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University of Arkansas, Fayetteville

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Immunomodulation of Breast Cancer Cells for Whole Tumor Vaccination

Kristina G. Maxwell

University of Arkansas, Fayetteville
May 2016

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1. Abstract

Hematogenous metastasis causes 90% of breast cancer-related deaths.[1] Current therapies include chemotherapy and irradiation following surgery. These therapies are very harmful to the human body and do not elicit an anti-tumor immune response. To create a novel therapeutic, an autologous vaccine increasing the immunogenicity of non immunogenic breast cancer cell lines has been proposed.

To create this vaccine, 4T1 mouse mammary breast cancer cells have been selected as the desired cell line to treat. They are non immunogenic and highly invasive. In order to increase their immunogenicity, first projected, was the addition of cytokines to 4T1 cells to increase the expression of immunostimulatory molecules on the cells. TNF-α and IFN-γ, two pro-inflammatory cytokines, were added individually at a concentration of 50ng/mL to 4T1 cells. This concentration was chosen based on an MTS viability assay and ensures that the cytokines themselves are not killing the cells before an immune response is stimulated. Flow cytometry was used to evaluate the surface marker expression of major histocompatibility complex (MHC) I, MHC II, B7.1, B7.2, ICAM, and Fas. There was a minimal effect on each MHC and co-stimulator molecule resulting from TNF-α. IFN-γ developed in an increase in ICAM and MHC II. Neither of the cytokines created a large enough increase to have a large effect on the immunogenicity of 4T1 cells. Therefore, protein expression of 4T1 cells was reviewed.

4T1 cells express G-CSF in large amounts which causes myeloid derived suppressor cells (MDSCs) to accumulate within the spleen of a mouse. These MDSCs produce mature T cells although with high levels of G-CSF expressed they remain in the spleen undifferentiated. G-CSF was knocked out using CRISPR/Cas9 methods in order to increase the immunogenicity of 4T1 cells. The 4T1.GCSF^{-} cell line underwent single colony expansion and the concentration of G-CSF within the cell line was examined using ELISA (n=3). After single colony expansion, G-CSF was undetectable in the 4T1.GCSF^{-} cell line.

Next, the 4T1.GCSF^{-} cell line will be used in an in-vivo prophylactic vaccination. 100,000 4T1.GCSF^{-} cells will be irradiated and subcutaneously inserted into the mice as a primary vaccine on day 0 and a booster on day 10. On day 20 the mice will be challenged with live 4T1 cells and the tumor volume will be measured three times per week.

2. Summary

The past two years I have worked in Dr. David Zaharoff's Laboratory for Vaccine and Immunotherapy Delivery (LVID). During my time in Dr. Zaharoff's lab I have been working under the direction of Ph.D. candidate, Sruthi Ravindranathan. Through my time in Dr. Zaharoff's lab I have worked on two projects, both geared toward creating an autologous breast cancer vaccine for triple negative breast cancer (TNBC) strands. "TNF-α and IFN-γ Immunomodulation of Breast Cancer Cells for Whole Tumor Cell Vaccine" was my first project which included adding cytokines to a triple negative mouse mammary cancer cell line, 4T1, in efforts to create a vaccine. The addition of cytokines to 4T1 cells was performed in hopes of increasing the immunogenicity of this cell line. An appropriate concentration at which the cytokines would be added was determined from MTS viability testing. Flow cytometry was used to evaluate the increase in immunogenicity of 4T1 cells by testing the surface marker expression of major histocompatibility complex I (MHC I), MHC II, and several co-stimulatory molecules. 4T1 cells alone, 4T1 cells with irradiation, 4T1 cells with 50ng/mL of the cytokine, and 4T1 cells with 50ng/mL of the cytokine and irradiation were all tested for each cytokine. It was concluded that TNF-α has a minimal effect on the surface marker expression of 4T1 cells and IFN-γ has an effect on several co-stimulatory molecules although not enough to increase the immunogenicity of the cell line.

"G-CSF Knockdown Immunomodulation of Breast Cancer Cells for Whole Tumor Cell Vaccine" is my second project. During this project I used CRISPR/Cas9 to knock out granulocyte colony stimulating factor (G-CSF) to create 4T1.GCSF^{-}. G-CSF was removed to decrease the amount of myeloid derived suppressor cells (MDSCs) accumulating at abnormally increased

rates in the spleen of a mouse when 4T1 tumor formation occurred. MDSCs differentiate into mature immune cells. By regulating G-CSF, the MDSCs will differentiate into monocytes or granulocytes at a normal rate, thus the 4T1 cell line would increase its immunogenicity. G-CSF was successfully removed from the cell line by use of CRISPR/Cas9 and single colony expansion. Next, 4T1.GCSF^{-} will be used in an in-vivo prophylactic vaccination to examine the effects of knocking out G-CSF on the cell line's immunogenicity within mouse models.

3. Introduction:

3.1 Significance of Work:

Hematogenous metastasis is responsible for 9 of 10 breast cancer-related deaths.[1] Currently, to reduce this large fraction of deaths, adjuvant therapies such as chemotherapy and radiation are given following a mastectomy or lumpectomy surgery.[2] These therapies are very harmful to the body, regarding their high levels of toxicity, hence a novel, safer therapy is needed.[2] Immunogenic therapies using one's own cancer cells to remove any residual tumor cells is an innovative approach for an original, personalized therapeutic.

3.2 Background:

4T1 mammary carcinoma is a highly aggressive and invasive mouse breast cancer cell line. These cells are a very useful tumor model and metastasize to multiple distant sites compared to many other breast cancer cell lines, such as lymph nodes, blood, brain, and bone.[2,3] 4T1 cells are very useful in the laboratory setting because they have many characteristics comparable with human mammary breast cancer including spontaneous metastases development and ease of transplanting into mammary gland.[3]

In addition, 4T1 cells are completely non-immunogenic.[4] Non-immunogenic cell lines are also known as triple negative breast cancer cells. They lack the three receptors that typically indicate the cause for breast cancer, estrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2 (HER2).[1] Several breast cancers that express one of these receptors are targeted based on the receptor. Due to 4T1 cells lacking all three, finding therapeutics, target genes, or target proteins is very difficult. Therefore, research is constantly

conducted in order to provide new innovative forms of treating this highly metastatic, non immunogenic form of breast cancer.

