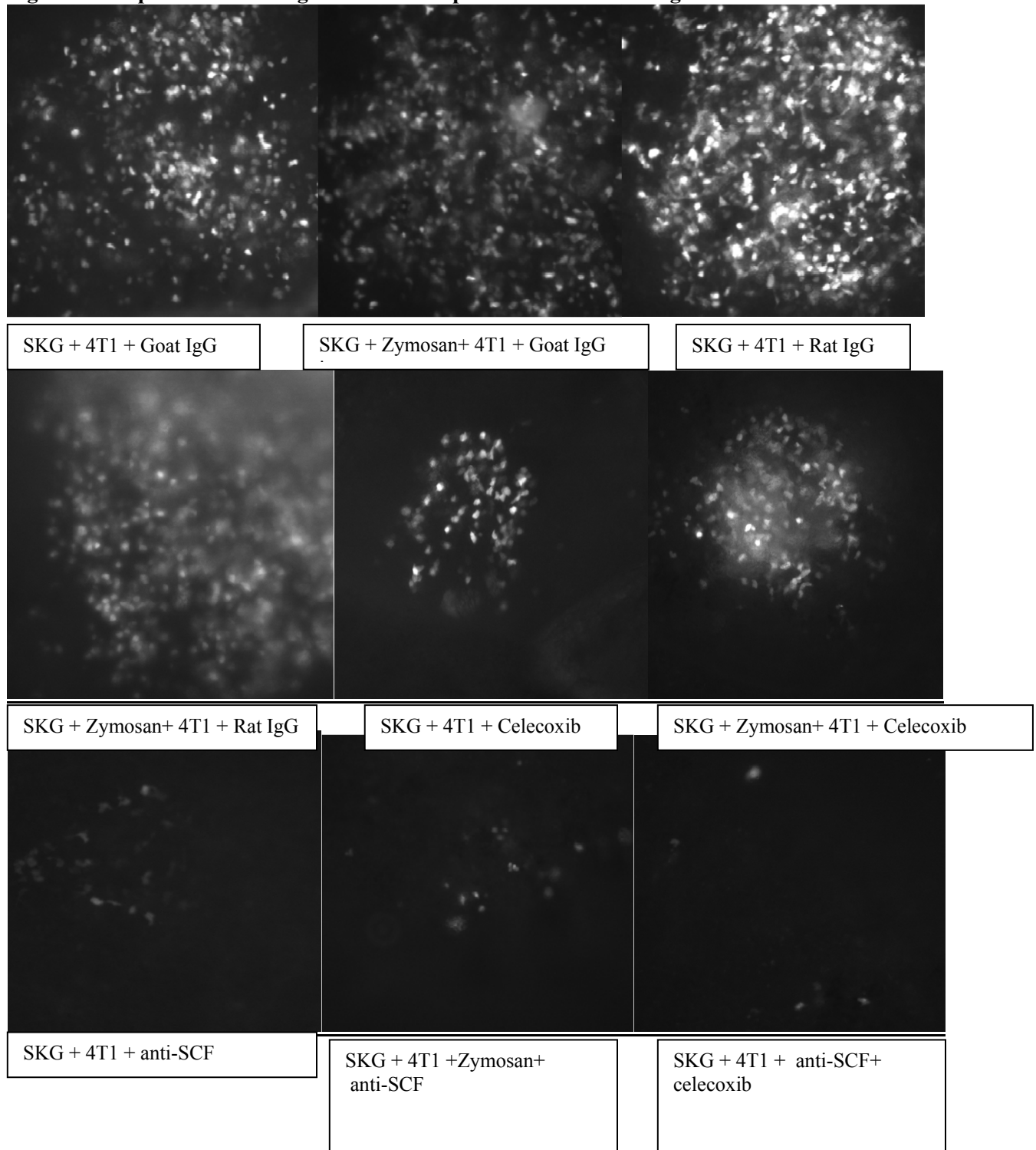
SKG + 4T1 +Zymosan + anti- SCF+ Celecoxib

