12-2014

Development and Characterization of an Autologous Whole Cell Breast Cancer Vaccine

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Biomedical Engineering

by

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University of Arkansas
Bachelor of Science in Biology, 2012

December 2014
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

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Abstract

Approximately 40,000 women will die from breast cancer in the United States in 2014. About 90% of these deaths will be due to metastases, rather than the primary tumor and majority of metastases are due to the recurrence and progression of non-metastatic disease. Current adjuvant treatments, such as chemotherapy and radiation, have severe side effects and may result in overtreatment and drug resistance.

Since greater than 90% of patients are diagnosed between stages I-III and have minimal residual disease after treatment, there is an opportunity to treat patients with an autologous breast cancer vaccine. Autologous vaccines under development have a multivalent antigen repertoire, nontoxic side effects and most importantly, allow for personalized, patient specific treatment. A vaccine may be able to eliminate remaining tumor cells following primary treatment, prevent a recurrence and result in improved survival.

We developed an autologous breast cancer vaccine using two murine cell lines, 4T1 and EMT6, to demonstrate the potential of vaccines for adjuvant treatment of breast cancer. We first tested two commonly used methods of inactivating cells, irradiation and freeze/thaw cycling, to see if either method was superior in establishing protective immunity. Next, we measured surface expression of MHC I, MHC II, Fas, ICAM-1, B7-1 and B7-2 and secretion of the immunosuppressive cytokines GM-CSF, IL-6, MCP-1, TGF-β, and VEGF by each cell line to better understand differences in immunogenicity.

In the EMT6 model, vaccination with irradiated cells provided protection from live tumor challenge in 80% of mice, while no protection was seen following vaccination with freeze/thawed cells. Furthermore, a minimum threshold of 250,000 irradiated cells was needed to elicit an anti-tumor response. In the 4T1 model, no protection was generated by irradiated or

freeze/thawed vaccines. After measurement of surface molecules, B7-1 was up-regulated following irradiation in EMT6 cells, but not 4T1 cells. IFN-γ was used to up-regulate surface markers on 4T1 cells. Additionally, EMT6 cells secreted higher levels of IL-6, MCP-1, TGF-β, and VEGF, while 4T1 cells secreted higher levels of GM-CSF. Expression of B7-1 and GM-CSF may potentially drive differences in immunogenicity.

Acknowledgements

I would like to thank my research advisor, Dr. David Zaharoff, for giving me the opportunity to explore the world of research and for his continuous support and guidance thereafter. This thesis wouldn't have been possible without his assistance and encouragement.

I would also like to thank my family for their support during the undertaking of this degree and for always believing in me.

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Chapter 1: Introduction to Breast Cancer

Diagnosis and Staging

About 295,240 women will be diagnosed with breast cancer in the United States in 2014. Majority of these cases, about 232,670, will be invasive breast cancer, while the other 62,570 cases will be carcinoma in situ, or pre-cancer. In addition to being the most diagnosed cancer, breast cancer is the second leading cause of cancer death in women. In 2014, about 40,000 women will die from breast cancer. Once a woman is diagnosed with breast cancer, the cancer is given a stage based on extent of disease. The cancer stage is one of the critical factors in determining prognosis and treatment. Staging of a cancer is evaluated on size of the primary tumor (T), number of nearby lymph nodes involved (N), and presence of metastasis (M). This is called the TNM system. The category T is divided into 4 subcategories: T1 is 2 cm or less in diameter, T2 is between 2 cm and 5 cm, T3 is more than 5 cm, and T4 is a tumor of any size growing into the chest wall or skin. The category N is also divided into 4 subcategories: N0 is no presence of tumor cells in lymph nodes, N1 is cancer has spread to 1-3 underarm lymph nodes and/or micrometastases in the internal mammary lymph nodes (N1mi), N2 is cancer has spread to 4-9 underarm lymph nodes or has enlarged mammary lymph nodes, and N3 is cancer has spread to 10 or more underarm lymph nodes with one area greater than 2 mm or cancer has spread to 4 underarm lymph nodes with one area greater than 2 mm and cancer is found in internal mammary lymph nodes. The category M is divided into two subgroups: the absence of metastatic disease (M0) or the presence of metastatic disease (M1)[1].

Information from the T, N and M categories are combined and given a single stage grouping. The stage ranges from Stage I (least advanced) to Stage IV (most advanced). A patient may be assigned a Stage 0 if the cancer is non-invasive. Most commonly this is ductal

carcinoma in situ (DCIS) and is considered pre-cancer. The TNM system is combined to the stage group as follows:

Stage I: T1, N0, M0

T0 or T1, N1mi, M0

Stage II: T0 or T1, N1, M0

T2 or T3, N0, M0

T2, N1, M0

Stage III: T0 to T2, N2, M0

T3, N1 or N2, M0

T4, N0 to N2, M0

Stage IV: any T, any N, M1

In general, an increase in the T, N, and M categories correlate with more advanced stage, but as soon as distant metastases are present the cancer is Stage IV[1]. Majority of patients are diagnosed with cancer between Stage 0 and Stage II. Approximately 20.3%, 41.4%, 23.8% of women are diagnosed with Stage 0, I, or II, respectively. Only 8.3% of women are diagnosed with Stage III cancer and 3.9% of women are diagnosed with Stage IV cancer[2]. Considering that early diagnosis of breast cancer is critical for survival, it is beneficial that most breast cancer patients are diagnosed at an earlier stage. As the number of tumor cells spreading through nearby lymph nodes and establishing in distant organs increases, the harder the cancer is to eliminate, which is why early diagnosis of breast cancer is key. For example, the relative 5 year survival rate for breast cancer patients is 100%, 93%, 72% and 22% for Stage I, II, III and IV, respectively[1]. The low survival rate for stage IV disease is directly related to the establishment of distant metastases and the inability to eradicate all of the tumor cells.

Subtypes

In addition to staging of breast cancer, presence of the hormone receptors, estrogen receptor (ER) and progesterone receptor (PR) and human epidermal growth factor receptor (HER2/neu) is also considered when giving a prognosis and treatment. Breast cancers can be divided into 4 subtypes: luminal A, luminal B, triple negative and HER2 type. The most common subtype is luminal A, which has presence of one or both hormone receptors and is HER2 negative. Luminal B contains one or both hormone receptors and is HER2 positive. Triple-negative subtype lacks all three receptors, while the HER2 subtype only expresses the HER2 and no hormone receptors[3].

Luminal tumors look most like the cells (luminal cells) on the inner lining of mammary ducts and are the most frequently diagnosed accounting for 40% (Luminal A) and 20% (Luminal B) of all diagnoses. Of all of the subtypes, luminal A usually has the best prognosis. Compared to luminal A, luminal B is associated with having larger tumors or positive lymph nodes. The prevalence of TNBC and HER2 type ranges from 15-20% and 10-15% of all diagnosed breast cancers, respectively. Both triple negative and HER2 type are often aggressive and associated with recurrence and metastases. The receptor status of each tumor must be considered when treating breast cancer patients because some treatments, like hormone or targeted therapy, will only work on patients with tumors that express ER, PR or HER2[3].

Treatment

The primary treatment of breast cancer is surgery and is usually followed by adjuvant therapy including chemotherapy, radiation, hormone therapy and/or targeted therapy. Surgery options include either a partial mastectomy or a mastectomy. A partial mastectomy involves

removal of the tumor and surrounding tissue, called a lumpectomy, or removal of up to a quarter of the breast, called a quadrantectomy. After tissue removal, edges of the tumor are examined by a pathologist for presence of tumor cells. If tumor cells are not found, the tissue has "negative margins" and no further surgery may be needed. If tumor cells are found, the tissue has "positive margins" and tumor cells may have been left behind. The surgeon may go back to remove more tissue or perform a mastectomy. Mastectomy involves whole breast removal, and possibly nearby tissues such as underarm lymph nodes and chest muscles behind the breast. A few lymph nodes may be resected during primary surgery or a biopsy may be performed to check for presence of cancer cells. Analysis of lymph nodes is important to determine the stage of breast cancer and potential treatment following surgery[1].

After surgery if no cancer is detectable, then patients are usually given adjuvant therapy in an effort to prevent a recurrence. It is thought that early in tumor development tumor cells leave the primary tumor and begin to spread throughout the body. Although these cells exist, they are undetectable and may begin to replicate and establish a second tumor elsewhere in the body. Adjuvant therapy is given in an effort to eliminate these lingering tumor cells following surgery. Chemotherapy is a systemic therapy given to approximately 80% of patients to eliminate tumor cells that have migrated from the tumor[4]. In advanced stage patients, chemotherapy may be the main treatment modality. Chemotherapy works by attacking quickly dividing tumor cells, but in addition attacks noncancerous quickly dividing cells such as those from our bone marrow, hair follicles, and lining of our mouth. This results in side effects such as hair loss, mouth sores, low blood cell counts along with toxic side effects like neuropathy, heart damage and "chemo-brain". Radiation therapy is a local treatment given at the tumor site. In breast conserving surgery, radiation treatment is given to lower the chance that the cancer will

come back in the breast or nearby lymph nodes. After a mastectomy, radiation treatment may be given if the tumor was larger than 5 cm or if cancer cells were found in the lymph nodes[1].

Hormonal therapy, such as Tamoxifen, may also be given as an adjuvant therapy to breast cancer patients. Estrogen promotes the growth of cancers that is hormone-receptor positive and hormonal therapy either lowering estrogen from acting on the cancer or lowering estrogen levels. Side effects of tamoxifen include fatigue, hot flashes, mood swings and possibly more severe side effects such as secondary cancers of the uterus and blood clots. However, this therapy is only given for hormone receptor positive tumors. Similar to hormonal therapy, targeted therapy, such as trastuzumab (Herceptin), relies on the presence of HER2 on the surface of tumor cells. Elevated HER2 expression is associated with an aggressive growing and spreading tumor[1]. Trastuzumab attempts to slow down cancer growth by use of a monoclonal antibody targeting HER2 to block the cell from receiving growth signals[1]. Compared to chemotherapy, side effects aren't as severe and include fever, chills, weakness, nausea and vomiting with a potential severe side effect of heart failure.

Some patients may receive chemotherapy or hormonal therapy prior to surgery, which is called neoadjuvant therapy. The goal of neoadjuvant therapy is to shrink the tumor and allow for a less invasive surgery. If a patient receives neoadjuvant therapy, they may not receive any adjuvant therapy.[1]

Recurrence

After treatment, if not all cancer cells are eliminated, the cancer may come back either locally or distantly (metastases) and this is called a recurrence. A local recurrence may come back as cancer in the breast or in the surrounding chest area. The treatment following a local

recurrence is similar to treatment of the primary tumor. If the patient originally had a lumpectomy, often the patient undergoes a mastectomy and receives some sort of adjuvant therapy. If the patient originally had a mastectomy, the secondary tumor is removed and often followed with radiation and/or other adjuvant therapies[1].

Distant recurrences most often occur in the bones, lung, liver or brain. Treatment after a diagnosis of metastases focuses more on the length and quality of life of the patient, rather than removing the cancer using the same therapies used to treat the primary tumor. Although metastatic cancer is treatable, it is not curable[1].

The possibility of a recurrence depends on a few different factors including the number of positive lymph nodes at the time of primary surgery and the breast cancer subtype. For stages I, II, and III the recurrences rates after 5 years are 6.9%, 11.2% and 12.7%, respectively. After 10 years, the recurrence rates increase to about 20%[5]. Additionally, a greater chance of recurrence is associated with more aggressive tumors, such as TNBC. For example, 6 years after diagnosis almost a third of TNBC patients have a recurrence compared to only 10% of all breast cancer patients. TNBC patients divided into their cancer stage at diagnosis show a distinct increase in recurrence corresponding with a higher cancer stage. The prevalence of recurrence 5 years after diagnosis in TNBC patients is about 7%, 33%, and 60% for stages I, II and III, respectively[6]. As evident by these statistics, an increase in recurrence is associated with extent of disease at time of diagnosis and more aggressive breast cancer subtypes.

Since 90% of breast cancer deaths are due to metastases[7] and only 3.9% of patients are diagnosed with stage IV cancer[1], majority of breast cancer deaths are due to the recurrence of non-metastatic disease. A non-toxic specific therapy is needed to target lingering tumor cells

following surgery in an effort to decrease breast cancer recurrence, and therefore increase survival rate.

Objective

The overall objective of this project was to develop and characterize an autologous tumor cell vaccine using a murine model that may be able to prevent recurrences and therefore result in improved overall survival. An autologous tumor cell vaccine is a form of active specific immunotherapy (ASI) that utilizes inactivated patient specific tumor cells to launch an anti-tumor immune response. Active stimulation of the immune system is one advantage of an autologous vaccine over current passive targeted therapies. By using patient-specific tumor cells, numerous known and unknown antigens may be targeted at once ensuring a personalized and potentially polyclonal immune response with minimal toxicity.

In this study, we used two murine breast cancer cells lines, 4T1 and EMT6, to develop an autologous vaccine. Both cell lines were used in order to compare their inherent immunogenic qualities relating to the vaccine and in vitro characterization. The 4T1 cell line is known as a non-immunogenic cancer representing human stage IV breast cancer, while the EMT6 cell line is relatively immunogenic. For each cell line, we first pursued the most immunogenic way to inactivate tumor cells. Two commonly used methods were used to inactivate cells, irradiation and freeze/thaw, and given as prophylactic vaccines to see if there was any difference in protection after a challenge with live tumor cells. An immunogenic profile was then created for each cell line based on surface molecule expression and immunosuppressive cytokine secretion. These are two characteristics that tumors use to evade the immune system, specifically T lymphocytes. We hoped to determine a few key differences in the immunogenic profile between

each cell line. Based on the differences, an immunogenic tumor may be engineered from a non-immunogenic tumor by manipulating various immunogenic characteristics and therefore promote an anti-tumor immune response.

Chapter 2: Autologous Whole Tumor Cell Vaccines for Breast Cancer

Introduction

Approximately 232,570 women will be diagnosed with invasive breast cancer and 40,000 women will die from breast cancer in the United States in 2014[1]. However, 90% of breast cancer deaths are due to metastases to the bone, brain, lung and liver, not the primary tumor[7]. Considering only 3.9% of breast cancer patients are diagnosed with metastatic Stage IV cancer, majority of these metastases and deaths are due to the recurrence and progression of non-metastatic disease[2]. For example, 7% of Stage I patients will experience tumor recurrence after 5 years, while 11% and 13% of patients with Stage II and Stage III, respectively will experience tumor recurrence. After 10 years, the overall breast cancer recurrence rate is about 20%[5].

