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Characterization of Murine Breast Cancer Cell Lines for Anti-Cancer Vaccine

An Honors Thesis submitted in partial fulfillment of the requirements for Honors Studies in Biology

By

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Biology
J. William Fulbright College of Arts and Sciences
The University of Arkansas

Acknowledgements

After three semesters at the Laboratory for Vaccine and Immunotherapy Delivery at the University of Arkansas, I have come away with a better understanding and a new appreciation for the work that must be done to discover, test, and develop cancer drugs and treatments. I am grateful to Dr. David Zaharoff, whose initial decision to let me join his lab was based on nothing but a resume and my persistence in asking for a position. I am exceedingly thankful to have had the opportunity to contribute to an exciting project and for the skills and knowledge that I have gained in your lab.

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Abstract

Breast cancer is the most commonly diagnosed cancer in women and the second leading cause of cancer death among women in the United States (1). While treatments involving radiation and chemotherapy currently exist, disease must be detected early in order for the treatments to be somewhat effective, and there is no effective treatment after metastasis occurs (2). Additionally, current therapies do not mitigate tumor immunosuppression. Decreasing the tumor-associated immunosuppressive conditions while activating antitumor immunity could prevent recurrence and metastasis, possibly leading to an effective treatment for cancer (3). Tumor cell vaccines could possibly address this issue and have become a recent topic of research. They have the potential to generate tumor regression and antitumor immune responses, but they have had low clinical response rates and poor immunogenicity so far (3, 4).

We suspect the failure of cancer vaccines to be due to the immunosuppression and heterogeneity of breast cancers. Thus, to determine how and why different breast cancers induce different levels of immunosuppression, we studied different cancer cell lines of varying levels of immunogenicity. The study included five murine breast cancer cell lines, 4T1, 4T07, 66cl4, 168FARN, and 67NR. These are sister cell lines that were isolated from a Balb/cfC3H mouse and that differ in aggressiveness and metastatic capability. The production of immunosuppressive cytokines GM-CSF, G-CSF, M-CSF, IL-6, MCP-1, TGF-β, and VEGF was quantified for each of these cell lines. We also studied the effect these cytokines have on the expansion of myeloid-derived suppressor cells (MDSCs), which are known to suppress the immune response, and found that high levels of G-CSF are correlated with high numbers of MDSCs. A correlation between G-

CSF levels and MDSC accumulation in these breast cancer cell lines could lead to future studies in which the effects of G-CSF are blocked in order to develop effective autologous breast cancer vaccines.

Introduction

I. Background

In recent years, breast cancer has become a topic of vigorous research. In the U.S., it is the most commonly diagnosed cancer in women with about 230,000 new cases reported every year (1, 5). It is also the second leading cause of cancer death among women in the U.S., affecting one in twelve women (1, 2, 6). Breast cancer patients have a five-year survival rate of 66% when the cancer is detected early. However, there is no effective cancer treatment after it metastasizes to sites such as the lungs, liver, and bones (2). About 10% to 15% of breast cancer patients develop metastases within three years of initial detection of the primary tumor, and metastases at distant sites can manifest as long as 10 years or more after the initial diagnosis (7). Metastasis is the dissociation of cancer cells from a tumor to other parts of the body. This process occurs in several steps: invasion into nearby tissues, intravasation into blood or lymphatic vessels, transport through the bloodstream or lymphatic system, arresting in small vessels, extravasation out of the vessels, growing in new tissue sites in micrometastases, and growing into larger macrometastases when the new conditions are favorable (6, 8). The high risk of metastasis makes breast cancer difficult to cure (7).

Upon diagnosis, breast cancer patients undergo primary treatment that involves surgical removal of the tumor. This is followed by chemotherapy or radiation therapy to

kill any residual cancer cells in an effort to prevent recurrence. However, metastasis differs in growth and establishment at differing sites, and each site may respond differently to treatment. Additionally, current treatments are usually unable to stop or reverse the metastasis cascade, and about 90% of breast cancer mortalities are due to cancer recurrence and metastasis (9). According to literature, such tumor recurrence could be due to immunosuppressive conditions induced by the cancer cells during their growth (3). These conditions provide a fertile environment for any residual cancer cells to grow and thrive in immunosuppressed microenvironments, resulting in recurrence or metastasis.

Decreasing the tumor-associated immunosuppressive conditions, while simultaneously activating antitumor immunity, could be an effective treatment for cancer, and it could also prevent recurrence and metastasis (3). Consequently, tumor cell vaccines have become a recent topic of research. Early clinical trials have proven that the activation of an antitumor immune response can lead to tumor regression (3, 4). However, antitumor vaccines and immunotherapies have demonstrated low clinical response rates thus far, possibly due to the immunosuppression and heterogeneity of breast cancers (3).

In a preliminary study in the Laboratory for Vaccine and Immunotherapy Delivery at the University of Arkansas, it was found that certain breast cancers induce immunosuppression more than others. If the reason for the differences in immunosuppression can be understood, one should be able to develop drugs to overcome tumor-associated immunosuppression and prevent breast cancer recurrence and metastasis. Thus, in this study, we aim to determine how and why different breast cancers

induce different levels of immunosuppression. For this purpose, we used five different murine breast cancer cell lines: 4T1, 4T07, 66cl4, 168FARN, and 67NR. These are sister cell lines that were isolated from a single Balb/cfC3H mouse and that differ in aggressiveness and metastatic capability (10). For instance, 4T1 and 66cl4 cells metastasize to the lungs, but the other three lines are not capable of completing all or part of the metastatic cascade (11). From each of these cell lines, the concentrations of immunosuppressive cytokines GM-CSF, G-CSF, M-CSF, IL-6, MCP-1, TGF-β, and VEGF were quantified. We also studied the effect these cytokines have on the expansion of myeloid-derived suppressor cells (MDSCs) in vivo, which could be resulting in tumor-associated immunosuppression (3, 4, 6, 9, 12-14).

II. Literature Review

a. Tumor Cell Lines

It is common for cell sublines from a single tumor to have heterogeneous metastatic potential (11). The murine sister cell lines used in this study were isolated from the same spontaneously arising Balb/cfC3H mammary tumor by Fred Miller at the Karmanos Cancer Institute (8, 9). They include 4T1, 4T07, 67NR, 168FARN, and 66cl4, and each of these cell lines differ in metastatic capabilities. 4T1 and 66cl4 are able to metastasize spontaneously and complete each step in the metastatic cascade, whereas 4T07, 67NR, and 168FARN are non-metastatic and are unable to complete one or more steps (8, 11).

The cell line 4T1 has the ability to metastasize hematogenously to sites such as the bones, lungs, liver, and brain and is one of a few types of breast cancer that are able to

do so effectively. It is one of the only cancers of any type that can metastasize spontaneously to the bone (2, 9). Line 66cl4 can spontaneously metastasize to the lungs and liver via the lymphatic system. The remaining cell lines are non-metastatic but still highly tumorigenic. They are unable to complete different steps of the metastasis cascade: 4T07 cells can disseminate via blood vessels and form micrometastases in the lungs but do not form visible metastases, 168FARN cells can form micrometastases in the lymph nodes but do not advance further to other sites, and 67NR cells are non-metastatic and cannot leave the primary tumor site (5, 8, 11). The metabolism of 4T1 is better able to adapt to new microenvironments than the metabolism of 67NR, which likely plays a role in 4T1's ability and 67NR's inability to metastasize (5).

