Macrophage Depletion at Early Murine Mammary Carcinoma Sites

Honors Thesis

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Biographical Sketch

Born and raised in Allendale, New Jersey, Amanda Lauricella attended Lafayette College in Easton, Pennsylvania to earn her undergraduate degrees, a Bachelor of Science in Biology and a Bachelor of Arts in Anthropology and Sociology. Amanda began research in immunology the summer going into her second year of college where she worked alongside Dr. Robert Kurt to study the innate immune response to murine mammary carcinoma. Outside of academia, Amanda works as an Emergency Medical Technician (EMT) in the sports medicine department at Lafayette, acted as Vice President of Risk Management for her sorority Pi Beta Phi during the COVID-19 pandemic and worked as a Teaching Assistant in General Biology and Molecular Genetics labs. In her free time, she also enjoys staying active through CrossFit as well as intramural sports such as soccer, floor hockey and flag football. Amanda's passion for immunology grew when she took Dr. Kurt's immunology course the spring semester of her junior year which encouraged her to pursue a career in healthcare. After graduation, Amanda will be earning a Master of Science in Physician Assistant Studies at Seton Hall University. Amanda hopes to work in Emergency Medicine in medically underserved areas in order to bridge healthcare disparities in the United States. Someday, Amanda would like to earn a Doctor of Medical Sciences to allow her to work on the board of a hospital and increase healthcare accessibility.

Abstract

It is known that macrophages play an essential role in the tumor microenvironment and are part of the innate immune response in patients with breast cancer as well as in murine mammary carcinoma. It is widely accepted that under certain conditions, macrophages that infiltrate the site during the later stages of tumor growth can either suppress or enable tumor growth and our lab has been working towards determining the conditions under which macrophages end up helping or inhibiting tumor growth. What we do not know, however, is whether macrophages at early tumor sites impact the growth of the tumor. In order to address this question, the primary goals of my project were to: validate a protocol to deplete macrophages at early tumor sites and determine whether macrophages at early murine mammary carcinoma sites contribute to tumor progression. In order to address these questions, I delivered 4T1 tumor cells to mice depleted of macrophages using clodronate liposomes. I found the clodronate liposomes effectively depleted F480+ cells as early as 24 hours after tumor delivery and for up to three weeks but did not impact the number of Ly6G+ cells suggesting the liposomes selectively depleted macrophages but not neutrophils. The macrophage depleted mice had smaller tumor volumes, smaller spleens and fewer lung metastasis than the control mice suggesting the presence of macrophages contributed to tumor growth and disease progression. Collectively, these data suggest that the macrophages present in the early tumor microenvironment exhibit M2 TAM characteristics by promoting tumor growth.

Background and Significance

In 2019, breast cancer impacted 270,000 patients in the United States taking the lives of 40,000 females and 500 males (Cancer facts & Figures 2019). Today, breast cancer is the leading diagnosed cancer type in females representing about 30% of all diagnosed cancers, and the second leading cause of cancer related deaths, at about 15% (Cancer facts & Figures 2019). The prevalence of breast cancer also continues to increase overtime. Between 2006 and 2015 the incidence of breast cancer in the United States increased by 0.4% and continues to rise (Cancer facts & Figures 2019). Furthermore, most patients with breast cancer have no family history of the disease making it particularly challenging to diagnose a patient in the early stages of the disease (Cancer facts & Figures 2019). Consequently, it is decidedly important to improve breast cancer diagnosis and treatments in order to improve patient prognosis. As of today, there is a great deal of research geared towards cancer progression and behavior in the later stages of the disease. Meanwhile, very little is known about what happens during the early stages of disease, including the function of macrophages in the first few days and weeks of breast cancer development.

When a patient is diagnosed with breast cancer their condition is classified as a certain stage of cancer based on disease progression which often determines a prognosis. A stage number is determined using three clinical characteristics which includes the size of the tumor and whether it has spread to nearby tissue, if it is in the lymph nodes, and if the cancer has metastasized to other parts of the body. Stage 0 is the first classification of breast cancer; it is used to describe a non-invasive small tumor in the breast that can typically be removed surgically and is associated with a fairly favorable prognosis (Wojciechowski 2020). If the cancer goes unnoticed and untreated it may progress to Stage I breast cancer where small clusters of cancer cells might be detected in nearby lymph nodes (Wojciechowski 2020). Stage II and Stage III involves extensive growth of the tumor and spread of the cancer into nearby lymph nodes, skin and chest wall (Wojciechowski 2020). Lastly, Stage IV breast cancer involves significant metastasis into other tissues in the body making it difficult to treat (Wojciechowski 2020). Consequently, patients with Stage IV disease have a poor prognosis; the 5-year survival rate for a patient with Stage IV Breast Cancer is about 20% (Wojciechowski 2020).

Macrophages in Breast Cancer

Macrophages, a specific type of white blood cell, can phagocytose pathogens as well as our own cells if they are abnormal or dying, and they also regulate lymphocyte activation and proliferation (Epelman et al. 2014). Macrophages play a significant role in fighting cancer by acting as phagocytes and antigen-presenting cells. As phagocytes, they perform phagocytosis, a specific type of endocytosis that can be used to remove and destroy threats to the body. In the context of cancer, the pattern recognition receptors (PRRs) on the surface of the macrophage bind to damage-associated molecular patterns (DAMPS) on the surface of cancer cells, causing the macrophage to engulf the cancer cells. As antigen-presenting cells, macrophages also activate T-cells that can then directly kill cancer cells (Yang et al. 2019). However, there are different types of macrophages and not all macrophages help to fight cancer.

Tumor-associated macrophages (TAMs) originate as monocytes in the bone marrow and are recruited through inflammatory signals that are released by cancer cells in the primary and metastatic tumor sites. Following recruitment these cells begin to differentiate into two common classifications of TAMs based on their functional response to cancer (Cassetta and Pollard 2018). M1 and M2 macrophages each express specific markers and exhibit differing functions. M1 macrophages are known to be pro-inflammatory by releasing cytokines like IL-6, IL-12 and TNF-alpha and are cytotoxic resulting in anti-tumor effects, whereas M2 macrophages are considered pro-tumor because they typically suppress inflammation, contribute to tumor progression and secrete anti-inflammatory cytokines such as TGF-beta and IL-10. (Ostuni et al. 2015). Because of these differing functions, TAM levels can be used as a predictor for the metastatic potential of human cancers and patient prognosis (Dandekar et al. 2011). Higher TAM levels are usually linked to a good prognosis in colon and stomach cancers but a poor prognosis in breast and ovarian cancers. In patients with primary invasive breast cancer, high infiltration of M2 TAMs has been associated with unfavorable clinicopathological components such as increased tumor cell proliferation and angiogenesis, as well as poor patient survival (Qiu 2018). Breast cancer cells have also been found to produce colony-stimulating factor (CSF-1) and CCL2 (a cytokine), both of which attract M2 macrophages that promote tumor growth and contribute to cancer cell proliferation (Mukhtar et al. 2011). The presence of intra-epithelial macrophages has also been correlated with the downregulation of E-cadherin, a tumor suppressor gene essential for cell-cell adhesion in epithelial tissues, which has been associated with progression to Stage IV disease by enhancing invasion and metastasis. This suggests macrophages may stimulate the loss of E-cadherin junctions, resulting in enhanced tumor growth and metastasis. Another way macrophages may contribute to tumor progression is by subverting the inflammatory response and recruiting suppressor cells which are lymphocytes responsible for suppressing antibody production, and inflammatory responses (Madera et al. 2015). These previous studies provide sufficient evidence that macrophages can contribute to breast cancer progression in several different ways. However, these studies have largely focused on macrophages at later stages of tumor growth and it remains to be determined whether macrophages at early tumor sites also contribute to tumor progression. Since macrophages are the most abundant white blood cell in the early stages of breast cancer, it is important to determine what function TAMs are exhibiting at the early tumor sites (Malfitano et al. 2020). If I find that macrophages at early tumor sites contribute to tumor growth, then my findings may provide information that could be used to design earlier treatment options possibly with the potential to prevent progression to Stage IV disease.