In an effort to combat tumor recurrence, majority of patients receive adjuvant therapy such as chemotherapy and/or radiation. Chemotherapy is routinely offered to about 80% of patients[4] even though the benefits are limited especially in older patients. Following chemotherapy treatment, patients younger than 50 years of age increase their 5 year survival rate by 4.7% while only a 2.6% increase is seen in patients older than 50[8]. Chemotherapy normally includes a combination of drugs, such as anthracyclines or taxanes, and has toxic side effects including hair loss, mouth sores, low blood cell counts, neuropathy and "chemo-brain"[1]. Radiation is also used an as adjuvant to treat breast cancer patients. A meta-analysis showed only a 5% decrease in 15-year breast cancer mortality risk [9]. Overtreatment with chemotherapy and/or

radiation can occur either by treating patients that do not need adjuvant therapy or administration of more aggressive treatment than necessary. A recent estimate suggests that 1 to 3 deaths occur due to overtreatment for every breast cancer death evaded [10]. Although chemotherapy and radiation increases survival rate for breast cancer patients, it comes with severe side effects, overtreatment results in unnecessary deaths and 1 in 5 patients still have a recurrence after 10 years. These recurrences result after adjuvant therapy due to the inability to eliminate all hidden tumor cells and may then acquire drug resistance [11, 12]. Due to severe side effects, overtreatment, and drug resistance with current adjuvant therapies there is a need for more effective interventions to limit recurrence and progression.

In 2010, the FDA approved sipuleucel-T (Provenge), an active cellular immunotherapy for the treatment of advanced prostate cancer. Since then, there has been a movement towards research of active specific immunotherapies (ASI) for the treatment of cancer. ASI is the use of tumor specific antigens to stimulate the adaptive immune system and launch an immune response against a tumor. In theory, after exposure to tumor antigens, tumor specific lymphocytes would be able eradicate all tumor cells and establish an immune memory. Breast cancer would be an ideal disease for the use of ASI due to the delay of recurrence after treatment and the thousands of patients eligible for this therapy. The time period before a recurrence would allow the immune system time to be stimulated by ASI, activate T lymphocytes to eradicate residual disease, and memory T lymphocytes to maintain immune memory of breast cancer. Since greater than 90% of patients are diagnosed between stages I-III and have minimal residual disease after treatment, ASI has the potential to benefit thousands of breast cancer patients from a recurrence, and therefore result in improved overall survival.

ASI can be accomplished using different vaccine formulations classified by the form of antigen and how they are administered. Antigens can be presented to the immune system as whole tumor cells [13], tumor specific peptides or tumor associated peptides [14, 15], carbohydrates [16] or cell lysates [17]. In addition, antigens can be delivered as inactivated whole cells [18, 19], pulsed with dendritic cells [20, 21], or delivered with cytokines or other immunomodulators [13, 21, 22].

Advantages of Autologous Tumor Cell Vaccines

Autologous tumor cells have a number of advantages when used as ASI, including having a multivalent antigen repertoire and patient specificity. An ideal cancer antigen has immunogenicity, oncogenicity, and specificity. Out of 75 cancer antigens analyzed by Cheever et al., none of the antigens satisfied all criteria for an ideal cancer antigen [23]. However, by using many antigens, both known and unknown, the ideal cancer antigen criteria may be satisfied among all of the available antigens to allow a higher immune system response. Additionally, by using autologous cells all potential antigens are being shown to the immune system. More than one potential antigen can be targeted at once, eliminating the problem of antigen loss and potentially activating a polyclonal immune response [13, 22]. Importantly, all of the potential antigens are directly from the patient's own tumor, therefore ensuring that patients are exposed to the antigens that their own tumor expresses [13]. There are an estimated 105 mutant genes in breast cancer and a personalized approach can be taken to ensure these mutations are recognized by a patient's immune system [24]. These inactivated whole tumor cells could enable a patient's own immune system to specifically destroy remaining cancer cells left after surgery and prevent the development of recurrence [25].

Furthermore, autologous whole tumor cells may be beneficial to even the aggressive triple negative breast cancer (TNBC). TNBC is a lethal subtype of breast cancer that has a greater risk of recurrence and shortened survival compared to hormone positive subtypes and has a 5 year survival of ~90%, ~78%, and ~35%, for stage I, stage II, and stage III-IV, respectively [26]. However, poor survival of TNBC can be partly explained by limited treatment options since hormonal and HER2 treatments do not benefit TNBC patients. By inactivating whole TNBC tumor cells, expression of current unknown antigens may be presented to the immune system and elicit a patient-specific adaptive immune response.

Disadvantages of Autologous Tumor Cell Vaccines

Preclinical and clinical studies have presented some challenges with development of autologous tumor cell vaccines. First, harvesting enough tumor cells can be inefficient and time consuming [13]. In a study by Avigan in 2004, only 16 of 32 vaccines were successfully generated. Vaccines were composed of fusions of dendritic cells and tumor cells which were then irradiated. Tumor cells did not expand ex vivo and tumor tissue smaller than 1cm did not provide adequate cell yields [27]. However, Elliott and Head mention obtaining 10,000,000 cells from a $1cm^3$ tumor [28]. The differences in tumor cell yield may be due to a lack of standardization of autologous tumor vaccine preparations which does not allow comparable results [29]. Tumors can be dissociated and then inactivated in a variety of ways. Tumors can be dissociated enzymatically with collagenase and DNAse [28], mechanically, or both [30]. Once in a single-cell suspension, tumor cells can be inactivated by irradiation [31, 32], freeze-thaw [30] or mitomycin-C [28]. Additionally, while some protocols mention separating tumor cells from other cells in the

suspension, others do not [28, 31-33]. It is uncertain which method(s) induces the most immunogenic vaccine.

Furthermore, tumor cells are mainly composed of normal antigens [22]. The tumor antigens present are only mildly immunogenic and self-like, therefore the immune response is not strong. Also, tumor cells do not express enough costimulatory proteins to produce a significant response [25]. Delivery systems and adjuvants are under exploration to increase immunogenicity.

Clinical trials with Autologous Breast Cancer Vaccines

To our knowledge, there have only been four clinical trials exploring autologous breast cancer tumor cell vaccines, two of which are ongoing. Majority of the clinical trials involving autologous vaccines for any cancer have only been phase I/II trials with small numbers of advanced stage patients. These patients have larger, metastatic tumors that secrete immunosuppressive factors and a poor immune status [34]. Since tumor cells alone have shown to be poorly immunogenic, there has been no trial testing of autologous cells alone. Instead, several trials using transfected autologous tumor cells have been explored. Autologous cells have often been genetically modified to express cytokines, chemokines or costimulatory molecules in order to make the vaccine more immunogenic [13]. GM-CSF has shown to be an effective costimulatory cytokine due to its ability to activate a tumor targeted T cell response by recruitment of dendritic cells. The dendritic cells are then able to uptake, process and present the antigen [13]. Although GM-CSF has been used as an immune adjuvant, if given at high doses over a sustained period of time it can cause immunosuppression by recruitment of MDSCs. A minimal dose to achieve desire immunogenic effects must be greater than 36 μg/day, but lower than 100-500 μg/day [35].

There are currently two active clinical trials registered in the NIH Clinical Trial database investigating autologous breast cancer cells that have been engineered to express GM-CSF. Both are phase I/II trials sponsored and collaborated by Dana-Farber Cancer Institute and Brigham and Women's Hospital. One trial is strictly for metastatic breast cancer patients[36], while the other is for breast cancer stages II-III[37]. The study completion date for metastatic patients is January 2015 and the study completion date for stages II-III is April 2015 with a primary completion date of April 2014.

A study conducted by Ahlert et al.[33] explores a vaccine comprised of autologous breast tumor cells infected with Newcastle disease virus (NDV). Primary breast cancer and metastatic breast cancer patients were treated with an autologous tumor cell vaccine infected with non-lytic NDV. Primary breast cancer patients started immunotherapy following surgery and were given at least two vaccinations. If applicable, chemotherapy and radiation was administered 28-35 days after the start of immunotherapy. Metastatic breast cancer patients followed this same protocol with the addition of IL-2/IFNα-2a and anti-suppressive drugs, such as cyclophosphamide and epirubicin. Vaccine quality was divided into high and low quality. The high quality group had a median count of $1.5x10^6$ viable tumor cells for each vaccine and median viability greater than 33%. The low quality group did not meet one of these qualifications. After 4 years, a significant difference was seen in patients with primary breast cancer: 96% of patients with a high vaccine quality were alive compared to 68% with a lower vaccine quality. An increase in survival was also observed in metastatic breast cancer patients with a high quality vaccine compared to a low quality vaccine, although the difference was not significant (P= 0.18) due to low patient numbers. [33]. It is not clear whether any follow-up studies were performed despite promising

results. This study also represents the importance of developing and standardizing vaccine preparations.

In a study using autologous cells with other antigens, Elliott and Head[28] used a vaccination with autologous breast tumor cells, allogeneic breast tumor cells, and 3 antigens combined with IL-2 and GM-CSF given to patients with depressed lymphocyte immunity. All patients underwent surgery and were finished with chemotherapy and/or radiation treatment prior to immunotherapy. Immunocompetency was determined at least 10 weeks after completion of adjuvant therapy and evaluated host reactivity to tumor antigens. Patients with depressed lymphocyte immunity were given a total of 6 vaccines on weeks 1, 2, 3, 7, 11 and 15. They concluded that disease specific survival of vaccinated patients with depressed immunity (89%) was significantly different than patients with depressed immunity that were unvaccinated (59%) [28].

Autologous Tumor Cell Vaccines for Other Cancers

Although there have not been many clinical trials reporting autologous vaccines for treatment of breast cancer, autologous whole tumor cell vaccines are being explored in numerous other cancers. Phase I clinical trials of autologous tumor cells administered with immunomodulators such as GM-CSF and BCG have been reported in colon cancer[32], hepatocellular carcinoma[38], melanoma[39], among other cancers. As a whole, these trials demonstrated the safety of this vaccine with a potential for clinical benefit [40]. Several of the intriguing results are highlighted to demonstrate the potential of autologous vaccines.

An autologous tumor cell vaccine for metastatic melanoma suggests a few notable conclusions. Patients were vaccinated with irradiated tumor cells weekly for 3 weeks and then

monthly for 5 months. A better overall survival was seen in patients with minimal disease, patients treated with GM-CSF or IFN-γ as adjuvant, or patients receiving an average of <7 million cells for the first three vaccinations. The 5 year overall survival for patients with minimal disease was 47% compared to 13% for patients with metastatic disease. The 5 year event-free survival for patients treated with adjuvant cytokines was 26% compared to 0% for patients without adjuvant treatment. In addition, patients with <7 million cells per vaccination had a 5 year event-free survival of 35% compared to 24% of patients with >7 million cells per vaccination. The vaccine produced minimal toxicity and was associated with long-term survival [39].

A study done by Peng et al. showed that an autologous tumor vaccine lowered the post-surgical recurrence rate of hepatocellular carcinoma. Patients treated with a vaccine had a 3 year recurrence rate of 54%, while the control group had a 72.1% recurrence rate [38]. All of these vaccines were safe and well-tolerated.

One Phase III trial using autologous whole cells was reported in colon cancer. This trial consisted of autologous colon cancer cells with BCG (OncoVax) and has shown a statistically significant improvement in recurrence-free survival and overall survival, specifically in Stage II patients [32]. Most importantly, the results showed a 61% decrease in recurrence rate with ASI compared to no treatment following curative surgery [41].

Preclinical Studies of Autologous Whole Tumor Cell Vaccines

Similar to clinical trials, not many pre-clinical studies have explored autologous breast cancer vaccines. To our knowledge, there has not been a single lab animal study using autologous vaccines in the therapeutic setting. However in the prophylactic setting, inactivated

tumor cells can elicit a protective immune response depending on the aggressiveness of the murine cancer cells. The immunogenic TS/A mammary cancer cell line has been shown to elicit protective immunity [42], while less immunogenic EMT6 mammary carcinoma cell line [43] and non-immunogenic mammary 4T1 cell line do not [42]. However, van Pel and Boon demonstrated in 1982 that protective immunity could be induced in mice with non-immunogenic tumors. The key point was that cells used to immunize the mice must have been exposed to a DNA damaging agent [44]. In addition, the lack of tumor immunogenicity was not a lack of tumor antigens, but rather an inability of the immune system to be stimulated due to immunosuppression [44]. In an effort to overcome immunosuppression, a variety of immune system adjuvants have been administered with cancer cells. By adding immune system adjuvants, most commonly GM-CSF and/or IL-2, a higher protective immunity is seen than by autologous cells alone [45]. Additionally, immunosuppression specifically occurs around a tumor microenvironment, but there is a "window of opportunity" 7-10 days after surgical removal of the tumor with decreased immune suppression [46]. The knowledge gained from van Pel and Boon's study has been applied to nearly every murine tumor system [44]. By decreasing immunosuppression through addition of adjuvants or tumor resection, we can elicit a high protective immunity. We know that autologous cell vaccines work in some murine tumors and this gives hope for successful human vaccines.

Although autologous whole tumor cell vaccines have shown substantial pre-clinical success, it is not evident why some murine cell lines elicit protective immunity while others will not. We want to continue studying in the murine system to improve our understanding of what is the right context for a vaccine to be most effective. Using two murine cell lines, we may compare the inherent immunogenic qualities and hope to determine some key factors that influence immunogenicity. By understanding factors that mediate the anti-tumor immune

response in the murine system, we may be able to apply our knowledge to the clinical setting and improve clinical success.

Chapter 3: Characterization of Immunogenicity in Two Breast Cancer Cell Lines

Introduction

Current adjuvant therapies for breast cancer are effective at prolonging survival, but still many patients relapse and toxicity is significant. Therefore, we sought to develop an autologous whole breast cancer cell vaccine due to the favorable safety record and patient-specific approach of breast cancer treatment. Autologous cells may specifically engage the immune system to eliminate lingering tumor cells following primary treatment. Furthermore, because lumpectomy or mastectomy is indicated for the vast majority of breast cancer patients, resected tumors offer a readily available, patient-specific source of tumor antigen. However, little clinical success has been observed due to the "self-like" quality of tumor cells, immunosuppression of tumor bearing patients and vaccinating in patients with extensive disease. In this study, we used two murine breast cancer cell lines, 4T1 and EMT6, as the basis for the development of an autologous vaccine.