The cell lines also differ in their aggressiveness. For example, cell lines 4T07 and 168 (precursor of 168FARN) are both non-metastatic from subcutaneous sites of injection (10). But when 168 and 4T07 are grown together in vivo and in vitro in monolayer, the resulting tumors consist primarily of the 4T07 line, even though line 168, when grown alone, has a faster tumor volume-doubling time than 4T07. This result occurs even when the ratio of 168 to 4T07 is 100:1 or greater. The inhibition of line 168 does not occur when 168 and 4T07 are injected on opposite sides of the mice, suggesting that growth inhibition requires cell contact or proximity. It has been suggested in some studies that 4T07 cells produce a growth-inhibitory factor that leads to the suppression of 168 cell growth (10). Additionally, line 168 is non-immunogenic (10).

Studying the differences in the immunogenicity, metastasis, and aggressiveness of these sister cell lines could assist the development of anti-cancer vaccines and

immunotherapies. For example, the selective steps of metastasis could serve as viable targets of therapeutic treatments (11).

b. Immunosuppressive Cytokines

Cytokines are secretory proteins involved in intercellular communications, specifically in the immune system. According to literature, some cytokines and their respective receptors are produced under certain pathological conditions, and it has been suggested that they are crucial to the induction, perpetuation, angiogenesis, and metastasis of breast cancer (12). Because cytokines are prominent in cancer development, they could serve as possible targets for therapeutic cancer treatments. Therefore, it is important to understand their presence and roles in different types of cancer. The cytokines observed and quantified in this study were TGF-ß, IL-6, VEGF, MCP-1, M-CSF, GM-CSF, and G-CSF.

Inflammation may play an important role in tumorigenesis. Activated immune cells and cytokine-secreting fibroblasts contribute to the inflammation of the microenvironments in which tumors can form, and cytokines help regulate tumor formation and proliferation. For example, interleukin-1 (IL-1), IL-6, IL-11, and transforming growth factor-ß (TGF-ß) stimulate the proliferation and invasion of cancer cells (12).

TGF-ß is secreted by breast cancer cells and can take on the role of either tumor suppressor or tumor promoter. In early stages of tumorigenesis, it inhibits proliferation of transformed cells. In later stages of disease, however, tumor cells are able to evade this inhibition. TGF-ß then regulates processes such as proliferation, differentiation,

migration, immunity, and apoptosis through mechanisms such as epithelial-mesenchymal transition (EMT), which promotes metastasis. It also stimulates angiogenesis by regulating the expression of other cytokines: vascular endothelial growth factor (VEGF) and monocyte chemotactic protein 1 (MCP-1). TGF-ß has been associated with aggressiveness and an earlier age of cancer onset. It has the capacity to bind to MDSCs, an action which suppresses natural killer cells and helps tumors evade the immune system (12).

IL-6 mediates humoral immunity and allergic responses (4). Like TGF-ß, however, IL-6 is also associated with aggressive cancers. It is secreted by breast cancer cells and activated fibroblasts in the breast tissue, and it initiates the proliferation and invasiveness of cancer cells. Overexpression of IL-6 further increases invasiveness and induces EMT. When secreted by tumor-infiltrating lymphocytes in large amounts, it can lead to chronic inflammation of breast cancer, which in turn can promote angiogenesis (12). It has been suggested that IL-6 is also connected to metastasis to the bone (2).

VEGF and MCP-1 both promote angiogenesis and the degradation of the extracellular matrix (5, 12). Additionally, MCP-1 attracts macrophages and induces an inflammatory response (15). Inflammation of breast cancer can lead to a higher expression of VEGF, VEGF receptors, and other proangiogenic molecules (12). According to literature, high levels of MCP-1 is also linked to tumor growth and lung micrometastases (15).

In neoplastic breast cancer cells, macrophage colony-stimulating factor (M-CSF, also known as colony-stimulating factor 1, CSF-1) expression is correlated with poor prognosis and is often associated with ipsilateral cancer recurrence. M-CSF promotes

angiogenesis, metastasis, and tumor cell invasion, and it is involved in recruiting macrophages to breast tumors (12). Tumor-associated macrophages are involved in cancer progression by promoting neoplastic transformation, immune evasion, and metastasis (16).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates the production of monocytes and granulocytes and is involved in the maturation of dendritic cells. It stimulates the T helper 1 (Th1) and T helper 2 (Th2) responses, as well as the local attraction of dendritic cells, macrophages, and granulocytes. In early trials of cytokine-secreting tumor vaccines, GM-CSF secreted by genetically engineered tumor cells was shown to induce tumor regression. The exact mechanism by which this happens is unclear, but GM-CSF may amplify the recruitment of antigen-presenting cells (APCs) to the vaccination site specifically, thereby increasing antigen presentation and stimulating a stronger immune response. But literature also shows that high systemic levels of GM-CSF, whether produced endogenously or introduced by vaccination, correlate with spontaneous metastasis and immunosuppression via MDSC expansion (4).

Granulocyte colony-stimulating factor (G-CSF) is responsible for regulating granulopoiesis, mobilizing neutrophils, and reversing neutropenia. However, it is also expressed by certain tumors, such as those of the head, neck, cervix, ovaries, breast, pancreas, bladder, and leukemia. Specifically, it is often overexpressed in ductal breast carcinomas (12, 14). Increasing tumor burden increases the levels of G-CSF, and overproduction is linked to inflammation, granulocytic MDSC accumulation, and tumor growth. The mechanism by which G-CSF causes this is unknown (13, 14). Tumor-

derived G-CSF and GM-CSF have both been linked to the development of MDSCs, but neither actually augments the immunosuppressive effects of MDSCs (14).

c. Myeloid-Derived Suppressor Cells

The accumulation of myeloid-derived suppressor cells in the blood, lymph nodes, spleen, and primary tumor site is a major mechanism of tumor progression and immune evasion and often manifests itself as splenomegaly (13, 14). MDSCs account for tumor-favoring microenvironments by suppressing innate and adaptive immunity, promoting angiogenesis, and assisting breast cancer cells in spontaneous metastasis (6, 14). They are the result of defective myelopoiesis: immature monocytes and granulocytes that fail to mature under pathological conditions such as tumor growth, trauma, infection, sepsis, and other inflammatory disorders (6, 14).

MDSC accumulation has been shown to hinder the immune system by increasing the production of reactive oxygen species (ROSs), which can suppress the T cell response (13, 17). MDSCs can also act by selectively activating enzymes in the L-arginine metabolic pathway and upregulating the production of arginase 1 to increase the metabolism of L-arginine. L-arginine supplements have been shown to inhibit tumor growth, decrease the number of MDSCs, and enhance the immune response, so increased metabolism of L-arginine understandably has the opposite effect (18). Finally, MDSCs act by impairing the cytotoxic T cell (CTL) response through upregulation of nitric oxide via inducible nitric oxide synthase (iNOS) and subsequent nitrosylation of T cell receptors (TCRs) and CD8 molecules. This inhibits T cell proliferation and leads to apoptosis (4, 6). A greater inhibition of T cells was correlated with a large number of

MDSCs in direct contact with the T cells (13). According to literature, it has also been suggested that there is a direct correlation between MDSC expansion and cancer cell aggressiveness, creating environments that promote the survival and replication of cancer cells and lead to spontaneous metastasis (6). iNOS inhibitors can reverse some of the immunosuppressive effects of MDSCs (4).

MDSCs have distinct surface markers that can be recognized when testing for MDSC accumulation: CD11b, Ly6C, and Ly6G. The monocytic MDSCs are $CD11b^{+}Ly6C^{high}$ $Ly6G^{-}$ and the granulocytic MDSCs are $CD11b^{+}Ly6C^{low}$ $Ly6G^{+}$. Up to 70 to 80% of MDSCs may be granulocytic, the accumulation of which is associated with large amounts G-CSF. Both cell types have similar immunosuppressive effects (14).