In the past, our laboratory has explored the immune environment associated with early murine mammary carcinoma sites. It has been discovered that macrophages and neutrophils are the primary immune cells infiltrating this site. There is an increased number of macrophages at the tumor site as early as 48 hours after introduction of 4T1 and EMT6 tumor cells when compared to controls (Zhang et al. 2019). Macrophages have also been observed at the early tumor sites in patients with breast cancer, usually in the areas of invasion during the development of early-stage cancer (Dandekar et al. 2011). This suggests that TAMs might be involved in the invasion of tumor cells (Dandekar et al. 2011). Based on all of this information, I want to further explore whether macrophages infiltrating the tumor site play a significant role in the growth or suppression of the tumor in the early stages of this disease.

Research Applications

Current treatments of breast cancer usually consist of a lumpectomy, mastectomy, radiation therapy, chemotherapy, anti-estrogen hormone therapy or targeted therapy (Cancer facts & Figures 2019). Unfortunately, these treatment options are not always successful, resulting in the need to explore alternative options. Immunotherapy is a contemporary cancer treatment option that includes the transfer of *ex vivo* activated CD8+ T cells and the administration of cytokines that activate CD8+ T cells, which eliminate cancer cells. However, most patients do not fully respond to these two types of immunotherapy and the overall outcome is often not significantly improved. One major barrier to improving success of immunotherapy is removing aspects of the defense system that promote tumor growth. Therefore, it is believed that a better understanding of tumor suppressing and tumor promoting cells is imperative to establish more efficient cancer immunotherapies.

Given their abundance in the tumor microenvironment, a better understanding of the involvement of TAMs in cancer progression may be necessary to enhance the effectiveness of immunotherapy. For instance, TAMs have been shown to have suppressive effects on immunotherapy, meaning it may be necessary to combine immunotherapy treatments with TAM interruption (Cassetta and Kitamura 2018). *Cassetta and Kitamura (2018)* found that various TAM targeting strategies such as TAM depletion, TAM reprogramming and targeting functional molecules of TAM can enhance immunotherapy using immune checkpoint inhibition. This therapeutic approach may be beneficial in treating certain types of cancers, including breast cancer. This means it is necessary to understand the role macrophages are playing at all stages of cancer progression to properly target these cells. In general, a high infiltration of macrophages in most solid tumors is correlated with a poor prognosis and chemotherapy resistance (Cassetta and Pollard 2018). It has been shown in mouse models that macrophages stimulate angiogenesis, increase tumor cell migration and suppress antitumor immunity to promote initiation of the cancer and aid in malignant progression (Cassetta and Pollard 2018). Additionally, it is believed that the premature recruitment of macrophages due to overexpression of CSF-1in mammary tumors promotes the transition from a benign to a malignant tumor (Cassetta and Pollard 2018). This transition is further enhanced by macrophages as they produce angiogenetic factors, secrete growth factors and produce proteases that facilitates tumor progression (Cassetta and Pollard 2018). Because of this, it is conceivable that targeting TAMs during various stages of cancer progression may improve patient outcomes.

Blocking colony-stimulating factor 1 receptor (CSF-1R) is a strategy commonly used to target tumor associated macrophages (Cassetta and Kitamura 2018). CSF-1 is responsible for the recruitment, differentiation, and survival of TAMs (Cassetta and Kitamura 2018). Using mouse models of breast cancer, it was found that the inhibition of CSF-1R with the use of antibodies or small molecule inhibitors results in a decrease in TAM infiltration or interruption of TAM development and progression in the late stages of breast cancer (Cassetta and Kitamura 2018). This blockage of CSF-1R results in the limitation of tumor growth by about 50% (Cassetta and Kitamura 2018). Although targeting TAMs in advanced cancers has been shown to be successful in decreasing tumor growth, it is still unknown if targeting TAMs in the early stages of cancer progression would also inhibit the advancement of breast cancer. As a result, my project focused on targeting macrophages at early tumor sites and determining whether this impacts disease progression.

Hypothesis and Aims

It is known that macrophages play an essential role in the tumor microenvironment and are part of the innate immune response in patients with breast cancer as well as in murine mammary carcinoma. It is widely accepted that under certain conditions, macrophages that infiltrate the site during the later stages of tumor development can either suppress or enable tumor growth. Through this research, I sought to discover whether macrophages at early tumor sites impacted the growth of the tumor. In order to address this question, the primary goals of my project were as follows:

Aim 1: **Validate a protocol to deplete macrophages at early murine mammary carcinoma sites.**

Aim 2: **Determine whether macrophages at early murine mammary carcinoma sites contribute to tumor progression.**

Previous research showed that a high infiltration of macrophages was correlated with poor prognosis in patients with breast cancer. More macrophages is often linked to enhanced tumor cell growth and proliferation as well as metastasis and angiogenesis. Therefore, **the hypothesis of my study was that macrophages at early stages of breast cancer also contribute to tumor progression.** In order to address this hypothesis, I used 4T1 tumor cells, an aggressive triple-negative murine mammary carcinoma model which is often used as a model for Stage IV breast cancer. I delivered 4T1 tumor cells in mice. In one set of mice, I depleted macrophages at the early murine mammary carcinoma site using anionic clodronate liposomes (Figure 1), another set of mice received control liposomes which do not deplete macrophages. I measured growth of the tumor over the course of three weeks in order to determine whether mammary tumors grew faster or slower without macrophages present in the early stages of development. At the end of the three weeks I removed the tumors to determine the number of macrophages present at the tumor site. In addition to removing the tumors I extracted the lungs from the mice and quantitated the number of lung metastases present since lung metastasis is an indication of Stage IV breast cancer in humans (Pietrangelo 2020). In addition, I harvested the spleens from the mice, measured the size and determined the number of splenocytes present since an enlarged spleen is also common at late-stage disease (Dunn and Goldwein 1975). I believed that macrophages present at the early tumor sites would foster tumor growth and therefore I expected to find smaller tumors, fewer lung metastases, and smaller spleens when macrophage were absent at the early tumor sites.

Materials and Methods:

Mice and Cells

4T1 cells were maintained in RPMI 1640 (supplemented with 10% heat-inactivated fetal bovine serum, VWR LifeScience Sanborn, NY), glutamine (2mM, VWR LifeScience), penicillin (100U/mL), streptomycin (100ug/mL, Lonza, Walkersville, MD), nonessential amino acids (Lonza), 2-mercaptoethanol ($5x10^{-5}$M, Lonza), and sodium pyruvate (1 mM, Lonza). BALB/c mice were bred on site and caged, 5 to a cage, in a Thoren caging system with food and water provided *ad libitum*. Each experimental group consisted of 5 mice and each experiment was repeated 3 times. All mice were used in accordance with an Institutional Animal Care and Use Committee approved protocol, following the guidelines for ethical conduct in care and use of animals.

Macrophage Depletion Over Time

Gelfoam Implantation, Injections and Recovery

Two different types of clodronate liposomes, anionic and neutral, were tested to determine which would be more effective at depleting macrophages. Additionally, various dosages of each lipid were tested prior to experiments. One a proper dosage was determined, mice were anesthetized with 2-3% isoflurane (Halocarbon, Peachtree Corners, GA) for 5 minutes and then maintained on isoflurane throughout surgery via nosecone delivery. After being anesthetized and shaved, mice were wiped with 70% isopropyl alcohol (Becton, Dickson and

Company, Franklin Lakes, NJ) and 10% povidone-iodine prep pads (Professional Disposables International Inc., Orangeburg, NY), then a small straight incision about 1 cm long was made on the back of the animals, close to the tail end. A 10 mm x 10 mm piece of gelfoam (Pfizer, New York, NY), soaked overnight in Hank's Balanced Salt Solution (HBSS, VWR LifeScience), was inserted under the skin, and a wound clip was used to seal the wound. Twenty-four hours after gelfoam implantation, the mice were anesthetized again and 100 μL control liposomes or 100 μL anionic clodronate liposomes were injected into the gelfoam using a 1 cc syringe with a 25-gauge needle. All liposomes were purchased from FormuMax (Sunnyvale, CA). 4T1 cells were detached from culture flasks by removing cell culture medium, washing the cells with 5 mL HBSS (subsequently referred to as saline), and then adding 1 mL trypsin- EDTA (VWR LifeScience). Cells were incubated at $37^{O}C$ for 5 minutes, then 5 mL cRPMI was added to each flask to collect the cells which were then spun down at 450 x g for 8 minutes. The cells were then resuspended in 10 mL saline, and 10 μL from this suspension was combined in a 1:1 ratio with trypan blue for the cell count using a hemocytometer. Cells were washed with 10 mL saline 3 times, centrifuged at 450 x g for 8 minutes, and finally resuspended at 500,000 cells/mL for injection. Forty-eight hours after gelfoam implantation, the mice were anesthetized again and 100 μL 4T1 cells (50,000) were injected into the gelfoam using a 1 cc syringe with a 25-gauge needle. Mice were sacrificed by CO_2 gas followed by cervical dislocation at 1, 3, 5, 7, 10 or 21 days after 4T1 injection. The gelfoam implants were removed and digested in a collagenase cocktail (1 mg/ml collagenase type IV, 10 U/ml hyaluronidase, 20 mg/L DNase, Worthington Biochemical Corp, Lakewood, NJ) for 2 hours at room temperature while rocking. The cells were filtered through 50μM nytex to remove undigested tissue, and red blood cells were lysed using 5ml lysis solution (NH_4Cl, $KHCO_3$, EDTA, in ddH_2O with pH 7.2-7.4 adjusted by 1.0M HCl) and 5ml cRPMI.

