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Membrane anchored immune stimulatory proteins enhance anti-tumor immunity in breast cancer models

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ABSTRACT

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Despite significant advances being made in the cancer field, breast cancer accounts for millions of deaths worldwide. Multiple strategies have been employed to combat this deadly disease such as chemotherapy, radiation therapy and various forms of immunotherapies. And while many are capable of reducing tumor burden or inducing tumor-specific immune responses, most are hampered by the development of therapeutic resistance over time. Our studies sought to investigate the efficacy of cellular based vaccines that were genetically modified to express glycosyl phosphatidylinositol immune stimulatory molecules (GPI-ISMs). The choice of ISMs, B7-1, GPI-IL-2 and GPI-IL-12, all play a critical role in the activation and cytotoxic capabilities of cellular immune responses, primarily mediated by T- and NK-cells. We demonstrate using multiple breast cancer models that the co-expression of B7-1 and GPI-IL-12 is highly effective at significantly reducing overall tumor burden and tumor incidence in prophylactic as well as therapeutic settings. In the non-metastatic, 4TO7 model, cellular vaccines expressing this combination of ISMs led to a significant reduction in myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs) locally within the tumor microenvironment as well as in the periphery. While in the HER-2 positive, D2F2/E2 model such tumor inhibition was accompanied by a reduction in HER-2 specific humoral immunity and an enhancement in HER-2 specific cellular immunity. Moreover in both models, long-lasting memory responses were induced following cellular vaccination with GPI-ISMs as evident by protection from secondary tumor challenges.

To further enhance the anti-tumor immunity that was induced following vaccination, combinatorial approaches with the cytotoxic agent Ukrain and an immunological blockade, anti-PD-L1 were investigated. The tumor-specific cytotoxicity of Ukrain minimally enhanced tumor inhibition in the 4TO7 model relative to vaccination alone while PD-L1 blockade served as an effective adjuvant in the D2F2/E2 model. Taken together, cellular vaccines expressing GPI-ISMs proved to be an effective approach in the battle against tumor development in multiple breast cancer models though the manipulation of immune suppression and the promotion of antigen-specific cellular immunity.

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CHAPTER I:

Introduction

Excerpts taken from the following publications:

Immunotherapeutic strategies for cancer treatment: A novel protein transfer approach as an alternative for cancer vaccine development. Med. Res. Rev. (2012) 32 (6): 1197-219. PMID: 23059764

Cancer vaccine development: Designing tumor cells for greater immunogenicity. Front Biosci. (2010) 15 (1):309-20. PMID: 20036822

Cancer is the second leading cause of mortality in the United States accounting for 23.7% of all deaths in 2010 [1]. While cancer attacks a variety of organs, tissues and cells within our bodies, breast tissue is the most prevalent site for cancer among women at 29% of estimated new cancer cases [2]. It was estimated that 226,870 women would be diagnosed with breast cancer in the United States while 39,510 women would die from this disease in 2012 [2]. While the mortality rates for several other diseases have declined substantially in the past 50 years including heart and cerebrovascular diseases [3] such a decline fails to be observed in the cancer field which speaks to the overwhelming necessity to develop more effective treatment options for cancer patients.

At present there are five approaches for the treatment of cancer: surgery, chemotherapy, radiotherapy, monoclonal antibody (mAb) therapy and immunotherapy. Surgical procedures are implemented in order to reduce overall tumor burden within patients and increase the efficacy of adjuvant therapies most notably chemotherapy and radiation therapy. While highly effective at eliminating tumor cells, the lack of specificity often leaves patients undergoing chemotherapy and/or radiation therapy severely immune-compromised and suffering from a wide host of side effects such as hair loss, fatigue and extreme nausea. As a result, several therapies have been recently developed and FDA approved that target specific proteins expressed or secreted by tumor cells. In addition, as many current investigations seek to unravel the dynamic relationship between cancer and the host immune system, the development of clinically effective immunotherapies and cancer vaccines is quite promising.

Cancer Immune Surveillance

Normal, healthy cells are responsive to intrinsic signals that direct them to divide, migrate or undergo apoptosis allowing the host to maintain a homeostatic state. However, when this responsiveness is lost, cells begin to proliferate uncontrollably and sustain this proliferative signaling, a fundamental hallmark of cancer development [4]. Other hallmarks include evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death [4, 5]. Our immune system is constantly under "surveillance", seeking out and effectively destroying these transformed cells, an idea first proposed by Paul Ehrlich in 1909 [6]. In the decades following Ehrlich, there was conflicting evidence suggesting that the immune system was incapable of recognizing malignant cells which were thought to be indistinguishable from self. In 1949 Frank Burnet published his theory of acquired immunological tolerance which stated that lymphocytes that are self-reactive are eliminated during the development of the host immune system. However, in the 1950s studies showed that mice were capable of rejecting a secondary transplantable tumor suggesting the host's recognition of tumor associated antigens (TAAs) and tumor specific transplantable antigens (TSTAs). The work of Burnet along with Lewis Thomas led to the development of the immune surveillance hypothesis [7, 8]. It was later demonstrated that immune surveillance was only one part of a larger immune editing process which consisted of 3 stages: elimination (immune surveillance), equilibrium and escape. Immune surveillance is primarily mediated by innate immune responses, most notably gamma-delta (γδ) T cells and natural killer (NK) and NKT cells [9, 10]. However, in the process of eliminating tumor cells, immune selective pressure is aiding in the

development of tumor variants that are progressively more resistant to immune attack. Within this "equilibrium" phase, over time the host immune response becomes increasingly incapable of controlling these tumor variants until finally the tumor cells "escape" the induced anti-tumor immune response completely.

Immune evasion mediated by secretory factors and modulation of surface proteins

Tumors have crafted numerous ways to evade the host immune response. These methods are quite varied and effective in inhibiting both the innate and adaptive immune responses that would otherwise be mounted. Numerous studies have shown that both innate and acquired immunity are capable of recognizing many tumor antigens and eliciting an anti-tumor response against developing tumors [11]. These anti-tumor responses are primarily mediated by T cells with minimum contribution from an antibody-mediated response towards the efficacy of immune surveillance under normal circumstances [12]. However, poorly immunogenic tumor cells escape from immune surveillance making the host unable to produce an adequate immune response towards the metastasis of the tumor. Taken together, stimulating the immune cells specifically to recognize the cancer cells is fundamental in the development of an efficacious therapeutic cancer vaccine.

The environment in which a tumor develops plays a critical role in the progression of a tumor. The type and frequency of infiltrating cells is a key determinant in whether the tumor has the appropriate microenvironment to thrive or whether it will be effectively eliminated by the host immune responses. The strategies that are employed by tumors to inhibit immune cell effector function, namely T cells and dendritic cells

(DCs), while simultaneously recruiting immune suppressive and regulatory cells into the microenvironment allows the tumor to colonize and form a niche within the host. As a result, numerous studies have sought to investigate this delicate balance between the host immune responses and the craftiness of a developing tumor locally at the microenvironment.

One method utilized by tumors to mediate immune escape is though the secretion of immune suppressive factors most notably vascular endothelial growth factor (VEGF) and transforming growth factor (TGF-β), interleukin-10 (IL-10) and tumor necrosis factor (TNF). VEGF is a known pro-angiogenic factor and has been shown to promote the formation of blood vasculature in the tumor microenvironment [13, 14]. The immune suppressive activity of TGF- β is wide reaching promoting an invasive phenotype by recruiting myofibroblasts and osteoclasts [15], shielding tumor cells from immune surveillance [16] and inducing the differentiation of regulatory T cells (Tregs) [17].

Tregs play a crucial role in the establishment of peripheral tolerance, thus minimizing the occurrence of autoimmune diseases [18]. However, within the tumor microenvironment, the presence of Tregs can suppress the effector functions of tumor-specific $CD4^+$ and $CD8^+$ T cells, thus promoting tumor development [19]. It has been shown that within tumor-bearing hosts, the frequency of Tregs is increased in the circulation as well as within the tumor itself [19]. The mechanism of immune suppression that is utilized by Tregs involves the secretion of IL-10 and TGF-β, which can inhibit DC maturation and cytotoxic T-cell activity. It has also been shown that Tregs can stimulate DCs using a CTLA-4-dependent mechanism to produce intracellular enzyme indoleamine 2, 3-dioxygenase, which converts tryptophan into kynurenines that

can induce apoptosis of T cells [18]. Tregs also express high levels of glucocorticoid-induced TNF-related protein (GITR) that when engaged with the ligand, GITR-L, expressed on endothelial and APCs, attenuates their suppressive activity [20]. Thus, agonist anti-GITR mAbs can lead to increased numbers of tumor-specific T cells and anti-tumor activity [20, 21]. On the other hand, tumor cells utilize the GITR-GITR-L interaction to inhibit NK cell-mediated anti-tumor immunity by expressing GITR-L as well as secreting the soluble form of GITR-L, which modulates the cytotoxic abilities of NK cells expressing GITR [22]. In conjunction with Tregs, the activation of myeloid derived suppressor cells (MDSCs), a phenotypically heterogeneous population of immature myeloid cells, contributes to the immunosuppressive nature of the tumor microenvironment [23].

Tumor-derived granulocyte colony stimulating factor (G-CSF) has been demonstrated in several mouse tumor models to promote the differentiation, accumulation and recruitment of MDSCs [24-26]. Upon activation, MDSCs exert their suppressive activity by expressing high levels of arginase I and inducible nitric oxide synthase which catabolize the amino acid L-Arginine (L-Arg) [27]. MDSCs are thus able to cause T-cell dysfunction in the tumor microenvironment by depleting extracellular L-Arg, which is required for optimal T-cell proliferation [28-30]. MDSCs can also enhance tumor angiogenesis and metastasis [31] and secrete IL-10 and TGF-β which induce Treg production [32]. It has also been shown that, through the recruitment of MDSCs, Tregs and lymphoid tissue inducer cells, tumors that secrete the chemokine CCL21 can establish a tolerogenic tumor microenvironment and promote immune escape by altering their stroma into lymphoid-like structures [33]. Due to their immunosuppressive role,

modulation of these regulatory cells can serve as an effective adjuvant therapy to other immunotherapies for the treatment of cancer [34]. The schematic representation of immunosuppression mediated by the tumor microenvironment is illustrated in Figure 1.

While it has been shown that the induction of an effective anti-tumor immune response is primarily cell-mediated, tumor cells have been shown to down-regulate the expression of major histocompatibility (MHC) molecules in an effort to avoid recognition by T cells. This modulation occurs at varying degrees including total deficiency of MHC, allelic and locus down-regulation and loss of MHC haplotype [35, 36]. This altered MHC expression prevents proper antigen presentation and recognition to T cells resulting in a deficiency of $CD8^+$ T cell-mediated immunity while making the tumor more susceptible to NK cell-mediated lysis [37, 38].

Because tumor cells are derived from the host's normal, healthy cells, often times the host immune system fails to recognize the developing tumor as "foreign" and thus fails to initiate an appropriate response. In some cases, tumor cells overexpress a specific protein or present that protein in an altered conformation on its cell surface, thus allowing this protein to serve as a target for therapies. Additionally, many proteins that are naturally expressed by normal cells are overexpressed by many tumors such as mucin 1 (MUC-1) [39] and human epidermal growth factor receptor 2 (HER-2) [40]. This aberrant protein expression provides potential targets for the development of more specific therapies in a clinical setting. Studies have also shown that tumors tend to up-regulate a number of inhibitory molecules such as programmed death ligand (PD-L1) that further leads to immune dysfunction by inhibiting the effector functions of T cells which express PD-1 and subsequently inducing T cell apoptosis (9).

Types of Breast Cancer

Breast cancer can be classified based on a number of factors, including protein receptor status, grade and stage of tumor development and histopathological appearance [41]. Using the receptor status nomenclature, there are 3 main categories of breast cancer. The majority (65%) of breast cancer is hormone receptor positive due to the expression of the estrogen (ER) and/or progesterone (PR) receptors on their cell surface. Patients with ER/PR positive tumors have a lower risk of mortality relative to patients with ER/PR negative tumors and are more likely to be responsive to available hormonal therapies such as tamixofen [42-44]. Approximately 20-30% of breast cancer is considered human epidermal growth factor receptor 2 (HER-2) positive based on an amplification of the HER-2/neu oncogene [45]. Due to the overexpression of this growth factor receptor, HER-2 positive breast cancer is highly aggressive and associated with a poorer clinical prognosis [46]. Still there are ~15% of breast cancers that lack the expression of ER and PR and fail to overexpress HER-2 and are classified as triple negative breast cancer (TNBC). While TNBC patients are non-responsive to most available, targeted therapies, these patients are typically more responsive to neoadjuvant chemotherapy than non-TNBC patients [47].

For those patients with HER-2 positive breast cancer, the mAb therapy trastuzumab (Herceptin) has been shown to be effective as a single agent as well as in adjuvant settings [48] . Trastuzumab mediates its action by inhibiting the homodimerization of HER-2 and the heterodimerization of HER-2 with other epidermal growth factor (EGF) family receptors, such as HER-1, HER-3 and HER-4, increasing the endocytic destruction of the receptor, promoting antibody-dependent cellular cytotoxicity

(ADCC) and inhibiting angiogenesis [49, 50]. In clinical trials, the patients receiving trastuzumab along with chemotherapy have shown higher response rates as compared to patients receiving chemotherapy alone [51]. The FDA also approved pertuzumab (Perjeta), a HER-2 specific mAb to be used in conjunction with trastuzumab and docetaxel chemotherapy for late-stage breast cancer disease [52]. Pertuzumab has been shown to bind a distinct region of the HER-2 protein, domain II , relative to domain IV that is recognized by trastuzumab [53]. However, over time patients who were once responsive to these therapies develop resistance ultimately causing relapse. More recently in February 2013, the FDA approved Kadcyla, a new antibody-drug conjugate that targets HER-2 in patients who have failed to respond to trastuzumab and a taxane [54]. Further efforts are being made by researchers to seek additional adjuvant therapies to combat this resistance [55, 56].

Immunotherapeutic approaches for cancer treatment

Demonstration that the immune system can be induced to respond to cancer dates back to the work of William Coley in the 1890s- early 1900s [57]. The development of "Coley's toxin", a mixture of killed bacterial cultures, served as one of the first examples of cancer immunotherapies, successfully curing >10% of patients who was afflicted with bone and soft-tissue sarcomas [58]. Nowadays, researchers have developed a number of immunotherapeutic approaches that seek to harness and augment the immune system's natural ability to eliminate emerging or established tumors. In order for this goal to be realized, advances must continue to be made in tumor immunology such that we gain a better understanding of how the immune system naturally responds to a tumor. The

current strategies seek to target and to boost specific components of the host's immune response to a developing or established tumor. The primary targets of immunotherapies include enhancing antigen targeting to APCs, enhancing T cell activation and removing the inhibitory signals that diminish the effectiveness of the anti-tumor immune response [59]. Pulsing DCs with tumor antigens, administering cytokines and using gene transfer technology to express various proteins on the surface of tumor cells have been found to be successful in eliciting effective anti-tumor immune response. However these therapies have been met with numerous clinical limitations including limited specificity, partial responses and systemic toxicity. Additionally, these therapies are often cumbersome and expensive to implement.

The identification and characterization of several MHC-restricted TAAs such as HER-2/neu, melanoma antigen 1 (MAGE-1) and glycoprotein 100 (gp100) has enabled more targeted immunotherapies to be developed [60]. While there are several therapeutic options geared toward cancer vaccine development currently under investigation, few such options demonstrate the dual potential to not only stimulate a robust anti-tumor immune response but to also be translational in human clinical trials as well.

Dendritic cell-based vaccines

Researchers are pursuing numerous strategies involving the use of DCs as cancer vaccines. One such strategy involves the use of "loaded" DCs. To achieve this, a population of DCs would first be genetically manipulated *ex vivo* to express tumor antigens prior to injection into the cancer patient. In theory these "activated" DCs would be able to present the tumor antigens, through MHC molecules, to $CD4^+$ and $CD8^+$ T

cells and thus elicit a robust immune response. In several models, vaccinating tumor-bearing mice with DCs loaded with autologous tumor-derived antigens in the form of peptides [61], heat shock proteins [62], tumor lysates [63] or mRNA [64] has proven to be highly effective. Additionally, the generation of DCs fused with tumor cells in order to induce anti-tumor immunity has been investigated in mouse models of lung carcinoma, melanoma and colon [65, 66]. However, the complications arise initially from the difficulty of properly activating the DCs *ex vivo*, as well as from determining the form, dose or types of antigens to load [67]. Such complications limit the overall efficacy and consistency of this approach. The tumor antigen, peptide-pulsed DCs would also only be capable of activating a peptide-specific repertoire of T cells. Due to the high mutation rate of tumor cells, the antigens presented by the tumor may differ greatly from those to which the immune cells have been previously primed upon vaccination thus leading to immune evasion. This approach is also limited only to tumor antigens that have been identified and characterized [68].

Despite these limitations, DC-based cancer vaccines have been used as a treatment option in several human clinical trials such as those for breast and prostate cancer [69, 70]. The most promising of these, is the FDA approved cancer vaccine sipuleucel-T (PROVENGE) for the treatment of asymptomatic or minimally symptomatic metastatic castrate resistant prostate cancer. The vaccine consists of autologous DCs pulsed with a fusion protein of GM-CSF and the prostate antigen prostatic acid phosphatase (PAP) which is expressed solely in prostate tissue and in 95% of prostate cancer cells [71].

Whole cell vaccines

Another cell based approach involves using irradiated whole tumor cells as potential cancer vaccines. By using whole tumor cells it allows for a wide repertoire of tumor antigens to be presented to the immune system leading to the induction of a more polyclonal immune response [72, 73]. Additionally, it has been shown that irradiation of whole tumor cells enhances immune recognition by T cells [74] as well as macrophages and DCs [75]. One such therapy is the development of GVAX, a whole cell based vaccine genetically engineered to secrete GM-CSF [76]. GVAX has been shown to decrease the serum levels of prostate specific antigen (PSA) in some patients in phase I and phase II clinical trials [77]. However, in phase III clinical trials comparing the clinical efficacy of GVAX immunotherapy in combination with Taxotere (docetaxel), a common chemotherapeutic agent, plus prednisone it was found that there was a higher incidence of death among the GVAX group relative to the Taxotere control group [77] . Because of this lack of survival benefit, it was determined that the study would not meet its primary survival endpoint and the prostate GVAX clinical trials for prostate cancer were terminated. The safety and efficacy of the GVAX platform is currently being investigated in clinical trials for pancreatic cancer [78]. While the use of irradiated whole tumor cells as vaccines holds great promise, several factors must be addressed before this promise is fully realized clinically. Nonetheless, the main limitations with whole cell vaccination are the difficulty in establishing cell lines from primary tumors and that the established cell lines may no longer be representative of the original primary tumor due to intratumoral heterogeneity [79].

Cytokines for tumor therapy

In order to overcome the immunosuppression mediated by tumors, the systemic administration of specific cytokines such as IL-2, IL-12, and IFN-α has been used to alter the tumor microenvironment to mediate tumor recognition by immune cells [80-82]. Additionally, the FDA has approved IL-2 to treat metastatic melanoma and renal cell carcinoma (RCC). Due to the potential synergy of specific cytokines, their use as combinatorial therapies is being evaluated in clinical trials. In a recent randomized clinical trial comparing the overall survival benefit among RCC patients following IFN-α2a monotherapy versus a combinatory therapy of IFN-α2a, IL-2 and the chemotherapy drug fluorouracil, there was found to be no significant difference between the two groups despite previous reports of higher response rates among those receiving the combination therapy [83]. Additionally, there was a higher incidence of toxicity among patients receiving the combination therapy which is a significant issue of cytokine therapies. A phase II study assessed whether GM-CSF could enhance the response rate to IL-2 and the chemotherapeutic drug thalidomide among metastatic RCC patients. These studies indicated that while the combination therapy was tolerated by the patients, the addition of GM-CSF did not lead to an improved response rate [84]. A high incidence of thrombotic occurrences was observed in phase I trials of high risk malignant melanoma patients when given GM-CSF and increasing doses of thalidomide following tumor resection [85]. The toxicities that develop in patients can range from mild cases of nausea and vomiting to more severe effects such as hypotension and systemic toxicity [80, 86]. Additionally, the elevated levels of cytokines in the circulation can lead to vital organ damage and ultimately death in some cases [87]. These and other studies indicate that

further evaluation of the interplay between cytokines is needed in order to determine the most optimal cytokine combination while minimizing the risks of life-threatening toxicities.

Gene therapeutic approaches

Gene therapy was primarily developed to correct defective genes to treat single gene disorders [88]. Gene therapy has also been used extensively in the cell-mediated treatment of head and neck, prostate and colorectal cancer [89-91], however it has failed in clinical trials due to the difficulty in establishing primary tumor cells. Currently two types of gene delivery systems are being used, viral and non-viral vectors and both are associated with substantial limitations. The use of adeno-, retro- and lenti-viral vectors yield relatively high gene transduction and expression efficiency compared to non-viral vectors and are used in the development of several cancer vaccines [92]. However, the major drawback of retro- and lenti-viral vectors is the probability of inducing mutagenesis and cancer gene activation since the viral genome can randomly incorporate into the host genome [93]. Adeno and fowl fox viral vectors are commonly used as an effective means to genetically modify tumor cells without the risk of incorporation into the host genome. However, adenoviral vectors are highly immunogenic and can lead to rapid clearance of the vector from the host which makes them undesirable for therapeutic use [94]. Non-viral vectors such as liposomes and polymers have been developed to deliver the gene of interest for cancer treatment. Though non-viral vectors are not immunogenic, their transfection efficiency is very poor making them unattractive for

gene delivery method [95]. Although gene therapy for cancer treatment is a feasible method, several limitations of gene transfer technology remains to be investigated.

Taken together, while these approaches are hampered by a number of clinically-significant issues, there exist ample opportunity for cancer researchers to develop more effective therapies and/or further optimize the current strategies. Herein, we have pursued the later through the investigation into the efficacy of genetically modified cellular vaccines expressing glycosyl phosphatidylinositol (GPI) immune stimulatory molecules (ISMs) using multiple breast cancer models.