In mice, 4T1 cell tumors are known to induce MDSC expansion, possibly due to the secretion of large amounts of G-CSF and GM-CSF (9, 14). The effects of MDSCs are not as well defined in humans as they are in murine models, as most of the research has been preclinical. However, cancer patients at all stages have significantly higher numbers of MDSCs than healthy people, and the highest number of MDSCs are found in patients with large metastatic tumor burdens, suggesting that MDSC accumulation is associated with the extent of disease (3, 13). Further clinical studies of MDSCs are needed to better understand cancer immune-evasion in humans.

d. Cancer Vaccines and Immunotherapies

Immunotherapies may become a viable alternative to chemotherapy and radiation. In clinical and preclinical studies, there is evidence that T and B cells can detect antigenic differences between normal cells and transformed cancer cells. Furthermore, tumor

regression can result from the activation of an antitumor immune response (3). However, normal adaptive immunity usually is not strong enough to prevent malignant tumors because it is inhibited by the tumor microenvironment. The immune system may also become tolerant to the tumor antigen because of antigen persistence, and the tumor may evade the immune system by downregulation of major histocompatibility complex (MHC) molecules or increased expression of Fas ligand (FasL), IL-10, TGF-ß, and other immunosuppressive molecules (3). By focusing on certain immunosuppressive targets, therapies have been shown to eliminate disseminated cancer cells by activating a systemic antitumor immune response (3).

Tumor cell vaccines can be autologous or allogeneic (4). Autologous tumor cell vaccines refer to those that aim to generate a cytotoxic T cell response using tumor cells isolated from the patient, killed exogenously, and then administered to the same person they were originally isolated from. Allogeneic tumor cell vaccines refer to those that are administered to a different patient than they were isolated from.

Another promising type of vaccine is the cytokine-secreting tumor vaccine, in which the tumor microenvironment is modified using gene transfer (4, 19). In preclinical trials, tumor cells in mice were genetically engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF), which proved to provoke a potent and long-lived systemic antitumor immune response. However, it was also discovered that high levels of GM-CSF impair antitumor immunity. These high-dose vaccines fail to generate antitumor immunity, fail to expand a preexisting population of T cells, and induce the expansion of MDSCs (4). Therefore, a GM-CSF vaccine can be immunostimulatory or

immunosuppressive, depending on the dose of GM-CSF delivered. It is only effective below a certain threshold of GM-CSF, after which it loses its efficacy.

When MDSC populations are expanded, the subsequent upregulation of ROSs, arginase 1, and nitric oxide are involved in immunosuppression. The constitutive expression of iNOS is associated with angiogenesis, tumor growth, metastasis, and drug resistance. Therefore, these could serve as possible targets of therapeutic agents in future studies (4).

Because immunosuppressive cytokines and MDSCs can serve as potential therapeutic targets, a better understanding of their roles in tumor growth could facilitate the efficacy of tumor vaccines and immunotherapies. Other possible therapies could focus on treating the metastatic sites in patients with late stages of cancer (9).

III. Preliminary Data

In a preliminary study to determine if certain breast cancers induce immunosuppression and affect antitumor immunity, two murine breast cancer cell lines, 4T1 and EMT6, were used. As is shown in literature, 4T1 is a highly aggressive and metastatic breast cancer cell line whereas EMT6 is comparatively less aggressive and non-metastatic (5, 6, 8, 9, 11).

This study found that irradiated EMT6 cells provided protective immunity to mice subsequently injected with live EMT6 cells. Balb/cByJ mice were vaccinated with an autologous tumor cell vaccine (ATCV) of irradiated EMT6 or irradiated 4T1 cells and then challenged with live EMT6 or live 4T1 cells, respectively. The EMT6 vaccine

resulted in tumor free survival in 100% of the mice for 70 days, while the 4T1 vaccine exhibited tumor growth similar to the unvaccinated control group (Figure 1).

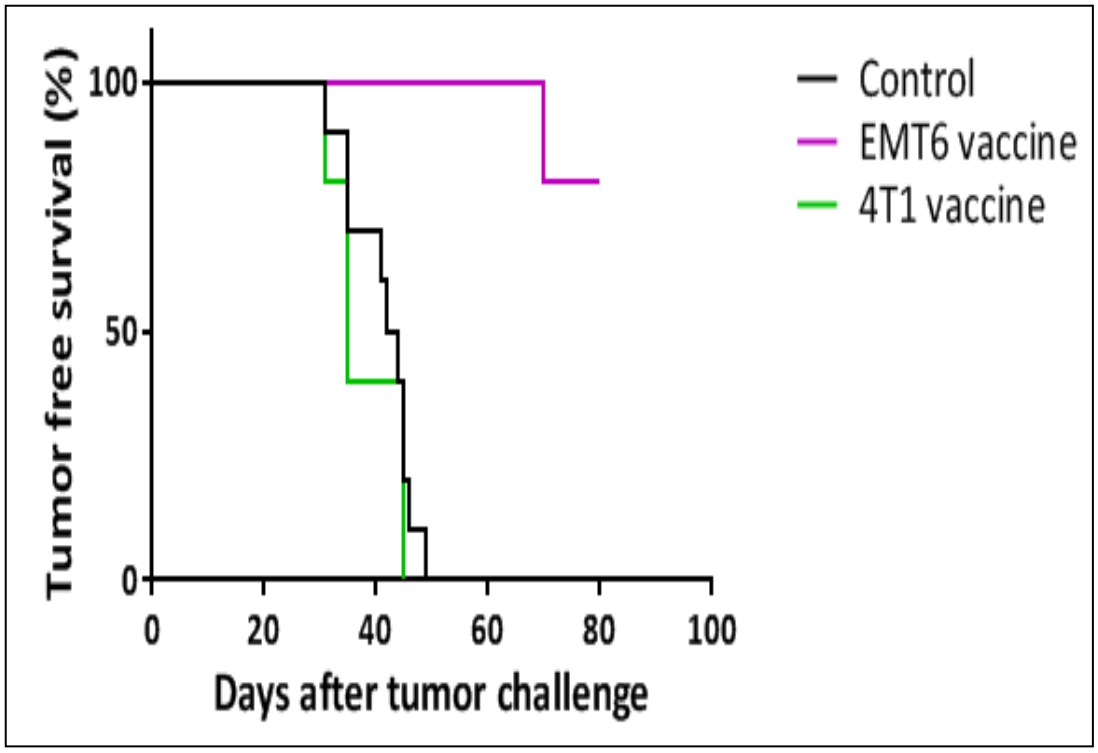

Figure 1: Irradiated EMT6 cells provide protective immunity. Balb/cByJ female mice were given priming and booster vaccination 10 days apart with irradiated 4T1 or EMT6 cells. 10 days after booster, the mice were challenged with live 4T1 or EMT6 cells and were monitored for tumor free survival over a period of 80 days. (Sruthi Ravindranathan)

When, in a related study, Balb/cByJ mice were vaccinated with irradiated EMT6 cells and challenged with live EMT6 cells, 80% of the mice rejected the live tumor challenge. However, when irradiated 4T1 cells were included, either on the same or opposite side as the EMT6 vaccine, the protective immunity reduced to 40% and 10%, respectively (Figure 2).