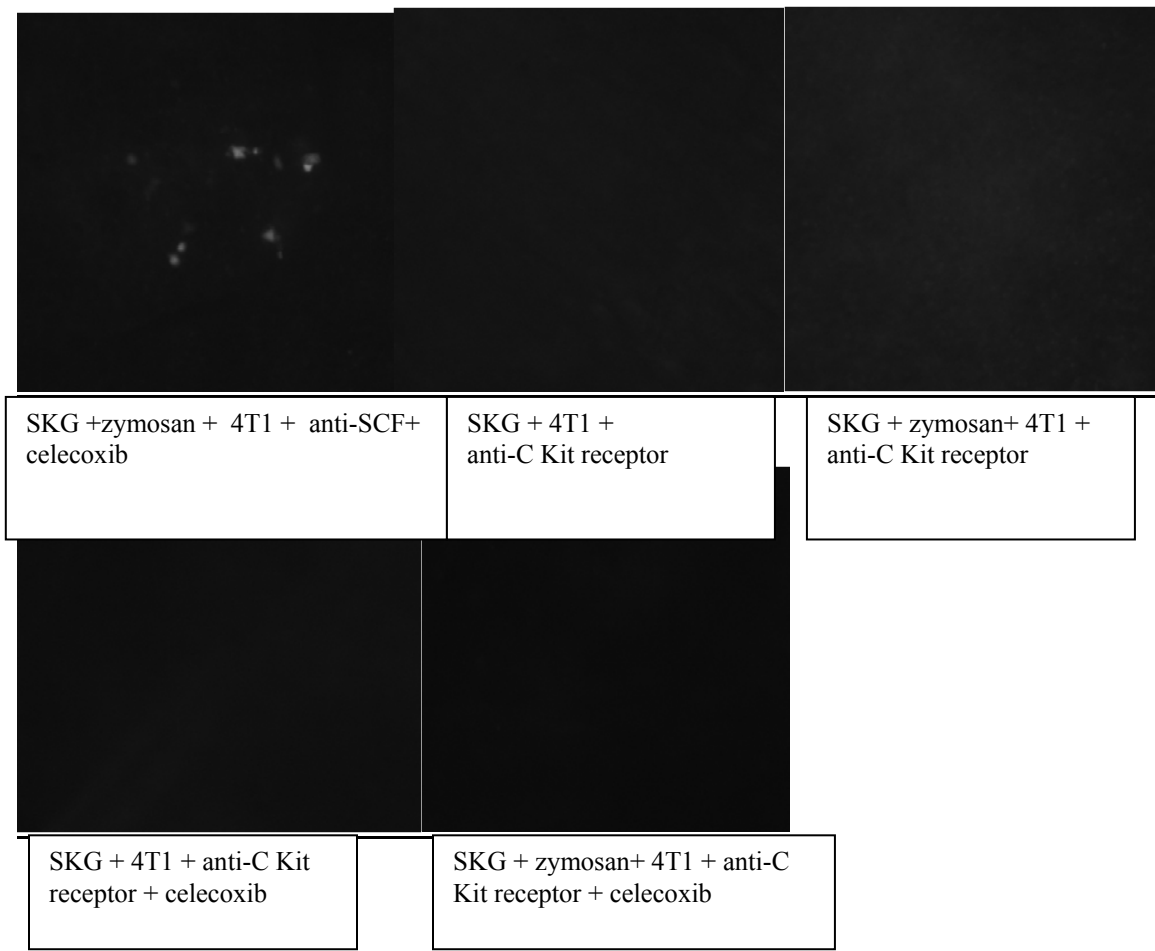


Since the mice were injected with 4 T1-GFP positive cells, so we looked for metastasis under the microscope. We found decreased metastasis in SKG mice induced with breast cancer and treated