Cell Staining and Flow Cytometry

Following isolation from the early tumor sites, cells were labeled with antibodies for macrophages (F4/80 (PE)) and separate samples were treated with the appropriate isotype control. Antibodies were purchased from BD Biosciences (San Diego, CA). For labeling, 1×10^6 cells/tube were resuspended in staining buffer (1× phosphate buffered saline, 2% fetal bovine serum, 0.09% sodium azide) and incubated with Fc block (anti-CD16/CD32, BD Biosciences) for 10 min on ice. Next, 1 µg of the specific antibodies were added and the cells were incubated for 20 min on ice. Following a wash with staining buffer the cells were resuspended in 0.5 ml HBSS and analyzed on a BD FACS Melody flow cytometer.

Testing Effect of Macrophage Depletion

Liposome Injections, 4T1 Injections, Measuring of Tumors

In order to study the effects of macrophage depletion on tumor progression, the lipids and tumors were delivered subcutaneously. Using a 1 cc syringe with a 25-gauge needle, 100 µL control liposomes or 100 µL clodronate liposomes were injected into the back of the right leg of each mouse. Twenty-four hours after liposome injection 100 µL 4T1 cells (50,000) were injected into the same site using a 1 cc syringe with a 25-gauge needle. The solid tumor mass was measured using a vernier caliper at 3, 5, 7, 10, 14, 17 and 21 days following injection and any significant necrosis at the tumor site was noted. Tumor volumes were calculated using the formula: length*width^2/2.

Tumor, Lung and Spleen Retrieval and Processing

Twenty-one days post 4T1 injections the solid tumor mass, lungs and spleen were retrieved. The solid tumor mass was diced and digested with a tumor digestion solution (1mg/mL collagenase IV, 20mg/L DNase I, 10 U/mL hyaluronidase in RPMI) at room temperature for two hours with rotation. The cell solution was filtered through 50μM nytex to obtain a single-cell suspension for red blood cell lysis. Cells were then stained with appropriate antibodies for macrophages (PE-conjugated anti-F4/80) and isotype control using the same protocol described above (see cell staining and flow cytometry). The number of macrophages present at the tumor site was determined using flow cytometry. Lungs were diced and digested with lung digest solution (1g/liter collagenase type IV, 100mg/liter elastase) at room temperature for one hour with rotation. The solution was filtered through 50μM nytex to remove undigested tissue, and red blood cells were lysed using 5ml lysis solution and 5ml cRPMI. The cells were resuspended in 10mL of cRPMI and cells were plated at 1/10, 1/100, 1/1000-fold dilutions with 10μL of thioguanine (0.2g thioguanine, 20mL of 0.1N NaOH) to make a 1000-fold dilution. The plates were incubated for 7 days at 37^{o}C. After 7 days the cells were stained with methylene blue and the metastatic colonies on each plate were counted to determine the number of lung metastases present. Extracted spleens were measured next to a 12-inch ruler and a photograph was taken to determine the size of the spleen. The spleen was placed in a petri dish and 5mL of HBSS was added. The flat end of a syringe plunger was used to expel the cells from the organ and the cells were filtered through 50μM nytex while being transferred to a 15mL tube. A cell count was performed with a 1/10 dilution with trypan blue using a hemocytometer to quantify the number of splenocytes present in each spleen.

Results

Depletion of Macrophages using Anionic Clodronate Liposomes

In order to deplete macrophages at early tumor sites, mice were given clodronate liposomes into gelfoam implants. After testing two different types of clodronate liposomes and various dosages it was determined that 100 µL of anionic clodronate was effective. Twenty-four hours later 4T1 tumors were injected into the gelfoam implants and then 1, 3, 5, 7 and 10 days later the sites were analyzed for the presence of macrophages using flow cytometry (Figure 1). At all five time points there was a decrease in the presence of macrophages in mice that received anionic clodronate liposomes relative to the control liposomes. Between 40% and 50% of the cells collected from mice that received control liposomes at all time points were macrophages, with a peak of about 75% on day 7 (Figure 3). Meanwhile, only 10% to 20% of the cells collected from mice that received anionic clodronate liposomes were macrophages at all time points (Figure 3). The percentage of macrophages at the 21-day tumor site was also determined using flow cytometry to discover how the depletion of macrophages early in the tumor microenvironment influenced the presence of macrophages later during tumor progression. There were significantly fewer macrophages at the 21-day tumor sites of mice that received anionic clodronate liposomes. About 6% of the cells present in the control tumors were macrophages while only about 2% of the cells in the experimental group were macrophages (Figure 3B). This suggested that clodronate liposomes decreased the number macrophages for a long period of time, at least up to 21-days following injection. Furthermore, the presence of neutrophils was analyzed using flow cytometry to determine whether anionic clodronate liposomes deplete this population of phagocytic cells. No significant difference was found in the percentage of neutrophils between the two groups suggesting liposomes do not deplete neutrophils (Figure 4). Overall, these data show evidence for successful macrophage depletion using anionic clodronate liposomes.

Slower Growth of Tumor Following Depletion of Macrophages

To determine whether the presence of macrophages at the early tumor site impacted growth of the tumors over time, tumor volume was measured every 2-3 days in the first twenty-one days following 4T1 delivery in mice that received the clodronate liposomes and controls. A difference in tumor volume was observed as early as day 9 following tumor delivery and tumor sizes in mice with macrophage depletion were significantly smaller than the control group at days 12, 14, 16, 18 and 21. The difference in tumor sizes between the two groups got increasingly larger over time (Figure 5). These data show that the presence of macrophages impacted tumor growth over time.

Additional Analysis of Cancer Progression in Mice with Macrophage Depletion

Since more lung metastasis has been linked to more advanced cancer, the number of metastases present in the lungs was acquired to determine if the presence of macrophages impacted the progression of the cancer (Figure 6A). Fewer lung metastases were observed in mice with macrophage depletion, although the data was inconsistent. The average number of lung metastases present in all three sets of mice that received control lipids was over 500 while there was an average of 40 lung metastases in all three sets of mice depleted of macrophages (Figure 6B). No significant difference in the number of lung metastases present was observed in any of the experiments. There was an average of 1241, 303 and 36 lung metastases respectively in the control groups while there was an average of 28, 66 and 21 lung metastases in the experimental groups 1-3 respectively (Figure 6B).

Furthermore, the size of the spleen was compared and the number of splenocytes was determined as enlarged spleens are associated with late-stage disease. The size of the spleens removed from the control mice were overall larger than the spleens in mice depleted of macrophages with the clodronate liposomes, but the data was not significant (Figure 7A). Furthermore, there was an average of 551 million splenocytes in all three sets of control mice but only an average of 316 million splenocytes in all three sets of mice that received clodronate liposomes. A significant difference was noted in experiment one, where there was an average of 609 million splenocytes in the control group and an average of 239 million splenocytes in the mice that received clodronate liposomes (Figure 7B). No significant differences were observed in experiments two and three. There were 522 million splenocytes in experiment two and 209 million splenocytes in experiment three in the control groups and 389 million splenocytes in experiment two and 305 million splenocytes in experiment three in the experimental groups (Figure 7B). Overall, the difference in lung metastases and splenocytes suggests less advanced cancer results in mice with fewer macrophages in the early tumor microenvironment.

