RAW 264.7 macrophages induce tumor-initiating behavior in metastatic but not nonmetastatic mammary carcinoma clones

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ABSTRACT

Macrophages promote primary tumor growth and metastatic dissemination through a variety of mechanisms. This thesis examined the ability of RAW 264.7 murine macrophages to elicit malignant behaviors from a series of murine mammary carcinoma cell lines (4T1, 66cl4, 4TO7, 67NR) that differ extensively in their metastatic potential when transplanted *in vivo*. It was hypothesized that the RAW 264.7 macrophages would evoke malignant behaviors from the carcinoma cells *in vitro*, but in a way that correlated with the metastatic nature of each line. Conditioned media experiments revealed that paracrine cues from the RAW 264.7s induced the expression of Sca-1, a murine surface marker known to represent tumor-initiating cells, in the most metastatic 4T1 and 66cl4 carcinoma cell lines. This effect was attainable in carcinoma cell co-culture with the RAW 264.7s, where the metastatic 4T1 and 66cl4 cell lines also formed spheroid structures amid direct cell contact from the macrophages. A subsequent gene expression analysis performed on 4T1/RAW 264.7 co-cultures suggested that the RAW 264.7s induced Sca-1 expression in the metastatic cell lines through anti-tumoral actions. Finally, a functional study of Sca-1(+) 4T1 cells indicated that they proliferated faster but tended to be less motile than Sca-1(-) cells in response to RAW 264.7-conditioned media, further supporting their tumor-initiating capability. By integrating these findings into a metastatic narrative, this thesis proposes that macrophages enhance metastasis through the expansion of a tumor-initiating subpopulation in metastatic mammary carcinoma cell lines.

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Abbreviations Used

(+)	positive subpopulation
(-)	negative subpopulation
CD24	heat stable antigen
CD49f	α6 integrin
CD61	β3 integrin
CSC	cancer stem cell
CSF-1	colony-stimulating factor-1
CSF-1R	colony-stimulating factor-1 receptor
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
HIF-1	hypoxia-inducible factor-1
iNOS	inducible nitric oxide synthase
M1	classical (anti-tumoral) macrophage activation
M2	alternative (pro-tumoral) macrophage activation
MACS	magnetic-activated cell sorting
MMR	macrophage mannose receptor
PARP	poly (ADP-ribose) polymerase
Sca-1	stem cell antigen-1
TAM	tumor-associated macrophage

PREFACE

Recent inclinations towards a Western lifestyle have led to increased breast cancer incidents on a global scale (1). This heightened prevalence of breast cancer is rather ominous, given that the disease is a complex amalgamation of not only diverse carcinoma cell types, but also the influential microenvironment in which these cell types reside. In fact, a plethora of research is now implicating the macrophage in having a significant effect on the many stages of tumor development. Dissecting out the role that macrophages play in cancer cell metastasis is therefore arguably crucial. After all, the metastatic process is a chief driver of patient mortality and many of its complex events remain enigmatic.

This thesis took advantage of an established *in vitro* system to further evaluate how macrophages promote breast cancer metastasis. The system consisted of a panel of mammary carcinoma cell clones, each of which were originally isolated from the same mammary tumor and retain differential metastatic capabilities when transplanted *in vivo*. By using these carcinoma cell lines to perform *in vitro* assays in conjunction with a macrophage cell line, it was revealed that the macrophages were able to elicit tumor-initiating ability from only the most metastatic carcinoma cell clones.

The ensuing thesis is divided into four major sections. First, current research on topics associated with this thesis is presented in the **Background**

and Significance section, where the themes of breast cancer metastasis and macrophages are examined in detail; these themes are then used to justify a hypothesis and experimental design. Second, the **Experimental Methods** section covers the techniques and reagents used in pursuit of this research. Third, the outcomes of this research are depicted and described in the **Results** section. Finally, the **Discussion** section includes a contemplation of the results, explains how the results integrate into the surrounding literature, and suggests areas for additional study.

BACKGROUND & SIGNIFICANCE

Breast Cancer in the Developed World

Breast cancer is a global problem that is not expected to subside in the foreseeable future. Each year, about one in every ten new cancer incidents is found within the female breast, and roughly 25% of these diagnoses will end in mortality (1). This disease maintains a worldly presence, and its increasing rate of detection in developed countries has raised some concern; the increase has been attributed to not only more sophisticated detection methods, but also recent changes towards a Western lifestyle. Suspected culprits for the increased rate of breast cancers include the following: behavioral alterations in childbearing, breastfeeding, food consumption, physical activity, and exogenous hormonal intake. Definitive causes cannot be identified with any certainty and, consequently, 2.7 million new breast cancer diagnoses are predicted to occur globally in the year 2030 alone.

Cancer Stem Cell Theory

Although the number of breast cancer incidents is growing, fortunately so is the scientific community's understanding of the disease. Through the examination of normal mammary gland morphogenesis, it has been demonstrated that distinct cell types exist within the mammary gland, and these

cell types retain varying degrees of differentiation potential (2). Moreover, it has been shown that an entire functional mammary gland can be reconstituted from a single cell (3). These findings have led to the identification of a panel of cell surface markers that represent the distinct hierarchy of cell types found within the breast (4, 5). This hierarchy applies to both humans and mice, and much work has been spent on finding markers to represent the less differentiated cell types. Some of these markers are identical for humans and rodents, but others are unique. The current markers used to pinpoint stem and progenitor cells within the murine mammary gland include various integrins used in combination with stem cell antigen-1 (Sca-1) (4) or aldefluor (6).

The notion of a cellular hierarchy is not solely an *in vivo* phenomenon. For instance, *in vitro*, cells can be grown in conditions that enrich for human mammary stem and progenitor subtypes. The method takes advantage of epithelial cell dependency on growth factors and adherence for differentiation. Thus, by propagating cells in culture media that is deprived of serum (7), and in tissue culture dishes that prevent cellular adherence (8), one can select for the less differentiated mammary cells. Mammary progenitor and stem cells grown under these conditions form “mammospheres,” these being spheroidal structures that mimic the culture morphology of normal embryonic stem cells (9). Cells harvested from mammospheres show a gene expression profile that is similar to mammary stem and progenitor cells (8), and also exhibit a greater capacity to form mammary glands upon transplantation into mice (9).

In cancer, it is currently accepted that cells with stem-like potency are the chief drivers of carcinogenesis (10). Evidence for this tenant has accumulated from studies whereby less differentiated cancer cells were first enriched *in vitro* and then transplanted *in vivo*. In essence, following transplantation, these less differentiated cells generate tumors at a higher rate than their non-enriched brethren (11). It is speculated that this enhanced tumor-forming ability is due to the capability of less differentiated cells to survive, proliferate, and recapitulate the tumor's original heterogeneity. Coinciding with their propensity for survival, these less differentiated cells have been found to be more resistant to chemotherapeutics, as they harbor drug efflux pumps (12). Of particular importance, in the murine model it has been discovered that these less differentiated cancer cells are signified by Sca-1 expression (13). Sca-1(+) cancer cells isolated from mice not only form tumors at limiting dilutions far lower than Sca-1(-) cancer cells when transplanted *in vivo*, but they are also inherently more resistant to chemotherapeutics (14, 15). Researchers now colloquially refer to cells like those that are Sca-1-positive as "cancer stem cells."

How these "cancer stem cells" (CSCs) are initially created has generated some contention. The more standard view is that CSCs are derived from the malignant transformation of a stem or progenitor cell, and CSCs therefore retain a cell type lineage within a tumor (16). It is believed that this fact explains why more differentiated cancer cells make up the bulk of a tumor, and why CSCs

represent such a minute subpopulation. However, recent work has indicated that the interconversion between mammary cell types can also take place and that cell lines strive to achieve equilibrium between subpopulations over time (17). The apparent plasticity of cancerous cell types has muddied the cancer stem cell theory, but researchers agree that understanding these stem-like cells is required to expand the effective treatment regimens available for breast cancer.

Metastasis as an Obstacle to Patient Survival

The current treatments for breast cancer are far more effective if the disease is discovered in its nascent stage. The past several years have been a boon for many individuals afflicted with breast cancer, as new treatments have been designed for subtypes of the disease that are traditionally more lethal. Novel recent advances in the realm of drug discovery are perhaps best exemplified by trastuzumab, a monoclonal antibody therapy that targets HER2/neu-positive breast cancer (18). This therapy has undoubtedly saved many lives since its implementation, and new therapies are currently in clinical trials. For example, orally active poly (ADP-ribose) polymerase (PARP) inhibitors are now in clinical trials to treat breast cancers with the BRCA1 and BRCA2 mutations, which are genetic aberrations that compromise DNA repair processes (19). PARP inhibitors work by preventing further DNA repair, a function that is still required for BRCA-associated cancers to grow, thus leading to death in cells that harbor the BRCA mutation. Given examples such as these, there is a

general optimism that, over time, treatments directed against breast cancer will become more effective. These expected advances, coupled with the fact that breast tumors are typically removed surgically after deterction, means that primary tumors do not usually cause patient mortality. Patient mortality is instead largely due to the cells that disseminate from the primary tumor and grow at distant sites (20).

Breast cancer metastasis is now recognized as a multistep process (21). During the metastatic cascade, a cancer cell must complete each of the following steps if it is to successfully establish itself at a distant organ. The first step in metastasis involves the escape of cancer cells from the primary tumor, an event that involves cancer cells invading through basement membrane and then intravasating into the circulatory system. Once in circulation, the cancer cells need to survive until they become arrested in capillaries, after which they must extravasate into the surrounding tissue. At the metastatic site, the cancer cells must then survive and proliferate in order to establish a new colony. Considered holistically, metastasis is a highly inefficient process that requires each of these events, and the odds are that cells will fail to complete each and every step during their invasive journey. Indeed, it is thus suggested that less than 0.1% of the cells that enter circulation are capable of creating a metastatic lesion (22).

Even with such a low probability for establishing a colony, metastases do form. In fact, 10% to 15% of individuals afflicted with the more aggressive

subtypes of breast cancer will develop metastases within 3 years after detection of the primary tumor (22). Furthermore, the metastatic lesions derived from breast cancers tend to preferentially localize to the bone, liver, lung, and pleura (23). This notion of a metastatic preference is an inherent feature of all cancer types and was traditionally attributed to stochastic migration through the circulatory system, but recent research has unearthed the role of chemokines in guiding metastasis. Just as a lymphocyte might use chemokine gradients to traffic through a healthy body, cancer cells co-opt this same mechanism during their own dissemination (24). In breast cancer, much emphasis has recently been placed on the role that CXCR4 plays in directing metastasis and it has been found that metastatic breast cancer cells up-regulate CXCR4 in order to bind to its cognate ligand CXCL12 (25). By following CXCL12 gradients through the body, breast cancers preferentially travel to tissues that are high expressers of CXCL12, such as the bone marrow, liver and the lungs. Additional evidence for the importance of CXCR4 in directing metastasis has been demonstrated in mice, as the knockdown of CXCR4 expression using RNAi was sufficient to not only reduce the growth of primary tumors, but also abrogate the formation of metastases (26).

Though CXCR4 has a well-defined role in metastasis, it is important to note that not all cells within a given tumor express CXCR4. The vast diversity of cancerous cells, in conjunction with the cellular hierarchy exhibited within tumors, has led researchers to propose that some cell types are inherently more effective

at metastasizing (27). This point is embodied in the process of the epithelial-mesenchymal transition (EMT). Under normal conditions, cells use EMT to transition between germ layers during embryonic development (28). But, in cancer, metastasizing cells located at the invasive edge of a tumor often use EMT to acquire mesenchymal features as they venture out into the tumor stroma (29). Along these lines, research has demonstrated that in pancreatic adenocarcinoma, a subpopulation of CXCR4(+) cancer stem cells is localized to the invasive front of the tumor (30). Importantly, depleting the CXCR4(+) cells greatly reduced metastatic capability, as evidenced by fewer tumor cells circulating in the blood, but did not affect the size of the original tumor. As insinuated by this study, the current belief is that EMT gives metastatic cells the features they need to be more motile; they are consequently endowed with increased survivability during the metastatic cascade.

Current research is elucidating the steps that occur early in dissemination, but many of the processes that govern cancer cell survival in the later metastatic stages remain enigmatic. Especially perplexing is the question of how disseminated cells manage to survive and establish new colonies at distant metastatic sites. While salient advances in the study of breast cancer have made the disease both easier to prevent and treat, understanding mysteries like this one should be a prime focus in metastasis research. The creation of new breast cancer treatment options like trastuzumab and PARP inhibitors that impair cancer cell proliferation and promote cancer cell death will certainly improve

patient prognosis, but understanding the mechanisms that control metastasis, and designing therapeutics to target these critical steps in carcinogenesis will be invaluable in reducing patient mortality.

The Tumor Microenvironment

Another prevalent theme that encompasses breast cancer is the instructive nature of the cellular microenvironment. The microenvironment of a mammary cell consists of the matrix in which the cell resides, in addition to an array of stromal cells, including fibroblasts, endothelial cells, adipocytes, and various immune cells (31). In recent years, researchers have begun to appreciate the extent to which a cell's surroundings can influence its behavior. For example, *in vivo* studies have shown that testicular (32) and neural cells (33) can be reprogrammed to create mammary glands following transplantation into the mammary microenvironment. Additional work has applied this concept to cancer cells *in vitro*, where mammary cancer cells placed into culture media derived from embryonic mesenchyme were differentiated into a more benign state (34). Given that a cell's stroma can influence its phenotype, it is reasonable to consider if the microenvironment has roots in the initiation of carcinogenesis.

Perhaps it is not surprising, then, that a wealth of research has implicated the inflammatory microenvironment in carcinogenesis. In a normal inflammatory response, there exist distinct physiological steps whereby acute inflammation is

established and then subsequently resolved. In cancer, which involves chronic inflammation, the steps that govern the inflammatory repair processes become disorganized (35). What ensues is the infiltration of fibroblasts, immune cells, and endothelial cells into the tumor site; the induction of angiogenesis by these stromal cells would normally repair the tissue but, amidst chronic inflammation, they instead end up promoting cancer cell survival and proliferation. In light of this repair dysregulation, researchers are increasingly referring to tumors as "wounds that do not heal" (36), using a phrase that pays homage to the intricate relationship that exists between carcinoma cells and their stroma.

The intricate relationship that exists between carcinoma cells and their stroma is a fundamental concept in metastasis research. It was recently discovered that the stromal cells implicated in resolving chronic inflammation not only support tumor growth, but also serve to fuel their dissemination (37). By inducing angiogenesis, secreting proliferative growth factors, and remodeling the extracellular matrix, stromal cells provide a means for cancer cells to escape from the primary tumor. But of all the stromal cells that support metastasis, a special focus has been given to the macrophage, as the macrophage density at a primary tumor site is positively correlated to metastases formation (38). Simply stated, the notion that macrophages can be co-opted by cancer cells to drive metastasis is quite alarming, and this idea is therefore an intense subject of current research.

The Tumor-Associated Macrophage

Since there exists many different types of macrophages within the human body, it is first necessary to describe which types interact with carcinoma cells and how they serve to benefit the tumor. When not associated with tumors, macrophages can be broadly placed into two categories: those that are classically activated and those that are alternatively activated (39). The classically activated (M1) macrophages are primarily stimulated by IFNγ and by ligands binding to Toll-like receptors. Once activated, the M1 cells release reactive oxygen species and nitric oxide to kill pathogens, in addition to secreting factors like IL-12 and TNFα to mediate inflammatory responses. Distinct from the M1 phenotype, alternatively activated macrophages (M2) are stimulated mostly by colony-stimulating factor-1 (CSF-1), IL-4, and IL-13. Following differentiation, M2 macrophages play a prominent role in tissue regeneration and humoral immunity. In essence, the M1 phenotype functions to kill off invaders whereas the M2 phenotype supports tissues by way of various wound healing processes.

When associated with the tumor microenvironment, each of these macrophage types can make a unique contribution in promoting malignancy. For example, by secreting factors like TNFα and IL-12, M1 macrophages induce inflammation around an epithelial lesion (40). The period that follows is marked by high cell proliferation as an array of growth factors are produced to stimulate the replacement of damaged cells. If the epithelial lesion contains dysregulated

cells, it too can benefit from the growth factors released, and can therefore turn into a neoplasia. This enhanced cell division subsequently triggers increased release of reactive oxygen species and nitric oxide by the M1 macrophages, as these immune cells attempt to curb the aberrant cell division. What ensues is a cellular milieu that is highly permissive to acquiring additional genetic defects, thus increasing the rate by which the surviving cancer evolves (41). In a fundamental sense, M1 macrophages are intended to promote healthy body function but inadvertently fuel the clonal evolution of cancer.

While it is accepted that M1 macrophages initially contribute to cancer progression through the inflammatory process discussed above, it is actually the M2-activated macrophages that make up the population known as tumor-associated macrophages (TAMs) (42). Monocytes are recruited in droves to sites of chronic inflammation by following chemokine gradients and upon arrival they are educated into M2 macrophages, which then perform functions that would normally support the wound healing process. Since malignant epithelium cannot be successfully removed by the adaptive immune system, the M2 phenotype ultimately carries out functions that serve to benefit the tumor. These functions fall into four main categories that include the production of growth factors, the suppression of adaptive immunity, the induction of angiogenesis and the degradation of the extracellular matrix (ECM). Each of these functions is essential for tumor growth and survival, but angiogenesis and ECM turnover are especially important for the metastatic dissemination of breast cancer cells.

The Role of Macrophages in Breast Cancer

The specific nature of tumor-associated macrophages varies based on the tissue type in which they reside. Interactive nuances are present in the breast, where TAM function involves intricate crosstalk between both carcinoma cells and infiltrating macrophages. Research has found that carcinoma cells release CSF-1 to attract monocytes to the hypoxic region of a tumor, where TAM differentiation is induced (43, 44). Once differentiated, the tumor-associated macrophages then secrete epidermal growth factor (EGF) and establish a paracrine loop with the resident carcinoma cells (*Figure 1*). There, in essence, EGF drives carcinoma cell proliferation and CSF-1 attracts additional TAMs. This interactive synergy is a common feature of the ductal carcinoma *in situ* and a transcriptional signature indicative of high CSF-1 expression often correlates with a poor patient prognosis (45).

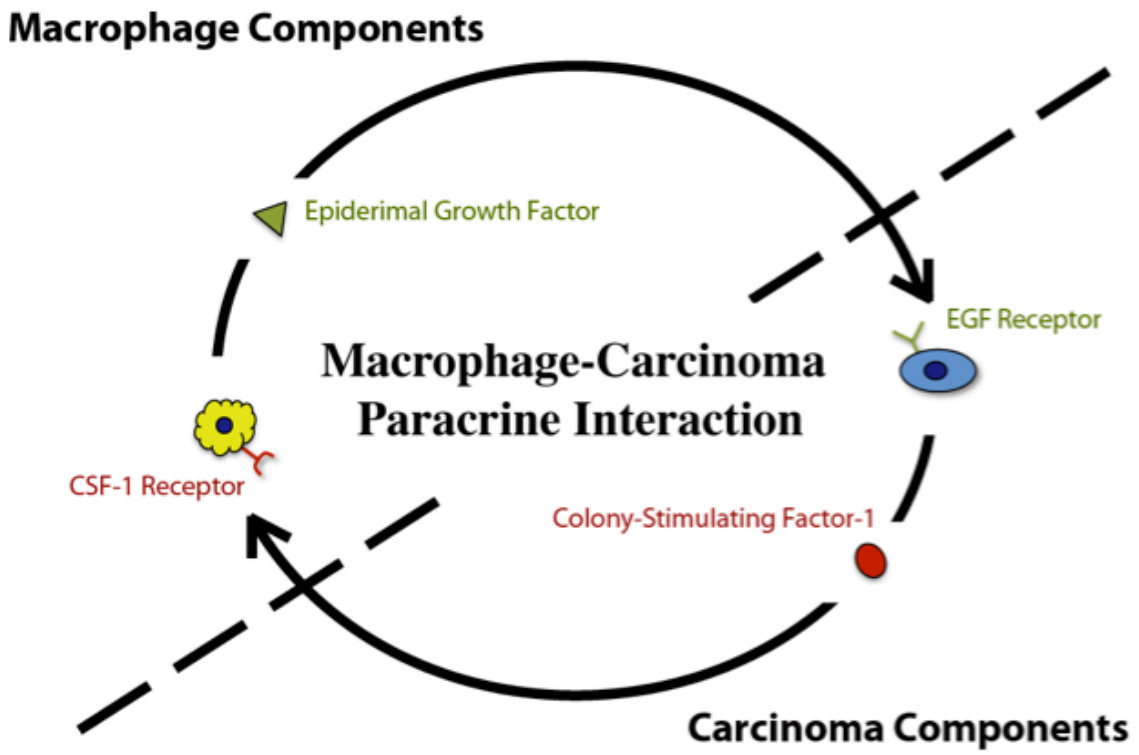

Figure 1. The macrophage-carcinoma paracrine interaction. Mammary carcinoma cells can establish a paracrine loop with tumor-associated macrophages (TAMs) in order to fuel tumor growth and metastasis. In this paracrine interaction, carcinoma cells produce CSF-1 to recruit macrophages and induce their differentiation into TAMs. In return, TAMs produce epidermal growth factor, which stimulates carcinoma cell proliferation. Each factor binds to its cognate receptor on the recipient cell. This figure was created based on a description in (44).