There are many forms of breast cancer cell lines and they are characterized by their metastatic potential and invasive properties. The immunology behind breast cancer can account for the metastatic potential of different cell lines. 4T1 cells are non immunogenic implying there is no immune response to 4T1 tumor growth or cell metastasis. Other cell lines such as MCF-7, which have a greater immune response and will not metastasize.[5] The innate immune system can detect MCF-7 cells using T cells in the bone marrow.[5] The innate immune system can detect multiple viruses and is non-specific utilizing macrophages and dendritic cells throughout the body.[7] The other form of immunity is the adaptive immune system controlled by mechanisms and interactions between T cells and B cells, two forms of lymphocytes. An adaptive immune response requires the innate immune system to detect viruses.[6] B cells have receptors which are able to detect viruses and microbes.[7] After attachment occurs between the virus and a B cell, the B cell transforms into a plasma cell to secrete an antibody. The antibodies attach onto virus and diseased cells to tag them for cytokinesis by macrophages.[6] T cells include T helper cells and cytotoxic T lymphocytes (CTLs). The T cells mediate the adaptive immune system by recognizing the presentation of specific peptides on the surface of macrophages' major histocompatibility complex (MHC) II surface marker receptors. These peptides are a portion of the virus' DNA.[6] The T helper cells will attach to the macrophage via the MHC II receptor and produce cytokines to mark the virus for phagocytosis. The recognition of MHC II by helper T cells also causes B cells to proliferate, in turn creating more antibody-secreting cells.[7] CTLs enforce automatic phagocytosis using MHC I receptors. Increased proliferation of

CTLs can cause autoimmune disorders due to non tumorigenic cells entering phagocytosis spontaneously.[6]

In my undergraduate honors thesis, two different methods of increasing the immunogenicity of 4T1 cells are described. One entails increasing the cytokine surface marker expression of 4T1 cells using either IFN-γ or TNF-α in order to recruit more T cells and enable macrophages to engulf 4T1 tumor cells. The other uses CRISPR/Cas9 to remove a protein, G-CSF, causing expansion of myeloid derived suppressor cells (MDSCs) within the spleen.

4. Project 1: TNF-α and IFN-γ Immunomodulation of Breast Cancer Cells for Whole Tumor Cell Vaccine

4.1 Introduction

Tumor necrosis factor-alpha (TNF- α) and interferon-gamma (IFN- γ) are two pro-inflammatory cytokines which have been shown to upregulate the expression of surface molecules involved in antigen presentation, such as major histocompatibility complex I (MHC I) and II (MHC II), and immune co-stimulators, such as B7.1.[8] IFN-γ directly regulates MHC II and TNF-α regulates MHC I, II, and B7 receptors.[9] By increasing the expression of these molecules, cells interact more strongly with cells of the immune system. These same cytokines can also induce apoptosis, or programmed cell death of the 4T1 cell and surrounding angiogenic endothelium to reduce further tumor vasculature.[10] An innate immune response is desired for production of an autologous vaccine. Through the addition of cytokines to non immunogenic 4T1 cells, the immune system may recognize the increased surface marker expression of MHC I, MHC II, or the several other co-stimulatory molecules expressed.

These surface markers directly correlate with T cells in the body. CTLs present the MHC I receptor along with CD8+, B7.1, and ICAM which bind with the tumor cells to increase bonding strength between the two cells.[6] T helper cells express the MHC I receptor and CD4+ which bind to cancer cells expressing MHC I, B7.2, and Fas (Figure 1).[6] When IFN-γ and TNF-α are secreted by T cells, it increases the MHC I and MHC II present on diseased cells. In turn CTLs will be activated and proliferated to account for the needed immune response.[10]

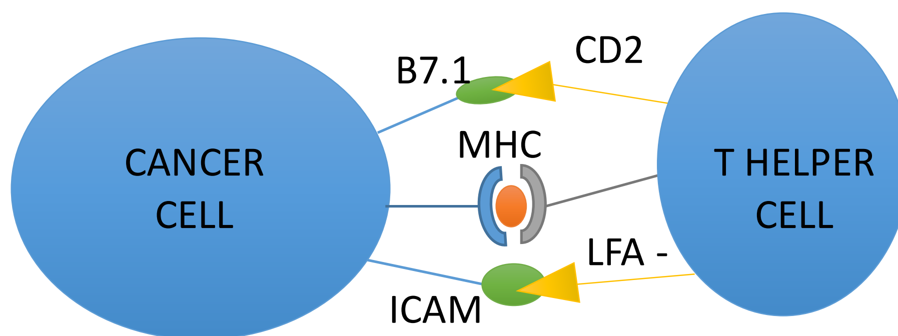


Figure 1. T helper cells recognize virus or disease-associated cells based on the presentation of MHC II on the surface of the virus. Co-stimulator molecules, B7.1 and ICAM, enhance the attachment strength between the T helper cell and the virus.

The objective of this study is to enhance the immunogenicity of a non immunogenic breast cancer cell line through treatment of IFN-γ or TNF-α. Cell viability will be analyzed at increasing concentrations of IFN-γ or TNF-α when added to 4T1 cells. The expression of specific surface molecules involved in antigen presentation will be measured; MHC I, MHC II, B7.1, B7.2, ICAM and Fas; in order to determine if the surface molecules are up-regulated with the addition of cytokines. Therefore, I hypothesize that exposing 4T1 cells to TNF- α or IFN- γ will enhance their immunogenicity and form the basis for a novel breast cancer vaccine.

4.2 Methods and Materials

4.2.1 Cell Culture.

The 4T1 cell line is a mouse mammary breast cancer purchased from ATCC. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) media containing 90% DMEM stock, 10% fetal bovine serum, 1% Penicilin Streptomycin, 4500mg/L glucose, 2mM L-glutamine, and 1% sodium pyruvate. The cells were grown in incubation at 37°C with monitored 5% CO2 and H20 levels. The cells are stored frozen in a Thermo Scientific Liquid Nitrogen container (Locator 4) until needed. The cell count doubles in about 24 hours.

4.2.2 CellTiter MTS Viability Assay.

To explore the effects of IFN-γ and TNF-α on viability, 4T1 cells were treated with increasing concentrations of IFN-γ and TNF-α cytokines. 2000 4T1 cells were seeded onto a 96 well plate with 100uL of DMEM and increasing concentrations of IFN-γ and TNF-α, for 24 hours. The concentrations included 50 ng/mL, 100 ng/mL, 200 ng/mL, 300 ng/mL, 400 ng/mL, and 500 ng/mL. The experiment was performed in triplicate.

A CellTiter MTS viability assay was performed on the 4T1 cells with IFN-γ and TNF-α, individually. The MTS reagent was thawed and the 96 well plate was equilibrated at room temperature for 30 minutes. 10 uL was added to each well separately with 100uL of cells and DMEM. The contents were mixed on the orbital shaker for 2 minutes without additional heat at a speed of 70. The plate was incubated for 1 to 4 hours to allow for proper mixture of the cells and substrate. The number of live 4T1 cells after 24 hours was assessed using a BioTek

Instruments Synergy 2 microplate reader (BioTek Instruments, Winooski, VT) and the luminescent assay. The plate reader was read at 490nm with Gen 5.1.09 computer software. A linear equation was created with standards in excel to ensure R^2>.95.

4.2.3 Live Cell Imaging of Cytokine-Treated 4T1 cells.

Images from a Nikon Eclipse Ti fluorescent microscope with a 10x objective were taken of the cells at each cytokine concentration and a percentage was assigned to each image to state each flask's confluency. The images revealed details on the dispersal of cells within the flasks and the visual interpretation of these cells viewed on a light microscope. Images were taken using Nikon NIS-Elements BR imaging software.