Discussion

Although the progression of tumors in the presence of macrophages has been thoroughly studied, the role macrophages play in the first 72 hours of tumor development is still not well understood. This study worked to determine how a reduction of macrophages in the early stages of tumor development impacts the growth of the tumor in the first three weeks in order to identify what types of TAMs are present early on, before tumors can be detected, and what function these macrophages are exhibiting; pro-tumor or anti-tumor. Clodronate liposomes allowed localized macrophage depletion to occur. By locally removing macrophages and introducing tumor cells 24 hours later I was able to observe how the presence of macrophages in the early tumor microenvironment contributes to the growth or inhibition of tumor progression.

Macrophage Depletion using Clodronate Liposomes

When validating a protocol to deplete macrophages at the tumor site, a sponge was implanted under the skin on the back of each mouse and anionic clodronate liposomes were introduced to the site 24 hours later. After about 24 hours tumors were delivered at the same site and then at days 1, 3, 5, 7 and 10 the gelfoam sponge was removed, digested into a single cell suspension and then cells were stained with F4/80 antibodies. F4/80 is a glycoprotein found on all macrophages. Significantly, there was a decrease in macrophages at these sites when compared to the control group. Overall, this finding suggests that anionic clodronate liposomes successfully depletes macrophages at the site of the tumor in the first 10 days of cancer progression. While both M1 and M2 macrophages would be depleted at the tumor site, the change in tumor volumes (Figure 5) suggests that the macrophages infiltrating the tumor site are primarily M2 macrophages.

Fewer macrophages were seen in both groups on day 21 when compared to earlier time points, about 40% of the cells present at the tumor site were macrophages on days 1-10 but fewer than 10% of the cells collected on day 21 were macrophages (Figure 3). This difference can likely be attributed to the different experimental design used to determine the number of macrophages present at the tumor site on day 21. The number of macrophages present on days 1, 3, 5, 7 and 10 was determined using a gelfoam implant model (Figure 1A). This is because a solid tumor does not usually form until day 9 following tumor cell injections, and as a result, a gelfoam sponge is needed to act as the tumor site to collect infiltrating cells for analysis.

Meanwhile a solid tumor model was used to obtain the number of macrophages infiltrating the tumor site on day 21 (Figure 1B). Tumor cells were injected directly under the skin of the mouse behind the right leg rather than into a gelfoam implant. This was necessary because measuring growth of the tumor was a crucial component of determining what impact depletion of macrophages would have on tumor progression. This could not be done using a gelfoam implant model because the gelfoam would interfere with the tumor measurements. These experimental design differences could explain the discrepancies between the number of macrophages present at the tumor site between the day 10 and day 21 time points.

Evidence for M2 Macrophages at Early Tumor Site

To observe how long the macrophages remain depleted, the percentage of cells at the tumor sites that were macrophages after three weeks of tumor progression was determined. In order to do this, tumors were removed on day 21 digested into a single cell suspension and then cells were stained with F4/80 antibodies. The cells at the tumor site were then analyzed using flow cytometry. The tumors in the mice that underwent macrophage depletion had consistently fewer macrophages suggesting that the effects of anionic clodronate liposomes can last up to three weeks after injection. These data were surprising because most of the literature suggests that the effects of the lipids only last about 7 days. My findings may be because the macrophages present at the early tumor site recruits more macrophages to the site over time (Dandekar et al. 2011). Since the number of macrophages are initially reduced there are fewer signals present to recruit macrophages to the site later in the progression of the tumor.

This study aimed to evaluate the growth of tumors over three weeks as well as the number of splenocytes and lung metastases to determine how the presence of macrophages in the early tumor microenvironment impacts tumor progression. It was predicted that macrophages present at the early stages of breast cancer express M2 functions which contribute to progression of the tumor (Mukhtar et al. 2011). Tumors in the presence of macrophages grew both faster and larger over the span of three weeks than tumors in the absence of macrophages suggesting that the macrophages present in the early tumor microenvironment contribute to tumor growth, and therefore exhibit M2 macrophage functions. My hypothesis was further supported through analysis of splenocytes. The number of splenocytes was higher in the control group, and as splenomegaly is associated with late-stage disease, this finding upholds the hypothesis that macrophages are contributing to growth of the tumor in the early stages of disease (Dunn and Goldwein 1975). A similar trend was seen when observing the number of lung metastases with more lung metastases found in the control group. Since a greater number of lung metastases are seen in more advanced cancers, this finding further emphasizes that the macrophages present exhibit M2 functions by enhancing tumor growth (Pietrangelo 2020). In order to validate these findings, it would be useful to perform gene expression profiling using microarray analysis or quantitative polymerase chain reaction (qPCR) to analyze genes that encode proteins linked with each TAM phenotype. Previous literature suggests M2 macrophages in mice will express *CXCL9, CXCL10, CXCL11* and *NOS2* while M1 macrophages will express *Mrc1, tgm2, Ym1/2* and *Arg1* (Martinez and Gordon 2014).

Clinical Applications

The findings of this study provide further evidence to support current therapeutic treatments being explored that specifically target macrophages to inhibit tumor progression. LY3022855, a CSF1R-blocking antibody, is one-way to target macrophages. A study by *Dowalti* *et al. (2021)* found that stable disease was a potential outcome in patients with breast cancer that received LY3022855. Similarly, *Papadopoulos et al. (2017)* used AMG 820, a drug that targets the CSF1R and inhibits TAM survival, and found some antitumor activity such as a reduction in tumor diameter and more stable disease in patients with breast cancer. Furthermore, targeting macrophages has also been linked to improvements in chemotherapy and radiation treatments. It has been suggested that TAMs contribute to tumor progression by secreting vascular endothelial growth factor (VEGF) which enhances the vasculature of tumors (Obeid et al. 2013). In fact, studies have showed that the addition of paclitaxel, a therapy that targets VEGF receptor 2, to chemotherapies decreases macrophage infiltration and increases progression-free survival for metastatic breast cancer (Miller et al. 2007, Miles et al. 2010). Additionally, some studies have been focusing on re-educating macrophages in order to alter their function so that they impair tumor progression rather than enhance it (Kowal et al. 2019). For instance, *Guiducci et al (2005)* reported that CpG plus anti-interleukin-10 receptor antibody with CCL16 treatment promptly switched infiltrating macrophages from M2 to M1 TAM. Moreover, this induced tumor shrinkage and resulted in more than half of the mice rejecting the 4T1 tumors. Likewise, *Perry et al. (2018)* used anti-CD40 antibody along with a CSF-1R inhibitor to increase the presence of M1 TAMs which resulted a significant decrease in tumor volume.

Additional studies are needed to determine the function of macrophages at early tumor sites

To further support the conclusion that macrophages present at the early tumor microenvironment are in fact contributing to tumor progression, these experiments should be repeated. Much of the data such as the number of lung metastases and splenocytes were inconsistent within each experiment and between all three experiments suggesting that more data is necessary to draw proper conclusions. For example, lung metastasis data in experiment 1 showed an average of about 1200 lung metastases in the control group but in experiment 2 there was less than 100 lung metastases in the control group; clear discrepancies between experiments are noted in the data. Only a weak correlation was found between tumor volumes and the number of lung metastases in the control group, and no correlation was noted between the tumor volumes and the number of lung metastases in mice that received clodronate liposomes (Figures 8A, 8B). Similarly, no correlation was found between the size of the tumors in the control and experimental groups and the size of their spleens (Figures 8C, 8D). Likewise, no correlation was found between the number of macrophages present at the tumor site and the number of lung metastases or splenocytes in mice that received clodronate liposomes (Figures 9B, 9D), and only a weak correlation between number of lung metastases or splenocytes and the number of macrophages at the tumor site (Figures 9A, 9C). It is unclear whether or not the number of macrophages present at the tumor site was impacting the spleen inflammation and metastasis, making the relationship between macrophages and disease progression unclear. In general, these findings show that more research is needed to determine what is causing an increase or decrease in the number of lung metastases and splenomegaly and whether the presence of macrophages is indeed influencing disease progression. Additionally, it would be useful to explore if different forms of cancer initiate the same immune response, specifically if the type of TAMs in the early tumor microenvironment are the same in the presence of different breast cancer cell lines. In other words, it would be beneficial to repeat the same experiments using less aggressive cancer cells such as 168 or EMT6 cell lines.