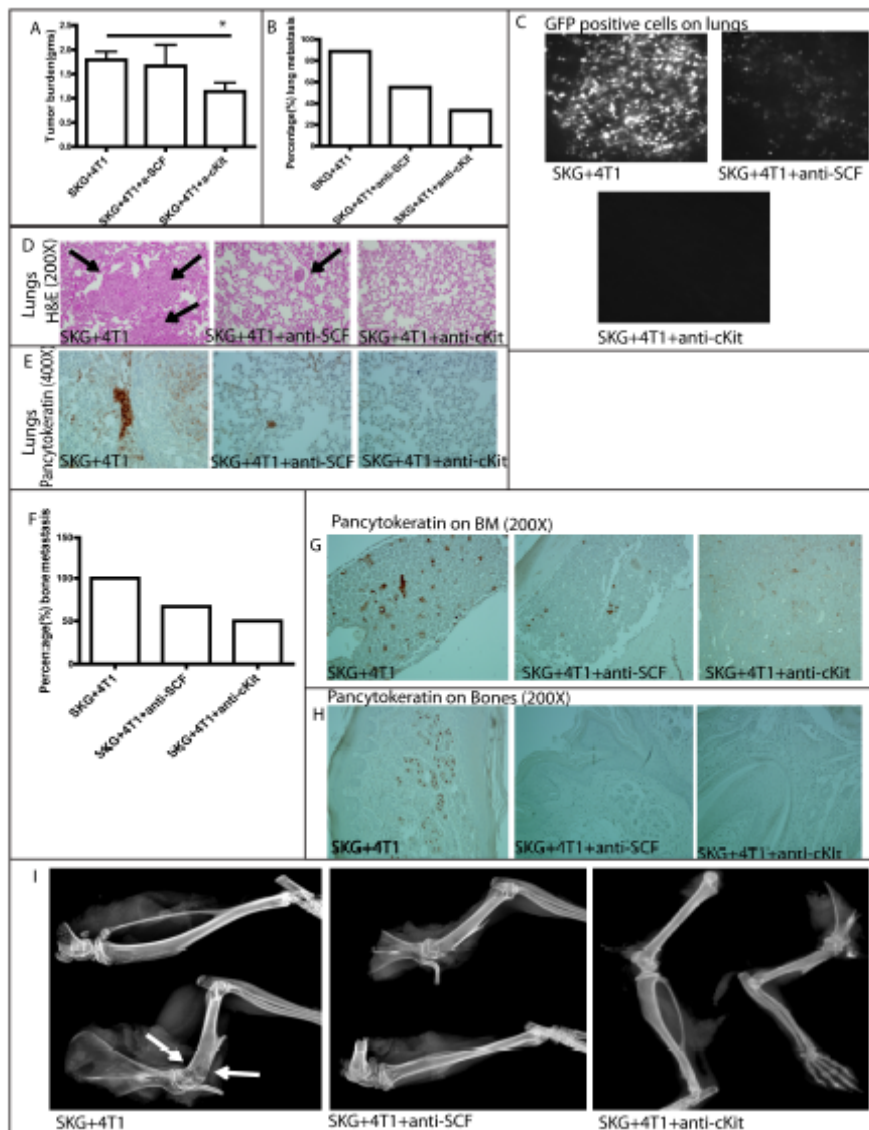
with anti-cKit or anti-SCF. The treatment did not make a difference in the Balb/c mice injected with 4T1 cells since the percentage of metastasis was significantly lower in Balb/c mice to begin with[6, 7].

Figure 13: Representative images of 4T1-GFP positive cells in the lungs of SKG mice.





From Aim 3, we have seen that SKG mice ± zymosan with breast cancer shows the same trend of results. The treatment with anti-cKit/anti-cKit+celecoxib and anti-SCF/anti-SCF+celecoxib shows the same trend. We did not see much difference when the mice were treated with celecoxib along with either anti-cKit or anti-SCF. Therefore in the figures below, we are showing representation of SKG mice challenged with breast cancer and treated with anti-cKit or anti-SCF. It is to be noted that we saw the same results with SKG mice with zymosan challenged with breast cancer. Mice treated with anti-cKit+celecoxib or anti-SCF+celecoxib showed the same results as mice treated with anti-cKit or anti-SCF alone. In the figures below, we are showing representation of SKG mice challenged with breast cancer and treated with anti-cKit or anti-SCF.



N=10 mice). Brown staining represents pancytokeratin positivity.

