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Immunotherapeutic Strategies to Reverse Tumor Associated Immunosuppression in Breast Cancer

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Immunotherapeutic Strategies to Reverse Tumor Associated Immunosuppression in Breast Cancer

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Abstract

Heterogenous cancers such as breast carcinoma, would highly benefit from personalized vaccine approaches such as autologous tumor cell vaccines (ATCVs). By using cancer cells that are isolated from self, this approach has the potential to generate a polyclonal immune response against tumor antigens, that are specific to the individual. However, so far, ATCVs against breast cancers have not had any clinical success due to the poor immunogenicity of the malignant cells.

In this study, for the first time, we determined tumor derived G-CSF to play a major role in affecting immune response against breast cancer ATCVs. By using CRISPR/Cas9 genome editing technology, we found that tumor derived G-CSF results in the accumulation of exceptionally high levels of myeloid derived suppressor cells (MDSCs) in the spleen and lymph nodes of immunized mice. For the first time, we demonstrated that by eliminating G-CSF in ATCVs, the overall efficacy of the vaccine can be significantly improved. Further, for the first time, by using breast cancer cells that naturally produce varying levels of G-CSF, we reestablished the effect of G-CSF on splenic MDSC accumulation.

Additionally, we addressed the MDSC associated immunosuppression, by investigating strategies to facilitate the maturation of immature immune cells. We considered different commercially available toll like receptor (TLR) agonists namely Pam3CSK4 (Pam), Poly (I:C), lipopolysaccharide, FLA-ST, FSL-1 and R848, and compared their effect on MDSC maturation in-vitro. Of all the agonists, we found that only Pam resulted in the complete maturation of MDSCs since it resulted in the upregulation of all four MDSC maturation markers, CD80, CD86, MHCII and F4/80. Additionally, Pam also significantly reduced the number of MDSCs in the treated whole splenocyte cultures.

Future studies that are discussed here will expand our knowledge on the effect of tumor derived G-CSF in clinical cancer burden and metastasis of breast cancer. Further, in-vivo studies with the TLR agonists, would help design an effective adjuvant therapy against aggressive and metastatic breast cancers, with improved survival and reduced rate of recurrence.

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I. INTRODUCTION

A. Introduction to breast cancer

Worldwide, breast cancer is the most commonly diagnosed cancer in women, accounting for 25% of all cases. In U.S alone about 230,000 new breast cancer cases are diagnosed every year. Overall, there are 2.8 million women in U.S who are currently being treated or have completed treatment for breast cancer. It is also the second leading cause of cancer death in U.S women with about 40,500 deaths in 2015 (1).

Breast cancer is the presence of malignant cells in the breast region. These cells either originate in the lobules, the milk producing glands or ducts, the tubes that carry milk to the nipple. Based on its ability to invade the surrounding normal tissue, the cancer is categorized as benign or malignant. Benign breast tumors are not life threatening since they remain enclosed within the region of origin. On the contrary, malignant tumors are cancerous and can be life threatening if left unchecked. Unfortunately, 1 in 8 women in U.S will develop invasive breast cancer in her lifetime. These malignant cells can eventually invade distant organs in the body such as the bone, liver, lungs or brain, in which case it is termed as metastatic breast cancer. It is estimated that 20-30% of all breast cancer cases will become metastatic (2).

B. Stages and types of breast cancer

Breast cancer is usually categorized into five stages from 0 to IV. Stage 0 denotes non-invasive breast cancer, where there is no evidence of the malignant cells breaching the surrounding normal tissue. On the contrary, stage IV denotes the advanced stages of the disease where the cancer has metastasized to distant lymph nodes and/or other organs of the body such as the lungs, brain, bone, skin or liver (3). Early diagnosis is paramount in breast cancer treatment since

once the cancer spreads to neighboring lymph nodes (stages III and IV), it becomes extremely challenging to eliminate the disease (4).

Further, invasive breast cancer is categorized into three different phenotypes, namely hormone receptor positive (HR+), human epidermal growth factor receptor positive (HER-2+) and triple negative breast cancer (TNBC) based on the surface marker expression. For instance, patients with breast cancer cells expressing estrogen or progesterone receptors are classified as hormone receptor positive. This is the most commonly diagnosed type of breast cancer with approximately 2 out of 3 patients testing positive for the hormone receptors (5). On the other hand, patients whose cancer cells overexpress HER-2 are classified as HER-2 positive. About 25-30% of breast cancer patients are HER2+ with significantly reduced disease free survival and poor prognosis (6). The third type, triple negative breast cancer (TNBC) is the most aggressive type accounting for 10-20% of the patients. Malignant cells of patients with TNBC do not express hormone or HER-2 receptors, rendering it extremely difficult to target these cells during therapy. Additionally, TNBC has extremely poor prognosis and tends to recur early when compared to the other subtypes of breast cancer (7).

C. Primary treatment

Primary treatment for breast cancer involves complete removal of the tumor via surgery. Based on the size of the tumor, patients undergo lumpectomy, where only part of the breast with cancer is removed or mastectomy where the entire breast tissue is removed (1). However, patients with invasive breast cancer usually always undergo axillary lymph node dissection (ALND), where some or all lymph nodes near the breast are removed. Sometimes ALND is also recommended in

patients with early stages of the disease as a preventive measure to minimize the chances of metastasis.

Unfortunately, patients undergoing ALND often suffer from lymphedema, where there is excessive swelling of the arm post-surgery. This condition is considered as an incurable side effect that leads to extreme discomfort and restricted arm movement (8). Thus, a comparatively new form of surgery, that overcomes this side effect is sentinel lymph node biopsy (SLNB). This technique involves the injection of radioactive drugs or dyes to the cancer, which help physicians to detect sentinel lymph nodes, the first lymph nodes that the tumor drains into. By first testing these lymph nodes for the presence of cancer cells, the need for any further surgery is assessed. Thus, SLNB is a minimally invasive procedure that prevents any unnecessary removal of lymph nodes from breast cancer patients. Studies comparing SLNB and ALND over the past few years have established the consistent low false negative rates associated with SLNB (9, 10).

D. Adjuvant therapy

Breast cancer cells that escape the breast and regional lymph nodes, if left unchecked have the potential to metastasize and result in recurrence after primary surgery. Thus, usually patients with invasive breast cancer after undergoing primary treatment, are recommended to undergo additional therapy to eliminate these residual cancer cells. Such therapies are collectively known as adjuvant therapy. Different types of adjuvant therapy are recommended based on the size of the tumor and the extent of its spread. The most commonly used adjuvant therapies for breast cancer are chemotherapy, radiation therapy and hormonal therapy (11).

Chemotherapy is a systemic therapy where medicines that destroy rapidly multiplying cancer cells are administered to the patient post-surgery. Though chemotherapy is associated with

reduction in recurrence in 35% of women under the age of 50 years, the short and long term side effects of the therapy cannot be overlooked. Some of the acute side effects of chemotherapy include neutropenia, nausea, vomiting, loss of appetite and alopecia. Premenopausal women also suffer from long term side effects such as damage of ovaries leading to premature menopause and/or infertility (1, 11, 12).

Radiation therapy uses high energy particles such as x-rays or gamma rays to kill the residual malignant cells. Unlike chemotherapy, radiation therapy is comparatively targeted at the malignant cells. Thus, the side effects of the therapy are limited to the treated region. It is offered to patients with stage I or II breast cancer, after lumpectomy. It is also recommended after mastectomy in patients whose cancer has spread to the lymph nodes. Despite being a comparatively targeted therapy, radiation therapy is not devoid of side effects. The most prevailing side effect is brachial plexopathy due to damage of nerves near the neck region. The intense radiation also results in a feeling of heaviness in the breast region and change in the color of skin over the breast (13).

Hormonal therapy on the other hand, is only given to patients whose cancers are hormone receptor positive. Here, drugs that either block the body's ability to release the hormones or those that interfere with the function of the hormone are used to stop tumor growth. Since the hormones are blocked during therapy, a major drawback of this form of treatment is interruption of the menstrual cycle. Additionally, patients also suffer from hot flashes, vaginal dryness, swelling of the breasts and decreased bone mineral density (14, 15). Also, studies have shown that, Tamoxifen, the most commonly used drug in hormonal therapy, increases the risk of endometrial cancer and venous thromboembolism (16).

From the discussion, it is evident that current adjuvant therapies are associated with numerous side effects that overshadow the treatment benefits. Additionally, since one cannot predict the fate of tumor after surgery, patients whose tumor would not have resulted in clinically apparent disease post-surgery, still undergo therapy and suffer from the side effects. Thus, the need to undergo adjuvant therapies is a lingering debate in patients with early stages of the disease (17). Also, it is important to note that 90% of breast cancer deaths are still due to recurrence and metastasis, corroborating the ineffectiveness of prevailing adjuvant therapies. Thus, it is indisputable that there is a compelling need to develop more effective, tumor specific and less toxic adjuvant therapies to improve survival in breast cancer patients.

E. Immunotherapy

Cancer immunotherapy is the technique of stimulating the patient's immune system to recognize and eradicate cancerous cells. Since this technique 'trains' the immune system to identify cancer cells, it is expected to have long lasting remission unlike most conventional therapies. Additionally, since immunotherapy specifically targets malignant cells, patients are less likely to suffer from adverse side effects. Thus, cancer immunotherapy is being considered a breakthrough in cancer treatment with promising potential in improving clinical outcomes. It is an umbrella term that includes different strategies such as antibody based therapy, checkpoint therapy and cell based vaccines. In breast cancer treatment, trastuzumab was the first antibody based immunotherapy approved in 1998. It is a monoclonal antibody that interferes with HER2/neu receptor, overexpressed by cancer cells in HER2+ patients (18). Since then, numerous immunotherapies have emerged and more are in preclinical studies or clinical trials.

For instance, pertuzumab and lapatinib are other HER2 directed antibody based drugs recently approved by FDA for treatment of advanced stages of HER2+ breast cancer. In these therapies, the efficacy largely depends on the tumor antigen chosen for treatment. If the chosen antigen is expressed homogenously and frequently by the malignant cells, the therapy is likely to be effective. Additionally, targeting an antigen that is exclusively upregulated in tumor cells, minimizes the chances of adverse side effects (19). However, it is important to note that the high specificity does not make these therapies devoid of adverse side effects. For instance, trastuzumab therapy increases the likelihood of heart damage leading to congestive heart failure. Moreover, the cardiac toxicity exacerbates when the drug is used in conjunction with other treatment regimens such as anthracycline (20).

Checkpoint therapy involves the use of drugs that specifically target checkpoint receptors or ligands. These receptors/ligands are literally 'checkpoints' of the immune system that assist in preventing autoimmune responses. Although this is an extremely important mechanism in preventing detrimental effects of autoimmunity, it greatly affects immunotherapeutic strategies. Thus, blocking them could provoke an anti-tumor response or improve its likelihood when used in combination with other therapies (21). Several checkpoint inhibitors such as Iplimumab, pembrolizumab and nivolumab, that were initially approved for melanoma treatment are under investigation for breast cancer (22).

Additionally, in 2010, FDA approved sipuleucel-T, an autologous cellular vaccine for patients with hormone-refractory metastatic prostate cancer (23). Since then different cellular vaccines are under clinical and pre-clinical studies for treatment of different cancers. Cellular vaccines usually involve the adoptive transfer of T cells that are engineered to enhance anti-tumor response. In an ongoing phase I trial, T cells engineered to target carcinoembryonic antigen

(CEA), that are often expressed on breast cancer cells are utilized (24). Similarly, there are also ongoing studies on using dendritic cell vaccines for breast cancer. These vaccines work by initiating the expansion of CD4+ and CD8+ T cells specific for the tumor (25).

Though peptide based vaccines and adoptive therapy are feasible strategies, they are limited by some disadvantages. For instance, peptide based vaccines only recognize a few epitopes on the cancer cells and therefore can only be used for patients with cancers expressing the specific antigen (23). Adoptive therapy on the other hand, is limited by the need for repetitive administration of the drug to sustain the anti-tumor response (26). An immunotherapeutic technique that overcomes these disadvantages is the whole tumor cell vaccine. These vaccines are made from either autologous or allogenic tumor cells. The allogenic tumor cell vaccine uses tumor cells isolated from multiple established tumor cell lines. Since various cell lines have overlapping antigen expression profile, the allogenic tumor cell vaccines have the potential to activate a tumor specific immune response via cross priming (23). On the other hand, autologous tumor cell vaccine (ATCV) is made from cancer cells obtained from the patient (27). ATCVs are a rich source of all tumor associated antigens (TAAs) unique to the patient's tumor. These vaccines thus have the advantage of activating a polyclonal immune response specific to the individual's cancer (28). Since ATCVs would highly benefit patients with heterogenous cancers such as the breast, in the past decade several pre-clinical studies have been focusing on developing effective ATCVs for breast cancer (29).

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II. IMMUNOSUPPRESSION IN ADVANCED STAGES OF BREAST CANCER

A. Introduction

Over the past few years, our knowledge of breast cancer has greatly improved and so have the treatment methods. Early detection along with new therapeutic strategies have decreased the mortality rate by about 37% over 25 years (30). However, unfortunately, most of the newly developed therapies are targeted only at a specific population such as the human epidermal growth factor 2 positive (HER2+) or estrogen receptor positive (ER+) or patients with non-invasive breast cancer. However, patients with the more aggressive forms such as triple negative breast cancer are not provided with enough treatment options. Unfortunately, these patients make for about 15-20% of breast cancers diagnosed every year. Additionally, 20-30% of patients with early stage breast cancer, go on to develop metastatic breast cancer and 6-10% of all invasive breast cancers are already metastatic at initial diagnosis (31). Unfortunately, the treatment options for these patients are extremely slim and is generally palliative.

Per literature, a major hindrance in the treatment of these aggressive forms of breast cancers is the tumor associated immunosuppression (32). Cancer immunosuppression involves the generation of an immunosuppressive environment mediated by various tumor derived factors such as transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) (33). These factors in turn cause the expansion of immunosuppressive immune cells such as regulatory T cells (Tregs) and immature myeloid cells that assist the tumors to evade the immune system. Not so surprisingly, such an immunosuppressive environment could also affect the success of several therapeutic strategies against these cancers. Thus, currently several studies are investigating the different immunosuppressive pathways associated with breast cancer. There are

also a few studies where new pathways of immunosuppression have been discovered and their correlation with tumor progression is being investigated.

Thus, this review highlights the major players of immunosuppression found in advanced stages of breast cancer and their correlation with poor prognosis or metastatic burden. Understanding these pathways, could help researchers develop better immunotherapeutic drugs or vaccines and improve overall survival of breast cancer patients.

B. Identifying major players of immunosuppression

In recent years, breast tumor microenvironment is widely studied to determine the different tumor infiltrating lymphocytes and growth factors/cytokines that affect the overall immune response. Interestingly, a few subsets of immunosuppressive cells have been recently identified in breast tumors, and were found to contribute to cancer progression and metastasis.

Regulatory T cells

FoxP3+ regulatory T cells (Tregs) are an immunosuppressive subset of T cells that have been greatly investigated in different cancer models (34-37). In breast cancer, several studies have reported an increased enrichment of tumor infiltrating lymphocytes (TILs) with Tregs to correlate with poor prognosis and high risk of cancer recurrence (38-40). For instance, in a study that included patients with invasive and ductal carcinoma in-situ (DCIS), Tregs were found at significantly higher frequencies in invasive tumor samples than in DCIS. Further, in the patients with invasive tumor, the higher levels of Tregs inversely correlated with positive node status, relapse free survival (RFS) and overall survival (OS). Additionally, a population of ER+ patients with high frequency of Tregs were found to be at a higher risk of shorter RFS and OS, when compared to ER positive patients with lower levels of Tregs (40). Similarly, in another study,

upon analysis of tumors removed from 100-treatment naïve breast cancer patients, it was found that the frequency of Tregs in triple negative breast cancer was the highest (38). Thus, these studies proposed that high risk breast cancer patients can be identified via quantifying Tregs in their tumor biopsies.

Recently, FoxP3, the transcription factor that plays a crucial role in the immunosuppressive property of Tregs, was also found to be expressed by breast cancer cells. This was surprising, since it was initially thought to be expressed only by cells of the lymphocyte lineage (41, 42). Additionally, the heterogeneous localization (nucleus/cytoplasm) of FoxP3 in cancer cells was found to have prognostic value, since the presence of cytoplasmic FOXP3 correlated with poor OS (43). However, the findings from this study completely contradicted another study where cytoplasmic FOXP3 expression improved OS and RFS in patients with HER2+ breast cancer (42). Nonetheless, it is important to note that the former study used treatment naïve breast cancer patients, whereas the latter collected samples from patients who had undergone neoadjuvant chemotherapy. Thus, the possibility of the discrepancy to be because of chemotherapy, cannot be ruled out until further investigation.

Further, in another study, a new subset of Tregs were found at significantly higher levels in TILs and peripheral blood of patients with advanced stages of breast cancer. These cells were found to express CCR6 and high levels of CD45RO and were termed as $CCR6^+$ Tregs. Unlike the $CCR6^-$ Tregs, the frequency of these cells in the TILs of patients with advanced stages (stage III and IV) of the disease was significantly higher than in patients with early stages (stage I and II). There was also a correlation between the presence of $CCR6^+$ Tregs and the decreased levels of IFN-γ producing $CD8^+$ T cells. Upon further analysis, this subset of Tregs was also found to inhibit CD4+ T cells at significantly higher levels than their $CCR6^-$ counterpart (44).

Regulatory B cells

Like the Tregs, recently another population of regulatory immune cells were identified in mouse models of chronic inflammation. These were termed as regulatory B cells (Bregs), since they were a population of B cells that produced IL-10 and/or TGF-β and were involved in immunosuppression (45). However, as of now, a unique marker for Bregs has not yet been identified. Therefore, among the different studies, there are slight differences in the markers used to identify Bregs. Most of the studies in human breast cancers identifies these cells as IL-10 (high) and TGF-β (high) producing B cells that are also $CD19^{+}$, $CD25^{high}$, $CD86^{high}$ and $CDd1^{high}$. In an in-vitro study, when these cells were co-cultured with stimulated $CD4^{+}$ T cells, they were found to significantly decrease the proliferation of $CD4^{+}$ T cells. Additionally, the Bregs were also found to increase the immunosuppressive capacity of Tregs by upregulating the expression of FoxP3 and CTLA-4. However, this effect on Tregs was contact dependent (46).

In the 4T1 murine breast cancer model, another unique subset of Bregs were recently reported. These cells belonged to CD19, CD25 and B220 expressing B2 lymphocytes that were found to be needed for metastasis of the cancer to lungs. These cells were labelled as tumor evoked Bregs (tBregs) and they were found to initiate the conversion of non-Tregs into Tregs in-vivo (47).