In addition to increasing the proliferative output of breast carcinoma cells, TAMs also work to counteract the immune cells that would otherwise keep this growth in check (46). The suppression of the adaptive immune system's anti-tumoral activity is due to two characteristics of TAMs. The first is that TAMs, being derivatives of the M2 type macrophage, possess low major histocompatability complex (MHC) expression and consequently lack the ability

to efficiently activate T cells (42). Secondly, the cytokines that TAMs produce have a tendency to recruit T cells that do not harbor any cytotoxic activity (46). For example, the secretion of IL-10 by TAMs results in the down-regulation of antigen presentation to immune cells, while concurrently limiting the production of pro-inflammatory cytokines. As one can see, the capability of TAMs to thwart the adaptive immune response is an unfortunate consequence of their developmental origin, and the stochastic outcomes that accompany high numbers of M2 macrophages infiltrating tumor tissue.

The ability of TAMs to promote angiogenesis and remodel the ECM pays a significant role in promoting breast cancer metastasis. When TAMs preferentially accumulate in the hypoxic region of a tumor, low oxygen tension triggers the activation of a transcription factor called hypoxia-inducible factor-1 (HIF-1) in both carcinoma cells and TAMs (47). In carcinoma cells that have undergone the Warburg effect, HIF-1 induction promotes anaerobic metabolism to allow for cell survival under harsh conditions (48). In TAMs, HIF-1 activation is responsible for triggering the release of pro-angiogenic factors such as vascular endothelial growth factor and platelet derived growth factor, which increase blood vessel density around the tumor (49). Paramount in this process is the ability of TAMs to degrade the ECM, in order to facilitate the growth of these new blood vessels. For instance, the production of matrix metalloproteinases causes turnover of the ECM, thereby releasing growth factors previously embedded within the matrix. These cytokines serve to further fuel cell proliferation and also

direct chemotaxis away from the tumor mass. Both of these functions are inextricably tied to metastasis, as angiogenesis provides the route for cancer cells to escape from the primary tumor while ECM degradation allows for easier entry into the surrounding blood vessels.

Macrophages, Metastasis, and Cancer Stem Cells

Within a developing mammary gland, M2 macrophages are localized to areas where ductal cells are expanding out into the mammary fat pad (50). Once there, these macrophages serve to remodel the ECM to facilitate epithelial arborization, as well as initiate processes to heal the associated inflammation. To achieve the proliferative output required to generate a functional mammary gland, epithelial cells found in the terminal end buds at the stromal interface tend to be less differentiated stem and progenitor cells. Thus, during the normal morphogenesis of a mammary gland, there exist close interactions between these less differentiated epithelial cells and macrophages. Visvader and colleagues showed the necessity of macrophages in mammary gland development, where the chemical ablation of macrophages was sufficient to halt mammary gland formation in mice (51). This was attained by co-implanting mammary stem cells with clodronate-liposomes, the latter of which induces apoptosis in macrophages when ingested. Studies like this one highlight the influence of macrophages on stem cell function *in vivo*.

In many ways a metastasizing tumor is a lot like a developing mammary gland. Just as a terminal end bud penetrates a stromal fat pad during mammary gland morphogenesis, a metastasizing tumor does much the same, albeit in a way that is more chaotic and deleterious to the organ. At the border between the tumor and its stroma, metastasizing cells interact with macrophages, as the latter attempts to either annihilate the former, or resolve the chronic inflammation associated with the growing wound; which function the macrophage chooses largely depends on the cytokines it has been exposed to, and its ensuing activation state. In addition, many of the cancer cells, through their interaction with the stroma, are utilizing EMT to generate stem-like properties in order to facilitate their dissemination. It is therefore possible that macrophages may also come in close contact with, and even influence the behavior of, cancer stem cells *in vivo*. Considering these events, it is reasonable for one to dissect out the steps associated with the interaction between macrophages and carcinoma cells. Given that tumors are heterogeneous masses consisting of multiple cancerous cell subtypes, it is likely that macrophages influence the dynamics of specific carcinoma subpopulations within a tumor. Of particular interest is the possibility that macrophages might interact with cancer stem cells to promote their metastasis.

An *In Vitro* System to Study Macrophage-Carcinoma Interactions

In this study, an *in vitro* system was devised to test the effects of macrophages on carcinoma cells. Traditionally, the vast majority of breast cancer research that is conducted *in vivo* involves the xenograft of human cancer cells into murine models. Such studies require immunocompromised mice to generate data, and this system lacks significant immune aspects of the tumor microenvironment that, as discussed above, play a crucial role in potentiating tumorigenicity and directing metastasis. The consequential goal of the current study is to use an *in vitro* syngeneic system where results can be quickly and effectively extrapolated into an *in vivo* setting. To accomplish this goal, murine cell lines originating from a similar genetic background were utilized so that, in the future, findings could be confirmed in mice possessing an intact immune system.

This project took advantage of a series of isogenic mammary cancer cell lines that are currently well established in the literature. Each of the cell lines originated from a single mammary tumor that spontaneously arose in a BALB/cfC_3H mouse and display varying degrees of metastatic potential when transplanted back into mice (52, 53) (*Figure 2*). The most metastatic of these clones is the 4T1 cell line. When transplanted into a mammary fat pad, the 4T1 line uses hematogeneous dissemination to colonize the bone, brain, lungs, and liver. Next, the 66cl4 line preferentially travels through the lymphatics and readily metastasizes to the lungs and liver, but at a slower rate than the 4T1s. While the 4T1s and 66cl4s can efficiently complete all metastatic steps, the remaining three

cell lines cannot. For example, the 4TO7 cell line has a proclivity towards hematogeneous dissemination via blood and can form small nodules on the lungs, yet these nodules fail to proliferate. Likewise, 168FARN cells can embark from the primary tumor and travel through the lymphatics, but these cells become arrested in the lymph nodes that surround the mammary gland. Finally, the 67NR cell line fails to disseminate from the primary tumor and is therefore the least metastatic of the clones.

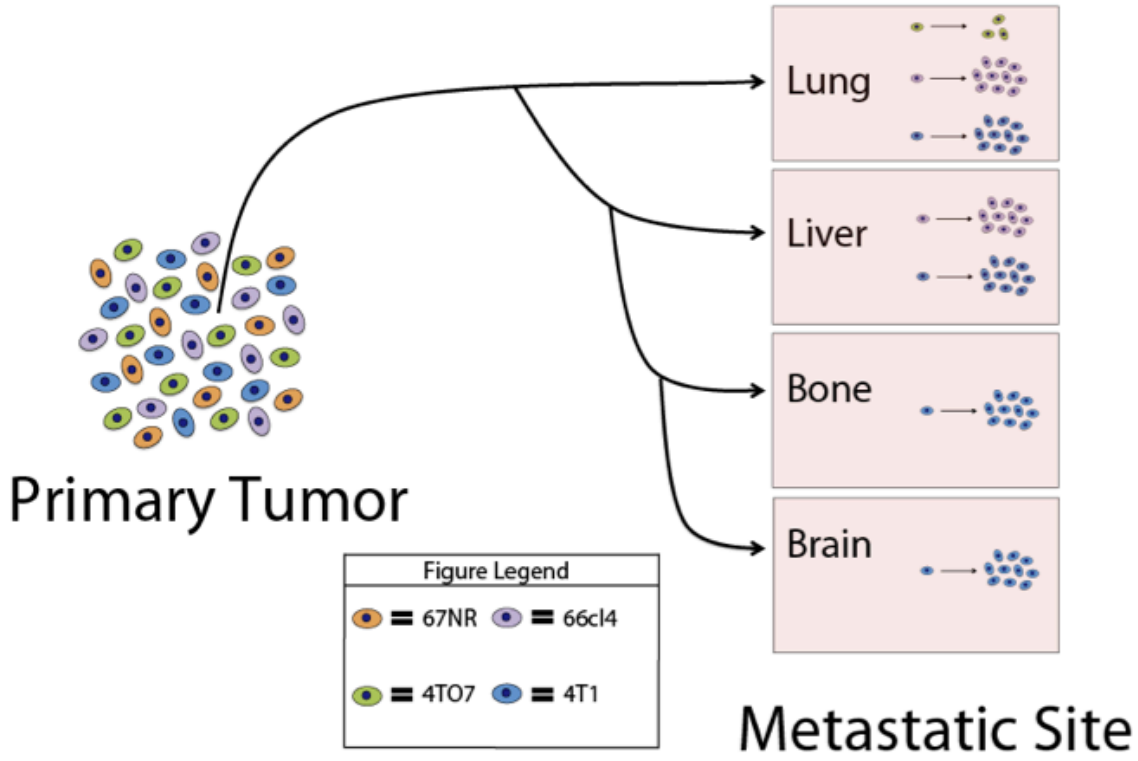

Figure 2. The metastatic propensities of the mammary carcinoma cell lines. Although each clone was isolated from the same mammary tumor, only the 4T1 and 66cl4 cell lines can complete every step in the metastatic cascade when transplanted *in vivo*. The 4T1s are considered the most metastatic cell line and can colonize the lung, liver, bone, and brain. The 66cl4s are the second-most metastatic and efficiently metastasize to the lung and liver. Next, the 4TO7s can disseminate from the primary tumor and seed the lungs, but only form small non-proliferative nodules. Finally, the 67NRs fail to disseminate from the primary tumor and are considered the least metastatic. This figure was created based on a textual description in (53).

Since each of the mammary cell clones differs extensively in their metastatic capability when transplanted *in vivo*, it was decided that this system could be used to examine interactions with macrophages. Studies using macrophages are heavily dependent on immune compatibility, as macrophages are adept at recognizing and destroying cells of foreign origin, and establishing an *in vitro* system with immune-compatible cells might be necessary to elicit pro-tumoral actions from the macrophages. In pursuit of this goal, the RAW 264.7 cell line was carefully chosen to model macrophages in this study for a number of reasons. First, RAW 264.7 macrophages were isolated from BALB/c mice and are therefore immune-compatible with the mammary cancer clones (54). Next, the RAW 264.7 cells have been used as a model for TAM interactions in colon cancer, where they were shown to elicit a motile phenotype from a colon cancer cell line (55). Finally, research has demonstrated that the RAW 264.7 cell line can be differentially activated into an anti-tumoral M1 or pro-tumoral M2 state using recombinant cytokines. The M2 activation of the RAW 264.7 cells was sufficient to promote primary tumor growth and metastasis of Lewis lung carcinoma cells in mice (56). Taken together, these points verify that RAW 264.7 cells can adequately model TAM behavior *in vitro* and *in vivo*. However, to date, studies using RAW 264.7 cells to study TAM functions in breast cancer are sparse.

Study Hypothesis and Design

This project examined the effect of RAW 264.7 macrophages on the 4T1, 66cl4, 4TO7, and 67NR mammary cancer cell lines. Since each of the mammary cancer cell lines differ in their inherent metastatic potential when transplanted *in vivo*, it was hypothesized that the RAW 264.7 macrophages would elicit malignant behaviors from the cell lines *in vitro*, albeit in a way that correlated with the inherent metastatic capability of each line. In pursuit of this hypothesis, the resulting study was divided into three major components:

1. To enable a study on how macrophages influence the subpopulation composition within the mammary cancer cell lines, subpopulations within those cell lines first needed to be identified. This aspect of the study involved a hierarchical surface maker characterization of the mammary cancer cell lines using flow cytometry. The expression of cytometric markers that are known to represent steps in metastasis was also examined.

2. The effect of macrophages on the carcinoma cells was then ascertained by employing conditioned media and co-culture assays. Analytical endpoints for this portion of the thesis included fractional changes in the Sca-1, CD61, and CXCR4 subpopulations, differential gene expression analyses, as well as morphological observations.

Experimental outcomes were then correlated to the activation state of the RAW 264.7 macrophages.

3. Sca-1(+) cells from the 4T1 cell line were examined in detail to further integrate these findings into a metastatic narrative. Specifically, the proliferation rate and motile nature of the Sca-1(+) cells versus Sca-1(-) cells were compared using *in vitro* techniques.

EXPERIMENTAL METHODS

Cell Culture Reagents and Techniques

The 4T1 and RAW 264.7 cell lines were purchased from the American Type Culture Collection (ATCC). The 67NR, 4TO7, and 66cl4 cell lines were acquired through the generous gift of Dr. Fred Miller of Wayne State University. All cell lines were maintained in a 1:1 solution of Dulbecco's Modified Eagle's Medium (Mediatech, Inc.) and Ham's F12 Nutrient Mixture (Gibco, Life Technologies), further supplemented with 10% FetalClone III (HyClone, Thermo Scientific) plus 1% Penicillin-Streptomycin (Mediatech, Inc.). Recommendations provided by the ATCC were followed during subculture, where the mammary cancer cell lines were dissociated via 0.25% Trypsin-EDTA solution (Gibco, Life Technologies) and the RAW 264.7 cells were dissociated by scraping cells off the tissue culture surface. Each cell line was generally kept within 10 passages of stock cultures in order to minimize genetic drift or other causes of biological change. All cultures were grown in a 37°C incubator that was injected with 5% CO_2.

Conditioned media was generated by application of fresh growth media to cultures at about 30% confluency and collected after 48 hours had passed. At the end of this period, cultures were roughly 80% confluent. Prior to use, the conditioned media was centrifuged at 300 x g for 10 minutes to remove residual

cellular debris, and further diluted to 90% strength using fresh growth media. The conditioned media was kept at -20°C in some instances, but for no longer than one week to minimize any potential ill effects from prolonged storage, and short-term storage did not appear to reduce its efficacy. Cells were grown in conditioned media for 24 hours before analysis unless stated otherwise.

Co-cultures involving the RAW 264.7 macrophages and mammary carcinoma cell lines were created in two distinct formats. In the first format, which was optimized for spheroid formation, RAW 264.7s were seeded into a tissue culture vessel with the carcinoma cells at a 3-to-1 ratio. The second format consisted of carcinoma cells being in the majority and they were seeded in a 5-to-1 ratio to the RAW 264.7 macrophages. Co-cultures were permitted to grow for 48 hours prior to experimental analysis.

Flow Cytometry

Flow cytometry was conducted using antibodies targeted against the following cell surface antigens: CD24 (BD Biosciences: FITC Rat Anti-Mouse), CD61 (BD Biosciences: PE Hamster Anti-Mouse), CD49f (R&D Systems: APC Rat Anti-Human/Bovine/Mouse), Sca-1 (BD Biosciences: FITC Rat Anti-Mouse, Clone D7), CXCR4 (R&D Systems: APC Rat Anti-Mouse), and F4/80 (AbD Serotec: RPE Rat Anti-Mouse). Cell suspensions were first subjected to FC Block (BD Biosciences) to prevent nonspecific antibody labeling. Next, antibody

targeting proceeded according to the manufacturer's recommendations, though labeling occurred at room temperature to improve Sca-1 antigenicity when appropriate. Experiments involving the CXCR4 marker required cells to undergo a trypsin recovery for 6 hours in a 37°C shaker prior to antibody use. Every data set was paired with an unstained and isotype control.

Flow cytometric labeling was generally executed on unfixed cells using a flow cytometric staining buffer that consisted of DPBS (Mediatech, Inc.) supplemented with 1% FetalClone III (HyClone, Thermo Scientific) and 0.09% sodium azide at pH 7.4. Analyses carried out on EdU retaining cells were fixed with 4% paraformaldehyde solution after antibody labeling, and then subjected to the manufacturer's Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Invitrogen) and the associated protocol. All flow cytometric analyses were performed on a BD FACSCalibur instrument running CellQuest software.

Gene Expression Analyses

Cells collected for gene expression analyses were dissociated from cultures that were between 50% and 80% confluent, depending on the experiment. Analyses conducted on conditioned media treated samples were always paired with an untreated control, whereas co-culture samples were contrasted with cells grown in monoculture. Cell types were purified from co-culture with a Magnetic-Activated Cell Sorting (MACS) apparatus and associated

protocol (Miltenyi Biotech), where the isolation proceeded by using Anti-PE MicroBeads (Miltenyi Biotech) targeted to the PE-conjugate of the antibody originally directed against the F4/80 macrophage marker. In essence, positive-selection of the RAW 264.7 macrophages occurred because the RAW 264.7s remained bound to the MACS column until eluted, while negative-selection of the 4T1s occurred by collecting the flow-through prior to the elution step. Flow cytometric analysis were conducted to determine the amount of contaminating cells after purification; these experiments revealed an average RAW 264.7 purity (n=5) of 98.2% and an average 4T1 purity (n=3) of 95.7% for downstream applications.

The first step in analyzing gene expression was an RNA extraction made possible by the RNeasy Mini Kit (Qiagen). After quantitation, cDNA was synthesized from the RNA using an AffinityScript cDNA Synthesis Kit (Agilent Technologies) and an Eppendorf Mastercycler. Next, quantitative PCR was carried out on the cDNA samples with Brilliant SYBR Green QPCR Master Mix (Agilent Technologies) and a Rotor-Gene 3000 (Corbett Research). Every qPCR sample was run in triplicate and each sample set was paired with a no reverse transcriptase and a no template control. Mean Ct values were recorded for each triplicate using the Rotor-Gene software and gene expression values were computed with Q-Gene software (BioTechniques). All genes in this study were normalized to the PUM1 housekeeping gene, which showed no discernable fluctuations between experimental and control samples.

Primer sets used for qPCR were obtained from Integrated DNA Technologies and designed to span multiple exons. The forward (F) and reverse (R) primers are listed for each gene in alphabetical order: CSF-1,F (5'-TAC AAG TGG AAG TGG AGG AGC CAT-3'), CSF-1,R (5'-AGT CCT GTG TGC CCA GCA TAG AAT-3;) CSF-1R,F (5'-ACC AAA TGG CCC AGC CTG TAT TTG-3'), CSF-1R,R (5'-TGC TTG GCA GGT TAG CAT AGT CCT-3'), EGF,F (5'-TGC CTT GGA TTA TGA CCC TGT GGA-3'), EGF,R (5'-AGG GCA AGA CCT TCA AGC GTA TCT-3'), EGFR,F (5'-TGC GTG GAG AAA TGC AAC ATC C-3'), EGFR,R (5'-TTG ACA CAG TGT GGG CCA TCA A-3'), MMR,F (5'-TGG GCT ACA GGA GAA CCC AAC TTT-3'), MMR,R (5'-GCA GTG GCA TTG ATG CTG CTG TTA-3'), NOS2,F (5'-CTG CTG GTG GTG ACA AGC ACA TTT-3'), NOS2,R (ATG TCA TGA GCA AAG GCG CAG AAC-3'), PUM1,F (5'-AGC AGC CTT AGC TAT TCC TCC TCT-3'), PUM1,R (5'-ATC TTC CAC TGC CGT TTG TGA GTC-3'), SCA-1,F (5'-TGC TGA TTC TTC TTG TGG CCC T-3'), SCA-1,R (5'-TCC ACA ATA ACT GCT GCC TCC T-3').

Coverslip Culture and Fluorescent Microscopy

Co-cultures with 4T1s and RAW 264.7s were grown in 6-well plates that contained sterile glass coverslips bathed in 2 ml of growth media. 4T1s and RAW 264.7s were grown at a 1-to-3 ratio and cell numbers were optimized for the smaller growth area, where the culture was seeded with a total of 1.5×10^5

cells. After 48 hours, the cultures were washed with 2 ml DPBS and then fixed with 4% paraformaldehyde for 20 minutes at room temperature. Once fixation was complete, the cultures were given 2 additional DPBS washes and then subjected to antibody labeling.

The FC Block and antibody steps were performed in a humidity chamber constructed to prevent evaporation of solutions from the coverslips. FC Block or fluorescent-conjugated antibodies were added to the fixed coverslips in a 200 µl total volume. The coverslips were rinsed 3 times between steps with flow cytometric staining buffer. When complete, a wet mount was prepared and imaged on a Nikon Eclipse TE300 fluorescent microscope running MetaMorph software.