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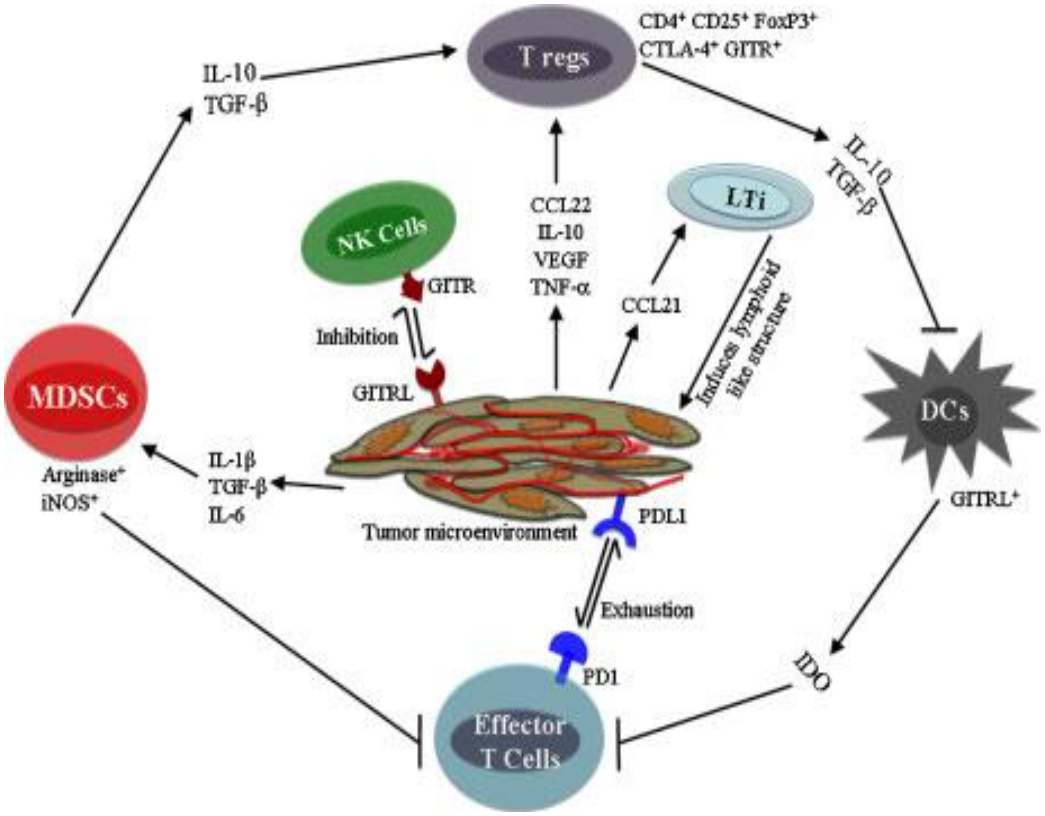
FIGURE LEGEND

Figure 1. Schematic representation of immunosuppression mediated by the tumor microenvironment to evade host anti-tumor immune responses. Tumors secrete a variety of immunosuppressive factors, such as cytokines, growth factors, and chemokines. These factors promote the differentiation, expansion, and recruitment of several regulatory cells, namely myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), which promote the growth of tumor cells by inhibiting the effector functions of cytotoxic T cells. The cytokines secreted and specific receptors expressed by tumor cells also inhibit the activity of immune cells (e.g. DCs, NK, and T cells). Tumor cells are also known to recruit lymphoid tissue inducer (LTi) cells that promote tumor growth by inducing a lymphoid-like structure surrounding the tumor cell.

FIGURE 1


T regs
CD4+ CD25+ FoxP3+
CTLA-4+ GITR+
IL-10
TGF-β
IL-10
TGF-β
NK Cells
GITR
Inhibition
GITRL
CCL22
IL-10
VEGF
TNF-α
CCL21
LTi
Induces lymphoid like structure
MDSCs
Arginase+
iNOS+
IL-1β
TGF-β
IL-6
Tumor microenvironment
PDL1
DCs
GITRL+
Exhaustion
PD1
IDO
Effector
T Cells

CHAPTER II

Expression of membrane anchored cytokines and B7-1 alters tumor microenvironment and induces protective anti-tumor immunity in a murine breast cancer model

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All figures in Chapter II are based on data generated by the Ph.D. candidate.
Data presented in Figures 1 and 2 were generated with the cooperation of Ashley Cimino-Mathews.
Data presented in Figure 4 (Immunohistochemistry) was generated with the cooperation of Deepa K. Machiah.

ABSTRACT

Many studies have shown that the systemic administration of cytokines or vaccination with cytokine-secreting tumors augments an anti-tumor immune response that can result in eradication of tumors. However, these approaches are hampered by the risk of systemic toxicity induced by soluble cytokines. In this study, we have evaluated the efficacy of 4TO7, a highly tumorigenic murine mammary tumor cell line, expressing glycosyl phosphatidylinositol (GPI)-anchored form of cytokine molecules alone or in combination with the costimulatory molecule B7-1 as a model for potential cell or membrane-based breast cancer vaccines. We observed that the GPI-anchored cytokines expressed on the surface of tumor cells greatly reduced the overall tumorigenicity of the 4TO7 tumor cells following direct live cell challenge as evidenced by transient tumor growth and complete regression within 30 days post challenge. Tumors co-expressing B7-1 and GPI-IL-12 grew the least and for the shortest duration, suggesting that this combination of immunostimulatory molecules is most potent. Protective immune responses were also observed following secondary tumor challenge. Further, the 4TO7-B7-1/GPI-IL-2 and 4TO7-B7-1/GPI-IL-12 transfectants were capable of inducing regression of a wild-type tumor growing at a distant site in a concomitant tumor challenge model, suggesting the tumor immunity elicited by the transfectants can act systemically and inhibit the tumor growth at a distant site. Additionally, when used as irradiated whole cell vaccines, 4TO7-B7-1/GPI-IL-12 led to a significant inhibition in tumor growth of day 7 established tumors. Lastly, we observed a significant decrease in the prevalence of myeloid-derived suppressor cells and regulatory T-cells in the tumor

microenvironment on day 7 post challenge with 4TO7-B7-1/GPI-IL-12 cells, which provides mechanistic insight into anti-tumor efficacy of the tumor-cell membrane expressed IL-12. These studies have implications in designing membrane-based therapeutic vaccines with GPI-anchored cytokines for breast cancer.

INTRODUCTION

Breast cancer is among the leading types of cancer among women in the United States, with an estimated 229,060 new cases in 2012 alone [1] and devising new strategies of breast cancer therapy remains a priority in medical research. While there have been numerous preclinical studies that have evaluated different methods and approaches to enhance the overall immunogenicity of tumor cells, few have been capable of inducing clinically relevant responses. One such approach includes the genetic modification of tumor cells to secrete cytokines including IL-12 and GM-CSF [2-4], or using GM-CSF secreting tumor vaccines with or without concomitant chemotherapy [5, 6].

In order for a cancer immunotherapy to be effective in a clinical therapeutic setting, the immune suppressive nature of the tumor microenvironment must be overcome [7, 8]. Tumor cells have been shown to up-regulate the expression of the inhibitory molecules PD-L1 and CTLA-4 [9-11]. Moreover, tumors also produce inhibitory cytokines and factors such as IL-10, vascular endothelial growth factor (VEGF), prostaglandins and transforming growth factor beta (TGF-β) that can induce immune tolerance by preventing dendritic cell (DC) maturation [12, 13] and promoting the differentiation and maturation of regulatory T cells (Tregs) [14, 15] and myeloid derived suppressor cells (MDSCs) [16].

To potentially address this issue, we have established glycosyl phosphatidylinositol (GPI) - anchored forms of the cytokines IL-2 and IL-12 for stable surface expression onto tumor cells along with the costimulatory molecule B7-1. The expression of these molecules allows for immune activation to take place locally at the vaccination site rather than systemic activation, which could potentially be toxic to patients [17-19]. This localized immune activation can

effectively manipulate or skew the tumor microenvironment towards being less immune suppressive. Additionally, direct targeting of tumor antigens to antigen presenting cells is more likely to occur following engagement of the modified tumor cells expressing IL-2 and IL-12 with their cognate receptors found on DCs.

Herein, we report for the first time the direct effects of membrane-anchored cytokines such as IL-2 and IL-12 on the tumorigenicity of a highly tumorigenic mouse mammary cancer model. Additionally, our studies provide insight into the potential mechanisms underlying the reduced tumorigenicity of these genetically modified cells as evidenced by a significant reduction in the local and peripheral immune suppressive microenvironment of tumor-bearing hosts.

MATERIALS AND METHODS

Cell culture and animals

4TO7 tumor cells, a kind gift of Fred Miller (Wayne State University), were cultured in DMEM media (Cellgro) with 10% FBS at 37°C. cDNA encoding GPI-anchored forms of murine IL-2, and IL-12 were constructed in our laboratory by attaching a GPI-anchor signal sequence as previously described [20, 21]. cDNA encoding murine B7-1 was kindly provided by Gordan Freeman (Boston, MA). The cDNAs were subcloned into the pUB6A expression vector (Invitrogen Corp). Cells were transfected using FuGene6 transfection reagent (Roche Molecular Biochemicals) and selected with blasticidin (10μg/ml). In order to select the population of cells expressing the GPI-molecules, the cells were subjected sequentially to a) magnetic activated cell sorting (MACS) (Dynal Biotech Dynabeads, Invitrogen), b) panning [22] and c) fluorescence activated cell sorting (FACS). The wild-type 4TO7 (4TO7-WT) cell population was later subjected to four rounds of *in vivo* passage following subcutaneous (s.c.) injection into BALB/c mice to yield more reproducible, aggressive tumor growth with palpable tumor development within 6 days (4TO7RG).

Female BALB/C mice 6-8 weeks of age were purchased from Jackson Laboratories and were maintained in accordance with IACUC approved institutional guidelines and protocols.

Characterization of tumor cells

Flow Cytometry. Surface expression of B7-1, IL-2 and IL-12 was determined by flow cytometry analysis. Briefly, cells were incubated for 30mins at 4˚C with directly-conjugated antibodies as follows: IL-2-PE (clone S4B6), IL-12-PE (clone 17.8), and B7-1-FITC (clone 1G10) (BD

Biosciences). The cells were then washed, formalin-fixed and analyzed using a FACSCaliber cytometer and analyzed with FlowJo software.

ELISA and Western blot analysis. Cell transfectants ($2x10^5$/well) were seeded in 24-well plates for 48h. After which, culture supernatant was collected, cells were washed and lysed using 2% octyl-β-glucoside, 50mM Tris-HCl pH8, 2mM PMSF, 5mM EDTA and protease inhibitor cocktail (1:100, Sigma). IL-2 and IL-12 in the cell lysate and culture supernatant was detected by sandwich ELISA according to the manufacturer's instructions (eBioscience) and western blotting techniques as previously described [23].

PIPLC (phosphatidylinositol phospholipase-C) treatment. Cell transfectants were treated with a 1:1000 dilution of the PIPLC enzyme (Glyko Prozyme, San Leandro, CA) in PBS/0.1% Ovalbumin and incubated for 45mins in a 37˚C water bath with slight agitation every 10mins. At the end of the incubation, the cells were centrifuged and washed with FACS buffer (PBS/1%CCS/1%EDTA) and stained for FACS analysis.

CFSE dilution. CFSE staining was used to determine the growth rate of tumor cells using adapted methods as previously described [24]. Cells were then washed with FACS buffer and either analyzed immediately to verify CFSE incorporation or cultured for FACS analysis at the specified time points.

Tumor challenge studies

Direct challenge Mice (n=5/group) were challenged subcutaneously (s.c.) in the rear hind flank with 4TO7-WT or transfected 4TO7 cells ($2x10^5$). Tumor size (mm^2) was measured using Vernier calipers every 2-3 days with 2x2 perpendicular measurements. Tumor-free mice were subjected to a secondary challenge with 4TO7-WT cells ($2x10^5$) 30-33 days later on the opposite

hind flank. Mice were monitored weekly for tumor growth. Mice were euthanized when the tumor size reached >2 cm^2.

Concomitant Immunity. Mice (n=5/group) were challenged with 4TO7RG cells ($2x10^5$) on the right hind flank and simultaneously challenged with each of the 4TO7 transfectants ($2x10^5$) on the opposite hind flank (s.c.). Mice were monitored as mentioned previously.

Therapeutic whole cell vaccination studies. Prior to vaccination, tumor cells were exposed to 80Gy of gamma irradiation. Mice were challenged subcutaneously with $5x10^4$ 4TO7RG cells on the right hind flank and vaccinated with $2x10^5$ irradiated cells on the opposite hind flank seven days later. Mice were monitored as previously mentioned.

Cellular phenotyping of immune infiltrates and Immunohistochemistry staining (IHC)

Tumor cells ($2x10^5$) were mixed in a 1:1 ratio with 250µL of Matrigel™ (BD Falcon) and injected into the hind flank of BALB/c mice (s.c.). Seven days post inoculation, the spleens, tumor-draining lymph nodes (TDLNs) and Matrigel were harvested. The Matrigel plugs were either digested using collagenase type III (Sigma) for 1h or formalin-fixed for IHC staining. Single cell suspensions were prepared from the digested Matrigel plugs and red blood cells were lysed. Cells were then washed, Fc blocked (clone 2.4G2) and stained with directly conjugated antibodies (eBioscience) for 25mins at 4^oC to detect T cells ($CD4^+$ and $CD8^+$), B cells ($B220^+$), DCs ($CD11b^+CD11c^+$), Tregs ($CD4^+CD25^+FoxP3^+$) and MDSCs ($CD11b^+Gr1^+$). Samples were analyzed as described previously. Formalin-fixed Matrigel plugs were embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin (H&E). Blood vessels were visualized by staining endothelial cells with a CD31 primary antibody at 1:200 dilution (Abcam).

Statistical analysis

Differences between tumor growth curves and spleen sizes groups were analyzed using ANOVA or the Student *t* test, respectively. Values of $p < 0.05$ were considered significant. For survival studies, Kaplan-Meier survival curves were plotted and analyzed. All graphs and statistical calculations were analyzed using Prism software (GraphPad).

RESULTS

Establishment of 4TO7 murine breast cancer cells expressing GPI-anchored IL-2 and IL-12

4TO7 murine mammary tumor cells were transfected to express transmembrane B7-1, GPI-IL-2, GPI-IL-12, B7-1 and GPI-IL-2, or B7-1 and GPI-IL-12. The GPI-linkage of the cytokine molecules on the surface of the cells was verified using the PIPLC enzyme, which specifically cleaves the glycosyl phosphatidylinositol lipid tail of the GPI-anchored molecule and leads to a decrease in surface molecule expression. After PIPLC treatment, the percentage of cells positive for B7-1 did not decrease as the B7-1 construct was not GPI-linked (Figure 1A-B). However, expression of IL-2 and IL-12 decreased following PIPLC treatment, verifying that the cytokines were anchored to the cell membrane by a GPI-linkage. The IL-12 GPI-linkage appears to be more susceptible to PIPLC cleavage, as its expression decreased by 94.5% while IL-2 expression decreased by 68.4% for the single transfectants (Figure 1A) and 96.7% and 77.5% for the double transfectants (Figure 1B), respectively. The sensitivity of the GPI-anchor to cleavage has been shown to vary depending on the molecule that it anchors and the cell type in which it is expressed [25].

Lastly, to determine whether GPI-IL-12 and GPI-IL-2 is found in the supernatant of 4TO7 transfectants, we analyzed cells and supernatants after culturing tumor cells for 48h. No measurable IL-12 was detected in the supernatant of transfected cells by Western blot, suggesting that GPI-IL-12 remains cell-associated and is minimally shed (Figure 1C). This is further supported by ELISA which indicates that 2.24ng/mL and 3.49ng/mL is cell-associated whereas only 0.35ng/mL and 0.411ng/mL is found in the supernatant of 4TO7-IL-12 and 4TO7-B7-1/IL-12, respectively. However, despite not being able to detect IL-2

by Western blot, we observed substantial amounts of IL-2 in supernatants of 4TO7-IL-2 cells by ELISA suggesting that IL-2 is expressed both as membrane-bound and secreted forms.

Membrane expression of IL-12 along with B7-1 leads to minimal tumor growth and rapid tumor rejection following direct challenge

While the expression of IL-2, IL-12 or B7-1 alone or in combination did not alter the *in vitro* growth properties of the 4TO7 tumor cells as indicated by CFSE dilution (Figure 1D), upon direct *in vivo* challenge, 4TO7-WT tumors grew progressively whereas the tumors from the transfected cell lines all completely regressed (Figure 2A). Thirty days post injection, none of the mice challenged with transfected cells had tumors while 100% of the mice challenged with wild-type cells had developed tumors. The onset of tumor formation of the 4TO7B7-1/IL-12 tumors was the slowest to appear and the smallest in size. This data indicates that the stable surface expression of B7-1 alone or in combination with IL-2 or IL-12 enhances the overall immunogenicity of 4TO7-WT tumor cells.

Transfected 4TO7 tumor cells are capable of inducing protective immune responses

All of the mice previously challenged with transfected 4TO7 cells were tumor-free 30 days after initial direct challenge (Figure 2B) and were then re-challenged with $2x10^5$ 4TO7-WT cells. All of the mice remained tumor-free, whereas all of the mice in the previously unchallenged group developed progressively growing tumors and had to be sacrificed within 30 days post challenge due to large tumor burden (Figure 2C). Taken together, protective anti-tumor immunity was induced by the transfected 4TO7 tumor cells and was sustained for up to

150 days of monitoring following re-challenge with 4TO7-WT cells (data not shown).

Splenomegaly correlates with tumor burden and MDSC

Throughout our studies, control mice challenged with 4TO7 cells displayed highly significant splenomegaly ($p<0.0001$) relative to naïve or tumor-free mice and overall tumor burden correlated with the spleen weight, $R^2= 0.943$ (Figure 3A). Consistent with previous reports that splenomegaly is associated with an increased infiltration of MDSCs [26], we also observed a significant increase in MDSCs from an average of 4.02% in tumor-free mice to 32.5% in tumor-positive mice with a corresponding percent decrease in other cell types including T and B cells (Figure 3B). There was no significant difference observed in spleen weight or splenocyte cell populations between naïve mice and mice that were tumor-free at the end of the aforementioned studies (data not shown).

The co-expression of B7-1 and GPI-IL-12 alters the tumor microenvironment of live 4TO7 cells

To gain insight into the early immune factors mediating the reduced tumorigenicity of 4TO7 B7-1/IL-12, tumor cells were mixed in a 1:1 ratio with Matrigel and transplanted subcutaneously into BALB/c mice. Staining for H&E and CD31, an endothelial cell marker, was used to visualize the angiogenesis in the Matrigel plugs. Reduced vasculature was observed with the co-expression of B7-1 and IL-12 relative to wild-type (Figure 4A). Among the cellular infiltrates found within the Matrigel, we observed that the co-expression of B7-1/IL-12 led to a significant decrease in MDSCs relative to wild-type challenged mice at the tumor site (13.5% to 5.2%) and in the spleen (17.0% to 5.98%) and a marked decrease in the TDLNs (9.9% to 6.5%) (Figure 4B). This reduction of MDSCs is somewhat surprising given that 4TO7 cells have been

shown to secrete granulocyte-colony stimulating factor (G-CSF), a factor that can promote the recruitment and mobilization of MDSCs to the tumor site as well as distant organs to establish pre-metastatic niches [27]. Additionally, we observed that within the Matrigel, 15.3% of total cells from wild-type challenged mice were Tregs whereas in 4TO7B7-1/IL-12 challenged mice there was a significant decrease in Tregs to 7.28% (Figure 4B). We also saw a notable decrease in Tregs in the TDLNs as well as in the spleen. The co-expression of B7-1 and IL-12 also led to an increased infiltration of $CD8^+$ T cells and DCs (Figure 4B, lower panels), as well as $CD4^+$ T cells and B cells (data not shown) relative to wild-type challenged mice. These observations suggest that the reduced tumorigenicity observed with the co-expression of B7-1 and IL-12 is based on a duality of inhibiting components of active immune suppression as well as increasing the infiltration of effector immune cell populations.

Simultaneous challenge with transfected 4TO7 tumor cells causes regression of a tumor growing at a distant site

Next, we wanted to determine whether the anti-tumor immune response elicited by the transfected 4TO7 cells is capable of acting systemically and induce regression of a 4TO7RG tumor, a highly aggressive variant of 4TO7,growing at a distant site. In this concomitant immunity model, we observed that the growth of the 4TO7RG tumor on the right flank was generally slower and less progressive in the mice challenged concomitantly with the 4TO7 transfectants (Figure 5A). The mice challenged with the double transfectants 4TO7-B7-1/IL-2 and 4TO7-B7-1/IL-12 showed a delay in overall growth kinetics and had the smallest average 4TO7RG tumor growth, with both groups remaining $<10mm^2$. Of the single 4TO7 transfectants, 4TO7-IL-12 was the most effective at reducing overall tumor burden with an average tumor size

of ~30mm^2 by day 27 post challenge. In contrast, 4TO7-B7-1 and 4TO7-IL-2 challenged mice developed wild-type tumors similar in size as the control mice. These findings indicate that the stable co-expression of B7-1 along with Th1 skewing cytokines IL-2 or IL-12 on the surface of 4TO7 tumor cells can overcome the immune suppression mediated by a developing, highly aggressive tumor.

Therapeutic vaccination with irradiated 4TO7 cells expressing cells B7-1 and GPI-IL-12 significantly reduces overall tumor burden

To assess the therapeutic efficacy of expressing GPI-anchored cytokines on the surface of breast cancer cells in a clinically relevant model, mice were vaccinated with irradiated 4TO7 transfectants on day 7 post challenge with 4TO7RG cells. We observed that after only one vaccination, mice treated with irradiated 4TO7B7-1/GPI-IL-12 cells had significantly lower tumor burden than unvaccinated mice with an average tumor size of 27.2mm^2 as compared to 165.3mm^2 (Figure 5B). Additionally, the tumor incidence was decreased to 40%. These results indicate that the co-expression B7-1 and GPI-IL-12 is capable of inducing anti-tumor immune responses against a highly aggressive tumor variant in a therapeutic setting.

DISCUSSION

The 4TO7 murine mammary tumor cell line was transfected to express GPI-anchored forms of cytokines IL-2 and IL-12 alone or in combination with the costimulatory molecule B7-1. Our challenge studies demonstrated that the transfectants had decreased tumorigenicity, induced long-term protective immunity and growth inhibition of a distant, concomitant tumor as well as significantly reduced overall tumor burden in a therapeutic setting. Interestingly, we also observed that irradiated 4TO7RG cells significantly inhibited 4TO7RG growth comparable to 4TO7 transfectants. The 4TO7 transfectants were established from 4TO7-WT cells and not the more aggressive, in vivo passaged variant 4TO7RG. For our concomitant and therapeutic vaccination studies we chose to challenge with 4TO7RG to test the stringency of the anti-tumor immune response that would be induced by the GPI-molecules. Due to the likely intratumoral heterogeneity [28] of the 4TO7RG cells, transfected clones may no longer be representative of the total antigenic population that must be presented to induce more complete tumor inhibition of 4TO7RG tumors. These findings have potential clinical implications such that clonally selected cell lines developed from a patient's highly heterogeneous tumor tissue that are modified with B7-1/GPI-IL-12 could be effective against the heterogeneous tumor.

Previous studies have evaluated the role of co-expressing B7-1 and secretory IL-12 [29-31]. Direct challenge of mice with A20 murine B cell lymphoma expressing B7-1 alone delayed onset of tumor appearance, whereas challenge with A20-IL-12 or A20-B7-1/IL-12 led to complete rejection [30]. Our challenge studies suggest that, like soluble IL-12, GPI-anchored IL-12 is also capable of synergizing with B7-1 in inducing anti-tumor immunity, but without the potential risks of systemic toxicities associated with soluble cytokines [17-19]. Pan et al

reported that a single chain murine IL-12 expressed as a fusion protein with the transmembrane domain and cytosolic tail of murine B7-1 is capable of reducing the tumorigenicity of CT26 murine colon cancer cells and upon intratumoral administration via an adenoviral vector is effective in a therapeutic setting [32]. Our approach is unique in that it modifies soluble cytokines with a GPI-anchor, which we have previously shown can spontaneously incorporate onto the lipid bilayers of cell membranes [21, 23].

The reduced tumorigenicity of 4TO7 B7-1/IL-12 tumor cells appears to correlate with a significant reduction in immune suppressive cell populations, namely MDSCs and $FoxP3^+$ Tregs. Furthermore, in human breast cancer, decreased Tregs has been associated with increased complete pathologic response [33] and improved overall survival [34] after neoadjuvant therapy. Similarly, it has been reported that among breast and colon cancer patients the presence of $Lin^-CD33^+HLA^-DR^{-/low}$ MDSCs is associated with a poor overall survival [35]. Thus, effective targeting of the development [36] or suppressive activities [37] of these cell populations, would be of great therapeutic benefit and have been investigated in several cancers [38].