Myeloid derived suppressor cells

Another immunosuppressive immune cell subset found in the tumor microenvironment of most solid tumors are the myeloid derived suppressor cells (MDSCs) (48). These cells express CD11b, CD33 and low levels of HLADR, in humans, whereas they are double positive for CD11b and Gr1 in mice (49). These cells are known to involve in immunosuppression by releasing different immunosuppressive factors such as arginase and reactive oxygen species (48).

In breast cancer patients, the presence of MDSCs in peripheral blood and its association with cancer stage and metastasis, has been widely investigated. Peripheral blood samples collected from breast cancer patients were found to have significantly higher levels of MDSCs when compared to healthy volunteers (50). Additionally, the levels of MDSCs were found to closely correlate with tumor burden (49, 50). Thus, it was also proposed that these cells could possibly provide predictive information on human breast cancer stage. Consequently, it was found that, patients with stage IV breast cancer had the highest peripheral blood level of MDSCs, which were sometimes as high as 25%. Corroborating this finding, in another study, patients with metastatic breast cancer with abnormal levels of MDSCs in peripheral blood were found to have decreased OS (51).

Further research on MDSCs revealed the presence of two subsets, namely granulocytic MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs). In mice, MDSCs that are $Ly6G^{+}$ $Ly6C^{low}$ are termed as granulocytic, whereas $Ly6G^{-}Ly6C^{high}$ are monocytic MDSCs. However, in humans MDSCs that are CD33+Lin- and $CD14^{+}$ $HLA\text{-}DR^{low/-}$ $co\text{-}receptor^{low/-}$ are termed as granulocytic and monocytic MDSCs, respectively (52). Though a few studies have compared the frequency and immunosuppressive capacity of these two subsets in breast cancer, the findings are contradicting. In some studies, M-MDSCs were found at elevated levels in the peripheral blood of breast cancer patients and it correlated with levels of dissemination of the disease (52). However, in a few other studies, granulocytic MDSCs were found at higher levels (50, 53). Thus, further investigation is required to characterize these subsets in human breast cancer and determine their effect on cancer progression.

Circulating tumor cells

Circulating tumor cells (CTCs) is another recently identified prognostic marker of breast cancer. The presence of CTCs in peripheral blood is often termed as 'carcinocythemia' and is frequently found in patients with metastatic breast cancer (54). In a few case reports, the presence of CTCs in peripheral blood correlated with cancer progression and OS (55-57). Though, it is not clearly understood how CTCs affect the immune response, a few studies have pointed out some noticeable differences in the anti-tumor immunity of CTC positive patients. In a study where peripheral blood from metastatic breast cancer patients were analyzed, it was found that patients with ≥ 5 CTCs/7.5ml of blood had decreased NK cell function when compared to patients with <5 CTCs (58, 59). Similarly, in another study, significant differences were found in the TLR expression of dendritic cells between patients with and without CTCs. Patients with CTCs had decreased expression of TLR3 and increased expression of TLR2, TLR4 and TLR8, when compared to patients without CTCs (60). Interestingly, in another study involving patients with primary breast cancer, no differences in number of cytotoxic T cells ($CD8^+$) and T-helper cells ($CD4^+$)/µl were found between CTC positive and negative patients. However, a significant upregulation in the expression of CD95(FAS) was observed in the $CD4^+$ T cells of CTC positive patients. Since the CD95/CD95L pathway is known to induce apoptosis, it is postulated that the cancer cells in CTC positive patients are inducing apoptosis of T helper cells, resulting in a dysfunctional immune response (61).

C. Conclusion

Tumor associated immunosuppression could be a major hindrance in developing immunotherapies against advanced stages of breast cancer. A review of the literature identifies

several immunosuppressive cell populations such as MDSCs, Tregs and Bregs in the tumor site or in the lymphoid organs of breast cancer patients. Additionally, recent research has identified unique subsets of immunosuppressive immune cells such as CCR6+ Tregs, that correlate with disease progression and/or response to therapy. However, it is important to note that not all subsets are fully characterized in human breast cancers. Additional investigations are required to reiterate their correlation with clinical cancer stage and determine their effect on immunotherapies. Also, understanding their pathways of development could help researchers identify molecules or drugs that could inhibit their generation and improve survival.

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III. G-CSF SECRETED BY AUTOLOGOUS TUMOR CELL VACCINE ABROGATES TUMOR SPECIFIC IMMUNITY

A. Introduction

Autologous tumor cell vaccines (ATCVs) have been widely investigated for the past few years for their ability to prevent cancer recurrence (1-3). ATCVs are generated by deriving malignant cells from patient's own tumor, that are processed and administered to initiate a tumor specific immune response. Since the malignant cells are isolated from self, ATCVs are HLA-matched and can facilitate a polyclonal tumor specific immune response. Additionally, unlike peptide based vaccines, ATCVs do not require pre-antigen selection. Thus, in heterogeneous cancers such as breast carcinoma, the ability of ATCVs to present all tumor associated antigens (TAAs) makes it highly likely to be a successful clinical strategy (4-6). Additionally, autologous vaccines open the possibility of treating triple negative breast cancer (TNBC), an extremely aggressive breast cancer type that lacks hormone and HER2 receptors which are otherwise the usual targets in breast cancer therapy (7).

However, despite being a promising strategy in breast cancer treatment, ATCVs suffer from a major drawback. Cancer cells isolated from patients, often tend to be poorly immunogenic, thereby lacking the ability to activate the immune system (8). The poor immunogenicity of the isolated cancer cells is unavoidable, since the malignant cells that survive and expand in the body are the ones that have developed mechanisms to evade the immune system (8). Generally, malignant cells escape immunosurveillance by downregulating the expression of major histocompatibility complex (MHCI/II) or other costimulatory molecules such as B7-1 and B7-2 (9). Hence, several studies are under investigation to enhance the immunogenicity of autologous cells by improving their expression of these costimulatory molecules. The most commonly used

method involves the use of cytokines such as interferons, GM-CSF or IL-12 to upregulate the expression of MHCII and B7-1 (4, 10). Another strategy is to genetically modify cancer cells to make them more immunogenic (11).

However, clinical success so far, in ATCV based vaccines have only been in naturally more immunogenic cancers such as melanoma or non-small cell lung cancer. On the other hand, for non-immunogenic cancers, they are usually tested only as a combination therapy along with checkpoint inhibitors (12). Thus, understanding the reason for the poor immunogenicity of non-immunogenic tumors, is a requirement for their successful clinical translation.

Thus, in this study, we set out to determine the reasons for the poor immunogenicity of ATCVs against breast cancer. To do this, we are using two murine breast cancer cells, 4T1 and EMT6, varying in their levels of metastatic ability. Here, 4T1 is highly aggressive and metastatic breast cancer model that imitates stage IV breast cancer in humans. On the other hand, EMT6 is comparatively less aggressive and non-metastatic breast cancer model (13, 14).

B. Materials and methods

Reagents

Cell culture media components including Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Hyclone laboratories (Marlborough, MA). Ammonium-chloride-potassium (ACK) buffer used in the lysis of red blood cells while isolating splenocytes, was purchased from Lonza (Allendale, NJ). All fluorochrome-conjugated antibodies used in the study were purchased from BD Biosciences (San Jose, CA).

Mice

All experimental procedures were approved by the Institutional Animal Care and Use Committee at University of Arkansas. Female Balb/cByJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were housed in microisolator cages. Mice were utilized for experiments at 8-12 weeks of age and animal care followed 'The Guide for Care and Use of Laboratory Animals' (National Research Council).

Breast cancer cell lines

Murine breast cancer cells, 4T1 and EMT6 were purchased from American Type Culture Collection (Manassas, VA, USA). The rest of the breast cancer cells, namely 4T07, 67NR, 66Cl4, 168 FARN were a generous gift from Dr. Fred Miller, Karmanos Cancer Institute, Detroit, MI. All cell lines except EMT6 were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. On the other hand, EMT6 cells were maintained in RPMI supplemented with 15% FBS and 1% penicillin/streptomycin. All cell cultures were maintained at 37˚C in a humidified CO_2 incubator with 5% CO_2.

CRISP/Cas9 genome deletion of G-CSF

Using the CRISPR design tool provided by Zhang lab (MIT, Cambridge, MA) a 20bp guide sequence targeting the granulocyte colony stimulating factor (G-CSF) gene in 4T1 cells was identified. Guide sequences were cloned into separate pCas-Guide-EF1a-GFP plasmid via Origene's cloning service. Plasmids were amplified in E. coli and isolated via QIAGEN Plasmid Maxi Kit. For transfection, plasmid encoding gRNA (10ug) was mixed with Lipofectamine 3000 reagent and added to $1x10^6$ 4T1 cells, that were pre-seeded on a 6 well plate 24 hours prior to transfection. 24-48 hours after transfecting, the cells that expressed GFP signal were sorted using

FACSAriaIII system (BD Biosciences). Sorted cells were subsequently cloned via limiting dilution. Further, by using G-CSF specific ELISA kit from R&D systems Inc. (Minneapolis, MN), clones producing lower than detectable levels of G-CSF were identified and used in experiments as 4T1.GCSF-KO.

Prophylactic vaccine studies

Tumor cell vaccines were generated by irradiating 4T1 or EMT6 cells at 100Gy using a Gammacell 1000 cesium irradiator. Mice were subcutaneously vaccinated with a primary and booster vaccine, 10 days apart with $1x10^6$ irradiated 4T1 cells (4T1 vaccine) or $5x10^5$ irradiated EMT6 cells (EMT6 vaccine). On the other hand, for ipsilateral and contralateral hybrid vaccines, $1x10^6$ irradiated 4T1 cells and $5x10^5$ irradiated EMT6 cells were injected on the same and opposite flanks, respectively. In some instance, 4T1.G-CSF KO cells were used in place of 4T1 cells. Vaccinated mice were challenged with $5x10^5$ 4T1, EMT6 or 4T1.G-CSF KO cells, 10 days after the booster vaccine. Tumor volumes were recorded 2-3 times per week using the formula V= (WxWxL)/2, where V is tumor volume, W is tumor width and L is tumor length.

In-vitro cytokine analysis

$5x10^5$ 4T1 or EMT6 cells were seeded on a T25 flask and cultured for 48 hours. Cell culture supernatants were collected and centrifuged to remove any non-adherent cells and stored at -80°C until analysis. Levels of cytokines monocyte-colony stimulating factor (M-CSF) and vascular endothelial growth factor (VEGF) in the cell culture supernatant were quantified via enzyme-linked immunosorbent assay kits (ELISA) from R&D systems Inc. (Minneapolis, MN). Whereas, transforming growth factor-β (TGF-β) was analyzed using latent TGF-β ELISA kit from Biolegend (San Diego, CA). On the other hand, cytokines interleukin-6 (IL-6), monocyte

chemotactic protein (MCP-1), GM-CSF and G-CSF were analyzed via cytometric bead array (CBA) on a FACSCantoII from BD Biosciences.

G-CSF in sera from mice

Blood from mice bearing 4T1, 4T1.GCSF-KO, 4T07, 67NR, 168FARN and 66Cl4 were collected in microcentrifuge tubes, when the tumor volume reached about 500-700mm^3. The blood samples were left at room temperature for 10-15 minutes and then centrifuged at 4000 rpm for 30 minutes. The sera were then carefully collected using a micropipette and tested for the levels of G-CSF using G-CSF specific ELISA kit from R&D systems Inc. (Minneapolis, MN).

Expression of MHC and costimulatory molecules

Irradiated and non-irradiated 4T1 and EMT6 cells ($5x10^5$ cells) were stained with fluorochrome-conjugated anti-CD80, anti-CD86, anti-H2-Kb (MHC I), anti-I-Ad/I-Ed (MHC II), anti-CD54 (ICAM-1) and anti-CD95 (FasR). The labeled and unlabeled cells were analyzed on a FACS Canto II. The differences in median fluorescence intensity (ΔMFI) between unstained and stained cells were determined using FlowJo software (Tree Star, San Carlos, CA).

Tissue collection and analysis of immune cell subsets

Spleens and draining lymph nodes (DLN) from 4T1 and 4T1.GCSF-KO tumor bearing mice were isolated when tumors reached a volume of about 500-700 mm^3. Single cell suspensions were prepared by mechanically dissociating both spleen and DLN samples and filtering through a 40µm nylon mesh cell strainer. Splenocytes were additionally treated with ACK buffer for 10 minutes to lyse red blood cells. Single cell suspensions were then blocked with purified rat anti-mouse CD16/CD32 monoclonal antibody (BD Biosciences, San Jose, CA) and stained with

fluorochrome-conjugated anti-CD11b, anti-CD19, anti-Ly6G and Ly6C, anti-CD3ε, anti-CD25 and anti-CD4 for 40 minutes at 4˚C. Cells were then rinsed, fixed and permeabilized with 1x perm/wash buffer from BD biosciences. The permeabilized cells were further incubated with fluorochrome-conjugated anti-FoxP3 for 40 minutes. The samples were then read on a BD FACS Canto II flow cytometer. Further, using FlowJo software, frequencies of myeloid derived suppressor cells (MDSCs), T cells, B cells and regulatory T cells (Tregs) were determined. For mice bearing 4T07, 67NR, 66Cl4 and 168FARN tumors, only the frequency of MDSCs in spleen were determined.

Statistical analysis

All data were analyzed using GraphPad Prism software, version 7 (La Jolla, CA). For all in vivo vaccine studies, Kaplan Meier survival curves were plotted and the comparisons were made using log rank test. For all other studies, the data is represented as mean ± standard error and the comparisons were made using ANOVA followed by Tukey's or Dunnett's posttest.

C. Results

4T1 cells are poorly immunogenic while EMT6 cells are moderately immunogenic

A standard prophylactic vaccine model was used to evaluate the immunogenicities of 4T1 and EMT6 breast cancer cell lines. Mice were vaccinated with irradiated 4T1 or EMT6 cells and challenged with live 4T1 or EMT6, respectively. As expected, vaccinated mice exhibited extended survival when compared to unvaccinated controls. However, when looking at the percentage of mice that remained tumor free, 70% of EMT6 vaccinated mice did not develop any tumor upon live EMT6 challenge. On the other hand, all 4T1 immunized mice developed tumors, upon live 4T1 challenge (Figure 1).

Costimulatory molecule and MHC expression on breast cancer cell lines

The elaboration of robust adaptive immunity requires antigen presentation in MHC I or MHCII complexes (signal 1) and simultaneous engagement of costimulatory molecules, such as B7-1, B7-2, ICAM-1 and FasR, on APCs, with their cognate receptors, T cell receptor (TCR), CD28, lymphocyte function-associated antigen1 (LFA-1) and Fas ligand (Fas-L) on lymphocytes. Thus, MHC and costimulatory molecules on 4T1 and EMT6 cells were evaluated to determine if differences in expression level could explain observed differences in immunogenicity. Since irradiated cells were found to naturally fluoresce more than non-irradiated cells, this was considered by comparing only the differences in mean fluorescence intensity (ΔMFI) between unstained and stained non-irradiated cells or unstained and stained irradiated cells (Table 1).

MHC I and MHCII are molecules that are required for presenting intracellular antigens to CD8+ and CD4+ T cells, respectively (15). Prior to irradiation, both 4T1 and EMT6 cells expressed MHCI at similar levels. Upon irradiation, though the ΔMFI values increased for both 4T1 (32 ± 11.3) and EMT6 (52.5 ± 9.19), there was no significant difference between the two cell lines. Likewise, the expression of MHCII molecules, that are required for presentation of antigen to CD4+ T cells (helper T cells) were comparable between the two cell lines before and after irradiation.

B7-1 and B7-2 are costimulatory molecules that would bind with CD28 on T cells. This costimulation provides the second signal that is required for antigen specific T cell response (16). Though there was no significant difference in the ΔMFI values of B7-2 between 4T1 and EMT6 cells before and after irradiation, EMT6 cells expressed higher levels of B7-1 upon irradiation.

Specifically, the ΔMFI of B7-1 for irradiated 4T1 cells was 38.2 ± 23, whereas it was 253 ± 1.3 for irradiated EMT6 cells.

ICAM-1 is a ligand for LFA-1, that is expressed on leukocytes. The high levels of expression of ICAM-1 would thus mean an increased level of transcellular migration of leukocytes to the tumor site. Additionally, ICAM-1 expression also acts as a costimulatory signal for CTL activation (17). Likewise, FasR is another costimulatory receptor, which when bound to Fas-L on CTLs, would cause apoptosis of the cell expressing FasR (15). Here, though there was no significant difference in the ΔMFI values of ICAM-1 between 4T1 and EMT6 before and after irradiation, the ΔMFI value of FasR for EMT6 cells was significantly higher than that of 4T1 cells, both before and after irradiation. Specifically, ΔMFI of FasR for 4T1 cells were only 366.7 ± 30.6 and 1197.7 ± 177, when compared to 2393.75 ± 56.2 and 4913.05 ± 155 for EMT6 cells, before and after irradiation, respectively.

Differences in cytokine release

Another factor that could influence the immunogenicity of a tumor cell vaccine is its release of immunosuppressive cytokines. To this end, IL-6, VEGF, TGF-β, MCP-1 and colony stimulating factors G-CSF, M-CSF and GM-CSF secreted by 4T1 and EMT6, before and after irradiation were compared (Figure 2).

Colony stimulating factors, G-CSF, GM-CSF and M-CSF in healthy individuals are known to initiate the proliferation and differentiation of immature myeloid cells. However, at higher levels, as is usually found in tumor microenvironment, these growth factors are associated with resulting in the expansion of immunosuppressive myeloid cells, known as MDSCs (18). We found that, all three colony stimulating factors were released by irradiated 4T1 cells were significantly higher

than irradiated EMT6 cells. Most strikingly, the levels of G-CSF released by 4T1 cells before irradiation (5765 ± 80.9 pg/10^5 cells) and after irradiation (5334 ± 114.2 pg/10^5 cells) were exceptionally high when compared to the levels released by EMT6 cells before (1100 ± 98.84 pg/10^5 cells) and after irradiation (1760 ± 145.1 pg/10^5 cells).

In tumor microenvironment, IL-6 is known to play a key role in promoting cancer cell proliferation and metastatic dissemination. It is also known to extrinsically act on other cells within the microenvironment, to induce angiogenesis (19). Here, IL-6 was found to be released at higher levels before irradiation (4T1: 62.6 ± 8.4 pg/10^5 cells; EMT6: 25.6 ± 3.7 pg/10^5 cells). However, upon irradiation, there was no significant difference in IL-6 release between the two cell lines (4T1: 21.3 ± 1.8 pg/10^5 cells; EMT6: 44.3 ± 6.6 pg/10^5 cells). Likewise, tumor derived MCP-1 and VEGF are associated with promoting angiogenesis (20, 21). Additionally, VEGF has also been shown to initiate the generation of MDSCs from $CD33^+$ mono-nuclear cells, in in-vitro studies (22). We found that 4T1 cells produce higher levels of MCP-1 only upon irradiation (4T1: 1596 ± 123.6 pg/10^5 cells; EMT6: 744.7 ± 58.91 pg/10^5 cells). On the contrary, EMT6 cells produced higher levels of VEGF before (833 ± 41.19 pg/10^5 cells) and after (371.3 ± 8.09 pg/10^5 cells) irradiation, when compared to 4T1 cells before (10 ± 1.1 pg/10^5 cells) and after (8.6 ± 0.6 pg/10^5 cells).