First, we determined the most immunogenic way to inactivate tumor cells using two common cell inactivation methods, irradiation and freeze/thaw cycling. After noticing differences in immunogenicity between the two cell lines, surface marker expression and immunosuppressive cytokine secretion was measured in hopes of identifying some key differences between each cell line. To further study the immunogenicity of these cell lines, a hybrid vaccine consisting of both cell lines was given to determine whether presence of 4T1 cells altered protection immunity against EMT6 cells. By identifying differences between each cell

line, manipulations may be made to better understand what significantly influences the immunogenicity of each cell line. Additionally, using the inherent immunogenic cell line, mice were vaccinated with varying cell doses to determine a minimum threshold of cells that will induce an anti-tumor immune response. Ultimately, our goal is to create immunogenic cells that will elicit an anti-tumor immune response against an inherently non-immunogenic cell line. The following data represent a significant first step in the development of a more effective autologous tumor cell vaccine for use as an adjuvant therapy for breast cancer.

Methods

Mice and Tumor Cell Culture

Female six to eight week old BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed and maintained under pathogen-free conditions in microisolator cages. Animal care was under compliance with the recommendations of The Guide for Care and Use of Laboratory Animals (National Research Council). 4T1 and EMT6 murine mammary carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). The 4T1 cell line was maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin and streptomycin, and 2mM L-glutamine. The EMT6 cell line was maintained in RPMI 1640 supplemented with 15% fetal bovine serum, 100 units/mL penicillin streptomycin, and 2mM L-glutamine. Cultures were maintained in a 37°C atmosphere with 5% CO_2.

Proliferation Assay

4T1 and EMT6 cells were suspended in medium and irradiated at varying doses. Cells were either not irradiated (0 Gy) or irradiated at 20, 40, 60, 80, or 100 Gy using a cesium irradiator. Cells were plated in triplicate in a 96 well plate for each irradiation dose with 200 µl of medium. After 24, 48, 72 and 96 hours of incubation, 20 µl of CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI) was added to each well and incubated for an additional hour. Absorbance was read at 490 nm using a Biotek Synergy 2 plate reader. This reagent is a colorimetric way to determine the number of viable cells in proliferation assays through conversion of MTS to formazan by metabolically active cells.

Tumor Vaccines & Challenge

Prior to all vaccines and challenges, mice were anesthetized with ketamine (75 mg/kg)/xylazine (15 mg/kg) injected intraperitoneally. Inactivated cells comprising the vaccine were injected subcutaneously on the right hind flank, while the live tumor cell challenge was administered subcutaneously on the left hind flank. Tumors were measured with calipers in two perpendicular diameters every 3 to 4 days. Tumor volume was calculated according to the equation $V(mm^3)= ½(\text{long width} * \text{short width}^2)$. Mice were euthanized by cervical dislocation when volume reached 2000 mm^3.

Irradiated versus Freeze/Thawed Vaccines

Cells were γ-irradiated at 100 Gy while suspended in medium at a concentration of 7,000 cells/µl. After irradiation, cells were centrifuged at 2000rpm for 5 minutes and resuspended in DPBS. For cell lysis, cells were subjected to 5 freeze/thaw cycles while suspended in DPBS using a 37°C water bath and liquid nitrogen. Mice were injected with $1x10^6$ irradiated or

freeze/thawed, 4T1 or EMT6 cells on days -20 and -10 prior to challenge. On day 0, mice were challenged subcutaneously with $5x10^4$ viable 4T1 cells or $5x10^5$ viable EMT6 cells.

Hybrid Vaccine

4T1 and EMT6 cells were taken from in vitro culture, trypsinized, counted and resuspended in medium at a concentration of 7,000 cells/μl. Cells were irradiated at 100 Gy using a cesium irradiator and centrifuged at 2000rpm for 5 minutes, then resuspended in DPBS. Each cell line was then combined in one tube and mixed to ensure uniform suspension. Mice were injected with $1x10^6$ 4T1 cells and $5x10^5$ EMT6 cells on days -20 and -10. On day 0, mice were challenged with $5x10^5$ EMT6 cells.

Irradiated Cell Dose Vaccines

EMT6 cells were taken from in vitro culture, trypsinized, counted and resuspended in medium at a concentration of 7,000 cells/μl. Cells were irradiated at 100 Gy and centrifuged at 2000rpm for 5 minutes, then resuspended in DPBS. Mice were injected with $5x10^5$, $2.5x10^5$, $1.25x10^5$, $6.25x10^4$, or $3.12x10^4$ irradiated EMT6 cells on days -20 and -10. On day 0, mice were challenged with $5x10^5$ EMT6 cells.

Detection of Secreted Cytokines

For detection of VEGF and latent TGF-β, $1x10^6$ tumor cells and irradiated tumor cells were plated in a 6 well plate. Supernatants were taken at 24, 48, 72 and 96 hours after plating and stored at -20°C (VEGF) or -80°C (TGF-β) until ready for use. The VEGF ELISA kit (R&D systems, Minneapolis, MN) was used with a 1:2 dilution for 4T1 samples and 1:12 dilution for

EMT6 samples. The latent TGF-β ELISA kit (BioLegend, San Diego, CA) was used without dilutions. Each ELISA was performed following manufacturer's protocol. For detection of GM-CSF, IL-6, IL-10, and MCP-1, supernatants were taken as described above and measured using a cytometric bead array kit (BD Biosciences, San Diego, CA) according to manufacturer's instructions and analyzed using FCAP Array v3 (BD Biosciences, San Jose, CA).

Antibodies and Flow Cytometry

Cells were suspended at a concentration of 500,000 cells /100 µl PBS per polypropylene tube and stained with 1µg of the appropriate antibody for an hour at room temperature. When a secondary antibody conjugated to a fluorophore was needed, samples were centrifuged and supernatant discarded before resuspension and incubation with the secondary antibody for an additional hour. Antibodies used were FITC-conjugated H-2Kb, PE-conjugated I-Ad/I-Ed, PE-Cy7-conjugated CD95, FITC-conjugated CD54, APC-conjugated CD80, and PE-conjugated CD86 (BD Pharmingen, San Diego, CA), ER, PR, ErbB2, PE-conjugated IgG1, and FITC-conjugated IgG2a (Abcam, Cambridge, MA). Samples were analyzed and median fluorescent intensity (MFI) was calculated using FlowJo software (BD Biosciences, San Jose, CA).

In Vitro Doubling Time

500,000 4T1 or EMT6 cells were plated in T25 flasks with 5 ml of medium and incubated for 8, 12, 16, 20, or 24 hours. At each time point, three flasks were removed from incubation. Cells were trypsinized, resuspended in 1 ml of medium, diluted in trypan blue and counted. Doubling time was then calculated using an online cell doubling time calculator[47].

Statistical Analysis

GraphPad Prism 6 was used for statistical analysis. For survival data, a log rank test was used to compare survival of vaccinated mice to unvaccinated mice. For doubling time, a two way ANOVA was used with a Bonferroni posttest. Means of each time point was compared for each cell line. For cytokine secretion, a two way ANOVA was used with a Tukey posttest. Groups compared were as followed: 4T1 vs. 4T1 irradiated, 4T1 vs. EMT6, 4T1 irradiated vs. EMT6 irradiated, and EMT6 vs. EMT6 irradiated. In figures, asterisks were used to denote the following significance levels: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Results

Dose of Irradiation has no Effect on Proliferation of Tumor Cells

Before vaccinating mice with irradiated cells, an appropriate dose of irradiation needed to be determined to adequately inactivate the cells in a timely manner. In 4T1 cells, no difference was found between cells not irradiated and cells irradiated with any dose up to 48 hours after seeding (Figure 1). Between 24 and 48 hours there was a slight increase in proliferation by all doses of irradiation (0-100), but after 48 hours cells that received any dose of irradiation showed no further increase in proliferation. Cells that received no irradiation showed a significant increase in proliferation after 48 hours. After 72 and 96 hours, there was a significant difference in proliferation of cells that received no irradiation and cells that received any dose of irradiation. Throughout all time periods of proliferation measurement, no difference was seen between any of the doses of irradiation.

For EMT6 cells, all cells (not irradiated and irradiated) showed a slight increase in proliferation until 48 hours after incubation, but the cells that weren't irradiated showed a

slightly significant increase in proliferation compared to cells that were irradiated (Figure 1). After 48 hours, cells that were irradiated no longer showed an increase in proliferation. However, cells that received no irradiation continued to show an increase in proliferation that was statistically significant from cells that received irradiation. Again, throughout all time periods of proliferation measurement, no difference was seen between any of the doses of irradiation. For each dose of irradiation, cells continued to slowly proliferate 48 hours after incubation and then showed no further increase. Based on the proliferation of both 4T1 and EMT6 irradiated cells, we concluded that dose of irradiation has no effect on the proliferation of cells.

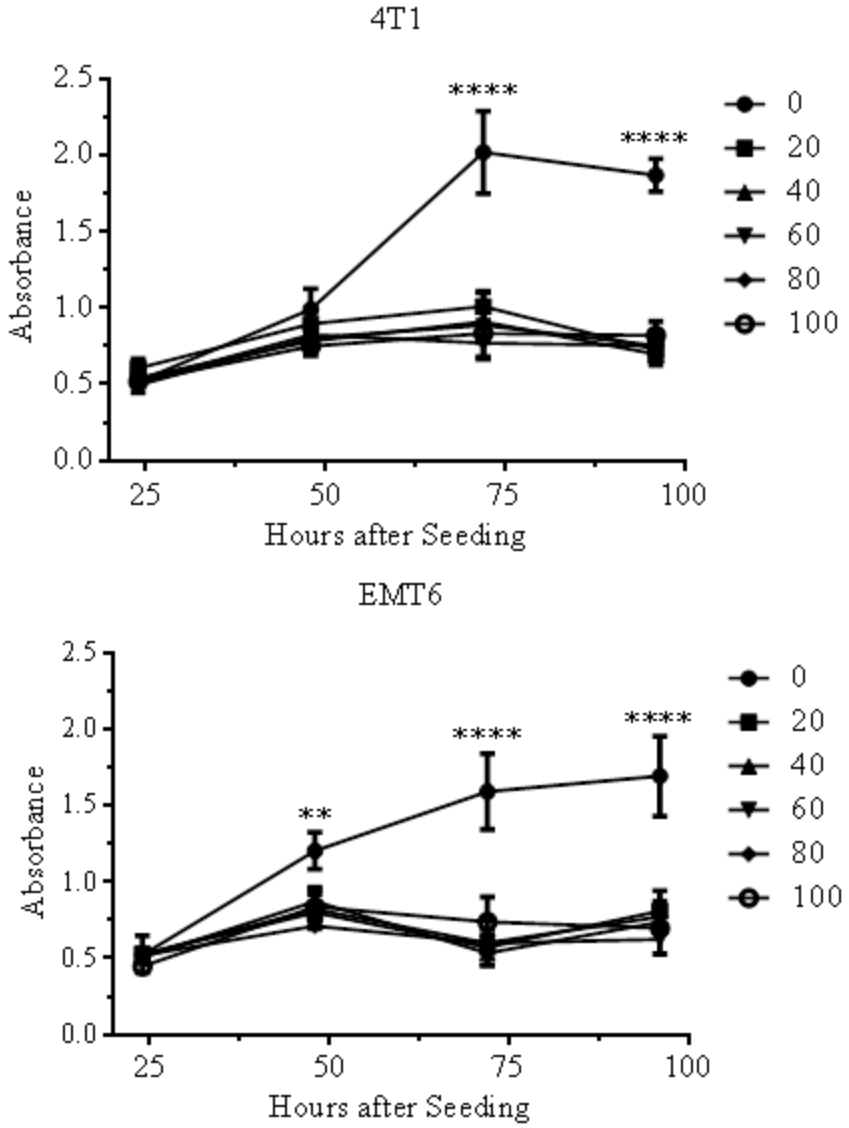

Figure 1: No difference in proliferation with varying irradiation doses. Both 4T1 and EMT6 cells were not irradiated (0 Gy) or irradiated with 20, 40, 60, 80 or 100 Gy and plated in a 96 well plate with 200µl of medium. At 24, 48, 72 or 96 hours after seeding, 20 µl of CellTiter 96 Aqueous One Solution Reagent was added to each well, incubated for an hour and read using a plate reader at 490 nm to determine cell viability. Statistical analysis was performed in GraphPad using a two way ANOVA with Tukey posttest. Error bars represent SEM. Asterisks were used to denote the following significance levels: **p≤0.01; ***p≤0.001; ****p≤0.0001.

Dose of Irradiation has no Effect on Cytokine Secretion

Since dose of irradiation showed no effect on proliferation of cells, dose of irradiation might have an effect on immunosuppressive cytokine secretion and therefore our vaccine. As

shown in Figure 2, there is a general increase in GM-CSF and IL-6 secretion by 4T1 cells for both not irradiated and irradiated cells as time increases. For both GM-CSF and IL-6, there is no significant difference between irradiated and not irradiated cytokine secretion until 96 hours after seeding. After 96 hours, there is a small significant difference in GM-CSF secretion between no irradiation and 20, 40 or 80 Gy. There is a higher significant difference between 0 Gy and 60 or 80 Gy. No significant difference is seen in GM-CSF secretion between any of the irradiation doses.

For IL-6, there is a general increasing trend in cytokine secretion as time after incubation increases however this increase is not as drastic as the increase GM-CSF. A statistically significant difference in IL-6 secretion between irradiated and not irradiated cells is not seen until 96 hours after seeding. There is a small statistically significant difference between 0 Gy and 60 or 80 Gy and a slightly higher statistically significant difference between 0 Gy and 100 Gy. Again, no difference is seen in IL-6 secretion between any of the irradiation doses. Since there was no significant difference between 20-100 Gy of irradiation, we performed a literature search to help decide an irradiation dose for a cell based vaccine. We chose 100 Gy based on the information found in our literature search[48,49].