Our results indicate that cytokine molecules expressed on the surface of 4TO7 murine cancer cells can induce protective immunity, inhibit progression of a distant tumor and effectively overcome the immune suppressive tumor microenvironment. Taken together these findings suggest that a cell or membrane-based vaccine containing GPI-anchored cytokines could be a viable immunotherapeutic approach to treat breast cancer.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. IL-2 and IL-12 are expressed on the surface of 4TO7 via a GPI-anchor. The enzyme PIPLC was used to verify the GPI-linkage of the membrane-bound B7, IL-2 and IL-12 molecules in the A) single and B) double transfectant cell lines. The shaded histogram represents staining with an isotype control antibody. The solid line represents protein expression at baseline and the dashed line represents protein expression following PIPLC treatment. C) Western blot analysis of IL-12 and ELISA of IL-2 and IL-12 in the cell lysate (Cell) and culture supernatant (Sup) of $2x10^5$ 4TO7 transfectants after 48 h culture. D) 4TO7 tumor cells were labeled with CFSE and analyzed using flow cytometry at 0, 8, 16, 24, and 48 h post culture. The mean fluorescence intensity (MFI) of each cell line was normalized relative to its initial (0 h) MFI. 100% was normalized as the 0 h time point for each sample.

Figure 2. Expression of B7-1 alone or in combination with GPI-IL-2 and GPI-IL-12 reduces the overall tumorigenicity of 4TO7 tumor cells. BALB/C mice (n=5/group) were challenged s.c. in the hind flank with $2x10^5$ cells and tumor growth was monitored. A) Mean tumor size was calculated as the average of the five tumor measurements per group. B) Each data line represents an individual mouse per group. C) Tumor-free mice were re-challenged with $2x10^5$ wild-type cells to determine the induction of protective immune responses. Mean ± SEM is depicted.

Figure 3. Significant increase in myeloid derived suppressor cell (MDSC) population correlates with observed splenomegaly. Spleens from naïve, tumor-free and 4TO7 challenged mice were weighed prior to single cell suspensions being prepared for flow cytometry analysis of splenic cell populations. A) Spleen weights of tumor positive mice were significantly higher than tumor-free mice and correlated with overall tumor burden. B) A significant increase in splenic MDSCs in tumor-positive (■) relative to tumor-free (●) mice. An unpaired Student's t test and ANOVA analysis was conducted to determine statistical significance among spleen weights and splenic populations, respectively. (****$p<0.0001$)

Figure 4. Significant reduction in MDSCs and regulatory T cells (Tregs) in mice challenged with 4TO7-B7-1/IL-12 cells. Tumor cells were mixed in a 1:1 ratio with Matrigel and inoculated s.c. into the hind flank of mice. Seven days later, the Matrigel plugs, spleens and tumor draining lymph nodes (TDLNs) were harvested. A) Reduced angiogenesis is present at 4TO7-B7-1/IL-12 tumor site relative to 4TO7-WT. Representative Matrigel plugs from two separate experiments are shown with H&E (10x) and CD31 endothelial cell staining (20x magnification). B) Co-expression of B7-1 and GPI-IL-12 decreased the prevalence of MDSCs ($CD11b^+Gr1^+$) and Tregs ($CD4^+CD25^+FoxP3^+$) locally as well as in the periphery, while increasing the prevalence of $CD8^+$ T cells and DCs ($CD11c^+CD11b^+$). White bar: PBS, Black bar: 4TO7-WT, Gray: 4TO7B7-1/IL-12. (*p<0.05, **p<0.01). Mean ± SEM is depicted.

Figure 5. Co-expression of B7-1 with GPI-IL-2 or GPI-IL-12 induces concomitant immune responses and inhibits tumor growth in a therapeutic setting. A) Groups of mice (n=5) were challenged with 4TO7RG cells ($2x10^5$) on the right hind flank and simultaneously challenged subcutaneously with each of the 4TO7 transfectants ($2x10^5$) on the opposite hind flank. Mice challenged with 4TO7 cells expressing B7-1/IL-2 or B7-1/IL-12 led to a significant inhibition of 4TO7-WT tumor growth at a distant site. B) Mice were challenged with $5x10^4$ 4TO7RG cells and vaccinated subcutaneously with irradiated (80Gy) 4TO7 transfectants 7 days later. Vaccination with 4TO7B7-1/IL-12 cells induced significant tumor inhibition. Statistical significance was determined using ANOVA analysis. (*p<0.05, *** p<0.001). Mean ± SEM is depicted.

FIGURE 1

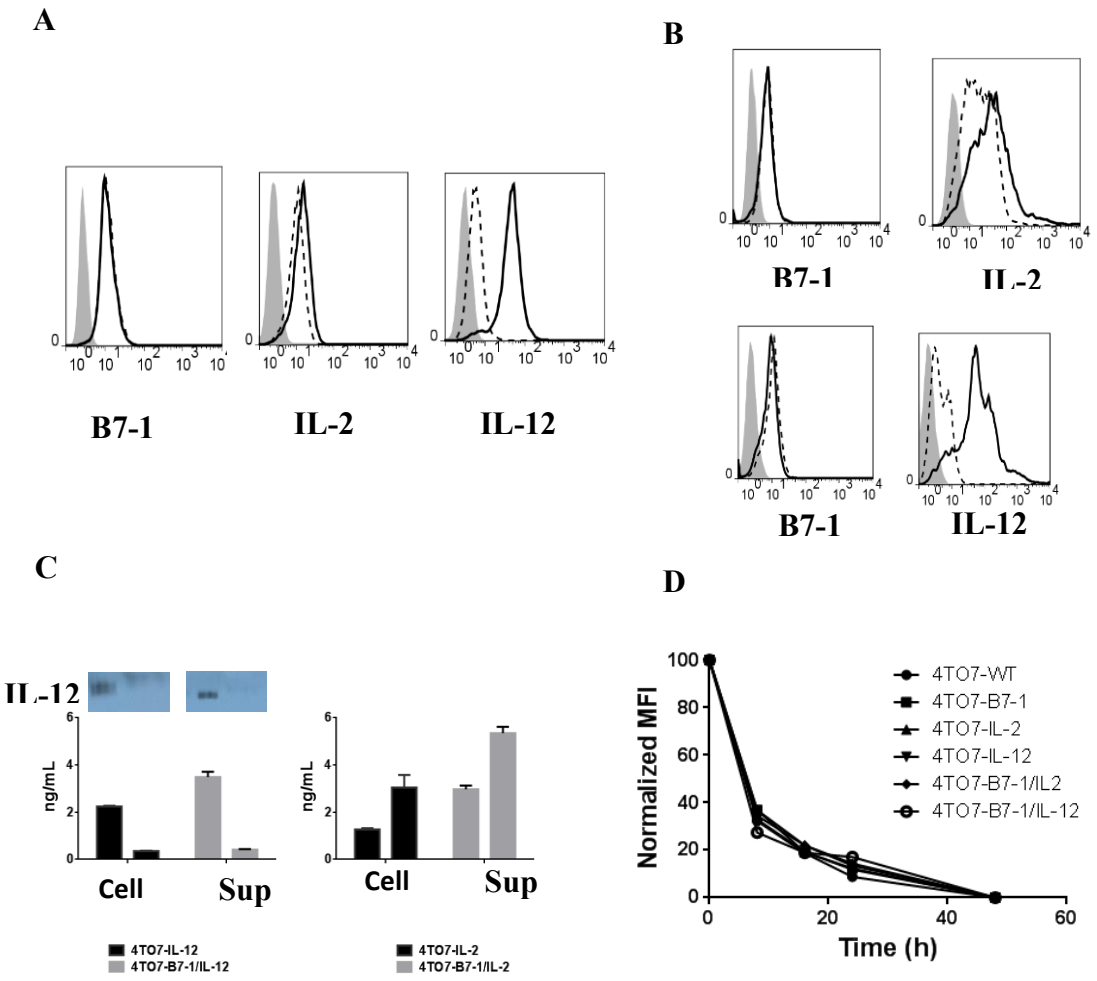
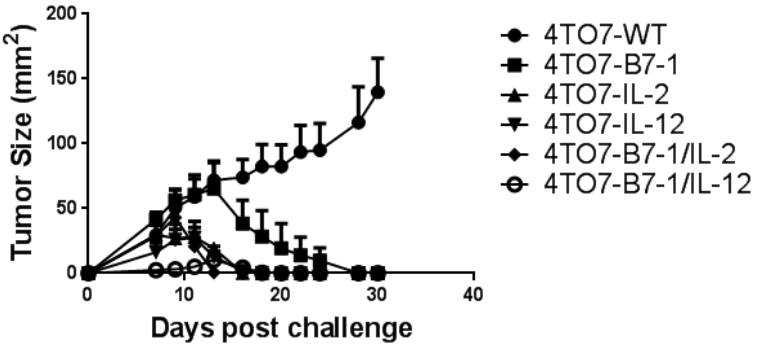
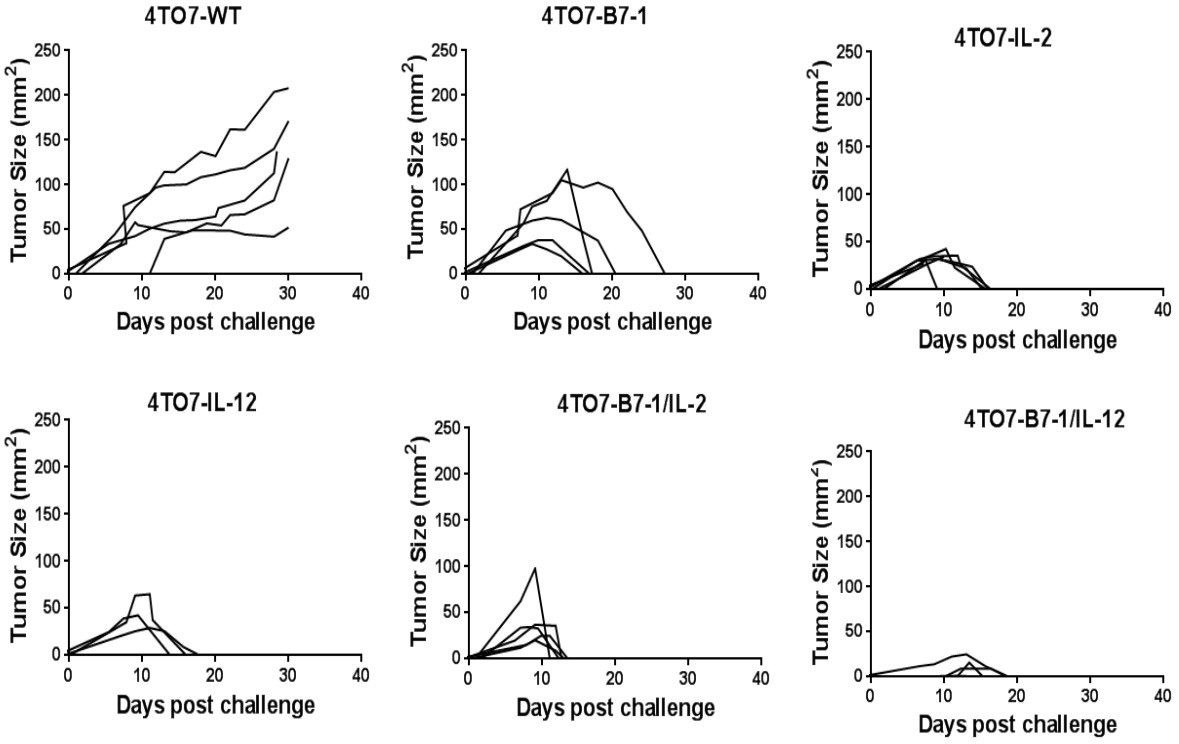

A
B7-1
IL-2
IL-12
B
B7-1
IL-2
B7-1
IL-12
C
IL-12
ng/mL
Cell
Sup
4TO7-IL-12
4TO7-B7-1/IL-12
ng/mL
Cell
Sup
4TO7-IL-2
4TO7-B7-1/IL-2
D
Normalized MFI
Time (h)
4TO7-WT
4TO7-B7-1
4TO7-IL-2
4TO7-IL-12
4TO7-B7-1/IL2
4TO7-B7-1/IL-12

FIGURE 2

A


Tumor Size (mm2)
Days post challenge
4TO7-WT
4TO7-B7-1
4TO7-IL-2
4TO7-IL-12
4TO7-B7-1/IL-2
4TO7-B7-1/IL-12

B


4TO7-WT
4TO7-B7-1
4TO7-IL-2
4TO7-IL-12
4TO7-B7-1/IL-2
4TO7-B7-1/IL-12
Tumor Size (mm2)
Days post challenge

C

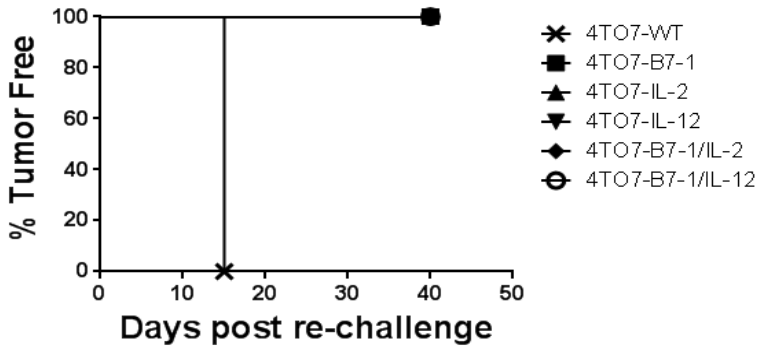
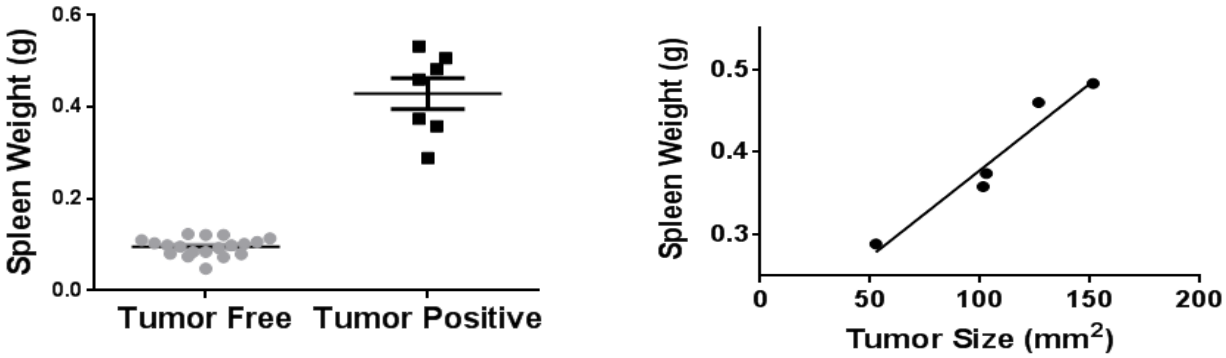

% Tumor Free
Days post re-challenge
4TO7-WT
4TO7-B7-1
4TO7-IL-2
4TO7-IL-12
4TO7-B7-1/IL-2
4TO7-B7-1/IL-12

FIGURE 3

A


Spleen Weight (g)
0.6
0.4
0.2
0.0
Tumor Free
Tumor Positive
Spleen Weight (g)
0.5
0.4
0.3
0
50
100
150
200
Tumor Size (mm2)

B

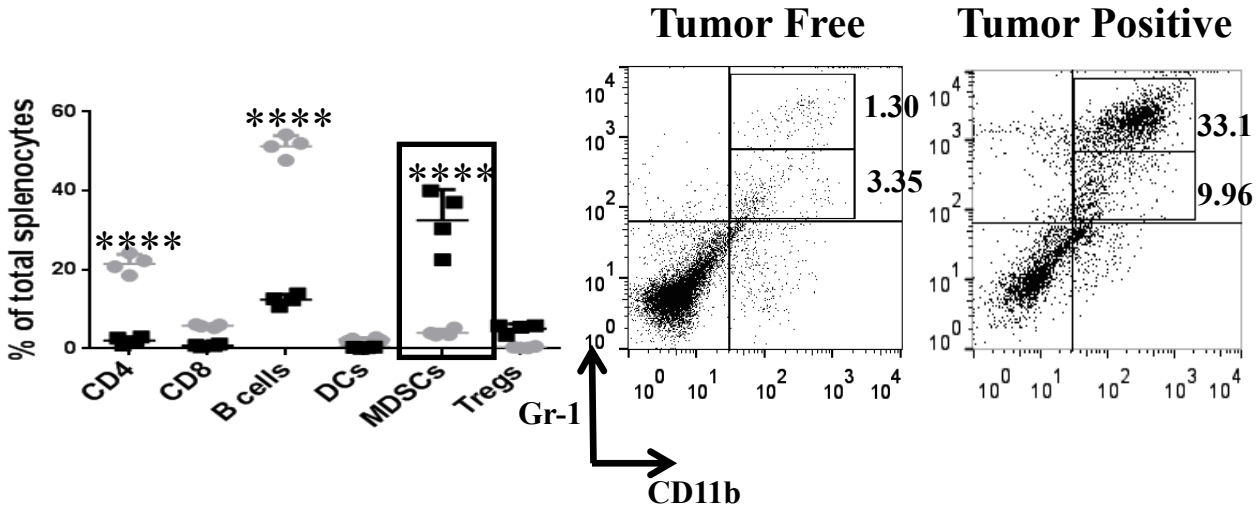
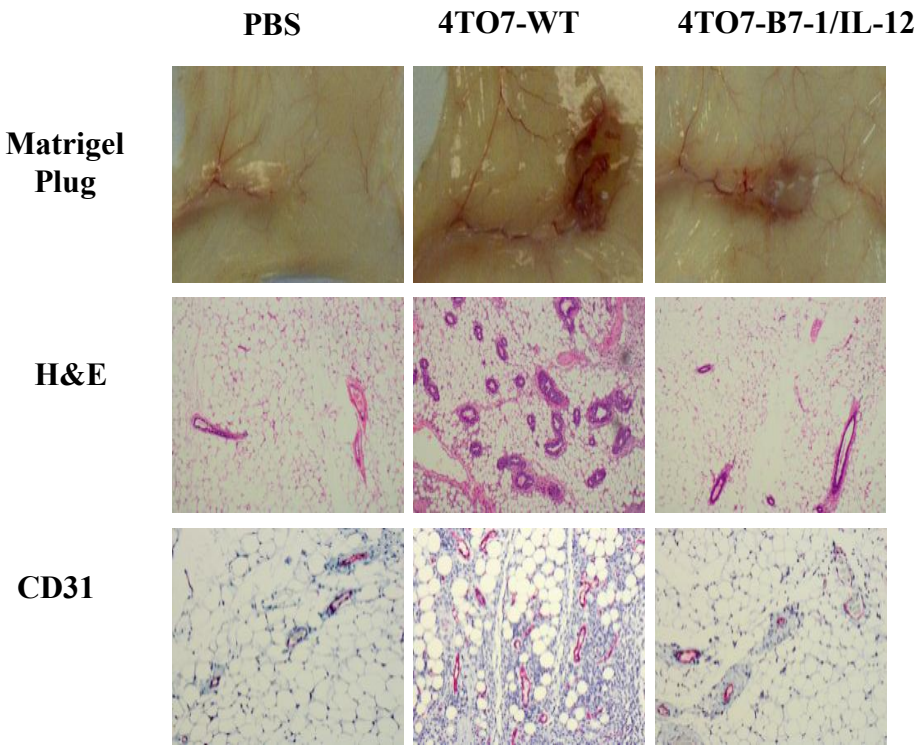

% of total splenocytes
60
40
20
0
****
****
****
CD4
CD8
B cells
DCs
MDSCs
Tregs
Tumor Free
Tumor Positive
1.30
3.35
33.1
9.96
Gr-1
CD11b

FIGURE 4

A


PBS
4TO7-WT
4TO7-B7-1/IL-12
Matrigel Plug
H&E
CD31

B

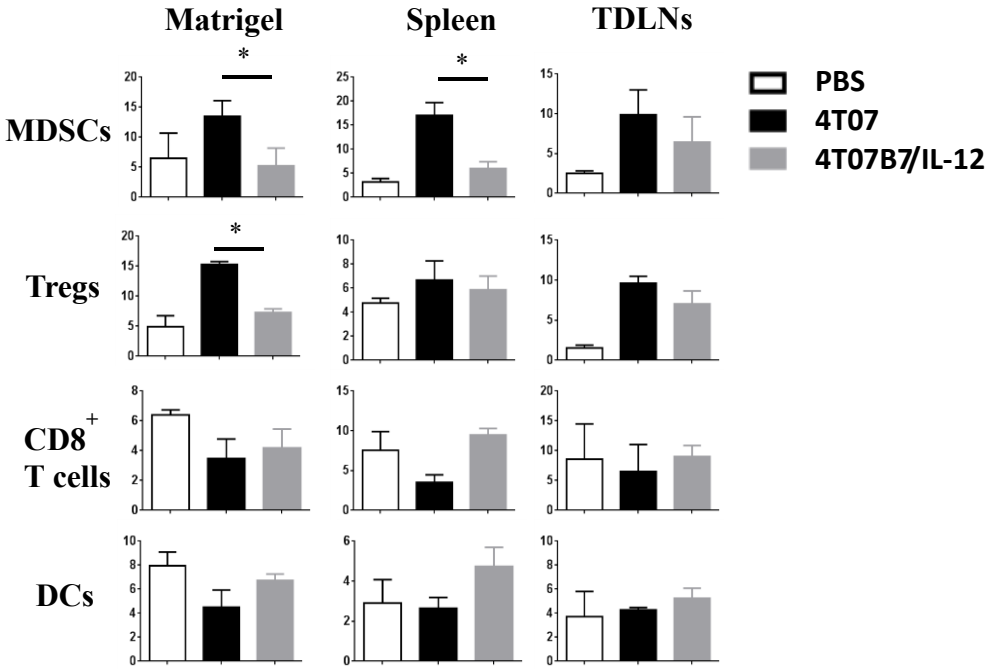
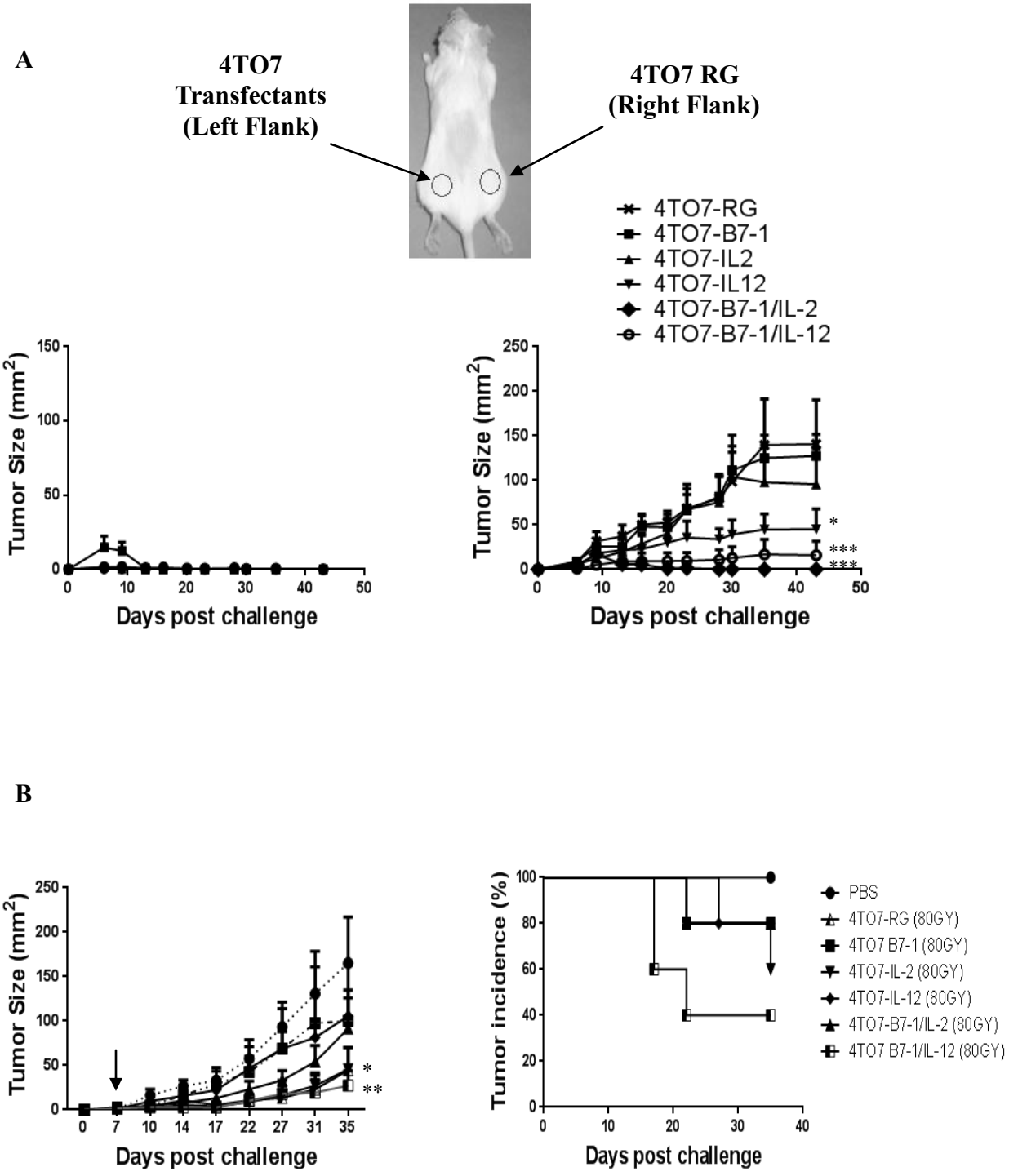

Matrigel
Spleen
TDLNs
MDSCs
Tregs
CD8+ T cells
DCs
PBS
4T07
4T07B7/IL-12

FIGURE 5


A
4TO7
Transfectants
(Left Flank)
4TO7 RG
(Right Flank)
4TO7-RG
4TO7-B7-1
4TO7-IL2
4TO7-IL12
4TO7-B7-1/IL-2
4TO7-B7-1/IL-12
Tumor Size (mm2)
Days post challenge
*
***
***
B
Tumor Size (mm2)
Days post challenge
*
**
Tumor incidence (%)
Days post challenge
PBS
4TO7-RG (80GY)
4TO7 B7-1 (80GY)
4TO7-IL-2 (80GY)
4TO7-IL-12 (80GY)
4TO7-B7-1/IL-2 (80GY)
4TO7 B7-1/IL-12 (80GY)

CHAPTER III

Irradiated cellular vaccines expressing B7-1 and GPI-cytokines promote the induction of tumor-specific cytotoxic activity and protective anti-tumor responses in a HER-2 positive murine tumor model

This data, as presented in this Chapter, is unpublished.