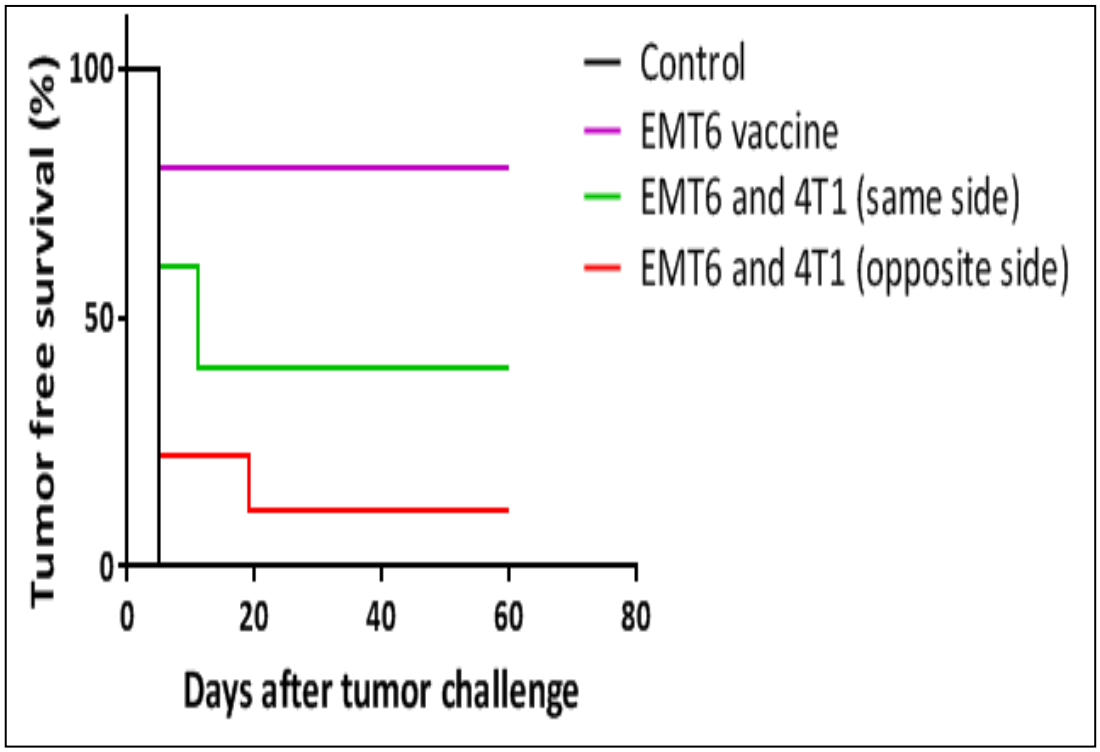

Figure 2: Irradiated 4T1 cells ruined protective immunity. Balb/cByJ female mice were given priming and booster vaccination 10 days apart with irradiated EMT6 alone or with irradiated 4T1 and EMT6 cells on the same or opposite flanks. 10 days after booster, the mice were challenged with live EMT6 cells and were monitored for tumor free survival over a period of 80 days. (Sruthi Ravindranathan)

Since the irradiated 4T1 cells abrogated the protective immunity even when injected on the opposite side, the effect is likely to be systemic rather than local. Hence, we looked at some of the immunosuppressive cytokines, namely GM-CSF, G-CSF, M-CSF, IL-6, MCP-1, TGF-β, and VEGF, produced by both 4T1 and EMT6 cells before and after irradiation (Figure 3).

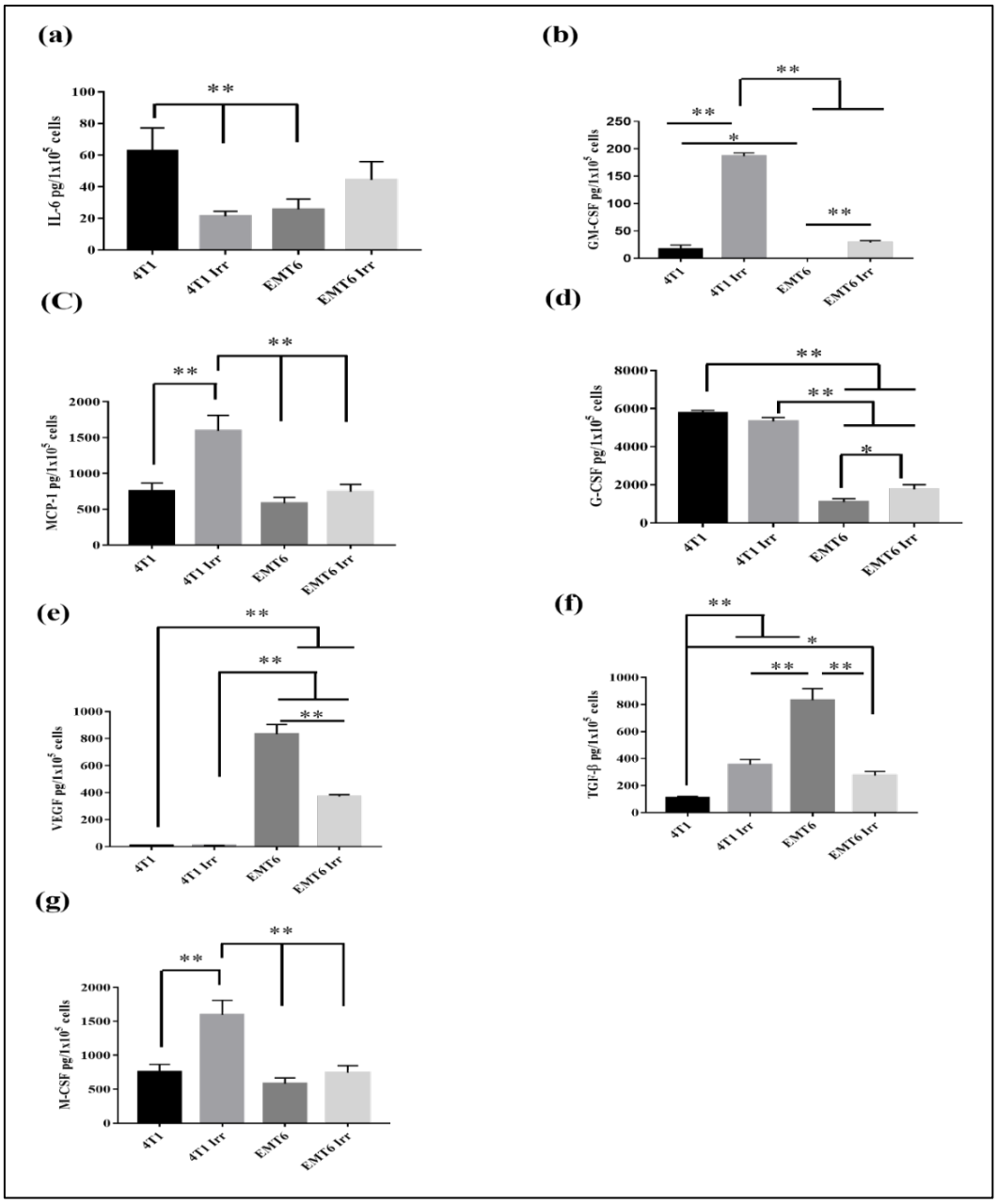

Figure 3. Cytokine release profile of 4T1 and EMT6 cells before and after irradiation. 4T1 or EMT6 cells were irradiated at 100 Gy using a Gammacell irradiator. $5x10^5$ non-irradiated (4T1 and EMT6) and irradiated (4T1 Irr and EMT6 Irr) cells were seeded on separate T25 flasks and cultured for 48 hours. The cell media was collected from each flask and centrifuged to obtain cell-free supernatants. Levels of cytokines IL-6 (a), GM-CSF (b), MCP-1 (c), and G-CSF (d) in the cell-free supernatant were measured using CBA. Cytokines M-CSF (e), VEGF (f), and TGF-β (g) were measured via ELISA. The experiment was repeated thrice and the results represent mean± standard error (**p<0.01, *p<0.05). The data were analyzed using GraphPad Prism software, version 7, and statistical differences were determined using one-way ANOVA tests followed by Tukey's tests. (Sruthi Ravindranathan)

IV. Objectives and Hypothesis

From preliminary studies, we know 4T1 and EMT6 cells are producing different levels of immunosuppressive cytokines, and 4T1 cells are able to recruit more MDSCs than EMT6 cells. It is important to note that 4T1 cells produce extremely high levels of colony stimulating factors G-CSF (both non-irradiated and irradiated) and GM-CSF (irradiated only), whereas EMT6 cells comparatively do not. The other cytokines released by 4T1 cells are at levels comparable to EMT6 cells or else very low (14). In literature, G-CSF and GM-CSF cytokines are often associated with the expansion of the immunosuppressive cells known as MDSCs (3, 4, 6, 14). Thus, the high levels of G-CSF and GM-CSF produced by 4T1 cells could be resulting in the expansion of MDSCs in the blood, peripheral lymphoid tissues, or tumor microenvironments, thereby affecting antitumor immunity and potentially limiting the effectiveness of an irradiated 4T1 cell vaccine.