Similarly, TGF-β, a cytokine known to promote the development of Tregs was produced at higher levels by EMT6 cells before irradiation (4T1: 108 ± 7.6 pg/10^5 cells; EMT6: 832 ± 49 pg/10^5 cells). However, upon irradiation, the difference was not significant (4T1: 355 ± 22.1 pg/10^5 cells; EMT6: 274 ± 17 pg/10^5 cells).

Local and systemic effects of 4T1 mediated immunosuppression

Based on differences in cytokine release (Fig 2), we explored if immunosuppressive cytokines released by 4T1 cells would abrogate the protective immunity established by the irradiated EMT6 vaccine. To explore the effect of a local immune suppression, we made a heterogeneous mixture of irradiated 4T1 and EMT6 cells (ipsilateral hybrid vaccine) and injected it subcutaneously to mice. On the other hand, to explore a possible systemic immune suppression mediated by 4T1 cells, we vaccinated mice with irradiated 4T1 cells and EMT6 cells on opposite flanks (contralateral hybrid vaccine). When both groups of mice were challenged with live EMT6 cells, the tumor free survival in the ipsilateral and contralateral vaccine groups dropped to 40% and 25% respectively. This suggested that the 4T1 cells in the vaccine have a systemic inhibitory effect on the immune response (Figure 3).

The immunosuppressive role of G-CSF

Due to the abnormally high levels of G-CSF produced by 4T1 cells with/without irradiation, we hypothesized that it plays a key role in inhibiting the efficacy of ipsilateral and contralateral vaccines. To test this hypothesis, we deleted the G-CSF gene via CRISPR/Cas9 genomic editing.

4T1 cells before G-CSF gene knock out released 4550 ± 604 pg of G-CSF per 10^5 cells, whereas after G-CSF gene knock out, they only released 386 ± 31 pg/10^5 cells. Furthermore, by limiting dilution, we selected a 4T1 colony that released lower than detectable levels of G-CSF in-vitro (4T1.G-CSF KO). Thus, we verified lack of G-CSF secretion in vitro by 4T1.G-CSF KO cells (Figure 4 a).

Further, to verify lack of G-CSF in-vivo in mice bearing 4T1.G-CSF KO tumors, we measured the G-CSF serum concentration when the tumor volume reached about 500-700 ^{3}. We found that

4T1.G-CSF KO bearing mice had only 10 ± 2.9 pg/ml of G-CSF, which was comparable to G-CSF in serum of naïve mice (59 ± 34 pg/ml). On the other hand, mice with comparable volume of 4T1 tumors, had 13096 ± 1947 pg/ml G-CSF (Figure 4 b). Additionally, we also isolated spleen and DLN from 4T1 and 4T1.G-CSF KO tumor bearing mice and determined the frequency of T cells, B cells, MDSCs and Tregs in the tissue samples. Here, we were specifically interested in MDSCs which are immature myeloid cells that have often been associated with high levels of colony stimulating factors (23, 24). Firstly, when the spleens were isolated, extreme splenomegaly was observed in spleens collected from 4T1 bearing mice (Figure 5). Secondly, we found that the spleen of 4T1 tumor bearing mice had significantly higher levels of MDSCs when compared to spleen from 4T1. G-CSF KO mice (4T1: 213 ± 21 MDSCs; 4T1.G-CSF KO: 26 ±10 MDSCs) (Figure 6 a). Similarly, 4T1 tumor bearing mice also had significantly higher numbers of T cells, B cells and Tregs, when compared to 4T1.G-CSF KO tumor bearing mice. (4T1: 82 ± 8 T cells, 37 ± 8 B cells and 4 ± 0.5 Tregs; 4T1.G-CSF KO: 28 ± 7 T cells, 14 ± 3 B cells and 1.5 ± 0.5 Tregs) (Figure 6 b, c and d). Additionally, the levels of all four cell types in 4T1.G-CSF KO were comparable with the levels in naïve mice.

Likewise, when the DLNs were analyzed, there was significant difference in the number of T cells between 4T1 and 4T1.G-CSF KO, whereas the levels were similar in 4T1.G-CSF KO and naïve mice (4T1: 335 ± 81 cells; 4T1.G-CSF KO: 194 ± 42 cells; 45 ± 5 cells) (Figure 7 b). However, when we compared the number of MDSCs, all three groups were significantly different from one another (4T1: 35 ± 0.5 cells; 26 ± 3.5 cells; naïve 1.2 ± 0.3 cells) (Figure 7 a). However, interestingly, the 4T1.G-CSF KO group had slightly higher number Tregs when compared to mice of 4T1 and naïve group (Figure 7 d). Additionally, 4T1 (35 ± 0.5 cells) and 4T1.G-CSF KO (26 ± 2 cells) mice had higher number of B cells, when compared to naïve mice,

which did not have any B cells. However, the difference was not statistically different (Figure 7 c).

Tumor associated G-CSF secretion correlates with MDSC accumulation in different breast cancers

To further establish the correlation between tumor secreted G-CSF levels and MDSC accumulation, we implanted mice with different breast cancer cell lines, namely 4T07, 67NR, 66Cl4 and 168 FARN, that are 4T1 sister cell lines that share a common origin (single, spontaneously arising breast tumor in balb/c mice), but different in their metastatic ability. 4T1 metastasizes to lung, liver, brain and bone; 66Cl4 to only lungs and liver and 168 FARN does not metastasize beyond the lymph nodes. While, 67NR are non-metastatic, 4T07 cells are frequently found in blood and lungs, but do not develop any visible metastatic nodules (25). We implanted each of these tumors in mice and measured the G-CSF in serum of mice when the tumor volumes were comparable (500-700mm^3). We found that these cell lines release varying levels of G-CSF. 4T1 and 4T07 bearing mice had the highest levels of serum G-CSF, 19100 ± 2274 pg/ml and 17600 ± 10220 pg/ml, respectively. On the other hand, 67NR, 66Cl4 and 168FARN bearing mice had 165 ± 53 pg/ml, 117 ± 16 pg/ml and 46 ± 6 pg/ml, of serum G-CSF respectively, which were not significantly different from G-CSF in serum of naïve mice (59 ± 34 pg/ml) (Figure 8 a).

Thus, to determine the correlation between serum G-CSF levels and frequency of MDSCs, we determined the number of MDSCs in the spleen of the tumor bearing mice. We found that, 4T1 and 4T07 tumor bearing mice had 1.27± 0.1 $x10^8$ and 1.9 ± 0.3 $x10^8$ MDSCs, respectively. These levels were significantly different from the number of MDSCs in the other three breast cancer

bearing mice (67NR: 4 ± 0.4 x10^6; 66Cl4: 3 ± 0.2 x10^6; 168FARN: 5 ± 0.7 x10^6 MDSCs) (Figure 8 b).

Effect of G-CSF secretion on protective immunity

From the above studies, we hypothesized that the high levels of G-CSF released by 4T1 cells in the vaccine were responsible for the poor immune response. To test this hypothesis, we repeated the contralateral hybrid vaccine study with irradiated 4T1.GCSF-KO plus EMT6 cells followed by a live EMT6 challenge. We found that in mice with vaccines containing 4T1.GCSF-KO cells, only 30% developed tumors. This was significantly different from contralateral vaccine with 4T1 and EMT6 cells, where 70% of the mice developed EMT6 tumors (Figure 9). Additionally, we recorded survival in mice that were vaccinated and challenged with 4T1 or 4T1.G-CSF KO cells alone. We found that in the 4T1 group, all mice developed tumors, like the control group (naïve mice with 4T1 challenge). On the other hand, none of the mice in the 4T1.G-CSF KO vaccine group developed tumors (Figure 10).

D. Discussion

It is well established that ATCVs comprised of poorly immunogenic tumor cells are not effective. Thus, in this study, we set out to determine the reason for poor immunogenicity against breast cancer ATCVs by using two murine breast cancer cells, 4T1 and EMT6. Here, 4T1 is inherently non-immunogenic when compared to EMT6 cells, and therefore, we aim at understanding the reasons for differences in immunogenicities by studying their immune response.

We initially confirmed the differences in ATCV response by vaccinating mice with irradiated 4T1 or EMT6 cells and subsequently challenging with live 4T1 or EMT6 cells. We found that the ATCV against the immunogenic EMT6 cells developed protective immunity to live EMT6

challenge. On the other hand, 4T1 vaccine failed to provide any protective immunity (Figure 1). Thus, to determine the reasons for the differences in immunogenicities, we first looked at the levels of expression of immunologically relevant surface molecules MHC I, MHC II, B7-1, B7-2, ICAM-1 and FasR, that would determine the robustness of T cell activation. We found that irradiated EMT6 cells express significantly higher levels of B7-1 and FasR, which could be responsible for the enhanced immune response to EMT6 vaccine (Table 1). However, when we studied the differences in some of the immunosuppressive cytokines released by these cells, we found that irradiated 4T1 cells release very high levels of GM-CSF, G-CSF and M-CSF (Figure 2).

Thus, to determine if the immunosuppression associated with 4T1 cells is ruining the vaccine response, we immunized mice with ipsilateral and contralateral hybrid vaccines and challenged them with live EMT6 cells. We found that the presence of irradiated 4T1 cells ruined the protective immunity to EMT6 challenge in both vaccine groups, implicating a systemic immunosuppressive response to irradiated 4T1 cells.

Of the different immunosuppressive cytokines released by 4T1 cells, we noticed that G-CSF is released at exceptionally high levels when compared to the release by EMT6 cells. At such high levels, G-CSF and other colony stimulating factors have been previously associated with expansion of MDSCs in-vivo (18). Thus, in our study, to determine if G-CSF is resulting in expansion of MDSCs, we knocked out G-CSF in 4T1 cells (4T1.G-CSF KO) and implanted it to mice. We compared the levels of MDSCs in spleen and DLN of mice with 4T1 or 4T1.G-CSF KO tumors (of comparable tumor volume). We found that mice with 4T1 tumors had increased frequencies of MDSCs in both spleen and DLN, when compared to mice with 4T1.G-CSF KO tumors. We further established the correlation between tumor derived G-CSF and frequency of

MDSCs by using breast cancer cells releasing varying levels of G-CSF (4T07, 67NR, 66Cl4 and 168 FARN).

Additionally, to determine if the tumor derived G-CSF is responsible for abrogating vaccine efficacy, we utilized 4T1.G-CSF KO cells in the contralateral vaccine group. We found that immunizing with G-CSF KO vaccine improved the percentage of tumor free survival. Thus, the findings from this study established a causal link between tumor-derived G-CSF and a loss of autologous tumor cell vaccine response. However, it is important to note that our findings do not eliminate the possibility of other mechanisms that could be involved in MDSC expansion. For instance, knocking out other colony stimulating factors such as GM-CSF could have a similar effect on vaccine efficacy.

For the first time, our study has completely knocked down G-CSF using CRISPR-Cas9 genome editing and established the relationship between tumor derived G-CSF and MDSC expansion. Additionally, by using other murine breast cancer cells of common origin, but naturally different levels of G-CSF secretion, we further confirmed that G-CSF is a key initiator in MDSC expansion. The findings from our study, agree with another study by Abrams et al, where the causal link between tumor derived G-CSF and splenic G-MDSC frequency was established (26). However, Abrams and group used RNA interference and transfection of cancer cells with G-CSF expression plasmid, to establish the correlation. Recently, in a clinical study involving breast cancer patients, high G-CSF expression was found in the tumors of patients with the more aggressive forms of breast cancer and significantly correlated with poor overall survival (27). With the findings from our study, we now know that the poor overall survival in these patients with aggressive cancers, could very well be due to the MDSC associated immunosuppression.

Additionally, our findings also imply that autologous vaccines against aggressive breast cancers, could be improved by targeting the tumor derived G-CSF expression.

E. Conclusion

Despite the plethora of studies on autologous tumor cell vaccines for heterogenous and non-immunogenic cancers, the clinical outcome has been extremely poor. This failure in developing an effective vaccine is often linked to the inherent poor immunogenicity of the autologous cancer cells. In this study, we used two murine breast cancer cells, 4T1 (metastatic) and EMT6 (non-metastatic), that are on the opposite sides of the spectrum of metastatic ability. We found that the ATCV against 4T1 was significantly ineffective when compared to EMT6.

Therefore, by comparing the immune response against 4T1 and EMT6 ATCV, we found that 4T1 cells release exceptionally high levels of G-CSF, when compared to EMT6. By eliminating G-CSF in these cells using CRISPR/Cas9 genome editing, we found that such high levels of G-CSF result in the accumulation of immunosuppressive MDSCs in spleen and draining lymph nodes. Further, the findings established a causal link between tumor derived G-CSF and poor immunogenicity of ATCVs against breast cancer. We also successfully reestablished the correlation between tumor derived G-CSF and MDSC accumulation by using four different 4T1 sister cell lines that naturally produced varying levels of G-CSF.

The findings from this study, thus established the need to quantify the levels of serum G-CSF before generating an ATCV against breast cancer, that can be used as an indicator to determine the overall efficacy of the vaccine. Also, our findings show that future studies that target MDSC populations in breast cancers, could improve the overall survival.

F. Tables and figures

Table 1. Difference in expression of MHC and costimulatory molecules in non-irradiated and irradiated breast cancer cells. 4T1 and EMT6 cells were exposed to 0 or 100 Gray. 24 hours after irradiation, cells were harvested and stained with fluorochrome conjugated anti-CD80, anti-CD86, anti-H2-Kb, anti-I-Ad/I-Ed, anti-CD54 and anti-CD95. The samples were analyzed on a FACSCantoII. The differences in mean fluorescence intensities between unstained and stained non-irradiated cells; and unstained and stained irradiated cells were tabulated. The experiment was repeated thrice and the results were represented as mean fluorescence intensity ± standard error.

Cells		B7-1	B7-2	ICAM-1	MHCI	MHCII	FasR
Non-Irradiated	4T1	5 ± 0.8	22.75 ± 7.9	13.25± 6.8	19.1 ± 6.6	31.9 ± 13.5	366.7 ± 30.6
	EMT6	38.2 ± 23	45.6 ± 35.4	16.75 ± 6	19.2 ± 7.3	60.8 ± 45.7	2393.75 ± 56.2
Irradiated	4T1	11.05 ± 1.6	45.3 ± 4.6	38 ± 8.4	32 ± 11.3	117.3 ± 28.7	1197.7 ± 177
	EMT6	253 ± 1.3	77.5 ± 19.9	19.5 ± 10.6	52.5 ± 9.19	135 ± 39	4913.05 ± 155

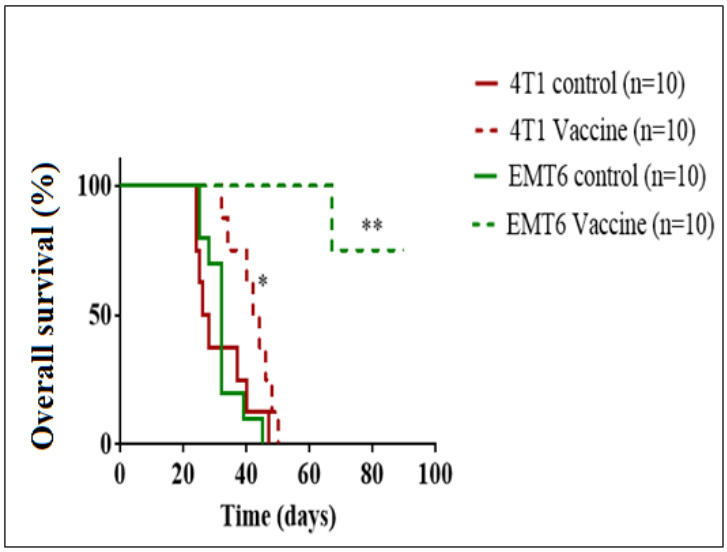

Figure 1. Differences in protective immunity induced by irradiated breast cancer cell lines. Balb/cByJ mice received $1x10^6$ irradiated 4T1 cells (4T1 vaccine) or $5x10^5$ irradiated EMT6 cells (EMT6 vaccine) twice, 10 days apart. 10 days after booster vaccination, mice were challenged with live $5x10^5$ 4T1 or EMT6 cells, respectively. Additionally, naive mice that received only $5x10^5$ live EMT6 cells (EMT6 control) or live 4T1 cells (4T1 control), served as controls for each group. Tumor growth was monitored and mice were sacrificed when the tumor volumes reached about $2000mm^3$. Kaplan-Meier curves were plotted for mice of the above groups (**$p<0.01$, *$p<0.05$).