Proliferation Assays

The Sulforhodamine B (SRB) assay was first used to examine cell proliferation in this thesis and the experimental methods were based on a published protocol (57). Briefly, 4T1s were seeded into a 24-well plate at 1 x 10^4 cells/well, in a total of 1 ml normal growth media. One well in each row was designated as a time point. After 24 hours had passed, wells for the first time point were aspirated and subjected to a DPBS wash to remove residual serum proteins; this set of wells served as a time zero control. Next, each of the three rows had their wells replaced with one of the following treatments: fresh growth

media, 4T1-conditioned media, or RAW 264.7-conditioned media. Each conditioned media type was generated from the seeding of roughly 1 million cells in a 10 ml culture vessel followed by collection after 48 hours of incubation. Once the wells were treated with its respective media, a series of wells were aspirated and washed every 24 hours for the next 5 days to make growth curves. Quantitation of protein within the wells was conducted according to the aforementioned protocol using the SRB reagent (Sigma), solubilized in a 1% acetic acid solution. Protein concentration was calculated by measuring absorbance at 540 nm on a SpectraMax M3 Multi-Mode Microplate Reader (MDS Analytical Technologies), and growth curves were created via normalization to the most confluent well on a given plate. In other words, the well with the highest protein amount was considered 100% confluent, and the percent confluency for the remaining wells were based off this number.

The Click-iT EdU proliferation assay (Invitrogen) was conducted using the manufacturer's protocol and incorporated some methodology from the SRB assay. In these experiments, 4T1s were treated with either 4T1- or RAW 264.7-conditioned media for 24 hours; conditioned media was created from cultures initially seeded with around 1 million cells. While being exposed to the conditioned media, specifically at the 20-hour mark, 4T1 cultures were given an additional 10% fresh growth media to stimulate proliferation and eventual EdU incorporation. After the 24-treatment with conditioned media had expired, the cultures were administered a 10 μM EdU pulse for either 16 hours or for 45

minutes. The 45-minute EdU pulse was initiated at the tail end of the 16-hour pulse, so as to synchronize the pulses for subsequent steps in the protocol. The detection of EdU within the 4T1s occurred as directed by the protocol. Though prior to detection, the 4T1 cells were either targeted with antibodies against Sca-1 or subjected to isotype labeling.

FluoSphere Motility Assay

The motile behavior of the 4T1 cells was determined using 1.0 μm red FluoSpheres (Invitrogen). The motility assays were optimized according to the analytical endpoint, whether it was fluorescent microscopy or flow cytometry, in the following manner. All cell culture vessels were initially pre-treated with DBPS containing 50 μg/ml poly-D-lysine for 30 minutes at room temperature. Next, different concentrations of FluoSpheres were added to DPBS, using a 1:200 dilution of the FluoSpheres for fluorescent microscopy and a 1:600 dilution for flow cytometry, and the mixtures were then added to the culture vessels. An incubation period of 2 hours at room temperature was allowed to facilitate FluoSphere adherence to the tissue culture surface, after which unbound FluoSpheres were removed via 2 subsequent DPBS rinses.

4T1 cultures destined for analysis by fluorescent microscopy were first grown in either normal culture media or RAW 264.7-conditioned media for 24 hours. The initial seeding of roughly 1 million RAW 264.7s into a 10 ml culture

vessel standardized conditioned media strength between experimental replicates. Next, 2 x 10^4 4T1s were added to 5 ml of fresh growth media and transferred to 60 mm culture dishes previously coated with the FluoSpheres. The 4T1s were permitted to migrate over the layer of fluorescent beads overnight, and microscopy photographs were acquired on a Nikon Eclipse TE300 fluorescent microscope running MetaMorph software.

4T1 cells intended for analysis by flow cytometry were subjected to similar treatment, albeit with a few modifications. Although the methods surrounding the conditioned media were the same, 6 x 10^4 4T1s were added in 5 ml of normal culture media to dishes coated with 1:600 FluoSpheres. 4T1s were allowed to migrate over the fluorescent beads for 8 hours and then cultures were dissociated for flow cytometry. 4T1 cell suspensions were administered either antibodies against Sca-1 or isotype-labeling prior to flow cytometric analysis.

Statistical Approach

This thesis used the Prism 4 software (GraphPad Software, Inc.) to graph data and conduct statistical analyses. Data sets produced by flow cytometric and gene expression experiments were log-transformed prior to analysis, due to the non-normal distribution that was an inherent feature of the untransformed data sets. When computed, paired two-tailed Student's t-tests were used to generate

p-values. Statistical significance was assessed using 0.05 as a cutoff for significance. All graphs are presented as untransformed data.

Flow Cytometric Example Analyses

The criteria used to denote positive flow cytometric labeling for Sca-1 was based on methods published in the surrounding literature (13, 15) and an example of these criteria is shown in *Figure 3*. In essence, cells positive for Sca-1 labeling were considered those cells that exhibited higher fluorescence than the negative isotype control. Past research has demonstrated that the selective transplantation of these Sca-1(+) cells into mice led to an enhanced rate of tumor formation (14, 15). In analyzing the Sca-1 marker in this thesis, flow cytometric quadrant statistics were carried out based on the isotype control. More specifically, the quadrant location was established so that roughly 1% of the isotype cells fell above the positive threshold; the quadrant location was applied to other samples in a given experimental replicate and theoretically includes at least 1% false-positive events. This method of quantitating positive events was also used for the CD24, CD61, and CD49f surface markers.

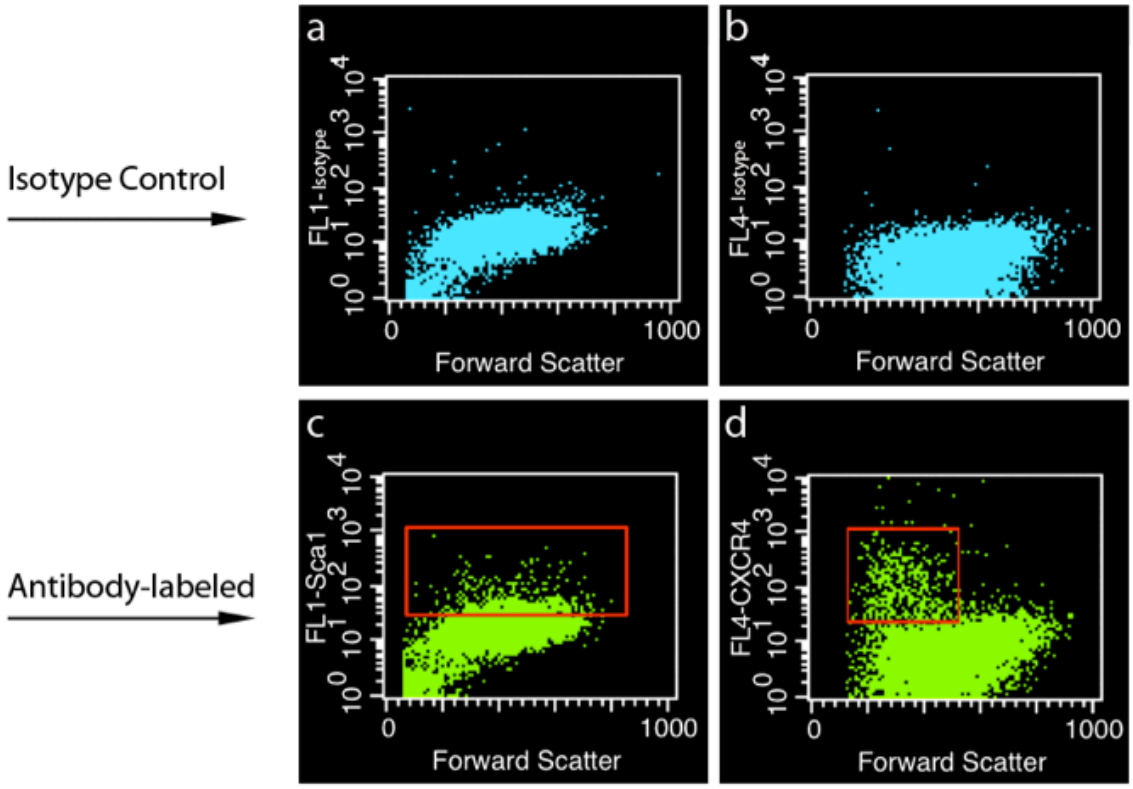

Figure 3. Example Analysis: Flow cytometry dot plots reveal more distinct Sca-1(+) and CXCR4(+) subpopulations within the 4T1 cell line. Panels **a** and **b** represent the negative isotype controls for Sca-1 and CXCR4, respectively. The lower panels show that a small subpopulation of 4T1s endogenously express Sca-1 (Panel **c**) and CXCR4 (Panel **d**). The criteria used to denote positive labeling were based on other studies for both Sca-1 (13, 15) and CXCR4 (58). In the plots shown above, these positive subpopulations are encapsulated in a red box.

The procedure used to quantitate CXCR4(+) cells was different and is also depicted in *Figure 3*. Rather than setting a 1% positive threshold using the isotype control, a flow cytometric gate was drawn to enclose the CXCR4(+) subpopulation. Flow cytometric region statistics were then utilized to count positive events within the established area. It was most accurate to analyze the marker this way because the CXCR4(+) cells were easily discernable on the

cytometric dot plots. This method of quantitation adhered to the procedure used in a separate study (58).

The flow cytometric analysis of carcinoma cells grown in co-culture with the RAW 264.7 macrophages took advantage of the F4/80 marker. F4/80 was solely expressed on the murine macrophages but not on cancer cell lines, and permitted the analysis of carcinoma cells or macrophages separately. Thus, cells from co-cultures were triple-labeled with antibodies directed against Sca-1, CXCR4, and F4/80. To analyze the carcinoma components discretely, flow cytometric analysis was performed on the F4/80(-) cellular fraction, where the F4/80(-) carcinoma cells were further examined for Sca-1 and CXCR4 expression using the methods discussed previously. An example of this entire process is illustrated in *Figure 4*.

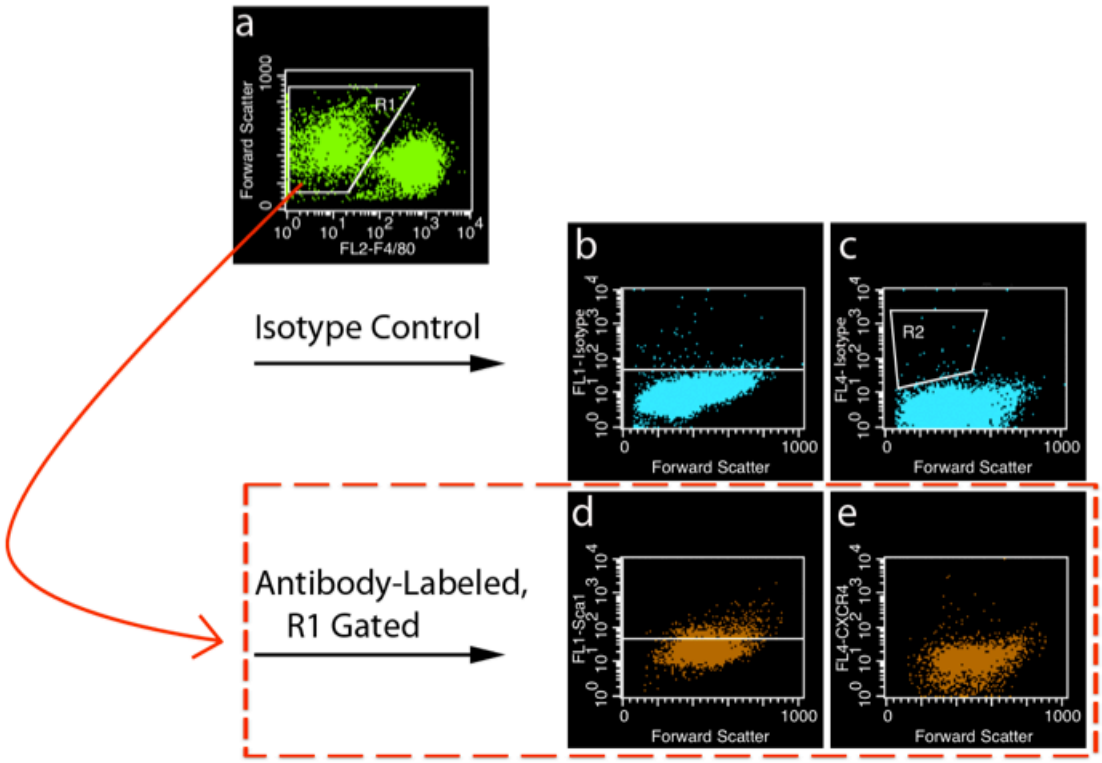

Figure 4. Example Analysis: F4/80-based flow cytometric gating allows the 4T1s to be analyzed separately from the RAW 264.7 macrophages after co-culture. The 4T1s and RAW 264.7 macrophages were permitted to grow in co-culture for 48 hours. Next, cells from the co-culture were labeled with fluorescent antibodies against Sca-1, F4/80, and CXCR4. Since only murine macrophages express the F4/80 receptor, the 4T1s were analyzed separately from the RAW 264.7s by flow cytometric gating of the 4T1s based on their negative expression of F4/80 (Panel **a**). Finally, surface marker expression was analyzed via comparison to the isotype controls (Panels **b**, **c**), where the Sca-1(+) cells are considered to be those that lie above the positive threshold (Panel **d**) while CXCR4(+) cells are considered those that fall within the R2 Region (gate shown in Panel **c**; region statistics applied to Panel **e**). In this example analysis, 18.3% of the 4T1s taken from co-culture express Sca-1 but only 2.3% of the 4T1s express CXCR4.

Flow cytometric evaluations utilized during the proliferation experiments brought an added degree of complexity. As demonstrated in *Figure 5*, gates for the Sca-1(+) and Sca-1(-) cellular fractions were first established using the

isotype control. Sca-1(+) and Sca-1(-) 4T1s were then analyzed separately for each EdU pulse length with flow cytometry quadrant statistics. The location of the flow cytometric quadrant was determined with a control, where 4T1 cells not pulsed with EdU were subjected to the detection protocol to identify background staining. Any background EdU staining was considered artifactual and eliminated from analysis by setting a positive threshold in accordance with this control. Once established, the threshold for non-artifactual EdU incorporation was applied to Sca-1(+) or Sca-1(-) cells for each pulse length. The number of Sca-1(+) or Sca-1(-) cells that fell above the threshold were considered positive for EdU uptake.

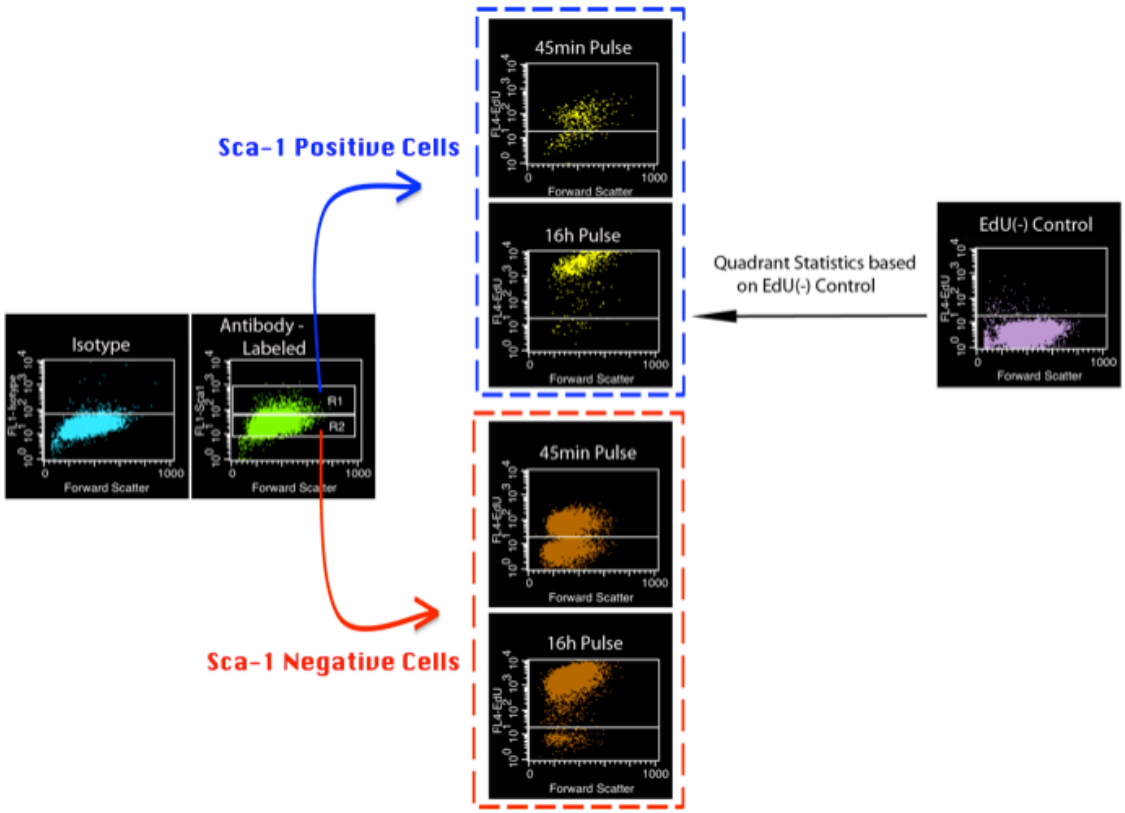

Figure 5. Example Analysis: The proliferation rate of the 4T1s in RAW 264.7-conditioned media is ascertained by comparing EdU uptake in Sca-1(+) versus Sca-1(-) cells. A 45-minute or 16 hour EdU pulse (10 μM) was

administered to 4T1s after being treated with RAW 264.7-conditioned media for 24 hours. Sca-1(+) and Sca-1(-) cells were distinguished by flow cytometric gating, where the gates were set in accordance with the isotype control. After cytometric gating, the number of cells positive for EdU incorporation were determined via EdU(-) control and flow cytometry quadrant statistics. In this example, 76.2% of Sca-1(+) cells have taken up EdU after a 45 minute pulse while 96.4% of Sca-1(+) cells are positive after a 16 hour pulse. This contrasts the Sca-1(-) cells, which show 53.0% or 96.8% EdU positivity after 45 minutes or 16 hours, respectively.

The flow cytometric analysis of 4T1s subjected to the FluoSphere assay proceeded much like the EdU analysis shown in *Figure 5*. Instead of Sca-1(+) or Sca-1(-) 4T1s being compared based on EdU incorporation, they were contrasted according to FluoSphere uptake. In these experiments, the negative control for FluoSpheres consisted of 4T1s grown in the absence of fluorescent beads. Consequently, the distinction between FluoSphere(+) and FluoSphere(-) cells was established using quadrant statistics on unstained 4T1s, and this location was applied to other members in the experimental set.

RESULTS

The objective of this project was to examine cellular interactions between macrophages and mammary carcinoma cell lines *in vitro*. Each of the mammary carcinoma cell lines differs extensively in their metastatic potential when transplanted *in vivo*, ranging from highly metastatic to completely nonmetastatic (52), and are therefore useful tools to assess macrophage influence. The identification of cellular subpopulations within the 4T1, 66cl4, 4TO7, and 67NR mammary carcinoma cell lines was employed as the first experimental goal in this thesis. The rationale for this goal was that prospective identification of subpopulations would enable a subsequent evaluation of how macrophages influence the proportion, morphology, and behavior of different cellular subtypes within the mammary carcinoma cell lines.

The surface marker characterization that resulted from the subpopulation search is depicted in *Figure 6*. All cell lines except for the 67NRs expressed the epithelial cluster of differentiation protein CD24. 67NR cells have a mesenchymal-like morphology *in vitro* that correlated with this finding, though the 4TO7s also have mesenchymal features yet still expressed the CD24 marker. The 4T1s and 4TO7s exhibited positive labeling for CD61, which is also known as β3 integrin, while every mammary carcinoma cell line expressed the α6 integrin CD49f. A small fraction of tumor-initiating Sca-1(+) cells was found within each carcinoma cell line and this was also the case with the CXCR4

chemokine receptor. Of notable interest, the characterization revealed that subpopulations existed within the 4T1 cell line for the CD61, Sca-1, and CXCR4 surface markers; these markers hence served as a central focal point for the remainder of this thesis.