4.2.4 Flow Cytometry Measures 4T1 Surface Marker Expression.

A BD FACSCanto II Flow Cytometer was used to evaluate the expression of MHC I, MHC II, Fas, ICAM, B7.1, and B7.2, before and after cytokine exposure. 4T1 cells alone were used as the control. The concentrations determined by the CellTiter viability assay to implement the optimal effect of TNF- α and IFN- γ was used, 50ng/mL of each cytokine. 2 million 4T1 cells were seeded in 4 T75 flasks. 24 hours later, 50ng/mL of either IFN-γ or TNF-α was added to 2 flasks. 24 hours after, a single flask of cytokine-treated 4T1 cells and 4T1 cells alone were irradiated with 100 gray. The day of irradiation, the flasks were all examined using flow cytometry. Each day the flasks were renewed with 10mL of DMEM media.

1 million cells from each experimental and control group were isolated for flow cytometer use. Flourochromes were used to detect each individual MHC or co-stimulatory

molecule. The flourochromes (BD BioScience, San Jose, CA) used include 2uL of FITC mouse anti-mouse H-2Kb, 5uL of PE rat anti-mouse I-Ad/I-Ed, 5 uL PE-Cy7 hamster anti-mouse CD95, 2uL of FITC hamster anti-mouse CD54, 5uL of APC hamster anti-mouse CD80, and 5uL of PE rat anti-mouse CD86, for MHC I, MHC II, Fas, ICAM, B7.1, and B7.2, respectively. A negative, isotype (PE Hamster IgG1, k Isotype control), and untreated cell sample were calculated for controls. The samples were incubated in dark for 1 hour before compensation controls were detected and accounted for. Then samples were measured after the supernatant was removed and 500uL of PBS was added to each tube. When not being sampled, the tubes were placed in ice to preserve the cells. A large increase in the expression of these markers is expected to correspond with the immunogenicity of the tumor cells. Images were provided by BD FACSCanto computer software (2.4). FlowJo software was used to combine data from the 4 groups and compare overlay images of the results.

4.3 Results

4.3.1 Effect of IFN-γ and TNF-α on 4T1 viability.

An MTS viability assay (CellTiter) was performed to evaluate the effect of IFN-γ and TNF-α on 4T1 cells. Varying concentrations were evaluated to determine which concentration would allow the cells to survive and generate an anti-tumor immune response. 2000 4T1 cells were seeded onto a 96 well plate and the cytokines were added to each well for 24 hours. The experiments began with concentrations of 0-100ng/mL, increments of 25, and little effect was shown. Concentrations were increased to 300ng/mL, increments of 50, and a larger difference in viability was viewed. Concentrations were also examined at varying time periods, 24, 48, and

72 hours, although there was no effect from prolonged exposure. The final viability assay was 0-500ng/mL which displayed the difference between IFN-γ and TNF-α (Figure 2). IFN-γ initially, at 100ng/mL, decreases the number of cells and levels off with a steady increase from 200-500 ng/mL. TNF-α shows a steady decrease after the initial decrease in cell number. TNF-α has a greater effect on killing 4T1 cells than IFN-γ. To have a comparable study between IFN-γ and TNF-α, the concentration of the cytokine should reveal a similar effect. Therefore, 50ng/mL was selected to treat the 4T1 cells with, to examine the effect of cytokines on surface marker expression of 4T1 cells.

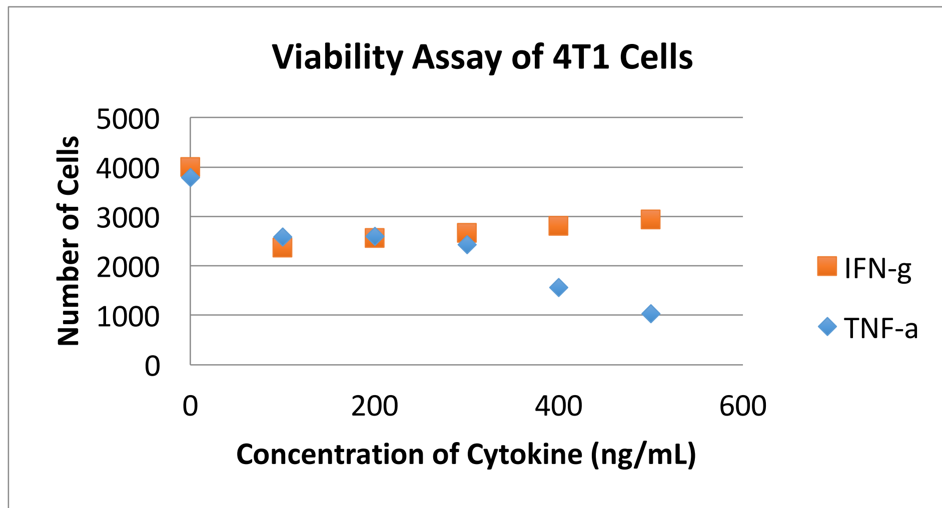


Figure 2. Comparison of IFN-γ or TNF-α cytokines and their effect on 4T1 breast cancer cell viability. As stated in methods section, there is an N=3 for the differing number of cells.

Images of each flask with 0-500ng/mL concentrations of IFN-γ and TNF-α were captured to examine the confluency of each flask (Figure 3). With increasing concentrations, the confluency appeared to increase in the mid range concentration of 300ng/mL and decrease again at 500ng/mL. The cells appear healthier in 0ng/mL and 300ng/mL compared to 500ng/mL

where several dark cells appear. The 0ng/mL images also appear well spread out and growing at normal rates whereas 300ng/mL appears very confluent for after only 24 hours of seeding the 4T1 cells.