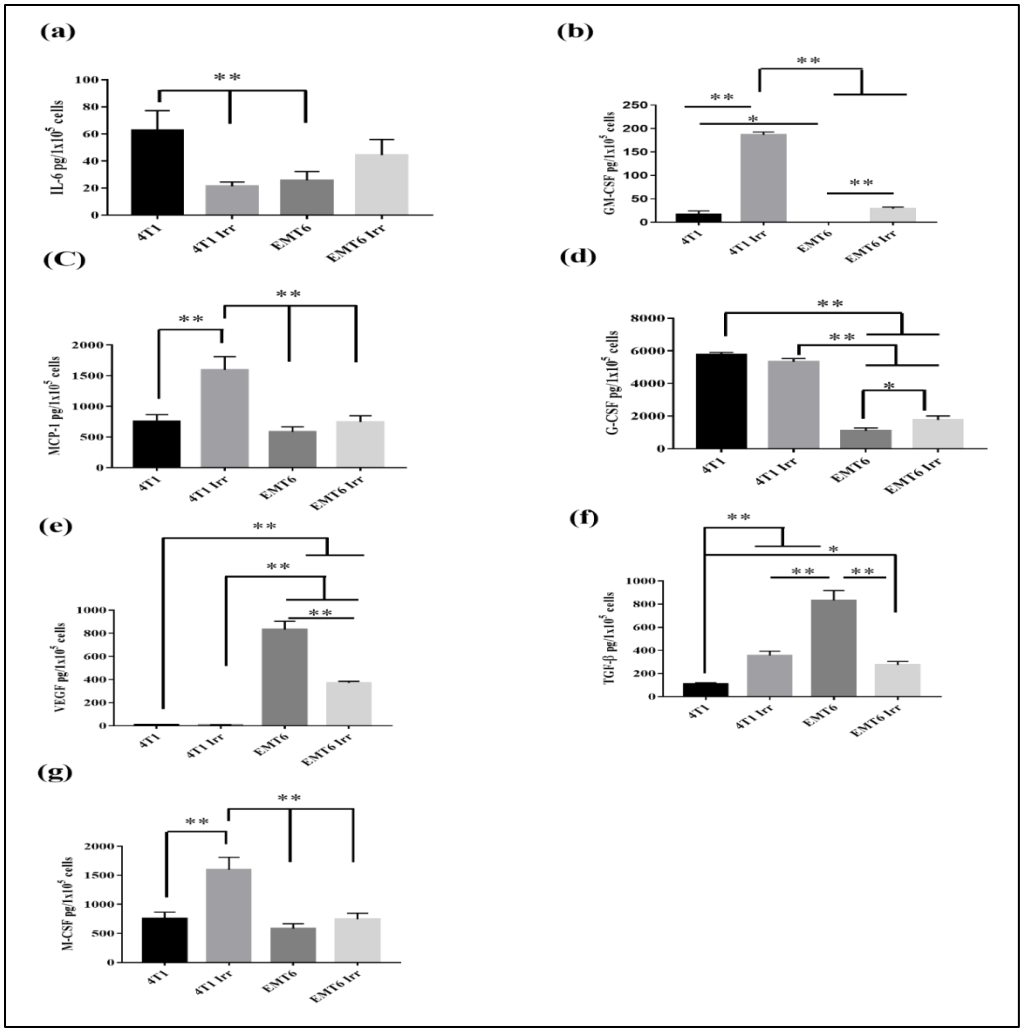

Figure 2. Cytokine release profile of 4T1 and EMT6 cells before and after irradiation. 4T1 or EMT6 cells were irradiated at 100Gy using a Gammacell irradiator. $5x10^5$ non-irradiated (4T1 and EMT6) and irradiated (4T1 Irr and EMT6 Irr) cells were seeded on separate T25 flasks and cultured for 48 hours. The cell media were collected from each flask and centrifuged to obtain cell-free supernatants. Levels of cytokines IL-6 (a), GM-CSF (b), MCP-1 (c) and G-CSF (d) in the cell free supernatant were measured using cytometric bead array. Whereas, cytokines M-CSF (e), VEGF (f) and TGF-β (g) were measured via ELISA. The experiment was repeated thrice and the results represent mean± standard error (**p<0.01, *p<0.05).

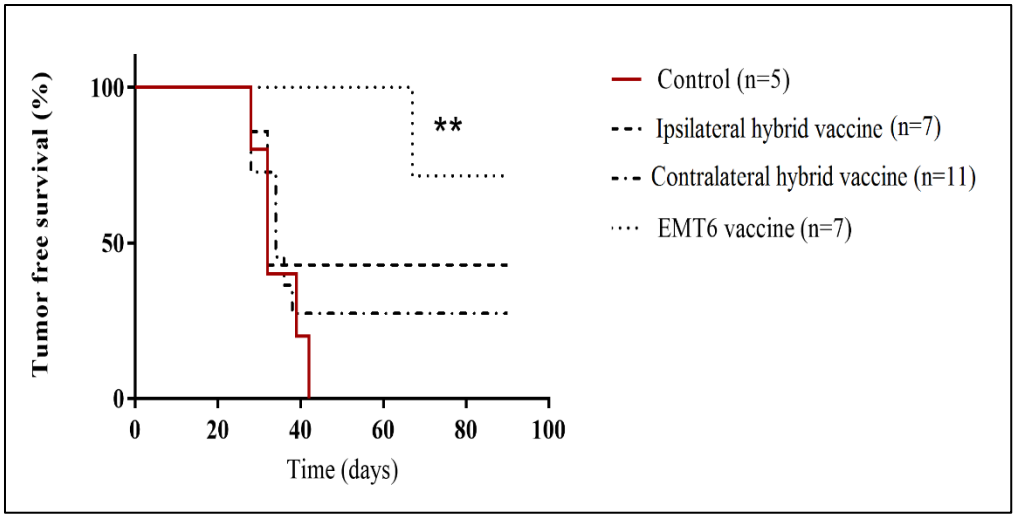

Figure 3. 4T1 vaccine abrogates EMT6 immunity. Female balb/cByJ mice were vaccinated with irradiated $5x10^5$ EMT6 cells (EMT6 vaccine) or homogenous mixture of irradiated $5x10^5$ EMT6 and $1x10^6$ 4T1 cells (ipsilateral hybrid vaccine) or irradiated $1x10^6$ 4T1 and $5x10^5$ EMT6 on opposite flanks (contralateral hybrid vaccine) twice, 10 days apart. Additionally, naïve mice that only received $5x10^5$ EMT6 cells served as control. 10 days after the booster vaccine, all mice were challenged with live $5x10^5$ EMT6 cells on the same side as the irradiated EMT6 cells. Tumor growth was monitored and mice were sacrificed when the tumor volume reached about $2000mm^3$. Kaplan-Meier curves were plotted for mice of the above groups (**p<0.01).

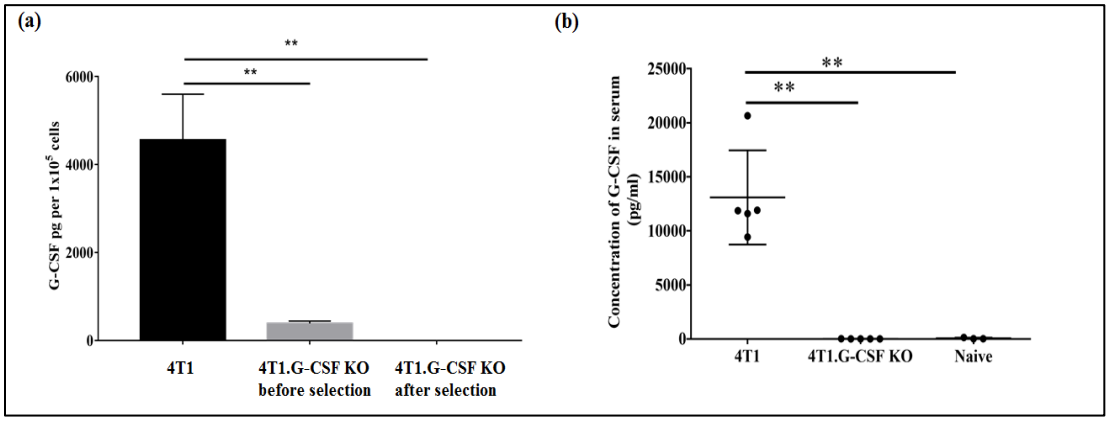

Figure 4. In-vitro and in-vivo G-CSF concentration. Female balb/cByJ mice were subcutaneously injected with $5x10^5$ 4T1 (n=5) or 4T1.GCSF-KO cells (n=5). The tumor growth was monitored and the serum samples were collected when the tumors reached 500-$700mm^3$. Serum from naïve mice were collected to serve as control. Levels of G-CSF in the collected samples where measured via G-CSF specific ELISA. **p<0.01.

Figure 5. Extreme splenomegaly in spleen of 4T1 bearing mice. $1x10^6$ 4T1 or 4T1.G-CSF KO cells were subcutaneously injected to balb/cByJ mice. Mice were sacrificed and their spleens were isolated when the tumor volume reached about 500-700mm^3. Spleen from mice bearing 4T1 (1) or 4T1 KO (2) tumors compared with spleen from naïve mouse (3).

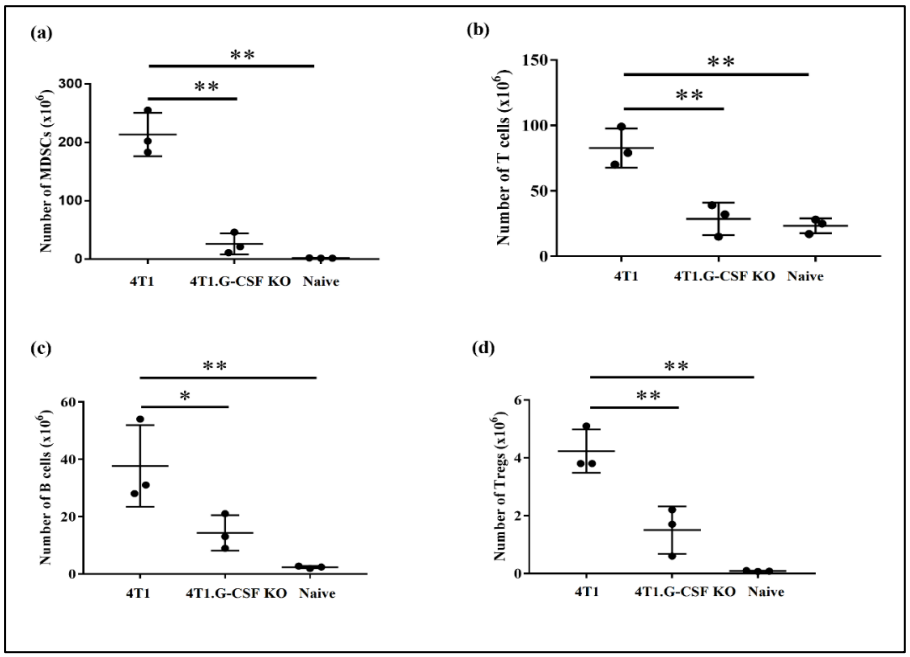

Figure 6. Comparison of immune cell subsets in the spleen of 4T1 and 4T1.G-CSF KO bearing mice. $1x10^6$ 4T1 or 4T1.G-CSF KO cells were subcutaneously injected to balb/cByJ mice (n=3). Once tumors reached a volume of 500-700mm^3, spleens were harvested and single cell suspensions obtained. Splenocytes isolated from naïve mice served as control (n=3). Cells were stained with fluorochrome-conjugated antibodies against CD3, CD4, CD25, FoxP3, CD11b, Ly6G and Ly6C and CD19. Flow cytometric analysis was performed to determine the percentage of MDSCs (CD11b+ and Ly6G and Ly6C+), B cells (CD19+), T cells (CD3+) and regulatory T cells (CD3+, CD4+, CD25+ and FoxP3+). Absolute numbers of MDSCs (a), T cells (b), B cells (c) and Tregs (d)were calculated and the results are represented as mean ± standard error (n=3, **p<0.01, *p<0.05).

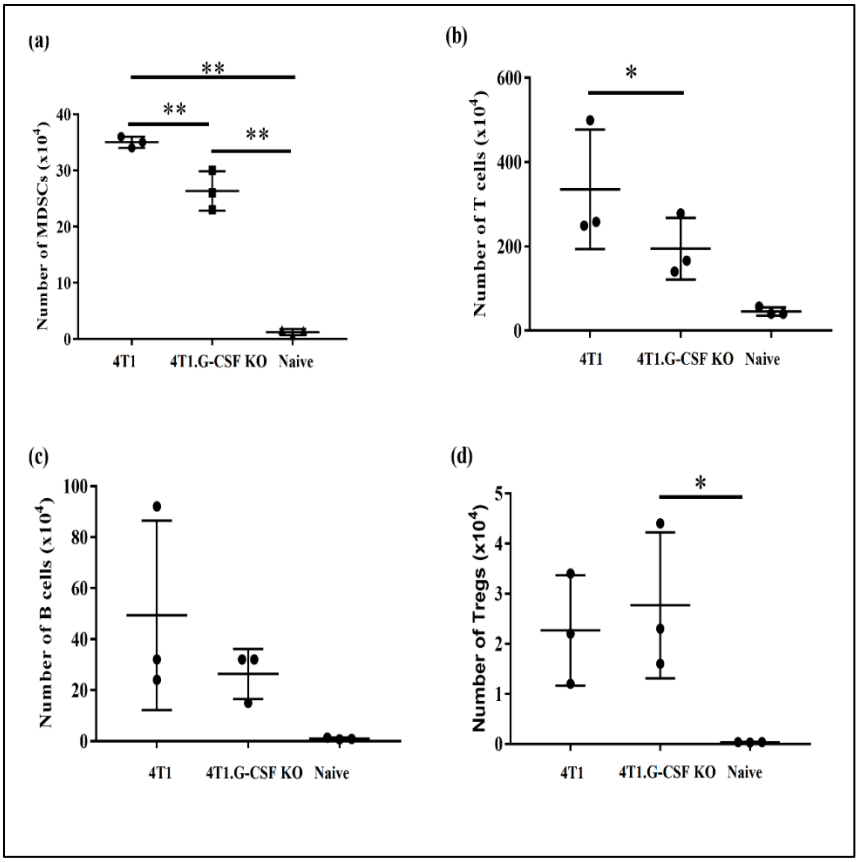

Figure 7. Comparison of immune cell subsets in the draining lymph nodes of 4T1 and 4T1.G-CSF KO bearing mice. $1x10^6$ 4T1 or 4T1.G-CSF KO cells were subcutaneously injected to balb/cByJ mice (n=3). Once tumors reached a volume of 500-700mm^3, draining lymph nodes were harvested and single cell suspensions obtained. Draining lymph nodes isolated from naïve mice served as control (n=3). Cells were stained with fluorochrome-conjugated antibodies against CD3, CD4, CD25, FoxP3, CD11b, Ly6G and Ly6C and CD19. Flow cytometric analysis was performed to determine the percentage of MDSCs (CD11b+ and Ly6G and Ly6C+), B cells (CD19+), T cells (CD3+) and regulatory T cells (CD3+, CD4+, CD25+ and FoxP3+). Absolute numbers of MDSCs (a), T cells (b), B cells (c) and Tregs (d)were calculated and the results are represented as mean ± standard error (n=3, **p<0.01, *p<0.05).

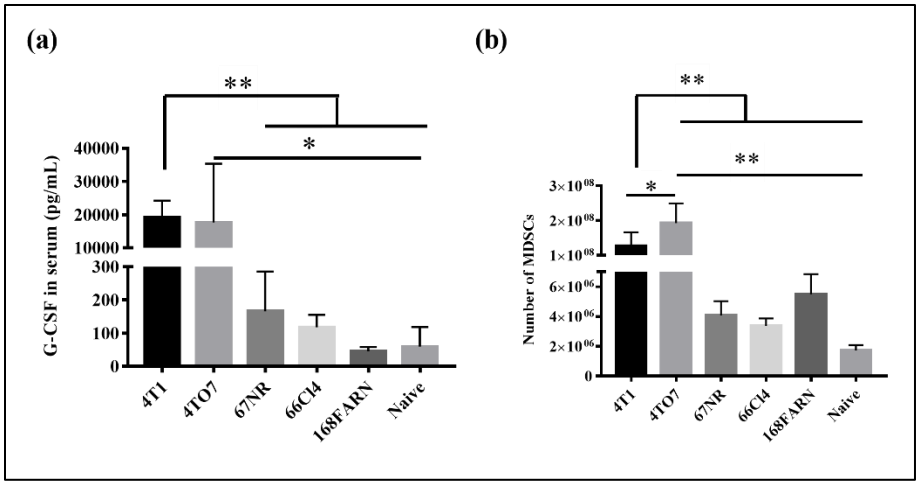

Figure 8. Concentration of G-CSF in sera and number of MDSCs in spleen of mice bearing different breast cancer cell lines. Balb/cByJ mice were subcutaneously injected with $1x10^6$ 4T1 cells (n=5), $5x10^6$ 4T07 cells (n=3), $1x10^6$ 168 FARN (n=3), $1x10^6$ 67 NR (n=5) and $3x10^6$ 66Cl4 (n=5). When the tumor volumes reached $500mm^3$, blood samples were collected via submandibular bleeding and centrifuged at 4000 rpm for 30 minutes to isolate sera. The concentration of G-CSF in sera were determined via G-CSF specific ELISA kit (a). Also, mice were sacrificed following submandibular bleeding, the splenocytes were isolated and single cell suspensions obtained. Cells were stained for MDSCs using fluorochrome-conjugated anti-CD11b and anti-Ly6G and Ly6C and flow cytometric analysis was performed to determine absolute number of MDSCs (b). Results are represented as mean ± standard error (**$p<0.01$, *$p<0.05$).

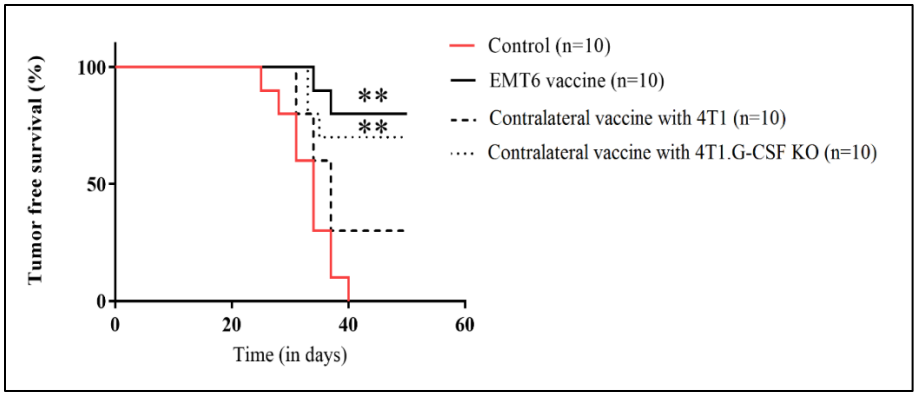

Figure 9. Contralateral vaccine with irradiated 4T1.G-CSF KO cells has improved vaccine efficacy. Balb/cByJ mice received $5x10^5$ irradiated EMT6 cells or $5x10^5$ irradiated EMT6 cells and $1x10^6$ irradiated 4T1 cells on opposite sides (contralateral vaccine with 4T1) or $5x10^5$ irradiated EMT6 cells and $1x10^6$ irradiated 4T1.G-CSF KO cells on opposite sides (contralateral vaccine with 4T1.G-CSF KO) twice, 10 days apart. 10 days after booster vaccination, mice of the above groups were challenged with live $5x10^5$ EMT6 cells. Additionally, naive mice that received only $5x10^5$ live EMT6 cells, served as control. Tumor growth was monitored and mice were sacrificed when the tumor volume reached about $2000mm^3$. Kaplan-Meier curves were plotted for mice of each group (**p<0.01).