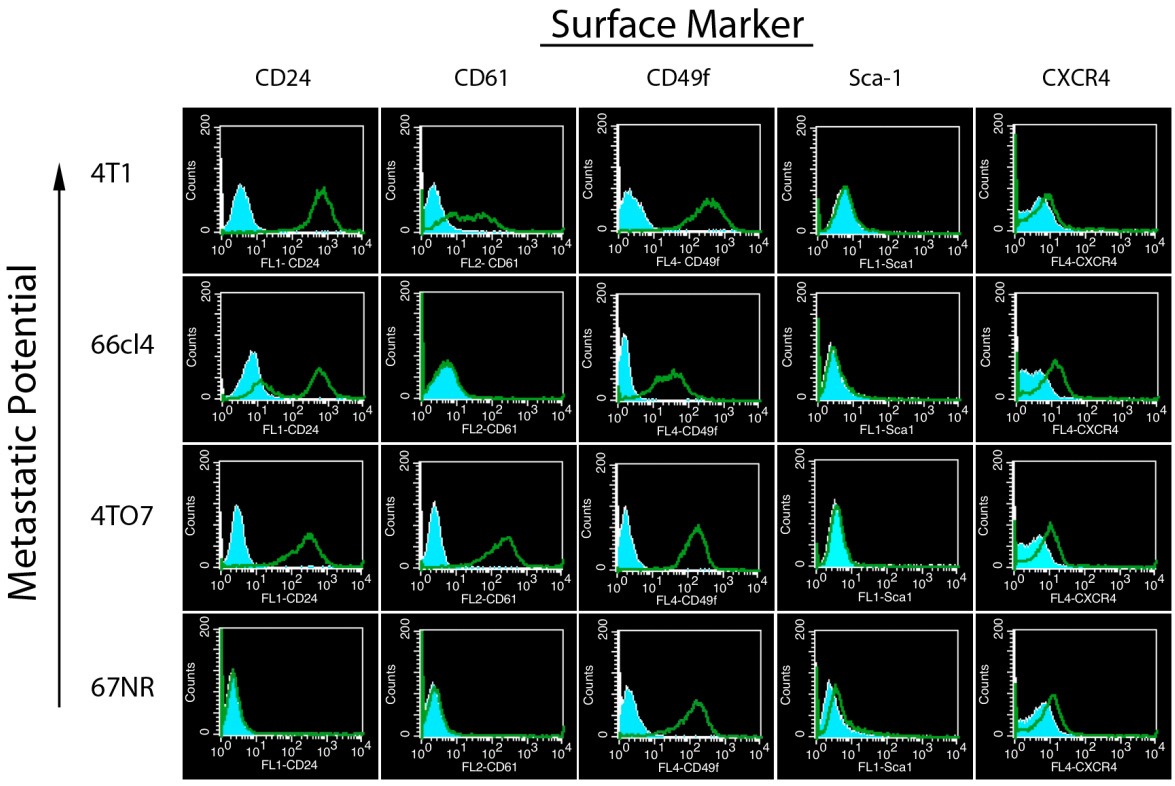

Figure 6. Flow cytometry histograms show a variable surface marker expression pattern in the mammary cancer cell lines. In each histogram plot, the surface profile of labeled cells (green line) is overlaid upon the negative isotype control (teal histogram). The 4T1s harbor positive and negative subpopulations for the following surface markers: CD61, Sca-1, and CXCR4.

A more in-depth characterization of the CD61, Sca-1, and CXCR4 subpopulations was conducted before considering macrophage influence. *Figure 7a* presents a cytometric dot plot that resulted when 4T1 cells were triple-labeled with antibodies targeted against each of the three surface markers. In doing so, it was determined that Sca-1(+) cells within the 4T1s concurrently expressed

CD61 but were also predominantly negative for CXCR4 expression. The miniscule Sca-1(+) and CXCR4(+) subpopulations therefore represent two distinct cell types within the 4T1 line. This relationship was preserved throughout the entire initial characterization, and *Figure 7b* demonstrates this in graphical form.

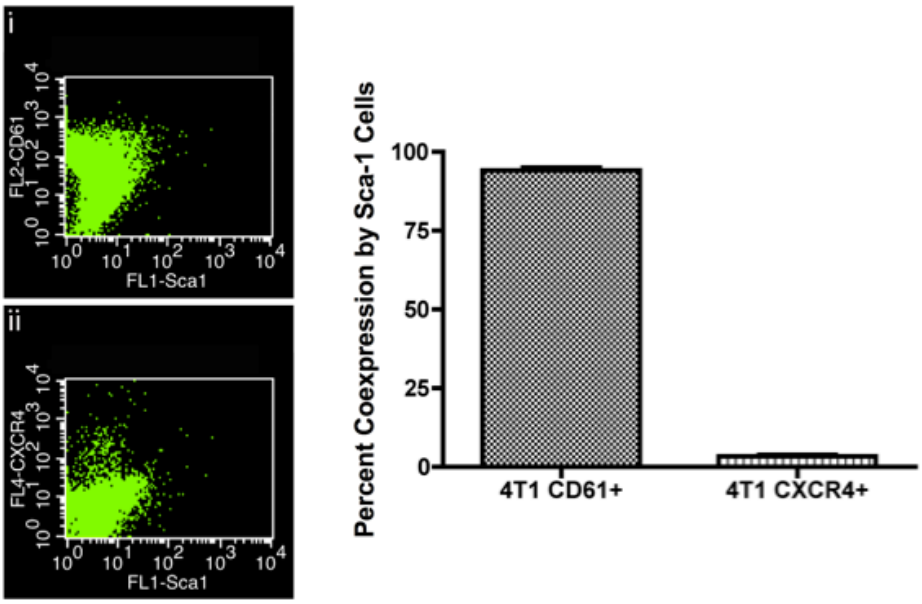

Figure 7. Most Sca-1(+) cells concurrently express CD61, while a small fraction also expresses CXCR4. (a) 4T1s grown in normal culture media were triple-labeled with antibodies against the Sca-1, CD61, and CXCR4 surface markers and analyzed by flow cytometery. In the flow cytometric dot plots, Panel **i** depicts Sca-1 vs. CD61 labeling whereas Panel **ii** depicts Sca-1 vs. CXCR4 labeling. **(b)** Marker coexpression was determined by first gating Sca-1(+) cells and then comparing the second marker's expression to an isotype control. Graphed data is Means ± SEM of three distinct flow cytometric analyses.

Now that cell subpopulations were identified and characterized using the 4T1 cell line, the influence of macrophages on the dynamics of these subpopulations was next ascertained. The RAW 264.7 cell line was utilized to achieve this end, largely because it harbored the BALB/c genetic background

and should be immune-compatible with the mammary carcinoma cell lines. Prior to allowing cells to engage in physical contact, however, a series of conditioned media experiments were first carried out. Data shown in *Figure 8* indicates that conditioned media harvested first from the RAW 264.7 macrophages and then transferred to the 4T1s was capable of inducing Sca-1 expression by over five-fold. Expression changes in the CD61 and CXCR4 markers in response to RAW 264.7-conditioned media were not observed. The fluorescent profile depicted in *Figure 8b* indicates that not only did more 4T1s display Sca-1 in response to the conditioned media, but individual cells also appeared to express higher levels of the marker.

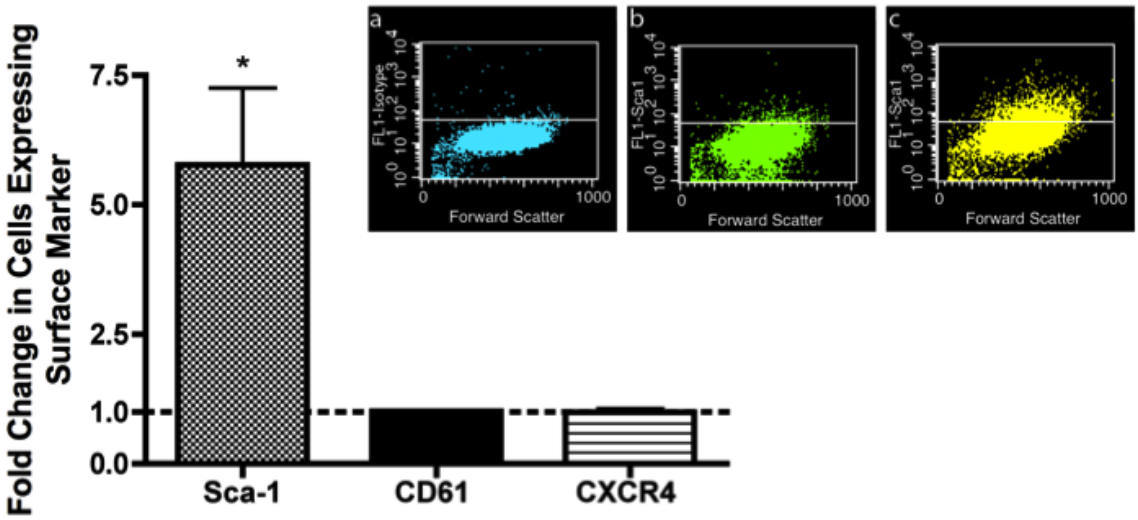

Figure 8. RAW 264.7 conditioned media induces Sca-1 expression in the 4T1s. (a) Conditioned media was collected from the RAW 264.7 culture after 48 hours of conditioning. RAW 264.7 confluency at the end of this period was approximately 80%. Surface marker expression of conditioned media-treated 4T1s was then compared to 4T1s grown in normal culture media by flow cytometry. Graphs shown above are represented as the fold change in the number of cells expressing the surface marker, using the Means ± SEM of three separate experiments. The average number of control 4T1s expressing a given surface marker was as follows: Sca-1 = 2.5%, CD61 = 76.0%, CXCR4 = 4.5%.

Statistics were carried out on log-transformed data using Student's t-tests to compare control versus conditioned media cultures (p-values: Sca-1 = 0.0258, CD61 = 0.5689, CXCR4 = 0.9568). **(b)** Flow cytometric dot plots: the percent of Sca-1 expressing cells was determined using flow cytometry quadrant statistics. Quadrant location was based on the isotype control (Panel **a**), and was set so 1% of the isotype cells fell above the positive threshold. In this experiment, 2.9% of 4T1s in normal growth media (Panel **b**) are considered positive for Sca-1 expression while 17.0% of the 4T1s are positive for Sca-1 after residing in RAW 264.7-conditioned media (Panel **c**) for 24 hours. These numbers include the 1% false-positive margin inherent in basing statistics off the isotype control.

Although the number of Sca-1(+) cells and the intensity of Sca-1 expression increased in the 4T1s following treatment with RAW 264.7-conditioned media, the CD61 and CXCR4 phenotype of the Sca-1(+) cells was not altered. A cytometric dot plot (*Figure 9a*) in addition to a graphical analysis of multiple experiments (*Figure 9b*) illustrates this point. Thus, even though RAW 264.7-conditioned media expands the number of Sca-1(+) 4T1s, the carcinoma cells remain predominantly CD61(+) and CXCR4(-).

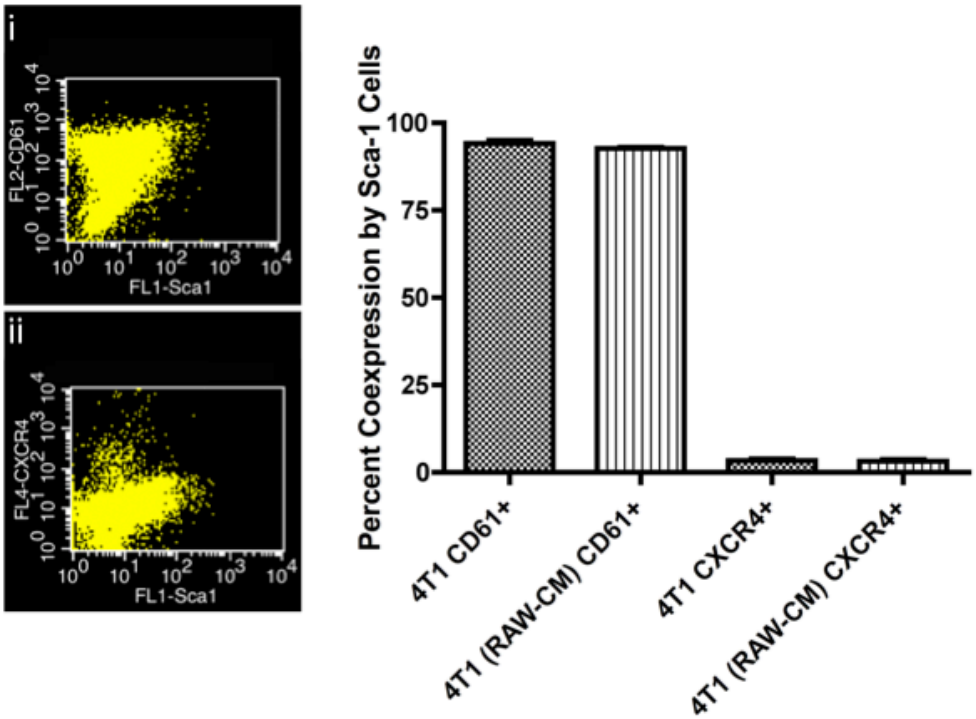

Figure 9. Treatment with RAW 264.7-conditioned media does not alter the predominant CD61(+)/CXCR4(-) status of Sca-1(+) cells. (a) 4T1s treated with RAW 264.7-conditioned media for 24 hours were triple-labeled with antibodies targeted against the Sca-1, CD61, and CXCR4 surface markers. Coexpression was determined by first gating Sca-1(+) cells and then comparing the second marker's expression to an isotype control. Compiling data from three distinct flow cytometric analyses paired with untreated controls created this graph. Data were statistically analyzed as described in the Methods (p-values: CD61 = 0.3638, CXCR4 = 0.8956). **(b)** Flow cytometric dot plots indicate that Sca-1(+) cells have similar expression patterns of CD61(+) but are mostly CXCR4(-) before and after treatment with RAW 264.7-conditioned media. In the images above, Panel **i** depicts Sca-1 vs. CD61 labeling while Panel **ii** depicts Sca-1 vs. CXCR4 labeling. Though the number of Sca-1(+) cells greatly increases, their coexpression profile remains unaltered.

When the conditioned media experiments were further applied to the 66cl4, 4TO7, and 67NR cell lines, it was discovered that Sca-1 induction only occurred in cell lines that were capable of completing metastasis (*Figure 10*). Moreover, the 4T1 cell line possesses greater metastatic potential than the 66cl4s and expectedly had the highest Sca-1 induction in response to the conditioned media. The lack of a detectable Sca-1 *increase* in the 4TO7 and 67NR cell lines correlated with their metastatic shortcomings, however, the 67NR cell line possessed a large static Sca-1(+) subpopulation that remained non-inducible.

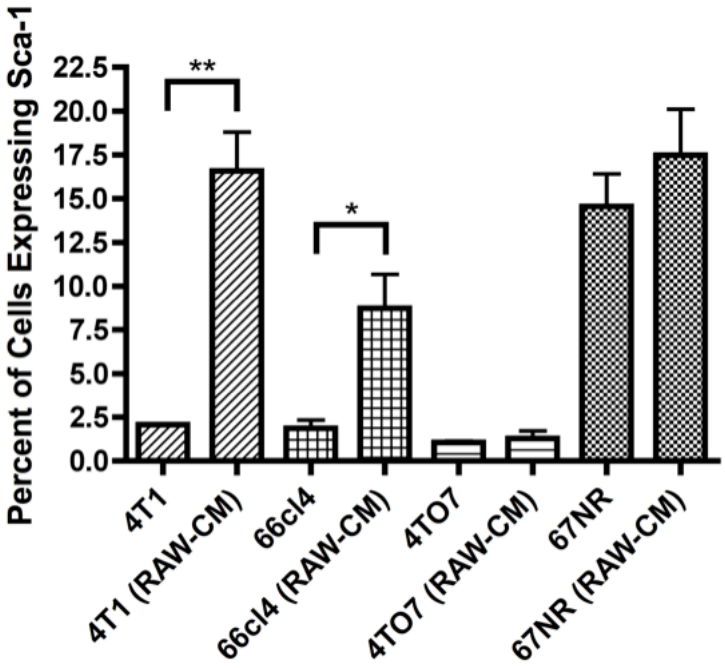

Figure 10. Conditioned media from the RAW 264.7 macrophages induces Sca-1 expression in the most metastatic carcinoma cell lines. RAW 264.7-conditioned media treatment elicits Sca-1 expression in the metastatic 4T1 and 66cl4 cell lines but not in the nonmetastatic 4TO7 and 67NR cell lines. Sca-1 expression was compared between monocultures of each mammary cancer cell line in normal growth media versus RAW 264.7-conditioned media. Graphed data is the Means ± SEM of three experimental replicates. Student's t-tests were preformed on flow cytometric data that was log-transformed (p-values: 4T1 = 0.0030, 66cl4 = 0.0383, 4TO7 = 0.8806, 67NR = 0.2200).

Given that RAW 264.7-conditioned media was able to increase Sca-1 expression in the 4T1s, the next step was to determine if conditioned media could also alter the transcription of genes associated with the macrophage-carcinoma paracrine loop involving CSF-1, a cytokine known to activate macrophages into a pro-tumoral state (M2 activation) and EGF, a known proliferation and migration signaling molecule (43). Data from this set of experiments showed that 4T1s treated with RAW 264.7-conditioned media did

not significantly up-regulate mRNA levels for the CSF-1 or EGFR genes (*Figure 11*).

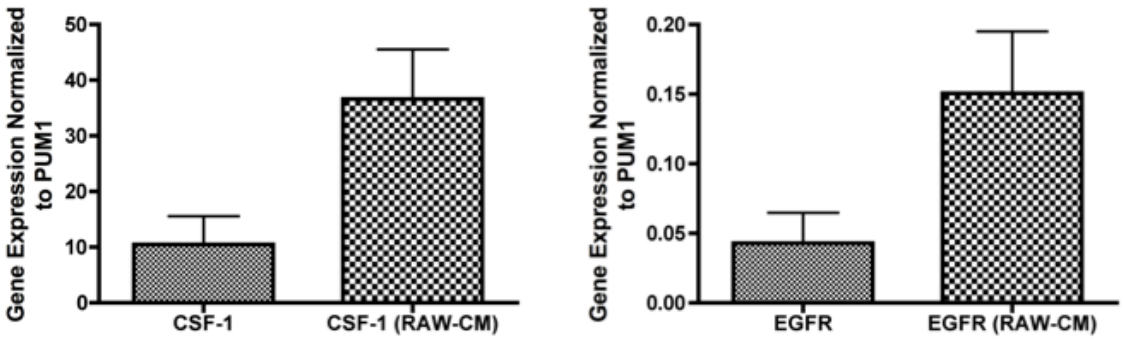

Figure 11. 4T1s treated with RAW 264.7-conditioned media do not exhibit altered expression of the colony-stimulating factor-1 (CSF-1) or the epidermal growth factor receptor (EGFR) genes. 4T1 cells were grown in either normal growth media or RAW 264.7-conditioned media for 24 hours. After this period, RNA was extracted and qPCR was performed in triplicate. The Means ± SEM of three independent experiments is depicted above and Student's t-tests were performed on log-transformed data (p-values: CSF-1 = 0.1915, EGFR = 0.1752).

The application of 4T1-conditioned media to the RAW 264.7 macrophages demonstrated that 4T1-conditioned media was also incapable of eliciting gene expression changes in the RAW 264.7s. *Figure 12* indicates that the transcription of CSF-1R and EGF, which are macrophage components of the macrophage-carcinoma paracrine interaction, remained unaltered by soluble factors from the 4T1s.

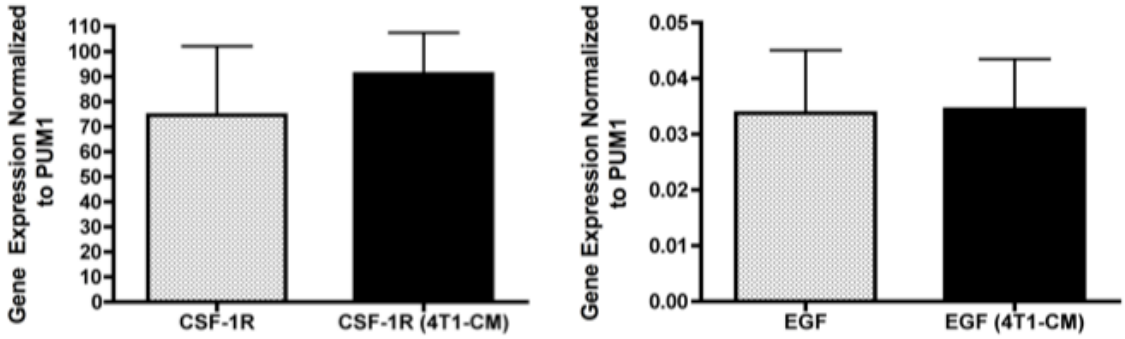

Figure 12. RAW 264.7 macrophages grown in 4T1-conditioned media do not show differential expression of the colony-stimulating factor-1 receptor (CSF-1R) or the epidermal growth factor (EGF) genes. RAW 264.7s were grown in either standard growth media or 4T1-conditioned media for 24 hours. Following incubation, RNA was extracted from the cultures and qPCR was run in triplicate. The graphs above present the Means ± SEM of four different experiments. Student's t-tests were performed on log-transformed data (p-values: CSF-1R = 0.4864, EGFR = 0.7275).

In addition it was found that 4T1-conditioned media was not activating the RAW 264.7s into either a pro-tumoral or anti-tumoral state, as the transcription levels of the inducible nitric oxide (iNOS) or macrophage mannose receptor (MMR) genes did not fluctuate when compared to a control (*Figure 13*).