All figures in Chapter III are based on data generated by the Ph.D. candidate.

ABSTRACT

Despite the clinical success of monoclonal antibody treatments for HER-2 positive breast cancer such as Trastuzumab, there is a growing incidence of resistance to these therapies. It has been proposed that therapies which induce T cell-mediated immunity with or without the accompaniment of humoral immune responses are more likely to circumvent this resistance. In order to investigate this possibility, we first characterized the role played by antibodies in a human HER-2 (hHER-2) positive murine breast cancer model, D2F2/E2. We observed that within this model, tumors grew progressively in the presence of high tumor-specific IgG levels, with the dominant subclass being IgG1, typically enhanced during the generation of Th2 responses. Closer examination revealed that the *in vivo* efficacy of these antibodies may be limited in this model due, in part, to the constitutive expression of CD47, which could inhibit antibody-mediated clearance, as well as the loss of the HER-2 antigen *in vivo*. As a result, in order to mediate D2F2/E2 tumor inhibition or eradication, we administered irradiated cellular vaccines genetically modified to express B7-1 or glycosyl phosphatidylinositol (GPI) anchored cytokines, GPI-IL-2, GPI-IL-2 or GPI-GM-CSF. We observed that co-injection of B7-1 and GPI-IL-12 expressing vaccines completely protected mice against subsequent D2F2/E2 tumor challenge potentially through the induction of robust HER-2-specific cytotoxic activity. More importantly, one dose of these cellular vaccines significantly reduced average tumor burden of day 7 established D2F2/E2 tumors. Because complete protection was not observed and PD-L1 is constitutively expressed by D2F2/E2 cells, we combined our cellular vaccines with anti-PD-L1 treatment. We

observed an augmentation of the efficacy of our cellular vaccination. Taken together, cellular vaccines expressing B7-1 and GPI-IL-12 are able to induce protective anti-tumor responses in prophylactic and therapeutic settings, potentially mediated by significant HER-2 specific cytotoxic activity, which can be further enhanced by adjuvant therapies such as PD-L1 blockade.

INTRODUCTION

Human epidermal growth factor receptor -2 (HER-2) positive breast cancer accounts for 20-30% of diagnosed breast cancer [1]. Due to the overexpression of this receptor, this form of breast cancer is highly aggressive and associated with a poorer clinical prognosis. Despite FDA approval of monoclonal antibody therapies such as Trastuzumab, Pertuzumab and the tyrosine kinase inhibitor lapatinib in conjunction with chemotherapy, many patients develop resistance to these therapies leading to relapse and more progressive disease [2, 3]. Thus, the use of vaccination strategies that generate T cell responses with or without accompanying antibody responses may serve to mitigate the problem of resistance [4]. The majority of pre-clinical studies have focused on HER2-plasmid DNA or peptide-based vaccines while less is known of the efficacy of cellular vaccines in inducing HER2-specific responses [5, 6].

With that knowledge we sought to investigate the efficacy of a whole cell vaccination strategy to induce HER-2-specific immune responses. Because tumor cells are inherently poorly immunogenic, we have modified the human HER-2 (hHER-2) positive mouse tumor cell line D2F2/E2 [7] with the following membrane anchored immune stimulatory molecules (ISMs): transmembrane B7-1 (B7-1), glycosyl phosphatidylinositol (GPI)-IL-2, GPI-IL-12 and GPI-granulocyte macrophage colony-stimulating factor (GM-CSF). Due to our choice of ISMs, which are primarily Th1 skewing [8] , we hypothesized that the induction of cellular immunity would be more prevalent in this model and confer protection. Additionally, the use of whole tumor cells as the vaccine material, allows for a wide repertoire of tumor antigens to be presented and

recognized by the host's immune system which is more likely to minimize tumor escape [9, 10].

MATERIALS AND METHODS

Tumor model and animals

The hHER-2 overexpressing murine breast cancer cell line D2F2/E2 was a kind gift of Dr. Wei (Wayne State University) and was cultured in DMEM media supplemented with 10% cosmic calf serum, 1% penicillin streptomycin and 400μg/mL G418. Stable transfectants expressing transmembrane B7-1 (B7-1) and GPI-IL-2, GPI-IL-12, GPI-GM-CSF was established as previously described [11] and maintained under 5μg/mL blasticidin selection (Invitrogen). Female BALB/c mice, 6-8 weeks of age, were purchased from Jackson Laboratories and were maintained in accordance with IACUC approved institutional guidelines and protocols.

Direct challenge and prophylactic vaccination settings

Mice were injected subcutaneously (s.c.) on the hind flank with $2x10^5$ live D2F2/E2 cellular transfectants for direct evaluation of GPI-ISM expression on the tumorigenicity of D2F2/E2 tumor cells. Tumor-free mice were re-challenged on day 37 with an additional $2x10^5$ live D2F2/E2 wild-type (WT) cells. For prophylactic vaccination studies, D2F2/E2 cellular transfectants were subjected to 80 Gy gamma (γ) irradiation using a Gammacell40 Caesium 137 irradiation unit. Irradiated cellular vaccines (Irr) were injected s.c. into mice at a dose of $2x10^5$ two weeks prior to tumor challenge with $2x10^5$ D2F2/E2 WT tumor cells on the opposite hind flank. Irradiated cellular vaccines expressing single ISMs were co-injected at a 1:1 ratio to create dual ISM expressing vaccines. Mice were monitored every 2-3 days for tumor growth. Mice

were euthanized when tumors became ulcerated or the tumor size reached 2 cm in diameter.

Therapeutic vaccination and program death ligand-1(PD-L1) blockade

Therapeutic vaccination with one dose of $2x10^5$ irradiated cellular vaccines was administered (s.c.) 1 week after D2F2/E2 tumor challenge. Irradiated cellular vaccines expressing single ISMs were co-injected at a 1:1 ratio to create dual ISM expressing vaccines. Anti-PD-L1 antibody (clone 9G2, BioXcell) or rat IgG antibody (Jackson ImmunoResearch) were given intraperitoneally (i.p.) on days 7, 10 and 13 post challenge at a dose of 100μg per injection. Mice were monitored as mentioned previously.

Assessment of humoral and cellular immunity following vaccination

Antigen-specific antibodies were measured by a cell ELISA using serum collected from mice 13 days post vaccination. D2F2/E2 cells ($5x10^4$/ well) were plated in a 96-well, V-bottom plate. Serum from naïve and vaccinated mice was diluted 1/1000 in FACS buffer (PBS, 1% CCS, 1% EDTA) and allowed to incubate with cells for 1 hr at 4°C with slight agitation. Cells were washed 3 times with FACS buffer. HRP-conjugated goat antibody against mouse total IgG or subclass-specific mouse IgG1 and IgG2a (Southern Biotech) was diluted 1/2000 in FACS buffer and added to cells for 1 hr incubation at 4°C with slight agitation. Cells were washed 3-5 times with FACS buffer. TMB Substrate (100μL) was added to each well and sulfuric acid (50μL) was added as the stop solution 10-15 mins later. Solution (100μL) was transferred to a 96-well, flat bottom plate for analysis of optical density (OD) at 415 nm or 450 nm.

Two weeks post vaccination, spleens were harvested, splenocytes isolated and red blood cells were lysed using RBC lysis buffer (Sigma). Pooled splenocytes from each vaccinated group were co-cultured for 5 days with mitomycin C-treated (50μg/mL) D2F2/2 as stimulator cells at a ratio of 5:1 in 6 well plates. Recombinant IL-2 (10U/mL) was added on day 2 of re-stimulation. Cytotoxic activity was quantified using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions with D2F2/E2, D2F2 and 4TO7WT cells as target cells at various effector: target ratios. Total cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100$$

Tumor infiltrating lymphocytes (TILs) isolation

Tumors from D2F2/E2 challenged mice were harvested and collagenase digested for 2 h at 37°C in a shaking incubator. Enrichment of TILs was achieved by passing the single cell suspension through a 67% Percoll gradient. TILs were collected from the gradient interphase and subsequently stained with directly conjugated antibodies specific for CD4, CD8 and PD-1 (eBioscience) at a dilution of 1:100 in FACS buffer for 30 mins at 4°C. Cells were washed twice with FACS buffer and fixed with 2% formalin for analysis using a FACSCalibur flow cytometer and FlowJo software.

Characterization of tumor cells and freshly isolated tumors

D2F2/E2 cells ($2x10^5$) were cultured for 48 h, supernatant was collected, diluted 1:10 and subjected to cytokine ELISAs (BD Biosciences) according to the manufacturer's instructions to quantify TGF-β and G-CSF secretion. Directly-conjugated

anti-mouse CD47 (eBioscience) or TA-1 culture supernatant was used to determine the surface expression of CD47 and HER-2 respectively, on cultured D2F2/E2 cells by flow cytometry as described above. D2F2/E2 tumors were harvested and collagenase treated for 2 h at 37°C in a shaking incubator. After which, a single cell suspension of D2F2/E2 tumor cells was subjected to flow cytometry for CD47 and HER-2 expression as previously mentioned.

Statistical analysis

Differences between tumor growth curves and spleen sizes groups were analyzed using ANOVA or the Student *t* test, respectively. Values of $p < 0.05$ were considered significant. For survival studies, Kaplan-Meier survival curves were plotted and analyzed. All graphs and statistical calculations were analyzed using Prism software (GraphPad).

RESULTS

D2F2/E2 tumors grow progressively despite high antigen-specific IgG responses

Previous studies have shown that the stable transfection of hHER-2 by 4T1 breast cancer cells and 38C13 B lymphoma significantly reduced the tumorigenicity of the parental tumor cell line and led to spontaneous tumor regression [12, 13]. We have observed a similar phenotype following hHER-2 transfection into 4TO7 cells (data not shown). However, hHER-2 expressing D2F2 tumors (D2F2/E2) grow progressively in BALB/c mice [14]. In our hands, tumors develop despite high tumor-specific IgG levels in the serum (Figure 1A). Upon assessment of specific IgG subclasses, we observed that IgG1 responses were dominant over IgG2a (Figure 1B). IgG1 responses are enhanced during Th2 responses which are typically undesirable in tumor settings [15, 16]. The induction of Th2 responses in the D2F2/E2 model is further supported by the high levels of TGF-β that are secreted by cultured cells *in vitro,* while minimal G-CSF was detected (Figure 1C). TGF-β has been shown to promote Th2 responses in tumor-bearing mice [17]. Taken together these findings suggest that D2F2/E2 tumors induce Th2 responses which must be overcome by a HER-2 targeted vaccination strategy.

Efficacy of humoral immunity is likely limited by CD47 expression or loss of HER-2 expression in vivo

We sought to investigate potential reasons for the lack of protection conferred by high antibody levels in the D2F2/E2 tumor model. Upon characterization of freshly isolated D2F2/E2 tumors, we observed that the expression of CD47, a surface protein that

functions as a “don’t eat me” signal to phagocytes, is maintained *in vivo* with a mean fluorescence intensity of approximately 35 (Figure 2A). The engagement of CD47 with its receptor signal regulatory protein-alpha (SIRPα) on phagocytes such as macrophages has recently been shown to form a barrier which inhibits antibody-mediated tumor cell destruction [18]. Additionally, there has been a correlation reported between CD47 and SIRPα expression in the bone marrow and peripheral blood of breast cancer patients and recurrence [19]. Further examination of D2F2/E2 tumor tissue revealed that approximately half of the original HER-2 expression is lost *in vivo,* with an MFI decrease from 251 to 154 (Figure 2B). This loss of HER-2 antigen is observed clinically with ~1/3 of patients with metastatic recurrence following Trastuzumab treatment no longer having detectable HER-2 amplification [20]. This suggests that tumors which express relatively low levels of HER-2 may no longer be susceptible to antibody-mediated killing thus leading to D2F2/E2 tumor progression.

Expression of membrane-anchored ISMs completely inhibits D2F2/E2 tumor growth

Since we have observed that humoral immunity (Th2 responses) is not protective in this hHER-2 positive tumor model, we investigated whether the expression of Th1 promoting ISMs could skew the induced responses and confer protection. D2F2/E2 cells were stably transfected with cDNA encoding B7-1, GPI-IL-2, GPI-IL-12 or GPI-GM-CSF. Surface expression of the ISMs was verified by flow cytometry analysis (Figure 3A). Mice were then challenged with each transfectant to directly assess the effect of each ISM on the tumorigenicity of D2F2/E2 cells. We observed that mice challenged with the transfectants were completely protected from tumor development while

D2F2/E2 WT tumors grew progressively (Figure 3B). This indicates that the tumorigenicity of this hHER-2 positive cell line can be abolished by the expression of membrane-anchored ISMs. Tumor-free mice were then re-challenged on day 37 with D2F2/E2 WT cells to determine if protective immunity was induced. There was, on average, a week delay in the tumor growth kinetics in mice previously challenged with D2F2/E2 transfectants. Mice previously challenged with D2F2/E2-GM-CSF cells were completely protected from secondary tumor challenge, whereas the tumor-free survival of mice challenged with D2F2/E2-B7-1 and D2F2/E2-IL-12 was 60% and 40% respectively (Figure 3C). GPI-IL-2 expressing D2F2/E2 cells conferred the least protection against the secondary tumor challenge with only 20% of mice being tumor free 70 days post D2F2/E2 WT challenge. This knowledge indicates that the expression of GPI-ISMs inhibits D2F2/E2 tumor growth and induces partial protective immune responses.

Prophylactic vaccination with irradiated D2F2/E2-B7-1 cells co-injected with D2F2/E2-GPI-IL-12 cells completely protects mice against subsequent tumor challenge

Due to the protection that was observed following direct challenge with the D2F2/E2 transfectants, we then wanted to determine whether irradiated cellular vaccines could confer protection in a prophylactic setting. Cellular vaccines were chosen based on observed efficacy in previous preliminary studies (data not shown). Two weeks after vaccination, mice were challenged with D2F2/E2 WT cells. We observed that mice vaccinated with a 1:1 mixture of irradiated D2F2/E2 B7-1 and D2F2/E2 GPI-IL-12 expressing cellular vaccines, Irr-(B7-1+GPI-IL-12), were completely protected against D2F2/E2 WT tumor challenge (Figure 4A), whereas all other vaccinated groups

developed tumors. Interestingly, cellular vaccines expressing GPI-GM-CSF (Irr-GPI-GM-CSF) were not as protective in the prophylactic setting as we had seen in the earlier direct challenge studies with only 20% remaining tumor-free 60 days post challenge. A possible reason for this observation is the disparate responses of myeloid cells within the tumor microenvironment induced by GM-CSF expression locally at the tumor site compared to its distal administration in the form of an irradiated cellular vaccine. Others have reported contrasting responses of GM-CSF-secreting vaccines (GVAX) based on the location of vaccination relative to the tumor site [21].

One day prior to challenge, serum was collected from vaccinated mice for analysis of IgG levels. Consistent with our earlier studies suggesting that IgG antibodies were not protective, vaccinated groups with the highest IgG serum levels prior to tumor challenge, Irr-GPI-GM-CSF and Irr-(B7-1 + GPI-GM-CSF), also had the highest overall tumor incidence (Figure 4B-C). Taken together, this further indicates that protection in this model is not mediated or correlative with the induction of antibody responses and suggests that the expression of GPI-ISMs could act by reducing humoral immunity and promoting cellular immune responses.

Co-injection of irradiated D2F2/E2-B7-1 and D2F2/E2-GPI-IL-12 and IL-12 cellular vaccines induce robust HER-2 specific cytotoxic activity

Since we have observed the protective effects of Irr-(B7-1 + GPI-IL-12) in a prophylactic setting, we investigated the induced cellular immune responses following vaccination. Two weeks post vaccination we observed no difference in the frequency of $CD4^+$ or $CD8^+$ T cells infiltration in the tumor-draining lymph node (data not shown).

Cytotoxic activity of splenocytes from vaccinated mice was assessed following 5-day *in vitro* stimulation with mitomycin-C treated D2F2/E2 cells. HER-2 specific cytotoxicity was determined after 4 h incubation with D2F2/E2 cells. HER-2- negative murine breast cancer cells, D2F2 and 4TO7WT, were used as specificity controls. Our data indicates splenocytes isolated from mice vaccinated with Irr-(B7-1 and GPI-IL-12) are able to kill approximately 50% of D2F2/E2 target cells at an effector to target ratio of 10:1, while there was minimal cytotoxic activity against the HER-2-negative target cell lines, D2F2 and 4TO7WT (Figure 5). Due to the antigen-specificity, the observed cytotoxicity is likely to be mediated by cytotoxic T lymphocytes (CTLs) rather than innate cells such as NK cells which would kill cells in a non-antigen specific manner. Additionally, splenocytes from mice vaccinated with irradiated D2F2/E2 WT cells demonstrated minimal cytotoxic activity against all target cell lines indicating that co-injection of B7-1 and IL-12 expressing cellular vaccines enhances HER-2 specific cellular immunity. These results clearly indicate that Irr-(B7-1 + GPI-IL-12) is able to induce significant levels of cytotoxicity capable of lysing D2F2/E2 tumor cells in a HER-2-specific manner.

Cellular vaccines expressing B7-1 and IL-12 significantly reduced average tumor burden in a therapeutic setting

To determine whether cellular vaccines expressing GPI-ISMs could induce tumor inhibition of established tumors, mice were vaccinated 7 days post tumor challenge. We observed that the Irr-(B7-1+GPI-IL-12) led to a significant reduction in the average tumor burden of challenged mice from 160.8 mm^2 to 25.63 mm^2 (Figure 6A).

Because only partial protection was observed following vaccination in a therapeutic setting, we sought the addition of a suitable adjuvant therapy to be used in combination with our cellular vaccines. Combinatorial therapies are thought to be the most promising strategy to induce optimal and sustainable anti-tumor immune responses. Recent investigations have demonstrated the adjuvant effect of blockade of immunological checkpoints such as CTLA-4 and PD-L1 [22, 23]. To determine the most appropriate therapy for our model, additional analysis of D2F2/E2 tumors was carried out assessing PD-L1 expression which was found to be expressed at substantial levels on cultured cells as well as freshly isolated D2F2/E2 tumors (Figure 6B). Further, we observed a significant up-regulation of PD-1 expression on $CD4^+$ and $CD8^+$ tumor infiltrating lymphocytes (TILs) but not in the spleens of tumor positive mice (Figure 6C) while we only observed a modest increase in intracellular CTLA-4 on TILs (data not shown). As a result of these findings we proceeded with testing the therapeutic efficacy of PD-L1 blockade in this HER-2 breast cancer model system.

Therapeutic vaccination was carried out as mentioned previously using Irr-(B7-1 and GPI-IL-12). Administration of 100 µg of αPD-L1 mAb or rat IgG was given i.p. on days 7, 10 and 13 post tumor challenge. Our data indicates that Irr-(B7-1 + GPI-IL-12) significantly ($p<0.05$) reduced tumor burden from 35.4 mm^2 to 19.6 mm^2, whereas αPD-L1 treatment alone and combined treatment significantly ($p<0.01$) reduced average tumor burden to 4.81 mm^2 and 4.2 mm^2, respectively (Figure 6C-left panel). Interestingly, despite the reduced overall tumor burden in mice treated with αPD-L1 mAbs, all of these mice were tumor-positive by day 13 similar to PBS-treated and Irr-WT vaccinated mice (Figure 6C-right panel). Whereas there was a slight delay in tumor development in mice

given the combined therapy of αPD-L1 mAb and Irr-(B7-1 +GPI-IL-12) relative to vaccine alone. These findings indicate that αPD-L1 treatment minimally enhanced the efficacy of cellular vaccination however, optimization of the dosing and timing schedule of the blocking antibody treatment is likely to enhance its therapeutic efficacy.