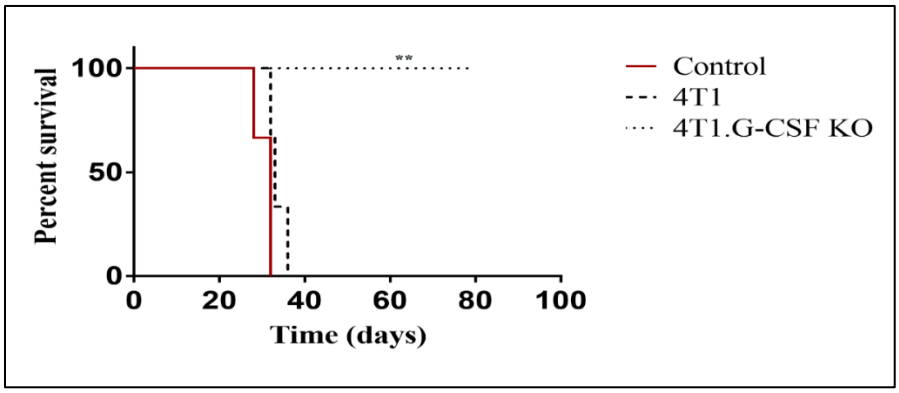

Figure 10. Protective immunity to 4T1.G-CSF KO tumor. Balb/cByJ mice were vaccinated with $1x10^6$ 4T1 cells or $1x10^6$ 4T1.G-CSF KO cells, twice 10 days apart. 10 days after booster vaccination, the two groups of mice were challenged with live $5x10^5$ 4T1 cells or $5x10^5$ 4T1.G-CSF KO cells, respectively. A third group contained naïve mice that received only a live challenge with $5x10^5$ 4T1 cells, served as control. Tumor growth was monitored and mice were sacrificed when the tumor volume reached about $2000mm^3$. Kaplan-Meier curves were plotted for mice of each group (**p<0.01).

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IV. COMPARISON OF DIFFERENT TLR AGONISTS ON REVERSING MDSC ASSOCIATED IMMUNOSUPPRESSION

A. Introduction

Myeloid derived suppressor cells (MDSCs), a population of immature myeloid cells, play a crucial part in the efficacy of cancer vaccines (1). In healthy individuals, immature myeloid cells arise in the bone marrow and differentiate into mature leukocytes namely, granulocytes, dendritic cells and macrophages. However, in the presence of cancer, these cells are prevented from differentiation, resulting in a heterogenous population of immature myeloid cells, collectively termed as MDSCs (2). In mice, these cells are identified by their expression of Gr1 and CD11b and include two unique subsets, namely monocytic MDSCs (M-MDSCs) and granulocytic MDSCs (G-MDSCs). Here, G-MDSCs are $Gr1^{high}$, $Ly6G^{+}$ and $Ly6C^{low}$, whereas M-MDSCs are $Gr1^{intermediate}$, $Ly6G^{low}$ and $Ly6C^{high}$ (M-MDSCs) (3). In humans MDSCs express CD11b, CD33 and low levels of human leukocyte antigen -antigen D related (HLADR). Further, G-MDSCs are $CD33^{+}Lin^{-}$ and M-MDSCs are $CD14^{+}$ $HLA\text{-}DR^{low/-}$ $co\text{-}receptor^{low/-}$ (4).

MDSCs create an immune suppressed tumor microenvironment via multiple methods that include releasing immunosuppressive factors, cytokines and chemokines. The MDSC based immunosuppression mainly targets CD8+ T cell response by releasing extremely high levels of arginase and nitric oxide. These molecules in turn, collectively cause the depletion of arginine, that is required for T cell activation and function (5). Additionally, it was also found that MDSCs inhibit natural killer (NK) cell responses by decreasing macrophage associated IL-12 release. This in turn results in poor stimulation of NK cells, which are immune cells of the innate system that have the potential to kill cancer cells without prior sensitization (2, 6, 7). Simultaneously, MDSCs also release high levels of IL-10 that affect the maturation of dendritic cells which are

involved in antigen presentation (8). Also, M-MDSCs increase the number of regulatory T cells (Tregs), another immunosuppressive immune cell subset (3). Thus, elevated levels of accumulation of MDSCs and their presence in peripheral blood are frequently associated with poor prognosis in cancer patients (9-11).

Therefore, in the recent past, several approaches have been explored to eradicate MDSC associated immunosuppression (12). Different therapeutic agents such as IL-12, all trans-retinoic acid, vitamin A, etc. have been investigated for their ability to cause MDSC differentiation and improve survival via enhanced T cell response (2, 13).

However, in 2007, for the first time it was found that MDSCs expand through signaling via MyD88, an adaptor required for toll like receptor (TLR) signaling (14). TLRs are type I membrane glycoproteins, that play an important role in innate immunity. They are expressed on different immune cells such as B cells, macrophages, dendritic cells, as well as on non-immune cells such as endothelial and epithelial cells. So far, researchers have identified 10 and 13 TLR members in mice and human, respectively (15).

Thus, after the effect of MyD88 signaling on MDSCs was determined, several researchers explored the effect of different TLR agonists (molecules that target TLRs) on MDSCs. Though, all agonists were expected to have a positive effect on MDSC expansion, researchers found that some agonists can initiate MDSC differentiation. For instance, while a study using LPS and IFN-γ found that levels of expression of dendritic cells and macrophage markers (F4/80, CD11c, DEC205) remains unchanged (16), another study using Poly (I:C), a TLR 3 agonist reported an upregulation of maturation markers on these cells. Here, an intraperitoneal injection of Poly (I:C) into 4T1 tumor bearing mice, was found to decrease the frequencies of MDSCs in blood, bone

marrow and tumor when compared to mice treated with saline (17). However, when the effect of two different TLR agonists, Pam (TLR 1/2 agonist) and R848 (TLR 7/8 agonist) on M-MDSC differentiation were compared in another study, the results were contradicting. When M-MDSCs were isolated from human peripheral blood mononuclear cells (PBMCs) and treated with Pam or R848, Pam was found to differentiate M-MDSCs into M2-like macrophages, that are immunosuppressive. On the other hand, R848 differentiated M-MDSCs to M1-like macrophages, that are known to have tumoricidal properties (18).

Thus, there is a need to understand the effect of different TLR agonists on MDSC differentiation. Expanded knowledge on the effect of these molecules would help develop immunotherapeutic strategies to improve efficacy of different cancer vaccines. Additionally, it would also help in reversing tumor associated immunosuppression, which is one of the major reasons for tumor recurrence in cancer patients (19).

Therefore, this study aims at comparing the effect of different commercially available TLR agonists on the differentiation and immunosuppressive activity of MDSCs. Here, unlike most studies where MDSCs are isolated to test the effect of the agonist, we are looking at the overall response while the cells are in the whole splenocyte culture. By doing so, we believe that we can understand the overall immune response better, since the MDSCs are in an environment that closely mimics in-vivo events. Here, though we also tested for the effect of IL-12 on MDSC maturation in-vitro, we did not observe any significant effect. The results from this study can be found in the appendix section.

B. Materials and methods

Reagents

Pam3CSK4 (Pam), Poly (I:C)-LMW (Poly IC), FSL-1, FLA-ST and R848 were purchased from InvivoGen (San Diego, CA). All fluorochrome conjugated monoclonal antibodies (mAb) used in the detection of different immune cell subsets were purchased from BD Biosciences (San Jose, CA). Recombinant mouse granulocyte-monocyte colony stimulating factor (rGM-CSF) used in maintaining the viability of MDSCs in culture, were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture media components including Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Hyclone laboratories (Marlborough, MA). Ammonium-chloride-potassium (ACK) buffer used in the lysis of red blood cells while isolating splenocytes, was purchased from Lonza (Allendale, NJ).

Cell culture

4T1, murine breast cancer cells, were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Splenocytes from tumor bearing mice were isolated following the procedure described below. Isolated splenocytes were treated with 10ng/ml rGM-CSF and/or TLR agonists (at concentrations as mentioned in Table 1) in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. All cell cultures were maintained in a humidified CO_2 incubator with 5% CO_2 at 37˚C.

Mice

Female Balb/cByJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were housed in microisolator cages. All experimental procedures were approved by the Institutional Animal Care and Use Committee at University of Arkansas. Mice were utilized for experiments at 8-12 weeks of age and animal care followed 'The Guide for Care and Use of Laboratory Animals' (National Research Council).

Splenocyte isolation

Mice were injected subcutaneously with $1x10^6$ 4T1 cells and tumor growth was monitored. Spleens from tumor bearing (500-700mm^3) mice were isolated and single cell suspensions were prepared by mechanical dissociation. Further, the cell suspensions were passed through a 40µm nylon mesh before treating with ACK buffer, to lyse red blood cells. The cells were then washed and resuspended in PBS buffer for further analysis.

Toll like receptor expression

Isolated splenocytes were incubated with purified rat anti-mouse CD16/CD32 monoclonal antibody (BD Biosciences, San Jose, CA) for 10 at 4˚C, to block any non-specific binding of Fc receptors This was followed by staining with fluorochrome-conjugated anti-mouse Ly6G and Ly6C, anti-mouse CD11b, anti-mouse TLR1, anti-mouse TLR2 and anti-mouse TLR4 from eBioscience (San Diego, CA); fluorochrome-conjugated anti-mouse TLR3 and anti-mouse TLR7 from BD Biosciences (San Jose, CA); fluorochrome-conjugated anti-mouse TLR5 and anti-mouse TLR8 from abcam (Cambridge, MA) and fluorochrome-conjugated anti-mouse TLR6 from R&D systems (Minneapolis, MN). Cells stained with fluorochrome conjugated mouse IgG1 kappa antibodies were used as isotype controls.

After subsequent wash and resuspension in PBS, the cells were acquired using BD FACS Canto II flow cytometer. Further, using FlowJo software, viable MDSCs were gated following the gating strategy as in Figure 3 and the expression of TLRs 1-8 by these cells were detected.

Flow cytometry

Using a cell scraper, adherent cells in splenocyte cultures treated with both concentrations of each TLR agonist (for 24 and 72 hours), were detached. The media containing non-adherent and adherent cells were collected, centrifuged and cell pellets obtained. Homogeneous single cell suspension of the cells was produced by resuspending the cells in PBS. To block non-specific FC receptor mediated binding, the cells were first incubated with purified rat anti-mouse CD16/CD32 monoclonal antibody for 10 at 4˚C. Further, the cells were stained with fixable viability stain 660 for 15 minutes to detect viable cells. The cells were then washed and stained with fluorochrome-conjugated anti-CD11b, anti-Ly6G and Ly6C, anti-CD19, anti-CD3ε, anti-CD25, anti-CD4, anti-CD80, anti-CD86, anti-I-Ad/I-Ed (MHCII) and anti-F4/80 for 40 minutes at 4˚C. This was followed by fixing and permeabilizing the cells using 1x fix/perm buffer from BD biosciences. The permeabilized cells were then incubated with fluorochrome-conjugated anti-FoxP3 for 40 minutes, washed and resuspended in PBS. The samples were then acquired using FACScantoII and the flow cytometric data was analyzed using FlowJo software. The number of myeloid derived suppressor cells (MDSCs), CD11c+ cells, T cells, B cells and regulatory T cells (Tregs) were determined. Further the levels of expression of CD80, CD86, MHCII and F4/80 by MDSCs were analyzed. The gating strategies for MDSCs, CD11c+ cells, T cells, B cells and Tregs are shown (Figure 3 and Figure 4).

Cytokine release study

Cell culture media from splenocytes treated with the two different concentrations (low and high) of each TLR agonist for 24 and 72 hours were collected. The samples were then centrifuged to obtain cell free supernatants and the concentration of cytokines IL-6, IL-10 and TNF were detected using CBA. Further, concentration of TGF-β and IL-12 were detected via cytokine specific enzyme linked immunosorbent assay (ELISA).

C. Results

Expression of TLRs 1-8 by MDSCs

Since we are testing for the effect of different TLR agonists on MDSCs, we first wanted to ensure the expression of the receptors by these cells. We found that all eight TLRs tested (TLRs 1-8), were expressed by MDSCs (Figure 1).

Effect of TLR agonists on MDSC maturation

To determine the effect of TLR agonists on MDSC maturation, we treated splenocytes with rGM-CSF(10ng/ml) plus each of the TLR agonists at two different concentrations (Table 1). Here, GM-CSF was used to keep the splenocytes viable in culture and the concentrations of TLR agonists were selected such that the 'low' concentration represents the standard dose as found in the literature, whereas the 'high' is ten times the standard dose.

We then gated for MDSCs in the culture and tested for the expression of common maturation markers namely, CD80, CD86, MHCII and F4/80 by these cells (Figure 2). Of all the TLR agonists, only Pam resulted in an increase in expression of all four maturation markers with 24 and 72 hours of treatment. On the other hand, agonists LPS and FSL-1 resulted in upregulation

of CD80 and F4/80 alone with 24 and 72 hours of treatment. The effect was observed with both concentrations of the agonists LPS and FSL-1. R848 caused an increase in expression of MHCII and F4/80, only 72 hours after treatment. Poly IC and FLA-ST did not have any effect on MDSC maturation.

Effect of TLR agonists on frequency of different immune cell subsets

When we looked at cells treated with the TLR agonists under the microscope, we found some dendritic-like looking cells in some treatments. Hence, in addition to analyzing T cells, B cells, MDSCs and Tregs upon treatment, we also included antibodies against CD11c, a dendritic cell marker.

Of all the agonists tested, only Pam and FSL-1 did not have any effect on the number of B cells, when compared to cells treated with rGM-CSF alone. All other agonists, significantly decreased the number of B cells at the 24-hour time point at both doses. Interestingly, the differences were no longer significant at the 72-hour time point, except for with R8484. The numbers of B cells in samples treated with the high dose of R848 significantly increased at the 72-hour time point (Figure 5, Figure 6).

Further, looking at the number of T cells, at the lower dose, only LPS had a significant effect on the T cell population, 72 hours after treatment. However, at the higher dose, both LPS and Pam had a significant effect at 72 and 24-hour time points, respectively. Similarly, only Poly IC significantly decreased the number of CD11c+ cells at the 24-hour time point with both doses.

We also found that of all the agonists, only Pam and FSL-1 resulted in a significant decrease in the number of MDSCs, when compared to cells treated with rGM-CSF alone. This effect was found at both doses of the agonists and at both time points. Though LPS also decreased the

number of MDSCs, the difference was significant only with the higher dose of LPS at 24-hour time point.

Difference in cytokines released by splenocytes treated with TLR agonists

To get an idea for the overall effect of each agonist on the immune response, we looked at the levels of different cytokines that are usually associated with tumorigenesis. We compared these levels with the levels of cytokines released by splenocytes treated with rGM-CSF alone (Figure 7 and Figure 8). IL-6, a cytokine known to induce proliferation of cancer cells and inhibit their apoptosis, were found to be released at significantly higher levels by splenocytes treated with Pam, LPS and FSL-1 at both concentrations and at both time points. Splenocytes treated with R848 also released slightly higher levels of IL-6 at all treatment conditions, except with the low dose at the 24-hour time point. Similarly, IL-10, a cytokine involved in expansion of Tregs, was also found to be released at significantly higher levels by Pam, LPS, FSL-1 and R848. However, the differences were significant at all treatment conditions for all four agonists.

On the other hand, Pam, LPS, FSL-1 and R848, at the lower dose resulted in a decrease in the levels of TGF-β, another cytokine associated with expansion of Tregs at the 72-hour time point. Whereas at the higher doses of agonists, Pam, LPS, FSL-1 and Poly IC caused a decrease in TGF-β levels at the 72-hour time point. Further, when we measured the levels of TNF with low doses of agonists, we again found that all agonists except FSL-1 released significantly higher levels of TNF. However, with the higher dose, FSL-1 also resulted in significantly higher levels of TNF. On the contrary, only cells treated with R848 released any detectable levels of IFN-γ and IL-12.

D. Discussion

MDSCs are immunosuppressive immune cells of the myeloid lineage that expand aggressively in the presence of some cancers. The rapid accumulation of these cells in the tumor microenvironment facilitate tumor growth and progression. Additionally, their immunosuppressive activity could be a serious threat to newly developing cancer vaccines and immunotherapies.

Researchers have investigated the use of a few TLR agonists such as LPS, R848 and Poly IC in differentiating or expanding these cells. However, the findings from these studies are contradicting, since not all agonists have similar effects on MDSC maturation. Therefore, we set out to compare the effect of different commercially available TLR agonists on the maturation of MDSCs in-vitro.

In this study, instead of isolating the MDSCs, we observed the effect of TLR agonists on MDSC maturation, while the cells were in the whole splenocyte culture. This helped us to not just analyze the MDSCs, but also understand the effect on other immune cell subsets such as T and B cells. Additionally, we determined levels of different cytokines released in the splenocyte culture, which provided additional information on the overall immune response. Also, we believe that studying the MDSCs in the whole splenocyte culture would better mimic the in-vivo events.

We found that, even though MDSCs express all eight TLRs (TLRs 1-8), not all agonists cause the maturation of MDSCs. When we looked at the expression of maturation markers CD80, CD86, MHCII and F4/80, we found that only Pam upregulated the expression of all four maturation markers, at both low and high concentrations. On the other hand, FSL-1 and LPS only upregulated CD80 and F4/80, whereas R848 only upregulated MHCII and F4/80. On the

contrary, FLA-ST and Poly IC did not have any effect on MDSC maturation as well as MDSC count. Additionally, when looking at the effect on the number of MDSCs, at the low concentration, only Pam and FSL-1 significantly decreased the number of MDSCs. However, at the higher concentration, LPS also significantly decreased MDSC levels.

Our study, thus showed that Pam can initiate maturation of MDSCs and decrease the MDSC count in-vitro. However, this contradicted the findings by Wang et al. Here, Pam when co-cultured with human M-MDSCs, increased the expression of CD200R and CD206, thereby increasing their immunosuppressive activity (18). Additionally, in our study we found Poly IC to have no effect on any of the four maturation markers on MDSCs. This contradicted the findings by Forghani et al, where MDSCs isolated from the spleen of 4T1 tumor bearing mice when co-cultured with 20µg/ml of Poly IC resulted in upregulation of MHCII, CD80 and CD86 (17). However, it is important to note that in both studies, the effect on maturation were determined on isolated M-MDSCs or MDSCs. The different cytokines released by other immune cells in the culture and their interaction with MDSCs in our study, could be the rationale for the differences in MDSC maturation.

In our study, though we also looked at the levels of other immune cells, such as T and B cells, the findings are preliminary. Additional experiments such as determining the frequency of regulatory B and T cells are required to understand the overall immune response.

Additionally, we also looked at the concentration of different cytokines in splenocytes co-cultured with the TLR agonists. Only cultures treated with Pam, Poly IC and LPS had significantly high levels of IL-6 and IL-10. Though these cytokines are usually associated with tumorigenesis, recent studies have revealed the dual role of both cytokines in tumor

microenvironment. IL-6 trans-signaling can attract lymphocytes to tumor and lymph nodes and activate them towards immunostimulatory phenotype (24). Similarly, growing evidence is revealing the role of IL-10 in T cell activation and tumor shrinkage (25). Additionally, we also found that splenocyte cultures treated with Pam, LPS, FSL-1 and R848 also had decreased levels of TGF-β. Per literature, TGF-β initiates the generation of Tregs, that are immunosuppressive and is a hindrance in the proliferation of effector T cells. Thus, the low levels of TGF-β could mean that there is decreased immunosuppression. However, additional experiments that involve co-culturing MDSCs and T cells with the agonists, would provide information on the overall immunosuppressive activity.