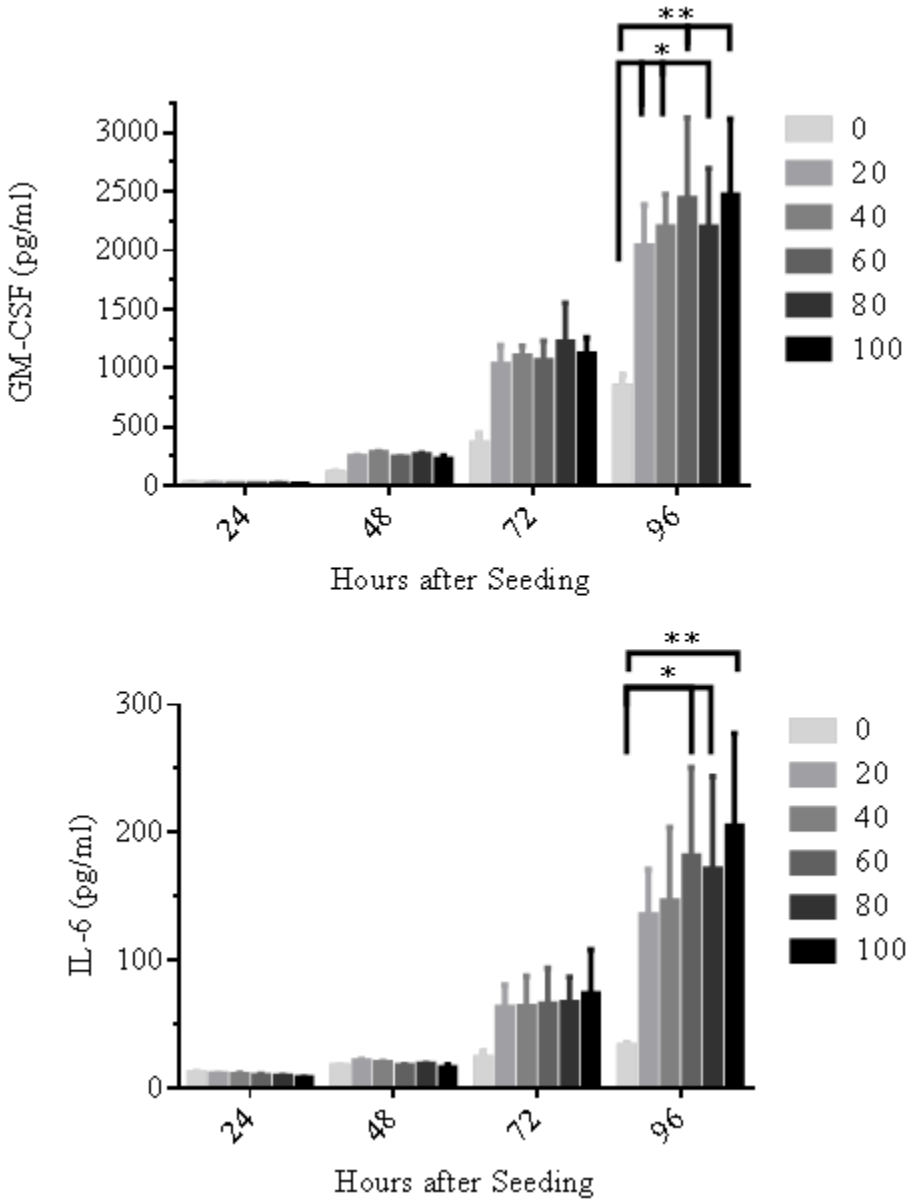

Figure 2: Cytokine secretion after varying doses of irradiation. 4T1 cells were not irradiated (0 Gy) or irradiated with 20, 40, 60, 80 or 100 Gy and plated in a 6 well plate with 5 ml of medium. At 24, 48, 72 and 96 hours after plating, samples were taken from the 6 well plate and store at -80°C. Once all samples were collected, cytokine levels (mean ± SEM) were measured with CBA. Statistical analysis was performed in GraphPad using a two way ANOVA with Tukey posttest. Asterisks were used to denote the following significance levels: *$p \leq 0.05$; **$p \leq 0.01$.

Irradiated Cells Provide Protection in Immunogenic Cell Line

To determine whether irradiated or freeze/thawed cells result in better protection when given as a prophylactic vaccine, $1x10^6$ irradiated or freeze/thawed cells were administered to

healthy Balb/c mice 10 and 20 days before viable tumor cell challenge. Vaccinations began with a tumor cell dose of 1,000,000 cells, which we thought was a reasonable amount of cells that we may be able to extract from a tumor and later be able to vaccinate in the therapeutic setting. Vaccinations were performed for both the 4T1 and EMT6 cell lines. As shown in Figure 3, no protection was observed after vaccinations with either irradiated or freeze/thawed cells in the 4T1 cell line. After each vaccination with irradiated 4T1 cells, some mice developed small tumors which regressed after a few days (data not shown). There was not a significant difference in survival of vaccinated mice and control mice, which received no vaccinations. The median survival was 35, 40 and 43 days for mice vaccinated with irradiated 4T1 cells, vaccinated with freeze/thawed 4T1 cells and control mice, respectively.

However, 80% of mice (4/5) vaccinated with irradiated EMT6 cells survived over 150 days following viable EMT6 tumor cell challenge. One mouse developed a tumor after a long delay between challenge with EMT6 cells and tumor growth. The tumor first appeared 42 days after challenge with EMT6 cells (Figure 4). Like the 4T1 cell line, there was no protection observed with freeze/thawed cells in the EMT6 cell line. The median survival for both mice vaccinated with freeze/thawed EMT6 cells and control mice was 35 days. These data confirm previous studies that irradiated cells provide better immune protection than freeze/thawed cells when administered as a vaccine [50, 51].

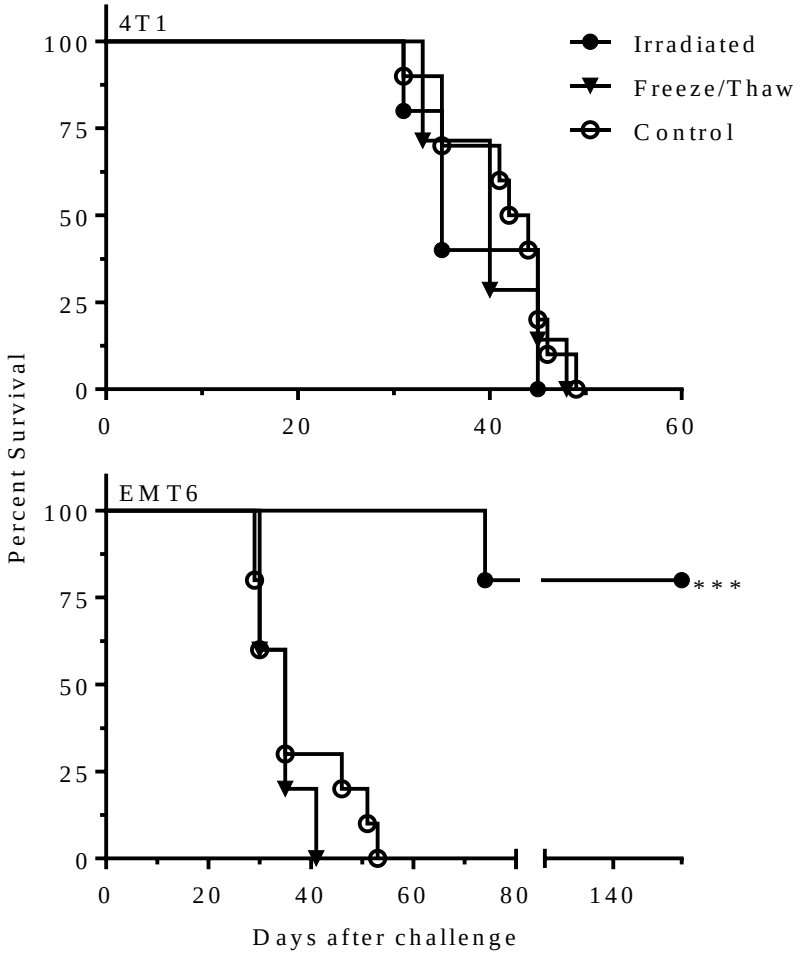

Figure 3: Irradiated EMT6 cells provide protection as a prophylactic vaccine. $1x10^6$ 4T1 and EMT6 cells were irradiated with 100 Gy or were subject to five freeze/thaw cycles with liquid nitrogen and a 37°C water bath. Vaccines were given on day 10 and 20 prior to vaccine challenge. On day 0, mice were challenged with $5x10^4$ 4T1 or $5x10^5$ EMT6 viable tumor cells and tracked for survival. Control mice were only challenged on day 0. Statistical analysis was performed in GraphPad using the Log Rank test. Asterisks were used to denote the following significance level: ***$p \leq 0.001$.

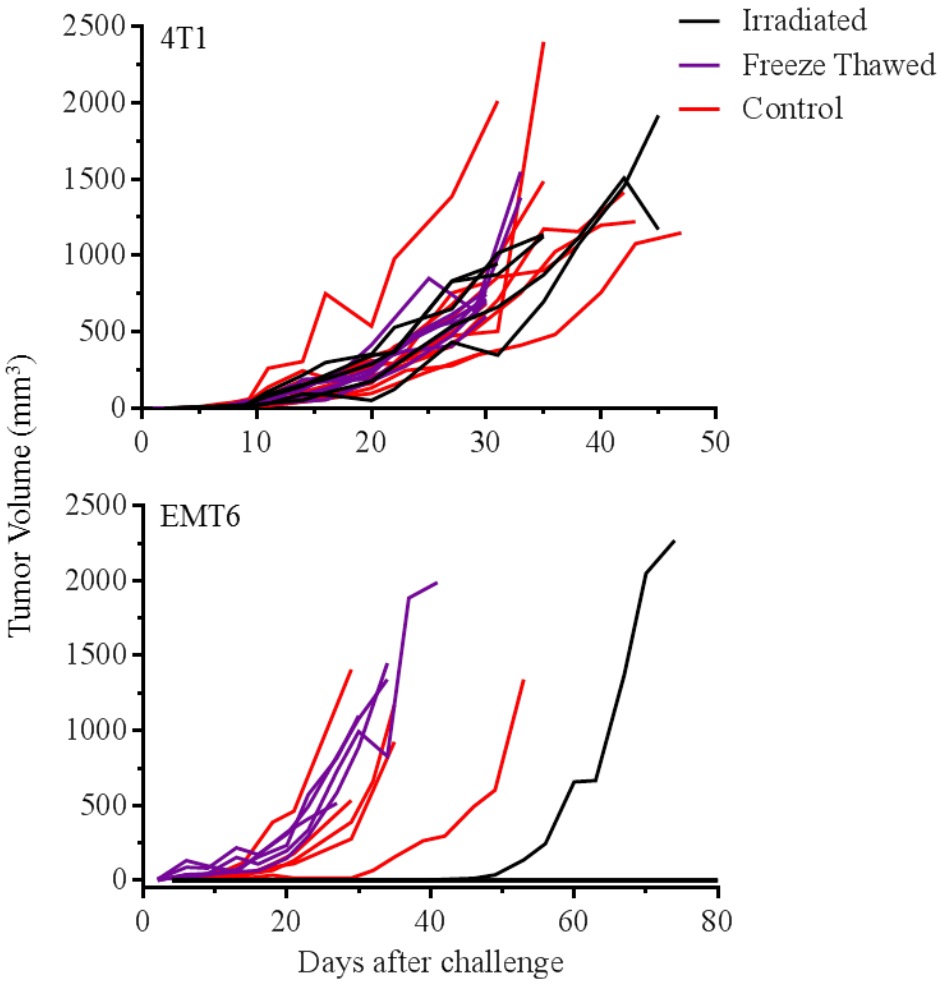

Figure 4: Irradiated EMT6 cells provide protection as a prophylactic vaccine. $1x10^6$ 4T1 and EMT6 cells were irradiated with 100 Gy or were subject to five freeze/thaw cycles with liquid nitrogen and a 37°C water bath. Vaccines were given on day 10 and 20 prior to vaccine challenge. On day 0, mice were challenged with $5x10^4$ 4T1 or $5x10^5$ EMT6 viable tumor cells and tracked for survival. Control mice were only challenged on day 0. Tumor volumes were measured every 3 to 4 days in two perpendicular directions using calipers. Statistical analysis was performed in GraphPad using the Log Rank test.

EMT6 Cells Have a Faster Doubling Time than 4T1

In order to eliminate the possibility that 4T1 tumors grew out due to a faster growth rate, doubling time for each cell line was calculated. Both cell lines showed no increase in the number of cells prior to 12 hours after seeding. The 4T1 cell line actually had a slight decrease in cell number before starting to increase after 16 hours. EMT6 cells showed a shorter lag phase

and started to slowly increase after 12 hours of incubation. As shown in Figure 5, as early as 16 hours after seeding, there are significantly more EMT6 cells than 4T1 cells. The calculated doubling time of EMT6 cells is 9.0 hours, while 4T1 cells have a 10.5 hour doubling time. Therefore, EMT6 cells grow slightly faster in vitro than 4T1 cells do. This eliminates the possibility that 4T1 escape protective immunity due to a faster growth rate.

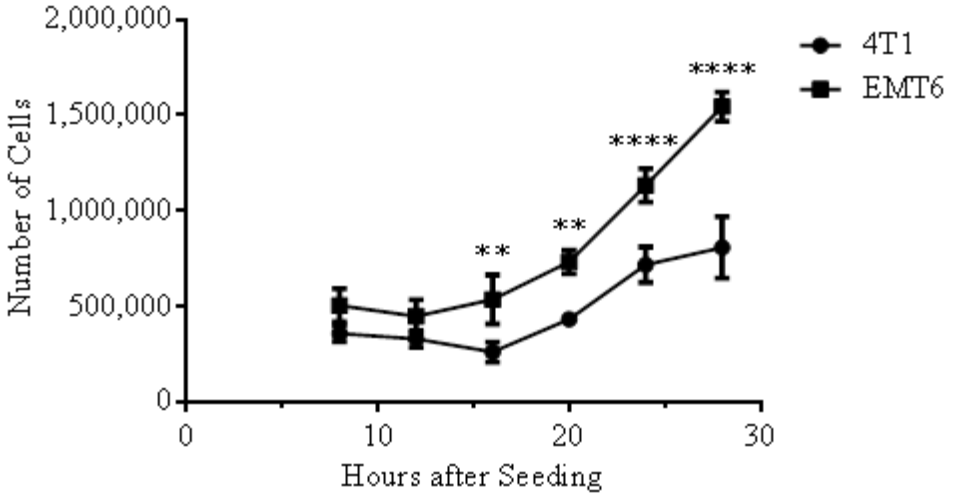

Figure 5: In vitro doubling time of 4T1 and EMT6 cells. 500,000 cells were plated in T25 flasks and cultured for various amounts of time. At 8, 12, 16, 20, 24 and 28 hours after seeding, flasks were removed from incubation, trypsinized, centrifuged and resuspended for cell counting. Flasks were counted in triplicate at each time point and number of cells reported is mean ± SEM. Statistics were calculated in GraphPad using a two way ANOVA with Bonferroni posttest. Asterisks were used to denote the following significance levels: *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001.

