Studying the Therapeutic Potential of Macrophages in Mouse Breast Cancer Models

Meghan Roberts Fall 2022 - Spring 2023

Research Advisor: Professor Robert A. Kurt
Thesis Committee: Dr. Khadijah Mitchell, Dr. Manuel Ospina-Giraldo, and Dr. John S. Shaw

Table of Contents

Abstract……2

Background……. 3

Hypothesis and Aims…….. 7

Materials and Methods…….. 8

Results……... 11

Discussion……..14

Limitations…….22

Conclusion…….23

Acknowledgements…….24

Figures and Tables……25

References……..33

Abstract

Involved in homeostasis maintenance and pathological defense, macrophages are crucial cells for the human innate immune system. Due to functional plasticity, the infiltration of macrophages into microenvironments are also associated with adverse outcomes, often playing a detrimental role in the tumor microenvironments (TME). Based on local stimuli, these tumor associated macrophages (TAM) can have either pro-inflammatory and anti-tumor effects (M1) or anti-inflammatory and pro-tumor effects (M2). Prior studies have focused on TAM-modulating strategies, using single stimuli to convert TAMs into M1 macrophages. The purpose of this study was to investigate macrophage polarization by evaluating the therapeutic potential of macrophages exposed to gamma interferon (IFN-γ), CD40 ligand (CD40L), polyinosinic-polycytidylic acid [poly(I:C)] (PIC), or combinations of the three stimuli. Using the EMT6 and 4T1 tumor models, the efficacy of the stimulated macrophages was assessed based on tumor progression and survival rates. For mice bearing EMT6 tumors, the following treatments had a significant effect on survival rate: CD40L+IFN-γ, IFN-γ+PIC, CD40L+PIC, and IFN-γ+CD40L+PIC. The IFN-γ+CD40L+PIC treatment group also had a significant effect on survival rate and metastatic spread for mice bearing 4T1 tumors. These findings suggest that combining two or more of these stimuli are effective at converting TAMs into M1 macrophages that have anti-tumor activity.

Background

Cancer is the second leading cause of death in the United States, and is the leading cause of death in the world (*Cancer.Net* 2022). In 2020, about 2.3 million women were diagnosed with breast cancer, accounting for 1 of every 3 cancer diagnoses annually (*Cancer.Net* 2022). Of those who were diagnosed, about 680,000 will die from breast cancer (*Cancer.Net* 2022). Due to the incidence, mortality rate, and economic cost of breast cancer, there is a global burden and concern, justifying the need for more research focused on earlier diagnoses and treatments.

Treatment options for women with breast cancer are largely dependent on the stage, histology, and biomarkers (Trayes and Cokenake *et al.* 2021). Stage 0 lobular carcinoma and ductal carcinoma is due to abnormal tissue growth in the lobules and milk ducts (Trayes and Cokenakes *et al.* 2021). This stage of cancer will either not progress or increase the risk of developing into a more invasive form of breast cancer (Trayes and Cokenakes *et al.* 2021). Therapies are not indicated as a necessary step, rather patients are recommended to annually undergo breast examination and mammogram for surveillance (Trayes and Cokenakes *et al.* 2021). Stage I and II include early-stage invasive breast cancers and are resolved through a modified radical mastectomy, removing the tumor without excess healthy breast tissue (Trayes and Cokenakes *et al.* 2021). Depending on the status of axillary lymph nodes, radiation therapy and adjuvant systemic therapy may be administered to reduce the recurrence rate (Trayes and Cokenakes *et al.* 2021). Chemotherapy and endocrine therapy are other modes of treatment for node-positive breast cancer and for women with larger tumors (Trayes and Cokenakes *et al.* 2021). Stage III breast cancer is considered locally advanced, extending larger than 5 cm with extensive regional lymph node involvement. This stage is treated with induction chemotherapy to destroy any fast-growing cells and then followed by local therapy, a secondary treatment that is directed to a specific organ or area of the body (Trayes and Cokenakes *et al.* 2021). The local

therapy could be one of the treatments previously mentioned or could be surgery, radiation therapy, or laser therapy (Trayes and Cokenakes *et al.* 2021). The combination of both ensures that the chemotherapy and local therapy has reached the affected tissues, decreasing the rate of local recurrence (Trayes and Cokenakes *et al.* 2021). Stage IV breast cancer is diagnosed when the cancer is metastatic and the five-year survival rate is less than 24%. The treatments discussed above can be used in combination, however, systemic treatment largely depends on the hormone receptor status, rate of disease progression, and the willingness of the patient to tolerate adverse effects of treatment given the survival rate of stage IV breast cancer (Trayes and Cokenakes *et al.* 2021). Due to growth in the areas of gene sequencing, targeted therapies, and molecular diagnostics, breast cancer treatments are becoming more selective towards specific tumor characteristics, rather than the specific stage at which the patient is diagnosed. One therapeutic strategy that may also work is reprogramming macrophages from pro-tumor to anti-tumor macrophages.

Macrophages are innate immune cells, belonging to the phagocytic mononuclear system, that circulate from peripheral tissues to sites of inflammation and tissue damage (Cendrowicz *et al.* 2021). These cells have three key functions: phagocytosis, exogenous antigen presentation, and immunomodulation through either cytokine or growth factor secretion (Boutilier *et al.* 2021). Macrophages also contribute to new tissue and blood vessel formation via angiogenesis (Boutiler *et al.* 2021). Another feature of macrophages is functional plasticity, also known as polarization, which enables different immune responses through changes in local environments and stimuli (Cendrowicz *et al.* 2021). Macrophages are often divided into two polarized phenotypes: M1 macrophages that have pro-inflammatory and anti-tumor effects, and M2 macrophages that have anti-inflammatory and pro-tumor effects (Yunna *et al.* 2020) (Figure 1).

There are multiple mechanisms and processes that influence the polarization of macrophages– microenvironment, tissue regeneration, cytokines and signaling pathways, transcription factors, inflammation, etc. (Yunna *et al.* 2020). The state of polarization is also not fixed to a singular point in time; most studies have found that TAMs waiver between pro- and anti-tumor phenotypes and then change based on the type and concentration of signals within the tumor microenvironment (Pan *et al.* 2020). At the early stages, the tumor microenvironment favors M1 TAMs, which quickly switches to M2 TAMs due to the influence of cancer cells (Boutiler *et al.* 2021). M2 TAMs contribute to the further development of tumors, modulating metastasis, latency, and immune suppression through the secretion of messenger molecules (Boutiler *et al.* 2021). As a case in point, Hachim *et al.* (2020) used transcriptomic data from 1,084 breast cancer patients and found that patients with higher levels of a M1 gene signature had less advanced disease, better patient outcomes, and a prolonged overall survival than patients with higher levels of a M2 gene signature. By supporting cancer proliferation, M2 TAMs can cause additional resistance to conventional treatments and immunotherapies (Hachim *et al.* 2020). Consequently, therapeutic strategies to target and reprogram TAMs from a pro-tumor to an anti-tumor phenotype have been under review as potential treatments for cancer (Li *et al.* 2021). One way to reprogram TAMs is known as priming. For instance, different types of TAMs (M1, M2) can be obtained through exposing macrophages to different stimuli (Li *et al.* 2021) (Figure 1). Previous studies have found multiple stimuli that produce anti-tumor M1 macrophages including gamma interferon (IFN-γ), CD40 ligand (CD40L), and polyinosinic-polycytidylic acid [poly(I:C)] (PIC) (Panni *et al.* 2014) (Figure 1).

IFN-γ, a type II cytokine produced by natural killer (NK) cells and CD4+ T-cells, induces M1 macrophages by binding to the IFN-γ receptor and transmitting signals in the

cytoplasm to start an innate immune response (Schroder *et al.* 2003). IFN-γ binds to either gamma interferon receptors, either IFNGR1 or IFNGR2, activating Janus Kinases (JAK1 and JAK2) through phosphorylation (Kak *et al.* 2018). The activated JAKs phosphorylate the transcription factor STAT1, which then binds to gamma activated sequences in the promoter region of genes that mediate an innate immune response (Kak *et al.* 2018). This signaling pathway enables macrophages to increase antigen processing and presentation as well as production of reactive oxygen species (ROS), nitric oxide species (NOS), and proinflammatory cytokines (TNF-α, IL-1, IL-6) (Kak *et al.*, 2018). All of which can help macrophages fight cancer.

CD40L, a type II transmembrane protein, is expressed by activated T cells, B cells, and platelets and enhances anti-tumor immune responses through macrophage and dendritic cell maturation (Elgueta *et al.* 2013). The stimulation of CD40 induces receptor trimerization via FcγRIIB, which leads to anti-tumor effects via the production of TNF-α and NOS as well as the promotion of antibody-dependent cellular cytotoxicity (ADCC) (Djureinovic *et al.* 2021). Once CD40L binds to its receptor, CD40, found on macrophages, the signaling also promotes the secretion of IL-12, which leads to the activation of NK cells (Djureinovic *et al.*, 2021). This further promotes tumor cytotoxicity by NK cell mediated apoptosis (Djureinovic *et al.* 2021).

PIC, a synthetic double-stranded RNA (dsRNA), is recognized by Toll-Like Receptor 3 (TLR3) found in macrophages (Hong *et al.* 2021). TLRs are pattern recognition receptors that elicit innate immune responses and can induce M1 macrophages (Hong *et al.* 2021). By binding to the extracellular domain of TLR3, PIC stimulates type I interferons and inflammatory cytokines, like IL-1β, which then activates transcription factors such as interferon regulatory factor (IRF) and nuclear factor kappa B (NF-κB) (Hong *et al.* 2021). Macrophages treated with

PIC also activate CD8+ T cells, which furthers the anti-tumor response through T-cell mediated tumor cytotoxicity (Hong *et al.* 2021).

Most studies have focused on a single mechanism to convert TAMs into M1 macrophages. In order for M1 macrophage polarization to be considered a therapy for breast cancer, research needs to explore different mechanisms of M1 macrophage activation and to determine which has the greatest therapeutic potential.

Hypothesis and Aims

Because of the positive association between M1 macrophages and survival rates in patients with breast cancer (Guo *et al.* 2019), the aim of this study was to find a stimulus or combination of stimuli that elevates the expression and activity of M1 macrophages within mouse models of breast cancer. I hoped to monitor tumor growth, and find slower progression of breast cancer in mice that received the M1 macrophage treatments. My hypothesis was that bone marrow-derived macrophages (BMDM) stimulated with multiple stimuli would improve M1 macrophage anti-tumor activity, decreasing the average tumor volume and increasing the survival rate compared to the controls.

In order to test this hypothesis, BMDM were exposed to IFN-γ, CD40L, and PIC alone and in various combinations. I expected to find a significant difference in tumor growth and survival rates in mice that received macrophages generated with multiple stimuli compared to mice that received macrophages that were generated with a single stimulus.