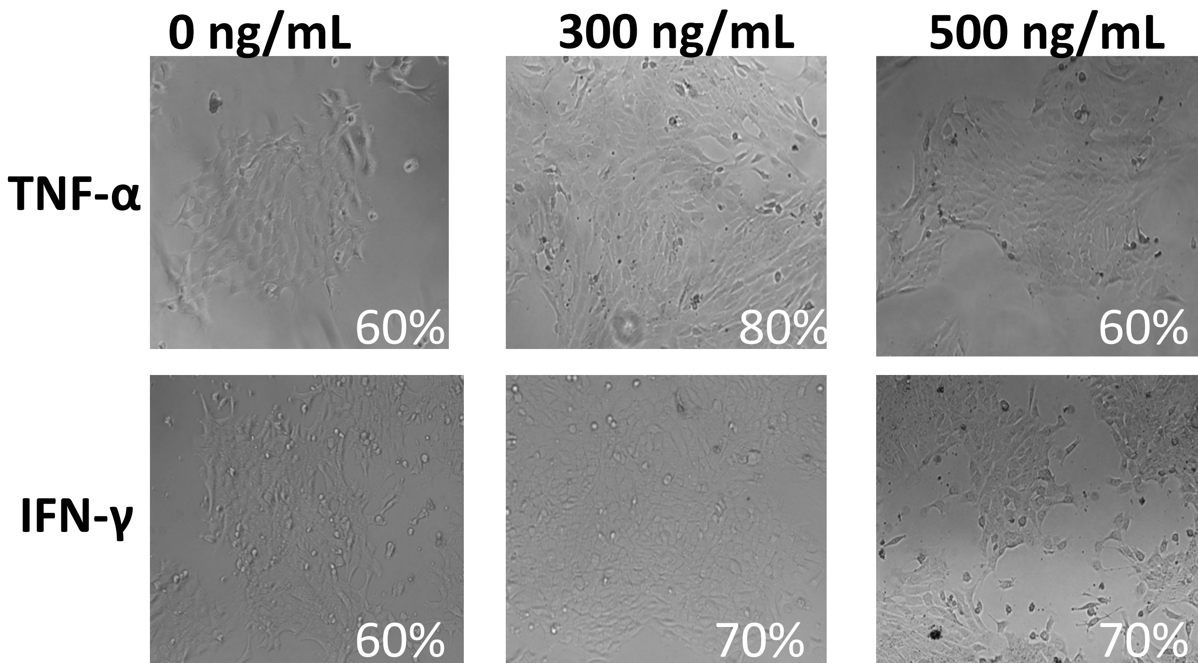


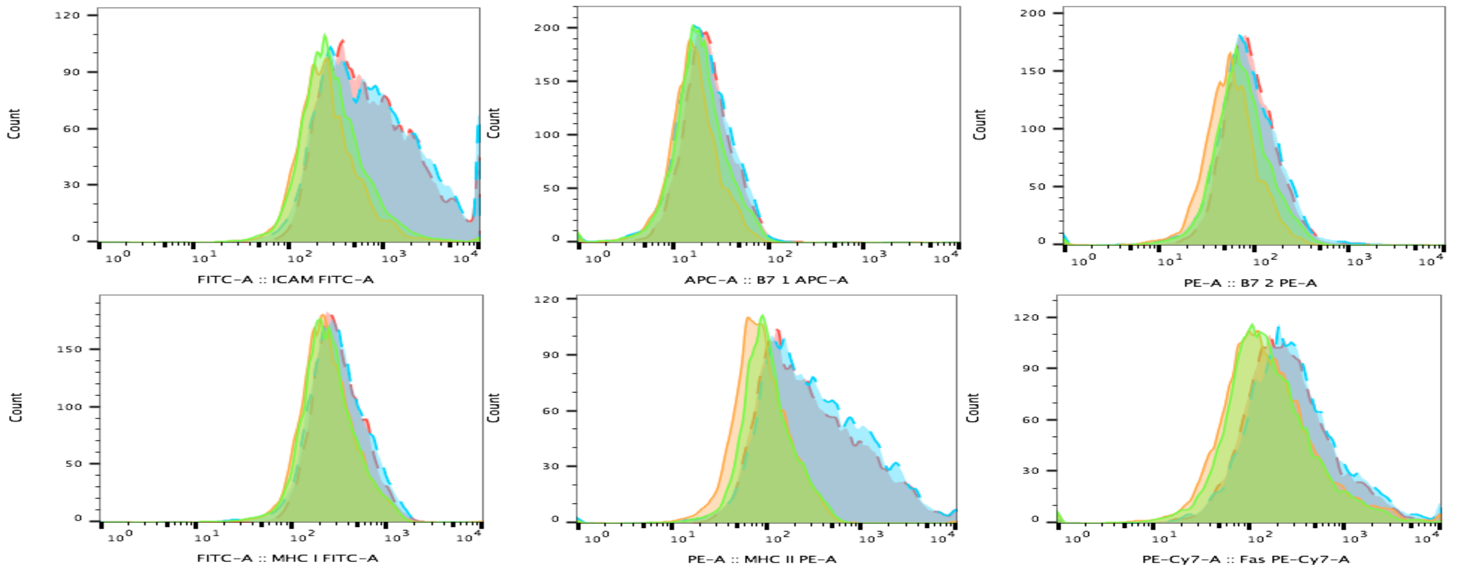
Figure 3. Confluency of each 4T1 well after 24 hours is shown with a corresponding confluency percentage. Both IFN-γ and TNF-α treated cells at different concentrations are compared with control cells. Images are taken with a fluorescent microscope, 10x lens.

4.3.2 Effect of IFN-γ and TNF-α on surface marker expression.

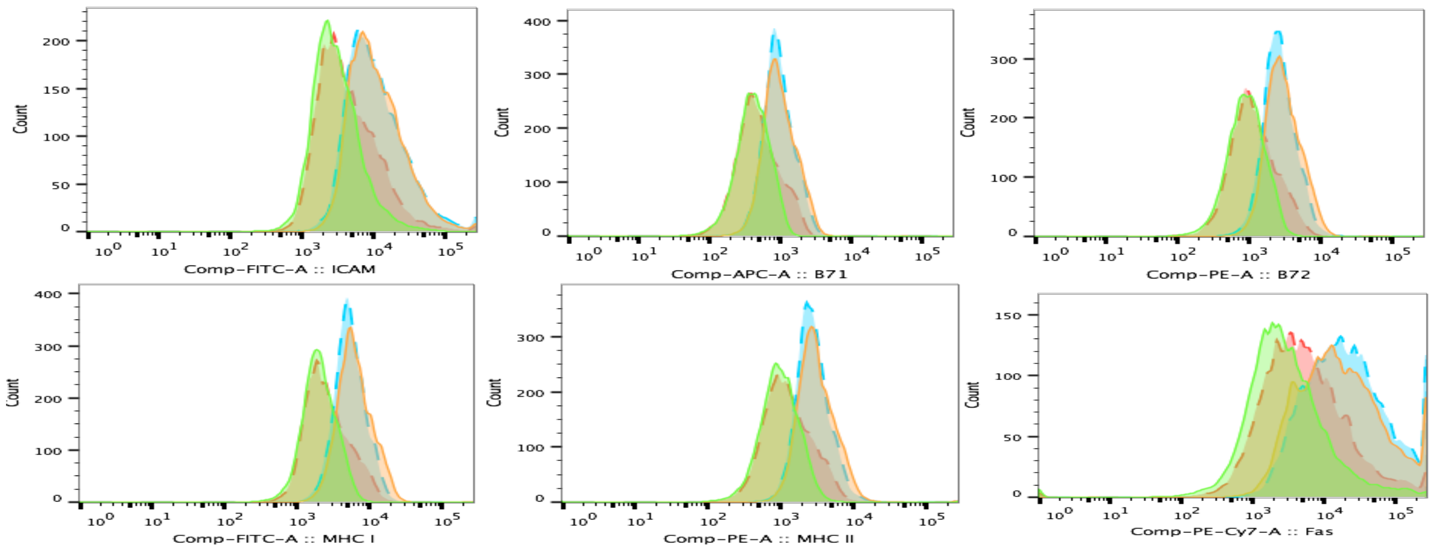
500,000 cells of each cytokine-treated cell line was used to test expression of surface molecules. 50ng/mL of each cytokine was added to 4T1 cells. A 6-color flow cytometer measured different fluorochromes (FITC, APC, PE, PE-Cy7) to test the surface marker expression

of B7.1, B7.2, ICAM, Fas, MHC I, and MHC II. The co-stimulator and MHC molecules were chosen due to their immune system connection with CTLs and T helper cells. 4T1 cells alone and 4T1 cells alone with irradiation were used as controls. 4T1 cells with 50ng/mL of the selected cytokine and 4T1 cells with 50ng/mL of the selected cytokine and irradiation were compared to the controls. IFN-γ provided an increase of FITC and MHC II (Figure 4). Based on the difference in the flow cytometry results there is not a large enough different in surface marker expression for the alternate molecules to have an effect on 4T1 cells' immunogenicity. An increase in two of the molecules tested will not elicit an immune response. TNF-α had a very minimal effect in increasing any surface marker expression (Figure 4). There is a small separation in peaks for all molecules tested although not enough to have a difference in 4T1 immunogenicity, as stated for IFN-γ. Therefore, 50ng/mL of neither cytokine-treated 4T1 cells had an effect on surface marker expression. Multiple time periods, 24 hrs, 48 hrs, and 72hrs, were tested to detect if there was a difference in surface marker expression although no difference was seen, similar to previously results seen in cell viability.