Therefore, the overall objective of this study is to determine if different breast cancers produce varying levels of immunosuppressive cytokines and if there is a correlation between cytokine release and MDSC expansion in vivo. If such a correlation exists, the immunogenicity of the cancer cells will be better understood, and it could lead to the production of more effective cancer treatments, therapies, and vaccines.

We hypothesize that different breast cancers produce varying levels of different immunosuppressive cytokines. We also hypothesize that these cytokines result in varying levels of MDSC expansion in the spleens of mice, therefore decreasing the possibility of an antitumor immune response.

V. Significance

The murine breast cancer cell lines used in this study have not been characterized before. Investigating the levels of immunosuppressive cytokines produced by these cell lines will help us understand the reasons for differences in their immunogenicity. Additionally, by determining the levels of MDSC accumulation in the spleens of mice bearing the different breast cancers, a relationship between cytokine release and MDSC accumulation could be determined. If a relationship is established, knocking out genes that are responsible for producing the specific cytokine could help in the development of better autologous tumor cell vaccines in the future.

Methods and Materials

I. Tumor Cell Lines

In this study, we used five different murine breast cancer cell lines, 66cl4, 168FARN, 4T1, 4T07, and 67NR, derived from a spontaneously arising mammary tumor of the Balb/cfC3H mouse. They were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with penicillin/streptomycin, L-glutamine, and 10% fetal bovine serum (FBS). They were passaged by trypsinization and subsequent centrifugation and resuspension in complete DMEM.

II. Cytokine Studies

Cells were passaged at least once before analysis and were irradiated using gamma radiation at 100 grays using a Gammacell irradiator. Cells before and after irradiation were cultured in four T25 flasks containing approximately 500,000 cells each

with 4 milliliters of complete DMEM. The cells were counted using a hemocytometer after staining them with trypan blue: the number of cells counted in the hemocytometer was multiplied by the dilution factor, then multiplied by the volume within the hemocytometer, and finally divided by the number of grids in the hemocytometer to yield cells per milliliter of media. Media from the T25 cultures of each cell line were collected 24, 48, 72, and 96 hours after seeding. The media was then centrifuged at 1400 rotations per minute (rpm) for 5 minutes to remove any dead cells or debris from the solution. The cell supernatants were tested for cytokines G-CSF, GM-CSF, M-CSF, IL-6, TGF-β, VEGF, and MCP-1 to determine cytokine levels produced by each cell line, before and after irradiation. Cytokines IL-6, GM-CSF, MCP-1, and G-CSF were measured using cytometric bead array (CBA) while M-CSF, VEGF, and TGF-β were measured via enzyme-linked immunosorbent assay (ELISA).

a. Cytometric Bead Array

Cytokines IL-6, GM-CSF, MCP-1, and G-CSF were measured using CBA, which allows for the simultaneous quantification of multiple proteins via flow cytometry. Media from each cell line culture (irradiated and non-irradiated) collected 24, 48, 72, and 96 hours after seeding were vortexed with cytokine-specific capture beads (BD Biosciences). Each sample was also mixed with PE detection reagent beads. The samples were run through a flow cytometer in duplicate or triplicate and compared to serial dilutions of a top standard to determine the concentration of cytokine. The detection and quantification of each cytokine was determined via flow cytometric analysis performed using BD FACSCantoII.

b. Enzyme-Linked Immunosorbent Assay

Since the cytokine-specific CBA beads were only available for four of the relevant cytokines, M-CSF, VEGF, and TGF-β levels were measured using ELISA (R&D Systems, Inc. and BioLegend). ELISA was also used to measure the concentration of G-CSF in serum of naïve and tumor-bearing mice. Each ELISA kit included flat-bottomed, 96-well plates pre-coated for cytokine-specific antibodies. The M-CSF, VEGF, and G-CSF ELISA kits also included control samples that indicated the assays were working correctly when their calculated control concentrations fell within a specified range. Serial dilutions of stock solution were included to create a standard curve for each cytokine. Standards, controls, and media from each cell line culture (irradiated and non-irradiated) collected 24, 48, 72, and 96 hours after seeding were added to the 96-well plate in duplicate or triplicate. Cytokine-specific antibodies with conjugated horseradish peroxidase were subsequently added, followed by substrate solutions of tetramethylbenzidine and hydrogen peroxide and a stop solution of hydrochloric acid. A plate reader set to 450 nm and then 540 nm was used to determine the optical density of each standard, control, and sample. The readings at the two wavelengths were subtracted in order to correct for optical imperfections in the plate and to ascertain more accurate measurements. The sample values were then read off the standard curve to determine the concentrations of cytokines in picograms per milliliter of solution.

III. In Vivo Tumor Growth Studies

Each cell line was injected subcutaneously into the right flank of female Balb/cByJ mice approximately 8 weeks old, and the tumor growth was monitored. Five

mice were injected per cell line, and depending on the aggressiveness of the cells, 1, 3, or 5 million cells suspended in phosphate-buffered saline (PBS) were injected per mouse. Some cell lines did not initially exhibit tumor growth, so new mice were subsequently injected with a larger number of cells. Anesthesia of 75 mg/kg ketamine and 15 mg/kg xylazine was administered intraperitoneally immediately before cell injection.

IV. Determining the Number of MDSCs in Spleen

Tumor volumes were calculated by measuring the length (longest dimension of the tumor, y) and the width (dimension perpendicular to the length, x) and using the equation $(x^2y)/2$. When the tumor volume reached about 500 mm^3, mice were terminally bled by suborbital bleeding. The mice were then sacrificed by cervical dislocation and the spleens were harvested. Splenocytes were then isolated by mechanical dissociation and centrifugation and treated with ammonium-chloride-potassium (ACK) lysis buffer to remove red blood cells. They were resuspended in Roswell Park Memorial Institute (RPMI) medium or PBS. The splenocytes were surface stained with fluorochrome-conjugated monoclonal antibodies (mAbs) against CD11b and Ly6G/Ly6C (MDSC surface markers), and the percentage of MDSCs was determined via flow cytometry. In this study, FITC Rat IgG2b,k and PE Rat IgG2B,k were used as isotype controls. Cells were surface stained with PE Rat Anti-Mouse Ly6G/Ly6C and FITC Rat Anti-Mouse CD11b, or APC-Cy7 Rat Anti-Mouse CD11b.