Materials and Methods

Mice and Cell Maintenance

BALB/c mice were bred and housed on campus, where water and food were provided *ad libitum*. Treatment of these mice were in accordance with IACUC-approved protocols, following the guidelines for ethical care and use of the mice.

EMT6 and 4T1 cell lines were maintained in complete RPMI (RPMI 1640 (Lonza, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (VWR, Radnor, PA), 5 mL of 2mM L-glutamine (Lonza), 5 mL of 100 U/mL penicillin/streptomycin (Lonza), 5mL of non-essential amino acid (Lonza), 5mL of 1 mM sodium pyruvate (Lonza), and $5x10^{-5}$ M β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO)) and incubated at 37℃, 5% CO_2. Every two to three days the cell lines in the flasks were split. For this purpose, the cRPMI was discarded and the cells were washed with 5mL of Hank's balanced salt solution (HBSS, VMR). The HBSS was discarded and 1 mL of 0.50% trypsin-EDTA (Thermo-Fisher Scientific, Hampton, NH) was added to each flask and incubated for 5 minutes at 37℃. Following incubation, 9 mL of cRPMI was added and 10mL of the solution was transferred to a 15mL centrifuge tube. The cells were centrifuged at 400 x g for 8 minutes and resuspended in 10 mL of cRPMI. On Mondays and Wednesdays, $2x10^5$ cells were added back to each flask and, on Fridays, $1x10^5$ cells were added back to each flask.

Generation of Bone Marrow-Derived Macrophages

BALB/c mice were sacrificed using CO_2 asphyxiation and spinal cord dislocation. The legs were removed, cutting the hind legs at the hip bone and removing the excess tissue and muscle. To collect the bone marrow from the tibia and femur, two cuts were made: the first where the femur meets the tibia and the second where the tibia meets the foot. Five mL of cRPMI was dispensed into a petri dish and bone marrow was extracted by injecting 1 mL of cRPMI into the ends of the bones using a 1 cc syringe with a 27 gauge needle. The injection was repeated on all ends of the joints, twice. The cells were transferred from the petri dish to a 15 mL centrifuge tube, and the petri dish was rinsed with 5mL of cRPMI that was then added to the centrifuge tube. Cells were centrifuged at 450 x g for 8 minutes and resuspended in 5mL of red

blood cell (RBC) lysis buffer (8.29 g NH_4Cl, 1 g $KHCO_3$, 37.2 mg EDTA in 1 L of H_2O, pH 7.2-7.4) and 5mL of cRPMI. The cells were centrifuged again and then 24 mL of cRPMI and 1 mL of 10 ng/mL M-CSF (PeproTech, East Windsor, NJ) was added. Next, one mL of cells was added to each well of a non-tissue culture treated 24 well plate, which was placed into the incubator at 37°C, 5% CO_2.

Every 2 to 3 days, 50% of the media was removed from each well and 1 mL of cRPMI with M-CSF was then added. After 7 days, the macrophages were treated with stimuli to convert them into anti-tumor macrophages.

Macrophage Activation

To activate the BMDM, the cells were treated with the following: 200 units/mL mouse IFN-γ (Thermo-Fisher Scientific), 200 ng/mL mouse CD40L (Thermo-Fisher Scientific), 100 ug/mL PIC (InvivoGen), a combination of two stimuli (IFN-γ and CD40L, IFN-γ and PIC, CD40L and PIC), or a combination of all three. For a control group, the cells were treated with HBSS. The cells were then incubated at 37°C, 5% CO_2 for 24 hours.

Tumor and Macrophage Injection

To collect BMDM, all media was transferred to a 50mL centrifuge tube and then each well was rinsed with 0.5mL of HBSS. Next, 100 µL of 1x PBS + 10 µM EDTA was added to each well and the cells were placed on ice for 5 minutes. The cells were then dislodged from the wells by pipetting the media up and down, in a circular motion, in each well. The cells were then collected and placed into the 50mL centrifuge tube. After centrifugation, the cells were resuspended in 5 mL of cRPMI and counted with a hemocytometer. The tumor cells were collected by trypsinization as described above. The tumor cells and BMDM were then mixed in a

1:1 HBSS solution, washed three times in HBSS, and resuspended so that the tumor cells were at $1.0x10^5$ cells/mL.

The mouse cages were labeled with the ratio of BMDM to tumor cells, the BMDM stimuli, tumor type (either EMT6 or 4T1), and the date of injection. Using a 1 cc syringe with a 25-gauge needle, 0.1 mL of BMDM ($1x10^5$) and tumor cells ($1x10^5$) were administered subcutaneously above the left rear leg.

Tumor Progression and Observation

To measure the efficacy of the BMDM, the growth of the tumors and the viability of the mice were followed every 2-3 days until the tumors reached 1500 mm^3. Any unexpected necrosis or death was noted. Using the formula $\frac{length\ (width^2)}{2}$, the volume of the tumors were calculated with a vernier caliper.

Analysis of Metastasis

After 21 days, the mice that bore 4T1 tumors were sacrificed, the lungs were removed, and then placed in a 15 mL tube. Lungs were then cut up and transferred back into the 15 mL tube with 2 mL of lung digest solution (1 g/L collagenase type IV (Worthington Biomed, Lakewood, NJ) and 100 mg/L elastase in HBSS (Worthington Biomed). The lungs were rotated and digested for 1.5 hours at room temperature. After the digestion process was completed, 1 mL of the solution was resuspended in 5mL of RBC lysis. The cells were spun down in a 5mL suspension of cRPMI, which was then plated at a 1/10-fold dilution, at a 1/100-fold dilution, or at a 1/1000-fold dilution in a 24 well plate. After 7 days of incubation, the media was poured off, the cells were fixed with methanol for one minute, and then stained with 0.03% methylene blue (Sigma-Aldrich) for five minutes. The metastatic colonies were counted.

Results

Effect of BMDM on Tumor Progression and Survival in Mice Bearing EMT6 Tumors

To determine if BMDM had an effect on the survival of mice bearing EMT6 tumors, BALB/c mice were given EMT6 tumors with or without BMDM. The mice that received EMT6 tumors without BMDM were considered the control group for this experiment. Seven days after injection, the growth of tumors was calculated and measured every 2-3 days until tumors reached above 1500mm^3. Once the first mouse within that particular treatment was euthanized, the survival rate was followed until the last tumor reached 1500 mm^3 and the mouse was euthanized.

Delivery of BMDM with the EMT6 tumor had little to no effect on tumor growth (Figure 2A). Although the mice that received EMT6 tumors with BMDM had smaller tumors compared to the control group, the difference was not significant. The survival rate for the control hit 0 at day 25, whereas the survival rate for the treatment group hit 0 at day 27 (Figure 2B). However, the difference in survival rates between the two groups was not statistically significant. These data show that the presence of unactivated macrophages did not impact tumor growth or survival of the tumor-bearing mice.

Effect of BMDM treated with a Single Stimuli on Tumor Progression and Survival in Mice Bearing EMT6 Tumors

To determine therapeutic potential of activated macrophages, BMDM were treated with IFN-γ, CD40L, or PIC and administered into mice with the EMT6 tumors. Mice that received EMT6 tumors with HBSS treated BMDM were considered the control group for this experiment. Seven days after the injection, the tumor growth and survival of the mice were followed.

The single agonist treated BMDM had no statistically significant effect on the tumor progression (Figure 3) even though mice that received PIC and CD40L treated BMDM had larger tumors compared to the control group (Figures 4A, 4B), and mice that received IFN-γ treated BMDM had smaller tumors compared to the control group (Figure 4C).

Interestingly, the survival rate for the control group was better than PIC and CD40L treatment groups, with the survival for both conditions reaching 0 on day 23 and the controls reaching 0 on day 25 (Figures 4D, 4E). Some of the mice that received IFN-γ treated BMDM survived to day 27 (Figure 4F). However, none of the treatment groups displayed a statistically significant difference in survival rate when compared to the control group (Figures 4D, 4E, 4F).

Effect of BMDM treated with Multiple Stimuli on Tumor Progression and Survival in Mice Bearing EMT6 Tumors

For BMDM treated with two stimuli, there was no significant decrease in tumor growth over time (Figure 5). While the mice that received IFN-γ+PIC treated BMDM and the CD40L+PIC treated BMDM had smaller tumors compared to the control group, the mice that received CD40L+IFN-γ treated BMDM had larger tumors than the control group (Figures 6A, 6B, 6C). None of the differences were statistically significant. However, the survival of the mice in all 3 treatment groups were significantly better than the controls. While the controls only survived to day 25, mice that received either CD40L+IFN-γ treated BMDM or IFN-γ+PIC treated BMDM survived to day 33 (Figures 6D, 6E). Mice that received CD40L+PIC treated BMDM survived to day 29 (Figure 6F).

For BMDM treated with the three stimuli, there was no impact on the tumor growth rate over time, but there was a statistically significant impact on survival. Although the mice that received IFN-γ+CD40L+PIC treated BMDM had smaller tumors at day 17, there was no significant difference compared to the control group (Figure 7A). There was a statistically significant difference in survival rate, as the mice that were treated with BMDM that received three stimuli lived significantly longer than the control group. Mice in the group that were given BMDM that received three stimuli survived to day 33, while the controls survived to day 25

(Figure 7B). Compared to the mice that received BMDM treated with two stimuli, mice that received BMDM treated with three stimuli had the greatest degree of statistical significance in regards to survival with a p-value of 0.010.

Effect of BMDM treated with Multiple Stimuli on Tumor Progression and Survival in Mice Bearing 4T1 Tumors

Due to the significant increase in survival rate for EMT6 tumor-bearing mice treated with BMDM stimulated with either two or three stimuli, those conditions were chosen as subsequent experiments for mice given 4T1 tumors. There was no statistically significant impact on 4T1 tumor growth rate over time (Figures 9A, 9B, 9C). Additionally, there was no statistically significant difference in survival rate for mice that received BMDM treated with variations of two stimuli. The controls survived to day 27. Mice that received CD40L+IFN-γ treated BMDM survived to day 29, mice that received IFN-γ+PIC treated BMDM survived to day 31, and mice that received CD40L+PIC treated BMDM survived to day 25 (Figures 9D, 9E, 9F). Overall, there was no statistically significant difference in tumor growth or survival rate for the two stimuli treatment groups bearing 4T1 tumors.