Moreover, it is interesting that only R848 treated splenocytes released detectable levels of IL-12 and IFN-γ in the whole splenocyte culture. However, the lower than detectable amounts of these cytokines could be due to the excessive levels of MDSCs in culture that is minimizing the number of antigen presenting cells, the source of these cytokines. By repeating the experiment using splenocytes isolated from a smaller tumor bearing mice, we could obtain conclusive evidence.

E. Conclusion

Despite growing research on the effect of TLR agonists on the immunosuppressive activity of MDSCs, literature lacks a direct comparison between the different agonists. Additionally, the effect of these agonists is usually tested only on isolated MDSCs, which fail to mimic the actual immune response in-vivo. By directly comparing the effect of different TLR agonists on MDSCs in splenocyte culture, we established the immunological differences between them. Though

further T cell activation and in-vivo studies are required to come to conclusions, we found that focus needs to be on Pam, LPS, FSL-1 and R848, which had direct impact on MDSC maturation.

F. Tables and figures

Table 1. List of TLR agonists and their concentrations that were tested. The low concentration for each agonist used in this study, is the standard concentration at which it is used in literature for in-vitro studies. On the other hand, the higher concentrations were selected such that they were ten times that of the standard.

TLR	TLR agonist	Concentration		Reference
		Low	High	
TLR1/2	Pam	20ng/ml	200ng/ml	(18)
TLR3	Poly IC	20ug/ml	200ug/ml	(17)
TLR4	LPS	0.1ug/ml	1ug/ml	(20)
TLR5	FLA-ST	10ng/ml	100ng/ml	(21)
TLR2/6	FSL-1	10ng/ml	100ng/ml	(22)
TLR7/8	R848	10ng/ml	100ng/ml	(18, 23)

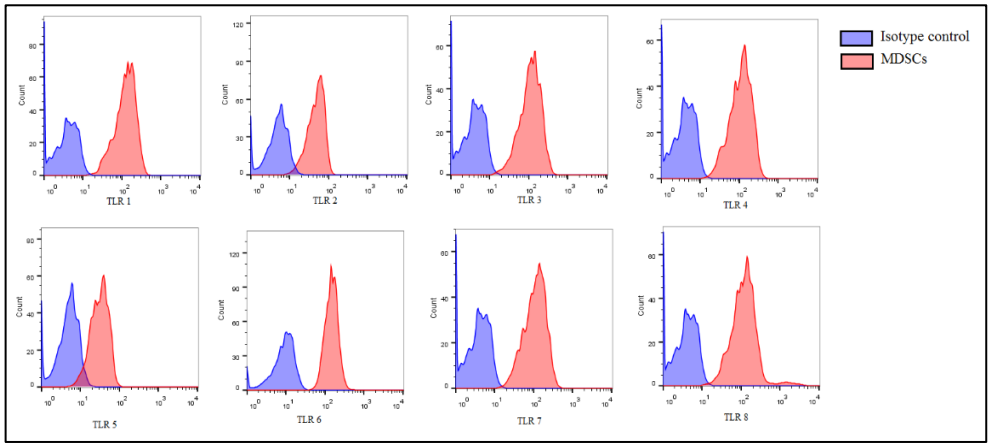

Figure 1. Expression of TLRs 1-8 by MDSCs. Balb/cByJ mouse was subcutaneously injected with $1x10^6$ 4T1 cells. When the tumor volume reached about 500-700mm^3, mice were sacrificed and splenocytes were isolated. Single cell suspensions of splenocytes were first incubated with purified rat anti-mouse CD16/CD32 for 10 minutes, to block non-specific Fc receptor mediated antibody binding. Further, the cells were stained with fluorochrome conjugated anti-CD11b, anti-Ly6G and Ly6C and all anti-TLRs from 1-8. Cells stained with fluorochrome-conjugated mouse IgG1 kappa antibodies served as isotype control. The samples were acquired using FACScantoII and the expression of TLRs 1-8 by MDSCs were determined using FlowJo software.

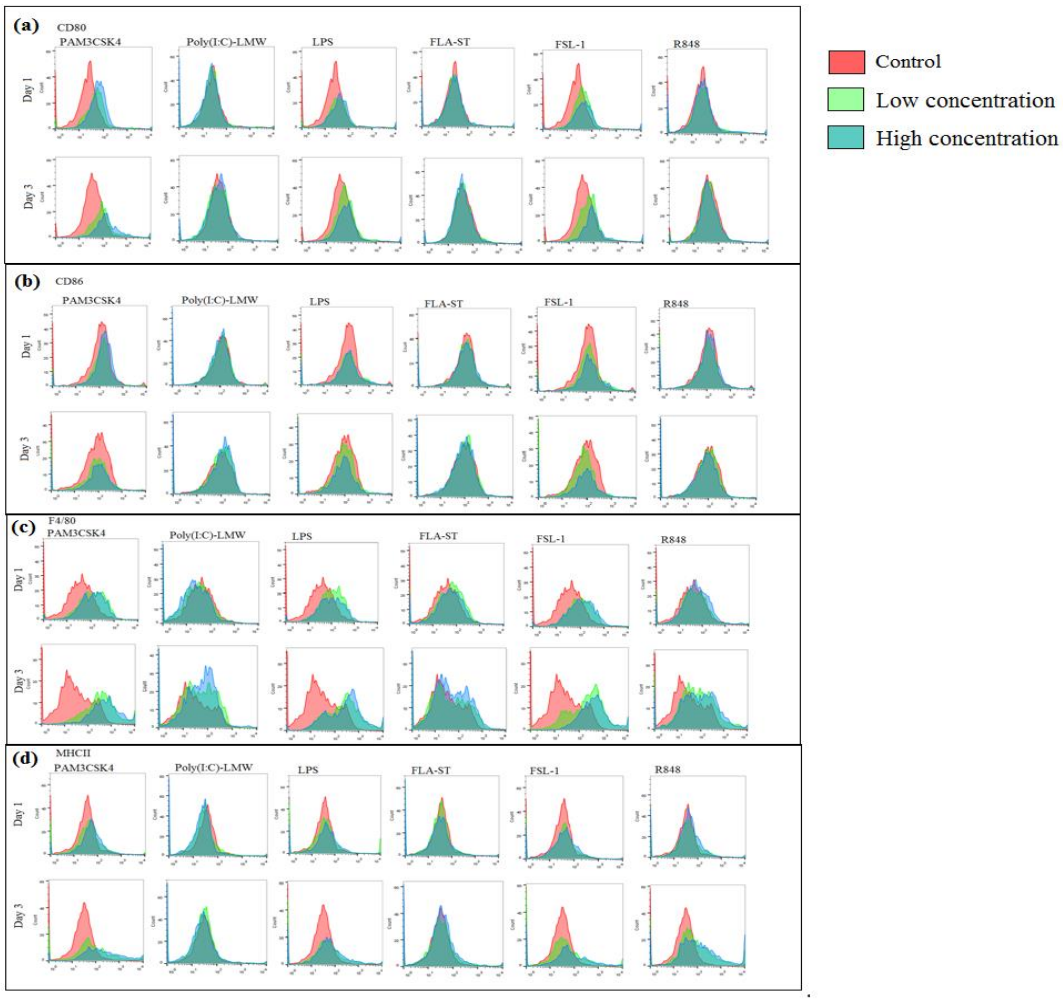

Figure 2. Expression of MDSC maturation markers upon treatment with TLR agonists. Balb/cByJ mice were subcutaneously injected with $1x10^6$ 4T1 cells and spleens were isolated when the tumor volumes reached about 500-700 mm^3. Splenocytes were isolated via mechanical dissociation and single cell suspensions obtained. Cells were then maintained in media containing 20ng/ml or 200ng/ml Pam; 20ug/ml or 200ug/ml Poly IC; 0.1ug/ml or 1ug/ml LPS; and 10ng/ml or 100ng/ml FLA-ST, FSL-1 or R848 for 3 days. 24 and 72 hours after treatment, cells were stained for MDSCs (anti-CD11b and anti-Ly6G and Ly6C) and maturation markers CD80, CD86, MHCII and F4/80. Cells treated with GM-CSF alone served as control. The samples were acquired using FACScantoII. The levels of expression of maturation markers CD80 (a), CD86 (b), MHCII (c) and F4/80 by MDSCs treated with each TLR agonist (low and high concentration) was determined using FlowJo software. The experiment was repeated thrice and a representative for each treatment is shown.

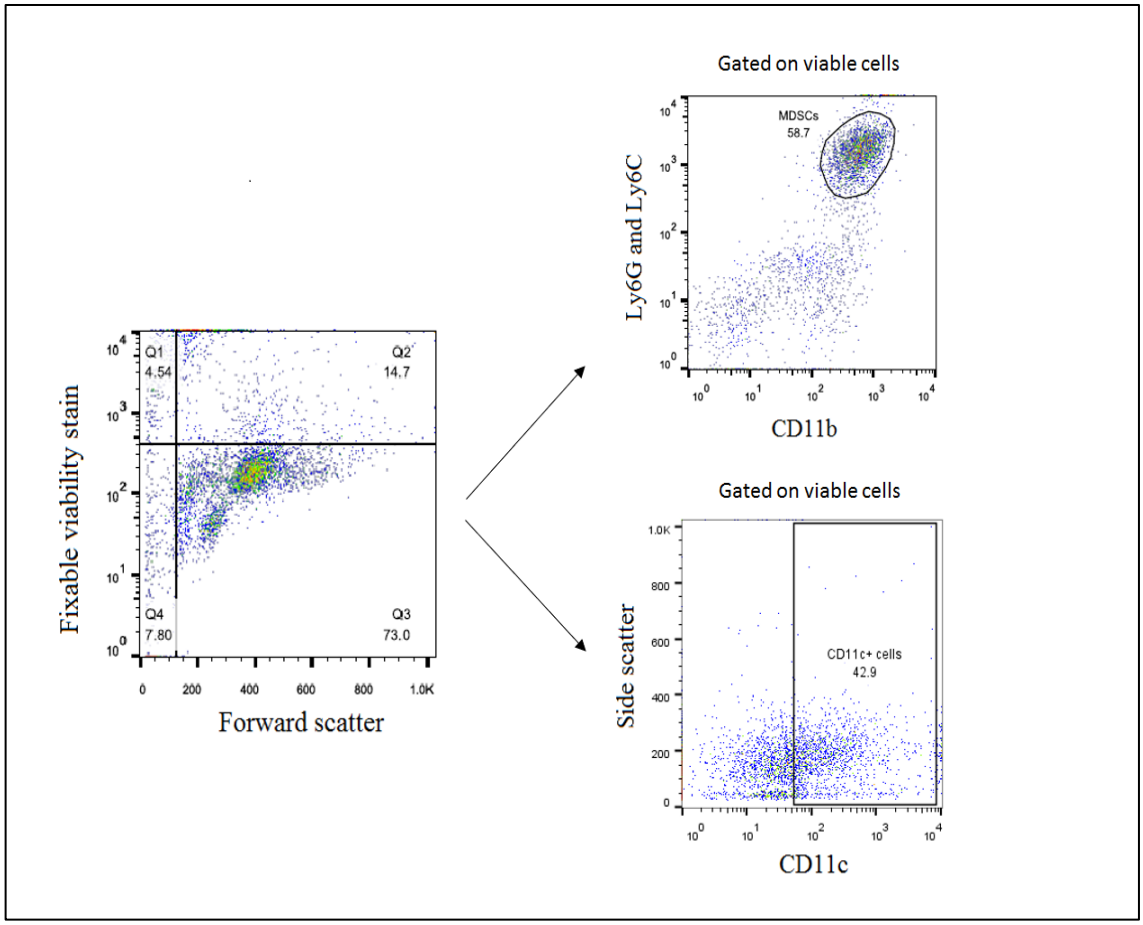

Figure 3. Gating strategy for MDSCs and CD11c positive cells. Balb/cByJ mice were subcutaneously injected with $1x10^6$ 4T1 cells and the spleen was isolated when the tumor volume reached about 500-700 mm^3. Splenocytes were isolated via mechanical dissociation and single cell suspensions obtained. The cells were stained with fixable viability stain 660 and fluorochrome-conjugated mAb against CD11b, CD11c and Ly6G and Ly6C. Using FACScantoII, the samples were acquired, followed by analysis using FlowJo software to gate for live MDSCs (CD11b+ and Ly6G and Ly6C+) and CD11c+ cells.

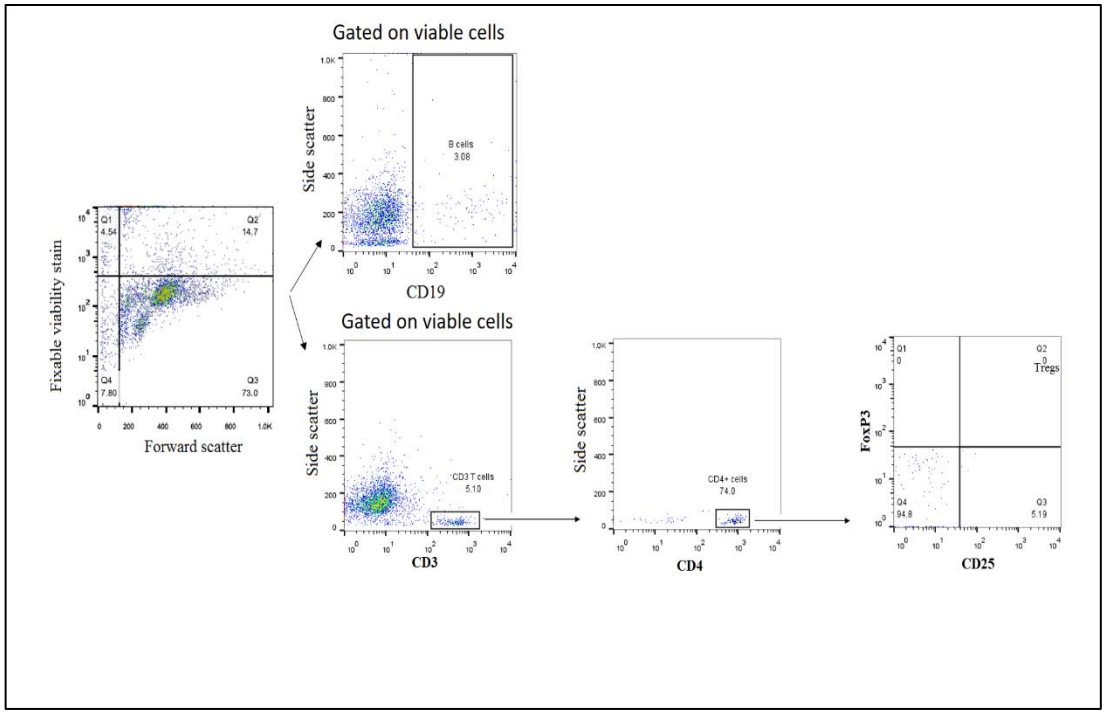

Figure 4. Gating strategy for B cells, T cells and Tregs. Balb/cByJ mice were subcutaneously injected with $1x10^6$ 4T1 cells and spleens were isolated when tumor volumes reached about 500-700 mm^3. Splenocytes were isolated via mechanical dissociation and single cell suspensions obtained. The cells were stained with fixable viability stain 660 and fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD25 and anti-FoxP3. Using FACScantoII, the samples were acquired and the positive gates for live B cells ($CD19^+$), T cells ($CD3^+$) and Tregs ($CD3^+$, $CD4^+$, $CD25^+$ and $FoxP3^+$) were determined using FlowJo software.

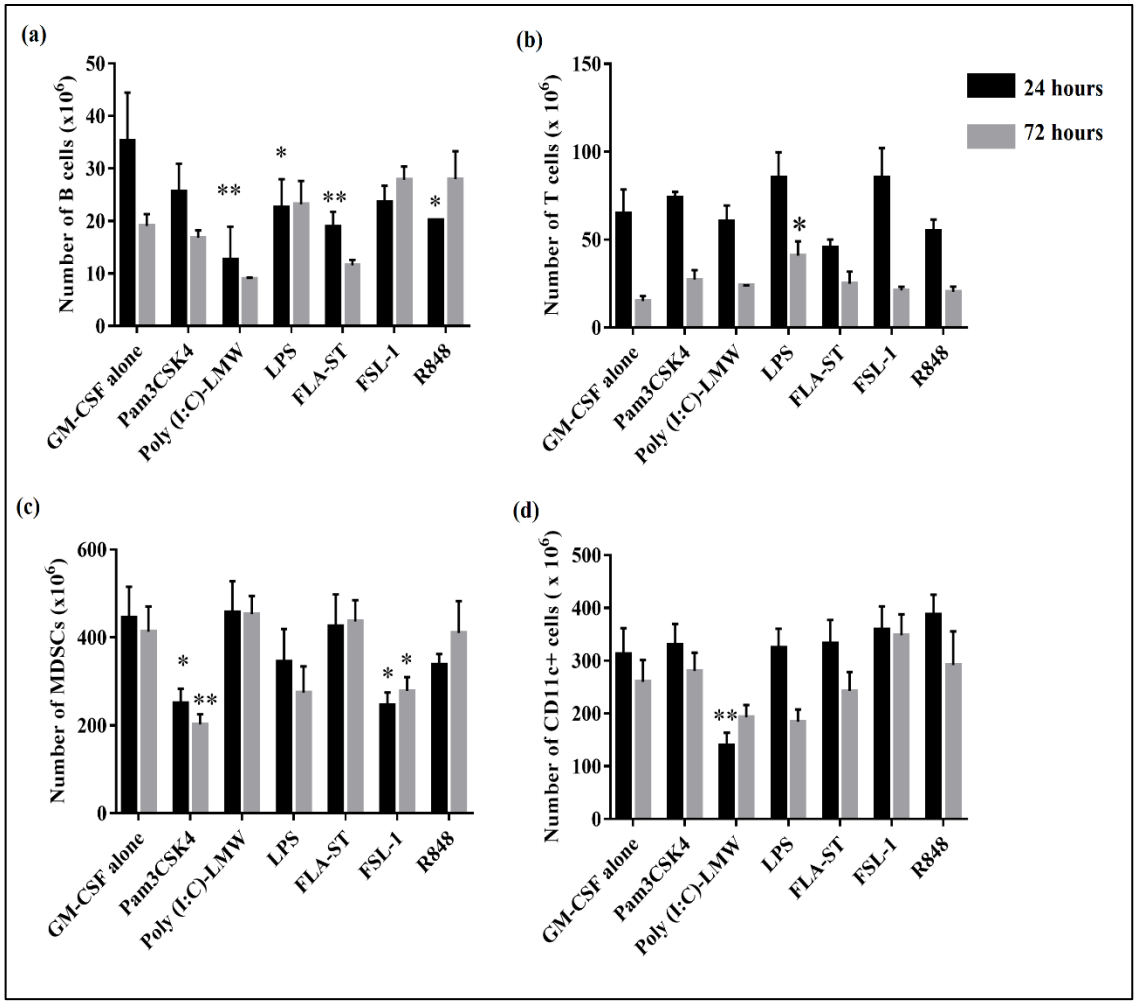

Figure 5. Effect of lower dose of TLR agonists on immune cell subsets Balb/cByJ mice were subcutaneously injected with $1x10^6$ 4T1 cells and spleens were isolated when tumor volumes reached about 500-700 mm^3. Splenocytes were isolated via mechanical dissociation and single cell suspensions obtained. Cells were then maintained in media containing 20ng/ml Pam, 20ug/ml Poly IC, 0.1ug/ml LPS and 10ng/ml FLA-ST, FSL-1 or R848 for 3 days. 24 and 72 hours after treatment, cells were stained for MDSCs ($CD11b^+$ and Ly6G and $Ly6C^+$), B cells ($CD19^+$), CD11c+ cells and T cells ($CD3^+$) and acquired using FACScantoII. Cells treated with 10ng/ml GM-CSF alone for 24 and 72 hours served as control. Absolute numbers of B cells (a), T cells (b), MDSCs (c) and CD11c+ cells (d) were determined upon analysis of the flow cytometric data using FlowJo software. The experiment was repeated thrice and the results are represented as mean ± standard error (*$p<0.05$, **$p<0.01$).