V. Serum Studies

When the tumor reached 500 mm^3, about 500 µl of blood was collected from the mouse via suborbital bleeding. The blood was allowed to be at room temperature for 10 minutes and then was centrifuged at 4600 rpm for 30 minutes. Serum was collected and stored at -80˚C until ready for quantification of G-CSF levels via ELISA (R&D Systems, Inc.). Each sample was diluted 1:50 with an ELISA calibrator diluent.

VI. Statistical Analysis

All data were analyzed using GraphPad Prism software, version 7. In this study, we were mostly interested in G-CSF, which was released in comparatively higher levels than other cytokines in aggressive cell types 4T1 and 4T07. Statistical differences were determined using one-way Analysis of Variance (ANOVA) tests followed by Tukey's tests for Figures 5, 7, and 8. Probability values (p-vales) of less than 0.05 were considered significant and are indicated on graphs by one asterisk. Two asterisks indicate p-values of less than 0.01.

Results and Discussion

Of the five cell lines, 67NR and 168FARN did not produce any G-CSF in the cytokine studies. However, 4T1 produced 4606 pg/10^5 cells and 4158 pg/10^5 cells before and after irradiation, respectively, after 48 hours of incubation. The cell line 4T07 produced comparatively less at 2695 pg/10^5 cells and 2631 pg/10^5 cells, and 66cl4 produced only 61 pg/10^5 cells and 60 pg/10^5 cells (Figure 4).

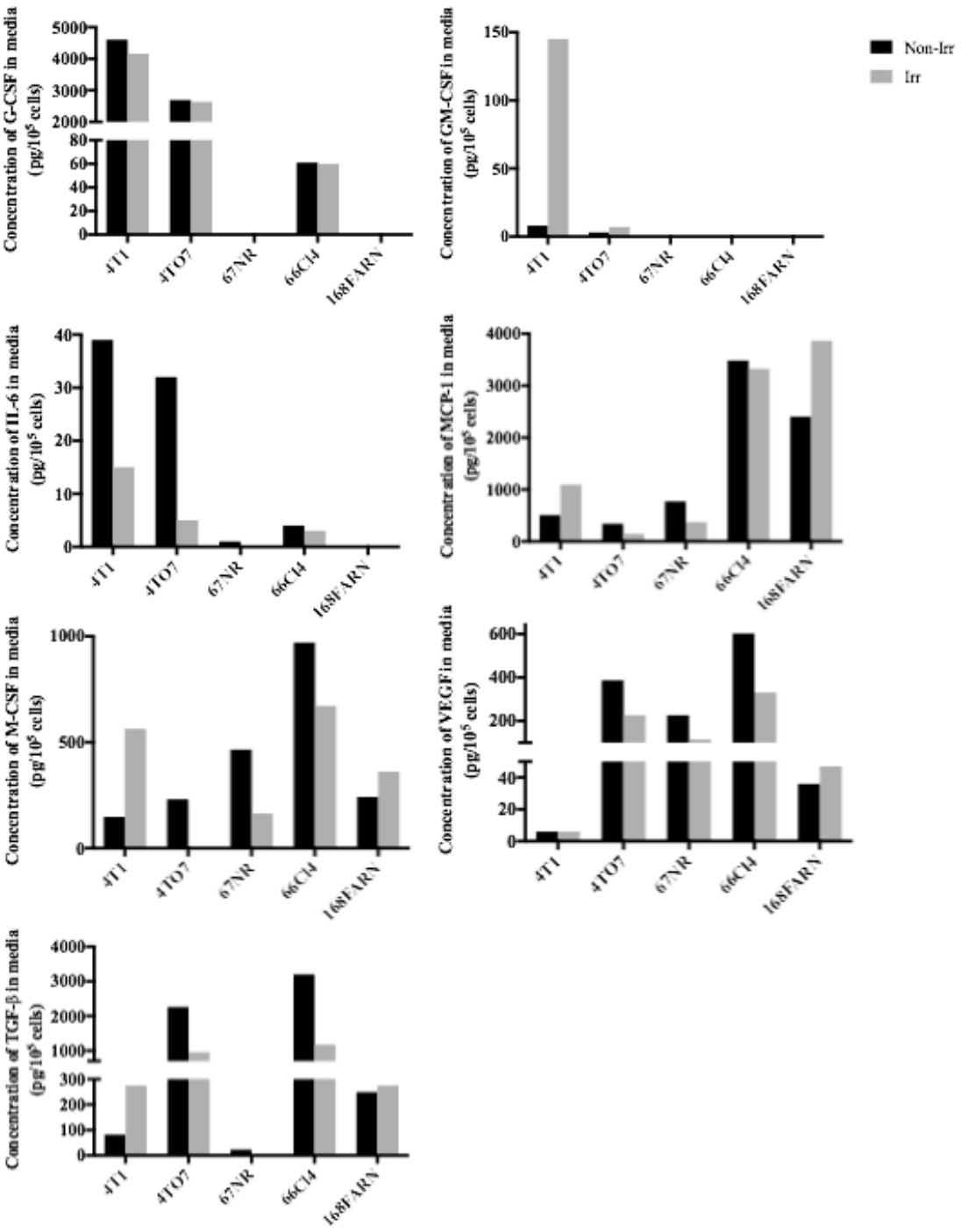

Figure 4: Cytokine release profile of 4T1, 4T07, 67NR, 66Cl4, and 168FARN before and after irradiation. Cells were irradiated at 100 Gy using a Gammacell irradiator. $5x10^5$ non-irradiated and irradiated cells were seeded on separate T25 flasks and cultured for 48 hours. The cell media was collected from each flask and centrifuged to obtain cell-free supernatants. Levels of IL-6, GM-CSF, MCP-1, and G-CSF were measured using CBA, and levels of M-CSF, VEGF, and TGF-β were measured via ELISA. The experiment was performed once and the results represent mean.

Though some cytokines were produced at higher levels by certain cell lines more than others, we focused our attention on G-CSF due to the extremely high concentrations released by the aggressive 4T1 cells, both irradiated and non-irradiated. In the in vivo tumor growth studies, each spleen was harvested and weighed to see if there was a correlation between the levels of G-CSF, the spleen mass, and the number of MDSCs in the spleen. From the data, it is evident that the mice bearing 4T1 and 4T07 tumors had significantly larger spleens, weighing an average of 350 mg and 658 mg, respectively. The other tumor types all displayed similar spleen sizes: 67NR was 104 mg, 66cl4 was 95 mg, and 168FARN was 124 mg (Figure 5).