Both 4T1 and EMT6 cells are classified as Triple Negative Breast Cancer

Since the subtype of breast cancer affects the aggressiveness and recurrence frequency of the disease, the molecular subtype of each cell line was classified. Cells were stained with ER, PR, and HER2 antibodies and analyzed using flow cytometry. Both 4T1 and EMT6 cells showed no surface expression of either hormone receptors or HER2 meaning they fall into the aggressive TNBC subtype (Figure 6). Since both cell lines are categorized as TNBC, the

difference in immunogenicity between each cell line must not be related to the hormone receptors or HER2 expression. Additionally, this means that we are working with two murine cell lines that are associated with poor prognosis and have a tendency to recur, but that vaccines may establish immune protection against even aggressive tumors that do not express any receptors targeted by current therapies as evident by vaccination with irradiated EMT6 cells.

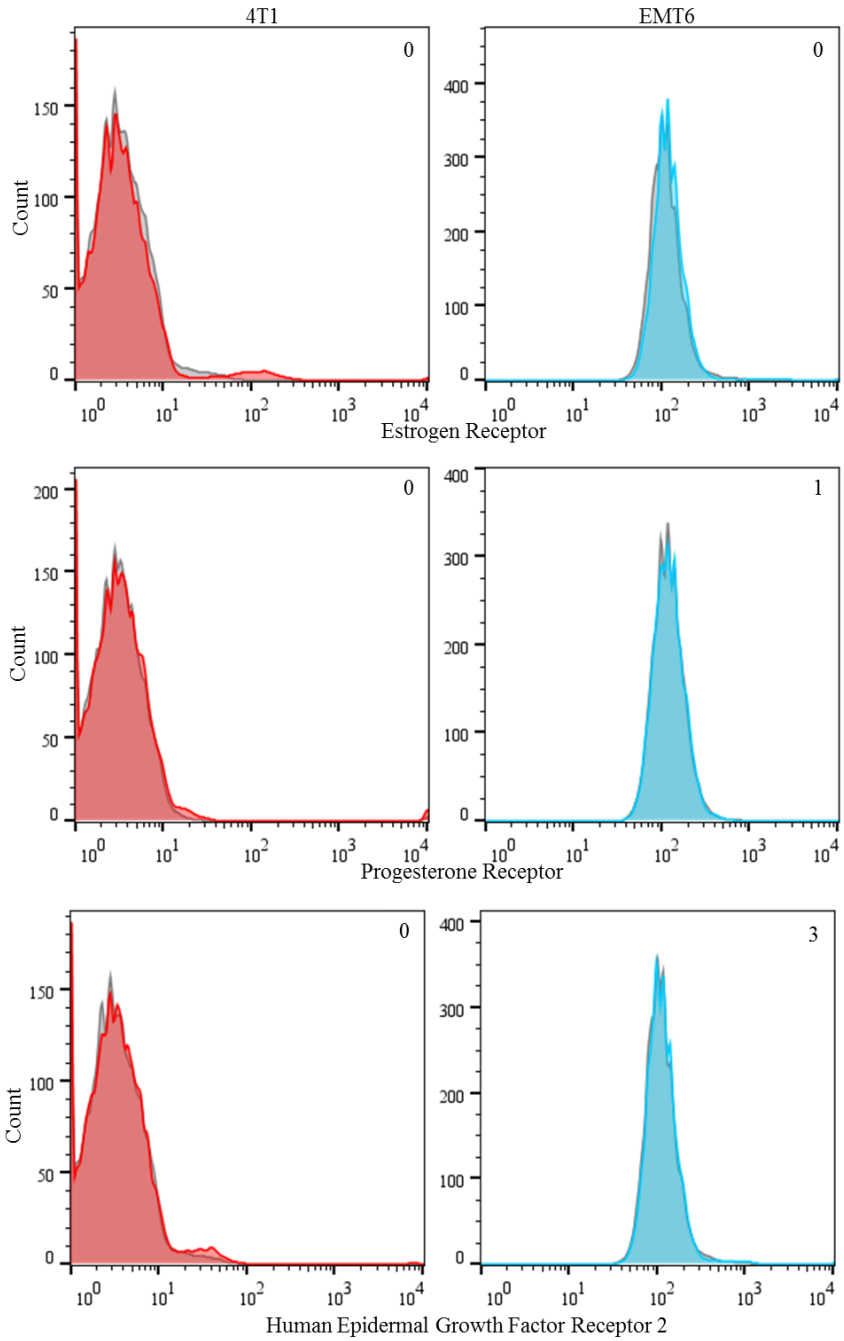

Figure 6: Both 4T1 (red) and EMT6 (blue) cells lines have triple negative receptor status. Cells were taken from culture and suspended in polypropylene tubes at a concentration of 500,000 cells/ 100µl PBS. Cells were stained with anti-ER, anti-PR, or anti-ErbB2 for an hour at room temperature. Following incubation, tubes were centrifuged, supernatant discarded and resuspended in PBS. Cells were then stained with PE anti-IgG1 or FITC anti-IgG2a for an hour at room temperature. Tubes were again centrifuged and supernatant discarded a second time. Cells were resuspended in PBS and acquired on a BD FACSCanto II flow cytometer. Data was analyzed using FlowJo_V10. Gray shaded areas represent cells stained with only the secondary antibody. Numbers represent the difference in median fluorescent intensity between the control and stained cells (ΔMFI).

Surface Marker Expression of 4T1 and EMT6 Cells

Surface molecule expression of each cell line was analyzed after a distinct difference was observed between vaccinating with irradiated 4T1 or EMT6 cells. The presence or absence of certain surface markers may influence activation and responses of effector T cells. Each surface marker tested and their function is summarized in Table 1. Surface marker expression on 4T1 and EMT6 cells was measured along with irradiated forms of each cell to understand what was presented to the immune system during both the vaccination and challenge. Irradiated cells were found to naturally fluoresce slightly higher than normal cells and this phenomenon was taken into account when considering up-regulation of surface markers following irradiation. One way tumors may evade the immune system is by down regulating MHC I expression on the tumor surface so that cytotoxic T lymphocytes (CTLs) cannot recognize the tumor cells[52]. Both 4T1 and EMT6 cells show similar expression of MHC I (Figure 7). After irradiation, both 4T1 and EMT6 cells show a two-fold increase in ΔMFI MHC I expression. This suggests that both cell lines should be able to activate CD8+ T cells. Additionally, CTLs should be able to recognize 4T1 cells as efficiently as EMT6 cells and that the cell lines are equally able to present antigen as an irradiated vaccine.

MHC II is needed for the activation of helper T cells, which in some cases are necessary for the differentiation of CTLs [52]. MHC II is expressed by both 4T1 and EMT6 cells, and show a distinct up-regulation of MHC II expression 24 hours following irradiation. A slightly higher up-regulation is seen in 4T1 cells (four-fold increase), compared to EMT6 cells (three-fold increase). Based on this information, both cell lines should be able to activate helper T cells.

Along with MHC I and II, costimulatory molecules like B7-1 and B7-2 are needed to initiate a strong effector T cell response [52]. Slight B7-1 expression is seen in 4T1 cells with a small up-regulation following irradiation. EMT6 cells express almost 5 times as much B7-1 as 4T1 cells, although the ΔMFI between the negative control and stained EMT6 cells is only 21.6 (Figure 7). However, irradiated EMT6 cells have an 11-fold increase in B7-1 expression compared to before irradiation (Figure 7). EMT6 cells also express significantly more B7-1 following irradiation than 4T1 cells. As for B7-2, 4T1 and EMT6 cells have similar expression and show some increase in expression following irradiation. This suggests that EMT6 cells may be able to induce a strong effector T cell response due to B7-1 expression during vaccination, while 4T1 cells are not as capable.

Another costimulatory molecule for the activation of T lymphocytes is ICAM-1, which is specifically important for the costimulation of CD8+ T cells[53]. Before irradiation, both 4T1 and EMT6 cells show little ICAM-1 expression with a ΔMFI of 8.4 and 12.1, respectively (Figure 7). However following irradiation, 4T1 cells have a 5-fold increase in ΔMFI. The ΔMFI stays the same before and after irradiation of EMT6 cells. This suggests that little costimulation signal may be provided by ICAM-1 in the EMT6 cell line. In the 4T1 cell line, normal cells may provide some costimulation while irradiated 4T1 cells provide slightly more costimulation.

After CD8+ T cells are activated, a granule-independent mechanism may be used by CTLs to kill target cells. After CTLs are activated, they express Fas ligand (FasL) which may then bind to a cell expressing Fas and induce apoptosis of the target cell [52]. We found that both 4T1 and EMT6 cells show a distinct expression of Fas (Figure 7). EMT6 cells show a much higher fluorescence intensity of Fas than 4T1 cells (8.8-fold increase). EMT6 cells show no histogram overlap between the stained cells and control, while a distinct overlap is seen between

4T1 stained cells and control. We can conclude that CTLs should be able to induce apoptosis of tumor cells based on Fas expression, but may be able to better eliminate EMT6 tumor cells through a granule-independent mechanism than 4T1 cells due to the distinctly higher Fas expression. However, this is dependent on the presence of CTLs.

Table 1. Summary of Surface Molecules and Functions that may Influence Immunogenicity of Tumor Cells.

Surface Molecule	Function
MHC I	Peptide presentation to CD8+ T cells Signal 1 for T cell activation
MHC II	Peptide presentation to CD4+ T cells Signal 1 for T cell activation
B7-1	Costimulatory signal (signal 2) for T cell activation
B7-2	Costimulatory signal (signal 2) for T cell activation
ICAM-1	Cell-cell adhesion Costimulatory signal, specifically for CD8+ T cells
Fas	Granule-independent method of inducing apoptosis by CTLs

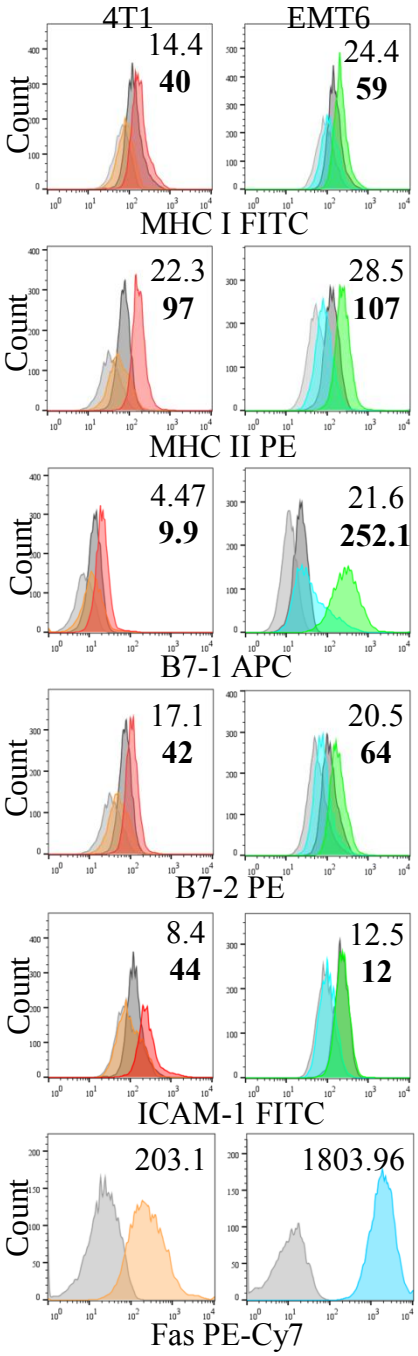

Figure 7: In vitro assessment of surface molecules on 4T1 and EMT6 cells before and after irradiation by flow cytometry. 4T1 (left column) and EMT6 (right column) cells were stained with MHC I, MHC II, Fas, ICAM-1, B7-1, and B7-2 surface markers before irradiation (orange and blue) or after irradiation (red and green). Cells were taken from culture and stained with antibody for an hour at room temperature or irradiated at 100 Gy and cultured for 24 hours before staining. Gray shading represents negative control of cells (light gray) and irradiated cells (dark gray). Numbers represent the difference of median fluorescence intensities (ΔMFI) between stained cells and the negative control (regular) or stained irradiated cells and the negative control (bold). This experiment was repeated three times and a representative from each group is shown.

Secretion of Immunosuppressive Cytokines varies between Cell Lines

Another way that tumor cells may evade the immune system is by secreting products that suppress the anti-tumor immune response. In order to evaluate the immunosuppressive effects secreted by each cell line, GM-CSF, IL-6, IL-10, MCP-1, TGF-β and VEGF cytokine levels were measured using CBA or ELISA.

High levels of GM-CSF promote expansion of myeloid derived suppressor cells (MDSCs)[54]. EMT6 cells secrete no GM-CSF before irradiation and show little secretion after irradiation (Figure 8A). There is no significant difference between before and after irradiation, and therefore no significant difference between vaccination and challenge with EMT6 cells. However, there is a significant difference before and after irradiation in 4T1 cells. An increase in GM-CSF secretion is seen over time and this increase is even further amplified in irradiated 4T1 cells. There is also a difference in GM-CSF secretion between 4T1 and EMT6 cells during both the vaccination and challenge with viable cells.

MCP-1 has chemotactic activity for monocytes and T lymphocytes and regulates the infiltration of macrophages into the tumor environment[55]. Like GM-CSF, EMT6 cells secrete significantly more MCP-1 than 4T1 cells during both the vaccination and challenge with viable cells (Figure 8C). Within each cell line, the irradiated cells secrete significantly more MCP-1 than viable cells.

One way that TGF-β exerts its immunosuppressive effects is by inhibiting proliferation and effector functions of T cells. TGF-β influences the development of T regulatory cells (Tregs), which inhibit the ability of APCs to stimulate T cells[56]. EMT6 cells were found to secrete significantly higher amounts of TGF-β than 4T1 cells (Figure 8D). This suggests that

EMT6 cells have a stronger inhibition of APCs than 4T1 cells. No significant differences were seen between irradiated and non-irradiated forms of each cell line indicating that relatively the same amount of TGF-β is secreted during the vaccination and challenge.