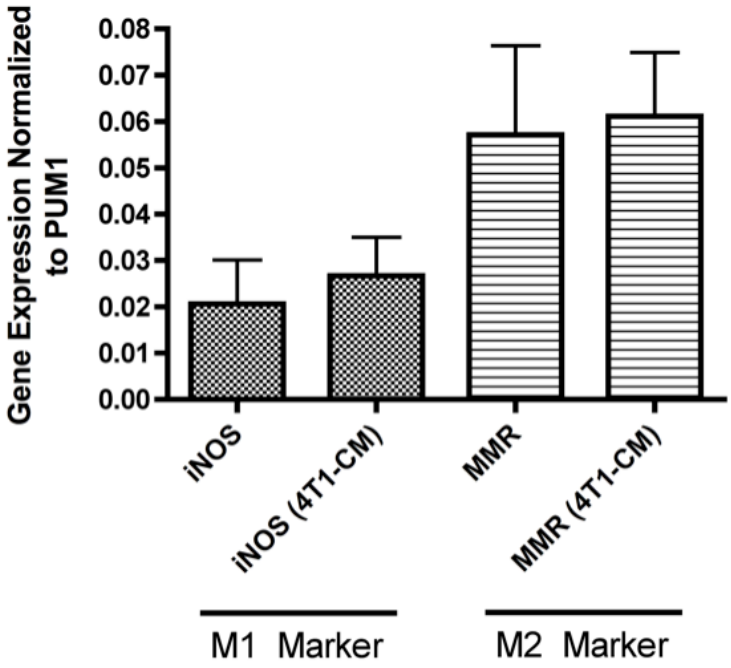

Figure 13. 4T1-conditioned media does not appear to activate the RAW 264.7 macrophages into either an anti-tumoral (M1) or pro-tumoral (M2) state. RAW 264.7 cells were permitted to grow in normal culture media or 4T1-conditioned media for 24 hours prior to RNA extraction and qPCR. There was no change in gene expression for inducible nitric oxide synthase (iNOS) or macrophage mannose receptor (MMR) between control and conditioned media-treated cells, indicating the lack of M1 or M2 macrophage activation. Graphed data represents the Means ± SEM of four separate experiments, and statistics were performed on log-transformed values using Student's t-tests (p-values: iNOS = 0.5766, MMR = 0.4315).

Interactions between the RAW 264.7 macrophages and the mammary carcinoma cell lines were next examined in a co-culture setting. This aspect of the thesis was thought to more appropriately reflect the communicative exchanges that would occur *in vivo*, because co-culture permits physical interactions and allows the cell lines to engage in crosstalk. Thus, in co-culture with the RAW 264.7s, these conditions led to the discovery that the RAW 264.7 macrophages induced the metastatic 4T1 and 66cl4 cell lines to form spheroid

structures (*Figure 14*). Notably, this spheroidal morphology was absent in RAW 264.7 co-cultures involving the 4TO7s and 67NRs.

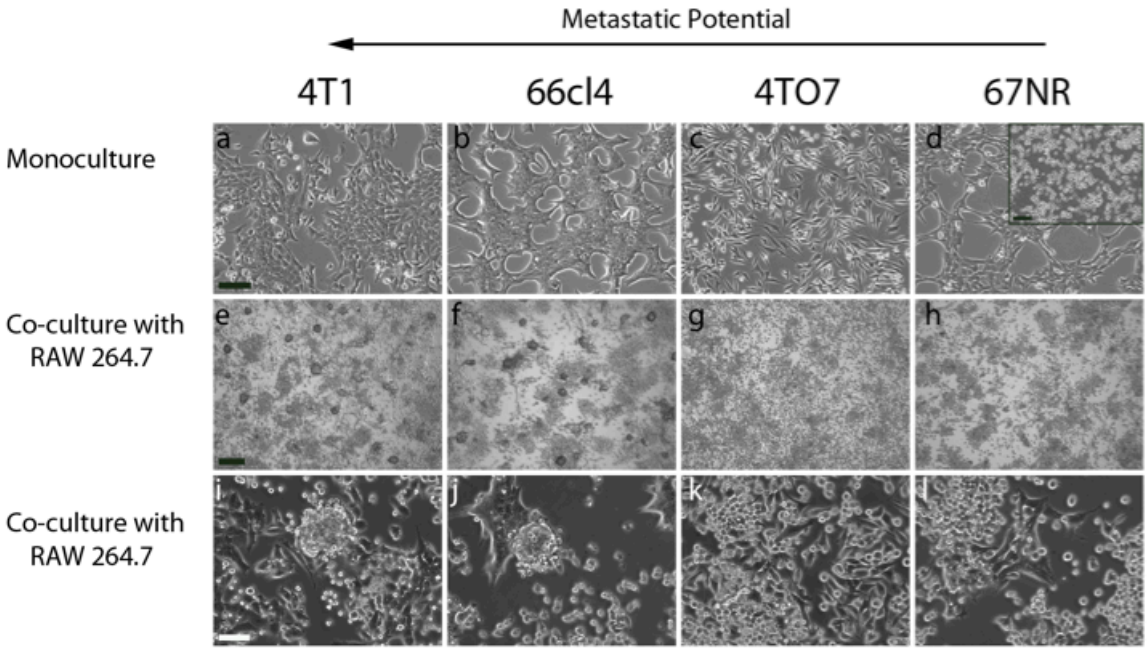

Figure 14. Metastatic mammary carcinoma cell clones form spheroids upon co-culture with RAW 264.7 macrophages. Brightfield microscopy photographs for each mammary cancer cell line were captured in a monoculture setting and in co-culture with the RAW 264.7s. The morphology of the RAW 264.7 macrophages is shown as an inset in Panel **d**. Cancer cell spheroids are formed upon co-culture with the RAW 264.7 macrophages in the 4T1 and 66cl4 mammary carcinoma cell lines. The central images (Panels **e** and **f**) use lower magnification to demonstrate that spheroid formation is a ubiquitous event in the 4T1s and 66cl4s, whereas Panels **i** and **j** present spheroid morphology at a higher magnification. Scale bars are found in the left-most panels and correspond to each row. Scale bar lengths: Panels **a**-**d**: 50 μm, Panels **e**-**h**: 100 μm, Panels **i**-**l**: 25 μm.

In order to assay Sca-1 expression in the spheroids, co-cultures of the 4T1s and RAW 264.7s were grown on glass coverslips and then labeled with fluorescently conjugated antibodies. The ensuing microscopy photographs in *Figure 15* suggest that the highest Sca-1 expression is retained in the cancer cells that compose the spheroids.

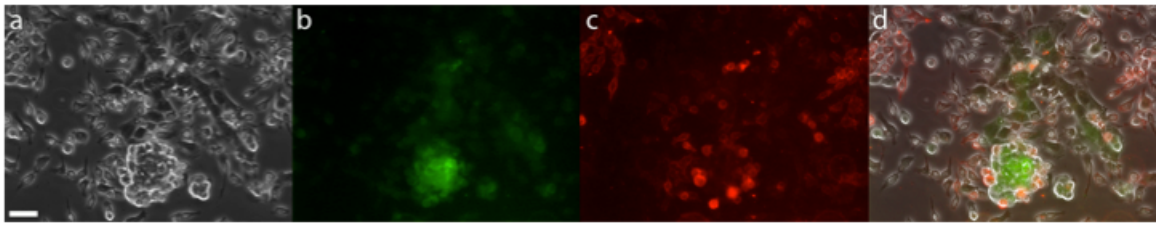

Figure 15. RAW 264.7 macrophages induce 4T1 cells into spheroids that are enriched for Sca-1 expression. Co-cultures of the 4T1 and RAW 264.7 cell lines were grown on glass coverslips, fixed with 4% paraformaldehyde, and then labeled with fluorescently-tagged antibodies against Sca-1 (Panel **b**; green) and the macrophage marker F4/80 (Panel **c**; red). Panel **d** represents an overlay of Panels **b** and **c**. The scale bar is shown in Panel **a** and corresponds to a length of 25 μm.

A subsequent array of experiments sought to determine if macrophage number was positively correlated to the expression of the Sca-1 or CXCR4 surface markers. These experiments were pursued using the 4T1, 4TO7, and 67NR mammary cancer cell lines, where the RAW 264.7 macrophages were added to create two types of co-cultures: one in which the carcinoma cells were in the majority and one in which the macrophages were in the majority. Distinct morphological changes between these conditions were found in co-cultures involving the 4T1s and RAW 264.7s, and the associated microscopy photographs can be seen in *Figure 16a*. Essentially, 4T1 spheroids were only formed when macrophages were seeded in the majority to the 4T1s. A comparison of Sca-1 and CXCR4 expression in the 4T1/RAW 264.7 co-cultures indicated that Sca-1 expression by the 4T1s was indeed correlated with macrophage number, although macrophage presence did not affect CXCR4 expression (*Figure 16b*). This theme carried over to the 4TO7/RAW 264.7 co-

cultures, where the 4TO7s were found to have a small inducible Sca-1 subpopulation that was discernible with a high macrophage number (*Figure 16c*). When 67NR/RAW 264.7 co-cultures were examined, it was found that macrophages influenced neither Sca-1 nor CXCR4 expression by the carcinoma cells (*Figure 16d*). There was a discrepancy in the amount of positive 67NRs in the conditioned media experiments (*Figure 10*) versus these co-culture experiments (*Figure 16d*), which was caused by varied locations of the positive flow cytometric threshold. In co-culture experiments involving the 67NRs, the positive threshold was set more conservatively to accommodate greater background fluorescence from the RAW 264.7s.

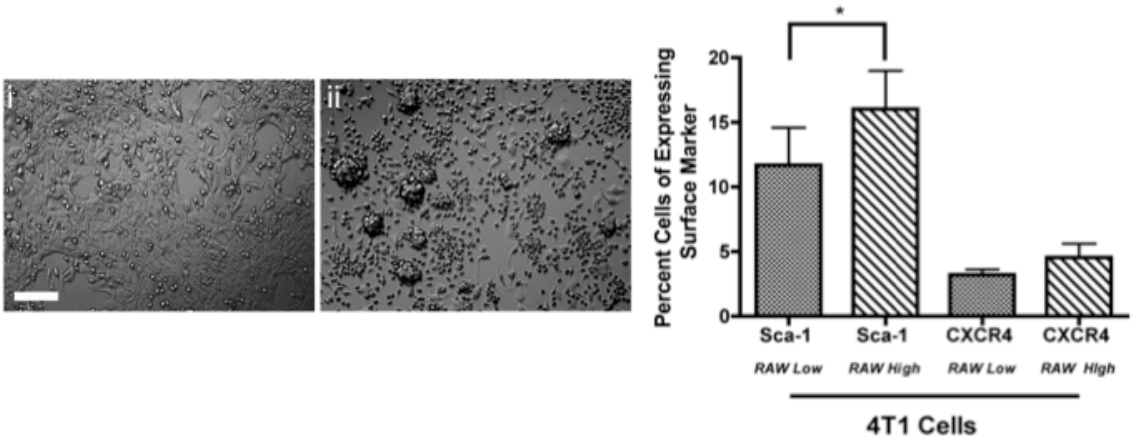

Figure 16. Higher macrophage number enhances spheroid formation and Sca-1 induction in responsive cell lines. (a) Spheroid formation in the 4T1s is a product of higher RAW 264.7 macrophage numbers in co-culture. 4T1 cells were placed into co-culture with the RAW 264.7 cells in two unique settings: one with a low macrophage presence and one with a high macrophage presence. In the first setting, the 4T1s outnumbered the RAW 264.7s by 5:1 (microscopy Panel **i**) and in the second setting the RAW 264.7s outnumbered the 4T1s by 3:1 (microscopy Panel **ii**). Placing the RAW 264.7s in the majority is not only necessary to induce higher Sca-1 expression in the cancer cells, but it is also required for spheroid formation (microscopy Panel **ii**). **(b)** The extent of Sca-1 expression in the 4T1s is positively correlated to RAW 264.7 macrophage number in co-culture. Data analysis was conducted via flow cytometric gating based on the F4/80 receptor. Graphed data is presented as Means ± SEM, and

Student's t-tests were performed on log-transformed data from five independent experiments (p-values: Sca-1 = 0.0495, CXCR4 = 0.3005). The scale bar in the microscopy images corresponds to a length of 50 μm.

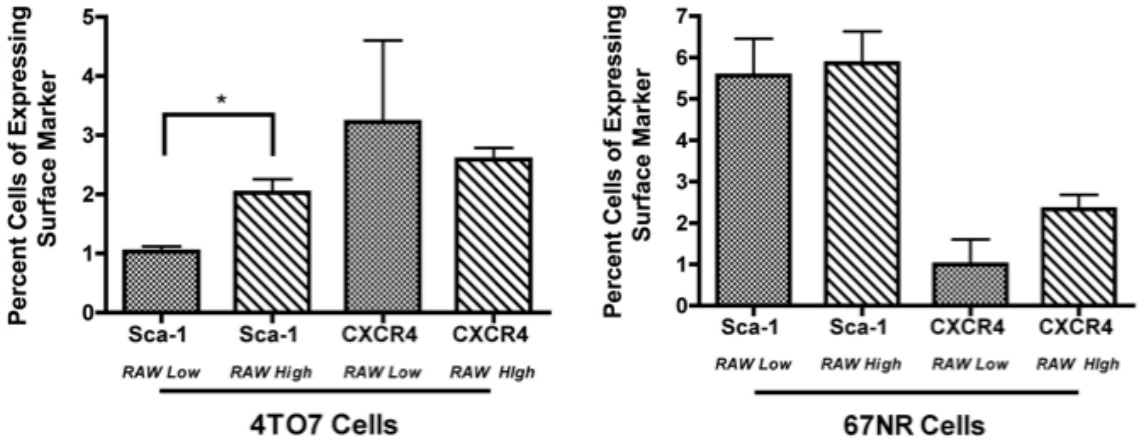

Figure 16 (continued). (c) Sca-1 induction in the 4TO7s is positively correlated to RAW 264.7 macrophage presence in co-culture, but the number of Sca-1-expressing cells remains low. 4TO7 cells were placed into co-culture with the RAW 264.7s, where the latter cell type had either a high or low presence. Cancer cells were analyzed by the flow cytometric gating of F4/80-negative cells. The data graphed above is Means ± SEM, and the product of three distinct experiments. Statistical analysis was conducted on log-transformed data using Student's t-test (p-values: Sca-1 = 0.0254, CXCR4 = 0.9355). **(d)** RAW 264.7 number is not correlated with Sca-1 expression in the 67NR cell line. 67NRs were co-cultured with the RAW 264.7s in two conditions: one where the 67NRs were in the majority, and one in which the RAW 264.7s were in the majority. 67NRs were analyzed by the flow cytometric gating of F4/80-negative cells. The data graphed above is Means ± SEM and the outcome of three different experiments. Statistics were conducted on log-transformed data via Student's t-test (p-values: Sca-1 = 0.6892, CXCR4 = 0.1202).

Because co-culture provides a milieu for crosstalk between cell lines, it was thought that this setting would be more ideal to examine the expression of genes associated with the macrophage-carcinoma paracrine loop. Consistent with this premise, 4T1s analyzed after 48 hours of co-culture with the RAW 264.7s were found to express lower levels of the CSF-1 gene when compared to a monolayer control, even as transcription of the Sca-1 gene increased (*Figure*

17a). In contrast, there was no difference found in this comparison when EGFR was analyzed (*Figure 17b*).

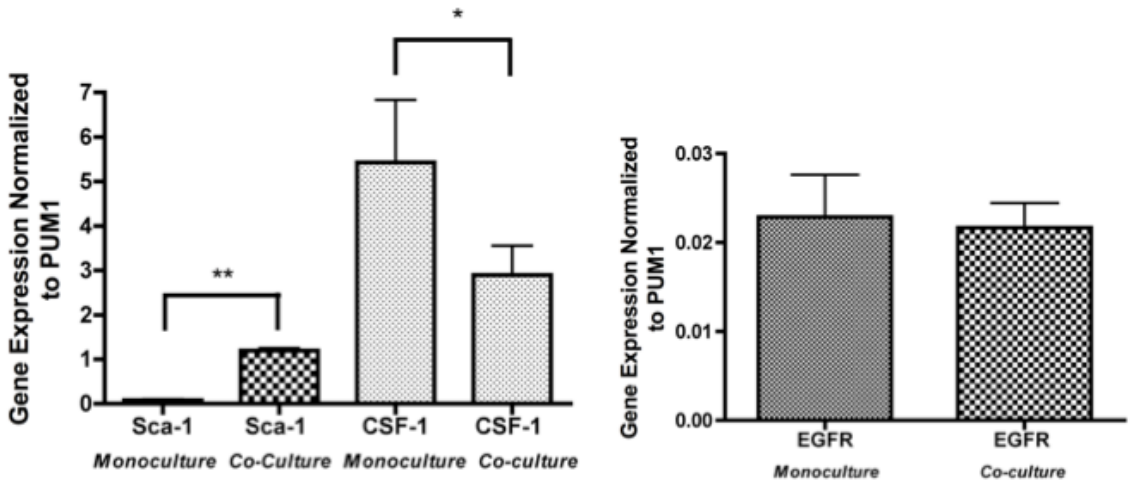

Figure 17. 4T1 co-culture with the RAW 264.7s leads to increased Sca-1 but reduced CSF-1 gene expression. (a) 4T1s exhibit increased Sca-1 mRNA but decreased CSF-1 expression following co-culture with the RAW 264.7 macrophages. The 4T1s were harvested from co-culture by negative selection using magnetic-activated cell sorting and the macrophage F4/80 marker. The expression of Sca-1 and CSF-1, a cytokine known to activate macrophages into a pro-tumoral state, was then examined by qPCR. The reduction in CSF-1 expression by the 4T1s is consistent with anti-tumoral (M1) activation seen in the RAW 264.7 cells. Means ± SEM were calculated from three distinct experiments. Data were log-transformed and analyzed using Student's t-tests (p-values: Sca-1 = 0.0080, CSF-1 = 0.0355). **(b)** 4T1s do not show changes in EGFR gene expression after co-culture with the RAW 264.7s. The lack of increased epidermal growth factor receptor expression suggests the absence of a macrophage-carcinoma EGF/EGFR paracrine loop in co-culture. Means ± SEM were calculated from three different experiments. Data were log-transformed and analyzed using the Student's t-test (p-values: EGFR = 0.9088).

RAW 264.7 macrophages isolated and analyzed from co-culture with the 4T1s showed that both of their CSF-1 and EGF genes were not differentially expressed (*Figure 18*). This data indicated that the EGF-based macrophage-carcinoma paracrine loop might not be operating at detectable levels in this co-

culture system given the methodology used, which did not involve an assessment of protein levels or phosphorylation states.

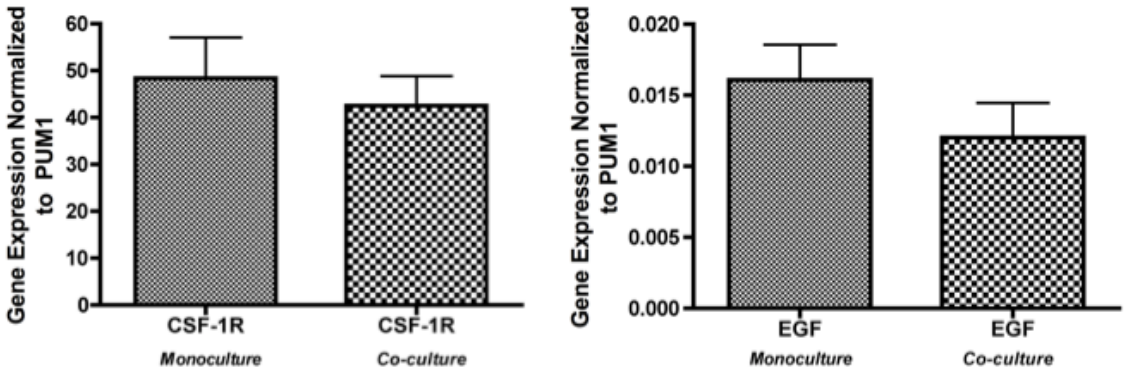

Figure 18. RAW 264.7 colony-stimulating factor-1 receptor (CSF-1R) and epidermal growth factor (EGF) mRNA levels are not altered in co-culture with the 4T1s. RAW 264.7 cells were first collected from co-culture via magnetic-activated cell sorting targeted against the F4/80 receptor. Next, RNA was extracted from the RAW 264.7s and qPCR was run in triplicate. RAW 264.7s taken from co-culture were compared to samples obtained from monoculture controls. The lack of differential expression in the CSF-1R and EGF genes signify the absence of a macrophage-carcinoma EGF-based paracrine loop in co-culture. The data graphed above is the Mean ± SEM from five separate experiments. Student's t-tests were carried out on log-transformed data (p-values: CSF-1R = 0.4613, EGF = 0.1125).

However, gene expression changes were discovered for the macrophage markers associated with both anti-tumoral (M1) and pro-tumoral (M2) functions. In co-culture with the 4T1s, the RAW 264.7 macrophages produced higher levels of anti-tumoral iNOS mRNA, which is the enzyme utilized by macrophages to mediate carcinoma cell destruction (*Figure 19*). Significant increased expression of the MMR marker that signifies pro-tumoral activities was also seen. Taken together, these findings indicated that, in co-culture, the 4T1s drove the RAW

264.7 macrophages to up-regulate markers associated with anti-tumoral as well as pro-tumoral functions.