DISCUSSION

The identification and characterization of the tumor associated antigen HER-2, has allowed for the development of more targeted therapies such as monoclonal antibodies that recognize different regions of the HER-2 protein, Trastuzumab and Pertuzumab. Even though the induction of tumor-specific immunity is considered essential for optimal therapeutic responses, the type of immunity that is induced is equally important. The balance between Th1 and Th2 responses plays a critical role in the type and efficacy of anti-tumor immune responses that are induced. While Th1 and Th2 responses are capable of inducing potent anti-tumor immune responses, they have been demonstrated to act by distinct mechanisms. Th1 responses promote anti-tumor immunity through cellular immunity and the induction of immunological memory that further promotes CTL activity [16]. Whereas Th2 responses initiate the production of humoral immunity and mediate tumor eradication via necrotic mechanisms [24].

Our data indicates that the dominant subclass of IgG antibodies found in the serum of tumor positive mice is IgG1, which is enhanced following Th2 responses [8]. However, with the expression of GPI-IL-2 and GPI-IL-12 which promote Th1 responses, the tumorigenicity of D2F2/E2 tumor cells was significantly reduced which strongly suggest a deviation from the endogenous Th2 response. However, cytokine profile analysis of isolated T cells from vaccinated mice and further serum isotyping will provide additional evidence for this *in vivo* skewing towards a Th1 phenotype.

Several potential explanations have been investigated as to why antibody responses fail to correlate with protection and, similarly, why the passive administration

of mAbs ultimately leads to the development of resistance for numerous patients. In fact it has been demonstrated that in some cases antibodies can promote tumor growth [25]. One potential means of $CD8^+$ CTL activity suppression is mediated by the formation of IgG-TGF-β complexes that are later endocytosed and processed by macrophages which deliver TGF-β directly to lymphocytes [26]. Additionally, the antibodies that are produced could be ineffective in mediating clearance of the tumor cells either indirectly through the lack of antibody-dependent cellular cytotoxicity (ADCC) or by the inability to directly kill the tumor cells. This can be further inhibited by the expression of CD47 on tumor cells which blocks phagocytosis. Moreover, antibodies must be able to bind its target antigen, thus down-modulation of the target antigen, as we observed, is highly likely to reduce the efficacy of induced humoral immune responses. Because of these and other factors , the induction of HER-2 specific T cell responses have been investigated by several strategies including peptide-specific and DNA-based vaccines [27, 28].

Our cellular vaccines expressing B7-1, GPI-IL-2, GPI-IL-12 or GPI-GM-CSF are well suited for the promotion of Th1 responses. While IL-12 is well known for its ability to directly promote Th1 (cellular) responses [29], it has recently been proposed to "reprogram" tumor infiltrating MDSCs to create an acute inflammatory environment which further enhances the induction of local cellular immunity [30]. Additionally, it has been shown that *in vivo* Th1 responses require the presence of IL-2 [31] and that blocking B7-1 interactions during T cell activation induces inactivation of Th1 cells [32]. Lastly, to a lesser degree, GM-CSF is reported to induce cellular responses indirectly through the activation of macrophages and DCs [33, 34].

Robust cytotoxic activity is generally thought to be essential for tumor rejection in many models [35, 36]. We observed that mice vaccinated with Irr-(B7-1 and GPI-IL-12) were able to induce significant HER-2 specific cytotoxicity that were able to lyse approximately 50% of target D2F2/E2 cells at an effector to target ratio of 10:1 in an antigen-specific fashion.

One of the proposed mechanisms of trastuzumab-mediated efficacy is the down-regulation of HER-2 [37], which is supported by the observation that 1/3 of patients with metastatic recurrence no longer having HER-2 amplification. In order to destroy the remaining tumor cells that now lack the HER-2 overexpression, adjuvant therapies would need to be administered. The PD1/PD-L1 signaling pathway is a critical immunological checkpoint and blockade of this pathway has been well characterized in several viral models including LCMV and HIV [38, 39]. Because PD-L1 is up-regulated by most cancers [40], PD-L1 blockade may serve as a promising therapeutic option. Within the D2F2/E2 model, we found that PD-L1 is constitutively expressed and TILs found within tumor-positive mice expressed significant levels of PD-1. In keeping with those observations, we combined our cellular vaccines expressing B7-1 and GPI-IL-12 with anti-PD-L1 mAb treatment and observed an augmentation in the efficacy of our cellular vaccines.

Taken together, the D2F2/E2 tumor system served as an appropriate HER-2 positive model to assess the non-protective role of humoral immune responses. Moreover, it allowed for the investigation and implementation of a cellular vaccination strategy that was capable of inducing cellular immunity which conferred significant protection in prophylactic and therapeutic settings.

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FIGURE LEGENDS

Figure 1. Th2 responses are induced following D2F2/E2 tumor challenge. Serum was collected from tumor-positive mice and analyzed for A) total IgG or B) IgG subclass-specific antibody production against D2F2/E2 using a cell-ELISA. C) Supernatant from $2x10^5$ D2F2/E2 cells cultured for 48 h was collected and subjected to ELISA for quantification of TGF-β and G-CSF secretion.

Figure 2. CD47 expression and loss of HER-2 expression in vivo may limit efficacy of humoral immunity. D2F2/E2 cells maintained in culture (*in vitro* cultured cells) and tumors harvested from D2F2/E2 mice (tumor-derived) were assessed for the expression of mCD47 and hHER-2 by flow cytometry analysis.

Figure 3. GPI-ISM expression completely abolishes the tumorigenicity of D2F2/E2 cells and induce partial protective immune responses following secondary tumor challenge. A) Surface expression of B7-1, GPI-IL-2, GPI-IL-12, GPI-GM-CSF or hHER-2 on D2F2/E2 cell transfectants. B) Mice were directly challenged with $2x10^5$ live D2F2/E2 WT cells or transfectants expressing B7-1, GPI-IL-2, GPI-IL-12 or GPI-GM-CSF. C) Tumor-free mice were subjected to a secondary challenge with $2x10^5$ live D2F2/E2 WT cells on day 37. Tumor growth and incidence was monitored.

Figure 4. Prophylactic vaccination with irradiated D2F2/E2-B7-1 cells co-injected with D2F2/E2-GPI-IL-12 cells leads to complete protection against subsequent

D2F2/E2 tumor challenge. Two weeks after vaccination (s.c.) with irradiated (Irr) cellular vaccines expressing GPI-ISMs ($2x10^5$) mice were challenged (s.c.) with $2x10^5$ live D2F2/E2 wild-type cells on the opposite hind flank. A) Average tumor growth and B) tumor incidence was assessed. C) Serum was collected from vaccinated mice 1 day prior to challenge to assess for total IgG antibodies against D2F2/E2 cells by a cell ELISA. Significance relative to naive (**) $p<0.01$

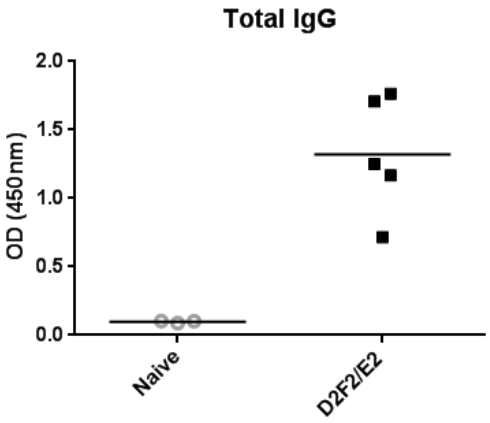
Figure 5. HER-2 specific cytotoxic activity is induced following co-injection of B7-1 and GPI-IL-12 expressing cellular vaccines. Splenocytes isolated two-weeks post vaccination with irradiated D2F2/E2 WT (Irr-WT) or D2F2/E2 B7-1 + D2F2/E2 GPI-IL-12, Irr-(B7-1 + GPI-IL-12) were re-stimulated *in vitro* for 5 days with mitomycin-treated (50μg/mL) D2F2/2 as stimulator cells at a ratio of 5:1. Stimulated splenocytes were cultured with D2F2/E2, D2F2 or 4TO7WT cells for 4 h at indicated effector: target (E:T) ratios. Percentage of specific lysis was quantified by LDH release using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Mean ±SEM is plotted.

Figure 6. GPI-ISM expression by irradiated cellular vaccines alone or in combination with PD-L1 blockade reduced average tumor burden of day 7 established D2F2/E2 tumors. A) Mice were challenged (s.c.) with $2x10^5$ live D2F2/E2 wild-type cells. One week later, mice received one dose of $2x10^5$ irradiated cellular vaccines on the opposite flank (s.c.). Tumor growth was monitored. B) Expression of PD-L1 on D2F2/E2 cells maintained in culture (*in vitro* cultured cells) and tumors harvested from D2F2/E2 mice (tumor-derived) were assessed by flow cytometry analysis.

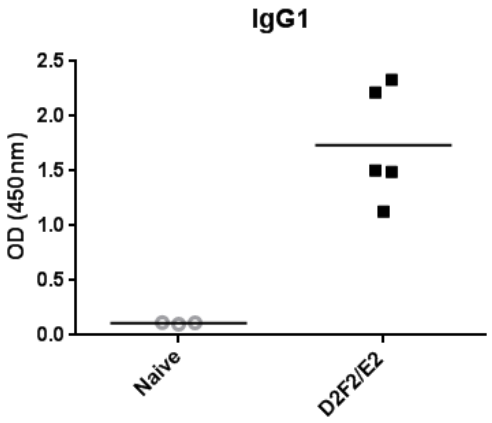
Tumor infiltrating lymphocytes (TILs) were isolated from D2F2/E2 tumors and analyzed for the expression of PD-1 by $CD4^+$ and $CD8^+$ T cells by flow cytometry. C) Mice were vaccinated (s.c.) with a single dose of Irr-(B7-1+GPI-IL-12) on day 7 post tumor challenge. Additional mice were treated (i.p.) with α-PD-L1 or rat IgG (100μg) on days 7, 10 and 13 post tumor challenge either alone or in combination with the cellular vaccine. Mean ±SEM is plotted. Significance relative to PBS-treated (*) $p<0.05$, (**) $p<0.01$

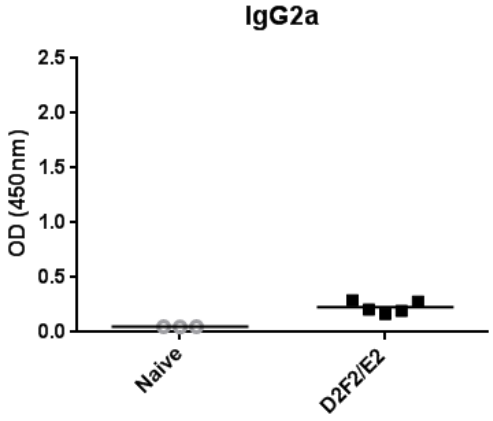
FIGURE 1

A


Total IgG
OD (450nm)
2.0
1.5
1.0
0.5
0.0
Naive
D2F2/E2

B


IgG1
OD (450nm)
2.5
2.0
1.5
1.0
0.5
0.0
Naive
D2F2/E2


IgG2a
OD (450nm)
2.5
2.0
1.5
1.0
0.5
0.0
Naive
D2F2/E2

C

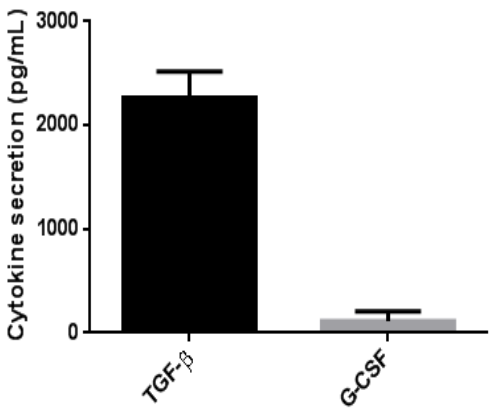

Cytokine secretion (pg/mL)
3000
2000
1000
0
TGF-β
G-CSF

FIGURE 2

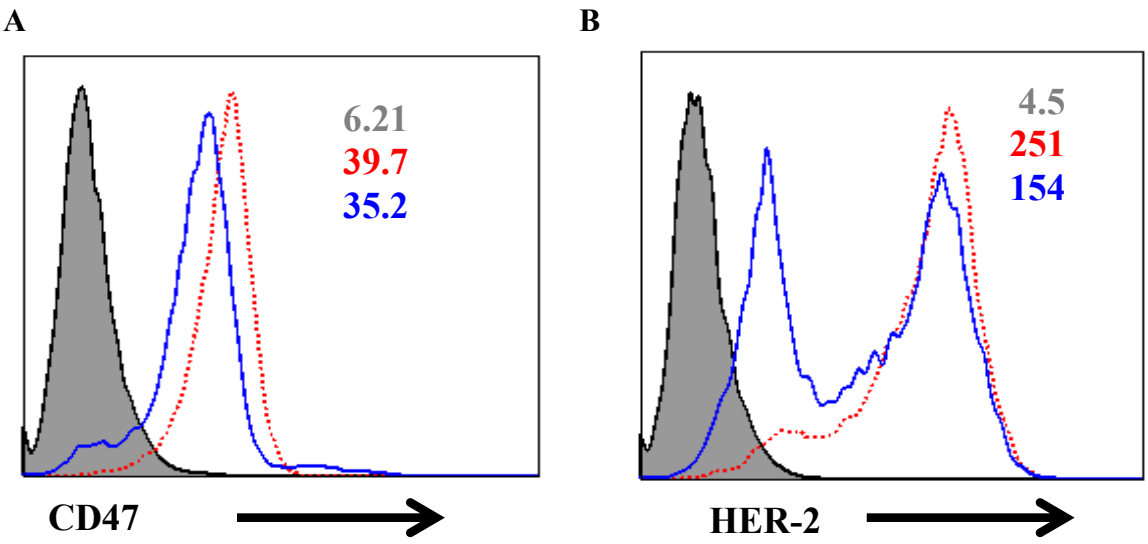
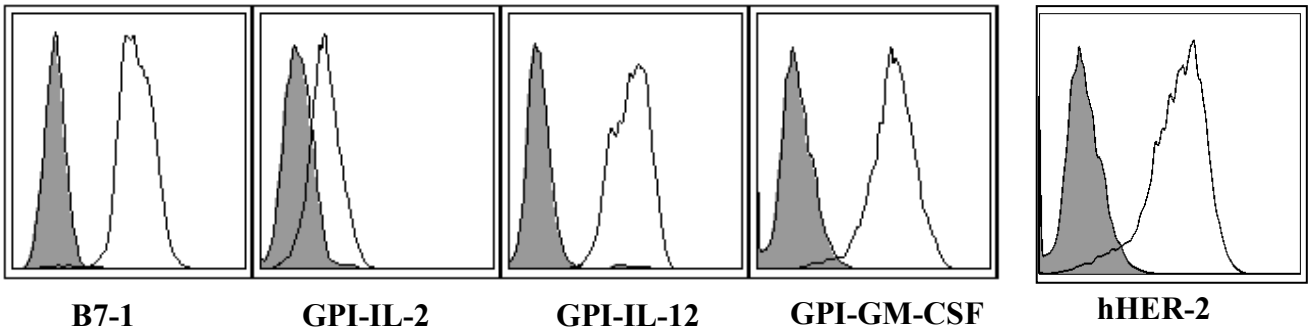
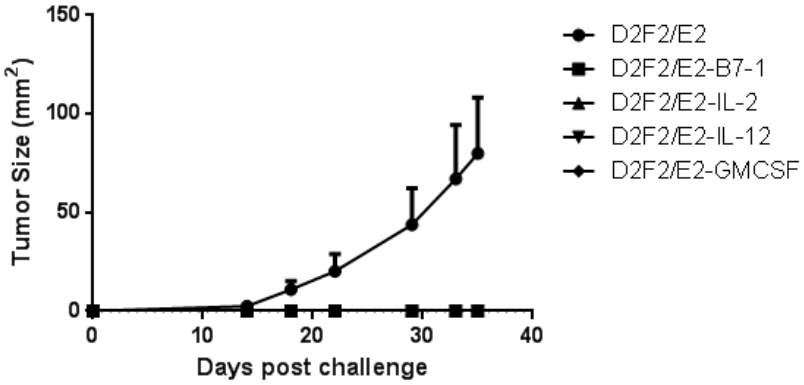

A
B
6.21
39.7
35.2
4.5
251
154
CD47
HER-2
Isotype
In vitro Cultured Cells
Tumor-Derived

FIGURE 3

A


B7-1
GPI-IL-2
GPI-IL-12
GPI-GM-CSF
hHER-2

B


Tumor Size (mm2)
150
100
50
0
0
10
20
30
40
Days post challenge
D2F2/E2
D2F2/E2-B7-1
D2F2/E2-IL-2
D2F2/E2-IL-12
D2F2/E2-GMCSF

C

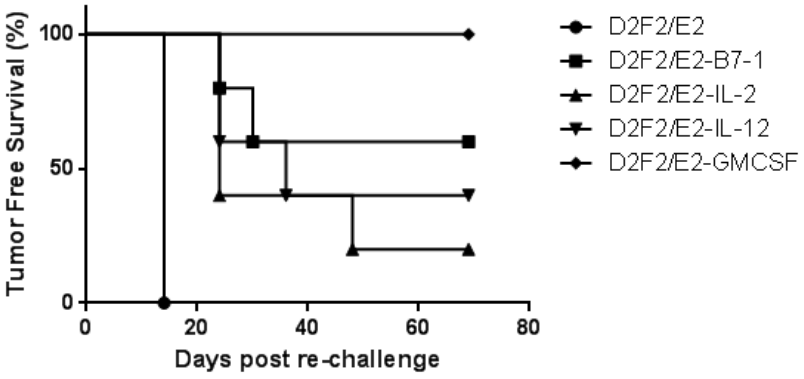
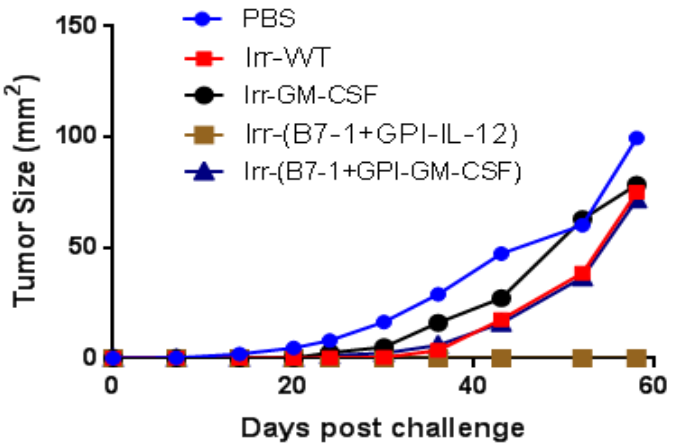
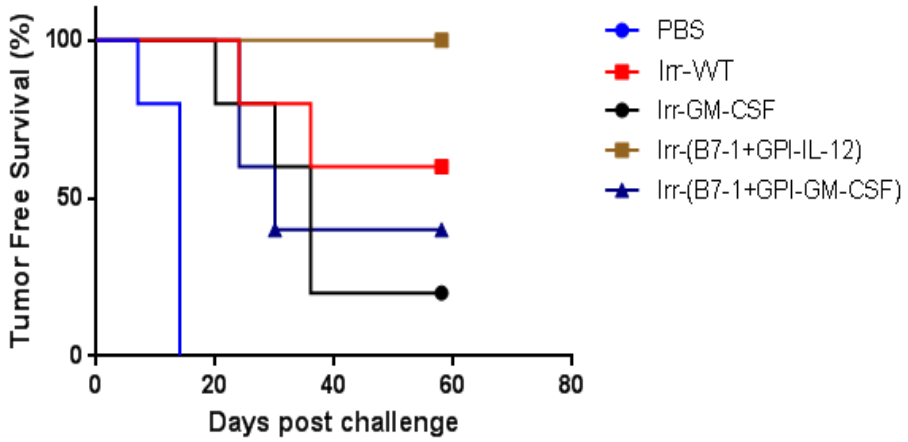

Tumor Free Survival (%)
100
50
0
0
20
40
60
80
Days post re-challenge
D2F2/E2
D2F2/E2-B7-1
D2F2/E2-IL-2
D2F2/E2-IL-12
D2F2/E2-GMCSF

FIGURE 4

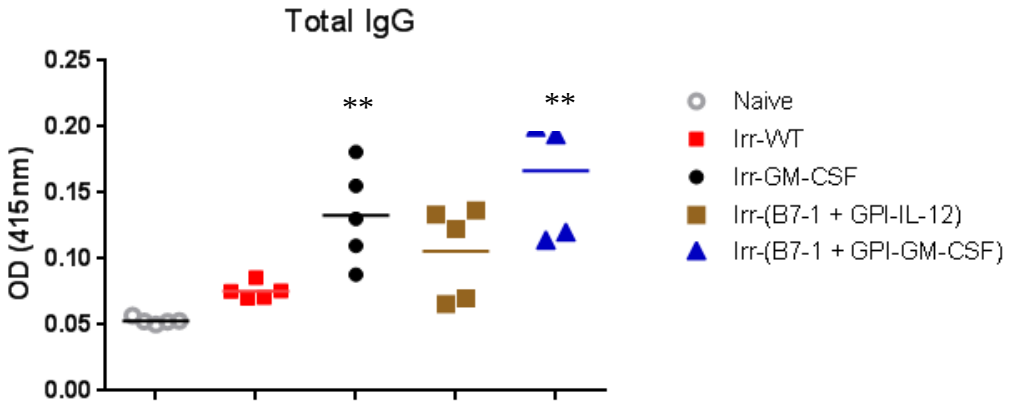
A


PBS
Irr-WT
Irr-GM-CSF
Irr-(B7-1+GPI-IL-12)
Irr-(B7-1+GPI-GM-CSF)
Tumor Size (mm2)
150
100
50
0
0
20
40
60
Days post challenge

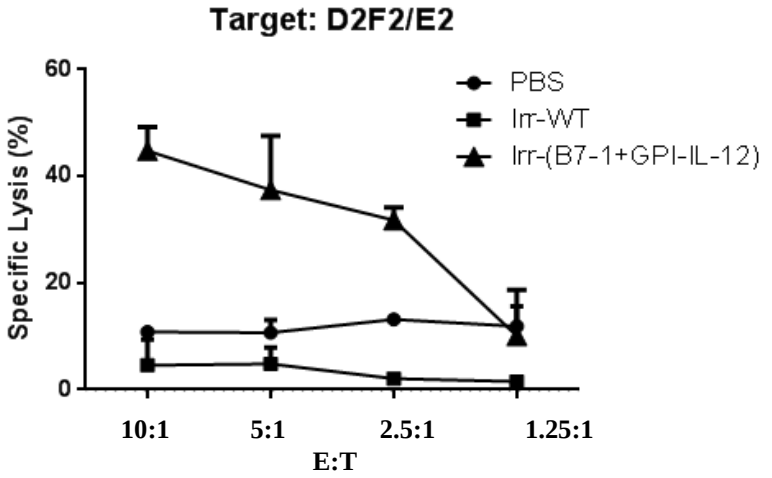
B

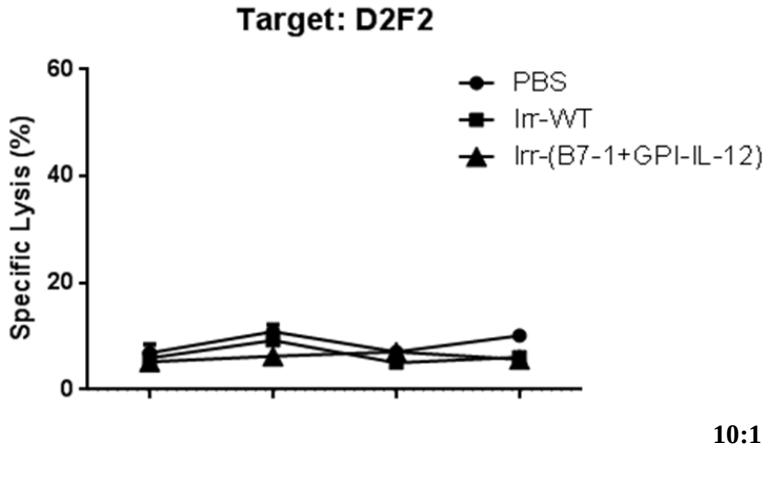

Tumor Free Survival (%)
100
50
0
0
20
40
60
80
Days post challenge
PBS
Irr-WT
Irr-GM-CSF
Irr-(B7-1+GPI-IL-12)
Irr-(B7-1+GPI-GM-CSF)