IL-6 and VEGF are other immunosuppressive cytokines that are responsible for the expansion and buildup of immature myeloid cells by blocking differentiation that inhibits the activity of CD4+ and CD8+ T cells [57]. The concentration of IL-6 produced by 4T1 cells increased slightly over time, while EMT6 mediated IL-6 production decreased over time (Figure 8B). Although EMT6 cells secrete a higher amount of IL-6, the difference was not significant. No significant difference was seen between irradiated 4T1 or irradiated EMT6 cells as well. There was a significant difference in IL-6 secretion before and after irradiation in each cell line. This indicates that there is significantly more IL-6 secreted during vaccination than during a challenge. VEGF production is significantly higher in EMT6 cells than 4T1 cells both before and after irradiation (Figure 8E). Unlike other cytokines, irradiation caused the down regulation of VEGF. This suggests that VEGF is secreted at much lower levels during vaccinations than a challenge with viable tumor cells.

We found no evidence of IL-10 secretion by either cell line (data not shown). We found it interesting that EMT6 cells secrete higher amounts of 4 of the 5 immunosuppressive cytokines since EMT6 cells are immunogenic. It is clear that immunogenicity is not completely influenced by cytokine secretion alone and that other factors, such as B7-1 expression, may be mainly responsible for the anti-tumor immune response.

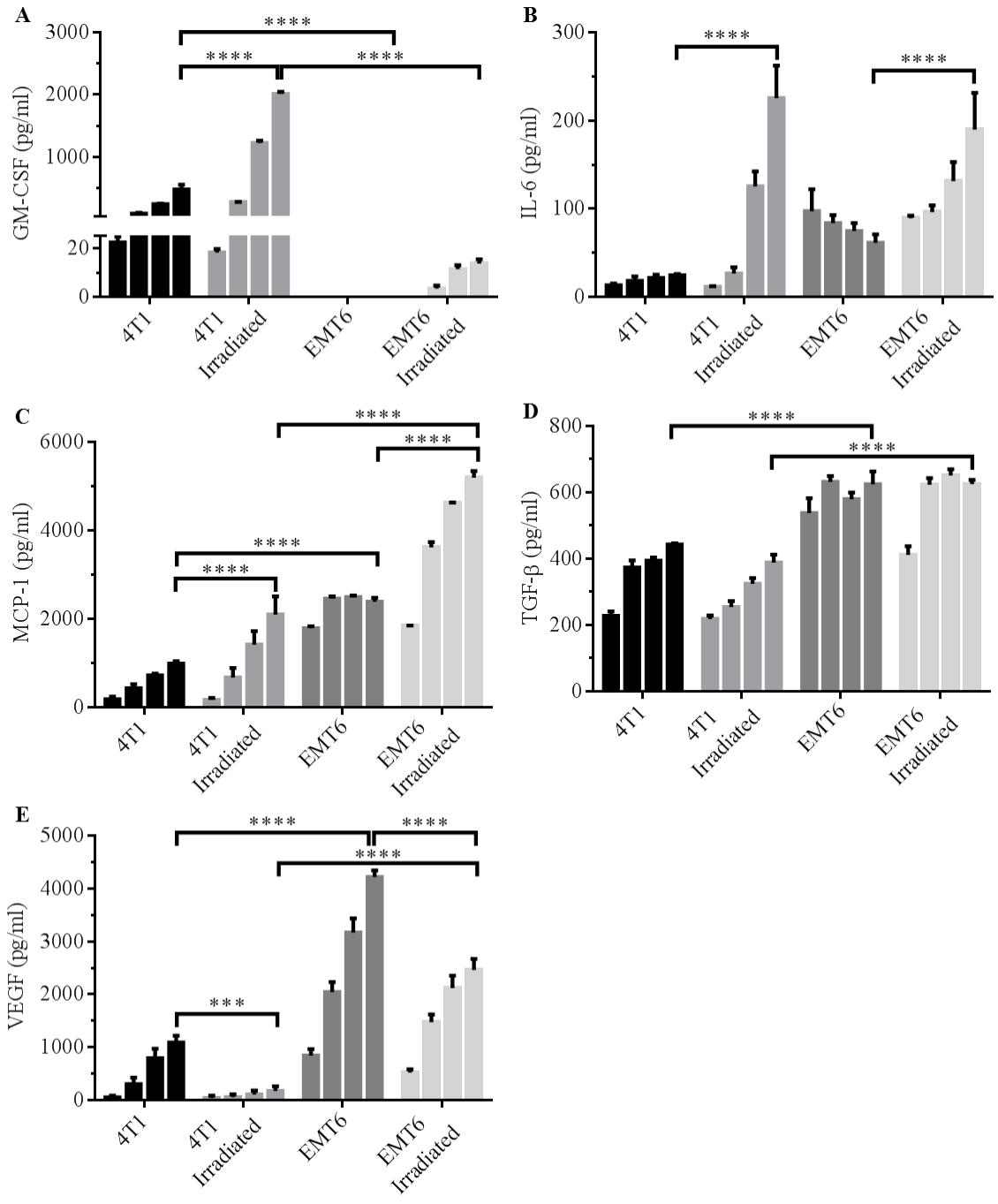

Figure 8: Assessment of in vitro immunosuppressive cytokine secretion by 4T1 and EMT6 cells. Supernatants were taken from culture 24, 48, 72, and 96 hours after incubation. Cytokines GM-CSF (A), IL-6 (B) and MCP-1 (C) were measured using CBA and TGF-β (D) and VEGF (E) were measured using ELISA. For each group, the four bars represent secretion after 24, 48, 72 and 96 hours, respectively. Experiment was repeated 3 times and data indicates mean ± SEM. Statistics were generated in GraphPad using a two way ANOVA with Tukey's posttest. Asterisks were used to denote the following significance levels: *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001

Hybrid Vaccine Inhibits Recognition of EMT6 Cells

An inhibitory signal during vaccination may be produced by 4T1 cells that prevents protection and an anti-tumor immune response. To test this, mice were vaccinated with both irradiated 4T1 and EMT6 cells and then challenged with EMT6 cells alone to determine whether irradiated 4T1 cells impacted protection immunity against EMT6 cells. As shown in Figure 9, protection was observed in only 40% of mice. This is half of the survival rate from vaccinating with irradiated EMT6 cells alone. Immediately after challenge, 5/5 mice grew tumors, but two of the mice later experienced tumor regression by day 15 and 25 after challenge (Figure 9). The largest tumor volume of mice that experienced a tumor regression was 29.16 mm^3 7 days after viable tumor challenge. However, tumor size during this time was not an appropriate indicator of whether the mouse would experience tumor regression because two control mice and three vaccinated mice had tumors smaller than 30 mm^3 7 days after challenge. Of the vaccinated mice that experienced large tumor growth, there was no difference in tumor growth between the control and vaccinated mice. This suggests that 4T1 cells produce some inhibitory signal that prevented some mice from rejecting the EMT6 cells.

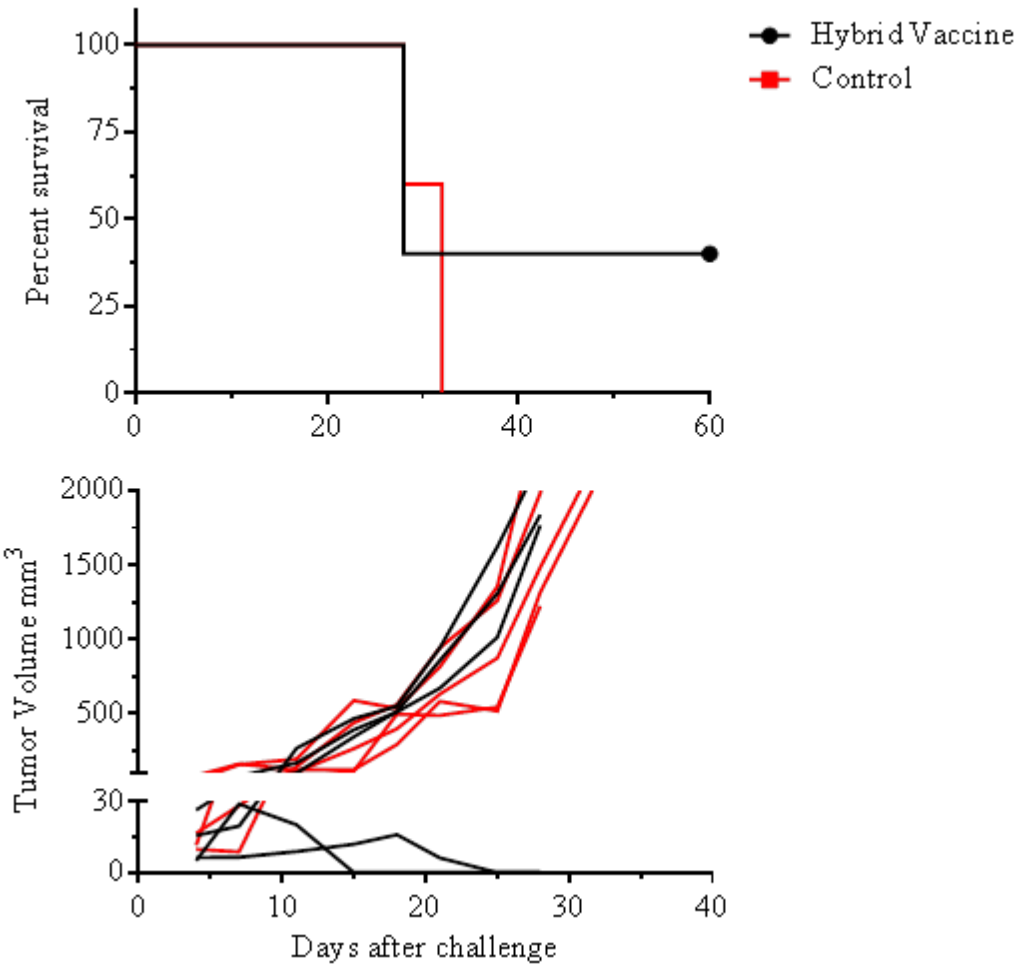

Figure 9: Hybrid vaccine generates little protection. Mice (n=5) were vaccinated on days 10 and 20 prior to tumor challenge with $5x10^5$ EMT6 and $1x10^6$ 4T1 irradiated cells. On day 0, mice were challenged with $5x10^5$ EMT6 viable tumor cells. Control mice (n=5) were only challenged on day 0. Tumor volumes were measured every 3 to 4 days with calipers in two perpendicular directions. (A) Survival of mice vaccinated with hybrid vaccine compared to controls. (B) Tumor volume of mice vaccinated with hybrid vaccine following challenge.

IFN-γ Exposure to 4T1 cells up-regulates MHC and B7-1

Cell-mediated immunity to tumors may be augmented by enhancing MHC and costimulation signals. By increasing MHC I and MHC II expression, tumor cells may be recognized by CTLs and recruit help from helper T cells. Costimulatory signals from B7 molecules may then induce an effector T cell response. In an effort to up-regulate MHC and costimulatory molecule expression, 4T1 cells were cultured with 1ng/ml of IFN-γ for 24 hours.

After incubation, cells were stained with antibodies or irradiated and stained with antibodies 24 hours later. Flow cytometric analysis shows that IFN-γ treatment up-regulates surface expression of MHC I, II and B7-1 (Figure 10). IFN-γ treatment affected MHC II expression the most with a 4.4-fold increase compared to normal cells, followed by B7-1 (2.6-fold increase) and MHC I (1.5-fold increase). Irradiation of IFN-γ treated 4T1 cells caused further increase in surface molecule expression, suggesting that IFN-γ treatment and irradiation work in a synergistic manner. After IFN-γ and irradiation, MHC I and II expression on 4T1 cells (Figure 10) was higher than expression seen on irradiated EMT6 cells (Figure 7). Although B7-1 expression on 4T1 cells was increased by IFN-γ and irradiation, the ΔMFI was still lower than the ΔMFI of before and after irradiation.

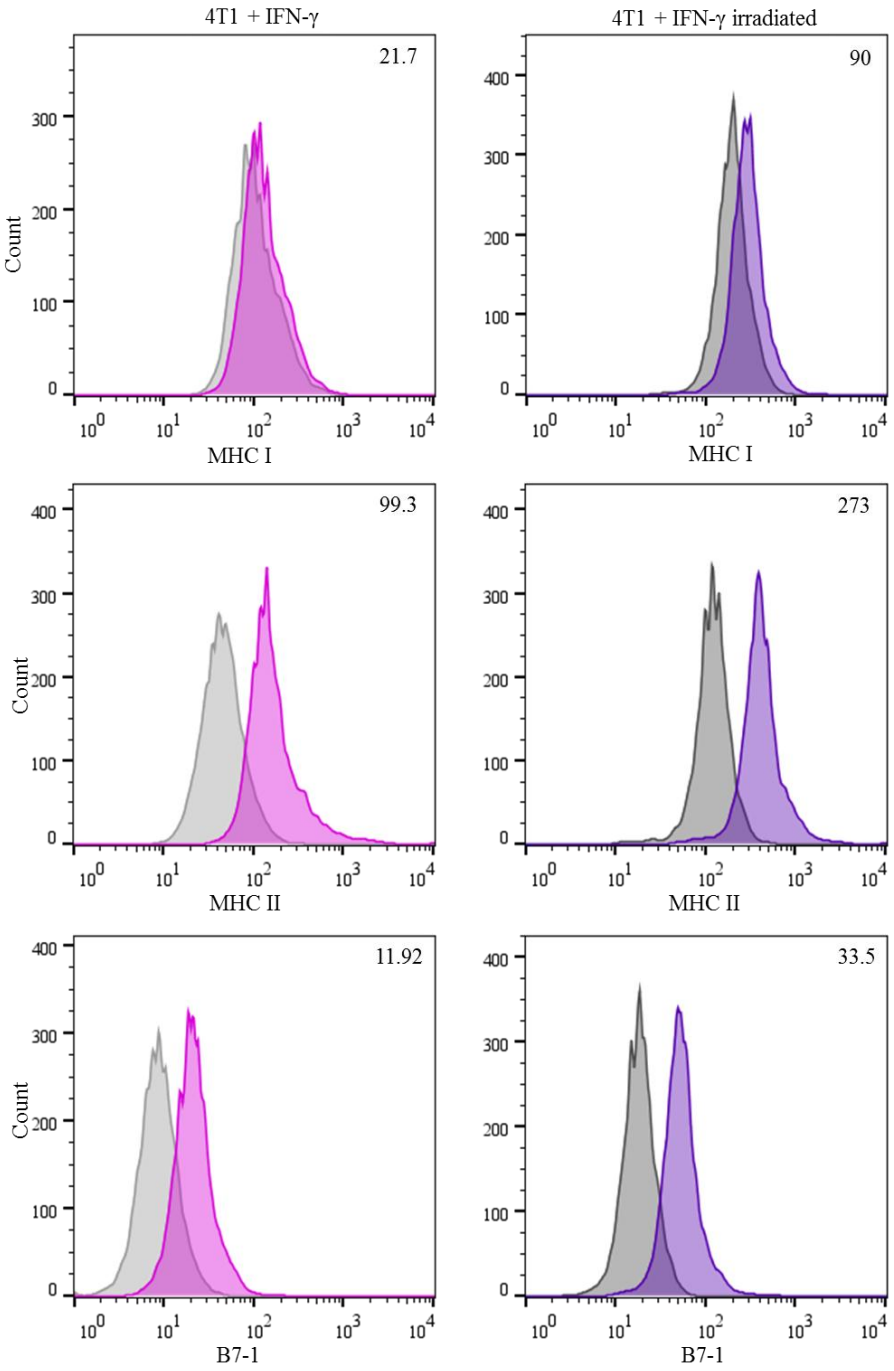

Figure 10: IFN-γ up-regulates surface MHC II and B7-1 of 4T1 cells in vitro. Cells were exposed to 1 ng/ml IFN- γ for 24 hours in vitro. Following incubation with IFN- γ, cells were taken from culture and stained with antibody for an hour at room temperature (pink) or irradiated with 100 Gy and cultured for 24 hours before staining (purple). Gray shading represents negative control of cells (light gray) and irradiated cells (dark gray). Numbers represent the difference of median fluorescence intensities (ΔMFI) between stained cells and the negative control. This experiment was repeated three times and a representative from each group is shown.