In order to understand how macrophages impact the progression of cancer in the early stages, it would also be useful to systemically deplete macrophages rather than only locally depleting them which was done in this study. This can be done by utilizing different methods to target macrophages. In this study, clodronate liposomes were used to locally target and deplete macrophages, but the liposomes could also be injected intravenously to systemically deplete macrophages. Other ways macrophages have been targeted in the tumor microenvironment is through macrophage depletion using trabectedin, targeting CCL2 and CSF1R, and through macrophage reprogramming using IFN-alpha, CD40, STAT6 and STAT 3 (Poh and Ernst 2018). For instance, *Qian et al. (2011)* targeted CCL2, a chemokine that recruits macrophages, and found it resulted in reduced tumor growth and metastasis, a similar impact as macrophage depletion in this study. Additionally, *Aharinejad et al. (2004)* found antiangiogenic and antimetastatic effects when targeting CSF1R, further supporting the antimetastatic findings of this study. Each of these methods have been found to have similar impacts on tumor progression but it would be important to explore these other methods of targeting macrophages in the 4T1 model, as well as other tumor models in order to determine whether targeting macrophages has widespread use. Additionally, it would be beneficial to look at these treatments at different stages of disease to determine the optimal time during disease progression for macrophage targeting.

Conclusion

This study identified the function of macrophages that are present in the early innate response to breast cancer in mice. Localized depletion of macrophages at the tumor sites resulted in slower tumor growth. Decreased lung metastasis and splenomegaly were also noted. Consequently, results from this study suggest that macrophages present in the early stages of tumor progression exhibit M2 functions by contributing to tumor progression.

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Figures

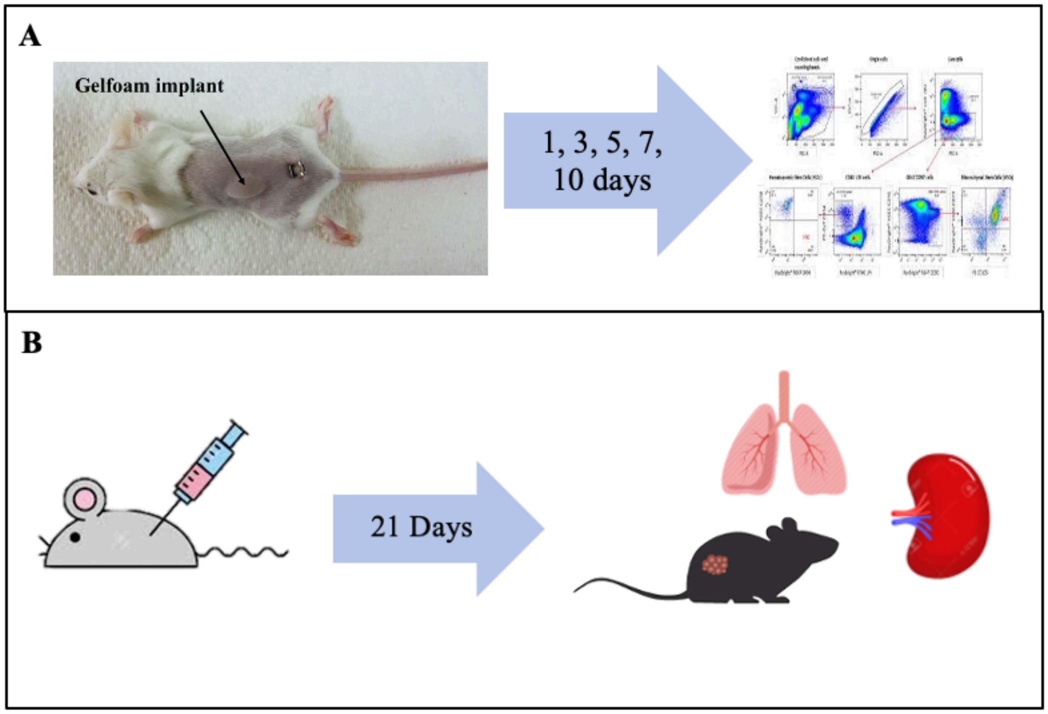

Figure 1: Experimental Design.

Two separate experimental designs were used in this study. (A) To address aim 1, a gel foam sponge was implanted under the skin into the back of a mouse, and 24 hours later clodronate liposome or control liposome was injected directly into the sponge. 4T1 cells were injected directly into sponge implant 24 hours post liposome injection. The sponges were removed at various time points and stained with F4/80 antibody and analyzed using flow cytometry to determine whether macrophage depletion via clodronate liposome was successful. (B) To address aim 2, macrophages were depleted using clodronate liposomes at a subcutaneous tumor site. 4T1 injections in the back of mouse leg followed macrophage depletion 24 hours later. Tumor volume was obtained periodically for 21 days. On day 21 tumors, lungs and spleens were removed to determine disease progression.

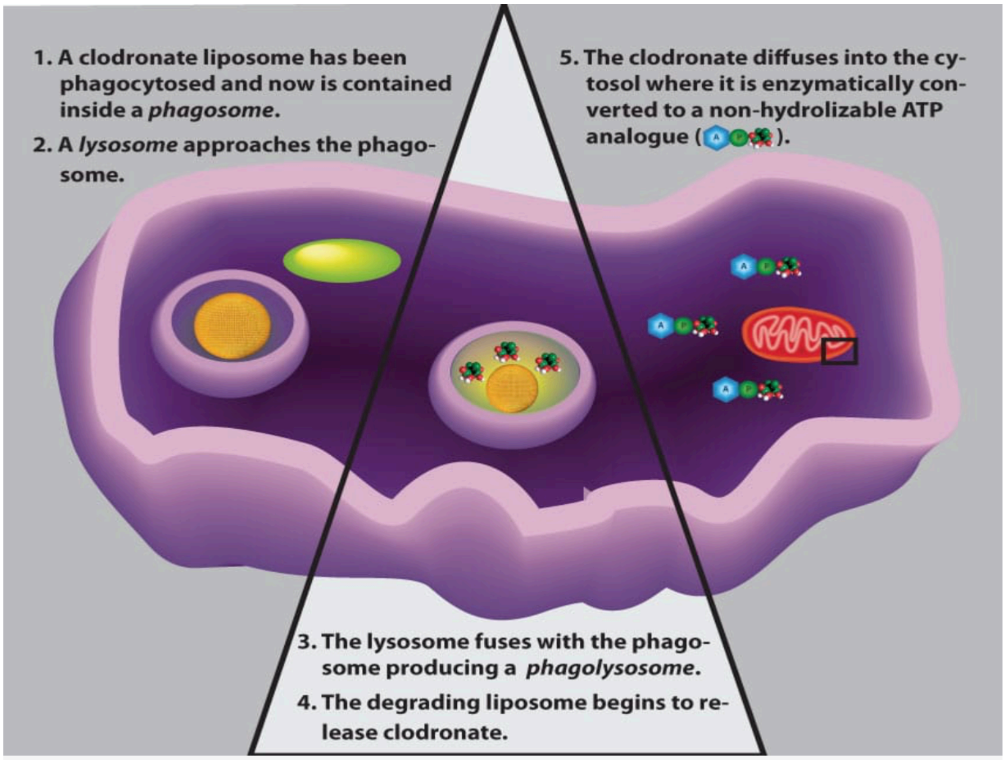

Figure 2: How clodronate liposomes work.

Macrophages will recognize the liposome as a foreign invader and phagocytose the liposome in an attempt to destroy it. The liposome is internalized in a phagosome that then fuses with a lysosome to breakdown the liposome. As the liposome gets degraded by the macrophage, clodronate is released and diffuses into the cytosol. Clodronate is mistaken for cellular pyrophosphate and used by Class-II aminoacyl-tRNA synthetases to produce a non-hydrolysable ATP analog (AppCCL2). AppCCL2 enters the mitochondrial outer membrane and irreversibly binds to ATP/ADP translocase in the mitochondria, resulting in the formation of pores in the mitochondria's inner membrane. Once the integrity of the membrane is compromised, depolarization occurs that allows molecular signals to be released by the mitochondria to initiate apoptosis (Mechanism of Depletion 2018).