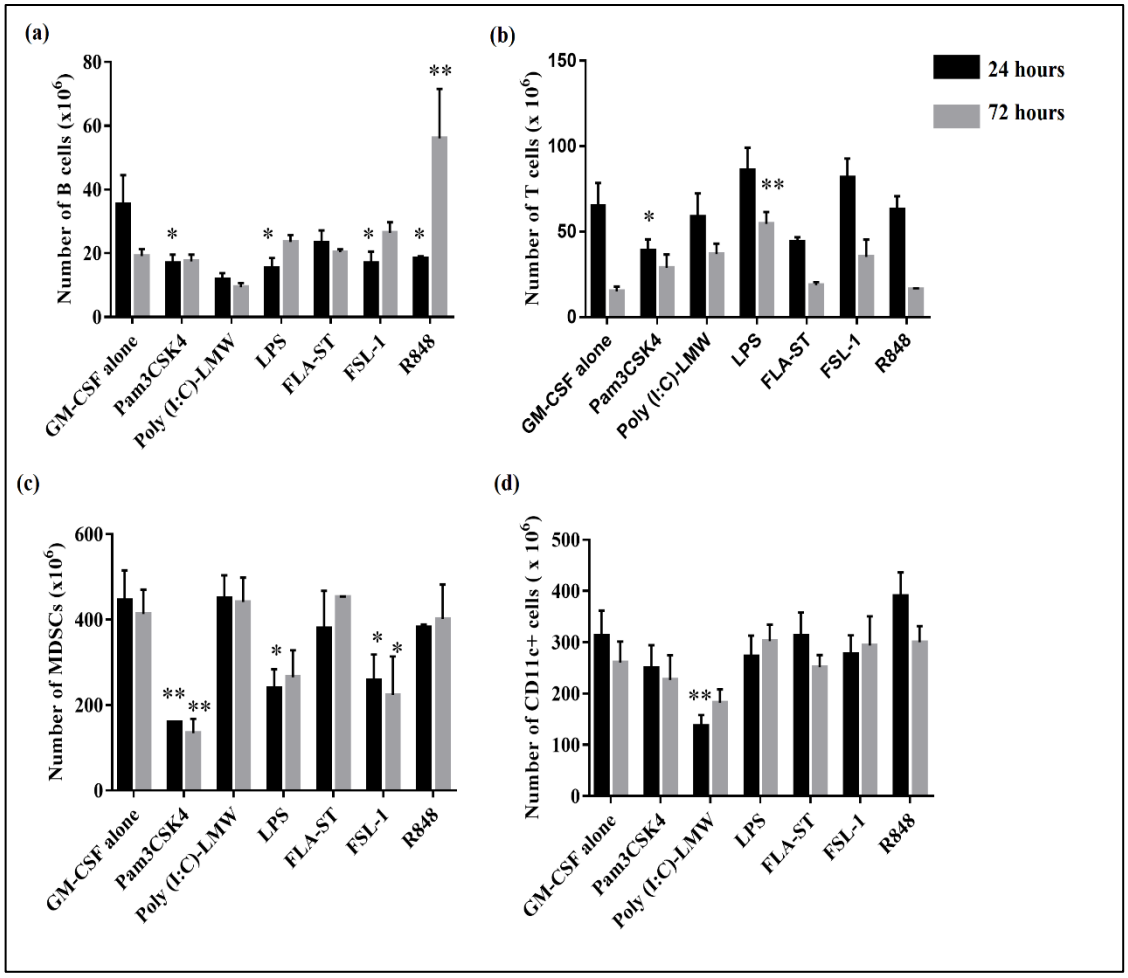

Figure 6. Effect of higher dose of TLR agonists on immune cell subsets. Balb/cByJ mice were subcutaneously injected with $1x10^6$ 4T1 cells and spleens were isolated when the tumor volumes reached about 500-700 mm^3. Splenocytes were isolated via mechanical dissociation and single cell suspensions obtained. Cells were then maintained in media containing 200ng/ml Pam, 200ug/ml Poly IC, 1ug/ml LPS and 100ng/ml FLA-ST, FSL-1 or R848 for 3 days. 24 and 72 hours after treatment, cells were stained for MDSCs ($CD11b^+$ and Ly6G and $Ly6C^+$), B cells ($CD19^+$), CD11c+ cells and T cells ($CD3^+$) and acquired using FACScantoII. Cells treated with 10ng/ml GM-CSF alone for 24 and 72 hours served as control. Absolute numbers of B cells (a), T cells (b), MDSCs (c) and CD11c+ cells (d) were determined upon analysis of the flow cytometric data using FlowJo software. The experiment was repeated thrice and the results are represented as mean ± standard error (*$p<0.05$, **$p<0.01$).

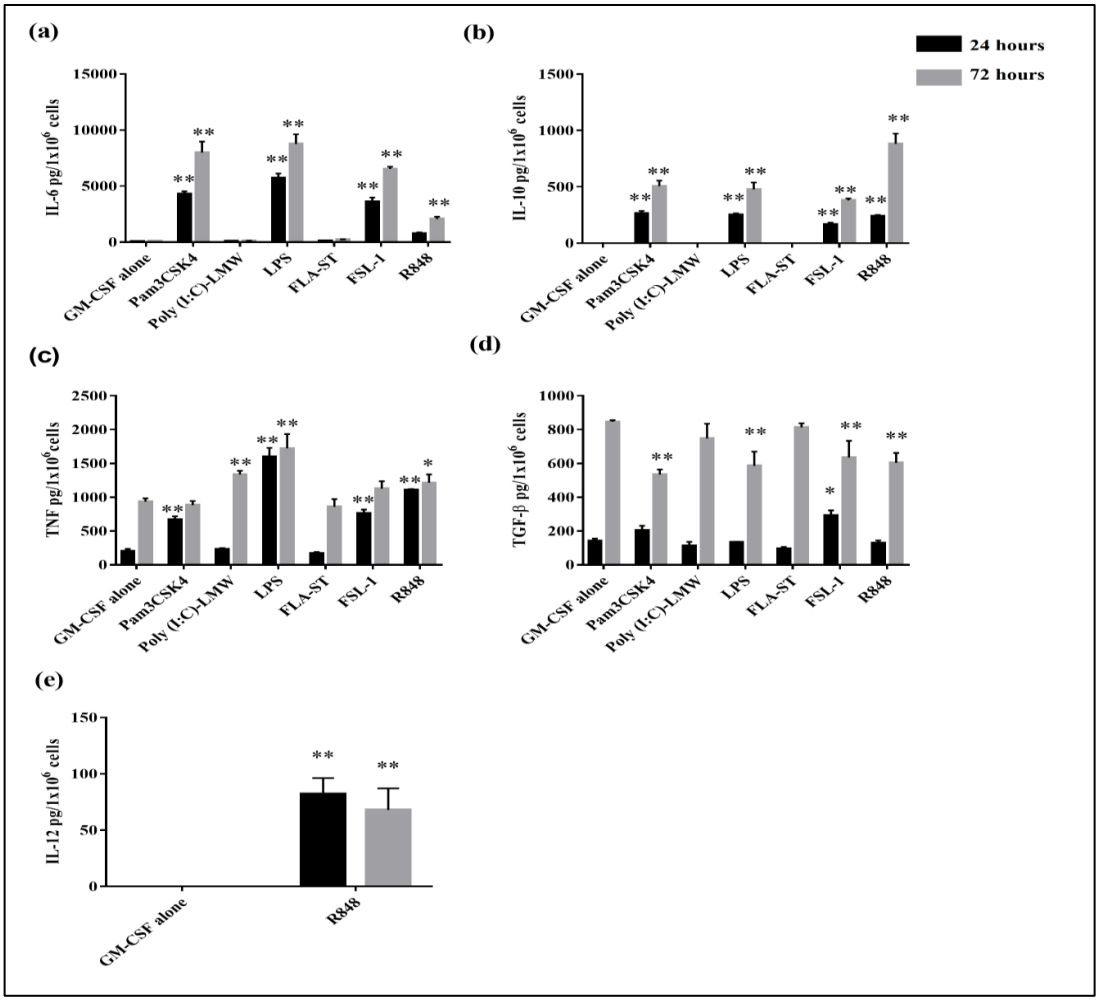

Figure 7. Cytokine release at lower dose of TLR agonists. Balb/cByJ mice were subcutaneously injected with $1x10^6$ 4T1 cells and the spleen was isolated when tumor volumes reached about 500-700 mm^3. Splenocytes were isolated via mechanical dissociation and single cell suspensions obtained. Cells were then maintained in media containing media containing 20ng/ml Pam, 20ug/ml Poly IC, 0.1ug/ml LPS and 10ng/ml FLA-ST, FSL-1 or R848 for 3 days. 24 and 72 hours after treatment, cell culture media was collected and centrifuged to remove any non-adherent cells. The cell culture supernatant was then tested for IL-6, IL-10 and TNF using CBA, whereas TGF-β and IL-12 were detected via cytokine specific ELISA. The experiment was repeated thrice and the concentration of IL-6 (a), IL-10 (b), TNF (c), TGF-β (d) and IL-12 (d) released per 10^6 splenocytes were represented as mean ± standard error (*$p<0.05$, **$p<0.01$).

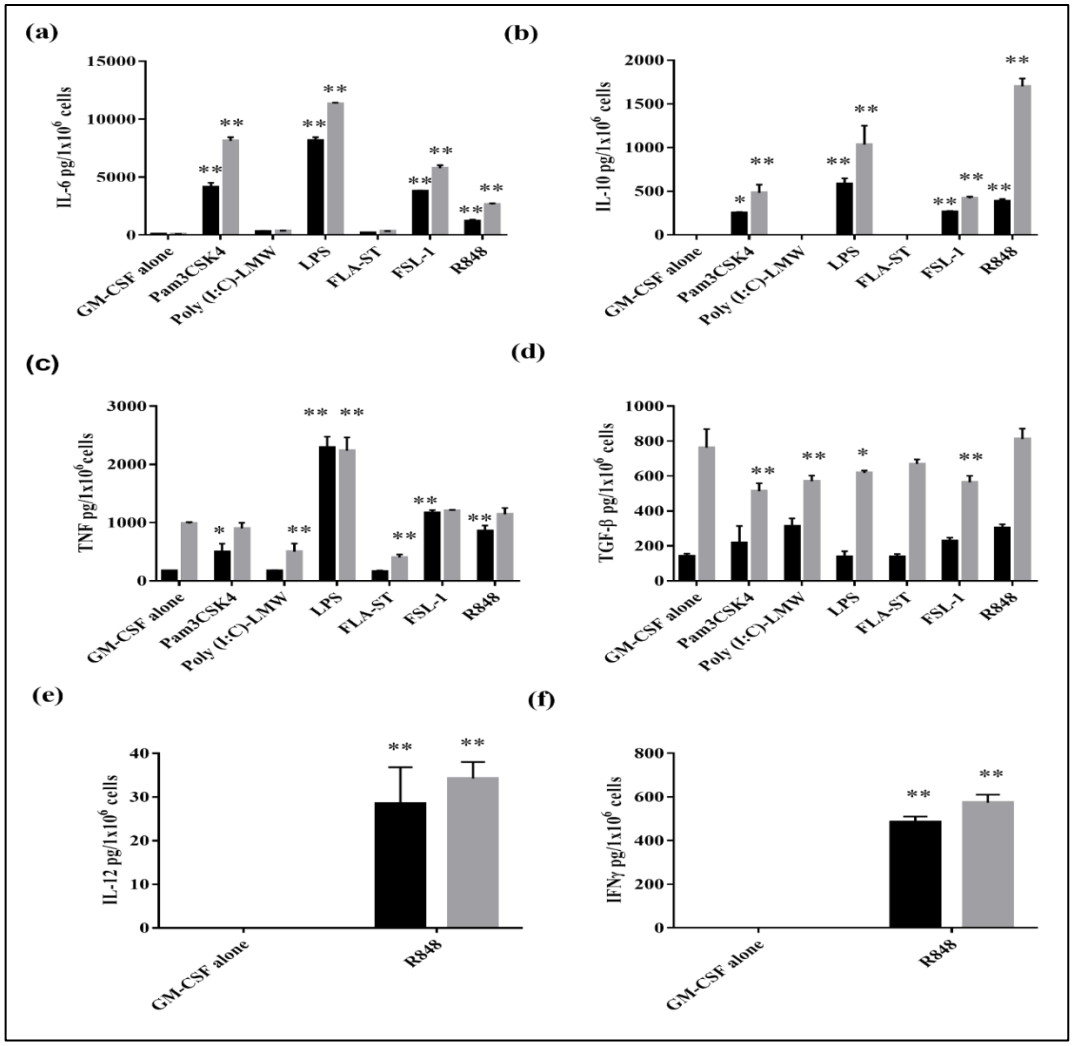

Figure 8. Cytokine release at higher dose of TLR agonists. Balb/cByJ mice were subcutaneously injected with $1x10^6$ 4T1 cells and spleens were isolated when tumor volumes reached about 500-700 mm^3. Splenocytes were isolated via mechanical dissociation and single cell suspensions obtained. Cells were then maintained in media containing media containing 200ng/ml Pam, 200ug/ml Poly IC, 1ug/ml LPS and 100ng/ml FLA-ST, FSL-1 or R848 for 3 days. 24 and 72 hours after treatment, cell culture media was collected and centrifuged to remove any non-adherent cells. The cell culture supernatant was then tested for IL-6, IL-10 and TNF using CBA, whereas TGF-β, IL-12 and IFN-γ were detected via cytokine specific ELISA. The experiment was repeated thrice and the concentration of IL-6 (a), IL-10 (b), TNF (c), TGF-β (d), IL-12 (d) and IFN-γ (e) released per 10^6 splenocytes were represented as mean ± standard error (*$p<0.05$, **$p<0.01$).

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V. CONCLUSION

Autologous tumor cell vaccines generated using inactivated tumor cells from self, is a promising strategy to treat highly heterogeneous cancers such as breast carcinoma. ATCVs contain all tumor associated antigens both known and unknown, thereby increasing the likelihood of a polyclonal immune response. Additionally, ATCVs also minimize the possibility of tumor escape through downregulation of tumor antigens, which is a common drawback of the other immunotherapies such as monoclonal antibody based therapy.

However, a major hindrance in the clinical translation of ATCVs is their poor immunogenicity. When tumor flourishes in the body, it develops a variety of mechanisms that help it from being recognized by the immune system. For instance, it downregulates the expression of major histocompatibility complexes (MHC I/II) and other costimulatory molecules that are required to generate a robust anti-tumor immune response. Since ATCVs are generated using these cancer cells, despite the potential to induce a polyclonal immune response, they greatly fail. Though several investigators have proposed the use of adjuvants such as GM-CSF to aid in enhancing the immune response, there has been no success so far in extremely heterogeneous and inherently aggressive cancers such as breast carcinoma.

Thus, in this research, we set out to determine the underlying reasons for the poor immunogenicity of ATCVs against highly aggressive breast cancers. To do this, we used two murine breast cancer cell lines, 4T1 and EMT6, that are on the opposite sides of the spectrum of metastatic capability. 4T1 is a highly metastatic and aggressive murine breast cancer cell line known to imitate human stage IV breast cancer. On the other hand, EMT6 is a non-metastatic murine breast cancer cell line. ATCVs against each cell line was generated upon irradiation at

100Gy using a cesium irradiator. When mice are vaccinated with the irradiated 4T1 or EMT6 cells and challenged with respective 4T1 or EMT6 live cells, we found that the vaccine efficacy of the two ATCVs greatly varied. With the 4T1 vaccine group, none of the mice developed any anti-tumor immunity to live 4T1 challenge. Whereas, 70% of the mice in the EMT6 vaccinated group, rejected a live EMT6 challenge. Thus, we set out to use these two murine breast cancer cells of varying levels of immunogenicity, to determine the underlying reasons for the poor immunogenicity of breast cancer ATCVs.

First to understand why 4T1 ATCV did not develop protective immunity, we determined the levels of expression of surface molecules MHC I, MHC II, B7-1, B7-2, ICAM-1 and Fas receptor expressed by both 4T1 and EMT6 cells. Since these surface molecules are essential for a robust immune response, differences in their levels of expression could result in the differences in their ATCV immunogenicity. We found that irradiated EMT6 cells express high levels of B7-1 and Fas receptor when compared to irradiated 4T1 cells. We initially suspected, upregulation of these molecules to be responsible for the improved protective immunity against EMT6 cells.

However, in addition to the above-mentioned surface molecules, an effective immune response would also depend on cytokines produced by tumor cells in the ATCV. If high levels of cytokines that promote cancer cell proliferation and increased immunosuppression are generated by an ATCV, it could greatly inhibit an effective anti-tumor immune response. Thus, in addition to the surface molecules, we also looked for differences in some of the cancer promoting or immunosuppressive cytokines produced by the irradiated cells. We found that 4T1 cells produce exceptionally high levels of immunosuppressive cytokines G-CSF and GM-CSF and significantly high levels of IL-6 and MCP-1.

Thus, based on the excessive levels of some of these cytokines, especially G-CSF by 4T1 cells, we suspected the differences in immunogenicity to be likely due to immunosuppression associated generated by these cytokines. We explored this possibility by generating ipsilateral and contralateral hybrid vaccines that included irradiated 4T1 cells along with EMT6 vaccine, on the same and opposite sides respectively and challenged with live EMT6 cells. Thus, a drop in the overall survival in any of the two groups when compared to EMT6 vaccine group, would imply the possibility of 4T1 based immunosuppression to affect the vaccine efficacy.