IFN-γ



TNF-α



	4T1 Cells Alone		4T1 Cells + 50ng/mL TNF-α
	4T1 Cell + Irradiation		4T1 Cells + 50ng/mL TNF-α + Irradiation

Figure 4. Flow Cytometry result comparison of 50ng/mL TNF-α and IFN-γ treated 4T1 cells. There was a control group for irradiated cells alone, cells alone, and 50ng/mL of the cytokine alone and with irradiation. Image overlay was created with FlowJo software.

4.4 Discussion and Future Work.

The 500ng/mL concentration of TNF-α results in a 73% lesser cell survival rate, compared to the control (Figure 4). This percentage indicates the cells will die as a result from cytokine treatment but not necessarily due to increased surface marker expression or an innate immune response. If the surface marker expression on these cells were to increase, it would likely produce an immune response[5,6,7]. If the concentration of cytokines were to increase more than 50ng/mL the cell survival would decrease (Figure 4). If enough cytokines are expressed, the cell will die although not due to an anti-tumor immune response but due to overproduction of cytokines. The in vitro study aims to mimic the immune system in the body and the large concentrations of cytokines are not realistic if the 4T1 cells are undetectable by the immune system. Also if the concentration is higher than 50ng/mL and the cells die more frequently, more cells will not survive the initial cytokine treatment and the body will not have the chance to generate an anti-tumor immune response. Therefore, no memory of the cells will be created by the adaptive immune system, similar to current treatments with chemotherapeutics. Chemotherapy is simply aimed to kill the cells, not to train the immune system in detection and removal of the cells on its own. The purpose of this project was to create an autologous vaccine to increase the immunogenicity of 4T1 cells therefore generating an anti-tumor immune response by the body for future tumor growth.

Due to the minimal or lack of increase in MHC and co-stimulatory molecules from either cytokine, a new method needs to be assessed to increase the immunogenicity of 4T1 cells. 4T1 cells need to be assessed in comparison to alternate non-immunogenic cell lines that have current effective treatments. Comparison methods should include surface marker expression

and protein expression in the two cell lines. EMT-6 is a non-immunogenic mammary breast cancer cell line with less invasive properties when compared to 4T1 cells.

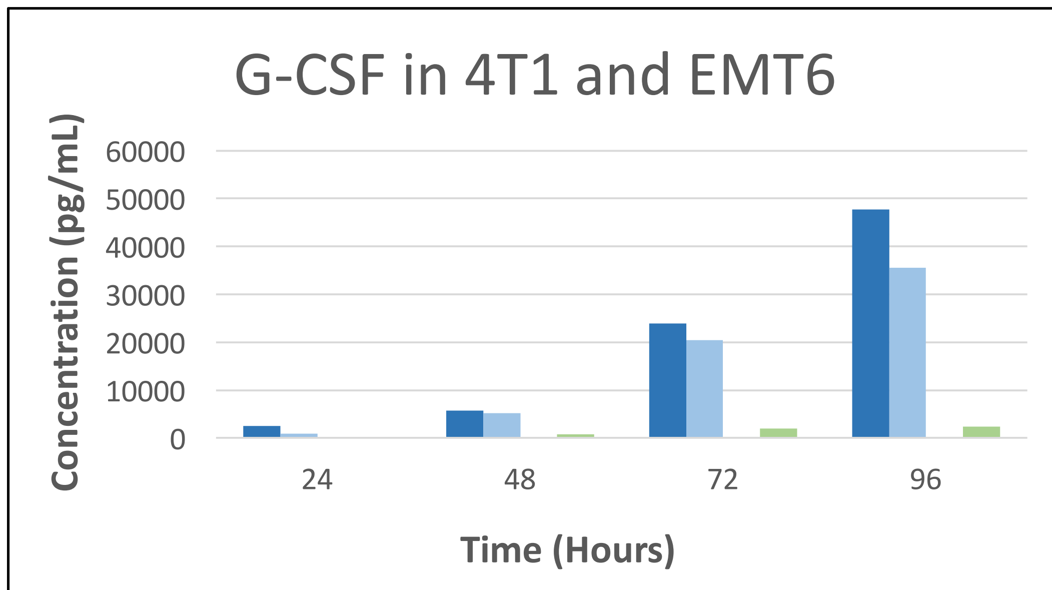
5. Project 2: G-CSF Knockdown Immunomodulation of Breast Cancer Cells for Whole Tumor Cell Vaccine

5.1 Introduction

EMT6 cells and 4T1 cells are both non immunogenic, TNBC, mouse cell lines. The difference between the two cell lines being 4T1 cells are more aggressive and invasive.[11] EMT6 cells created a vaccination model by irradiating EMT6 cells to create a primary and booster vaccine delivered 10 days apart. After given a live tumor challenge 10 days after the booster vaccine, 80% of mice survived the tumor challenge.[11] Through the same protocol for a 4T1 vaccine, all mice failed to survive the live tumor challenge.[12] A combination of EMT6 and 4T1 cells injected on the same flank as well as opposite flanks has also been performed. Although the live tumor challenge resulted in 60% and 88% of mice developing tumors, respectively.[12] Therefore, it was clear the 4T1 cell line is expressing a specific protein that diminishes the effect of an irradiation 4T1 vaccine.

Due to the addition of cytokines not having a large effect on surface marker expression of 4T1 cells, the next route of experiment to analyze was protein expression. This was verified by previous studies performed by Ravindranathan, et al. 4T1 cells and EMT6 cells express immunosuppressive cytokines including G-CSF, GM-CSF, M-CSF, IL-6, MCP-1, VEGF, and TGF-β. The cytokines are expressed in different amounts as well as comparing before and after irradiation, for both cell lines.[12] Cytometric bead array and ELISA measured the amount of cytokines within each cell line's media at 24, 48, and 96 hours.[13,14] IL-6, MCP-1, TGF- β, and VEGF are secreted at higher levels in EMT6 cells while GM-CSF and G-CSF are secreted at

increased levels in 4T1.[11] The data revealed increased levels of GM-CSF, M-CSF and G-CSF expressed by 4T1 cells.[12] These studies were used to determine which protein to knock out of the 4T1 genome. G-CSF displayed the largest increase in both irradiated and non-irradiated 4T1 cells when compared to EMT6 (Figure 5).[12]



	4T1 Cells Alone		EMT-6 Cells Alone
	4T1 Cells + Irradiation		EMT-6 Cells + Irradiation

Figure 5. Sandwich ELISA results from examining the concentration of G-CSF (blue) in EMT-6 (green) and 4T1 cells both alone (darker color) and irradiated (lighter color). ELISA performed for multiple time periods: 24, 48, 72, and 96 hours.[12]

Granulocyte colony-stimulating factor (G-CSF) is a protein within cells that inhibit myeloid derived suppressor cells (MDSC) from differentiating into mature immune cells. G-CSF has many functions involving immune response, including the ability to overcome neutropenia, neutrophil mobilization, and regulating granulopoiesis.[15] It has also been used in combination

with chemotherapeutics to treat advanced cancer.[16] Its ability to regulate basal and neutrophil production allows chemotherapy to be used without dose-limiting neutropenia.[16] Although it also activates and repopulates dormant breast cancer stem cells.[16] G-CSF has many effects in the body relating to breast cancer regulation although I focused on its effect on MDSCs.