Within the group that received BMDM treated with three stimuli, there was no impact on the tumor growth rate over time, but there was a statistically significant impact on survival rate. Mice that received IFN-γ+CD40L+PIC treated BMDM survived to day 29 (Figure 10B). Up to day 25, the IFN-γ+CD40L+PIC treated BMDM group had 100% survival rate, which then dropped to 60% by day 25 and then below 25% by day 27 (Figure 10B). For the 4T1 tumor-bearing mice, the three stimuli treatment group was the only group that displayed a significant difference in survival rate when compared to the control group (Figures 9D, 9E, 9F, 10B).

Effect of BMDM treated with Three Stimuli on Lung Metastasis in Mice Bearing 4T1 Tumors

As a result of the statistically significant improvement in survival for mice that received 4T1 tumors with IFN-γ+CD40L+PIC treated BMDM, the condition was selected to observe the impact of the treatment on metastatic spread (Figure 11A, 11B). BALB/c mice were given 4T1 tumors with HBSS treated BMDM (control) or with IFN-γ+CD40L+PIC treated BMDM. After 21 days, the mice were sacrificed in order to count the number of metastatic colonies found in the lungs. Five out of the 8 mice for the IFN-γ+CD40L+PIC treatment group had fewer lung metastases than the control group, with two mice having 0 lung metastases (Figure 11B). There was an average of 87.1 ± 68.7 lung metastases in the control group while there was an average of 12.3 ± 23.7 lung metastasis in the IFN-γ+CD40L+PIC treatment group (Figure 11). The differences in metastasis between the control and the BMDM treated groups were statistically significant.

Discussion

Prior studies have found that mononuclear phagocytes acquire properties of pro-tumor macrophages in tumor microenvironments, promoting tumor proliferation, metastasis, immune suppression, and chemoresistance. Cytokines, like IL-4 and IL-10, in tumor microenvironments cause macrophages to switch from an M1 phenotype to an M2 phenotype (Odegaard and Chawla 2011; Nguyen *et al.* 2011). Hypoxia, the inadequate supply of oxygen, is induced by tumor and stromal cells can also cause macrophages to remain in the M2 state (Lewis *et al.* 2000). In response to hypoxia, the macrophages release vascular endothelial growth factor (VEGF-A), which plays a critical role in regulating angiogenesis, the formation of new blood vessels (Korsisaari *et al.* 2007). Additionally, these macrophages can secrete other pro-tumor mediators, including basic fibroblast growth factor, thymidine phosphorylase, and adrenomedullin (Chen *et*

al. 2011). Adremedullin acts similarly to VEGF-A, as it produces nitric oxide, which promotes blood vessel formation and growth factor production (Chen *et al.* 2014). Macrophages are believed to also indirectly affect the metastatic spread of tumor cells through modulation of the tumor microenvironment. As tumor cells synthesize CSF-1, a hemopoietic growth factor, it promotes migration of macrophages and secretion of epidermal growth factor to further enhance tumor cell invasion (Wyckoff *et al.* 2004). M2 macrophages are also suspected to suppress other immune responses in the tumor microenvironment through the release of cytokines, such as IL-10, CCL22, and TGF-β (Kryczek *et al.* 2006). With these cytokines present, the immune response of CD8+ T-cells, which bind and kill cancerous cells, is inhibited (Kryczek *et al.* 2006). Overall, macrophages have direct and indirect interaction with the tumor microenvironment, leading macrophages to release growth factors, cytokines, chemokines, and ECM components that promote tumor growth.

In a previous study using a triple-negative breast cancer mouse model, Steenbrugge *et al.* (2018) used intraductal tumor cell inoculations to investigate the interactions between 4T1 tumor cells and RAW 264.7 macrophages, a mouse macrophage cell line. They found that tumor cells produced high levels of the anti-inflammatory cytokine, IL-10, which induced the polarization of macrophages from an M1 to M2 phenotype (Steenbrugge *et al.* 2018). Observing increased intracellular production of TGF-β1, the M1 to M2 polarization hypothesis was confirmed because TGF-β1 is a cytokine that is secreted by M2 macrophages (Steenbrugge *et al.* 2018). Thus, the decreased production of the M1-related cytokine IL-12 and the increased production of the M2-related TGF-β1 by RAW 264.7 macrophages supported the M1 to M2 polarization hypothesis, as these macrophages supported tumor progression and metastatic spread into the axillary lymph nodes (Steenbrugge *et al.* 2018).

To investigate the M1 to M2 polarization hypothesis with BMDM, BALB/c mice were initially given EMT6 tumors with and without non-polarized BMDM. Although there was no significant difference in tumor growth or survival rate, the mice with non-polarized BMDM had smaller EMT6 tumors and died sooner than the mice without BMDM. With prior studies suggesting that BMDM in tumor microenvironments are pro-tumor (Chanmee *et al.* 2014; Steenburgge *et al.* 2018), the small difference in survival rates between mice bearing EMT6 tumors with and without non-polarized BMDM may be explained by the unactivated macrophages displaying an M2 phenotype. Later in the discussion, I touch upon various methods that could be used in future studies to test this hypothesis, using either gene expression analysis, assessment of cytokine and chemokine production, or expression of cell surface markers.

This study worked to manipulate macrophages toward the M1/anti-tumor phenotype through a combination of pro-inflammatory stimuli. I examined how IFN-γ, CD40L, and PIC influenced macrophages and impacted their anti-tumor activity. I predicted that macrophages treated with these stimuli would express M1 functions, suppressing growth of tumors and increasing survival of tumor-bearing mice. I further hypothesized that there would be a significant difference in tumor growth and survival for the mice that received BMDM treated with multiple stimuli compared to a single stimulus. Findings from the survival rates of mice bearing EMT6 and 4T1 tumors support my hypothesis. Mice that received EMT6 tumors and BMDM treated with a combination of two and three stimuli showed reduced tumor progression and enhanced survival. BMDM treated with all three stimuli also decreased the growth of 4T1 tumors and significantly prolonged survival of the 4T1 tumor-bearing mice.

To confirm that the BMDM treated with two or three stimuli that impacted the survival of tumor-bearing mice were M1 macrophages, additional experiments that assess macrophage

phenotype, such as gene expression analysis (Jablonski *et al.* 2015), assessment of cytokine and chemokine production (Luo *et al.* 2006), and analysis of protein expression and cell surface markers (Qian *et al.* 2009), would be useful. Jablonski *et al.* (2015) performed an analysis of the transcriptional signatures of murine BMDM, confirming results by real-time polymerase chain reaction (PCR) and flow cytometry. They found that the polarization of M1 and M2 macrophages can be distinguished based on the expression of CD38 and Early growth response protein 2 (Egr2), as the results indicated that CD38 labels 71% of M1 macrophages and Egr2 labels 70% of M2 macrophages (Jablonski *et al.* 2015). CD38 gene is upregulated in response to pro-inflammatory stimuli and encodes a transmembrane glycoprotein that is involved the regulation of macrophage phagocytosis and removal of apoptotic cells (Jablonski *et al.* 2015). Egr2 is upregulated in response to anti-inflammatory stimuli and encodes a transcription factor that is involved in cell cycle progression, cell transformation, and clearance of apoptotic cells (Jablonski *et al.* 2015). Jablonski *et al.* (2015) validated the findings from the gene expression analysis through flow cytometry, which was also capable of distinguishing M1 and M2 macrophages based on the expression of CD38 and Egr2. Based on these findings, future studies could study CD38 and Egr2 in order to determine whether BMDM treated with two or three stimuli are M1 or M2 macrophages. This could be studied by collecting macrophages from the tumor sites after injections for RNA isolation and flow cytometry. Real-time PCR can be used to determine CD38 and Egr2 gene expression found within the mice that received stimulated BMDM. Macrophages could also be strained with antibodies specific for CD38 and Egr2 for the flow cytometric analysis. If CD38 is upregulated and Egr2 is not in BMDM treated with two or three stimuli, then this would support that these are M1 macrophages. Another method that could be used to determine whether the macrophages are M1 or M2 would be to use RNA sequencing,

looking for significant differences in *Cd38* for CD38 and *Knox20* for Egr2 gene expression. By collecting macrophages found in these tumor microenvironments and sequencing the cells' mRNA, the results from these experiments would further validate whether the BMDM treated with the different stimuli are M1 or M2 macrophages.

Another method that could be used to determine whether the stimuli treated BMDM are M1 macrophages would be to look at interleukin 1 beta (IL-1β) production. Using supernatants from the treated BMDM, the expression of IL-1β can be assessed through enzyme-linked immunosorbent assay (ELISA). IL-1β is considered to be a central mediator for innate immunity, specifically in response to inflammation (Pelegrin and Suprenant 2017). Previous studies have found high levels of IL-1β present in M1 polarized macrophages due to the activation of the NF-κB and MAPK cascades (Dinarello 1996; Martinez *et al.* 2006). On the contrary, M2 polarized macrophages do not produce IL-1β (Scotton *et al.* 2005). The release of IL-1β is a regulated process that involves a multiprotein complex, caspase-1 inflammasome, which is only found in stimulated M1 macrophages, not unstimulated M1 macrophages or M2 macrophages (Pelegrin and Suprenant, 2017). The expression of IL-1β and activation of M1 macrophages are closely linked, creating a continual feedback loop (Pelegrin and Suprenant 2017). If IL-1β is overexpressed by BMDM treated with IFN-γ, CD40L, and PIC, then the results would further support that these treatments create M1 macrophages.

Previous breast cancer studies have observed the antimetastatic effects of M1 macrophages, citing a decrease in tumor-associated angiogenesis and fibrosis as well as an increase in phagocytosis (Ahirwar *et al*. 2021). Thus, in addition to observing tumor progression and survival in mice bearing EMT6 and 4T1 tumors, the number of metastatic colonies was analyzed from the lungs of mice that were injected with 4T1 tumors. This part of the thesis was

to determine if activated macrophages impacted the metastasis of cancer to the lungs, which would signify the BMDM were functioning as M1 macrophages. The number of lung metastases was significantly higher in the control group than the treatment group. Five out of the eight mice that were given BMDM treated with IFN-γ+CD40L+PIC had fewer lung metastasis than the control group. Two out of those five mice had no metastasis. This finding demonstrates that IFN-γ+CD40L+PIC treated macrophages decreased metastasis and functioned as M1 macrophages.

While BMDM that were exposed to IFN-γ, CD40L, and PIC did not affect the tumor progression in mice bearing EMT6 and 4T1 tumors, the combination of two or three stimuli contributed to enhanced survival of mice bearing EMT6 tumors and all three stimuli contributed to the survival rates of mice bearing 4T1 tumors. In mouse breast cancer models, previous studies have found a negative correlation between tumor progression and survival rate when targeting tumor-associated macrophages (Luo *et al.* 2006; Steenbrugge *et al.* 2018; Binnemars-Postma *et al.* 2018). The lack of correlation between tumor growth and increased survival within my study may be due to the design of the study. Although each treatment group had between six to eleven mice, the entire experiment was only conducted one time and the tumor volumes within each treatment group showed a great set of variation. In future studies, a larger sample size is necessary; ten mice per group, two biological replicates would be beneficial, as the larger sample size would allow outliers and mice that experienced premature death to be removed while still receiving an adequate sample size for statistical analysis.