We found that in both the hybrid vaccine groups, the overall survival drastically dropped upon inclusion of irradiated 4T1 cells. Only 40% and 25% of mice in the ipsilateral and contralateral vaccine groups developed protective immunity to EMT6 challenge, respectively. Since, the contralateral hybrid vaccine also affected the immune response, it implied that the 4T1 based immunosuppression is systemic, rather than local. Thus, we suspected that the immunosuppressive cytokines and not the surface molecules, expressed by the irradiated 4T1 cells is affecting the overall vaccine efficacy. Here, of the different immunosuppressive cytokines, G-CSF was produced at exceptionally high levels (20 times more) by 4T1 cells. Thus, we hypothesized that it plays a key role in determining the overall efficacy of the vaccine.

To test this hypothesis, we used the loss of function approach, where we eliminated G-CSF in 4T1 by knocking out the gene via CRISPR/Cas9 technology (4T1.G-CSF KO). Successful knock out was first verified in-vitro by measuring the levels of G-CSF in cell free culture media collected from these cells. It was further verified in-vivo, by analyzing the concentration of serum G-CSF isolated from mice bearing 4T1.G-CSF KO cells. The serum G-CSF levels were significantly less in 4T1.G-CSF KO mice when compared to serum collected from mice with

comparable 4T1 tumor volumes (500-700mm^3). Thus, in this study, the 4T1 and 4T1.G-CSF KO cells were used as a model to test for the effect of G-CSF in ATCV efficacy.

By implanting 4T1 and 4T1.G-CSF KO cells in mice, we found that tumor derived G-CSF causes immunosuppression via the accumulation of immunosuppressive myeloid cells known as MDSCs, in spleen and DLN of tumor bearing mice. Further, a significant difference in the levels of T cells, B cells and Tregs were also found in the spleen of 4T1.G-CSF KO bearing mice, when compared to mice with 4T1 tumors. Though there was decrease in the levels of these immune cells in the DLN too, the difference was significant only for the T cells.

However, colony stimulating factors such as G-CSF are often associated with resulting in the excessive expansion of MDSCs in tumor microenvironment. Thus, to determine a correlation between G-CSF and MDSC levels, we used four other breast cancer cell lines, namely 4T07, 67NR, 168FARN and 66Cl4 that naturally released varying levels of G-CSF. We found that the levels of serum G-CSF in mice bearing these tumors, closely correlated with the frequency of splenic MDSCs. Hence, we further established the effect of G-CSF on MDSC expansion in breast cancers.

Therefore, we then set out to determine if the tumor derived G-CSF was also responsible for affecting the vaccine efficacy of these cells in an ATCV setting. To do this, we included irradiated 4T1.G-CSF KO cells in the contralateral vaccine instead of 4T1 cells and challenged the mice with live EMT6 cells. We found that the inclusion of 4T1.G-CSF KO cells instead of 4T1 cells, greatly improved the vaccine efficacy as only 30% of the mice developed tumors. Additionally, when we vaccinated and challenged mice with 4T1.G-CSF KO cells, none of the

mice developed tumors. Thus, for the first time, the findings from this study established a causal link between tumor derived G-CSF and poor anti-tumor immune response to ATCV.

Further, in this research, we wanted to address this MDSC based immunosuppression in breast cancers, by determining strategies to induce maturation of immature myeloid cells into mature monocytes and dendritic cells. As a step in this direction, we compared the effect of different TLR agonists namely, Pam, Poly IC, LPS, FLA-ST, FSL-1 and R848 on MDSC maturation in-vitro. However, unlike previous studies where the MDSCs were isolated to study their maturation, we treated whole splenocyte cultures with the TLR agonists and studied their effect on MDSC maturation. We believe that this would closely mimic in-vivo events where MDSCs would receive signals and cytokines from other immune cells that would play an important role in deciding the fate of these cells.

Our studies showed that not all TLRs have similar effect on MDSC maturation. Pam, which is a TLR1/2 agonist, was found to be the most effective of all the TLRs tested, since it upregulated the expression of all four markers of maturation on the MDSCs. Other agonists, namely FSL-1 and LPS, only upregulated CD80 and F4/80 expression, whereas R848 upregulated MHCII and F4/80. On the other hand, agonists Poly IC and FLA-ST did not have any effect on MDSC maturation. Additionally, we also found that there was significant decrease in the number of MDSCs only in cultures treated with Pam, LPS and FSL-1.

Further, to get more information on the effect of the agonists on immune response, we also determined the number of other immune cells, namely T cells, B cells, Tregs and CD11c+ cells in the splenocyte cultures treated with the agonist. Though we found LPS to decrease number of T cells; Poly IC to decrease the number of CD11c+ cells and all agonists to decrease the number

of B cells at either the low or high concentration, we couldn't make any conclusive comments on their overall effect on the immune response.

Additionally, when we looked at the levels of different cytokines in the cultures treated with the agonists, we found that only the agonists that had any effect on MDSC maturation markers (Pam, LPS, FSL-1 and R848), had significantly high levels of IL-6, IL-10, TNF and low levels of TGF-β. Since all these cytokines have both pro-tumor and anti-tumor response, further studies are required to determine their overall effect on the immune response.

Thus, to summarize, the findings of this study have established the significant role played by tumor derived G-CSF in abrogating the anti-tumor immune response to ATCVs in breast cancer. Since this was found to be largely due to immunosuppression via MDSC accumulation, agonists such as Pam and FSL-1 were identified to potentially eliminate the immunosuppression via initiating the maturation of these cells into monocytes and macrophages.

Considering some of the future directions that this research could take, involves exploring the likelihood of using serum G-CSF as an indicator of tumor burden or clinical cancer stage in breast cancer patients. Additionally, in this study, we also noticed a close correlation between tumor derived G-CSF levels and the metastatic ability of the cancer cells. Thus, we believe that there is a possibility for the existence of a causal link between tumor derived G-CSF and metastasis in breast cancer. Further, since MDSCs also affect the expansion of Tregs, previous studies that have determined a correlation between tumor burden and levels of Tregs could be due to high G-CSF secretion.

Additionally, the scope of this study was limited, as only in-vitro studies were performed with the TLR agonists. Studies that compare the effects of these agonists in-vivo would help us pick

out an agonist that would have the highest potential to eliminate the MDSC associated immunosuppression in breast cancer. Further, treatment strategies involving the agonist in an adjuvant setting, could improve the overall survival of breast cancer patients.

VI. APPENDIX

A. Materials

Reagents

Mouse recombinant IL-12 (rIL-12) and 2-mercaptoethanol used in maintaining 2D6 culture was obtained from Sigma Aldrich (St. Louis, MO) and University of Arkansas Biologics center (Fayetteville, AR), respectively. Recombinant mouse granulocyte-monocyte colony stimulating factor (rGM-CSF) used in maintaining the viability of MDSCs in culture, were purchased from Sigma-Aldrich (St. Louis, MO). All fluorochrome conjugated monoclonal antibodies (mAb) used in the detection of different immune cell subsets were purchased from BD Biosciences (San Jose, CA).

Cell culture media components including Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Hyclone laboratories (Marlborough, MA). Ammonium-chloride-potassium (ACK) buffer used in the lysis of red blood cells while isolating splenocytes, was purchased from Lonza (Allendale, NJ).

Cell culture

4T1 (murine breast cancer cell line) was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. 2D6 cells were a generous gift from Dr. David Klinke, West Virginia University and maintained in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin and 2μM of 2-mercaptoethanol. Splenocytes from tumor bearing mice were isolated following the procedure described below. Isolated splenocytes were treated with

10ng/ml rGM-CSF and with/without IL-12 (20ng/ml or 200ng/ml) in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. All cell cultures were maintained in a humidified CO_2 incubator with 5% CO_2 at 37˚C.

Mice

Female Balb/cByJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were housed in microisolator cages. All experimental procedures were approved by the Institutional Animal Care and Use Committee at University of Arkansas. Mice were utilized for experiments at 8-12 weeks of age and animal care followed 'The Guide for Care and Use of Laboratory Animals' (National Research Council).

B. Figures

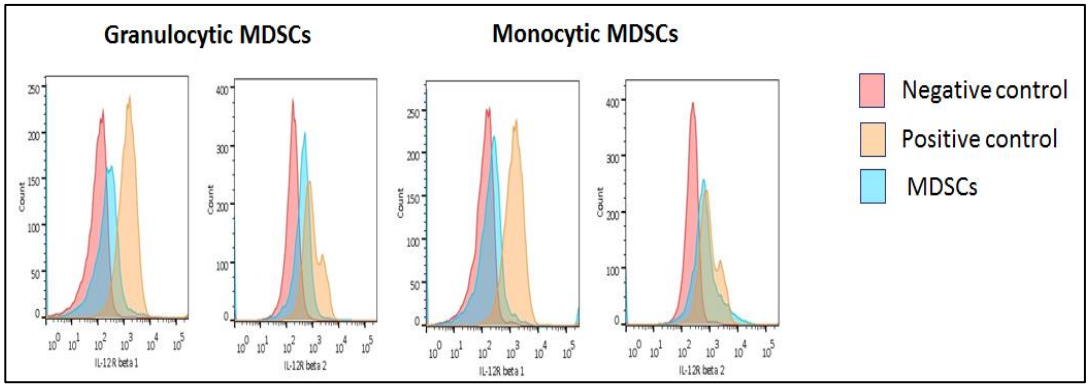

Figure 1. IL-12 receptor expression by granulocytic and monocytic MDSCs. Balb/cByJ mouse was subcutaneously injected with $1x10^6$ 4T1 cells. When the tumor volume reached about 500-700 mm^3, mice were sacrificed and splenocytes were isolated. Granulocytic ($Gr1^{high}$ $Ly6G^+$) and monocytic ($Gr1^{dim}$ and $Ly6G^-$) MDSCs were isolated using the myeloid derived suppressor kit from Miltenyi Biotech. The sorted cells were incubated with fluorochrome-conjugated anti-IL-12 receptor β1 (IL-12Rβ1) and a combination of anti-IL-12 receptor β2 antibody (IL-12Rβ2) and fluorochrome-conjugated secondary antibody. Cells were blocked with 1% BSA. 2D6 cells cultured in media containing 250pg/ml recombinant IL-12, were washed and incubated with fluorochrome-conjugated anti-IL-12 receptor β1and a combination of anti-IL-12 receptor β2 antibody and fluorochrome-conjugated secondary antibody. These cells served as positive control. Unstained sorted granulocytic and monocytic MDSCs served as negative control. The samples were acquired using FACScantoII and the expression of IL-12Rβ1 and IL-12Rβ2 by granulocytic MDSCs (a) and monocytic MDSCs (b) were analyzed using FlowJo software.

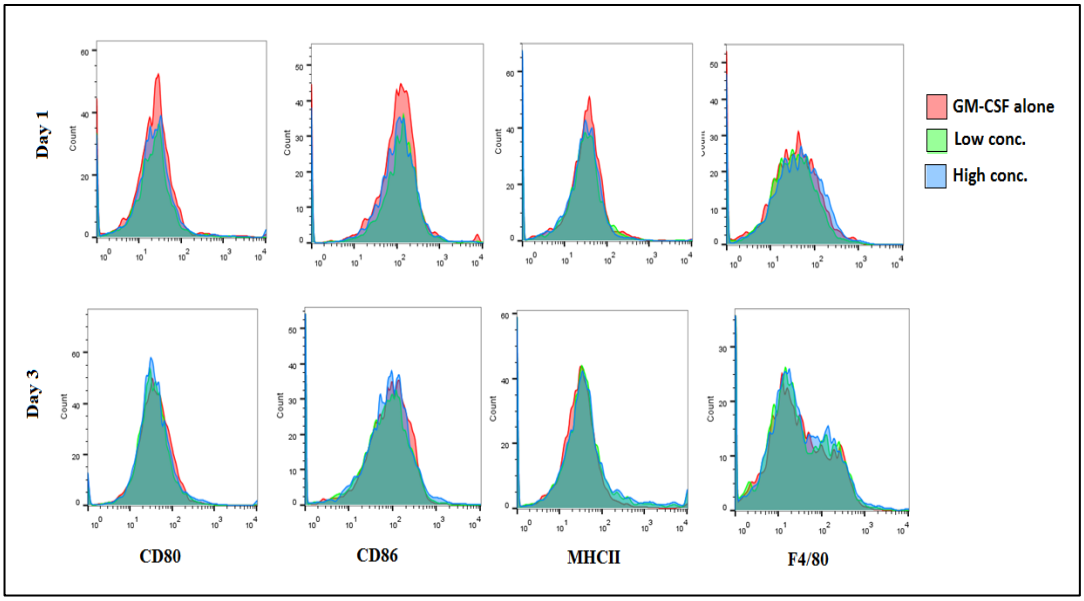

Figure 2. Levels of expression of maturation markers on MDSCs. Balb/cByJ mice were subcutaneously injected with $1x10^6$ 4T1 cells and spleens were isolated when tumor volumes reached about 500-700 mm^3. Splenocytes were isolated via mechanical dissociation and single cell suspensions obtained. Cells were then maintained in media containing 20ng/ml (low conc.) and 200ng/ml (high conc.) IL-12 for 3 days. 24 and 72 hours after treatment, cells were stained for MDSCs (anti-CD11b and anti-Ly6G and Ly6C) and maturation markers CD80, CD86, MHCII and F4/80. Cells treated with GM-CSF alone served as control. The samples were acquired using FACScantoII and the levels of expression of the maturation markers by MDSCs were analyzed using FlowJo software.

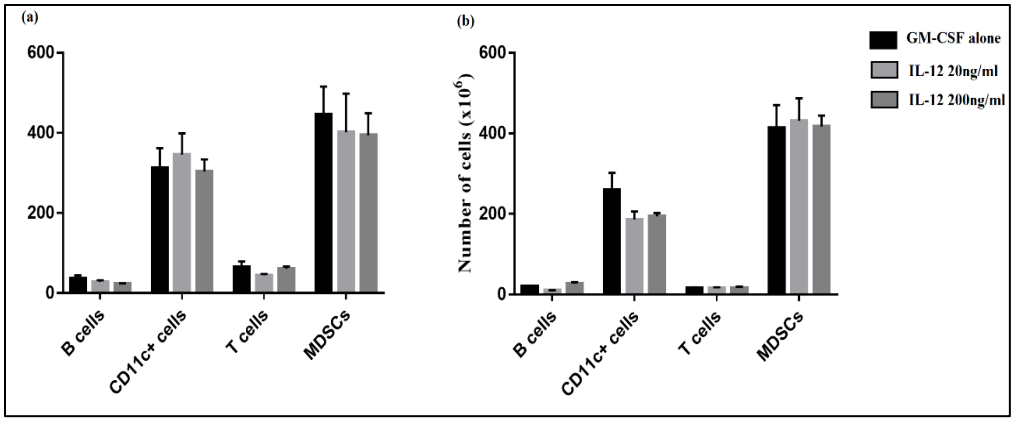

Figure 3. Number of B cells, T cells, MDSCs and CD11c+ cells in splenocytes treated with IL-12 in-vitro. Balb/cByJ mice were subcutaneously injected with $1x10^6$ 4T1 cells and the spleen was isolated when the tumor volume reached about 500-700 mm^3. Splenocytes were isolated via mechanical dissociation and single cell suspensions obtained. Cells were then maintained in media containing 20ng/ml and 200ng/ml IL-12 for 3 days. 24 and 72 hours after treatment, cells were stained for B cells (CD19+), MDSCs (CD11b+ and Ly6G and Ly6C+), T cells (CD3+) and CD11c+ cells. The samples were acquired via FACScantoII and analyzed using FlowJo software. Number of B cells, T cells, MDSCs and CD11c+ cells with 24-hour (a) and 72-hour (b) treatment with IL-12 were determined. The experiment was repeated thrice and the results represent mean ± standard error.

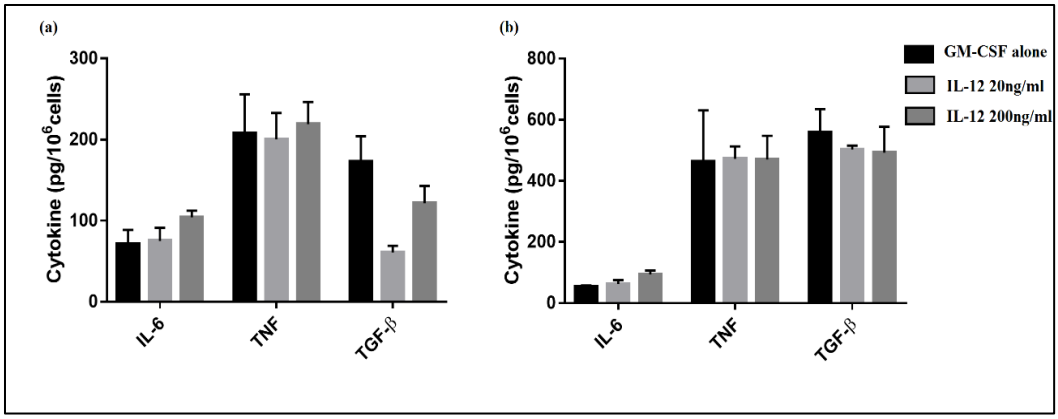

Figure 4. Cytokines IL-6, TNF and TGF-β released upon treatment with IL-12 for 24 and 72 hours. Balb/cByJ mice were subcutaneously injected with $1x10^6$ 4T1 cells and the spleen was isolated when the tumor volume reached about 500-700 mm^3. Splenocytes were isolated via mechanical dissociation and single cell suspensions obtained. Cells were then maintained in media containing 20ng/ml and 200ng/ml of IL-12 for 3 days. 24 and 72 hours after treatment, cell culture media was collected and centrifuged to remove any non-adherent cells. The cell culture supernatant was then tested for IL-6 and TNF using CBA, whereas TGF-β was detected via TGF-β specific ELISA. The experiment was repeated thrice and the 24-hour (a) and 72-hour (b) cytokine release were represented as mean ± standard error.

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: David Zaharoff

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: December 10, 2012

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **December 9, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13024 - "Immunotherapy of Breast Cancer"**. You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **10-09-2015**, you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at atime.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

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Fax: 479-575-3846 • http://vpred.uark.edu/199

UNIVERSITY OF ARKANSAS

Office of Research Compliance

June 24, 2014

MEMORANDUM

TO: Dr. David Zaharoff

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 14043

Protocol Title: "Response of murine cell lines to antigens, cytokines and delivery vehicles"

Approved Project Period: Start Date: June 12, 2014
Expiration Date: June 11, 2017

The Institutional Biosafety Committee (IBC) has approved Protocol 14043, "Response of murine cell lines to antigens, cytokines and delivery vehicles" You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199.php