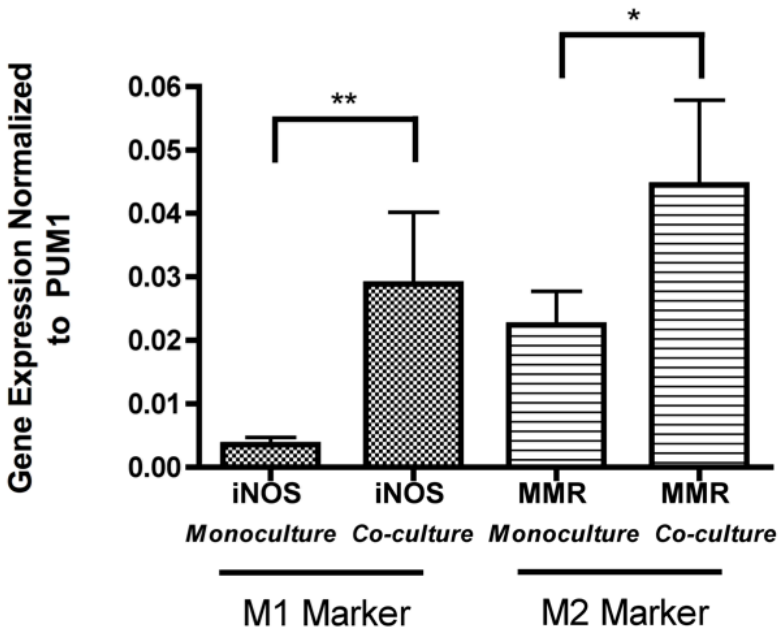

Figure 19. RAW 264.7 macrophages show increased iNOS and MMR gene expression following co-culture with the 4T1s. RAW 264.7 cells were collected from co-culture by magnetic-activated cell sorting of F4/80-positive cells. The expression of markers associated with anti-tumoral (M1) and pro-tumoral (M2) states was then evaluated in the macrophages using qPCR. The inducible nitric oxide synthase (iNOS) gene was indicative of M1 activation while the macrophage mannose receptor (MMR) gene served as a surrogate for M2 activation. Graphed data represents the Mean ± SEM of qPCR results taken from five independent experiments. Student's t-tests were performed on log-transformed data (p-values: iNOS = 0.0021, MMR = 0.0241).

At this point in the thesis, experimental findings showed that RAW 264.7-conditioned media or physical interactions from the RAW 264.7s could induce Sca-1 expression in metastatic mammary carcinoma cell lines. Furthermore, elevated Sca-1 levels in the 4T1s appeared to accompany less CSF-1 expression by those cells, in addition to higher iNOS gene transcription in the RAW 264.7s. Though RAW 264.7 macrophage MMR levels also increased in

co-culture, the net reduction in 4T1 CSF-1 mRNA in conjunction with enhanced iNOS expression in the RAW 264.7s was most consistent with anti-tumoral macrophage function. This notion is further supported by the direct involvement of iNOS in facilitating macrophage killing of pathogenic cells, whereas MMR functions as a receptor in extracellular debris clearance. This data fit well into a paradigm whereby a subset of RAW 264.7 macrophages were driven into an anti-tumoral state by the 4T1s in co-culture, which led to Sca-1 induction and spheroid formation in the carcinoma cells. Now that they had been described, these findings needed to be enveloped around a metastatic story. Proliferation and motility assays were next conducted to accomplish this aim.

The first of these experiments looked at the proliferation rate of Sca-1(+) cells in 4T1- versus RAW 264.7-conditioned media. The SRB assay was initially used to compare the proliferation rate of the 4T1s in normal growth media to each of the conditioned media types. *Figure 20* shows that each conditioned media type retarded cell proliferation rate, but the differences were most stark after 48 hours in conditioned media. The next set of experiments was designed with this caveat in mind.

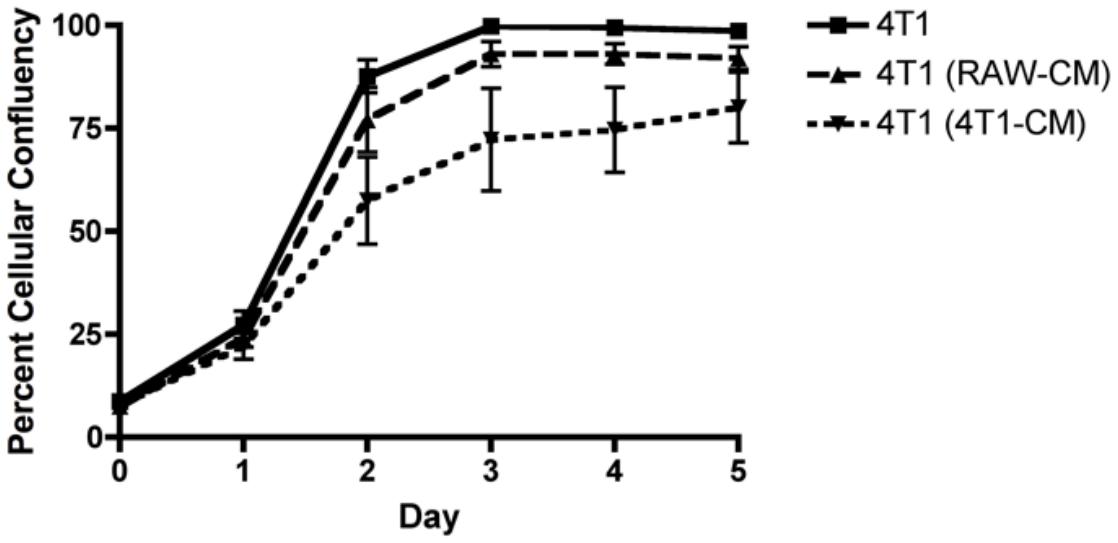

Figure 20. 4T1 cells exhibit slower proliferation when grown in conditioned media derived from either the 4T1s or the RAW 264.7s. Growth curves for the different experimental conditions were generated using the sulforhodamine B (SRB) assay. In short, fresh growth media or conditioned media was added to cell culture plate wells on Day 1, and then a time point was taken every 24 hours until Day 5. Total protein per well was quantitated using the SRB assay, where percent cellular confluency was determined by normalizing plate data to its most confluent well. The graphed data represents the Mean ± SEM of three independent experiments. Statistics were performed using Student's t-tests to compare the Mean of normal 4T1s to the Means of the conditioned media samples (p-values: 4T1 (RAW-CM) = 0.0074, 4T1 (4T1-CM) = 0.0159).

The thymidine analog called EdU was used to compare the rate at which Sca-1(+) cells divide with the proliferation rate of Sca-1(-) cells. First, 4T1s were treated with either 4T1- or RAW 264.7- conditioned media for 24 hours. Next, the 4T1s were either administered a 10μM EdU pulse for either 16 hours or for 45 minutes. Though no difference in EdU incorporation was seen after the 16 hour pulse, it was learned that more Sca-1(+) cells took up EdU following the 45 minute pulse (*Figure 21a*). This data suggested that Sca-1(+) cells proliferated faster than Sca-1(-) cells, and this phenomenon was independent of conditioned

media type. A flow cytometric dot plot example of increased EdU incorporation by Sca-1(+) cells can be seen in *Figure 21b*.

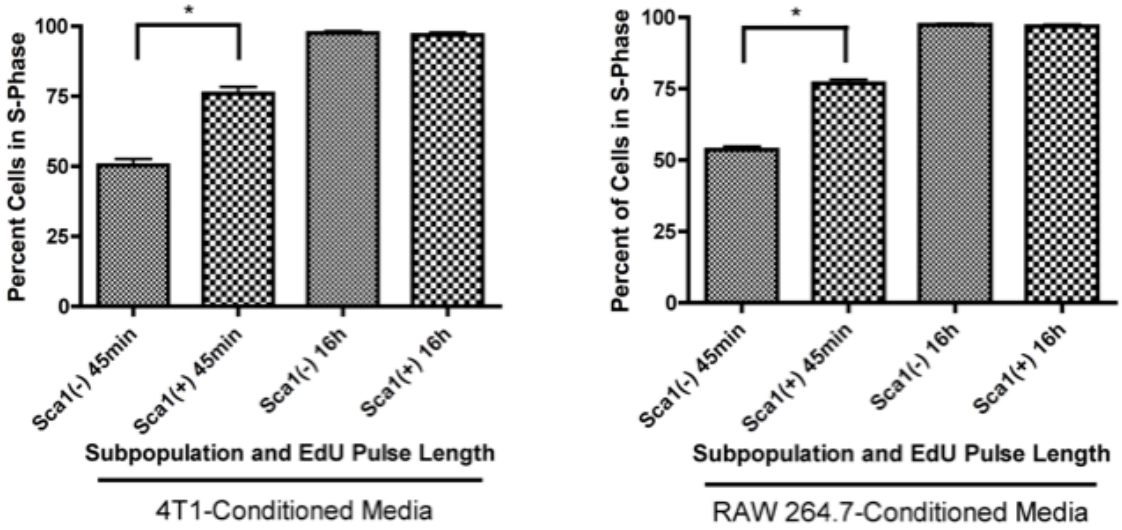

Figure 21. 4T1 Sca-1(+) cells proliferate faster than their Sca-1(-) counterparts. (a) 4T1-conditioned media and RAW 264.7-conditioned media induces the Sca-1(+) subpopulation in the 4T1 cell line to proliferate faster than the Sca-1(-) subpopulation. The 4T1 cell line was subjected to 4T1-conditioned media or RAW 264.7-conditioned media for 24 hours and then pulsed with 10 μM EdU, a thymidine analog that is incorporated during DNA synthesis, for either 45 minutes or 16 hours. The percent of EdU(+) cells was then determined using flow cytometry and compared over three individual experiments. Data are presented as Means ± SEM. Statistical analysis was carried out using Student's t-tests on log-transformed data (4T1-conditioned media p-values: 45min = 0.0251, 16h = 0.4387; RAW 264.7-conditioned media p-values: 45min = 0.0104, 16h = 0.5864).

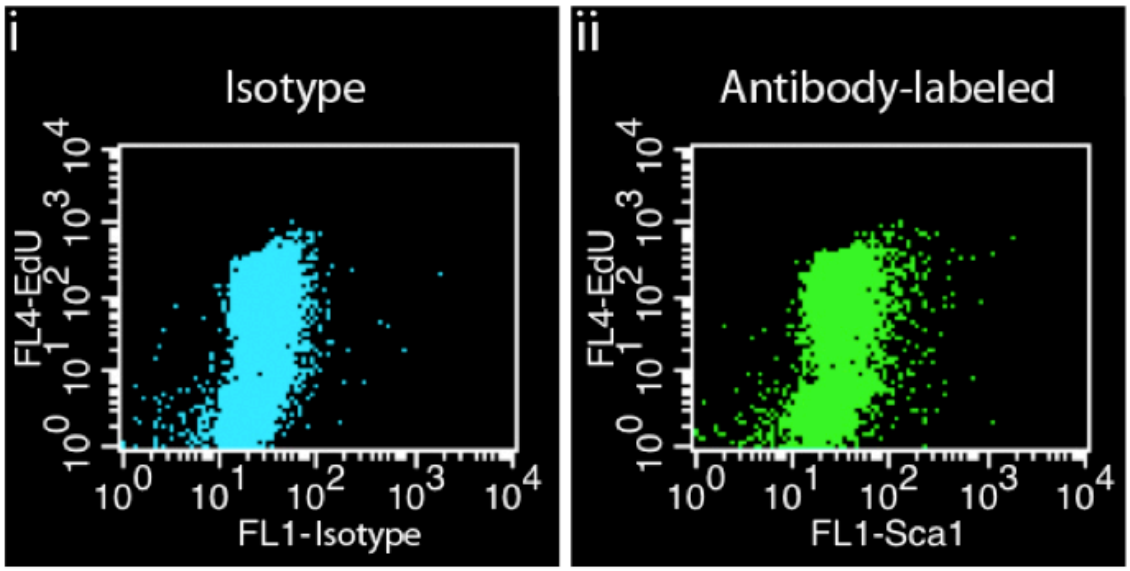

Figure 21 (continued). (b) A flow cytometric dot plot comparison shows that Sca-1(+) cells have a tendency to incorporate higher levels of EdU. 4T1s were grown in RAW 264.7-conditioned media and then pulsed with 10 μM EdU for 45 minutes. When the isotype control (Panel **i**) is compared with 4T1s labeled with Sca-1 antibodies (Panel **ii**), it can be seen that the Sca-1(+) cells incorporate higher levels of EdU than Sca-1(-) cells. This data indicates that Sca-1(+) cells proliferate faster in RAW 264.7-conditioned media than Sca-1(-) cells.

A fluorescent phagokinetic bead assay was used to assess the motile nature of the 4T1s. First it was acknowledged that 4T1s had an overall tendency to be less motile when exposed to RAW 264.7-conditioned media. This was discerned because 4T1s treated with RAW 264.6-conditioned media, and then permitted to migrate over a layer of fluorescent beads, moved shorter distances as shown by the fluorescent microscopy images in *Figure 22a*. It was subsequently confirmed by flow cytometry that 4T1s treated with RAW 264.7-conditioned media engulfed fewer fluorescent beads than those left in control media (*Figure 22b*).

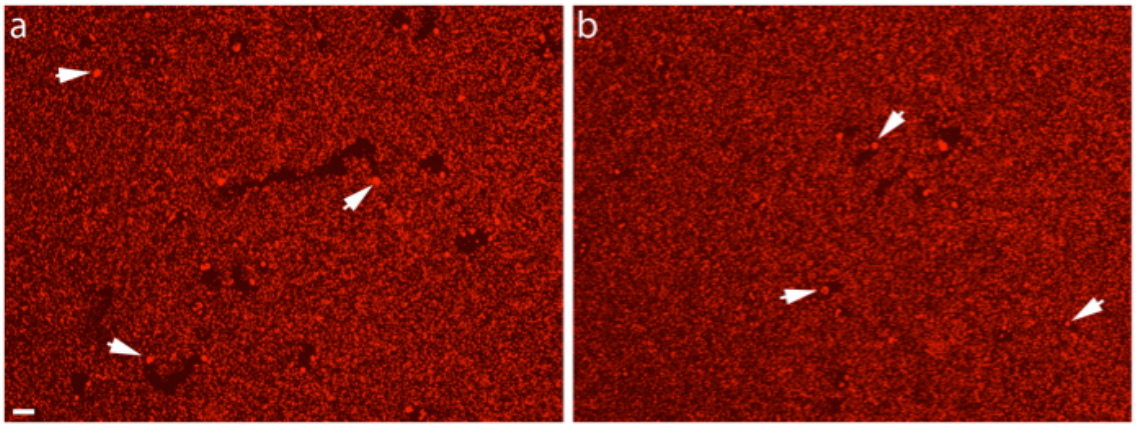

Figure 22. 4T1s are less motile when treated with RAW 264.7-conditioned media versus normal growth media. (a) 4T1s were treated with normal growth media or RAW 264.7-conditioned media for 24 hours, and then permitted to migrate over a layer of fluorescent beads for 12 hours. The 4T1 cells collected from control cultures (Panel **a**) show greater motility than 4T1s taken from RAW 264.7-conditioned media (Panel **b**), as evidenced by increased phagokinesis of fluorescent beads. In the above microscopy photographs, arrowheads are used indicate example 4T1 cells. The scale bar corresponds to a length of 100 µm.

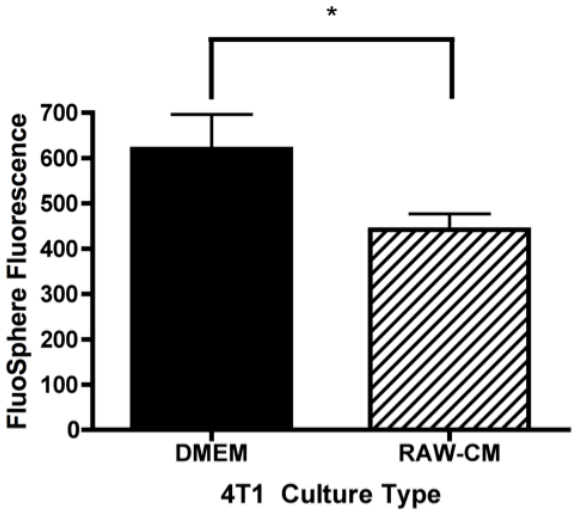

Figure 22 (continued). (b) Flow cytometric analysis confirms that 4T1s exhibit less motility after treatment with RAW 264.7-conditioned media. 4T1s were either grown in normal media or RAW 264.7-conditioned media for 24 hours, then permitted to migrate over a layer of fluorescent beads for 8 hours. The phagokinetic activity was then compared using flow cytometry, where high fluosphere fluorescence is positively correlated with cellular bead uptake. Graphed data shows the Means ± SEM of three distinct experiments and a Student's t-test was performed on the log-transformed data (p-value = 0.0393).

When cultures akin to the one in *Figure 22a* were subjected to Sca-1 labeling and flow cytometry, the motile behavior of Sca-1(+) 4T1s versus Sca-1(-) 4T1s could be further distinguished. These experiments demonstrated that fewer Sca-1(+) cells engulfed beads than Sca-1(-) cells, although the Sca-1(+) cells that moved migrated further than Sca-1(-) cells, but only when in control media (*Figure 23a*). The flow cytometric dot plots in Figure 23b further exemplify the fact that Sca-1(+) 4T1s are generally less motile than Sca-1(-) cells.

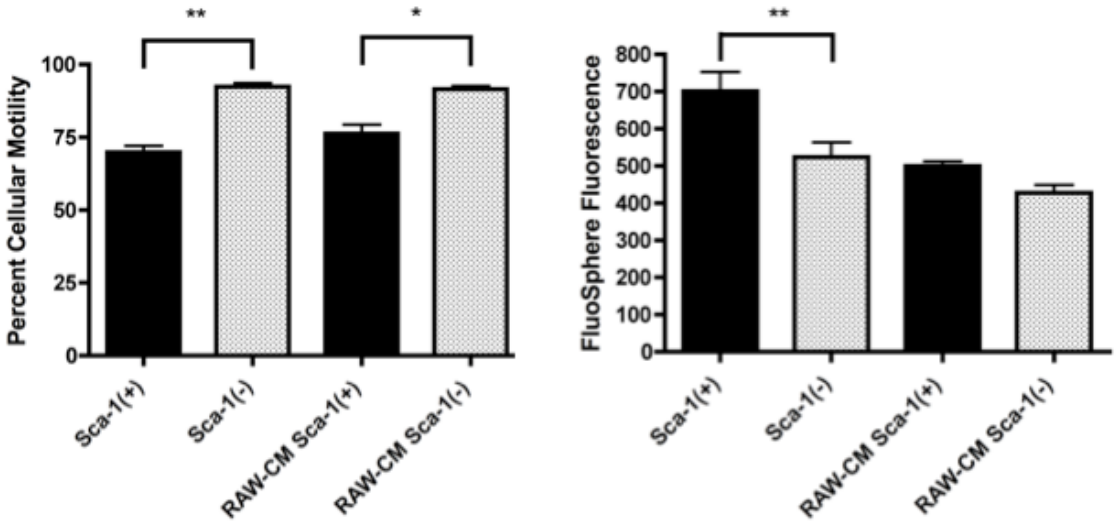

Figure 23. Fewer Sca-1(+) cells exhibit motility than Sca-1(-) cells in normal growth media and RAW 264.7-conitioned media. However, the motile population of Sca-1(+) cells move further than Sca-1(-) cells in normal growth media. (a) 4T1s were treated with normal media or RAW 264.7-conditioned media for 24 hours, and then allowed to migrate over fluorescent beads for 8 hours. After this period, the 4T1s were labeled with antibodies against Sca-1, and the phagokinetic ingestion of fluorescent beads was compared between Sca-1(+) cells and Sca-1(-) cells by flow cytometry. Graphs represent the Means ± SEM of three independent analyses. Statistical comparisons were made on log-transformed data using Student's t-tests (Percent motility p-values: control media = 0.0047, RAW 264.7-conditioned media = 0.0287; FluoSphere fluorescence p-values: control media = 0.0018, RAW 264.7-conditioned media = 0.0140).