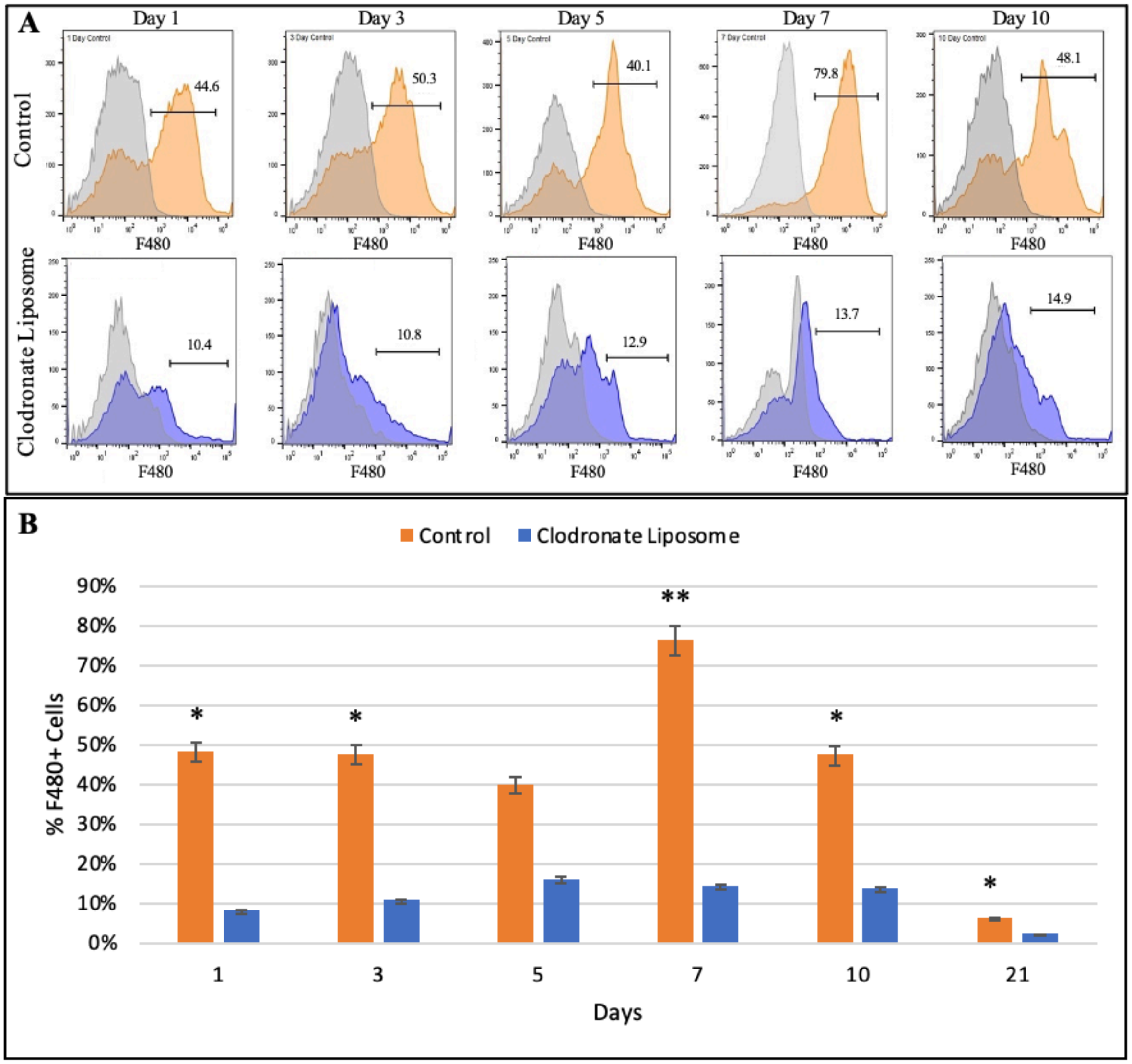

Figure 3: Macrophage depletion via clodronate liposomes.

Following liposome and tumor delivery, sponge implants (days 1, 3, 5, 7, 10) and tumors (day 21) were removed, and infiltrating cells were stained with F4/80 antibodies to determine the percentage of macrophages present by flow cytometry. (A) The histograms show the isotype control in gray and F480 stained cells in orange or blue with the gate ([—]) showing the F480+ cells. (B) These data represent the average and standard deviation of three separate experiments. Where indicated * $p<0.05$ and ** $p<0.01$ there were significant differences between the number of macrophages present in each group using a two-tailed t-test with unequal variances.

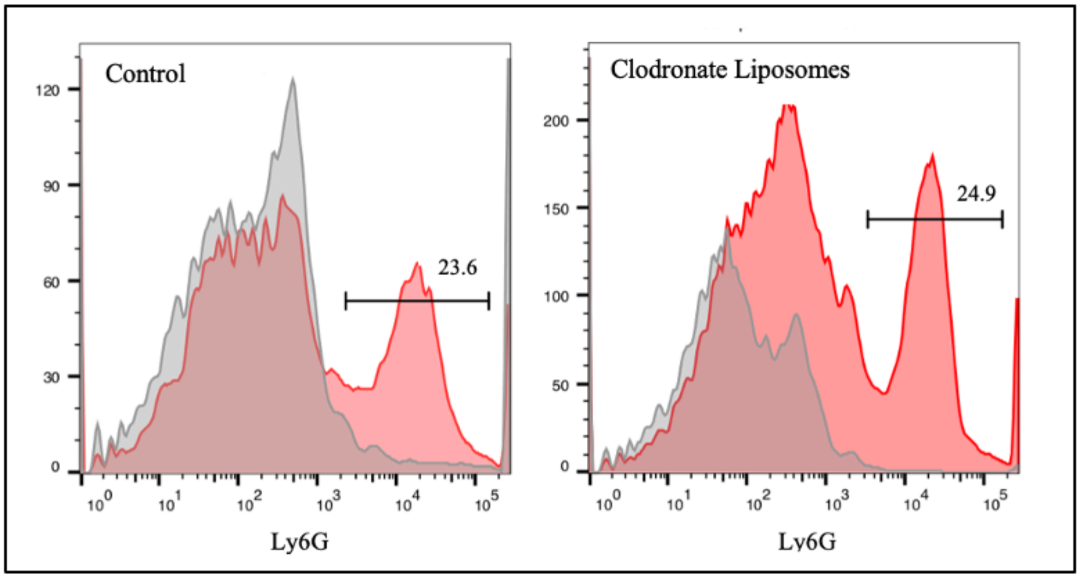

Figure 4: Neutrophils were not depleted by liposomes.

Following liposome and tumor delivery, sponge implants were removed at 24 hours to look for neutrophil depletion. Cells were stained with Ly6G antibodies to determine the percentage of neutrophils present at the tumor site with isotype control shown in gray and Ly6G stained cells in red. The gated population ([—]) shows the percent of Ly6G+ cells. The was no significant difference in the number of neutrophils between the two groups.

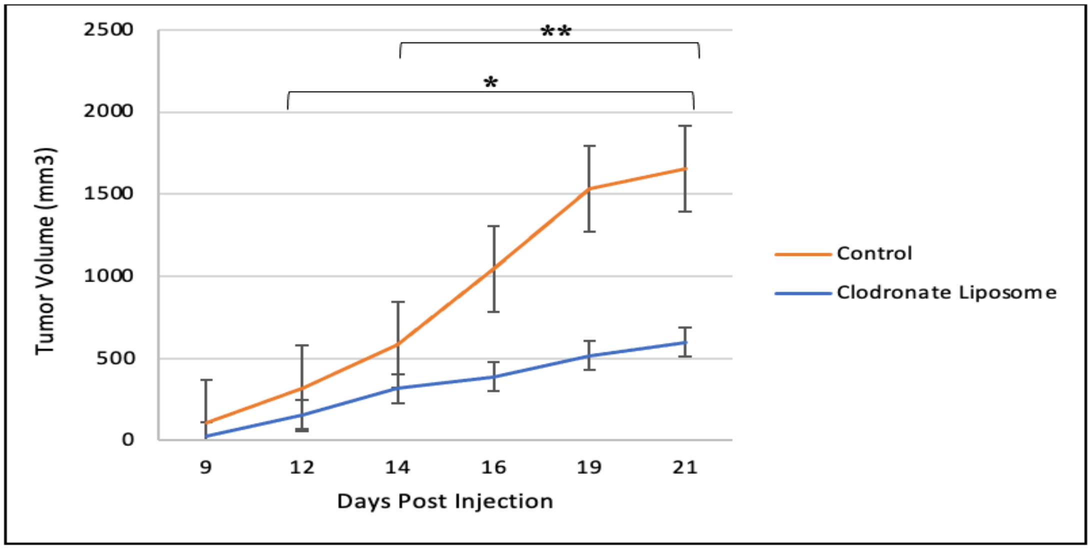

Figure 5: **Tumor growth over time.**

Tumor volume was determined at specific time points following 4T1 injections. The control group received control liposomes 24 hours prior to 4T1 injections, and the experimental group received clodronate liposomes in order to deplete macrophages prior to 4T1 injections. These data represent the average and standard deviation of three separate experiments. Where indicated (┌┐) * $p<0.05$ and (┌┐) ** $p<0.01$ there were significant differences between the size of the tumors in each group using a two-tailed t-test with unequal variances.