Prior studies have found each stimulus used in my study effective in reducing tumor growth and metastasis by itself (An *et al.* 1996; Fang *et al*. 2022; Zhu *et al.* 2014). Although the results from these studies found that each treatment impeded the growth of tumors (An *et al.* 1996; Fang *et al*. 2022; Zhu *et al.* 2014), the methods and cell lines from these studies were

different from my project. Each study used discrete mechanisms to deliver IFN-γ, CD40L, and PIC to the TME, whether it was stem cells (Zhu *et al.* 2014) or nanoparticles (Fang *et al*. 2022). For my thesis, I mixed the stimulated BMDM with tumor cells before injecting them into the mice. It is possible the M1 macrophages were converted to M2 macrophages within the tumor microenvironment, leading to a decrease in efficacy. In order to explore other methods of IFN-γ, CD40L, and PIC delivery, future studies could try using nanoparticles to stimulate macrophages found within the tumor microenvironment and the results could be compared to the BMDM to evaluate the most effective method for decreasing tumor progression.

The results of my study also showed that the impact of BMDM treated mice differed depending on the tumor type. Mice bearing EMT6 tumors survived longer when treated with BMDM that received two stimuli, while survival of 4T1 tumor-bearing mice only occurred when the mice were treated with BMDM that received all three stimuli (Figure 10B). These results were not anticipated because EMT6 tumor cells secretes high levels of IL-6 and transformation growth factor-β1 (TGF-β), which promotes polarization toward M2 phenotype (Maxwell 2016). Consequently, one would expect EMT6 to be harder to treat than 4T1 tumors. The same study found 4T1 tumor cells secreting high levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), which promotes polarization toward M1 phenotype (Maxwell 2016). Based on these results, the cytokine release by 4T1 would be expected to polarize macrophages to M1, making two stimuli an ample amount to improve survival of mice bearing those tumors. This was, however, not what my study showed, as 4T1 were harder to treat than EMT6 tumor-bearing mice. The results of my project may have been affected by the aggressiveness and invasiveness of 4T1 tumors (Maxwell 2016).

There may not have been enough BMDM delivered to the tumors to offset the invasiveness of 4T1 tumors (He *et al.* 2022).

Another study found variation in macrophage polarization between estrogen receptor-positive (ER+) and triple-negative breast cancer (TNBC) cell lines (Hollmen *et al.* 2015). For TNBC cells, the stimulated macrophages down-regulated citrulline metabolism and differentiated into M2-like phenotype, displaying an increased MMR protein expression and CCL2 secretion (Hollmen *et al.* 2015). CCL2 further stimulates the recruitment of monocytes to the tumor microenvironment and promotes of tumor growth (Hollmen *et al.* 2015). EMT6 cell lines are ER+ and 4T1 cell lines are TNBC. The biological differences between the two tumor types may also explain the differences in the impact of BMDM that was found in my study. Even if the BMDM treated with two stimuli expressed M1 functions originally in the 4T1 tumors, the macrophages may have been repolarized to display the opposite effect, promoting the proliferation of cancer cells. The three stimuli treatment may have fully reinforced the M1 phenotype, causing there to be a significance in survival for BMDM treated with three stimuli and no significance for BMDM treated with two stimuli.

Immunotherapy, such as using macrophages to fight cancer, has several advantages over other cancer treatments (Aminin and Wang 2021). It displays high specificity and low side effects, enabling cooperation with other conventional therapies (Aminin and Wang 2021). The invasiveness of the procedure is also minimal compared to other therapeutic strategies, as it only involves collecting TAMs, modifying the macrophages, and then reintroducing the macrophages (Aminin and Wang 2021). Additionally, clinical studies have found significant success with postoperative treatments as well, preventing metastases and recurrences (Aminin and Wang 2021). Macrophage polarization may also impact racial disparities found in breast cancer

incidence, as studies have found that a patient's race is significantly associated with the number and the types of TAMs found in tumor microenvironments (Koru-Sengul *et al.* 2016). Among Black, Hispanic, and non-Hispanic White patients, Black women have the highest number of TAMs detected in tumors that were associated with macrophage proliferation, a M2 function (Koru-Sengul *et al.* 2016). The potential application of M1 polarization may address breast cancer disparities in minority race groups, as M1 killing activities may decrease the number of M2 TAMs and increase survival in all racial groups (Koru-Sengul *et al.* 2016).

The results of this project demonstrate IFN-γ, CD40L, and PIC having immunomodulatory activity upon TAMs, as combinations of the stimuli prolonged the survival of mice bearing EMT6 and 4T1 tumors. Overall, macrophages stimulated with multiple agonists have significant potential in becoming an immunotherapeutic option for patients with breast cancer as highlighted by my study.

Limitations

The design of this project may have allowed for some bias within the data collecting process. Due to lab constraints, the experiment could not have been conducted as a double-blind study. In the future, different students should stimulate the BMDM, inject the mice, measure the tumors, and analyze the data. By following this procedure, it may help decrease bias when collecting and evaluating the data. Future experiments should also use ten mice per group and conduct two biological replicates.

Conclusion

With current studies demonstrating macrophages playing an important role in TMEs, wavering between pro- and anti-tumor phenotypes, a further understanding of macrophage polarization is needed for the success of tumor immunotherapy (Pan *et al.,* 2020). In this study, the findings demonstrated anti-tumor functions of BMDM treated with multiple stimuli, slowing EMT6 and 4T1 tumor progression as well as increasing survival rate. The implications of these data demonstrate an immunotherapy approach to breast cancer treatment involving the activation of M1 macrophages.

Acknowledgements

I would like to express appreciation to my thesis advisor, Professor Kurt. Besides being an instrumental mentor for my research conducted at Lafayette College, he also introduced me to two of my science passions– immunology and cancer biology.

Towards the end of my first-year, I felt tremendously apprehensive about my future with biology, especially not being placed into BIOL 113 during my sophomore fall. Thank you for allowing me to take BIOL 245 during this time; it became a pivotal moment in my life. I would not be completing a thesis without that opportunity. Thank you again for the consistent encouragement and advice throughout this honors thesis journey, words cannot express my gratitude.

An additional thank you to my committee members, Dr. Mitchell, Dr. Ospina-Giraldo, and Dr. Shaw, your knowledge and expertise on how to conduct and create a publishable scientific paper was tremendously helpful. My thesis would not have been completed without our bi-monthly meetings– your feedback was essential to my success. I want to also thank Professor Ho for helping me to organize and display my large dataset. Thanks should also go to Pippa Moody, our organismal support specialist, who ensured the well-being of my mice when I was unable to do so.

Finally, I would like to thank my lab cohort members, Thalia and Josh, for collecting different parts of my data. Without their generosity and moral support, I would not have been able to successfully conduct this project.

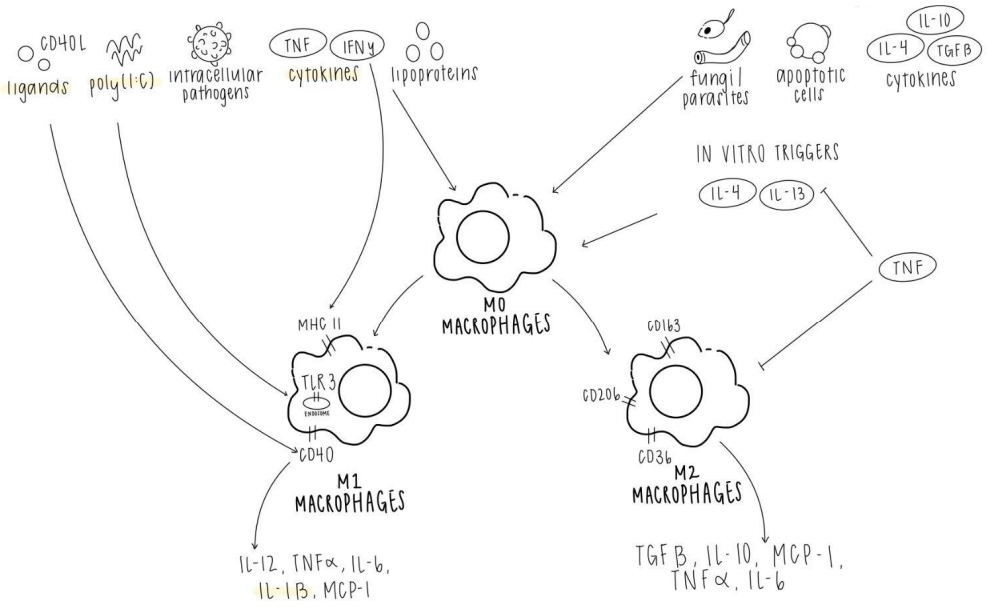

Figure 1: M1 and M2 Macrophage Polarization
Macrophages are differentiated into M1 macrophages in response to stimulation with pathogen-associated molecular patterns (PAMPs) such as cytokines, lipopolysaccharides, and ligands. M1 macrophages have high expression of the following receptors: MHC II, TLR3, and CD40. M1 macrophages produce nitric oxide synthase, reactive oxygen species, IL-β, IL-6, IL-12, TNFα, and MCP-1. Macrophages are differentiated into M2 macrophages in response to stimulation with cytokines and growth factors such as IL-4, TGF-β, IL-10, and IL-13. M2 macrophages have high expression of the following receptors: CD36, CD163, and CD206. M2 macrophages produce TGF-β, IL-6, IL-10, MCP-1, and TNFα. (Ahmed and Ismail, 2020)

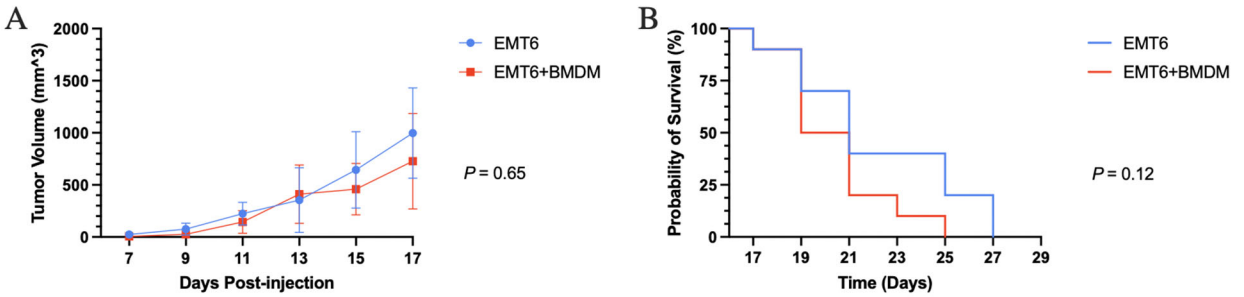

Figure 2: Impact of BMDM on growth and survival of EMT6 tumor-bearing mice
To determine if BMDM had an effect on the survival of mice bearing EMT6 tumors, BALB/c mice were given EMT6 tumors with (n=10) or without (n=6) BMDM. A. Tumor growth was measured starting 7 days after injections and continued every two-three days. The data shows the mean tumor volumes and the standard error of mice that grew tumors. B. Survival was followed after the first mouse within each group was euthanized (started at day 17) and continued until the last mouse within each group was euthanized.