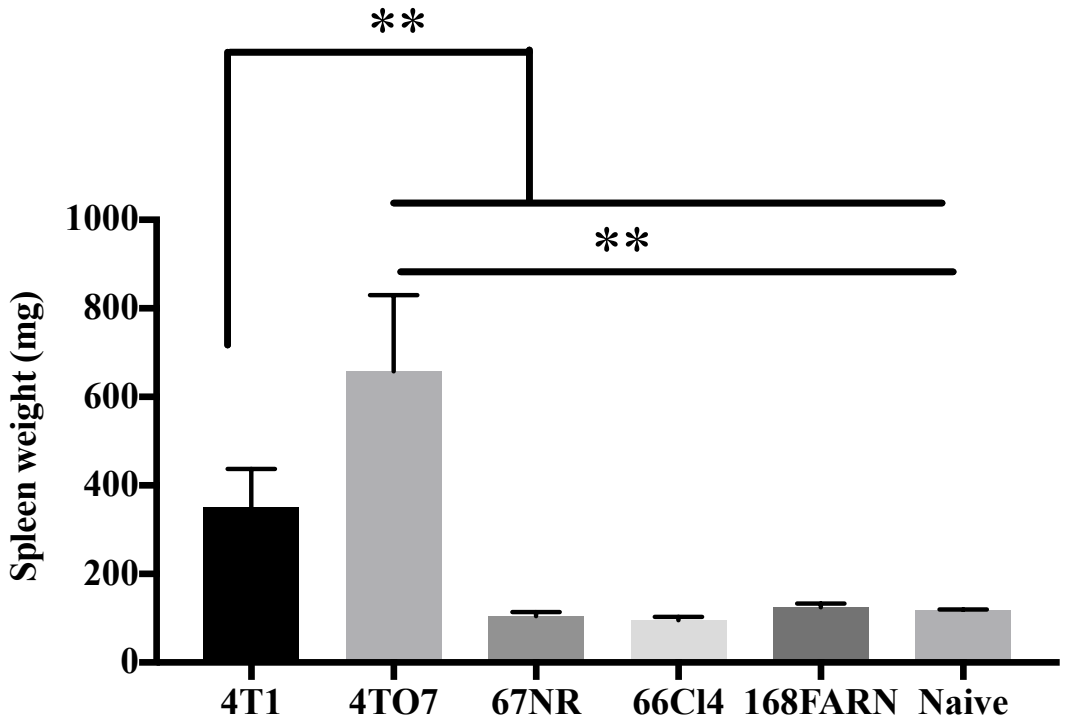

Figure 5: Average spleen masses of mice inoculated with 4T1, 4T07, 67NR, 66Cl4, and 168FARN. Mice were injected subcutaneously with 1, 3, or 5 million cells. When the tumors reached about 500 mm^3, the mice were sacrificed and their spleens were harvested. The results represent mean± standard error (**p<0.01, *p<0.05).

The splenocytes were isolated mechanically, and the number of MDSCs was quantified via flow cytometry. Dead cells and debris were removed via forward scatter and protein-binding fixable viability stain performed by FlowJo. MDSCs in quadrant 3 were gated as live cells and subsequently analyzed for CD11b and Ly6C/Ly6G. The gating strategy is shown in Figure 6.

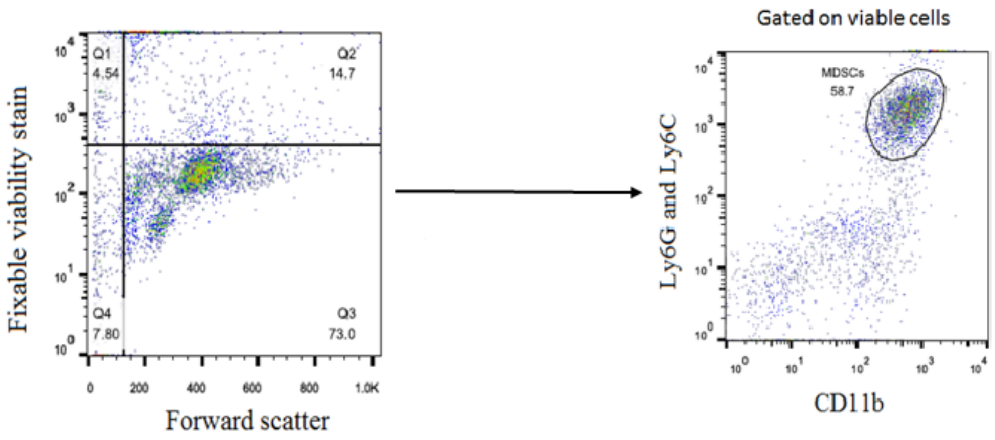

Figure 6: Gating strategy for MDSCs. Balb/cByJ mouse were subcutaneously injected with 1, 3, or 5 million cells and the spleen was harvested when the tumor volume reached about 500 mm^3. Splenocytes were isolated and stained with fluorochrome-conjugated mAbs against CD11b, Ly6G, and Ly6C, then were analyzed using flow cytometry. FlowJo software was used to gate for live MDSCs (CD11b+ and Ly6G/Ly6C+).

Mice bearing 4T1 and 4T07 tumors also had significantly high numbers of MDSCs. The spleens of 4T1 tumor-bearing mice contained about $1.3x10^8$ MDSCs (34% of the total number of splenocytes), and the spleens of 4T07 tumor-bearing mice contained about $1.9x10^8$ MDSCs (33% of the total number of splenocytes). Since these cell lines were found to produce high levels of G-CSF, we suspect a direct correlation. The percentages of MDSCs in the spleens of 67NR, 66cl4, and 168FARN tumor-bearing mice were 5%, 6%, and 7%, respectively (Figure 7).

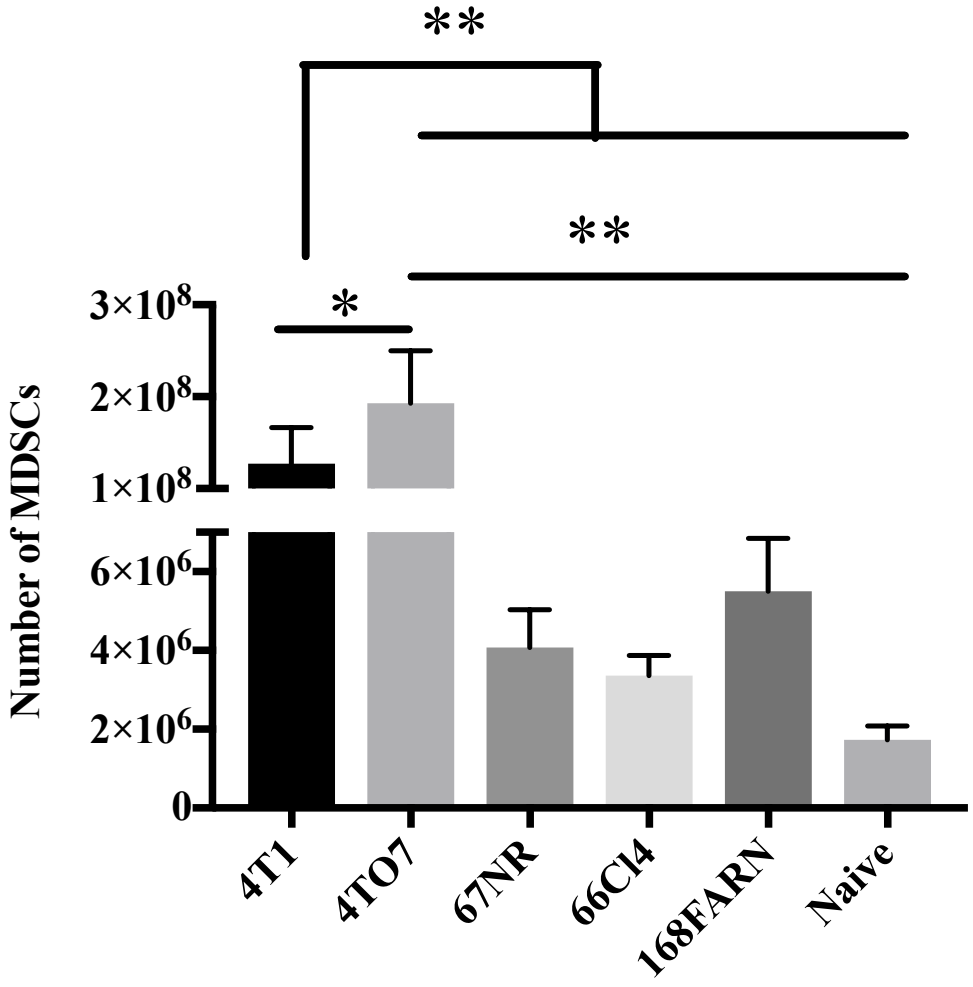

Figure 7: Average number of MDSCs in spleens of mice inoculated with 4T1, 4T07, 67NR, 66Cl4, and 168FARN. Mice were sacrificed and their spleens were harvested when the tumors reached about 500 mm^3. Splenocytes were isolated via mechanical dissociation and ACK lysis buffer, and then they were surface stained for Ly6G/Ly6C and CD11b using PE Rat Anti-Mouse Ly6G/Ly6C and FITC Rat Anti-Mouse CD11b, or APC-Cy7 Rat Anti-Mouse CD11b. MDSCs were detected and quantified using flow cytometry. The results represent mean± standard error (**$p<0.01$, *$p<0.05$).