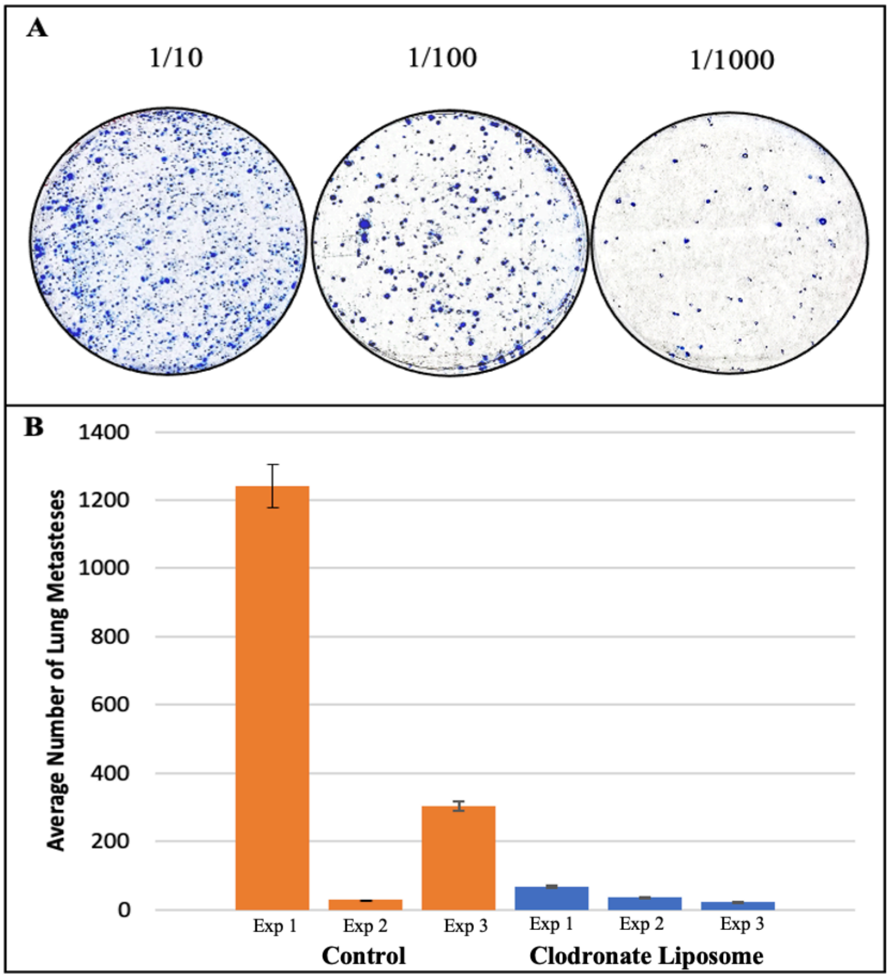

Figure 6: Analysis of lung metastases in tumor bearing mice.

As a measure of cancer progression, I analyzed lung metastasis in the tumor bearing mice that received clodronate liposomes and the control group. (A) At day 21, lungs were extracted, digested and plated at 1/10, 1/100 and 1/1000-fold dilutions and stained using methylene blue. The number of metastatic colonies present were counted. (B) These data represent the averages and standard deviations of three separate experiments with five mice in each experiment. None of the data was found to be significant using a two-tailed t-test with unequal variances.

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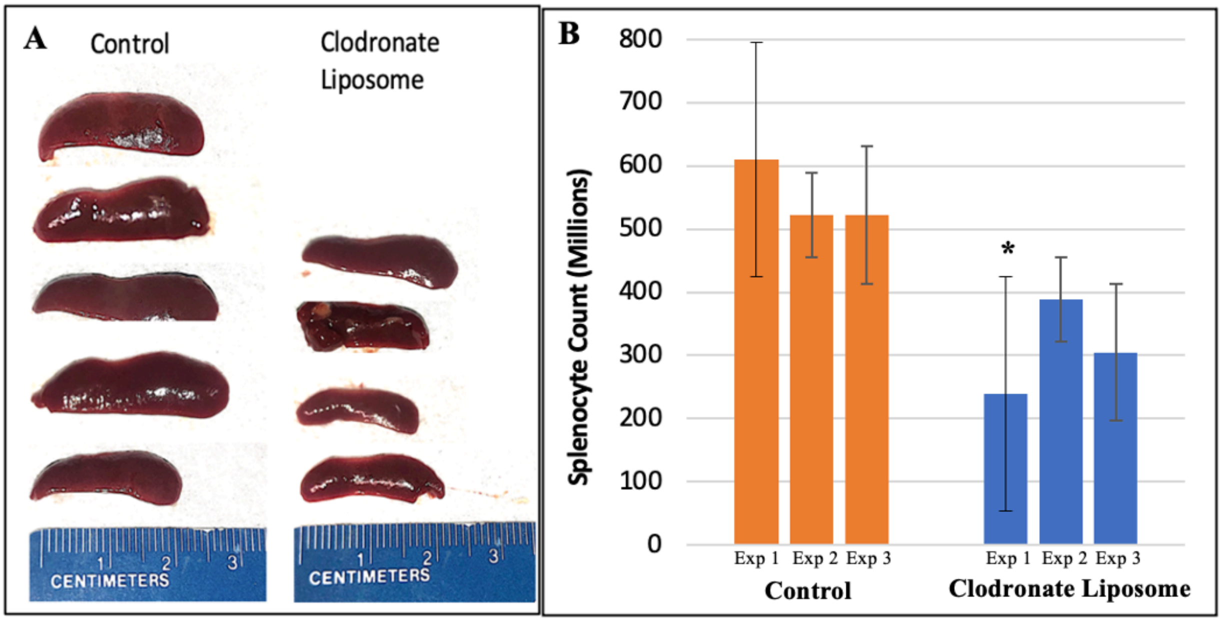

Figure 7: Analysis of spleens in the tumor bearing mice.

As another measure of cancer progression, I analyzed splenomegaly in the tumor bearing mice that received clodronate liposomes and the control group. (A) Spleens were removed from the control and the experimental groups and the size of spleens were measured. The spleens shown in this image are from one experiment. (B) Cells were expelled from the spleens and the number of splenocytes was counted using a hemocytometer. These data represent the average and standard deviations of each experiment with five mice in each experiment. Where indicated * $p<0.05$ and ** $p<0.01$ there were significant differences between the number of splenocytes present in each group using a two-tailed t-test with unequal variances.