The MDSCs are prevalent in innate and adaptive immune response due to their granulocytes and monocytes production.[15] MDSCs down regulate antitumor immunity by inhibiting T cell responses and supporting tumor progression.[17] MDSCs also produce factors to promote tumor angiogenesis and lymphangiogenesis.[18] The accumulation results from their inability to differentiate into mature immune cells, caused by expression G-CSF in 4T1 cells.[18] 4T1 cells' G-CSF-causing accumulation of MDSCs causes 4T1 cells to be non immunogenic. There may be other factors which cause the non immunogenicity as well.

Due to the protein, G-CSF, exhibiting a large increase in 4T1 cells, the removal of the protein will have an effect on the cell line's immunogenicity. CRISPR/Cas9 will remove the protein and single colony expansion will generate the $4T1.GCSF^{-}$ cell line. Therefore, I hypothesize that knocking down the G-CSF protein within 4T1 cells will enhance their immunogenicity and form the basis for a novel breast cancer vaccine.

5.2 Methods and Materials

5.2.1Cell Culture.

The 4T1 cell line is a mouse mammary breast cancer purchased from ATCC. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) media containing 90% DMEM stock, 10% fetal bovine serum, 1% Penicilin Streptomycin, 4500mg/L glucose, 2mM L-glutamine, and 1% sodium pyruvate. The cells were grown in incubation at 37°C with monitored 5% CO2 and H20 levels. The cells are stored frozen in a Thermo Scientific Liquid Nitrogen container (Locator 4) until needed. The cell count doubles in 24 hours.

5.2.2 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9.

Gene editing technology, CRISPR/Cas9, was used to edit the genome of 4T1 cells. A protein, G-CSF, was removed from the genome of 4T1 mouse mammary breast cancer cells. Cas9 is a CRISPR-associated protein with two nuclease domains encoded to cleave DNA at a specific site.

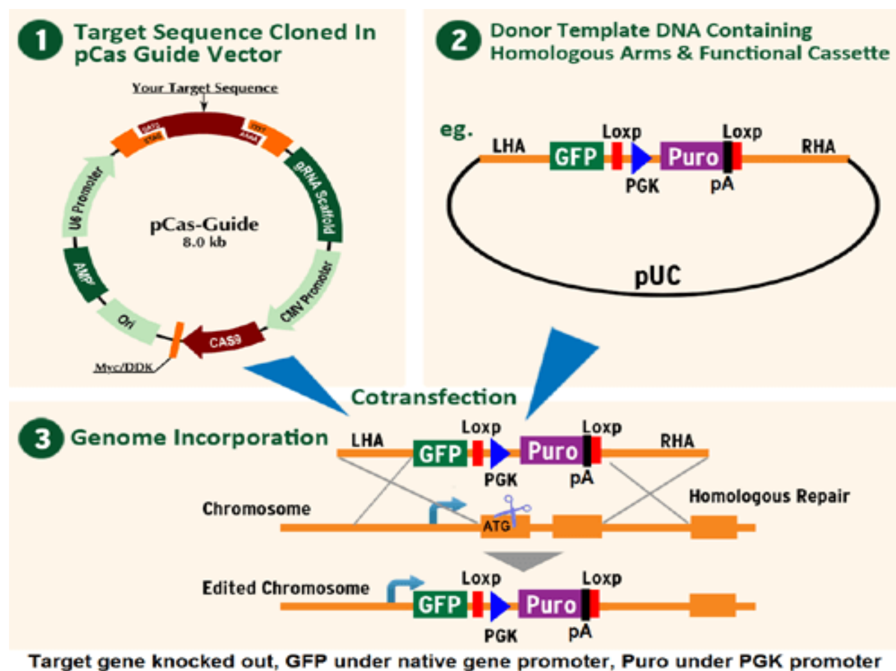


Figure 6. CRISPR/Cas9 schematic provided from OriGene.[19]

5.2.2.1 G-CSF Protein Sequence

The amino acid base sequence which codes for G-CSF within 4T1 cells was found from NCBI and the guide sequence for the CRISPR/Cas9 guide RNA was provided online at crispr.mit.edu. Once the protein was found, CSF3-mouse gene knockout kit via CRISPR (KN303891) was purchased from Origene (Figure 6 (1)).

5.2.2.1 Amplify Plasmid and Transfect 4T1 Cells

The plasmid will be amplified within bacteria using a *maxiprep kit (*Qiagen, Inc.. The 298B G-CSF plasmid comes on top of agar in a vile. It is spread on an agar plate and incubated for 24 hours to allow for replication. A single colony is selected and added to 20mL of Lysogeny Broth (LB) medium. It is placed on a shaker plate for 24 hours to ensure proper mixing.

Nucleic acid quantification will isolate the RNA plasmid from the e.coli. The supernatant of the solution is filtered through a spin column and centrifuged for 10 minutes then 1 minute at 13000rpm, 4 times. A *spectrometer* machine measures the DNA absorbance at OD260nm and protein at OD 280nm. A purified plasmid, lacking the desired protein inside, is confirmed with the equation:

$$\frac{OD260}{OD280} = A$$

$$Purified\ protein: 1.8 < A < 2.0$$

The plasmid is visualized using *gel electrophoresis* techniques and a UV gel reader. The amplified plasmid will be added into a flask with 4T1 cells where the plasmid will inject itself into the nucleus of the 4T1 cells via *lipofection*. Lipofectamine 3000

(Invitrogen, Carlsbad, CA) is used to transfect the 4T1 cells with the plasmid DNA and Cas9 enzyme. 1 million cells/well were seeded in a 6 well plate for 24 hours. Cells were 75% confluent in a T75 flask upon transfection. Lipofectamine 3000 reagent was used, diluted in optimum medium. DNA plasmid was diluted to become 1ug/uL, recommended 2uL P3000/1mg DNA. Diluted DNA plasmid and diluted lipofectamine 3000 reagent were combined and incubated at room temperature for 5 minutes. The DNA-lipid complex was added to the 4T1 cells in the 6 well plate and incubated for 24 hours.