Blocking the SCF-cKit interaction significantly decreases the metastasis of 4T1 tumors towards the lungs and bones of SKG mice

A key finding from this study indicates that the SCF/c-Kit signaling was significantly increased in the tumor-bearing arthritic versus non arthritic mice. This set the stage for examining the effects of blocking this signaling. When the mice were treated with a therapy to target the c-Kit receptor or SCF, there was a significant decrease in tumor burden in the mice treated with anti-cKit antibody but no difference in tumor burden was observed in mice with anti-SCF therapy (Figure 14A). Nevertheless, metastasis to the lungs (Figure 14B-E) and bone (Figure 14F-I) was significantly reduced in both the treatment groups. We observed ~3 fold and ~2 fold decrease in lung metastasis in mice treated with anti-cKit and anti-SCF respectively (Figure 14B). Figure 8C shows representative images of GFP positive 4T1 cells in the lungs of SKG mice injected with 4T1 cells with no treatment versus the treated groups. Figure 14D shows the metastatic lesions in the lungs and the pancytokeratin staining confirms that the metastatic patches seen are epithelial cancer cells (Figure 14E). Similarly, we observed ~3 fold and ~2 fold decrease in metastatic bone lesions in mice treated with anti-cKit and anti-SCF respectively (Figure 14F). Pancytokeratin staining in the BM and bone tissues (Figure 14G-H) and representative radiographic x-ray bone images

(Figure 14I) confirmed the presence of epithelial tumor cells in the bones of these mice. *Note:* c-Kit is expressed in HSCs, MCs, melanocytes, and germ cells. Therefore, in our study, we identified if metastasis was attenuated by blocking the SCF/cKit interaction through downregulation of MCs as cKit is also the MC receptor and MC population is observed to be significantly high in BC mice with arthritis (Figures 6-9).