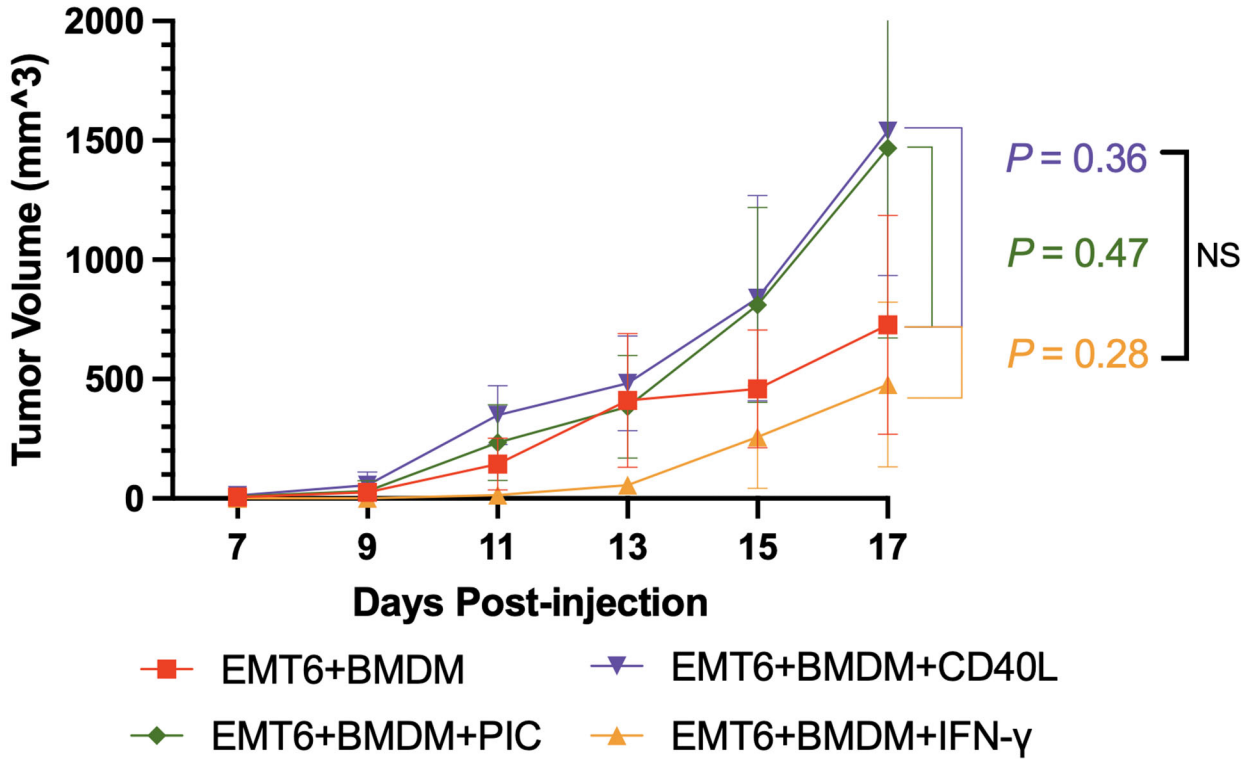

Figure 3: Impact of BMDM treated with one stimuli on EMT6 tumor growth
To determine if BMDM treated with one stimuli had an effect on the progression of EMT6 tumors, the tumor volume was measured starting 7 days after injections and continued every two-three days. Tumor progression was followed until the first tumor from the condition reached above 1500 mm^3, which was day 17. The data shows the mean tumor volumes and the standard error of mice that grew tumors. NS = Not significant (Student's t-test, unequal variances)

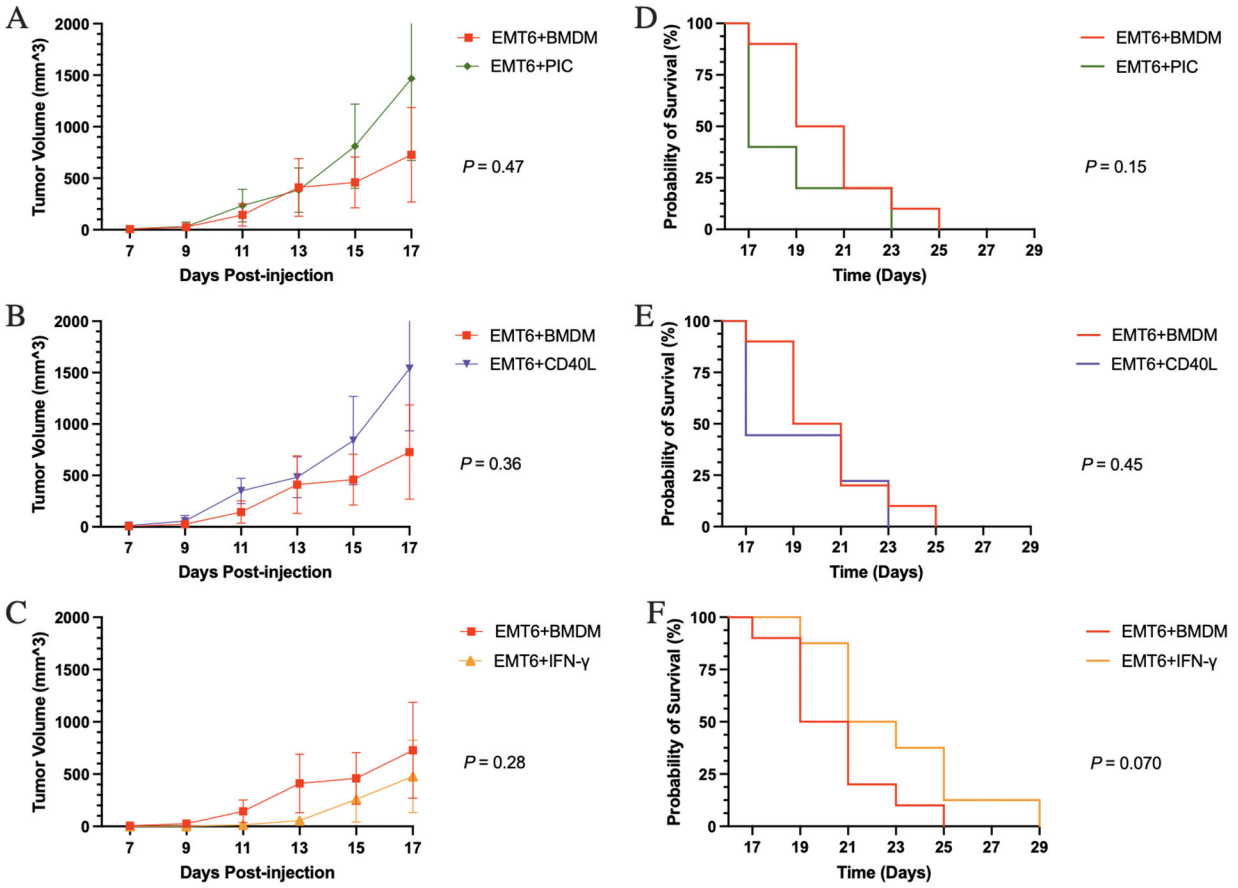

Figure 4: Impact of BMDM treated with one stimuli on growth and survival of EMT6 tumor-bearing mice
To determine if BMDM treated one stimuli had an effect on the survival of mice bearing EMT6 tumors, BALB/c mice were given BMDM treated with PIC (n = 9) (A), CD40L (n = 9) (B), or IFN-γ (n = 8) (C) and compared to the control (n = 10). A-C. The data shows the mean tumor volumes and the standard error of mice that grew tumors. D-F. Survival was followed after the first mouse within each group was euthanized (started at day 17) and continued until the last mouse within each group was euthanized.

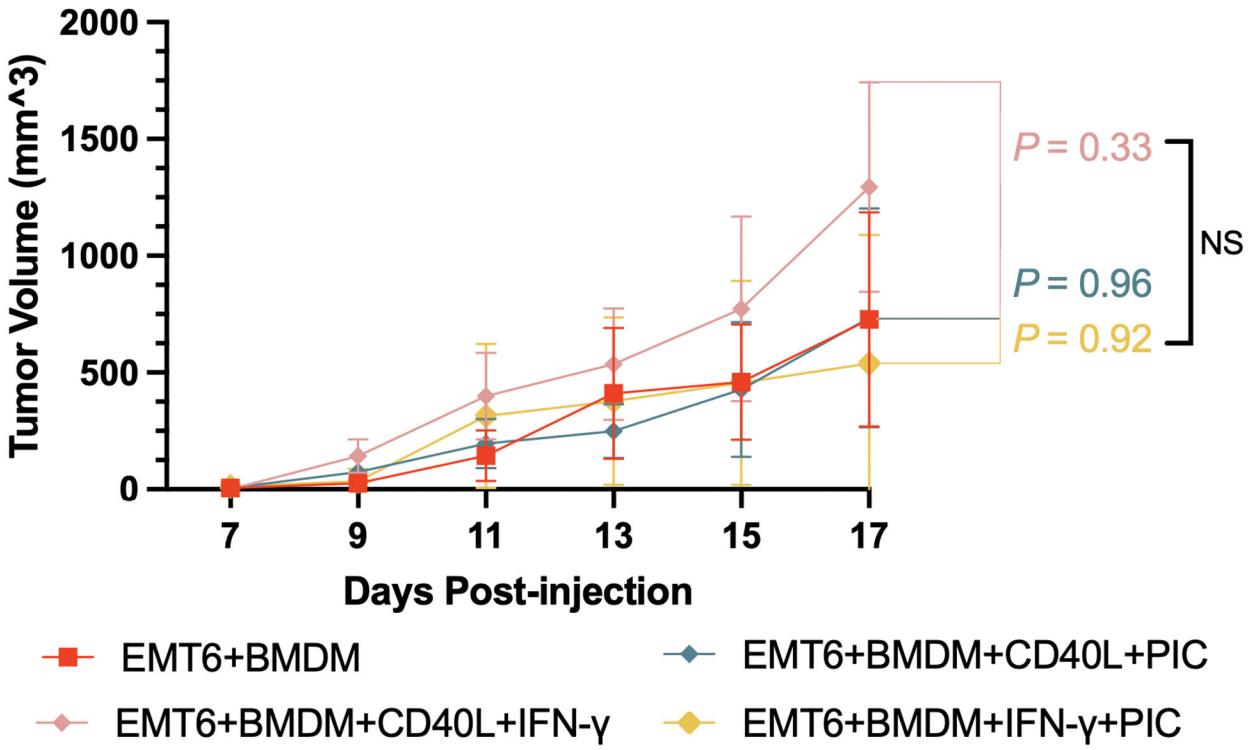

Figure 5: Impact of BMDM treated with two stimuli on EMT6 tumor growth
To determine if BMDM treated with two stimuli had an effect on the progression of EMT6 tumors, the tumor volume was measured starting 7 days after injections and continued every two-three days. Tumor progression was followed until the first tumor from the condition reached above 1500 mm^3, which was day 17. The data shows the mean tumor volumes and the standard error of mice that grew tumors. NS = Not significant (Student's t-test, unequal variances)