C


Total IgG
OD (415nm)
0.25
0.20
0.15
0.10
0.05
0.00
**
**
Naive
Irr-WT
Irr-GM-CSF
Irr-(B7-1 + GPI-IL-12)
Irr-(B7-1 + GPI-GM-CSF)

F IGURE 5


Target: D2F2/E2
PBS
Irr-WT
Irr-(B7-1+GPI-IL-12)
Specific Lysis (%)
60
40
20
0
10:1
5:1
2.5:1
1.25:1
E:T


Target: D2F2
PBS
Irr-WT
Irr-(B7-1+GPI-IL-12)
Specific Lysis (%)
60
40
20
0
10:1
5:1
2.5:1
1.25:1
E:T

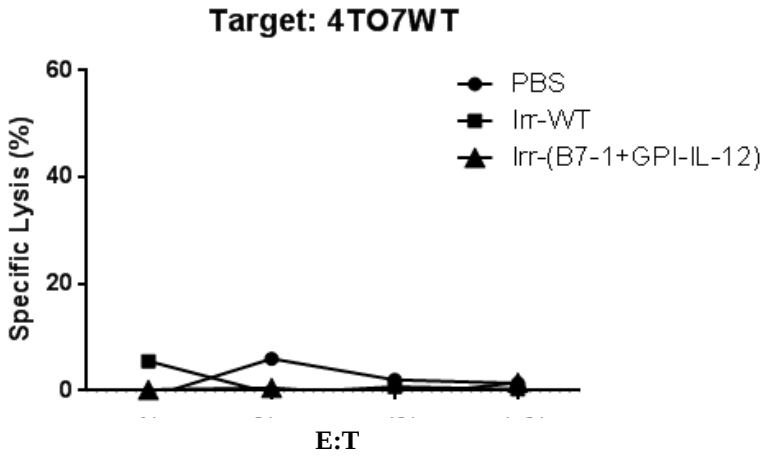
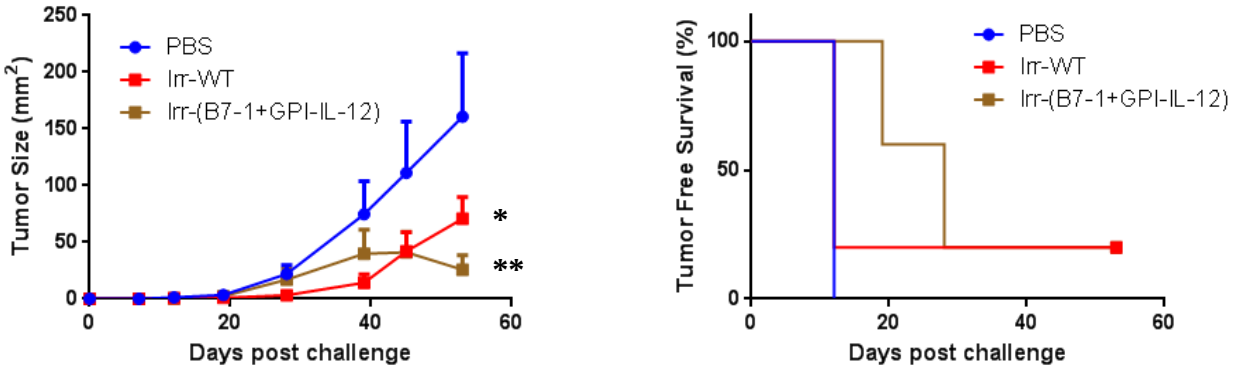
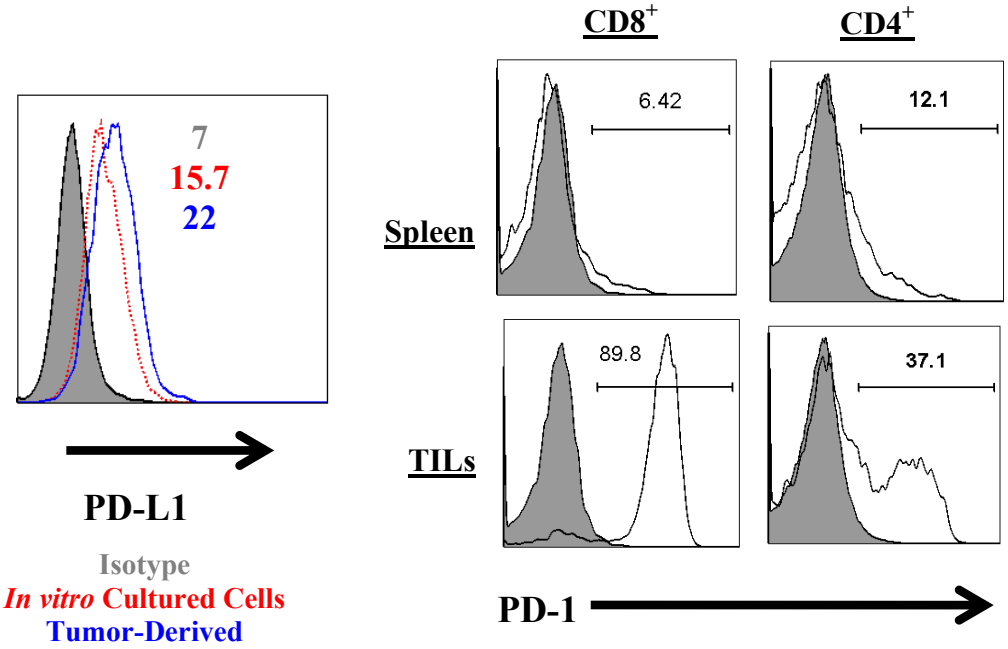

Target: 4TO7WT
PBS
Irr-WT
Irr-(B7-1+GPI-IL-12)
Specific Lysis (%)
60
40
20
0
E:T

FIGURE 6

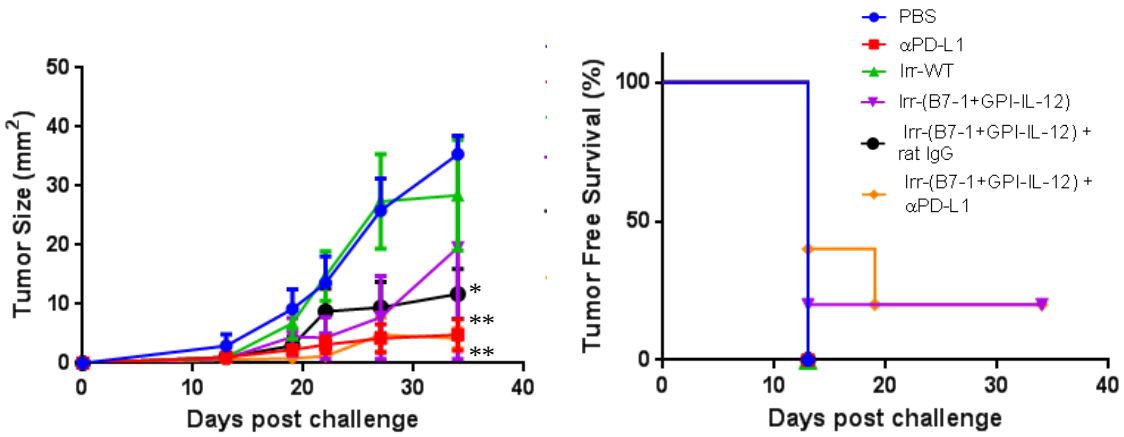
A


PBS
Irr-WT
Irr-(B7-1+GPI-IL-12)
Tumor Size (mm2)
Days post challenge
*
**
Tumor Free Survival (%)

B


CD8+
CD4+
Spleen
TILs
6.42
12.1
89.8
37.1
7
15.7
22
PD-L1
PD-1
Isotype
In vitro Cultured Cells
Tumor-Derived

C


PBS
αPD-L1
Irr-WT
Irr-(B7-1+GPI-IL-12)
Irr-(B7-1+GPI-IL-12) + rat IgG
Irr-(B7-1+GPI-IL-12) + αPD-L1
Tumor Size (mm2)
Days post challenge
Tumor Free Survival (%)
*
**
**

CHAPTER IV

Ukrain, a plant-derived semi-synthetic compound, exerts anti-tumor effects against murine and human breast cancer and induce protective anti-tumor immunity in mice

The data (Figures 1-6), as presented in this Chapter, has been published in *Experimental Oncology*, 2012; 34 (4): 340-347. PMID: 23302993

Figure 7 is unpublished data.

All figures in Chapter IV are based on data generated by the Ph.D. candidate.

ABSTRACT

Despite the recent advances in anti-cancer therapies, breast cancer accounts for the highest percentage of estimated new cases among female cancer patients. The anti-cancer drug Ukrain, a plant-derived semi-synthetic compound, has been shown to be effective in a variety of tumor models including colon, brain, ovarian, melanoma and lymphoma. However, the direct cytotoxic effects of Ukrain have yet to be investigated in breast cancer models. Herein, we investigated the *in vitro* and *in vivo* cytotoxicity of Ukrain using murine (4TO7 and TUBO) and human (SKBR-3) breast cancer cell lines. Cells were treated with varying concentrations of Ukrain for up to 72 h and analyzed for viability by trypan blue exclusion, apoptosis by intracellular caspase 3 and Annexin V staining, and proliferative potential by a clonogenic assay. Female BALB/c mice were challenged subcutaneously (s.c.) with 4TO7-RG cells and administered 5mg/kg or 12.5mg/kg body weight Ukrain intravenously (i.v.) on the same day and 3 days later. Protective immune responses were determined following re-challenge of tumor-free mice 35 days post primary challenge. Ukrain exposure induced apoptosis in a dose and time-dependent manner with 50 μg/mL Ukrain leading to >50% cell death after 48 h exposure for all three breast cancer cell lines. Ukrain administration (12.5mg/kg) led to significant inhibition of 4TO7 tumor growth *in vivo* and sustained protective anti-tumor immunity following secondary challenge. Our findings demonstrate the *in vitro* and *in vivo* cytotoxic effects of Ukrain on breast cancer cells and may provide insight into designing Ukrain-based therapies for breast cancer patients.

INTRODUCTION

Cancer is among the leading causes of mortality in the United States, second only to heart diseases [1]. While the mortality rates associated with many other diseases have seen a significant decline in the past 50-60 years, a similar reduction has yet to be seen in the cancer field [2]. These statistics speak to the overwhelming necessity to develop more effective treatment options for cancer patients. Closer examination of these statistics contribute to the high mortality reveals that breast cancer is the second leading cause of cancer-related deaths among women and accounts for the highest percentage of estimated new cancers among female cancer patients [3]. While several treatment options are currently available to cancer patients such as surgery, chemotherapy and radiation therapy either alone or in an adjuvant setting, each approach is associated with a wide range of clinical challenges. The most significant drawback to chemotherapeutic drugs, the most widely used anti-cancer treatment option, is their toxicity towards non-malignant cells [4].

The anticancer drug Ukrain (NSC 631570), a semisynthetic compound derived from the extract of the plant *Chelidonium majus* L., has been shown to exert selective cytotoxic effects towards a variety of malignant cells including colon, brain, ovarian, melanoma and lymphoma without harmful side effects on healthy human cells[5, 6]. The cytotoxic selectivity of Ukrain is thought to be based on the differential membrane potentials of malignant cells and normal cells. It has been reported that the positive charge of the celandine alkaloids comprising the Ukrain allows malignant cells to absorb more Ukrain than healthy cells [7].

To date several clinical trials have assessed the efficacy of Ukrain in patients with an assortment of malignancies such as colorectal, pancreatic, bladder and breast cancers [8]. In each of these trials, patients treated with Ukrain had a more favorable clinical response compared to control groups (placebo or other therapies) which included either longer survival [9], extended periods of non-progression[10] and/or symptomatic improvements [11]. Previously, two groups [12, 13] have assessed the potential effects of Ukrain in breast cancer models citing that Ukrain fails to enhance the radiosensitivity of MDA-MB-231 human breast cancer cells and Ukrain treatment can enhance the cytolytic activity of peritoneal exudate macrophages towards DA-3 mammary tumor cells. However, to the best of our knowledge, a thorough investigation of the direct cytotoxic effects of Ukrain on a variety of breast cancer cells has yet to be reported.

Herein, we demonstrate the *in vitro* effectiveness of Ukrain to selectively kill both mouse and human breast cancer cells. Additionally *in vivo* experiments indicate that Ukrain can induce tumor inhibition in a breast cancer model and for the first time that Ukrain can induce protective anti-tumor immunity. Taken together, our findings provide evidence of the effectiveness of Ukrain in breast cancer models.

MATERIALS AND METHODS

Cell Lines

The 4TO7 cell line, a non-metastatic clone derived from a spontaneous adenocarcinoma in BALB/cfC3H mice (410.4), was a kind gift from Fred Miller (Wayne State University) [14]. While cells can be recovered from the lungs of mammary pad inoculated mice, 4TO7 fails to colonize the lungs and form metastatic nodules [15]. TUBO cells, derived from a spontaneous carcinoma in BALB-neuT mice [16], are also non-metastatic and were provided by John C. Morris (National Institutes of Health). The 4TO7, TUBO and NIH 3T3 (mouse embryonic fibroblasts) cell lines were cultured in DMEM F12 media, supplemented with 10% cosmic calf serum (Hyclone, Logan, Utah) and 1% penicillin streptomycin (Mediatech, Manassas, VA). The human breast cancer cell line, SKBR-3, was cultured in RPMI 1640 media supplemented with 10% cosmic calf serum and 1% penicillin streptomycin and was obtained from American Type Culture Collection (ATCC, Manassas, VA). The TUBO and SKBR-3 cell lines constitutively express the HER-2/neu oncogene, which is overexpressed in 30% of human breast cancer patients. All cells were maintained at 37^{o}C in a humidified 5% $C0_2$ incubator.

Mouse Model

Female BALB/c mice (8-12 week old) were purchased from Jackson Laboratories (Bar Harbor, Maine) and used for all *in vivo* studies. Mice were housed and maintained

according to institutional guidelines and protocols approved by the Institutional Animal Care and Use Committee (IACUC) for Emory University in Atlanta, GA.

Reagents

Ukrain was kindly provided by Dr. Wassil J. Nowicky and Stephen Karoly from Nowicky Pharma (Vienna, Austria). The stock solution was supplied at a concentration of 1mg/mL. Apoptosis was assessed using the Annexin V-FITC Apoptosis Detection Kit (Sigma, St. Louis, MO), BD Cytofix/Cytoperm Fixation and Permeabilization solution and FITC rabbit anti-active caspase-3 antibody (BD Pharmingen, La Jolla, CA). Antibodies used to phenotype isolated splenocytes by flow cytometry included anti-CD4-FITC, anti-CD8-FITC, anti-B220-PE, anti-CD11b-FITC, anti-Gr-1-PE and CD16/32 Fc-block (eBioscience, San Diego, CA).

In vitro drug treatment assay

Murine and human breast cancer cells were seeded at a density of $5x10^4$ cells per well in 24-well plates (in duplicate) in appropriate culture media. After being allowed to adhere, tumor cells were treated with 0, 25, 75, 100 µg/mL Ukrain for 24, 48 and 72 h. At the specified time point, cells were analyzed for viability, apoptosis, and clonogenic potential. As specificity controls, concanavalin A (ConA)-stimulated mononuclear cells, isolated from the spleens of naive BALB/c mice and recovered by Ficoll-Hypaque density centrifugation, and NIH 3T3 cells were treated with Ukrain at the aforementioned concentrations for 24, 48, and 72 h and cellular viability was subsequently assessed by the trypan-blue exclusion method.

Detection of apoptosis using Annexin V, PI staining

Ukrain-treated cells were washed once with PBS, re-suspended in 1X Binding Buffer and Annexin V-FITC (5μL) and Propidium Iodide-PE (10μL) (Sigma, St. Louis, MO) was added to each cell suspension. Cells were incubated for 10 minutes at room temperature in the dark. Staurosporine, 2.5μM, (Sigma) was used as a positive control for apoptosis induction. Stained cells were analyzed immediately using a FACSCaliber (Beckman Coulter, Fullterton, CA) and FlowJo software.

Quantification of active caspase-3

Following Ukrain treatment, cells were washed, fixed and permeabilized for 20 min at 4^{o}C using BD cytofix/cytoperm solution (BD Pharmingen). Permeabilized cells were washed and stained for intracellular caspase-3 using the FITC rabbit anti-active caspase-3 antibody (clone C92-605) for 30 min at 4^{o}C in the dark (BD Pharmingen). Cells were then analyzed using flow cytometry as described previously.

Clonogenic Assay

The long-term effects of Ukrain treatment on murine and human breast cancer cells were assessed using a clonogenic assay. Following 24, 48 and 72 h treatment with Ukrain, cells were plated in 60 mm tissue culture plates at 100 cells/plate. Cells were allowed to form colonies for two weeks. The colonies were then gently washed with PBS, fixed and stained with crystal violet (0.5% w/v in 90% ethanol) and counted by light

microscopy. The surviving fraction of colonies was calculated as previously described [17].

Tumor growth inhibition studies

4TO7 cells were passaged 4 times *in vivo* to obtain reproducible tumor growth with palpable tumor development within 6 days (4TO7RG). To assess the *in vivo* cytotoxicity of Ukrain in a mouse model, female BALB/c mice were challenged subcutaneously (s.c.) with $5x10^4$ 4TO7RG cells on the shaved right hind flank. Mice were then administered 5mg/kg or 12.5mg/kg body weight Ukrain via tail vein injection (i.v.) on the same day. The mice were then administered an additional dose of Ukrain 3 days later. Tumors were measured 2-3 times a week using a Vernier caliper. Mice were sacrificed once the tumors became ulcerated or exceeded 1 cm^2 in diameter. At the conclusion of the experiment (day 35), tumor-free mice were re-challenged with $5x10^4$ 4TO7RG cells and monitored for tumor growth for an additional 35 days to determine whether Ukrain is capable of inducing protective anti-tumor immunity.

Therapeutic administration of 12.5mg/kg Ukrain was administered i.v. on days 1 and 3 post tumor challenge. Mice were then vaccinated subcutaneously (s.c.) with $2x10^5$ irradiated (80Gy) 4TO7 cellular vaccines expressing the immune stimulatory molecules B7-1 and GPI-IL-12 on day 7 post challenge as previously described [18].

Cellular phenotyping of splenocytes

Spleens were harvested and weighed from Ukrain treated mice on day 35 following re-challenge with 4TO7RG cells. Single cell suspensions were prepared and

red blood cells were removed by hypotonic lysis. Splenocytes were then washed in FACS buffer (PBS/1%CCS/1%EDTA), incubated in Fc block (CD16/32, clone 2.4G2) and stained for 25 minutes at 4^{o}C with the appropriate antibodies to detect T cells (CD4 and CD8), B cells, and myeloid derived suppressor cells (MDSCs). Antibodies used for analysis were anti-CD4-FITC, anti-CD8-FITC, anti-B220-PE, anti-CD11b-FITC, anti-Gr-1-PE. Sample readings were collected using a FACSCaliber and analyzed with FlowJo software.

Statistical analysis

All *in vitro* experiments were performed in duplicate or triplicate and were repeated at least twice. Differences between caspase-3 activation following Ukrain treatment as well as tumor growth curves were assessed using ANOVA analysis. For survival curves, *P* values were determined using the Log-rank (Mantel–Cox) test. $P < 0.05$ was considered statistically significant. All graphs and statistical calculations were done using GraphPad Prism software (GraphPad Software Inc., San Diego).

RESULTS

Ukrain exhibits cytotoxic effects specifically on breast cancer cells not non-malignant cells

Human and murine breast cancer cells were treated with 25, 50, 75 and 100 µg/mL Ukrain for 24, 48, and 72 h. At each time-point, viability was determined using the trypan-blue exclusion method. We began to observe morphological changes of all three breast cancer cell lines after 48 hours of Ukrain exposure. The Ukrain-treated cells began to shrink in size, undergo membrane blebbing, loose cell-to-cell contact and adherence to the tissue culture plate (Figure 1A). Additionally, we observed a dose and time-dependent decrease in viability with 50 µg/mL Ukrain leading to 50% cell death after 48 h exposure for all three breast cancer cell lines tested (Figure 1B). Following 72 h Ukrain exposure at 100 µg/mL maximal cell death was observed with the viability of 4TO7, TUBO and SKBR-3 cells being 12.9%, 17.53% and 30.8% respectively. As specificity controls, mouse fibroblasts (NIH 3T3 cells) and ConA-stimulated mononuclear cells were treated with similar doses of Ukrain. We observed minimal cytotoxic effect of Ukrain on mouse fibroblast cells (Figure 1C) and ConA-stimulated lymphocytes (data not shown) even at higher exposure doses with the viability remaining above 90%. These findings indicate that the cytotoxic effects of Ukrain are specific to the breast cancer cells while not affecting non-malignant cells.

Ukrain treated tumor cells fail to regain proliferative capacity

To determine whether Ukrain treated cells have a reduced ability to proliferate, a clonogenic assay was conducted. Following Ukrain treatment for 24, 48 or 72h, cells were washed and replated in fresh culture media lacking Ukrain. After two weeks of culture, colonies were washed, stained with crystal violet and counted by light microscopy. The surviving fraction is defined as the number of colonies derived from pre-treated tumor cells following two week removal of Ukrain expressed in terms of plating efficiency as previously described [17]. The pre-treated cells showed a reduced ability to form colonies relative to untreated cells (Figure 2). Following 24h pre-treatment with 50 µg/mL Ukrain, 4TO7, TUBO and SKBR-3 tumor cells exhibited a comparable inability to form colonies with surviving fractions of 0.0024, 0.0039 and 0.0019 respectively. Minimal differences were observed in the surviving fractions of 4TO7 and TUBO cells exposed to 50 µg/mL Ukrain for 24, 48 or 72 h. However, 72h exposure to 50 µg/mL Ukrain led to a 10-fold reduction in the surviving fraction of SKBR-3 cells (0.000135) relative to 24h and 48h from exposure from 0.0019 and 0.001315, respectively. Both murine and human breast cancer cells that were pre-treated with Ukrain for 72h with 100 µg/mL Ukrain, were unable to form any visible colonies (denoted by †). This data illustrates the long-term effects of Ukrain treatment on the clonogenic potential of breast cancer cells and indicates that upon removal of Ukrain, the treated tumor cells are unable to recover from the drug's cytotoxic effects.