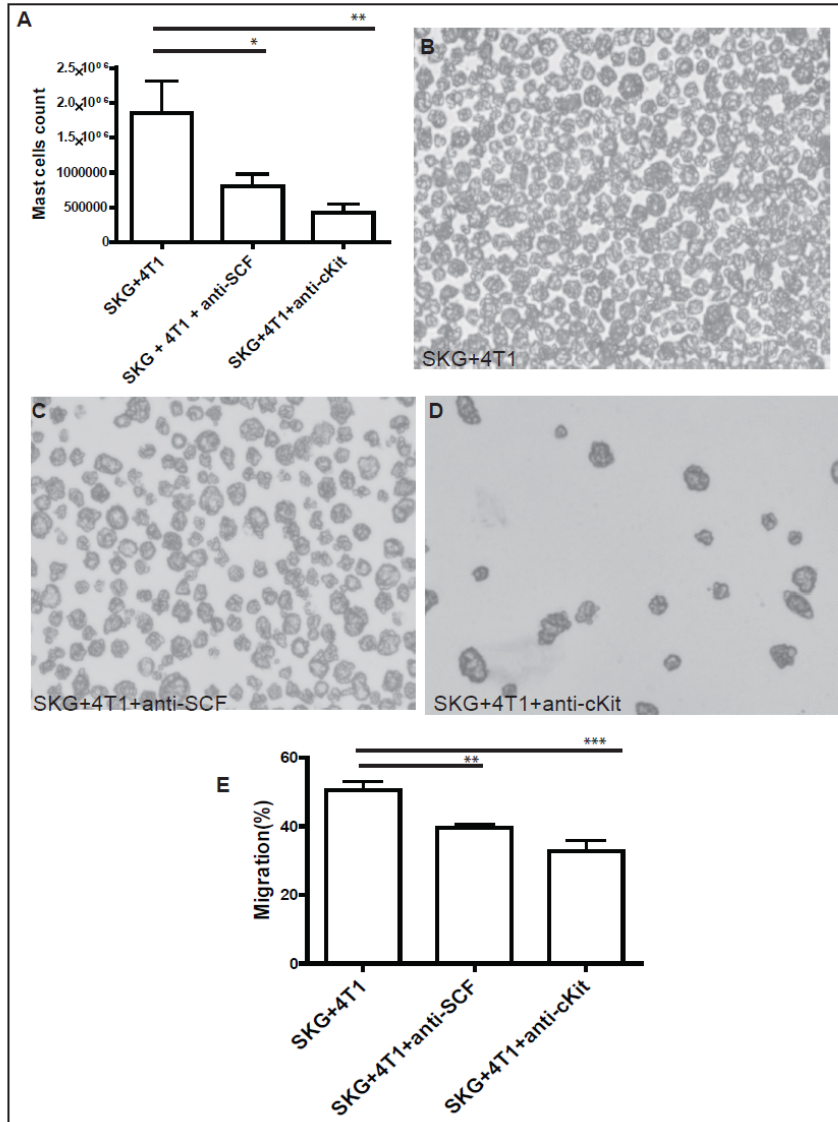


Figure 15: Decr eased differentiation of MCs from BM precursors in mice treated with anti-SCF or anti-C-kit antibody

A: Significant reduction in the differentiation of BMMCs in mice treated with neutralizing SCF or cKit antibodies (*P<0.05, **P<0.01). **B-D:** Representative images of BMMCs showing diminution of MC population in treated group (**A-D:** N=10 mice). **E:** Significant reduction in migration of 4T1 cells towards MCs from mice treated with anti-SCF or anti-cKit (**P<0.01, ***P<0.001).

Significant decrease in differentiation of MCs from BM precursors from the mice treated with anti-cKit and anti-SCF therapy.

To determine the effect of the therapy on MC differentiation, we plated 2x10⁶ cells from each treatment group of mice.

Post ~30 days of culture with IL-3 and SCF, we observed significant decrease in MC population from the BM of mice treated with anti-cKit and anti-SCF (Figure 15A-D) indicating that the BM cells destined to differentiate to MCs are affected by this therapy. Next, we performed migration assay by keeping the 4T1 cells on the upper chamber and MCs derived from the mice with and without treatment in the lower chamber. We saw a significant decrease in migration of 4T1 cells towards the MCs derived from the mice treated with anti-cKit and anti-SCF (Figure 15E). This significant finding illustrates that this therapeutic intervention not only reduces the differentiation of MCs from BM precursors but also affects migration of the MCs towards tumor cells. In all instances, treatment with anti-c-kit was significantly better than anti-SCF. The reason for this is unknown at this time but one can speculate that the anti-c-kit antibody may have a superior neutralizing effect.

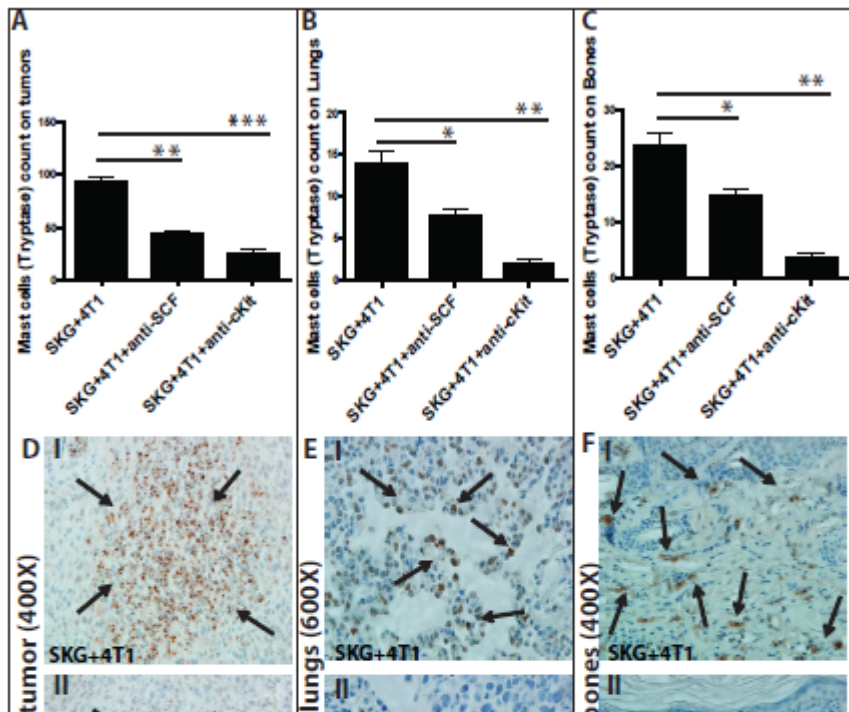


Figure 16: Significant reduction in number of MCs (tryptase staining) in the tumor, lungs and bones of mice treated with anti-SCF or anti-ckit antibody