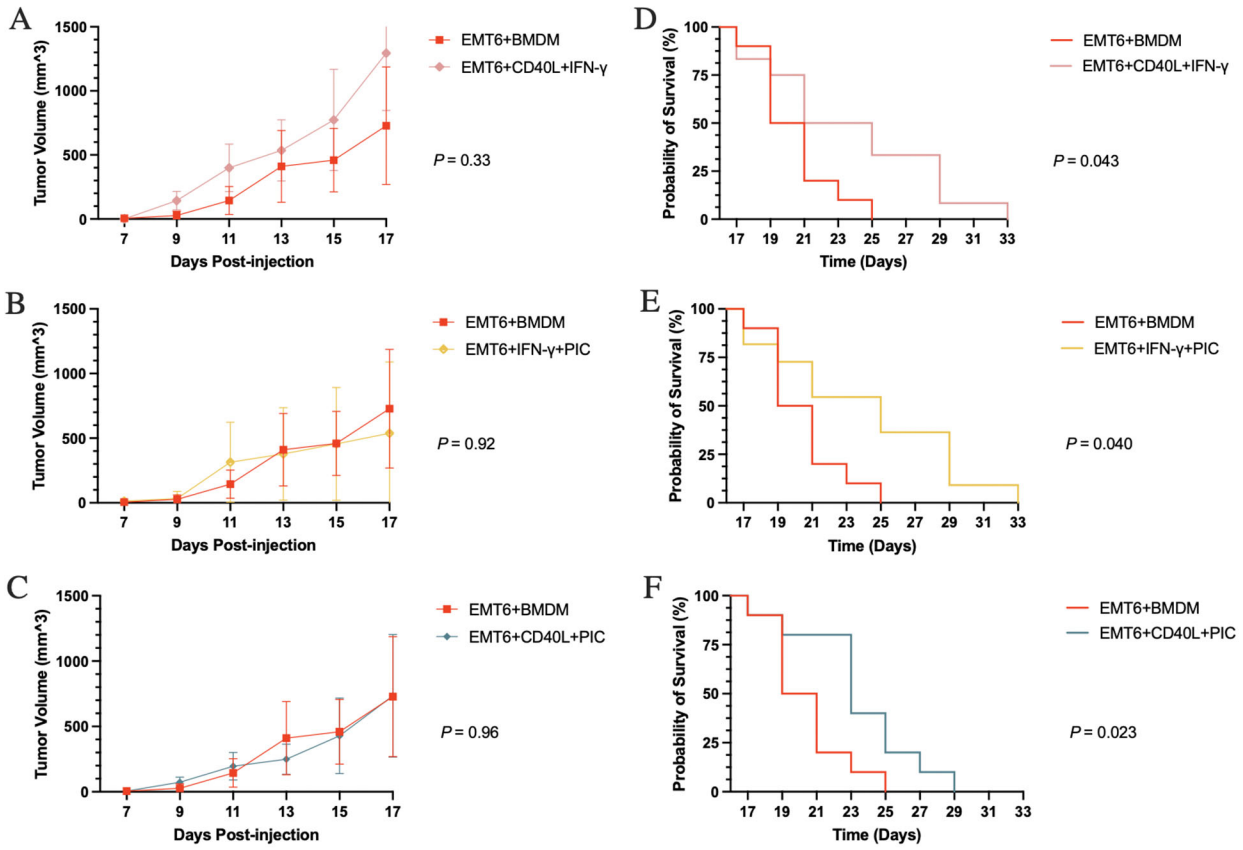

Figure 6: Impact of BMDM treated with two stimuli on growth and survival of EMT6 tumor-bearing mice
To determine if BMDM treated with two stimuli had an effect on the survival of mice bearing EMT6 tumors, BALB/c mice were given BMDM treated with CD40+IFN-γ (n = 10) (A), IFN-γ+PIC (n = 11) (B), or CD40+PIC (n = 10) (C) and compared to the control (n = 10). A-C. The data shows the mean tumor volumes and the standard error of mice that grew tumors. D-F. Survival was followed after the first mouse within each group was euthanized (started at day 17) and continued until the last mouse within each group was euthanized.

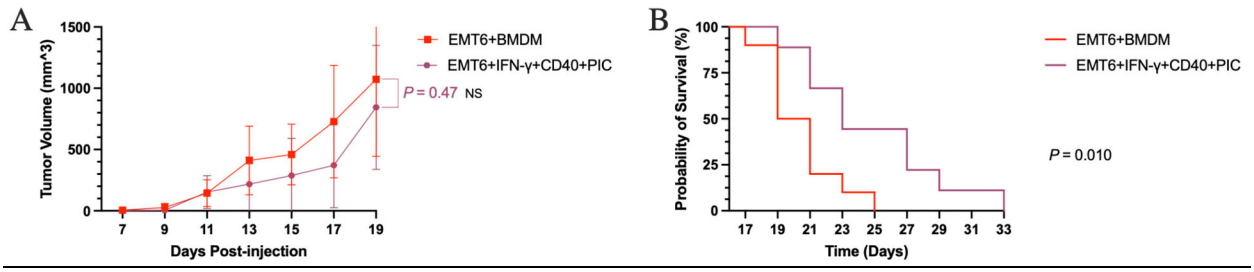

Figure 7: Impact of BMDM treated with three stimuli on growth and survival of EMT6 tumor-bearing mice
To determine if BMDM treated with three stimuli had an effect on the survival of mice bearing EMT6 tumors, BALB/c mice were given BMDM with IFN-γ+CD40+PIC (n = 9) and compared to the control (n = 10). A. The data shows the mean tumor volumes and the standard error of mice that grew tumors. NS = Not significant B. Survival was followed after the first mouse within each group was euthanized and continued until the last mouse within each group was euthanized. Euthansia started for the control group at day 17 and the IFN-γ+CD40+PIC group at day 19. $p < 0.05$ (Mantel-Cox test)

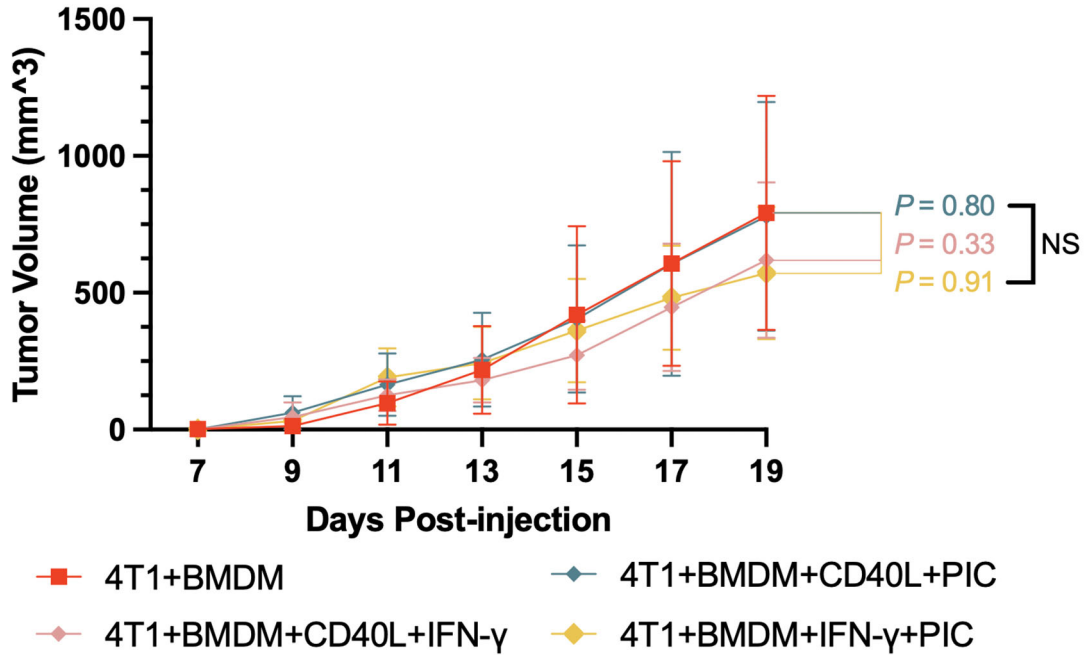

Figure 8: Impact of BMDM treated with two stimuli on 4T1 tumor growth
To determine if BMDM treated with two stimuli had an effect on the progression of 4T1 tumors, the tumor volume was measured starting 7 days after injections and continued every two-three days. Tumor progression was followed until the first tumor from the condition reached above 1500 mm^3, which was day 19. The data shows the mean tumor volumes and the standard error of mice that grew tumors. NS = Not significant (Student's t-test, unequal variances)