Ukrain exerts anti-tumor activity through apoptosis induction

Ukrain has previously been shown to induce apoptosis of Jurkat T cell lymphoma through mitochondrial membrane depolarization and caspase activation [19] and HeLa

cervical cancer cells through the activation of the intrinsic pathway (caspase-9 cleavage) [20] . Therefore to investigate whether the observed cytotoxic effects of Ukrain on breast cancer cells are due to the induction of apoptosis we carried out an Annexin-V binding assay as well as intracellular staining for active caspase-3. As shown in Figure3A, there was a 10-15 fold increase in activated caspase-3 among 4TO7 and TUBO cells and a 2-3 fold increase among SKBR-3 within 48 h of drug exposure. Similarly, 80-90% of the Ukrain-treated tumor cells were apoptotic as indicated by AnnexinV/PI dual staining after 48h (Figure 4A). The kinetics of apoptosis induction indicates a dose and time dependent increase in activated caspase 3 and Annexin V/PI staining (Figures 3B and 4B). Our findings indicate apoptosis as the cytotoxic mechanism of action which is consistent with previous reports in other tumor model systems [19-22].

Systemic administration of Ukrain significantly reduces breast cancer tumor growth in mice

To assess the in vivo efficacy of Ukrain in causing tumor inhibition in a murine breast cancer model, mice were challenged with $5x10^4$ 4TO7RG cells (s.c.) and administered varying doses of Ukrain (5mg/kg or 12.5mg/kg) on the same day (i.v.). Three days later, mice received an additional dose of Ukrain (i.v.). It has previously been reported that following subcutaneous or intraperitoneal injection of Ukrain, no inhibitory effect on the growth of established DA-3 mammary adenocarcinoma was observed whereas significant inhibition was observed following intravenous Ukrain administration [12]. We observed that treatment with two doses of 12.5mg/kg Ukrain led to a significant reduction in overall tumor burden by day 31 relative to untreated mice with an

average tumor burden of 22.7 mm^2 compared to 78.9 mm^2 for untreated mice (Figure5A). While all mice had palpable tumors by day 7 post challenge, we began to observe the effects of the Ukrain treatment regimes on day 14 post challenge (11 days after the last Ukrain administration). The mice that received 5mg/kg Ukrain, on average, also had smaller tumors that developed more slowly than the untreated mice with a mean tumor burden of 47.7 mm^2. By the end of the experiment, tumors in several mice treated with 5mg/kg and 12.5mg/kg had completely regressed with tumor incidences being 60% and 40%, respectively (Figure 5B). Importantly, we observed no visible signs of morbidity such as weight loss among the Ukrain treated groups (Figure 5C). Interestingly, when the Ukrain dose was increased to 20mg/kg, the treated mice failed to be protected and developed progressive tumor growth similar to the untreated mice (data not shown). While 25mg/kg Ukrain has been reported to effectively inhibit metastases of murine Lewis lung carcinoma [23], our observation suggests that similarly high doses of Ukrain may not be effective *in vivo* in breast cancer models. Taken together, these findings indicate that moderate doses of Ukrain can inhibit tumor progression and induce tumor regression in a highly tumorigenic mouse breast cancer model.

Ukrain treatment induces protective immunity

Next, to determine whether Ukrain administration can provide protection against a secondary tumor challenge, thirty-five days post initial challenge, tumor-free mice from each Ukrain-treated group were re-challenged with $5x10^4$ 4TO7RG cells. We observed that all mice remained tumor free up to day 35 post re-challenge indicating that protective immunity was induced following Ukrain treatment (Figure 6A).

Interestingly, we observed that untreated, tumor-bearing mice developed significant splenomegaly (1.16g) relative to the Ukrain treated mice (0.1g) whose spleens were comparable in size and weight to naïve, unchallenged mice (Figure6B, photograph). To gain insight into potential immune modulatory effects of Ukrain that could be responsible for the observed protective immunity, we conducted cellular phenotyping of cells that were found within the spleens of Ukrain treated mice. Previous reports have shown that splenomegaly in the 4T1 mouse breast cancer model, derived from the same parental clone as 4TO7, to be associated with an increased infiltration of myeloid derived suppressor cells (MDSCs) [24]. Our observations indicate that in the 4TO7 tumor model system, wild-type challenged mice develop a similar splenomegaly which correlates with a 12-15 fold increase in the percentage of $CD11b^{+}Gr\text{-}1^{+}$ MDSCs relative to the Ukrain treated, tumor-free mice (Figure 6C). Interestingly, when we compared the percentage of MDSCs in naïve mice to the Ukrain-treated, tumor-free mice, we noticed a 2-3 fold decrease in the splenic population of these cells. Taken together, these studies demonstrate that Ukrain treatment results in persistent protective immunity in a highly immunosuppressive and tumorigenic breast cancer model.

Cellular vaccination enhances tumor inhibition induced following Ukrain treatment

We have previously reported that vaccination with irradiated 4TO7 cellular vaccines co-expressing the costimulatory molecule B7-1 and the Th1-promoting cytokine IL-12 is effective in reducing overall tumor burden and tumor incidence following 4TO7RG tumor challenge [18]. Because we observed that Ukrain was effective as a monotherapy against 4TO7RG tumor challenge, we then evaluated whether combining

Ukrain with an irradiated 4TO7 cellular vaccine co-expressing B7-1/GPI-IL-12 could lead to more complete protection in a therapeutic setting. Following tumor challenge, mice were administered two doses of Ukrain (12.5mg/kg) on days 1 and 3 either alone or in combination with the irradiated cellular vaccine on day 7. We observed significant tumor inhibition in mice receiving combined Ukrain treatment and vaccination relative to PBS-treated mice with average tumor sizes of 6.4mm^2 and 43.5 mm^2, respectively (Figure 7A). However, there was no significant difference in average tumor burden in mice vaccinated with the irradiated 4TO7 cellular vaccine alone and the combined treatment group. Due to severe tumor ulceration, PBS-treated mice had to be sacrificed on day 21 post tumor challenge. Tumor incidence was decreased to 40% in the combined treatment group, while 80% of mice treated with Ukrain alone developed tumors. Taken together, this data indicates that, within a therapeutic setting, cellular vaccination can serve to augment the anti-tumor responses elicited following Ukrain treatment leading to greater protection as evidenced by smaller tumors and a reduction in overall incidence.

DISCUSSION

The anticancer drug Ukrain has been assessed for potential clinical efficacy in Europe in patients suffering from colorectal, pancreatic, bladder and breast cancers as reviewed in [8]. I*n vitro* studies have demonstrated the anti-proliferative and cytotoxic effects of Ukrain in a wider variety of tumor models including colon, brain, ovarian, Ewing, melanoma, lymphoma and glioblastoma [5, 25-28]. The property that distinguishes Ukrain from typical therapies, such as radiation therapy and chemotherapy, lies in its unique ability to specifically target and kill malignant cells while leaving healthy cells unharmed [5]. However, the direct anti-tumor effects of Ukrain in breast cancer models remain obscure. Therefore, in this report we investigated the *in vitro* and *in vivo* efficacy of Ukrain in murine and human breast cancer models. Consistent with previous reports in other tumor models, we observed that Ukrain induces apoptosis of breast cancer cells through caspase 3 activation. Additionally, after a short exposure time, 24h, Ukrain treated cells have reduced clonogenic potential in the absence of the drug and are unable to form colonies following longer exposure (72h) at 100µg/mL Ukrain. Most importantly, *in vitro* cytotoxicity is translatable *in vivo* in the form of significant tumor inhibition and regression. Interestingly, we observed that Ukrain is also capable of inducing protective anti-tumor immune responses which is consistent with clinical observations of patients achieving sustained/long-term remissions for years following Ukrain treatment [29-31].

At present, the mechanism(s) responsible for these protective anti-tumor responses induced by Ukrain remain unclear. However, it is possible that Ukrain could

potentially be working through two related mechanisms: 1) direct cytotoxic effects on tumor cells leading to a significant reduction in overall tumor burden and 2) as a result of the direct killing of tumor cells, antigen-presenting cells such as dendritic cells (DCs) could endocytose tumor-derived apoptotic bodies and in turn elicit a robust tumor-specific CTL response as reported in other tumor models [32]. The induction of "immunogenic cell death" has been demonstrated following treatment with chemotherapeutic agents such as doxorubicin and cyclophosphamide such that due to their enhanced antigenicity, apoptotic tumor cells are more efficiently phagocytosed by DCs [33, 34]. The characteristics of immunogenic apoptosis of cancer cells include: phosphatidylserine (PS) exposure, caspase activation, and mitochondrial depolarization [35]. Additionally, this type of cell death is associated with the surface expression of certain molecules including calreticulin and HSP90 as well as the ability to elicit a protective immune response against tumor cells [36, 37]. As we observed in our studies, Ukrain treatment led to the activation of effector caspase 3 and PS surface expression as detected by positive Annexin-V staining in all three breast cancer models tested *in vitro*. Further, Ukrain has been shown to induce mitochondrial depolarization in Jurkat cells [19]. Our study also demonstrated the *in vivo* efficacy of 5mg/kg and 12.5mg/kg Ukrain administration in the form of tumor inhibition and protective immunity. However, at a higher dose, 25mg/kg, these responses were not observed which suggest the induction of a less immunogenic, potentially tolerogenic, form of cell death as reported following treatment with other anticancer drugs such as alkylating agents and cisplatin [37, 38]. Taken together our findings, along with others, are in support of the

potential induction of immunogenic tumor cell death following 'low dose' Ukrain treatment.

Alternatively, Ukrain could also act directly on immune cells and thus mediate the induction of anti-tumor immune responses. It has been reported that Ukrain can enhance the cytolytic activity of macrophages and lymphocytes *in vitro* [12, 39]. We observed that following secondary challenge, Ukrain treated mice remained tumor free, failed to develop splenomegaly and correspondingly had a significantly lower prevalence of splenic MDSCs relative to untreated mice. Additionally, the Ukrain treated mice had increased percentages of $CD4^+$, $CD8^+$ and $B220^+$ cells in the spleen relative to the untreated, tumor-bearing mice (data not shown). Interestingly, we also observed a 2-3 fold decrease in the percentage of MDSCs in the spleens of Ukrain treated mice when compared to naïve mice. These findings suggest that Ukrain could potentially inhibit the expansion of splenic $CD11b^+Gr\text{-}1^+$ MDSCs thus reducing the overall level of immune suppression present within the treated mice. This reduction in immune suppression could ultimately lead to protection against subsequent challenge and the splenomegaly observed in untreated, tumor-bearing mice. While chemotherapeutic drugs, namely gemcitabine and 5-fluorouracil, have been shown to effectively deplete MDSCs [40, 41], additional studies would be needed to determine whether Ukrain has any direct effects on this immune suppressive cell population *in vivo* particularly in tumor-bearing mice.

Finally, we assessed whether the use of 4TO7 cellular vaccines co-expressing B7-1 and GPI-IL-12 could augment anti-tumor immune responses induced by Ukrain administration. The combination of immunotherapeutic approaches and cytotoxic agents (i.e. chemotherapy) have been previously investigated [42]. Surprisingly, our studies

indicate that Ukrain treatment minimally enhanced the efficacy of cellular vaccination. In contrast, we observed the most profound enhancement in tumor inhibition between Ukrain treatment alone and the combinatorial approach suggesting that the cellular vaccines served as an adjuvant to anti-tumor responses mediated by Ukrain. Further, while Ukrain was able to induce significant tumor inhibition when administered on the same day as tumor challenge, delaying treatment one day failed to have any inhibitory effects on 4TO7RG tumor development. The reason for these observations is currently unknown. However, it is likely that within one day of transplantation, tumor cells have begun to form a niche, several days prior to palpable tumors being formed. Disruption of this niche may thus require a higher dose of Ukrain than was used in previous studies to enhance its therapeutic efficacy.

Taken together, our findings provide insight into the direct cytotoxic effects of Ukrain on breast cancer cells and the *in vivo* efficacy of Ukrain administration on the reduction of overall tumor burden and the induction of protective anti-tumor immunity. Based on these observations, it is likely that the immune modulatory effects following Ukrain administration *in vivo* are a result of enhanced phagocytosis of apoptotic tumor cells either alone or in combination with direct effects on the relative abundance/prevalence of effector and suppressive immune cell populations.

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FIGURE LEGENDS

Figure 1. Dose and time dependent cytotoxic effects of Ukrain on breast cancer cells. A) Morphological changes are observed for murine and human breast cancer cells following 48hr exposure to Ukrain by light microscopy (10X magnification). B) Following 24h, 48hr 72hr Ukrain exposure, the viability of B) murine (4T07 and TUBO) and human (SKBR-3) breast cancer cells and C) mouse fibroblast cells were assessed for viability by trypan blue exclusion. Data shown is mean ± SD of three individual experiments.

Figure 2. Survival Fractions following Ukrain treatment. Following 24h, 48h, and 72h exposure, Ukrain treated cells were plated for two weeks to allow for colony formation. Colonies were washed, fixed and stained with crystal violet and counted by light microscopy. The surviving fraction was then calculated as the ratio of the plating efficiency of drug-treated cells to the plating efficiency of untreated cells. (†) denotes a surviving fraction of zero. Data shown is mean ± SD of two individual experiments.

Figure 3. Ukrain induces the activation of intracellular caspase-3 in a dose and time dependent manner. Following Ukrain treatment, cells were washed, fixed, permeabilized and stained for intracellular caspase 3 with FITC rabbit anti-active caspase-3 antibody A) Representative plots of intracellular caspase 3 activation following 48h Ukrain exposure by flow cytometry. B) Dose response and kinetics of intracellular caspase 3 activation following 24h and 48h Ukrain treatment. Data shown is mean ± SD

of two individual experiments. Statistical analyses of caspase 3 activation P values were calculated using ANOVA. (*) $p < 0.05$, (**) $p<0.01$, (***) $p< 0.001$ relative to untreated.

Figure 4. Cytotoxic effects of Ukrain are mediated by apoptosis. Ukrain treated cells were washed and stained with Annexin-V-FITC and PI-PE and analyzed by flow cytometry. A) Representative plots of Annexin V/PI staining following 48h Ukrain exposure by flow cytometry. B) Mean ± SD of two individual experiments. B) Dose response and kinetics of Annexin V/PI staining following 48h and 72h Ukrain treatment. Mean ± SD of two individual experiments.

Figure 5. Ukrain administration led to significant tumor inhibition. Mice (n=10) were challenged with $5x10^4$ 4T07RG cells. Ukrain was administered (i.v.) on the same day and day 3 post challenge. A) Average tumor size of mice treated with Ukrain. B) Tumor incidence C) Average Body Weight. Statistical analyses of tumor growth *P* values were calculated using one-way ANOVA. (*) $p < 0.05$ relative to untreated. Data shown is mean ± SEM. Representative of 2 independent experiments.

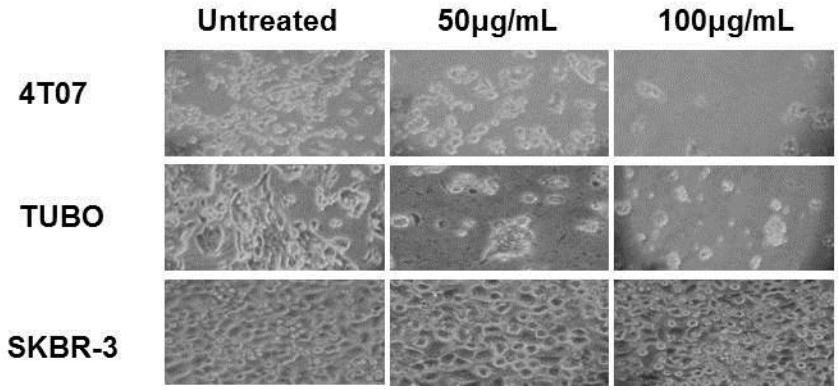
Figure 6. Ukrain administration induced protective anti-tumor immunity. On day 35 post primary challenge, Ukrain treated, tumor-free mice (n=10) were re-challenged with $5x10^4$ 4T07RG cells and monitored for an additional 35 days for tumor development (A). On day 35 post re-challenge, spleens were harvested from Ukrain treated mice and analyzed for MDSCs ($CD11b^+$, $Gr\text{-}1^+$). Protective immunity induced by Ukrain

correlates with a reduced spleen weight (B) and reduced prevalence of splenic MDSCs (C).

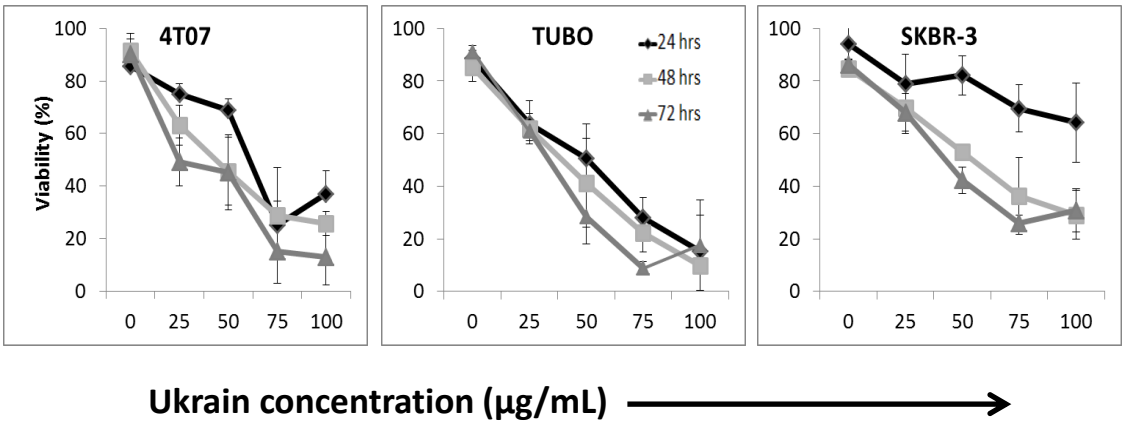
Figure 7. Combinatorial strategy of Ukrain and a cellular vaccine led to greater tumor inhibition than Ukrain treatment alone in a therapeutic setting. Mice received two doses of Ukrain (12.5mg/kg) on days 1 and 3 post tumor challenge and a single dose of irradiated 4TO7 cellular vaccines expressing B7-1/GPI-IL-12 (Irr-vaccine) on day 7. Mice were monitored for A) tumor growth and B) tumor incidence. Mean ± SEM is plotted.

FIGURE 1

A


Untreated
50µg/mL
100µg/mL
4T07
TUBO
SKBR-3

B


4T07
TUBO
SKBR-3
24 hrs
48 hrs
72 hrs
Viability (%)
100
80
60
40
20
0
0
25
50
75
100
Ukrain concentration (µg/mL)

C

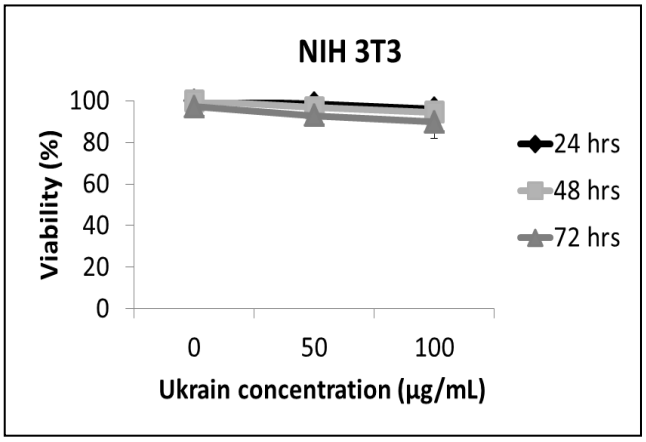

NIH 3T3
Viability (%)
100
80
60
40
20
0
0
50
100
Ukrain concentration (µg/mL)
24 hrs
48 hrs
72 hrs

FIGURE 2

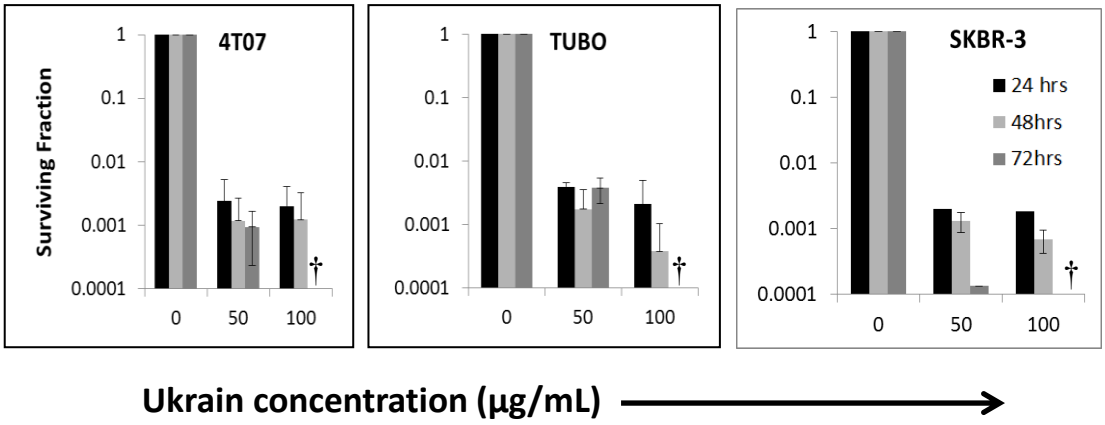
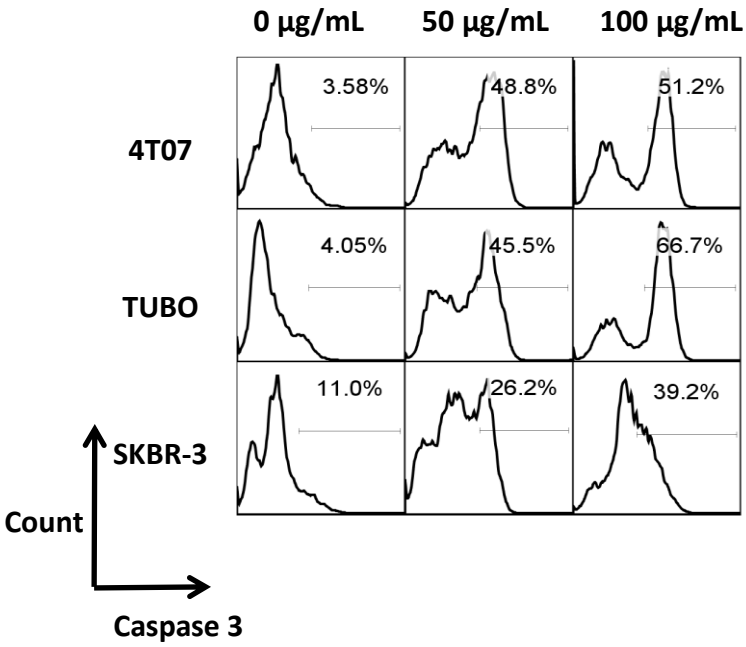

Surviving Fraction
4T07
TUBO
SKBR-3
24 hrs
48hrs
72hrs
1
0.1
0.01
0.001
0.0001
0
50
100
†
Ukrain concentration (µg/mL)

FIGURE 3

A


0 µg/mL
50 µg/mL
100 µg/mL
4T07
TUBO
SKBR-3
3.58%
48.8%
51.2%
4.05%
45.5%
66.7%
11.0%
26.2%
39.2%
Count
Caspase 3