Protection from Irradiated Cells is Dose Dependent

A minimum threshold of irradiated cells required to induce immune memory was measured in the EMT6 cell line. Mice were vaccinated with $1x10^6$, $5x10^5$, $2.5x10^5$, $1.25x10^5$, $6.25x10^4$ or $3.12x10^4$ irradiated EMT6 cells on days 10 and 20 prior to viable EMT6 tumor challenge. After the challenge, mice were tracked for tumor growth and tumors were measured every 3 to 4 days. With this tumor model, the data suggests a minimum vaccine dose of 250,000 cells is required to induce immune memory. Mice that were vaccinated with lower than 250,000 cells experienced small tumor formation immediately after viable tumor cell challenge, but the tumor later regressed as shown in Figure 11. This phenomenon occurred in 2, 2 and 3 mice for doses $1.25x10^5$, $6.25x10^4$ and $3.12x10^4$, respectively. Although these mice experienced tumor regression, we did not find it positive that mice experienced any tumor formation. Vaccinating with 250,000 irradiated EMT6 cells was the only vaccination dose that no tumor growth was observed so this was determined to be the minimum threshold to completely inhibit tumor formation.

For vaccination doses higher than 250,000 irradiated cells, 1/5 mice for each dose experienced tumor growth and were sacrificed once the tumor reached 2000 mm^3. The mouse vaccinated with $1x10^6$ irradiated cells showed a delayed tumor growth starting 42 days after challenge, while the mouse vaccinated with $5x10^5$ irradiated cells showed no delay and experienced tumor growth similar to control mice. Larger sample sizes are needed before conclusive statements can be drawn about effect of tumor cell dose.

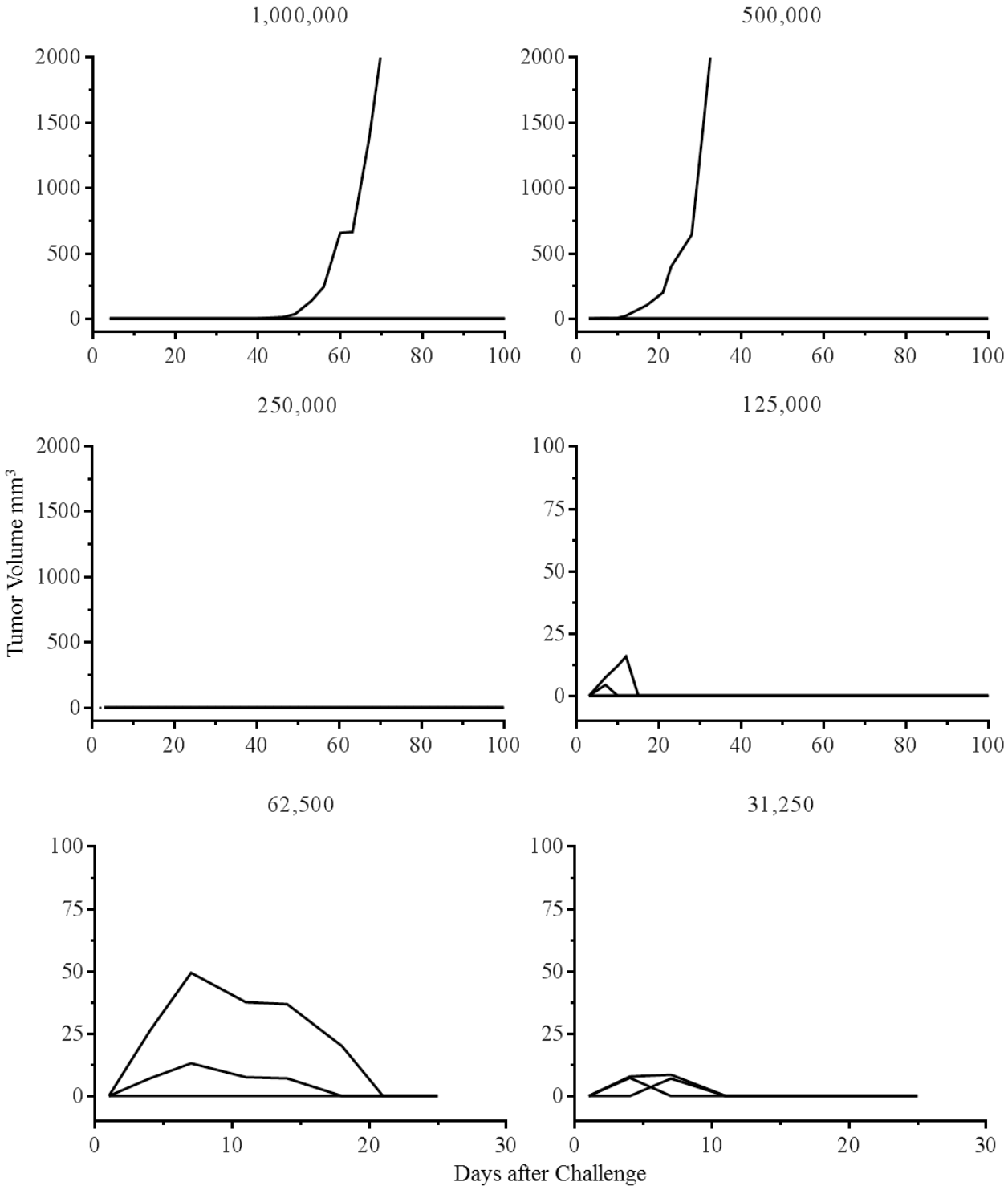

Figure 11: Limiting EMT6 dose study. Mice were vaccinated on days 10 and 20 prior to tumor challenge with $5x10^5$ (n=5), $2.5x10^5$ (n=5), $1.25x10^5$ (n=5), $6.25x10^4$ (n=3), or $3.12x10^5$ (n=3) irradiated EMT6 cells. On day 0, mice were challenged with $5x10^5$ EMT6 viable tumor cells. Tumors were measured every 3 or 4 days in two perpendicular directions.

Discussion

Autologous cells as a form of breast cancer immunotherapy have numerous advantages over current adjuvant therapies including patient specificity, minimal toxicity, and a potential of maintaining long term immunity. Since tumor resection is indicated for the majority of breast cancer patients, a source of tumor antigen is readily available for vaccination in the adjuvant setting. A vital component of inducing protective immunity from cancer using cellular vaccines is first inactivating tumor cells. Two common methods of inactivating cells are irradiation and freeze/thaw. We found a distinct difference in the ability of irradiated and freeze/thawed cells to establish immune memory. In an immunogenic cell line, prophylactic vaccination with irradiated cells elicited protective immunity in 80% of mice, compared to 0% protection using freeze/thawed cells. This is consistent with other reports in various mouse models [50 51]. The difference in protective immunity between each antigen form indicates a preference in the way antigen is presented to the immune system. When administering cellular vaccines, inactivating cells with irradiation will elicit stronger responses than freeze/thawed cells.

Although we did not analyze any further why irradiated cells provided better protection, others have reported the in vivo effects and mechanistic results of vaccinating with both forms of antigen. The in vivo effects of each vaccine, as reported by Scheffer et al., show that freeze/thawed cells failed to induce a potent immune response that may be due to the infiltration of various cells. Irradiated cells were able to bring strong infiltration of CD4+, CD8+, and dendritic cells (DCs) at the injection site while freeze/thawed cells had no presence of these cells 10 days past injection and instead resulted in a strong macrophage infiltration [50]. Furthermore, a detailed in vivo mechanistic study with irradiated and freeze/thawed cells reports that both forms of antigen are taken up by APCs and result in the cross-priming of naïve T cells. However while

CD8+ T cells primed by irradiated cells expand and express full effector function, CD8+ T cells primed by freeze/thawed cells do not accumulate and a smaller proportion become functional [58]. The accumulation of different cells, particularly the lack of CD8+ accumulation by freeze/thawed cells, may be responsible for the differences in immune protection.

While vaccination with irradiated cells from an immunogenic cell line (EMT6) is able to elicit protection, no protection was observed when vaccinating with a non-immunogenic cell line (4T1). There must be a difference between 4T1 and EMT6 cells that prevents 4T1 cells from inducing an anti-tumor immune response. Some surface markers, such as those used to classify breast cancer subtype, may have an indirect influence on the anti-tumor immune response by indicating aggressiveness of the cancer and potential of breast cancer recurrence. Presence of hormone receptors and/or HER2 influences the prognosis and treatment options available to breast cancer patients. Both 4T1 and EMT6 cells lack the ER, PR, and HER2 and therefore are classified as TNBC. This subtype is known to have aggressive characteristics such as early relapse and decreased survival. However, there is a lack of therapeutic treatments for TNBC due to the absence of these receptors which may influence the survival trend [59]. Since both the immunogenic and non-immunogenic cell lines are triple negative for the ER, PR, and HER2, we can assume that while these markers may influence the aggressiveness of cancer, they do not determine immunogenicity. Protective immunity elicited by vaccinating with EMT6 cells demonstrates the presence of immunogenic antigens other than HER2. Approximately only 40% of women have HER2 positive breast cancer (Luminal B and HER2+ subtypes) and will benefit from HER2 targeted therapy. Autologous cells may serve as a promising treatment option for all breast cancer patients, but notably women with TNBC, due to presence and immunogenicity of unknown antigens, as evident by EMT6 cells.

Surface marker expression and cytokine secretion by each cell line may directly influence the anti-tumor immune response by evading recognition by T cells or inhibiting their activation. Tumors may down regulate MHC I to evade recognition, and therefore apoptosis, by CTLs. Tumors may also lack MHC II expression, which is needed to activate helper T cells and aid in differentiation of CTLs[52]. Both the 4T1 and EMT6 cell lines express MHC I and II at low levels and should be able to carry out these processes.

Costimulators are also needed to initiate T cell responses[52]. The best characterized costimulatory molecules are B7-1 and B7-2 which bind to CD28 on a naïve T cell. Expression of B7 is imperative because through multiple mechanisms, engagement of CD28 induces differentiation of effector and memory cells [52]. The most notable difference observed between the surfaces of each cell line was the up-regulation of B7-1 in EMT6 cells after irradiation, while irradiated 4T1 cells only saw a small up-regulation. This suggests that EMT6 cells may provide a strong costimulatory signal through the B7-1 molecule and enhance T cell activation, while 4T1 cells are not.

IFN-γ is a known regulator of B7 molecule expression on APCs. In the 4T1 tumor microenvironment, there is not enough IFN-γ in the tumor microenvironment to up-regulate genes until 15 days after implantation[60], after tumor cells have already metastasized. Gene transfer of MHC II or B7-1 reduces metastases and tumorigenicity of 4T1 cells [61]. Treatment of 4T1 cells with only 1ng/ml IFN-γ for 24 hours at least doubles MHC II and B7-1 expression and a further increase is seen after irradiation. However, increase in B7-1 expression still is not comparable to B7-1 expression on irradiated EMT6 cells. It is not clear how much B7-1 expression is needed to benefit the anti-tumor immune response or if any protection would be observed in mice vaccinated with IFN-γ treated irradiated 4T1 cells.

Once CTLs are activated, they may perform granule-independent killing, through Fas/FasL interaction, or granule-dependent killing, through ICAM-1/LFA-1 interaction. Once FasL on the surface of the CTL attaches to the death receptor Fas on the target cell, apoptosis is initiated in the Fas expressing target. Both 4T1 and EMT6 express Fas and show an up-regulation following irradiation suggesting that CTLs, which express FasL, should be able to eliminate both tumor cell lines through a granule-independent mechanism as long as CTLs are present. ICAM-1 is a cellular adhesion molecule and functions as a costimulator, specifically for CD8+ cells. ICAM-1 is hardly expressed on 4T1 or EMT6 cells, but irradiation results in an increase in ICAM-1 expression on 4T1 cells. Since both cell lines express little ICAM-1 and high levels of Fas, we assume that each cell line is likely killed in a granule-independent manner.

Immunosuppressive cytokine secretion is another way that tumor cells may escape immune defenses. Cytokines can influence the immune system to promote immunosuppression and tumor growth by inhibiting T cell activation and regulating expansion, recruitment and differentiation of various cells. MCP-1 regulates the infiltration of macrophages in the tumor environment and also influences T cell differentiation through IL-4, which inhibits a Th1 CD4+ T cell response critical for the elimination of cancer [55]. TGF-β also inhibits a Th1 response, along with inhibiting NKs, CTLs, and macrophages that results in prevention of anti-tumor immunity [56, 62]. However, TGF-β has both tumor promoting and tumor suppressive effects, which is dependent upon the stage of cellular transformation. As a tumor suppressor, TGF-β increases apoptosis of cancer cells and inhibits expression of growth factors, including GM-CSF [56, 63]. IL-6 and VEGF are other immunosuppressive cytokines that are responsible for the expansion and buildup of immature myeloid cells by blocking differentiation that inhibits the activity of CD4+ and CD8+ T cells [57]. VEGF prevents DC precursors from developing into

antigen-presenting DCs and is associated with the accumulation of tumor associated macrophages[64]. We show that EMT6 cells produce higher amounts of each immunosuppressive cytokine tested than 4T1 cells, except for GM-CSF. We found it interesting that the immunogenic cell line actually secretes more immunosuppressive cytokines than the non-immunogenic cell line. It is not clear if there is a dominant immunosuppressive cytokine or if certain cytokines synergize to create a more suppressed immune system. Since GM-CSF production is the major difference between each cell line, future studies will focus on the knock-out or knock-in of GM-CSF in 4T1 and EMT6 cells, respectively.