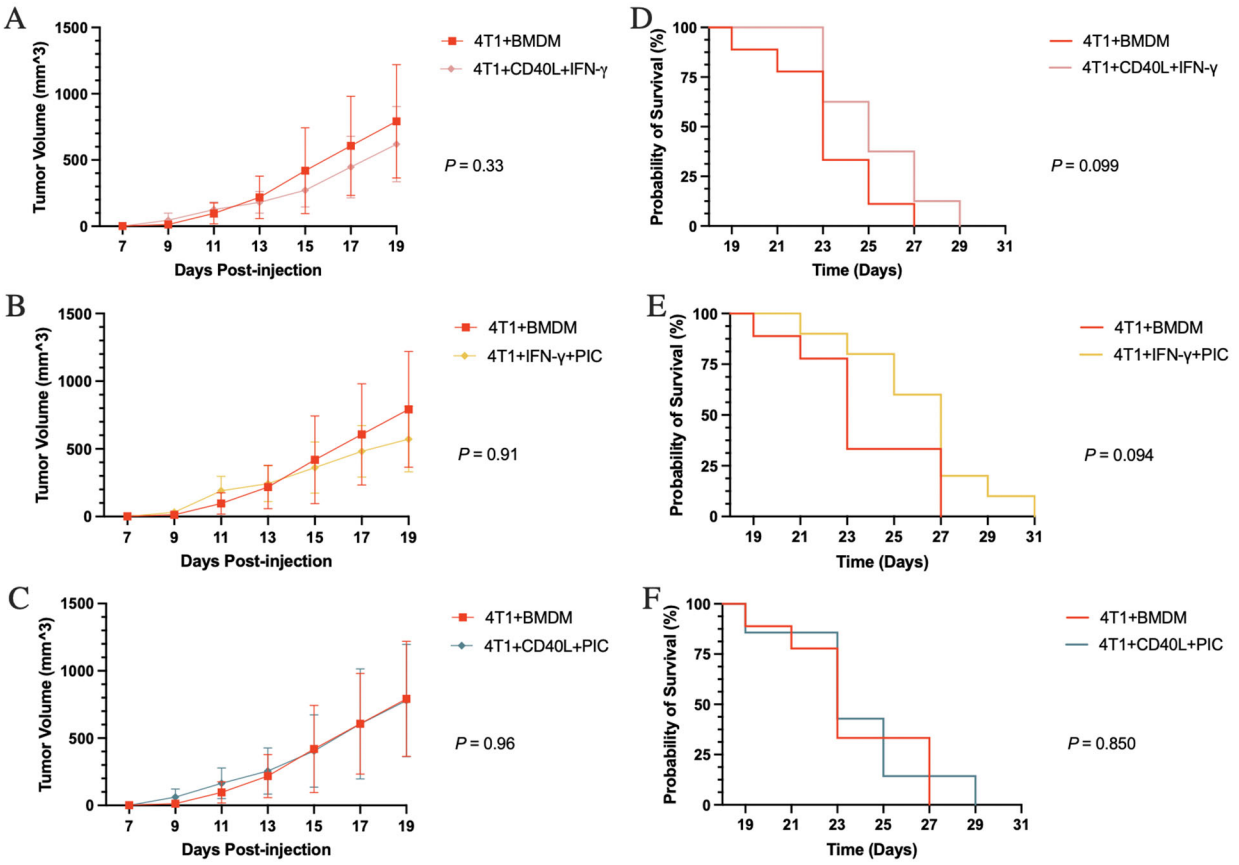

Figure 9: Impact of BMDM treated with two stimuli on growth and survival of 4T1 tumor-bearing mice
To determine if BMDM treated with two stimuli had an effect on the survival of mice bearing 4T1 tumors, BALB/c mice were given BMDM treated with CD40+IFN-γ (n = 8) (A), IFN-γ+PIC (n = 10) (B), or CD40+PIC (n = 7) (C) and compared to the control (n = 9). A-C. The data shows the mean tumor volumes and the standard error of mice that grew tumors. D-F. Survival was followed after the first mouse within each group was euthanized and continued until the last mouse within each group was euthanized. $p < 0.05$ (Mantel-Cox test)

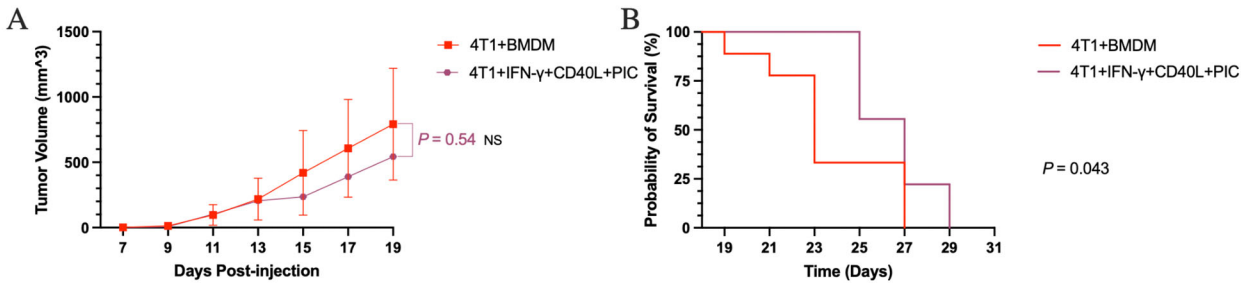

Figure 10: Impact of BMDM treated with three stimuli on growth and survival of 4T1 tumor-bearing mice
To determine if BMDM treated with three stimuli had an effect on the survival of mice bearing 4T1 tumors, BALB/c mice were given BMDM with IFN-γ+CD40+PIC (n = 9) and compared to the control (n = 9). A. The data shows the mean tumor volumes and the standard error of mice that grew tumors. NS = Not significant B. Survival was measured after the first mouse within each group was euthanized and continued until the last mouse within each group was euthanized. Euthansia started for the control group at day 19 and the IFN-γ+CD40+PIC group at day 25. $p < 0.05$ (Mantel-Cox test)

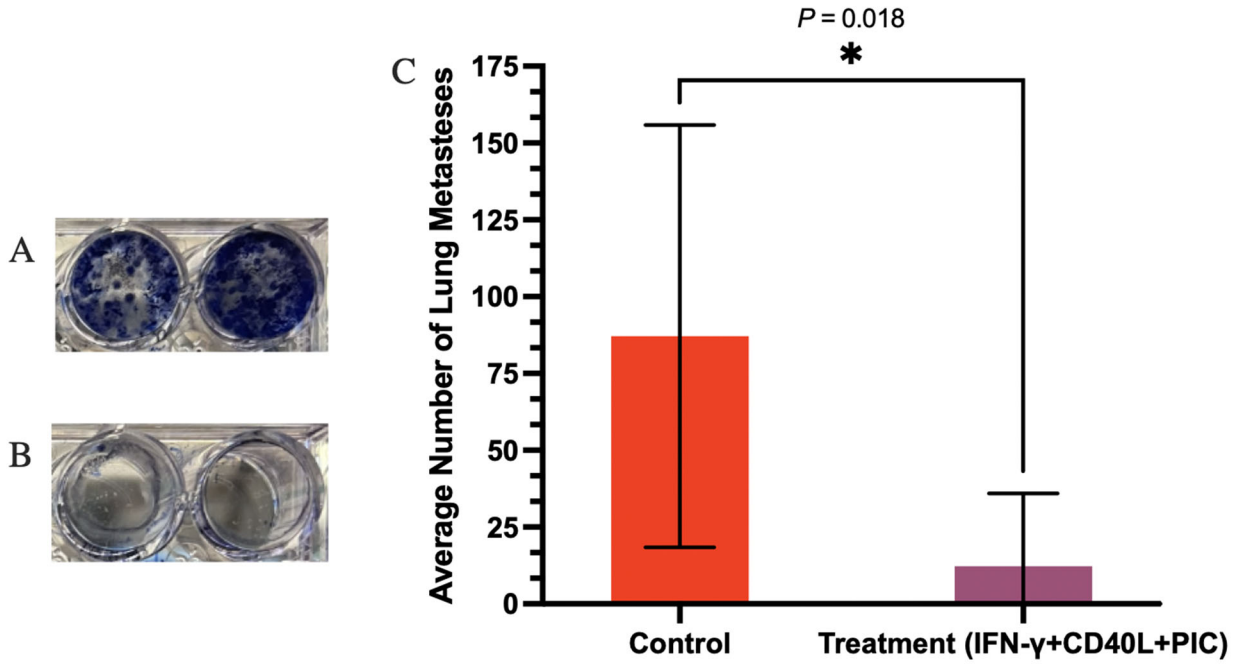

Figure 11: Analysis of lung metastases in 4T1 tumor-bearing mice
To determine if BMDM treated with three stimuli had an effect on the metastatic spread in mice bearing 4T1 tumors, BALB/c mice were given BMDM treated with IFN-γ+CD40+PIC (n = 8) and compared to the control (n = 8). After 21 days, the lungs of the mice were harvested to analyze lung metastasis. A. Two wells plated from the 1/10-fold dilution from the control group. B. Two wells plated from the 1/10-fold dilution from the treatment group. C. The data shows the average and standard deviation of the control and treatment group. *$p < 0.05$ (Student's t-test, unequal variances)

References

Ahmed, I. & Ismail, N. (2020). M_1 and M_2 Macrophages Polarization via $mTORC_1$ Influences Innate Immunity and Outcome of *Ehrlichia* Infection. *Journal of Cellular Immunology, 2(3)*, 108-115. https://doi.org/10.33696/immunology.2.029

Ahirwar D.K., Charan M., Mishra S., Verma A.K., Shilo K., Ramaswamy B., Ganju R.K. (2021). Slit2 Inhibits Breast Cancer Metastasis by Activating M1-Like Phagocytic and Antifibrotic Macrophages. *Cancer Res*, 20, 5255-5267. https://pubmed.ncbi.nlm.nih.gov/34400395/

Alexander, C., & Rietschel, E. T. (2001). Bacterial lipopolysaccharides and innate immunity. *Journal of endotoxin research*, *7*(3), 167–202.