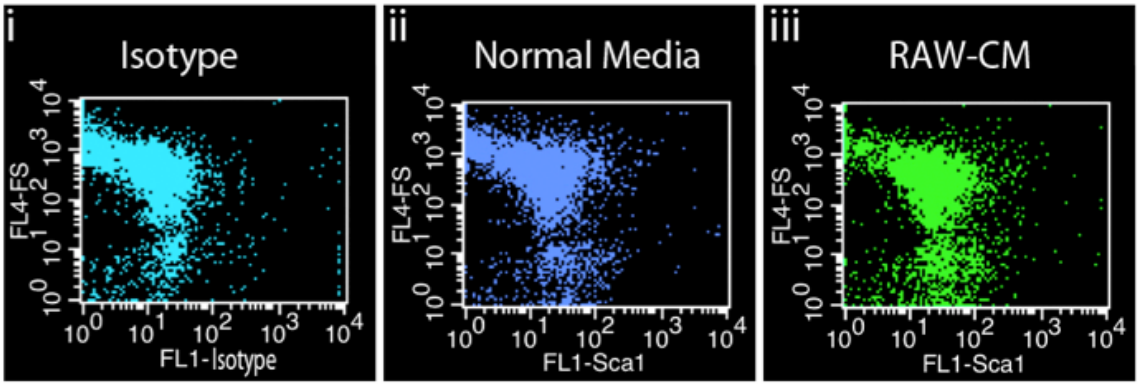

Figure 23 (continued). (b) Flow cytometry dot plots corroborate that the Sca-1(+) subpopulation in the 4T1 cell line generally exhibits less motility than Sca-1(-) cells. 4T1s were grown in normal media or RAW 264.7-conditioned media for 24 hours and then allowed to migrate on a layer of fluorescent beads for 8 hours. A comparison of 4T1s from normal media (Panel **ii**) versus RAW 264.7-conditioned media (Panel **iii**) is shown above, and related to the negative isotype control (Panel **i**). The flow cytometric dot plots reveal that the Sca-1(+) cells tend to exhibit less FluoSphere uptake than the Sca-1(-) cells.

When assembled together, the experimental work in this thesis showed that RAW 264.7 macrophages induced Sca-1 expression in metastatic but not nonmetastatic mammary carcinoma cell lines. The induction of Sca-1 in the metastatic cell lines occurred via RAW 264.7 paracrine cues, as evidenced by conditioned media experiments, and the RAW 264.7s induced metastatic cell lines into spheroids upon co-culture. A gene expression study on 4T1/RAW 264.7 co-cultures indicated that the macrophages might elicit Sca-1 expression in the carcinoma cells through anti-tumor mechanisms in conjunction with elevated MMR levels. This inference is made because iNOS was up-regulated in co-culture, and it is an enzyme that is used by macrophages to mediate targeted cell destruction by oxidative burst. Finally, a functional evaluation of Sca-1(+) cells in the 4T1s revealed that Sca-1(+) cells proliferated faster than Sca-1(-) cells but tended to be less motile overall, although a small subpopulation was quite motile.

DISCUSSION

Subpopulation Characterization

A requisite precursor to examining how macrophages influence the surface marker characteristics of carcinoma cells was the identification of subpopulations within the cell lines. Recent work by various groups has identified a stem-like component within mammary breast cancer cell lines, as well as more heterogeneous cell types that arise by differentiation (6). In conjunction with the herculean effort being employed by some to apply the mammary gland hierarchy to cancerous cell lines (4, 5), the first portion of this study characterized the 4T1, 66cl4, 4TO7, and 67NR cell lines based on their expression of hierarchical integrins in addition to other markers associated with tumorigenicity and metastasis. The flow cytometric histograms that resulted from this characterization are shown in *Figure 6*.

In the literature it is reported that CD24 represents heat stable antigen and functions as an adhesion molecule for mammary epithelial cells (4, 59). The tissue culture morphology of the 4T1 and 66cl4 cell lines is epithelial in nature, so their expression of the CD24 marker was not surprising. The culture morphology of the 4TO7 and 67NR cell lines is more mesenchymal so, while the lack of CD24 in the 67NR cells is expected, the discovery that the 4TO7s robustly express CD24 is peculiar. The discrepancy between culture morphology and surface characteristics can be potentially explained by the epithelial-

mesenchymal transition (EMT) phenomenon. Some groups have described that a mesenchymal phenotype can arise from cell lines during extended tissue culture (60, 61). Sure enough, it is reported that the 67NR cells express markers associated with EMT, such as vimentin, and do not possess epithelial junctional proteins like E-cadherin (62). The 4TO7 cell line was not examined in that study but, judging from its morphology, it is possible that this cell line also expresses markers associated with an EMT phenotype.

In a normal mammary gland, the CD61 marker represents β3 integrin and is often found on progenitor cells (4). Researchers have discovered that CD61 also represents an enriched pool of cancerous cells in the murine model, as evidenced by their increased ability to initiate tumors (63). The data from the characterization in *Figure 6* shows that not all of the cell lines express CD61, with the exception of the 4T1 line having cells that are either negative or positive for the marker. The CD61 expression profile among the cell lines is further elucidated when one considers an alternative function of the marker. In fact, CD61 is normally displayed on endothelial cells and is purported to have a role in interactions associated with blood vessels (64). Thus, the revelation that the cell lines that follow a hematogeneous route during metastasis, namely the 4T1 and 4TO7 cells, are positive for the β3 integrin may hold significance. The 66cl4s, which metastasize through the lymphatics, and the 67NRs, which do not generally disseminate from the primary tumor, are both entirely negative for the CD61 marker. Given these results, it is plausible that the 4T1 and 4TO7 cell

lines may co-opt some endothelial function to facilitate travel through blood vessels, particularly during intravasation or extravasation.

The final cell adhesion surface marker studied was CD49f, or α6 integrin. Like CD24 and CD61, CD49f is thought to mediate cell adhesion, but most specifically by acting as a receptor for laminin (4). In studying the mouse mammary gland, the Stingl lab found that mammary epithelial stem cells express high levels of this integrin, but the purification of stem cells required additional surface markers to be used in conjunction with CD49f (5). In this thesis, however, CD49f was shown to exhibit minimal selectivity as every mammary carcinoma cell line expressed high levels of the integrin. Though it was not ascertained in this thesis, it would be interesting to establish a specific advantage that high CD49f expression might provide to mammary cancer cells. Past research has shown that the knockdown of CD49f in the MCF-7 breast cancer cell line prevents mammosphere formation and reduces cell tumorigenicity (65), so it is possible that this marker aids cancer stem cell function.

With respect to the Sca-1 marker, the distinction between Sca-1(+) cells and Sca-1(-) cells involves some ambiguity, but the standard used in this thesis was a modeled after previous studies (13, 15). Based on the published approach, the static Sca-1 subpopulation within the carcinoma cell lines is indeed present but small, and difficult to ascertain, that is, with the exception of the 67NR cell line. The static Sca-1(+) population within the 67NRs is roughly 14%

of cells within the line, and this Sca-1(+) subpopulation exhibits a clearer distinction from the Sca-1(-) subpopulation when compared to the 4T1s, 66cl4s, and 4TO7s. Nevertheless, when juxtaposed with the isotype control, it is believed that each of the mammary cancer cell lines harbors Sca-1(+) tumor-initiating cells.

The last surface marker compared between the cell lines was CXCR4. Previously, researchers have correlated the size of this subpopulation to the metastatic potential of the 4T1, 4TO7, and 67NR cell lines, where the 4T1s have the most CXCR4(+) cells and the 67NRs have the least (58). This thesis was able to corroborate these findings, as the static CXCR4(+) subpopulation is ~4.5% of total cells in the 4T1s and <0.5% of the total cells in the 67NRs. Furthermore, past research also reported that growing the 4T1s in conditions that enrich for cancer stem cells also enriched for CXCR4 expression (58). Consequently, their study suggests that CXCR4(+) cells are either a unique stem-like subpopulation, or that this marker is capable of being expressed on pre-existing CSCs. Perhaps in contrast to this discovery, *Figure 7* suggests that the vast majority of Sca-1(+) cells and the CXCR4(+) cells represent distinct cell types within the 4T1 cell line. However, there was a very small fraction of dual positive cells. Though the idea that the Sca-1(+) cells represent CSCs is currently established in the literature, the nascent claim that CXCR4(+) cells are also CSCs needs confirmation. The fact that these subpopulations are both miniscule adheres to the prevailing notion that CSCs are few in number, so it is

possible that the 4T1s harbor two distinct CSC subpopulations. If corroborated, such a finding would not entirely be novel in cancer research, as researchers recently identified two distinct CSC subpopulations within oral squamous cell carcinoma lines (66). Although both subpopulations had hallmarks of CSCs, one subpopulation was found to be motile and the other was proliferative.

Moreover, the finding that Sca-1(+) cells coexpress CD61 coincides with the previous claim that the CD61(+) cancerous progenitor pool exhibits increased tumorigenicity (63). But, in that study, it is likely that the greater ability of CD61(+) cells to form tumors is mainly due to the Sca-1(+) tumor-initiating cells that reside within the CD61 subpopulation. *Figure 7* also indicates that CXCR4(+) cells are concurrently negative for CD61 expression. If CD61 is truly required for cancer cell associations with blood vessels, then one might expect the more motile CXCR4(+) cells to benefit by coexpressing the CD61 marker. In reality, however, metastasis is a highly complex event. Attempting to make concrete sense of metastatic propensities using only a few cell lines and surface markers oversimplifies the true biology behind the phenomenon.

Nevertheless, this surface marker characterization revealed that cellular subpopulations existed within the 4T1 cell line for the Sca-1, CD61, and CXCR4 markers. These markers were hence chosen for further study not only because measuring changes in surface maker expression by cancer cell subpopulations would provide a way to assess macrophage influence, but also because each of

the markers are relevant to the topic of breast cancer metastasis. In pursuit of this theme for the remainder of this thesis, it was decided that CXCR4 would broadly represent motility, Sca-1 would denote CSCs as well as tumor-initiating ability, and CD61 would identify potential blood vessel interactions.

Macrophage Influence on Subpopulation Dynamics

Conditioned media experiments were utilized as the first step in examining macrophage influence on carcinoma cells. The objective of these experiments was to determine if carcinoma cell surface marker expression could be altered by secreted factors from the macrophages. *Figure 8* indicates that conditioned media harvested from the RAW 264.7 macrophages is capable of inducing Sca-1 expression in the 4T1 cell line, but expression of the CD61 and CXCR4 markers is not affected by the treatment. Moreover, the Sca-1(+) cells that are generated by exposure to RAW 264.7-conditioned media retain their CD61(+)/CXCR4(-) phenotype as shown in *Figure 9*. The ostensible retention of the CD61(+)/CXCR4(-) phenotype in the inducible Sca-1 subpopulation implies that the newly derived Sca-1 cells may not be entirely unique per se but, rather, the product of expanding a subpopulation already present within the cell line. Taken together, this data suggests that soluble factors endogenously secreted by the RAW 264.7 macrophages are capable of expanding the Sca-1(+) subpopulation, which is reportedly endowed with CSC features and enhanced tumor forming ability (13-15), in the 4T1s.

The time frame involved in Sca-1 induction is rapid and warrants some discussion. *Figure 8* additionally shows that the Sca-1(+) subpopulation can increase just over five-fold in response to a 24-hour treatment with RAW 264.7-conditioned media. If the current dogmatic view of the cancer stem cell theory is to be accepted, the Sca-1(+) cells should only arise by symmetrical division from pre-existing Sca-1(+) cells. The finding that the Sca-1(+) subpopulation can increase from ~2% to ~16% in merely 24 hours can be used to test this model. The more traditional view of the CSC theory would require the 4T1s to complete 3 successive doublings to increase the Sca-1(+) subpopulation from 2% to 16%. The standard 4T1 doubling time is around 13 hours, and therefore the expansion of the Sca-1(+) subpopulation from 2% to 16% should theoretically need at least 39 hours to complete. Obviously, the 16% Sca-1(+) subpopulation is achieved in a much shorter time period than 39 hours after exposure to RAW 264.7-conditioned media.

Further support for this rapid Sca-1 expansion without substantial cell division comes from observations of carcinoma cells treated with the conditioned media, where these cultures often exhibited 20% less confluency than the control carcinoma cells after treatment. So, how does one reconcile this finding with the CSC theory? There are two major potential explanations. First, Sca-1(+) cells may be generated by the dedifferentiation of Sca-1(-) cells to form CSCs. If true, this would challenge the current rigid conception of the CSC theory. The second

potential explanation is that the newly generated Sca-1(+) cells do not faithfully represent CSCs, but instead they may simply be differentiated cells that have acquired the marker. While this latter explanation has some merit, it does challenge other studies that implicate Sca-1 as a marker for dedifferentiated mammary progenitor or stem cells in mice (67, 68). Future work should tease these possibilities apart.

When the ability of RAW 264.7-conditioned media to up-regulate Sca-1 expression was examined across all of the mammary cancer cell lines, it was learned that RAW 264.7-conditioned media was capable of eliciting Sca-1 expression only in the more metastatic cell lines (*Figure 10*). Even though the 67NRs are the least metastatic cells, this assay showed that, while they have a large static Sca-1(+) subpopulation, their Sca-1 expression remains non-inducible by RAW 264.7-conditioned media. Previously, it has been reported that the 67NRs are quite aggressive at the primary tumor when transplanted *in vivo* despite their ensuing lack of metastatic dissemination (53) and this may be a product of their endogenous tumor-initiating Sca-1(+) subpopulation. In regards to the 4T1 and 66cl4 cell lines, the extent to which Sca-1 induction was achieved by the conditioned media correlated positively with their metastatic potential. It is possible that, after dissemination, the larger induction of Sca-1(+) tumor-initiating cells in the 4T1s versus the 66cl4s facilitates greater metastatic colonization by the former. Also consistent with this premise, the inability of the RAW 264.7-conditioned media to induce a measurable tumor-initiating subpopulation within

the 4TO7s may correlate with their formation of non-proliferative metastatic lung nodules *in vivo*.

The revelation that the inducible Sca-1(+) subpopulation in the 4T1s achieves the same size as the static Sca-1(+) subpopulation in the 67NRs may at first seem like a substantial obstacle to drawing any correlative to metastatic potential. However, despite the sizes of these subpopulations being similar, the inducible nature of the 4T1 Sca-1(+) subpopulation implies that it is not the same as the 67NR's static Sca-1(+) subpopulation, even if both types of Sca-1(+) cells are endowed with tumor-initiating ability as the literature states (13). In addition, the mere fact that the 4T1s have a more flexible Sca-1(+) subpopulation may be a crucial requirement for metastatic success. The capacity of a cell to change its phenotype to accommodate a diverse array of conditions or selective pressures may increase the chance for survival during the variable steps of the metastatic cascade. In accordance with this idea, there remains an open question about the other phenotypes that an inducible Sca-1(+) cell might possess when it is in a Sca-1(-) state.

Given the Sca-1-evoking effect of the RAW 264.7 macrophages on the metastatic carcinoma cell lines, the next step was to determine if RAW 264.7-conditioned media also stimulated the expression of genes associated with the macrophage-carcinoma paracrine loop (*Figure 2*). The data in *Figure 11* shows that an increase in Sca-1 surface marker expression on the 4T1s does not alter

transcription levels of the CSF-1 and EGFR genes. Indeed, this trend was also observed in the RAW 264.7 cells in response to 4T1-conditioned media (*Figure 12*). Though the non-responsiveness of genes associated with the macrophage-carcinoma EGF-based paracrine loop was initially perplexing, further analysis of the RAW 264.7 macrophages indicated that 4T1-conditioned media was not sufficient to drive them into either a M1 or M2 activation state (*Figure 13*). Thus, these observations support the assumption that the RAW 264.7 cells not only constitutively produce a soluble factor that is capable of inducing Sca-1 expression in the 4T1s, but the production of this factor may be independent of the RAW 264.7 activation state.

Evidence for a cause of Sca-1 induction in the metastatic cell lines was obtained through a variety of observations associated with this thesis project. Additional work completed in this thesis indicated that Sca-1 expression was modulated by a number of different factors. Treating 4T1 cultures with HCl for 24 hours to lower the media's pH (<7.0) or exposing the cells to 1 mM hydrogen peroxide for 24 hours was sufficient to induce Sca-1 expression without cell death (data not shown). In addition, preventing cellular adherence to a tissue culture surface for two days was sufficient to induce Sca-1 expression (data not shown). In these experiments, 4T1s were grown in culture media drops that hung from the roof of a petri dish in order to harness gravity to create non-adherent spheroids. In response to these observations, it was speculated that Sca-1 induction correlated with processes that may lead to cell stress. In support

of this idea, past work completed with other mammary cancer cell lines found that there was a tight correlation between Sca-1 expression and cell stress (69). Evidence such as this is particularly applicable to studies using conditioned media, where discerned effects could be the byproduct of using culture media that has been deprived of essential nutrients and growth factors during the conditioning phase. Consistent with this possibility, a Western blot carried out on the 4T1s following incubation in RAW 264.7-conditioned media showed that the 4T1 cells down-regulated cyclin-D1 (data not shown), which is a protein required for progression through the cell cycle.

Work conducted in this thesis project also found that Sca-1 induction by RAW 264.7-conditioned media is a dose-dependent effect. It is believed that this points to a RAW 264.7 soluble factor as the main potential inducer of Sca-1 expression, as opposed to the side effects of using conditioned media. Although it should also be noted that macrophages are adept at producing inflammatory molecules and factors associated with cell stress, and the secretion of these may be responsible for inducing Sca-1 expression in the metastatic cell lines. For instance, gene expression work conducted in this thesis found that RAW 264.7 macrophages produce IL-6 as well as iNOS, the latter of which is involved in the generation of reactive oxygen species, and either of these molecules may cause carcinoma cell stress and subsequent Sca-1 induction. In support of this idea, other research has unveiled IL-6 as having a role in eliciting a cancer stem cell phenotype from MCF-7 cells (70). RAW 264.7 macrophages also produce high

levels of TGF-β mRNA and, though this thesis did not experimentally test its ability to induce Sca-1, TGF-β is known to have a substantial role in the generation of CSCs (71). Recent work conducted by a separate research group has shown that Sca-1 may interfere with anti-proliferative signaling through the TGF-β receptor, and its up-regulation by mammary cancer cells thus enables escape from anti-proliferative signals (72). Taken together, these various observations should imply that there is still much to be discovered about the factors that modulate Sca-1 activity.

The spheroid morphology of the 4T1 and 66cl4 cell lines following co-culture with the RAW 264.7 macrophages correlates with the inherent metastatic capability of the carcinoma cell lines (*Figure 14*). While the spheroids first emerge after just 24 hours of co-culture, the spheroids begin detach from the tissue culture surface or burst within the next 48 hours. Whether this is due to the cells rapidly exhausting their media, the formation of a necrotic core within the spheroid as it grows, or macrophage-directed killing of the cancer cells remains unclear. However, when spheroids are collected and transferred to a new tissue culture vessel, the cells flatten out and continue to grow with minimal cell death. Presumably, the absence of spheroid morphology after the transfer is a consequence of macrophage loss during the carry over. It is also essential to point out that 4TO7s and 67NRs are not induced into spheroids by the macrophages, which may be due to the more intrinsic mesenchymal morphology exhibited by those cell lines. Perhaps epithelial junctional proteins like E-

cadherins are required to form spheroids, and the EMT-like phenotype of the 4TO7 and 67NR cell lines prevents 3D spheroid morphology.

Growing 4T1 and RAW 264.7 co-cultures on glass coverslips permitted a more detailed examination of the spheroids. Targeting Sca-1(+) carcinoma cells or F4/80(+) macrophages with fluorescent antibodies without culture dissociation suggested that the spheroids are enriched for Sca-1 expression (*Figure 15*). Fluorescent visualization of F4/80(+) macrophages confirmed that they were localized to the spheroid surface. The finding that Sca-1 expression is enriched in the spheroids is supported by other data in this thesis. First, specific isolation of spheroids using EDTA and agitation produced the highest Sca-1 expression observed in this project; cells dissociated from four independent spheroid collections were ~41% positive for Sca-1 expression on average. This level is far higher than the Sca-1 expression found in co-cultures without enrichment. Next, inducing spheroid formation without the macrophages using the hanging drop method led to Sca-1 induction as well. These data suggest that the morphological changes associated with RAW 264.7 interaction is sufficient to up-regulate Sca-1 expression in the metastatic mammary carcinoma cell lines. This conclusion is further supported in the literature, where it was discovered that Sca-1 expression is correlated with less cellular adhesion (73). So, while Sca-1 expression can clearly be elicited by morphological cues, spheroid formation may simply be an artifact of increased Sca-1 expression.