A-C: Significant reduction in number of MCs in A) tumors (**P<0.01,***P<0.001) B) lungs (*P<0.05,**P<0.01) and C) bones (*P<0.05,**P<0.01) of treated mice. D-F: Representative images of MCs tryptase in D) tumors (400x) E) lungs (600x) and F) bones (400x magnification) (n=6 mice per experimental group and 10 fields per section per organ). Brown staining represents MCs tryptase expression.

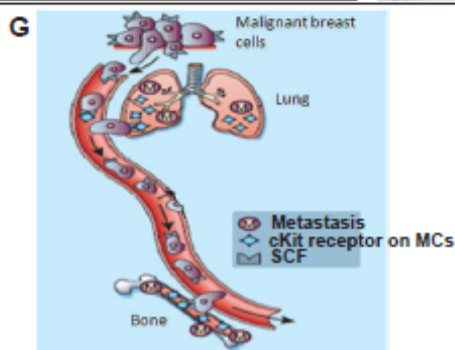
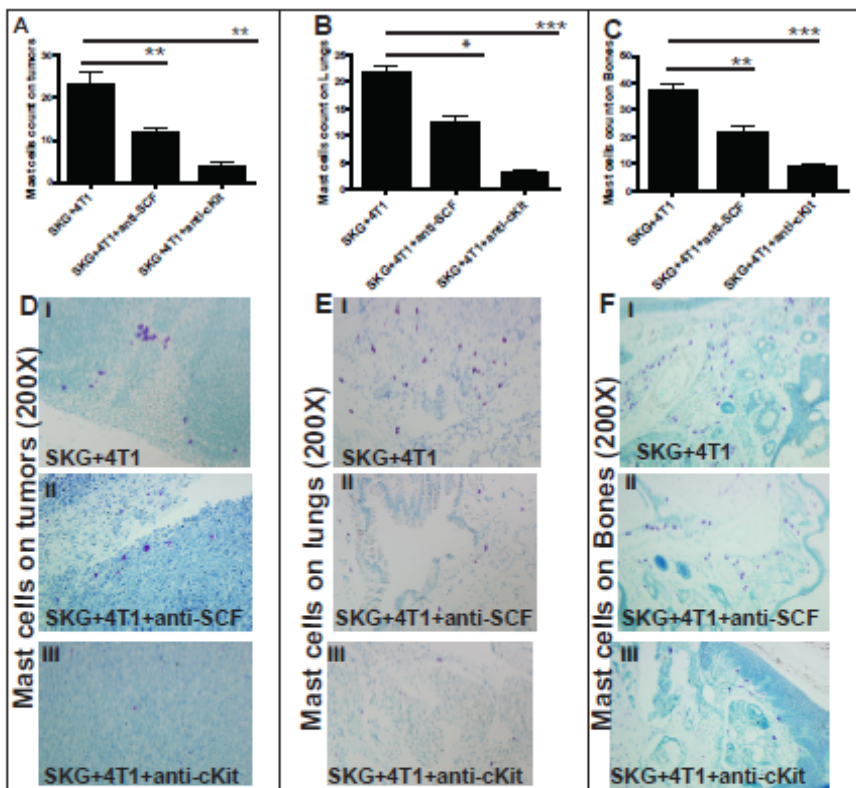


Figure 17: Significant reduction in number of MCs (toluidine staining) in the tumor, lungs

and bones of mice treated with anti-SCF or anti-C-kit antibody

A-C: Significant reduction in number of MCs in **A**) tumors (**P<0.01) **B**) lungs (*P<0.05, ***P<0.01) and **C**) bones (**P<0.01,***P<0.001) of treated mice. **D-F:** Representative images of MCs toluidine in **D**) tumors **E**) lungs and **F**) bones (200x magnification) (n=6 mice per experimental group and 10 fields per section per organ). Purple-blue staining represents MCs toluidine expression.