Aminin, D. & Wang Y.M. (2021). Macrophages as a "weapon" in anticancer cellular immunotherapy. *The Kaohsiung Journal of Medical Sciences,* 37(9), 749-758. https://doi.org/10.1002/kjm2.12405

An, Z., Wang, X., Astoul, P., Danays, T., Moossa, A.R., & Hoffman, R.M. (1996). Interferon Gamma is Highly Effective Against Orthotopically-Implanted Human Pleural Adenocarcinoma in Nude Mice. *Anticancer Research*, 16, 2546-2552. http://www.metamouse.com/links/Lu11.pdf

Bianchini, G., Balko, J.M., Mayer, I.A., Sanders, M.E., & Gianni, L. (2016). Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. *Nature Reviews Clinical Oncology,* 13, 674-690. https://doi.org/10.1038/nrclinonc.2016.66

Binnesmars-Postma, K., Bansal, R., Storm, G., & Prakash J. (2018). Targeting the Stat6 pathway in tumor-associated macrophages reduces tumor growth and metastatic niche formation in breast cancer. *The FASEB Journal*, 32(2), 969-978. https://doi.org/10.1096/fj.201700629R

Boutilier, A., & Elsawa, S. (2021). Macrophage Polarization States in the Tumor Microenvironment. *International Journal of Molecular Sciences,* 22(13). https://doi.org/10.3390/ijms22136995

Cancer.Net. (2022). Breast Cancer: Statistics. https://www.cancer.net/cancer-types/breast-cancer/statistics

Cendrowicz, E., Sas, Z., Bremer, E., & Rygiel, T. P. (2021). The Role of Macrophages in Cancer Development and Therapy. *Cancers*, 13(8), 1946. https://doi.org/10.3390/cancers13081946

Chanmee, T., Ontong, P., Konno, K., & Itano N. (2014). Tumor-Associated Macrophages as Major Players in the Tumor Microenvironment. *Cancers,* 6(3), 1670-1690. https://doi.org/10.3390/cancers6031670

Charan, J. & Kantharia, N.D. (2013). How to calculate sample size in animal studies? *Journal of Pharmacology & Pharmacotherapeutics*, 4(4), 303-306. https://doi.org/10.4103/0976-500X.119726

Chen, P., Huang, Y., Bong, R., Ding, Y., *et al*. (2011). Tumor-associated macrophages promote angiogenesis and melanoma growth via adrenomedullin in a paracrine and autocrine manner. *Clinical cancer research : An official journal of the American Association for Cancer Research*, *17*(23), 7230–7239. https://doi.org/10.1158/1078-0432.CCR-11-1354

Cheng, Y. S., & Xu, F. (2010). Anticancer function of polyinosinic-polycytidylic acid. *Cancer biology & therapy*, 10(12), 1219–1223. https://doi.org/10.4161/cbt.10.12.13450

Debien, V., *et al.* (2023). Immunotherapy in breast cancer: an overview of current strategies and perspectives. *npj Breast Cancer*, 9(7). https://doi.org/10.1038/s41523-023-00508-3

Djureinovic, D., Wang, M., & Klunger H.M. (2021) Agonistic CD40 Antibodies in Cancer Treatment, *Cancer, 13*, 1302. https:// doi.org/10.3390/cancers13061302

Egea, L., Hirata, Y., & Kagnoff, M. F. (2010). GM-CSF: a role in immune and inflammatory reactions in the intestine. *Expert review of gastroenterology & hepatology*, *4*(6), 723–731. https://doi.org/10.1586/egh.10.73

Elgueta, R., Benson, M. J., de Vries, V. C., Wasiuk, A., Guo, Y., & Noelle, R. J. (2009). Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunological reviews*, *229*(1), 152–172. https://doi.org/10.1111/j.1600-065X.2009.00782.x

Guo, L., *et al.* (2019). *Induction of Breast Cancer Stem Cells by M1 Macrophages through Lin-28B-Let-7-HMGA2 Axis*. *Cancer Letter, 452,* 213-225. https://doi.org/10.1016/j.canlet.2019.03.032

Hachim M.Y., Hachim I.Y., Talaat I.M., Yakout N.M., & Hamoudi R. (2020). M1 Polarization Markers Are Upregulated in Basal-Like Breast Cancer Molecular Subtype and Associated With Favorable Patient Outcome. *Front. Immunol, (11)*, 560074. doi: 10.3389/fimmu.2020.560074

Hollmén, M., Roudnicky, F., Karaman, S., & Detmar, M. (2015). Characterization of macrophage--cancer cell crosstalk in estrogen receptor positive and triple-negative breast cancer. *Scientific reports*, *5*, 9188. https://doi.org/10.1038/srep09188

Hong, Y., Lee, J., Vu, T. H., Lee, S., Lillehoj, H. S., & Hong, Y. H. (2021). Immunomodulatory effects of poly(I:C)-stimulated exosomes derived from chicken macrophages. *Poultry science*, *100*(8), 101247. https://doi.org/10.1016/j.psj.2021.101247

Jablonski, K.A., *et al.* (2015). Novel Markers to Delineate Murine M1 and M2 Macrophages. *PLOS ONE*, 10(12). https://doi.org/10.1371/journal.pone.0145342

Jorgovanovic, D., Song, M., Wang, L., & Zhang, Y. (2020). Roles of IFN-γ in tumor progression and regression: A Review. *Biomarker Research*, *8*(1). https://doi.org/10.1186/s40364-020-00228-x

Kak, G., Raza, M. & Tiwari, B. (2018). Interferon-gamma (IFN-γ): Exploring its implications in infectious diseases. *Biomolecular Concepts*, *9*(1), 64-79. https://doi.org/10.1515/bmc-2018-0007

Korsisaari, N., Kasman, I. M., Forrest, W. F., Pal, N., Bai, W., *et al*. (2007). Inhibition of VEGF-A prevents the angiogenic switch and results in increased survival of Apc+/min mice. *Proceedings of the National Academy of Sciences*, *104*(25), 10625-10630. https://doi.org/10.1073/pnas.0704213104

Koru-Sengul, T., Santander, A. M., Miao, F., Sanchez, L. G., Jorda, M., Glück, S., Ince, T. A., Nadji, M., Chen, Z., Penichet, M. L., Cleary, M. P., & Torroella-Kouri, M. (2016). Breast cancers from black women exhibit higher numbers of immunosuppressive macrophages with proliferative activity and of crown-like structures associated with lower survival compared to non-black Latinas and Caucasians. *Breast cancer research and treatment*, *158*(1), 113–126. https://doi.org/10.1007/s10549-016-3847-3

Kryczek, I., Zou, L., Rodriguez, P., Zhu, G., *et al*. (2006). B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. *The Journal of experimental medicine*, *203*(4), 871–881. https://doi.org/10.1084/jem.20050930

Lewis, J.S., Landers, R.J., Underwood, J.C., Harris, A.L., & Lewis, C.E. (2000). Expression of vascular endothelial growth factor by macrophages is up-regulated in poorly vascularized areas of breast carcinomas. *J. Pathol. 192*, 150–158.

Li, C., Xu, X., Wei, S., Jiang, P., Xue, L., Wang, J., & Senior Correspondence (2021). Tumor-associated macrophages: potential therapeutic strategies and future prospects in cancer. *Journal for immunotherapy of cancer*, *9*(1), e001341. https://doi.org/10.1136/jitc-2020-001341

Locati, M., Curtale, G., & Mantovani A. (2020). Diversity, Mechanisms, and Significance of Macrophage Plasticity. *Europe PubMed Central, 15*, 123-147. https://doi.org/10.1146/annurev-pathmechdis-012418-012718

Luo, Y., *et al.* (2006). Targeting tumor-associated macrophages as a novel strategy against breast cancer. *The Journal of Clinical Investigation*, 116(8): 2132-2141. https://doi.org/10.1172/JCI27648

Martinez F. & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000 Prime Reports, 6-13.* https://doi.org/10.12703/P6-13

Meda Spaccamela, V., *et al*. (2019). High Levels of IL-18 and IFN-γ in Chronically Inflamed Tissue in Chronic Granulomatous Disease. *Frontiers in immunology*, *10*, 2236. https://doi.org/10.3389/fimmu.2019.02236

Müller, E., Speth, M., Christopoulos, P. F., Lunde, A., Avdagic, A., Øynebråten, I., & Corthay, A. (2018). Both type I and type II interferons can activate antitumor M1 macrophages when combined with TLR stimulation. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.02520

Nguyen, K., Qiu, Y., Cui, X. *et al.* (2011). Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. *Nature* 480, 104–108. https://doi.org/10.1038/nature10653

Odegaard J.I., Chawla A. (2011). Alternative macrophage activation and metabolism. *Annu Rev Pathol,* 6, 275-97. https://doi.org/10.1146/annurev-pathol-011110-130138.

Pan, Y., Yu, Y., Wang, X., & Zhang, T. (2020). Tumor-Associated Macrophages in Tumor Immunity. *Frontiers in immunology*, *11*, 583084. https://doi.org/10.3389/fimmu.2020.583084

Panni, R. Z., Linehan, D. C., & DeNardo, D. G. (2014). Targeting tumor-infiltrating macrophages to combat cancer. *Immunotherapy*, *5*(10), 1075–1087. https://doi.org/10.2217/imt.13.102

Pelegrin, P. & Surprenant, A. (2009). Dynamics of macrophage polarization reveal a new mechanism to inhibit IL-1β release through pyrophosphates. *The Embo Journal,* 28(14), 2114-2127. https://doi.org/10.1038/emboj.2009.163

Qian, B., *et al.* (2009). A Distinct Macrophage Population Mediates Metastatic Breast Cancer Cell Extravasation, Establishment, and Growth. *PLOS ONE*, 4(8). https://doi.org/10.1371/journal.pone.0006562

Shatsky, Rebecca A. (2023). Immunotherapy for Breast Cancer. *Cancer Research Institute*. https://www.cancerresearch.org/cancer-types/breast-cancer

Schroder, K., Hertzog, P., Ravasi, T., & Hume, D. (2003). Interferon-γ: an overview of signals, mechanisms, and functions. *Journal of Leukocyte Biology, 75(2)*, 163-189. https://doi.org/10.1189/jlb.0603252

Sieve, I., Ricke-Hoch, *et al*. (2018). A positive feedback loop between IL-1β, LPS and NEU1 may promote atherosclerosis by enhancing a pro-inflammatory state in monocytes and macrophages. *Vascular pharmacology*, *103-105*, 16–28. https://doi.org/10.1016/j.vph.2018.01.005

Steenburgge, J., Breyne, K., Demeyere, K. *et al.* (2018). Anti-inflammatory signaling by mammary tumor cells mediates prometastatic macrophage polarization in an innovative intraductal mouse model for triple-negative breast cancer. *J Exp Clin Cancer Res,* 37, 191. https://doi.org/10.1186/s13046-018-0860-x

Tau, G., & Rothman, P. (1999). Biologic functions of the IFN-gamma receptors. *Allergy*, *54*(12), 1233–1251. https://doi.org/10.1034/j.1398-9995.1999.00099.x

Trayes, K. P., & Cokenakes, S. E.H. (2021). Breast Cancer Treatment. *American Family Physician,* 104(2)*,* 171-178.

Wyckoff, J., Wang, W., Lin, E. Y., Wang, Y., *et al*. (2004). A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer research*, *64*(19), 7022–7029. https://doi.org/10.1158/0008-5472.CAN-04-1449

Yunna, C., Mengru, H., Lei, W., & Weidong, C. (2020). Macrophage M1/M2 polarization. *European Journal of Pharmacology*, *877*, 173090. https://doi.org/10.1016/j.ejphar.2020.173090

Zhu, D., *et al.* (2014). Induced Pluripotent Stem Cell-Derived Neural Stem Cells Transduced with Baculovirus Encoding CD40 Ligand for Immunogene Therapy in Mouse Models of Breast Cancer. *Mary Ann Liebert, Inc.,* 25(8). https://doi.org/10.1089/hum.2013.160

Zhou, J., Tang, Z., Gao, S., Li, C., Feng, Y., & Zhou, X. (2020). Tumor-Associated Macrophages: Recent Insights and Therapies. *Frontiers in oncology*, *10*, 188. https://doi.org/10.3389/fonc.2020.00188