The next set of co-culture experiments conducted on the 4T1, 4TO7 and 67NR cell lines showed that macrophage density in co-culture was positively correlated to increased Sca-1 expression in the 4T1 and 4TO7 cell lines (*Figure 16*). Interestingly, bright field microscopy photographs show that the 4T1 spheroids preferentially form in cultures that contain a high macrophage presence rather than a low macrophage presence (*Figure 16a*). Although the up-regulation of Sca-1 expression in the 4TO7s in co-culture first appears inconsistent with data from conditioned media experiments (*Figure 10*), the initial lack of detection was most likely due to limited sensitivity of the conditioned media assay. Both *Figure 10* and *Figure 16b* identifies the induced 4TO7 Sca-1(+) subpopulation as being small, and this feature presumably makes subpopulation fluctuations difficult to detect. It also remains possible that crosstalk with the RAW 264.7s is required for Sca-1 induction in the 4TO7s, which is why surface marker fluctuations were only observed in co-culture. Regardless, there is a stark difference in the induced Sca-1(+) subpopulation between the 4T1 and 4TO7 cell lines. The co-culture data also supported the initial finding that the 67NRs have a static Sca-1(+) subpopulation that remains unaffected by the macrophages. Finally, the data in these experiments confirm the original finding that macrophage factors do not govern CXCR4 expression in the 4T1, 4TO7, and 67NR cell lines, despite other research showing that the RAW 264.7s are known producers of CXCR4's cognate ligand CXCL12 (55). Granted, future experiments involving cellular crosstalk in the midst of a chemokine gradient may be fundamental to entirely rule out CXCR4 modulation

by the RAW 264.7s. The transwell assay could thus be employed to assuage lingering uncertainty.

In any case, the notion that macrophage number is related to Sca-1 expression has some *in vivo* implications and these co-culture experiments were designed with this parallel in mind. It was believed that using different densities of macrophages *in vitro* might model distinct settings *in vivo*. For example, one might expect that, at the location of the primary tumor, the cancer cells would generally be in the majority to the macrophages. This relationship might be reversed at certain primary tumor regions such as the stromal-tumor interface or at areas near blood vessels where macrophage accumulation is high. Furthermore, one might also expect to see macrophages in the majority to cancer cells during the later stages of metastasis after a cancer cell has broken away from the network of a primary tumor. With these regional differences in mind, it becomes clearer to see how Sca-1 induction may occur circumstantially *in vivo* and specifically in areas that are inundated with macrophages. Again, it appears that only the more metastatic mammary carcinoma cell lines could adjust their phenotype into a Sca-1(+) state to coincide with these regional variations.

It was next decided that if the macrophage-carcinoma paracrine loop and RAW 264.7 activation were to be seen, they would manifest themselves in co-culture. This is mainly because co-cultures allow the cell types to engage in

cellular crosstalk, which is an interplay that more accurately reflects an *in vivo* setting and provides cell types with the opportunity to educate one another. Amid this crosstalk it was learned that, while Sca-1 mRNA levels increased, the 4T1s down-regulated transcription of the potent M2 activator CSF-1, though EGFR expression remained unaltered (*Figure 17*). For the RAW 264.7 macrophages, genes implicated on their side of the paracrine loop were unaffected (*Figure 18*), but both of the M1 and M2 activation markers were expressed at higher levels (*Figure 19*).

At first, the significant increase in both anti-tumoral iNOS and pro-tumoral MMR in the RAW 264.7s following co-culture with the 4T1s may appear paradoxical. In truth, the classification of macrophage activation by tumor cells is evolving and bears many possibilities that are not solely restricted to the M1 or M2 state. The Pollard lab argues that the binary M1/M2 system oversimplifies the true spectrum of activation states held by macrophages and it is often the case that both M1 and M2 markers are concurrently displayed on a single macrophage (74). These individuals also suggest that the scientific community should forgo the M1/M2 system and instead group macrophages into the following categories using associated markers: activated, immunosuppressive, angiogenic, metastasis-associated, perivascular, and invasive. In this system, iNOS is considered a marker for the activated subtype, which is involved in pathogen destruction and inflammation, while MMR is not involved in the categorization.

Alternatively, if the M1/M2 classification system is to be accepted, then a couple of potential explanations for the results in this thesis exist. First, RAW 264.7 cells isolated from co-culture with the 4T1s may signify an activation state previously uncharacterized that involves both iNOS and MMR expression. Even if this were potentially true, RAW 264.7 macrophage expression of additional activation makers would need to be tested before this conclusion is accepted. Second, it is possible that pro-tumoral (M2) and anti-tumoral (M1) macrophages exist in co-culture as two distinct subpopulations. This is plausible given the observation that not all of the 4T1s are induced into spheroids during co-culture, and M1 versus M2 macrophages may be associated with spheroidal versus non-spheroidal 4T1s, respectively.

At any rate, the use of iNOS as marker is this thesis is far more telling of macrophage function than MMR. Since iNOS is an enzyme involved in mediating pathogen destruction by macrophages (39), its up-regulation suggests that the RAW 264.7s are attempting to destroy the 4T1s in co-culture. The MMR gene is more of a surrogate for M2 activation, as it produces a receptor that is used by macrophages for debris clearance (75), a function that is often carried out by TAM during ECM remodeling. In reality, both of the iNOS and MMR markers could have a role in 4T1 destruction, specifically where iNOS produces the reactive oxygen species needed to kill the 4T1s and MMR permits the macrophages to phagocytose the resultant cellular debris. This possibility,

coupled with the reduced CSF-1 expression by the 4T1s, lends credence to the idea that the RAW 264.7 macrophages were pushed into an anti-tumoral state in co-culture. Nonetheless, future evaluations of macrophage activation in this cell culture system should incorporate arginase as an additional marker, which is apparently more indicative of M2 activation than MMR (39).

Various results from the co-culture experiments fit well with a paradigm whereby RAW 264.7 macrophages create cellular stress in the 4T1s, which leads to Sca-1 induction and subsequent spheroid formation. Evidence produced by carcinoma cells treated with RAW 264.7-conditioned media also appears to corroborate this idea. Though general phenotypic descriptions have been drawn between the metastatic cell lines and nonmetastatic cell lines using the RAW 264.7 macrophages, the integration of these findings into a functional metastatic narrative has yet to be achieved. The generation of a functional metastatic narrative involving Sca-1 is the primary goal of the next section.

Functional Metastatic Narrative

In order to assess the functional significance of Sca-1 induction in the metastatic carcinoma cells by the macrophages, two general types of assays were performed: a proliferation assay and a motility assay. Since Sca-1(+) cells are believed to harbor an enhanced tumor-initiation ability, a series of experiments were conducted to determine if Sca-1(+) cells proliferate at a

different rate than Sca-1(-) cells. It was predicated that if Sca-1(+) cells proliferated slower, this behavior might be further associated with stem cell cycling and chemoresistance; the slower rate of division used by CSCs is thought to render them therapeutically indolent (76). Conversely, if Sca-1(+) cells were found to proliferate faster, then this behavior might be relatable to tumor aggressiveness and metastatic colony formation. But, prior to answering this question, the rate at which the 4T1s proliferate in conditioned media was sought because subsequent proliferation assays would utilize similar culture conditions. *Figure 20* indicates that conditioned media from either the 4T1s or the RAW 264.7s most significantly affects cell proliferation after 48 hours, a phenomenon likely attributable to media exhaustion. In response to this fact, proliferation assays were designed so the 4T1s would not reside in conditioned media for more than 48 hours.

The proliferation rate of the 4T1s was ascertained with EdU, a thymidine base analog that is incorporated into cells during DNA synthesis. The underlying assumption is that cells will replicate their DNA during S-phase in preparation for mitosis. Thus, the proportion of cells that take up EdU indicates the amount of cells in S-phase, which further translates to the number of cells actively dividing in culture. A longer EdU pulse serves as a control in this experiment because it exceeds the average cell cycle time for 4T1 cells and, consequently, it should provide both Sca-1(+) and Sca-1(-) subpopulations with ample opportunity to incorporate the base analog; one would expect there to be no difference in EdU

incorporation between cell types after this pulse. *Figure 21* demonstrates that, when the 4T1s are administered a 45 minute EdU pulse, more Sca-1(+) cells take up EdU than Sca-1(-) cells. As expected, there is no difference in EdU incorporation among cell subpopulations following a 16-hour pulse. This observation is the same regardless of conditioned media type used, and suggests that the 4T1 Sca-1(+) cells cycle faster than the Sca-1(-) cells.

A logical question in response to these findings would be the following: if Sca-1(+) cells proliferate faster than Sca-1(-) cells, should not the former cell type overrun and replace the latter in culture? Additional work performed in this thesis has shown that, when RAW 264.7-conditioned media is removed, the Sca-1(+) subpopulation eventually returns to a basal level. This observation suggests that the induced Sca-1(+) subpopulation is of a transient nature and dissipates after its stimulus is removed. The notion that cell types trend toward equilibrium has been observed in other research (17) and suggests that homeostatic mechanisms are present within cancerous cell lines. If Sca-1(+) cells are truly CSCs, then it is quite plausible that they undergo a differentiation program into Sca-1(-) cells over time. Alternatively, as suggested previously in this thesis, Sca-1(+) cells may not be true CSCs, and these differentiated cells may revert to a Sca-1(-) phenotype after the initial stimulus is removed.

Even if only transient, it is easy to ponder how a rapidly proliferating cancer cell might facilitate the formation of metastases. For a cancerous cell to

endure all steps in the metastatic cascade, the ability to enter a highly proliferative state could beget a huge survival advantage. The step in which such a state would be most advantageous is during the final stage in dissemination, where a metastatic cell has arrived at a new organ and attempts to establish a colony. There the metastatic cell is more vulnerable in its newfound foreign soil, being away from the protective guise of the tumor and its associated permissive microenvironment. The induction of Sca-1 and the subsequent entry into a more proliferative state could allow the metastatic cell to outcompete neighboring cell types in the new microenvironment, or potentially escape destruction by immune cells. The end result would be the successful formation of a metastatic colony.

Though it has begun to take shape, this model becomes even more clear when the motile essence of Sca-1(+) cells is considered. The exposure of 4T1s to RAW 264.7-conditioned media and then subjection to a phagokinetic fluorescent bead assay revealed that the conditioned media reduces general cellular motility (*Figure 22*). Additionally, Sca-1(+) cells tend to be less motile than Sca-1(-) cells although, when they do move, Sca-1(+) cells may migrate over longer distances at least when not treated with conditioned media (*Figure 23*). It is possible that this more motile Sca-1(+) subpopulation may represent the tiny fraction of Sca-1(+) cells that concurrently express the CXCR4 marker. But taken overall, these results bolster the presumption that Sca-1(+) cells maintain a state that favors tumor cell expansion rather than motility, and further

highlights their potential significance in that last stage of metastatic dissemination. Therefore, if the purpose of the Sca-1(+) status is to faithfully recapitulate a tumor at a distant organ by acting in colony formation, then motile features may not be necessary to achieve success. It is entirely reasonable that a more proliferative cell would be less motile, and this observation mirrors findings in the literature. For example, other researchers claim that more motile cells generated via EMT are less proliferative partly due to the cytoskeletal features that accompany the phenotypic shift (77). It is believed that the Sca-1(+) cells examined in this thesis describe the other end of the spectrum.

The tradeoff between proliferative and motile cellular states may seem convincing, but a final caveat must be acknowledged. A separate research group has determined that the 67NR cell line, although the least metastatic, actually exhibits the most motile behavior on a tissue culture surface when compared to other cell lines *in vitro* (78). This relationship is reversed, and more accurately mimics its true *in vivo* behavior, when 67NR motility is assayed in a collagen matrix. Even though the motile behavior of the 67NRs was not examined in this thesis, studies like the one mentioned here emphasize that testing motility on a 2D culture surface may not accurately model *in vivo* settings. This notion is a common critique of 2D cell culture, and there is now a prevailing trend towards using 3D culture systems to better model the *in vivo* environment (79). As a result, the discovery that Sca-1(+) cells are less motile than Sca-1(-) cells would be ideally tested *in vivo* using grafted cells. However, since

transplanted cells are difficult to analyze *in situ* and in real time, future assessments of motility might involve alternative *in vitro* conditions where cells are evaluated amid a 3D collagen matrix or in Matrigel.

CONCLUSION AND EXTRAPOLATION

Data from this thesis can be assembled into a speculative model in an attempt to explain how macrophages facilitate breast cancer metastasis. Based on initial findings, it was determined that macrophages induce Sca-1 expression only in metastatic mammary carcinoma cell lines, perhaps by inducing stress in those cell lines through anti-tumoral mechanisms. The Sca-1(+) cells that are elicited by the macrophages appear to proliferate at a higher rate but at the cost of reduced motility. This interaction between macrophages and carcinoma cells may have a particular relevance *in vivo*, especially at the end stage of metastasis. It is therefore suggested that after a metastatic cell survives in circulation and extravasates into the novel environment of the metastatic site, the Sca-1(+) phenotype may be utilized by the metastatic cells to establish a new tumor. In the model depicted in *Figure 24*, this Sca-1(+) phenotype arises when cells of the innate immune system detect and attempt to destroy the metastatic invader. By inadvertently eliciting a tumor-initiating state from the metastatic cell, macrophages may transiently provide the nascent tumor with the proliferative edge that it needs to survive immune destruction and outcompete neighboring cell types, as well as to reestablish a microenvironment that is conducive to tumor growth (*Figure 24*).

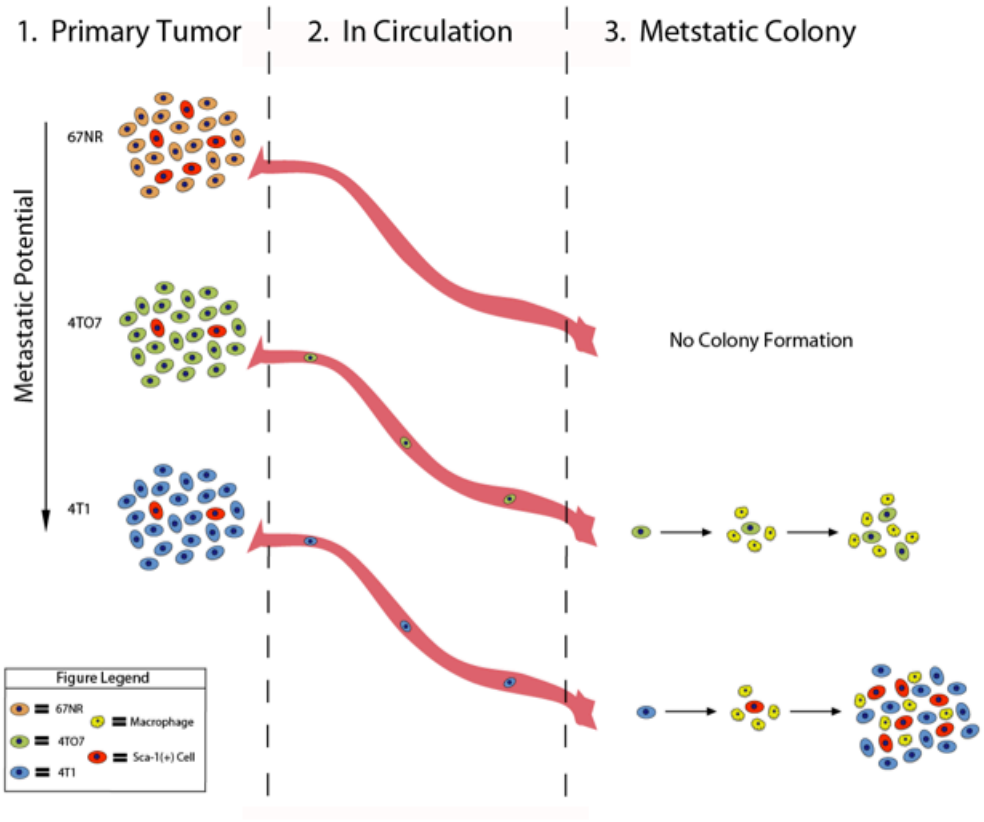

Figure 24. Macrophages may enhance metastasis by generating tumor-initiating cells. The metastatic tendencies of the mammary cancer cell lines are well documented in the literature (52). The 4T1s can efficiently complete all steps in metastasis when transplanted into mice, but the 4TO7 and 67NR cell lines exhibit metastatic shortcomings. Specifically, the 4TO7s can seed the lungs, where they form small non-proliferative nodules, and the 67NRs show a limited exodus from the primary tumor. This model integrates the metastatic nature of the cell lines with the results from this study. It is speculated that macrophages are localized to metastatic cells and, by inducing Sca-1 expression in the cancer cells, they endow the most metastatic cells with the proliferative output needed to establish a new colony.

Of the mammary cancer cell lines studied in this thesis, it is speculated that the 4T1 and 66cl4 cell lines are able to form metastatic colonies because they have an inducible Sca-1 phenotype in their repertoire. In fact, the lack of a large inducible Sca-1(+) subpopulation in the 4TO7 cell line may suggest why these cells form non-proliferative nodules after arriving at a metastatic site.

Though the 67NRs have a large static Sca-1(+) subpopulation, these cells do not disseminate from the primary tumor, so their tumor-initiating potential is not realized at a distant metastatic site. The flexible nature of the tumor-initiating subpopulation in the metastatic cell lines may act as a boon for disseminating cells. Utilizing a more motile Sca-1(-) or CXCR4(+) phenotype in the early stages of metastasis and then converting to a more proliferative Sca-1(+) phenotype in the later stages may greatly increase the potential for metastatic success by providing unique survival advantages for each stage. Clinicians should take such plasticity into account when designing therapeutic regimens to treat patients with breast cancer metastasis.

FUTURE DIRECTIONS

A core feature of this study was to produce *in vitro* data that could be efficiently corroborated *in vivo* using syngeneic mice. Thus, the overall significance of this study's *in vitro* data is incumbent upon the confirmation that, in an immune-competent tumor microenvironment, M1 activated macrophages elicit Sca-1 expression and tumor-initiating potential from metastatic mammary carcinoma cells. Preliminary data collected by a different group suggests that the realization of this goal is indeed possible, as 4T1 tumors are known to recruit a sizeable myeloid infiltrate to the primary tumor site (80). With respect to this thesis work, the Rose-Hellekant lab is currently carrying out an *in vivo* analysis using the 67NR, 4TO7, and 4T1 cell lines. In this analysis, each of the mammary carcinoma cell lines was transplanted into the cleared mammary fat pads of BALB/c mice. When the primary tumor reaches 1 cm in diameter, flow cytometric and histological evaluations will then be carried out on 67NR, 4TO7, and 4T1 primary tumors. At the primary site, the size and location of the macrophage infiltrate will be correlated to the induction of Sca-1 in the mammary cancer cells. Because the influence of macrophages on metastatic lesions is currently not well understood, metastatic 4TO7 and 4T1 lung nodules will also be examined for macrophage presence and Sca-1 expression using histological techniques.

With respect to the Sca-1(+) cells and their inherent capacity to initiate tumors, further research must resolve whether the Sca-1(+) cells generated by

macrophages faithfully represent cancer stem cells. This research project discovered that the amount of Sca-1 expressing cells could increase five-fold in a 24 hour period; this feat is not adequately explained by the more rigid premise of the cancer stem cell theory, which emphasizes that cell types follow an irreversible differentiation lineage. It is plausible that inducible Sca-1 expression either signifies cancer stem cells created by dedifferentiation, or that they are differentiated cells with a heightened tumorigenic nature. Future work involving the Sca-1 marker must better examine its relevance to the cancer stem cell story. For instance, additional markers for stem-like cells, such as aldefluor and an array of cytoskeletal elements, could be employed to examine if the endogenous Sca-1(+) subpopulation has the same features as the inducible Sca-1(+) subpopulation.

This thesis generated data that suggests the RAW 264.7 macrophages were activated in a M1 fashion and their anti-tumoral behavior may lead to increased Sca-1 expression in metastatic cancer cells. In regards to the macrophages, further teasing apart their activation status seems highly important. Though data from this project stresses macrophage M1 activation, the 4T1 cell line was found to produce basal levels of CSF-1, which is involved in promoting the pro-tumoral M2 macrophage state. Moreover, a significant increase in M2 marker expression by the RAW 264.7s was observed following co-culture with the 4T1s. Given these findings, it would be helpful to identify the specific factor that induces Sca-1 expression in the 4T1s, and discern if this

factor is unique to M1-activated macrophages. Since it is known that Sca-1 induction in the mammary cancer cells is associated with cell stress (69), candidate factors include reactive oxygen species as well as pro-inflammatory cytokines produced by M1 macrophages like IL-6 and TNFα. In addition, the demonstration that M2-activated macrophages have a reduced ability to cause Sca-1 expression in cancer cells, in conjunction with a recapitulation of the Sca-1 phenotype *in vitro* via 4T1 treatment with *ex vivo* inflammatory factors seem like crucial steps in accepting this project's major conclusions.

In the literature surrounding tumor-associated macrophages, many scientists have suggested that targeting the tumor microenvironment may be an effective approach in treating breast cancer (81). A greater understanding about how macrophage function governs carcinogenesis and metastasis should provide new and exciting ways to save patient lives. For example, if it is confirmed that M1 macrophages elicit a tumor-initiating phenotype from metastatic cancer cells *in vivo*, then perhaps the discrete removal of macrophages from the tumor microenvironment should accompany standard chemotherapeutic regimens. To test these ideas in the murine model is scientifically feasible with current technology, and the purpose of this thesis project was to lay the *in vitro* groundwork for such studies. With models like these at the scientific community's fingertips, the future of breast cancer metastasis research should be marked by both creativity and progress.

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