G: Schematic model depicting the vicious interaction between metastatic breast tumors and the inflammatory microenvironment in the bone and lung through SCF/cKit-MC signaling.

Interrupting the SCF/c-kit signaling significantly reduces the numbers of MCs infiltrating the tumor sites

We observed a significant decrease in MC infiltration in the tumor, lungs and bones of SKG mice treated with anti-cKit receptor antibody (Figure 16 and 17). Representative tryptase and toluidine staining of tumor, lung, and bone tissue section is shown in Figures 16D-F and 17D-F respectively. Interestingly, mice treated with anti-SCF also showed significant decrease in MC expression in the tumor, lung, and bone (Figure 16D(ii) E(ii) F(ii) and Figure 17D(ii) E(ii) F(ii) respectively) suggesting that SCF plays a significant role in MC proliferation. Taken together, these findings confirm that the decrease in metastasis in the treated groups (Figure 14) was driven by low MC accumulation. Our data indicate that the anti-cKit and anti-SCF therapies were effective and must be developed further for clinical application.

Taken together, our study shows for the first time that in an arthritic condition with BC, MC is the master regulator inducing metastasis. MC population is highly augmented and the chemotactic nature of MCs helps in the recruitment of the tumor cells and facilitates the efficient metastasis of the BC cells in bone and lung tissue through SCF/cKit axis (as shown in the model Figure 17G). AA creates a systemic proinflammatory milieu that recruits activated MCs from BM to the primary tumor site by SCF. The SCF-MC interaction remodels the primary tumor and enables the malignant SCF-expressing BC cells to invade the extracellular matrix and circulate in the blood. Concurrently, in the BM, there is an increase in MC precursors and activated MCs expressing the c-kit receptor. Synchronously, MCs are also recruited to the lungs. The circulating BC cells are attracted to leave the circulation when they encounter the cKit receptor on MCs. SCF triggers the cKit signaling pathway for the differentiation, migration, maturation, and survival of MCs, remodeling the microenvironment by intensifying inflammation and releasing the pro-inflammatory cytokines involved in metastasis. This vicious cycle continues.

Specific Aim 3 (B) PyV MT mice induced with arthritis at premetastatic stage: We injected CII at 14 weeks of age and post one month of collagen injection, we started the treatment.

6 mice: no treatment; 3 mice: celecoxib alone (20mg/kg in 10% DMSO in 100ul, gavage); 6 mice: anti-SCF (10ug/ml of saline); 6 mice: anti-SCF + celecoxib; 10 mice: control Goat IgG antibody + 10% DMSO; 6 mice: anti-C kit receptor; 6 mice: anti-C kit receptor+ celecoxib; 6 mice: control rat IgG antibody

Results: 1. Decreased tumor burden was observed in PyV MT mice induced with arthritis and treated with anti-cKit receptor/anti-cKit receptor+ celecoxib and anti-SCF/anti-SCF+celecoxib.

2. Decreased lung and bone metastasis in PyV MT mice induced with arthritis and treated with anti-cKit or anti-SCF ±celecoxib versus PyV MT mice with arthritis with no treatment.

3. Decreased differentiation of Mast cells from Bone marrow in the presence of IL-3 and SCF from the arthritic PyV MT treated with anti-cKit or anti-SCF versus arthritic PyV MT mice with no treatment.

Therefore, from Specific Aim 3 (B), we generated similar data as generated from Specific Aim 3 (A).

KEY RESEARCH ACCOMPLISHMENTS:

We confirmed that the bone marrow cells differentiating into mast cells by addition of IL-3 and SCF is significantly higher in arthritic SKG mice versus non-arthritic Balb/c mice when induced with breast

cancer. Simultaneously, the bone marrow cells differentiating into mast cells by addition of IL-3 and SCF is significantly higher in PyV MT mice induced with arthritis versus non-arthritis PyV MT.