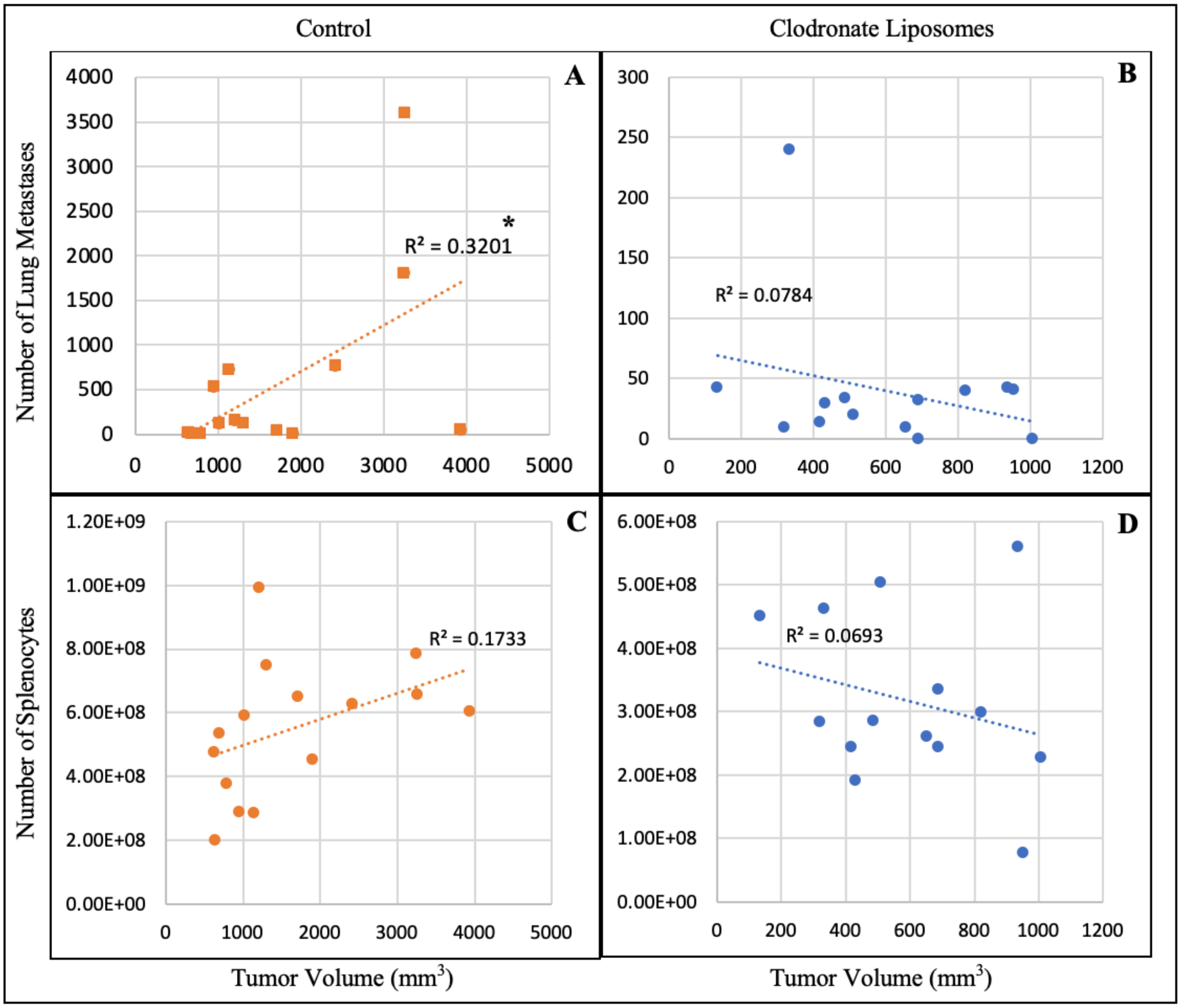

Figure 8: Correlation coefficients between tumor volume and lung metastasis or the number of splenocytes.

Correlation coefficients were calculated to determine whether disease progression correlated with the number of lung metastases and spleen inflammation. R^2 represents correlation coefficient where (A) tumor volume (mm^3) is compared to the number of lung metastases in control mice, (B) tumor volume (mm^3) is compared to the number of lung metastases in mice with macrophage depletion, (C) tumor volume (mm^3) is compared to the number of splenocytes in control mice, and (D) tumor volume (mm^3) is compared to the number of splenocytes in mice with macrophage depletion. Where indicated * $0.8>R^2>0.1$ identifies a weak correlation.

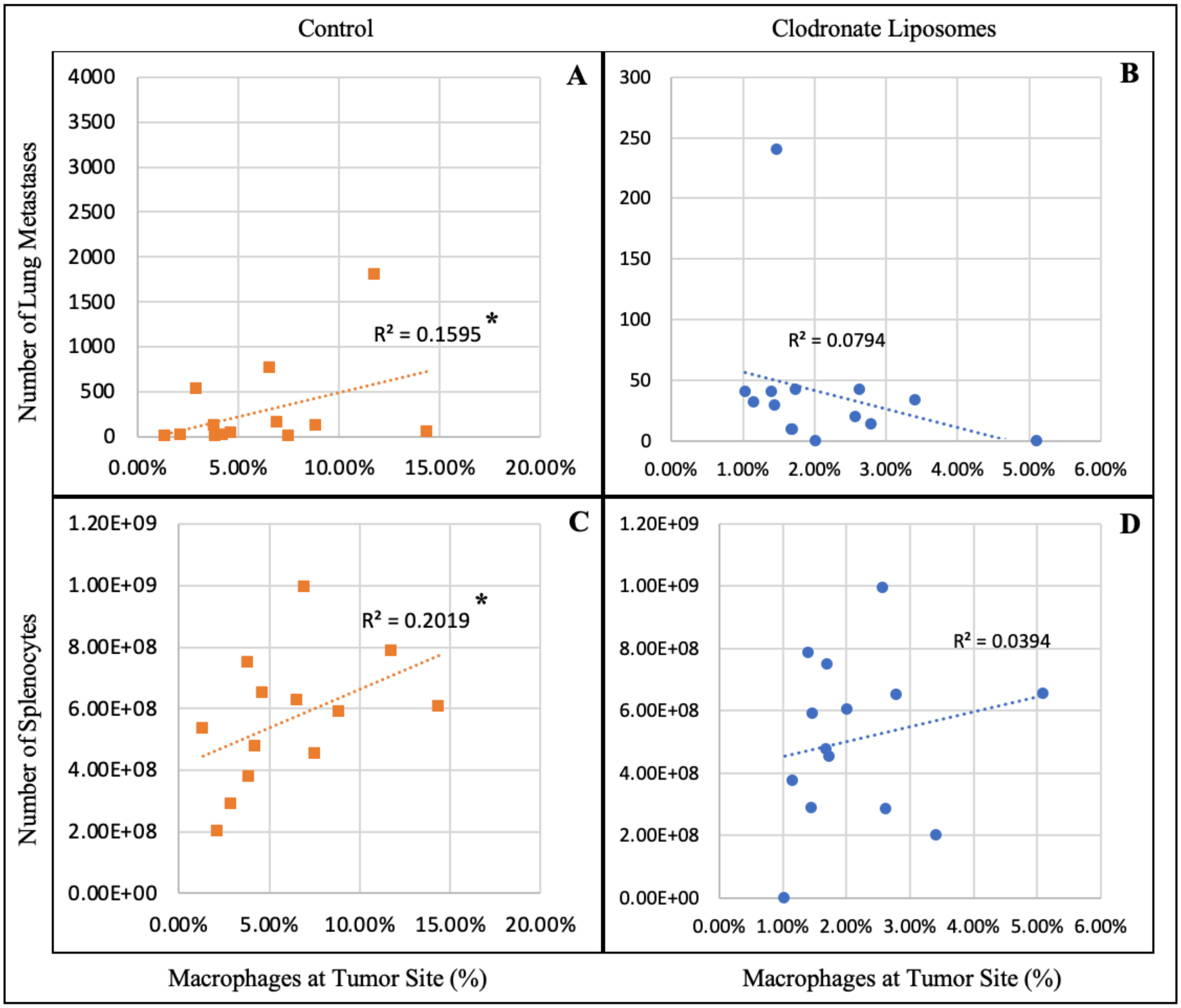

Figure 9: Correlation coefficients between macrophages at tumor site and lung metastasis or the number of splenocytes.

Correlation coefficients were calculated to determine whether the number of macrophages present at the tumor site correlated with the number of lung metastases and spleen inflammation. R^2 represents correlation coefficient where (A) percentage of cells at the tumor site are macrophages is compared to the number of lung metastases in control mice, (B) percentage of cells at the tumor site are macrophages is compared to the number of lung metastases in mice with macrophage depletion, (C) percentage of cells at the tumor site are macrophages is compared to the number of splenocytes in control mice, and (D) percentage of cells at the tumor site are macrophages is compared to the number of splenocytes in mice with macrophage depletion. Where indicated * $0.8>R^2>0.1$ identifies a weak correlation.