The Cas9 enzyme places a green fluorescent protein (GFP) where the target gene is knocked out (Figure 6 (3)). The *GFP signal was measured* using a Nikon Eclipse Ti fluorescent microscope and images were captured using Nikon NIS-Elements BR imaging software. The GFP signal will verify if the CRISPR/Cas9 knocked out G-CSF from 4T1 cells.

The GFP emitted by transfected cells will allow a BD FACS ARIA III 6-color cell sorting flow cytometer to *sort the 4T1 and 4T1.GCSF$^-$ cells.* The remaining 4T1 altered genome cell culture will be grown and tested for immunogenicity.

5.2.3 Single Colony Selection.

Single colony expansion was used to select a colony developed from one cell of the 4T1.GCSF$^-$ cell line. 1000 4T1.GCSF$^-$ cells were placed on a petri dish and cultured for 24 hours. Colonies were viewed under a microscope and selected for removal. Grease was applied to the end of a plastic tube and placed to surround a specific colony to isolate it from the remaining cells in the petri dish. Trypsin was used to remove the cell colony from the petri dish, using

typical trypsin protocol. The colony was selected and placed in a 24 well plate to expand further. The colony was transferred to a 6 well, T25 flask, and finally a T75 flask once each container reached a confluent layer of the 4T1.GCSF$^-$ colony. Multiple microcentrifuge tubes containing the multiple colonies are stored at -80ºC in a Thermo Scientific Liquid Nitrogen container (Locator 4).

5.2.4 Enzyme Linked Immunosorbent Assay (ELISA).

Sandwich ELISA was used to verify that G-CSF was knocked out of the 4T1.GCSF$^-$ cell line. This experiment was performed in triplicate. The G-CSF (CSF3) mouse ELISA kit was obtained from R&D Systems, Inc. (EMCSF3, Minneapolis, MN). The kit includes pre-coated, pre-calibrated 96 well plates. Standards were created using RD5-16 and a serial dilution from stock (900 pg/mL). 50uL of RD1-54 and 50uL of standard, control, and sample are added to the pre-assigned wells and placed in incubation at room temperature for 2 hours covered with an adhesive strip. The plate contents are discarded and washed 5 times with wash buffer. 100uL of mouse G-CSF conjugate is added to each well and plate is incubated for 2 hours at room temperature covered with an adhesive strip. The plate contents are discarded and washed 5 times with wash buffer. 100uL of the substrate solution is added to each well and incubated at room temperature for 30 minutes covered by an adhesive strip and aluminum foil (light sensitive). 100uL of stop solution is added to each well and a BioTek Instruments Synergy 2 microplate reader (BioTek Instruments, Winooski, VT) measures the O.D. at 450nm and 540nm. The 540nm O.D. reading is subtracted from 450nm and a standard curve is created using excel (R^2 >.95). The concentration of G-CSF samples is calculated using the standard curve equation.

5.3 Results

5.3.1 CRISPR/Cas9

CRISPR/Cas9 removed the G-CSF protein from the 4T1 mammary mouse breast cancer cell line. The amino acid base sequence which codes for G-CSF in 4T1 cells was identified and an RNA sequence in plasmid form was created. The plasmid was amplified within e.coli using a maxiprep kit and added to a flask of 4T1 cells. 6 million cells were transfected with CRISPR/Cas9 targeted genome editing technology. Fluorescent GFP images are displayed to verify cells express the Cas9 enzyme which has removed G-CSF from the genome (Figure 7). The large amount of GFP fluorescence correlates with a large efficiency of cells being transfected with the Cas9 enzyme. Bright field and GFP fluorescent images are taken to compare the number of fluorescent cells. The transfected and non transfected 4T1 cells were separated via flow cytometry cell sorter with an efficiency of 3%. This efficiency is rather low compared to the fluorescent images although the flow cytometer may have detected a low signal for GFP in cells and sorted them as non transfected.

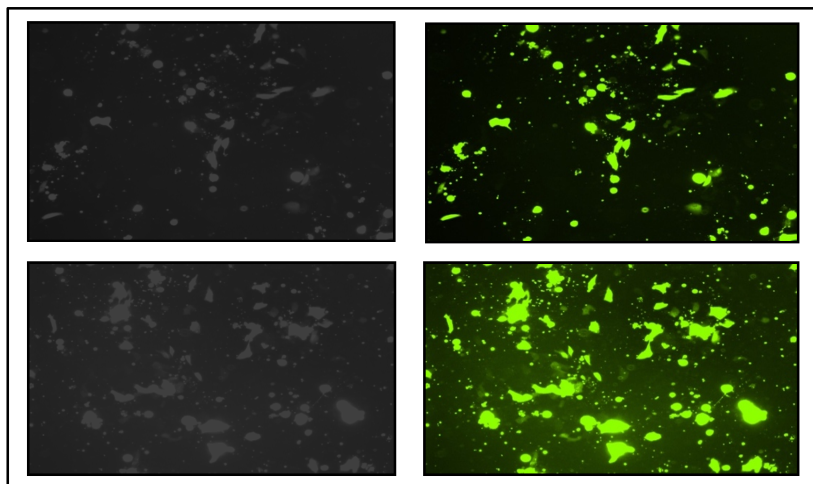


Figure 7. Fluorescent and bright field images captured using Nikon Imaging Software. GFP was expressed in 4T1 cells that up took the RNA plasmid.

Sandwich ELISA was performed to verify the removal of G-CSF from the 4T1 genome after the sorted 4T1.GCSF$^-$ cell line had doubled several times. The cells were examined and had decreased the expression of G-CSF by almost 12 fold (Figure 8). The results reveal several cells lack the G-CSF protein although some 4T1.GCSF$^-$ continue to have the protein expressed. Therefore, it was necessary to continue with single colony selection of the 4T1.GCSF$^-$ cell line.

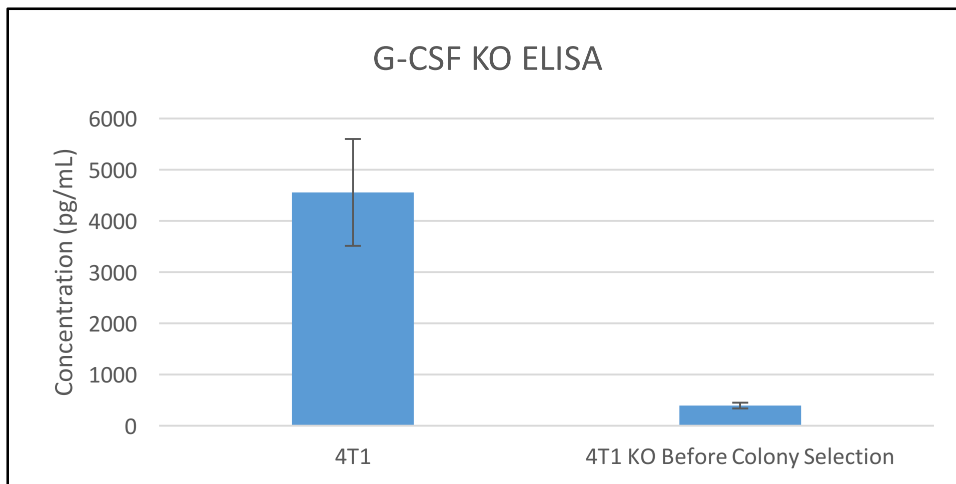


Figure 8. Sandwich ELISA performed on 4T1 and 4T1 KO cell lines to compare concentration of the protein G-CSF in the cells (n=3). 4T1 KO cells were tested before colony selection.