- The mast cell population is seen to be significantly higher in the bone, lung microenvironment of arthritic mice with breast cancer as compared to non-arthritic mice with breast cancer.
- We found that the SCF/c-Kit signaling is significantly upregulated in the arthritic microenvironment, hence up-regulating the population of mast cells through c-kit receptor expressed on the surface of mast cells. Hence, we can assume that Mast cells are activated by breast tumor-derived SCF via c-Kit signaling in the arthritic versus non-arthritic mice
- We found that treating the SKG mice induced with breast cancer with anti c-Kit receptor or with anti-SCF significantly reduces the differentiation of BM cells to mast cells. We saw the same results when the PyV MT mice induced with arthritis was treated with anti-cKit or anti-SCF.
- We found that treating the SKG mice induced with breast cancer with anti cKit receptor or anti-SCF significantly reduces lung and bone metastasis. We saw the similar results when the PyV MT mice induced with arthritis were treated with anti-cKit receptor or anti-SCF neutralizing antibodies.

REPORTABLE OUTCOMES:

1. **Manuscript:** Das Roy, L ., Curry, J., Sahraei, M., Kidiyoor, A., Besmer, D., Gruber, H.E and Mukherjee, P. (2012). Arthritis augments breast cancer metastasis: Role of mast cells and SCF/c-Kit signaling. **Manuscript submitted to Breast Cancer Research on 10/10/12. Manuscript ID # 1681497456823229.**

5. **Press Releases:**

Websites:

1. http://www.aacr.org/home/public--media/aacr-press-releases.aspx?d=2748#.T33R3jty_EM.email
2. <http://publicrelations.uncc.edu/news-events/news-releases/research-news-mechanisms-found-connecting-metastatic-breast-cancer-and-art>
3. <http://www.sciencedaily.com/releases/2012/04/120401134939.htm>
4. <http://cancerdiscovery.aacrjournals.org/content/early/2012/03/29/2159-8290.CD-NB2012-031.full>
5. <http://www.breastcancer.co/news/research/breast-cancer-metastases-link-identified-possibly-treatable>
6. <http://www.dailyrx.com/news-article/breast-cancer-metastasis-associated-arthritis-18333.html>
7. http://www.newsmaxhealth.com/healthwire/breast_cancer_arthritis/2012/04/09/444146.html

8. <http://www.northdrugstore.com/news/2012/04/scientists-find-link-between-arthritis-and-breast-cancer/>
9. <http://in.news.yahoo.com/intimate-relationship-between-metastatic-breast-cancer-arthritis-identified-044034701.html>
10. http://www.newsmaxhealth.com/healthwire/breast_cancer_arthritis/2012/04/09/444146.html
11. <http://publicrelations.uncc.edu/news-events/news-releases/research-news-mechanisms-found-connecting-metastatic-breast-cancer-and-art>
12. <http://uncclnews.blogspot.com/2012/04/mechanism-found-connecting-metastatic.html>

CONCLUSION:

From these above data we confirmed that the population of mast cells is significantly higher in spontaneous model of arthritis induced with breast cancer and in spontaneous model of breast cancer induced with arthritis as compared to the non-arthritic mice with breast cancer. The SCF/c-Kit signaling is also significantly upregulated in the arthritic microenvironment, hence upregulating the population of mast cells through C-kit receptor expressed on the surface of mast cells. We also confirm that treating the SKG mice with C-Kit receptor significantly reduces the differentiation of bone marrow cells to mast cells. The secondary metastasis to lungs and bones is also significantly decreased in SKG mice challenged with breast cancer followed with treatment with C-Kit receptor as well as in the PyV MT mice induced with arthritis and treated with anti-cKit or anti-SCF antibodies.

The clinical implications of this research are huge. We show that interrupting the SCF/cKit-MC signaling significantly reduces BC associated bone and lung metastasis. This research may enable us to design a therapy that will block the SCF/c-Kit signaling and reduce BC-associated metastases to the bone and lungs. Our data provide clear evidence for the first time that MCs play a critical role in not only remodeling the tumor microenvironment but also the metastatic niche to facilitate efficient metastasis through SCF/cKit -MC interaction in BC with **arthritis**.

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