GM-CSF production is a double-edged sword. In the B16 melanoma mouse model, GM-CSF was shown to be the most potent stimulator of anti-tumor immunity out of IL-2, IL-4, IL-5, IL-6, IFN-g, IL-1RA, ICAM, CD2, and human TNF-a [45]. Vaccines in conjunction with GM-CSF as an adjuvant result in enhanced tumor antigen presentation by dendritic cells and macrophages [65]. However, GM-CSF has also been reported to promote the expansion of MDSCs in the mouse, characterized by the CD11b and Gr1 markers [54]. Upon investigation, GM-CSF gives either a stimulating or inhibitory signal to the immune system depending on the level of cytokine secretion. In a model by Serafini, the upper threshold of stimulating the immune system through GM-CSF secretion was 206 pg/ml [54]. We show that not only do 4T1 cells produce a significantly greater amount of GM-CSF than EMT6 cells both before and after irradiation, but that the secretion level is well above the 206 pg/ml upper threshold. Therefore, 4T1 cells may be recruiting a large number of MDSCs resulting in immunosuppression rather than immune stimulation.

We confirmed production of an inhibitory signal by 4T1 cells with creation of a hybrid vaccine that consisted of both irradiated 4T1 and EMT6 cells. After a challenge with viable

EMT6 cells, 60% of mice developed a tumor. Importantly, immediately after the challenge with EMT6 cells all mice grew a tumor, but two mice later had tumor regression. Half of the protection is observed when vaccinating with both cell lines rather than vaccinating with EMT6 cells alone (40% vs. 80%). Therefore, an inhibitory signal must be produced by 4T1 cells during vaccination to prevent the recognition of EMT6 cells. It is not clear what the immunosuppressive signal is that prevents recognition of EMT6 cells. We predict that over production of GM-CSF takes some role in the immunosuppressive signal since this is the only immunosuppressive cytokine that we tested that EMT6 cells did not produce. We are not sure whether GM-CSF is acting alone or synergizing with the other immunosuppressive cytokines or surface molecules to prevent immune protection from 4T1 cells and EMT6 cells in the hybrid vaccine.

Secretion of large amounts of immunosuppressive cytokines leads to splenomegaly, which is observed due to massive granulocytic infiltrates [66]. These infiltrates, such as MDSCs, are an obstacle to active immunotherapy because they block the activation of tumor reactive T cells and induce accumulation of Tregs [67], another splenocyte population associated tumor-associated immune suppression [46]. Almost 50% of cells in the spleen of a 4T1 tumor bearing mouse are CD11b+Gr1+ [66], whereas the same cell population in the spleen of an EMT6 tumor bearing mouse is 20%[57]. The lower concentrations of MDSCs in the spleen of EMT6 mice may partly be attributed to the lack of GM-CSF secretion.

Additionally, there is a minimum limit in which irradiated cells are able to confer immunity and in the EMT6 model the limit is 250,000. In order to administer a successful therapeutic vaccine at least 250,000 cells must be obtained following tumor resection in the EMT6 model. Using a triple enzyme digestion method (See Appendix), we are able to obtain

approximately $2x10^6$ tumor cells per 0.5 g of tumor. Therefore, we are able to obtain more than enough cells needed for a therapeutic vaccine. Therapeutic studies are critical for future studies for a more accurate representation of the clinical setting.

Our studies confirm that irradiated cells serve as a better vaccination than freeze/thawed cells and provide a possible explanation for the lack of protection from 4T1 cells when administered as a vaccine. Based on the differences in the immunogenic profile of each cell line, a direction for future studies involves the manipulation of 4T1 cells through changes in surface molecules and cytokine secretion in hopes of developing a more immunogenic tumor.

Chapter 4: Future Perspective

Introduction

A majority of breast cancer patients will undergo surgery following diagnosis of breast cancer. Following surgery, majority of patients are given adjuvant therapy in an attempt to eliminate tumor cells that may have disseminated from the primary tumor. Adjuvant therapy, such as chemotherapy, radiation therapy, hormonal or targeted therapy, have toxic side effects and benefit only a subset of patients. Still, 1 out of 5 patients go on to have a recurrence after 10 years. Since the majority of breast cancer deaths are due to the recurrence and progression of non-metastatic disease, an intervention is needed that promotes the immune system to eradicate lingering tumor cells after surgery. The use of autologous whole tumor cells as adjuvant therapy has shown pre-clinical and clinical success for promoting an anti-tumor immune response.

Future Pre-Clinical Studies

Pre-clinically, we have demonstrated the effectiveness of an autologous whole cell breast cancer vaccine using a murine model. In the EMT6 cell line, 80% of mice vaccinated with autologous cell later reject a challenge with live cells. However, vaccinations were only successful in an immunogenic cell line. Mice vaccinated with irradiated 4T1 cells, showed no protection or delay in tumor growth. Using the differences observed between the immunogenic profiles, each cell line may be manipulated to change expression of surface molecules or secretion of cytokines in an effort to understand their role in immunogenicity. The advancement of immunotherapy for 4T1 cells is dependent on the understanding of various characteristics of the immunogenic profile and the influence they have on immunogenicity.

By altering the immunosuppressive cytokine profile and therefore the various cell infiltrates in the spleen and tumor, we may be able to change the "immunogenic profile" of a 4T1 tumor into an immunogenic tumor that is capable of immune protection. We believe that down regulation of GM-CSF secretion shows the most potential in eradicating the immunosuppressive signal secreted by 4T1 cells. EMT6 cells secrete no GM-CSF and other studies have shown that GM-CSF levels above 206 pg/ml induce a suppressive immune signal, so we believe that significantly lowering or knocking out GM-CSF in 4T1 cells will promote the anti-tumor immune response. Significant, but not total, knockout of GM-CSF in 4T1 cells results in reduction of CD11b+Gr1+ splenocytes [68]. Since MDSCs are a hindrance to immunotherapy, by reducing GM-CSF and therefore MDSCs accumulation, the immune system may be able to elicit some tumor protection. Another study may include a gene encoding GM-CSF to be inserted in EMT6 cells to experiment whether GM-CSF secretion by EMT6 cells would hinder protection.

Other immunosuppressive cytokines may also be knocked out in 4T1 cells. Treatment of a 4T1 tumor with an anti-TGF-β antibody also showed a decrease of CD11b+Gr1+ splenocytes. When mice were treated therapeutically with an anti-TGF-β antibody, approximately 30% of mice survived[56]. Future studies may explore a knockout of TGF-β in a prophylactic vaccine of 4T1 cells alone or in combination with GM-CSF knockout.

In addition to cytokine secretion, surface molecules may influence the anti-tumor immune response. Gene transfer of MHC II and B7-1 reduces the metastatic and tumorigenic capability of 4T1 cells. Vaccinating with 4T1 cells that have been transduced with MHC II and/or B7-1 may increase initiation of T cell responses supplying a stronger immune response following challenge. We show that IFN-γ up-regulates MHC I, II and B7-1 in vitro and the increase in expression of these surface markers may be enough to elicit some tumor protection. Additionally, vaccinating with a B7-1 knockout in the EMT6 cell line may also indicate the influence of B7-1 in immunogenicity of tumor cells.

In addition to the future studies mentioned above, therapeutic vaccinations may be implemented to more accurately represent the current clinical setting. After surgery, tumor cells may be left behind because either the whole tumor may not have been removed or tumor cells have already travelled to other local or distant parts of the body. Prophylactic vaccinations do not take into account existing tumor cells, and possible immunosuppression, while therapeutic vaccinations do. Since the primary treatment of breast cancer is surgery, it is important to study vaccinations following tumor resection.

Implementation in the Clinic

Clinically, less success has been observed compared to pre-clinical models. Three key questions need to be addressed regarding development autologous tumor cell vaccines to improve clinical results. First, immunogenicity of the cells will need to be improved through better delivery and inactivation of the cells. It has been shown pre-clinically that GM-CSF is a strong immune system stimulator. However, GM-CSF and IL-2 works synergistically to provide an immune response stronger than each adjuvant individually[69]. Different adjuvant formulations should be investigated to determine what best stimulates a patient's immune system. For example, 67% of mice treated intratumorally with chitosan/IL-12 prior to tumor resection became tumor free. All mice were later protected following a challenge with viable 4T1 cells, suggesting that this treatment made 4T1 cells immunogenic.

Second, a signature of what makes a tumor immunogenic would be helpful in identifying patients that will benefit from vaccination. Tumor antigen expression, number of T cell infiltrates, immunosuppression all could have an impact on how well a patient responds to vaccination. For example, establishment of an immune score based on cytotoxic and memory T cells in colorectal cancer has shown to improve prediction of tumor recurrence[70, 71]. An immune score should be applicable to most human tumors[72], including breast cancer. T-cell infiltration in breast cancer has been associated with favorable prognoses[73, 74], although extent of recurrence-free and overall survival with high T-cell infiltration is not known. By identifying patients that respond to vaccination and characterizing their tumor and tumor microenvironment, we may be able to determine patients with a high-risk of recurrence and patients that may benefit from immunotherapy. Additionally, by identifying what makes a tumor immunogenic we may be able

to manipulate tumor cells for patients who originally would not respond as well to immunotherapy in an effort to increase immune system activation.

Third, a standardization of preparing vaccines is important for consistent results. Once a tumor has been removed from a patient, standard protocols are needed for dissociating the tumor. Common methods include enzymes such as DNAse and collagenase. Then cells must be either cryopreserved or cultured for the duration of immunotherapy. Finally, cells must be prepared to give back to the patient. Often cells are either irradiated or treated with Mitomycin C. The numerous methods of preparing autologous tumor cells must be optimized so that they give the greatest immune response and give reliable results. After preparation, the location of administration and duration of the immunotherapy should be standardized as well. By solving these three key challenges, autologous whole tumor cell vaccines may produce clinically significant results that build on the success of improved over-all and recurrence free-survival that has already been achieved.

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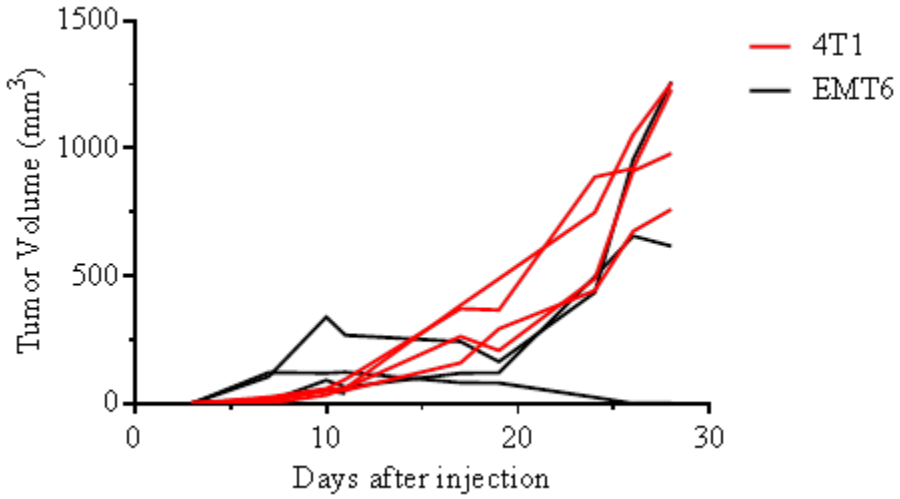
Appendix

Freeze/Thaw cells with -80°C Ethanol



In an effort to vaccinate mice with cell lysates, cells were subjected to 5 freeze/thaw cycles while suspended in DPBS using a 37°C water bath and -80°C 70% ethanol and distilled water mixture. Mice were injected with $1x10^6$ freeze/thawed 4T1 (n=4) or EMT6 (n=4) cells. However, this model did not serve as an appropriate method for freeze/thawing cells. Shortly after vaccination, mice began to grow tumors suggesting that a significant number of cells survived the freeze/thaw cycles. 100% of mice injected with 4T1 cells grew tumors, while 75% of mice injected with EMT6 cells grew tumors.

Triple Enzyme Mouse Tumor Digestion

Preparation

10X Triple Enzyme Stock Solution:

Collagenase 1 g f.c. [10 mg/ml]

Hyaluronidase 100 mg f.c. [1 mg/ml]

DNase 20,000 Units f.c. [200 mg/ml]

HBSS 100 ml

Sterile filter (0.22 μm) and store 5 ml aliquots at –20°C.

Thaw at RT (NOT 37°C) before use.

Procedure

1. Remove tumor and place into a 60 or 100 mm petri dish and add 5-10 ml HBSS

2. Quickly mince tumor into fragments small enough to be pulled into a 5 ml pipette without getting stuck.

3. Transfer to 50 ml conical tube.

4. Rinse petri dish with up to 40 ml HBSS and transfer to tube. Total volume in tube should be 45 ml (if not, bring up to volume with HBSS).

5. Add 5 ml 10X Triple Enzyme Mix to the tube and place on rotisserie for 1 hr.

6. Pass cells through 70 μm nylon mesh filter unit into a second 50 ml conical tube.

7. Pellet cells at 1200 rpm for 8 min.

8. Aspirate supernatant and resuspend cells in 15ml cell medium.

9. Carefully layer the cell suspension on top of 15ml Histopaque.

10. Centrifuge the sample at 400xg for 30 minutes with acceleration set at “1” and deceleration set at “0”.

11. At the end of the spin, an opaque layer of cells should be observed at the interphase of the histopaque and cell medium layers. Transfer these live cells carefully to a 15ml tube. Bring total volume to 15ml with cell medium if needed.

12. Centrifuge 15ml tube at 2000rpm for 10 minutes.

13. Aspirate supernatant and resuspend cells in 1ml medium.

14. Count cells.