The levels of G-CSF in the blood serum of tumor-bearing mice were also quantified. The serum of mice bearing 4T1 tumors contained an average of 19,119 pg/ml G-CSF, and the serum of 4T07 tumor-bearing mice contained 17,635 pg/ml. The mice with the other three tumor types had significantly less G-CSF present in the blood serum (67NR had 165 pg/ml, 66cl4 had 117 pg/ml, and 168FARN had 40 pg/ml). This data is

consistent with the amounts of G-CSF released by each cell line in the cytokine studies, and we suspect direct correlation between the tumor cell type and the concentration of G-CSF in the blood serum (Figure 8).

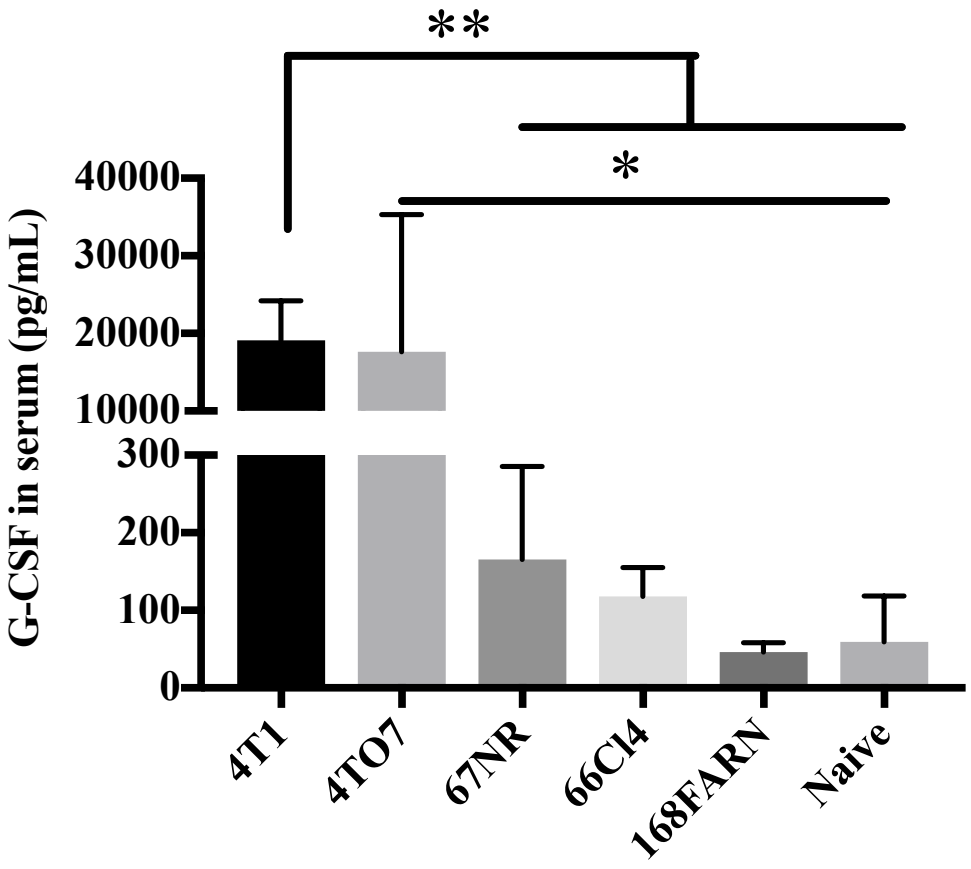

Figure 8: Serum cytokine release profile of 4T1, 4T07, 67NR, 66Cl4, and 168FARN in vivo. When the tumor reached about 500 mm^3, about 500 µl of blood was collected from the mouse via suborbital bleeding. The blood clotted at room temperature and then was centrifuged at 4600 rpm for 30 minutes. Serum was collected from the supernatant and analyzed for G-CSF via ELISA. The results represent mean± standard error (**$p<0.01$, *$p<0.05$).

These findings suggest that the cytokine G-CSF may be a key factor in cancer cell immunogenicity. Cell line 4T1 produced high amounts of G-CSF and demonstrated high MDSC accumulation in murine spleens. This agrees with literature suggesting that the

overproduction of G-CSF is linked to MDSC expansion and immunosuppressive effects (13, 14). The high levels of G-CSF and MDSC accumulation in 4T07 also agree with previous findings that G-CSF is linked to immunogenicity: although unable to complete the metastatic cascade, the aggressive 4T07 cell line can form micrometastases in the lungs and is also highly tumorigenic (5, 8, 10, 11). However, metastatic cell line 66cl4 and tumorigenic cell lines 168FARN and 67NR demonstrated low levels of G-CSF and few MDSCs. This suggests that there are other key factors involved in the metastasis and tumorigenicity of these cell lines (8, 11).

Immunotherapies have demonstrated the potential to activate antitumor immune responses and eliminate disseminated cancer cells (3). Because G-CSF appears to be strongly correlated with the immunogenicity of cancers, it could serve as a potential focus of future tumor cell vaccines. By eliminating the genes responsible for producing G-CSF, thereby blocking the effects of the cytokine, an autologous tumor cell vaccine could be created. The vaccine would, presumably, induce a cytotoxic T cell response against the growing tumor cells without providing an additional source of G-CSF (4). Additionally, since G-CSF correlates with the accumulation of MDSCs and subsequent immunosuppression, the effects of MDSC expansion could also be targeted by future immunotherapies. Possible targets of MDSC expansion include L-arginine metabolism as well as nitric oxide, iNOS, and ROS expression (4, 6, 13, 14).

Conclusion

G-CSF may play a significant role in the aggressiveness and metastatic capabilities of cancers. Of the five cell lines, 67NR and 168FARN did not produce any

G-CSF before or after irradiation. The aggressive 4T1 cells, however, produced comparatively more G-CSF than the other cell lines. Therefore, although certain cell types produced other cytokines at high levels, G-CSF became the focus of the study. After the spleens of tumor-bearing mice were harvested, it became evident that the mice bearing 4T1 and 4T07 tumors had significantly larger spleens than the mice bearing other tumor types. The 4T1 and 4T07 mice also had significantly higher numbers of MDSCs present in the spleens, as well as higher concentrations of G-CSF in the blood serum. Since the 4T1 and 4T07 cells were also found to produce high levels of G-CSF, we suspect a direct correlation between G-CSF production, spleen mass, and number of MDSCs present in the spleen. According to literature, MDSC expansion is linked to immunosuppression (13). Therefore, high G-CSF levels could be affecting antitumor immunity and limiting the effectiveness of anti-cancer treatments and vaccinations.

Blocking the effects of G-CSF, either by removing its coding genes or blocking downstream effects, could lead to better autologous tumor cell vaccines in the future. In order to block the downstream effects of G-CSF, more research may need to be done on the mechanism by which it leads to MDSC expansion. In addition, further study of G-CSF concentration in the serum of tumor-bearing mice could provide more insight into the immunogenicity of different types of breast cancer. Continued research of the 4T1, 4T07, 66cl4, 67NR, and 168FARN cell lines could lead to a better understanding of their immunologic differences and facilitate the production of more effective cancer treatments, therapies, and vaccines.

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