5.3.2 Single Colony Expansion

Single colony expansion was performed using 1000 4T1.GCSF$^-$ cells in a petri dish. A colony of cells resulted from a single cell. 5 colonies were selected and removed to continue growth in a larger space. Small rubber tubes and grease was used to separate the colonies while assembling them for continued culture (Figure 9). The cells were trypsinized and placed in separate flasks to grow for use in ELISA to test for complete knock out of G-CSF.

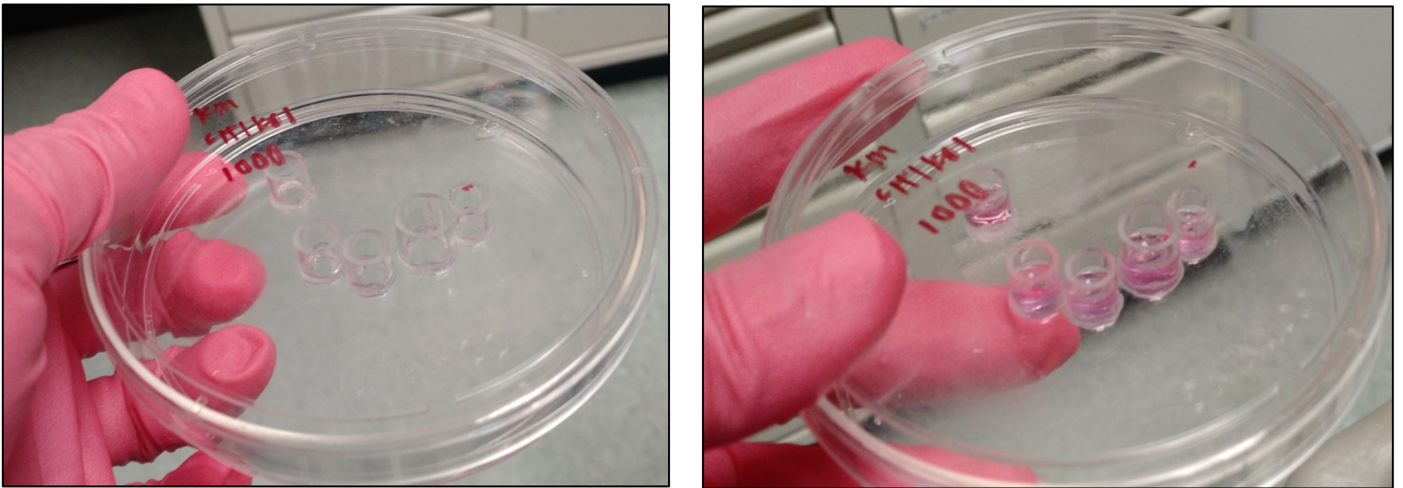


Figure 9. Single cell colonies were isolated inside a petri dish and typsinized for uptake and culture in larger flasks. 5 colonies were selected.

From the 5 colonies selected for continued culture, 1 colony was used for ELISA after colony selection. ELISA verified the removal of G-CSF from the 4T1 cell line (Figure 10). Each time ELISA was performed, the 4T1.GCSF^{-} after selection group appeared lower than detectable. A 4T1.GCSF^{-} cell line was successfully created.

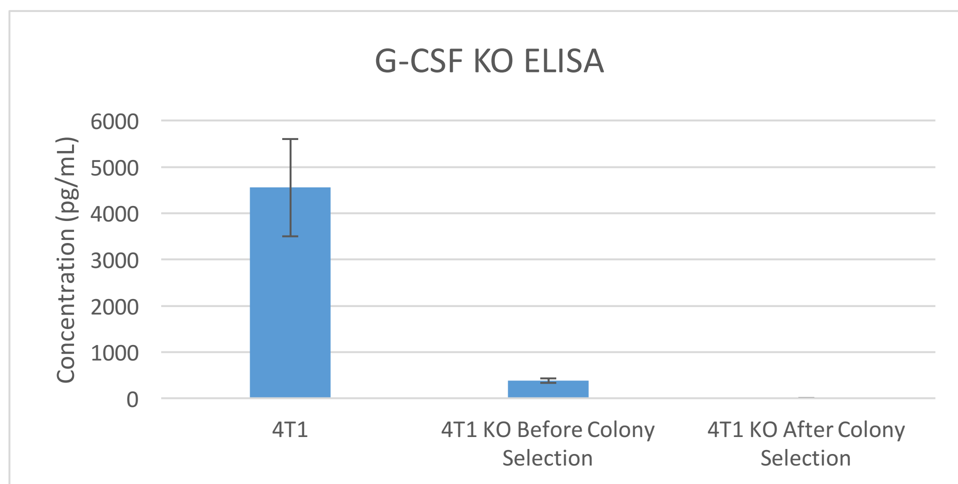


Figure 10. Sandwich ELISA performed on 4T1 and 4T1.GCSF^{-} cell lines both before and after colony selection compared concentrations of the protein G-CSF in the cells (n=3).

5.4 Discussions and future work

CRISPR/Cas9 is revolutionary technology that allows removal of specified genes in any genome.[20] The use of this method generated the 4T1.GCSF$^-$ cell line. In the future, 4T1.GCSF$^-$ and 4T1 cells will be implanted in balb/c female mice to determine if knocking out G-CSF inhibited the ability of 4T1 cells to induce MDSC proliferations. The cell line will be tested to see if the % of MDSCs in the spleen of balb/c female mice injected with 4T1.GCSF$^-$ has decreased compared to 4T1. This experiment will be done using flow cytometry to measure the % of cells in the spleen that are characterized as MDSCs.

After it has been verified that there is a decrease in the number of MDSCs in the spleen, an in-vivo prophylactic vaccination will occur. 4T1.GCSF$^-$and 4T1 will be compared in a prophylactic vaccination study. Groups of mice will receive 100,000 irradiated 4T1.GCSF$^-$ or 4T1 cells on days 0 and 10. Mice will be challenged with live tumor cells on day 20 and tumor volumes will be recorded three times per week.

If the 4T1.GCSF$^-$ fails to give a similar result as EMT-6 vaccinated mice, an alternate experiment may be performed using TNF-α and the 4T1.GCSF$^-$ cell line. G-CSF negates the effect of TNF-α in the body.[21] The previous experiments performed, adding cytokines to the 4T1 cells, should be repeated with 4T1.GCSF$^-$ to see if a different result is observed. There was a minimal difference in the addition of TNF-α to 4T1 therefore with the removal of G-CSF there could be a greater and more impactful effect on the immunogenicity of the cell line. If the 4T1.GCSF$^-$ cell line does not provide desired results as predicted for the in-vivo prophylactic vaccination, an additional in-vivo prophylactic vaccination should be performed with the 4T1.GCSF$^-$ cell line with cytokines.

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