B

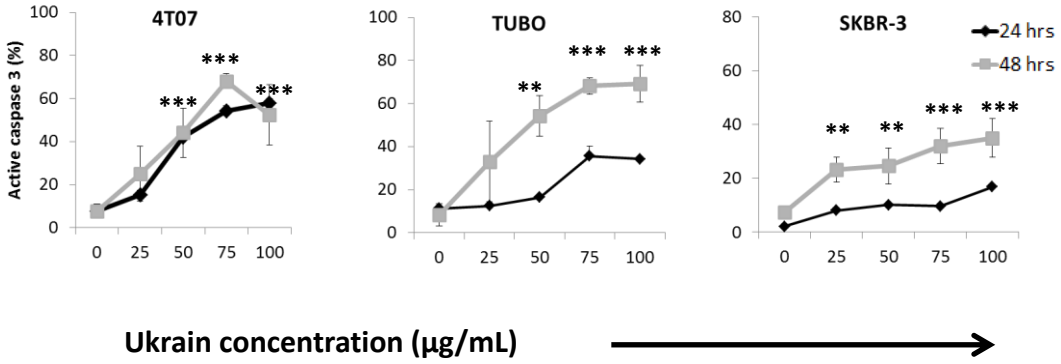
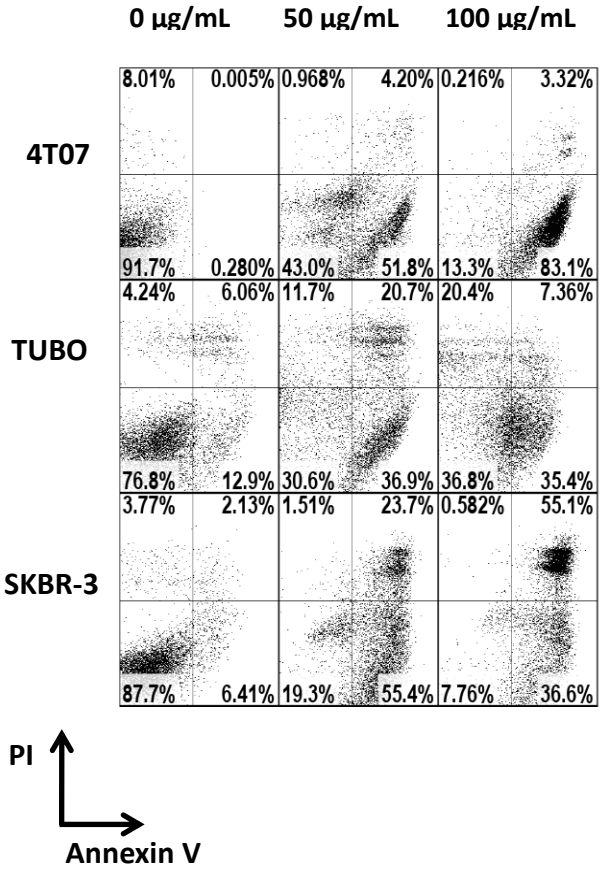

4T07
TUBO
SKBR-3
24 hrs
48 hrs
Active caspase 3 (%)
Ukrain concentration (µg/mL)

FIGURE 4

A


0 µg/mL
50 µg/mL
100 µg/mL
4T07
TUBO
SKBR-3
8.01%
0.005%
0.968%
4.20%
0.216%
3.32%
91.7%
0.280%
43.0%
51.8%
13.3%
83.1%
4.24%
6.06%
11.7%
20.7%
20.4%
7.36%
76.8%
12.9%
30.6%
36.9%
36.8%
35.4%
3.77%
2.13%
1.51%
23.7%
0.582%
55.1%
87.7%
6.41%
19.3%
55.4%
7.76%
36.6%
PI
Annexin V

B

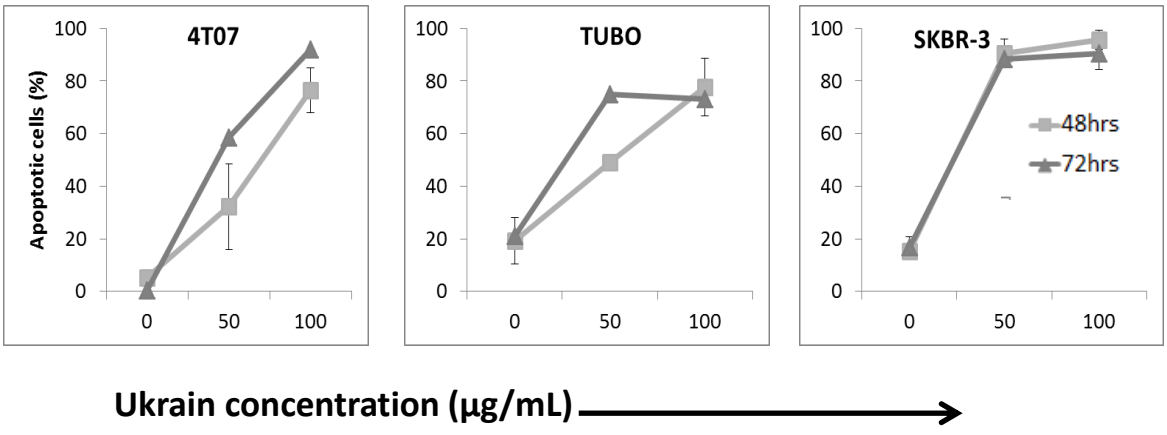
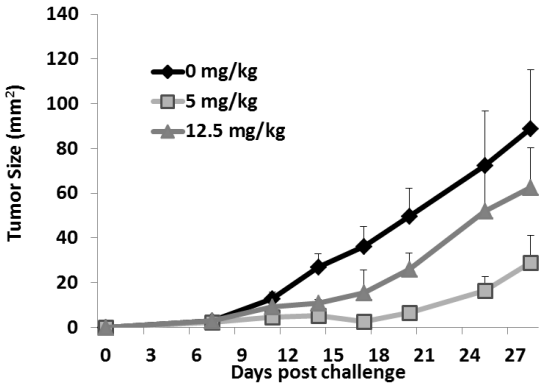
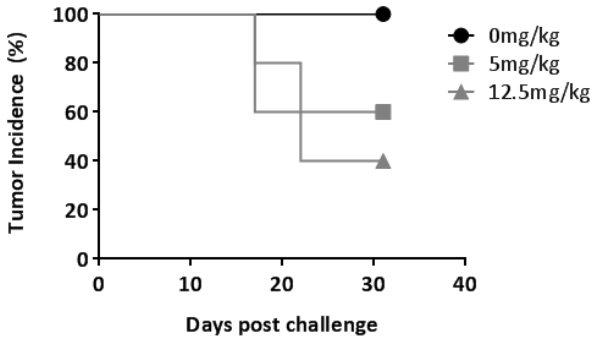

4T07
TUBO
SKBR-3
Apoptotic cells (%)
100
80
60
40
20
0
0
50
100
48hrs
72hrs
Ukrain concentration (µg/mL)

FIGURE 5

A


Tumor Size (mm²)
0 mg/kg
5 mg/kg
12.5 mg/kg
Days post challenge

B


Tumor Incidence (%)
0mg/kg
5mg/kg
12.5mg/kg
Days post challenge

C

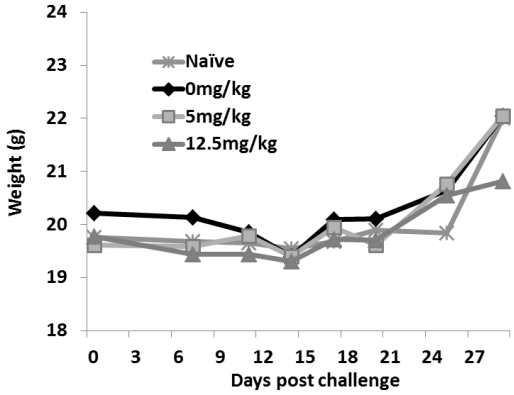

Weight (g)
Naïve
0mg/kg
5mg/kg
12.5mg/kg
Days post challenge

FIGURE 6

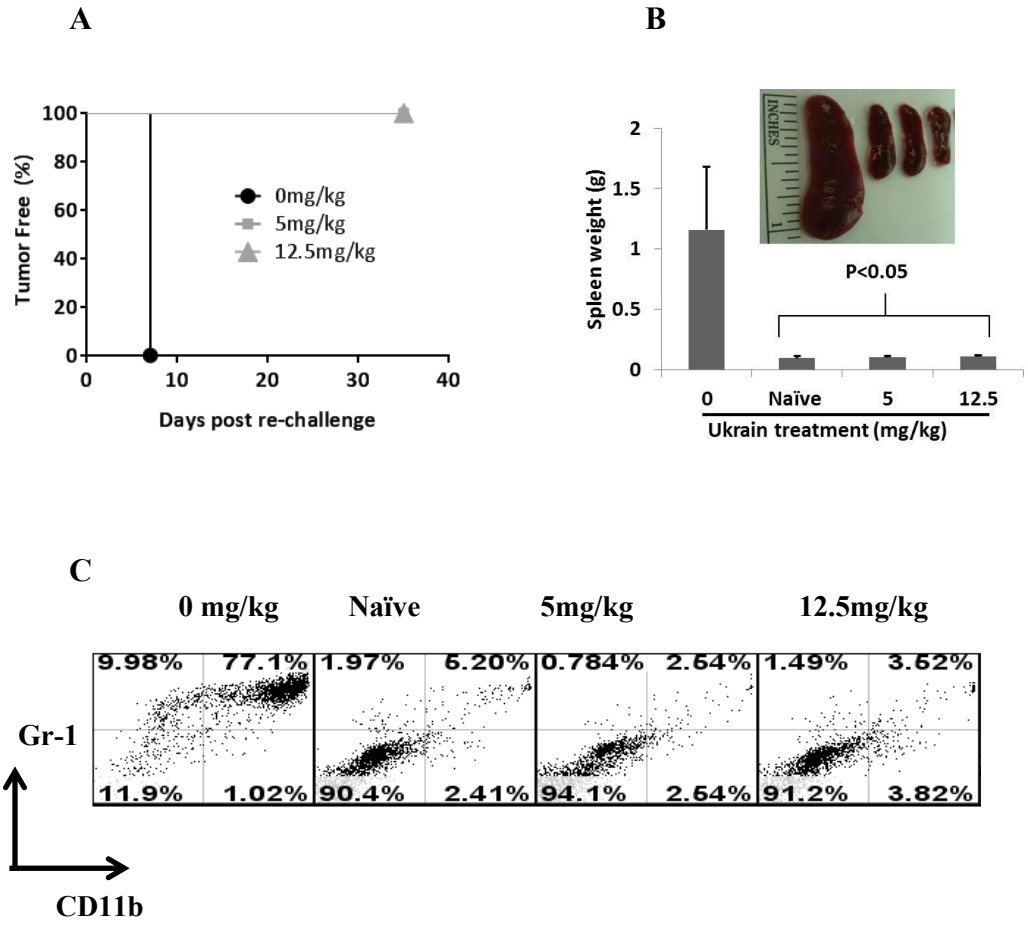
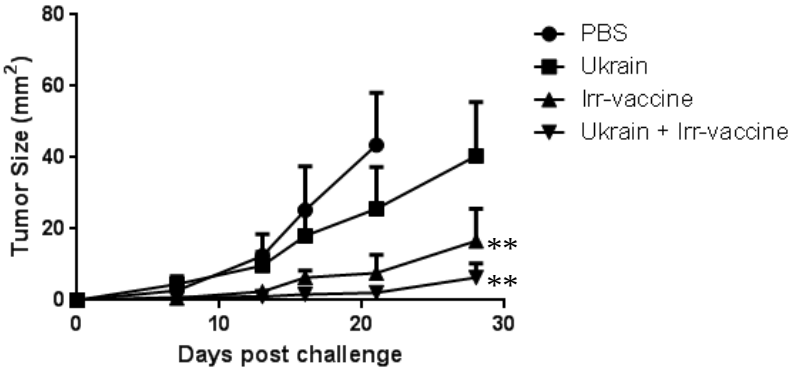
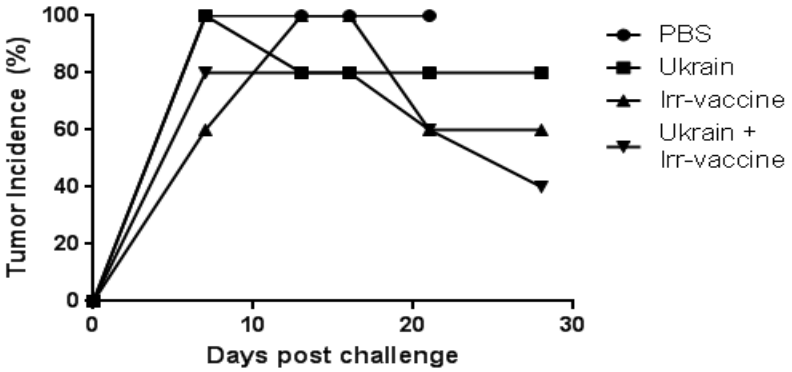

A
B
C
Tumor Free (%)
Days post re-challenge
0mg/kg
5mg/kg
12.5mg/kg
Spleen weight (g)
P<0.05
Naïve
Ukrain treatment (mg/kg)
0 mg/kg
Naïve
5mg/kg
12.5mg/kg
9.98%
77.1%
11.9%
1.02%
1.97%
6.20%
90.4%
2.41%
0.784%
2.64%
94.1%
2.64%
1.49%
3.62%
91.2%
3.82%
Gr-1
CD11b

FIGURE 7

A


Tumor Size (mm2)
80
60
40
20
0
0
10
20
30
Days post challenge
PBS
Ukrain
Irr-vaccine
Ukrain + Irr-vaccine
**
**

B


Tumor Incidence (%)
100
80
60
40
20
0
0
10
20
30
Days post challenge
PBS
Ukrain
Irr-vaccine
Ukrain +
Irr-vaccine

CHAPTER V:

Discussion

Despite our best efforts as cancer researchers, breast cancer continues to claim hundreds of thousands of lives each year [1]. With the gold standard of treatment being surgery with adjuvant chemotherapy and/or radiation therapy, new strategies for combatting this deadly disease are in desperate need. As more knowledge has been gleaned about the hallmarks of cancer including sustained angiogenesis, apoptosis evasion and tissue invasion/metastasis, more targeted therapies have been developed. The development of numerous drugs, some of which has gained FDA approval, has led to clinically-relevant responses, extending the mean survival for many patients with advanced or metastatic disease. However, many patients eventually develop resistance to these drugs rendering them no longer effective.

Tumor heterogeneity: a major therapeutic hurdle

Due to the heterogeneity of breast cancer, whether it is between patients (intertumoral) or within a given tumor mass (intratumoral), the task of developing effective treatments becomes even more daunting. While breast cancer has been categorized based on a number of factors including hormone receptor status, stage of tumor development and tissue location, there exists a substantial degree of heterogeneity within each group which poses a significant hurdle to determining the appropriate treatment regime for a given classification of breast cancer [2, 3]. While the passive administration of monoclonal antibodies (mAbs) such as Trastuzumab and Pertuzumab has proven to be among the most successful immunotherapies to date for HER-2 positive breast cancer, therapeutic resistance is frequently observed in these patients as well. In these cases, the source of resistance is thought to be compounded by either epitope

masking, signaling through alternate pathways, down-modulation of targeted proteins, overactive downstream tyrosine kinase activity, or the inability of the mAbs to trigger the appropriate immune mechanism to destroy HER-2 positive tumor cells [4, 5]. Additionally, individual tumor samples may not be representative of the whole tumor due to the high degree of intratumoral heterogeneity that exists in most cancers [6]. This heterogeneity, which can be genetic or phenotypic, further contributes to disparate therapeutic responses among patients as well as within a patient during his/her course of treatment [7].

Induction of anti-tumor immune responses in the many faces of immune suppression

The concept of augmenting the host's natural ability to eliminate tumors is the goal of immunotherapy. This can be achieved through various methods such as the adoptive transfer of effector cells, namely dendritic cells (DCs) or T cells, that were *ex-vivo* primed against specific tumor antigens [8, 9]. Additionally, the use of cellular vaccines provides the host with a vast repertoire of tumor antigens which potentially minimizes the likelihood of resistance or tumor escape. Modification of otherwise poorly immunogenic tumor cells with immune stimulatory molecules (ISMs) such as costimulatory molecules and cytokines, aids in augmenting the induced anti-tumor immune response. The host fails to respond appropriately to a developing tumor, in part, because tumors often lack the expression of costimulatory molecules such as B7-1 thus leading to the induction of anergy and/or apoptosis of T effector cells [10, 11]. Additionally, due to the critical role played by T cells in anti-tumor immunity, this is particularly problematic. Lastly, genetic modification of cellular vaccines with the

cytokines IL-2 and IL-12 can help promote the activation and cytotoxic capabilities of T cells and natural killer (NK) cells, both of which are key in many tumor models [12, 13].

Tumors further evade the host's immune response through the perpetuation of multiple layers of suppression systemically as well as locally within the tumor microenvironment. Intrinsic characteristics of the tumor itself including the expression of inhibitory molecules such as PD-L1 and CD47 as well as the lack of costimulatory molecule expression, as previously mentioned, well-equips tumors to develop in an otherwise immune-competent host. The engagement of these molecules with their cognate receptors on T cells (PD-1) and phagocytes (SIRPα) directly inhibits the effector functions of these immune cells. Further immune suppression is mediated via secretory factors such as TGF-β, VEGF and G-CSF that acts to promote the recruitment and maintenance of regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs). The studies presented in this dissertation provide strong evidence that cellular vaccines expressing GPI-anchored ISMs (GPI-ISMs) are capable of overcoming these layers of suppression, to some degree, in order to induce effective and long-lasting anti-tumor immunity.

Genetic modification of otherwise soluble proteins such that they become membrane associated via a GPI-anchor poses several advantages. The primary advantage is that rather than the proteins acting systemically in a potentially harmful manner (i.e. induction of systemic toxicity), the GPI-ISMs remain locally at the vaccination site. Our data shows that while GPI-IL-2 and GPI-IL-12 are expressed on the surface of breast cancer cells, partial shedding of these molecules from the cell surface occurs. This slow release of molecules at the site of vaccination allows for the local and sustained priming

of effector cells. When B7-1 and GPI-IL-12 are co-expressed at the tumor site, we observed an enhancement in the infiltration of effector cells such as DCs and $CD8^+$ T cells. Moreover, when these molecules are co-expressed, the immune suppressive nature of the tumor microenvironment is significantly altered as evident by a reduction of MDSCs and Tregs. Additionally, while we observed that the breast cancer models used in these studies secrete high levels of immune suppressive factors such as G-CSF and TGF-β, the expression of GPI-ISMs are able to overcome this layer of immune suppression as well.

While we demonstrate the local effects of GPI-ISMs, we also observed significant inhibition of tumors growing at a site distant from the vaccination area. These concomitant responses are often hindered by the presence of suppressor cells such as Tregs [14, 15]. This finding indicates that the anti-tumor effects of GPI-ISM expression extends beyond the local vaccination site and can act systemically either by entering the circulation or, more likely, by priming cells locally and promoting cell migration towards the developing tumor.

One of the more striking observations from our studies is the induction of anti-tumor immunity by our cellular vaccines against a more aggressive tumor variant (RG) that was selected by several rounds of *in vivo* passage. While our vaccines were established from the parental tumor cell line (WT), the expression of GPI-ISMs still allowed for the significant reduction of tumor burden and tumor incidence of RG tumors similar to cellular vaccines derived from RG cells. Our findings suggest that modification of vaccines prepared from a patient's initial tumor with GPI-ISMs could

potentially remain as an effective therapy for patients over an extended period of time when tumors typically become resistant to primary treatment options.

Sustained protective immunity induced by cellular vaccines expressing GPI-ISMs

Cancer recurrence is an ever-present concern for patients undergoing cancer treatment. Due to the cellular heterogeneity that is present within tumors, it is almost impossible to predict which individuals will relapse or when the relapse will occur. While many treatments are able to de-bulk the tumor mass, cancer stem cells (CSCs), which are thought to possess the intrinsic ability to initiate tumor development, are likely to remain after treatment eventually leading to tumor relapse [16, 17]. Pre-clinical mouse models seek to recapitulate this relapse scenario by subjecting tumor-free or "cured" mice to subsequent tumor challenges. Our data indicates that GPI-ISM expression by cellular vaccines, in both breast cancer models, is capable of inducing durable memory responses as evident by complete or partial protection for up to 60 days following secondary tumor challenges.

Attacking breast cancer with multiple strategies

Due to the immense degree of immune suppression present during tumor development, optimal anti-tumor immunity is more likely to be achieved through the use of multiple approaches. Our studies sought to assess the potential synergy between our cellular vaccines with the cytotoxic agent Ukrain or with the blockade of the program death ligand-1 (PD-L1) inhibitory checkpoint utilized by tumors. The plant-derived compound Ukrain had previously been shown to be highly cytotoxic towards lymphoma,

colon, brain, melanoma and brain cancer cells while non-malignant cells remain viable. The data presented in this dissertation extends the cytotoxic scope of Ukrain to breast cancer, both murine and human, at doses comparable or lower than standard chemotherapeutic drugs such as doxorubicin, paclitaxel and cyclophosphamide [18, 19]. Additionally, Ukrain treatment was well tolerated as evident by maintenance of body weight and no visible signs of morbidity. Further, our data provide insight for the first time into the immune modulatory effects of Ukrain administration on MDSC recruitment. Similar immune modulatory effects on suppressor cells have been demonstrated by chemotherapeutic drugs [20, 21] and the combination of chemotherapy prior to vaccination has been shown to enhance vaccine efficacy [22]. In our studies, Ukrain treatment followed by cellular vaccination led to greater tumor inhibition than Ukrain treatment alone, however only a minimal enhancement was seen relative to vaccination alone. It is likely that pre-administration of Ukrain at higher, more frequent doses could further enhance the therapeutic efficacy of cellular vaccines and provide further support for the use of Ukrain as a less harmful, potential alternative to chemotherapy.

Additionally, we established, consistent with other tumor models, that D2F2/E2 tumor cells express PD-L1. We utilized that knowledge to incorporate blocking antibodies against PD-L1 into our vaccination strategy as an adjuvant to our cellular vaccines. We observed an enhancement in the efficacy of our cellular vaccines when administered in conjunction with αPD-L1 mAbs. It is likely that upon further optimization of the dosing schedule (both amount and duration) this enhancement can be maximized. These studies strengthen the view that analysis of tumor characteristics in

conjunction with the host immune response to a given tumor can provide greater insight into therapies that are more likely to induce an effective response.

Current studies provide a solid "spring-board" for future investigations

The work presented herein has formed a strong foundational basis for a more potentially translational vaccine design, the use of protein transferred GPI-ISMs onto isolated tumor membrane vesicles (TMVs). The GPI-anchor allows for proteins to spontaneously incorporate onto the plasma membrane of cells. Protein transfer utilizes this unique characteristic of GPI-proteins. Due to the difficulty in establishing cell lines from primary tumor cells, the use of TMVs circumvents that requirement. These studies demonstrate that the combination of the costimulatory molecule B7-1 and the cytokine IL-12 whether co-expressed by the same cell or co-administered on different cells significantly reduces the overall tumor burden in HER-2 negative and HER-2 positive breast cancer models. We observe similar findings upon administration of TMVs co-expressing B7-1 and GPI-IL-12 (unpublished data). Our data also further supports data previously published from our laboratory indicating that TMVs expressing B7-1 or IL-12 can be effective in EG7 lymphoma or P815 mastocytoma tumor models, respectively [23, 24].

Potential pitfalls of cellular vaccines

With the main pitfall limiting the clinical use of whole cell vaccines being the difficulty in establishing cell lines from primary tumors, cellular vaccines expressing GPI-ISMs would potentially benefit roughly 10% of cancer patients by which cell lines

can be developed [25]. However, the success rate of establishing cell lines from primary colon and pancreatic cancers has been increased following xenotransplantation of primary tumors into immune-compromised mice prior to *in vitro* culture [26, 27]. While established cell lines may no longer be fully representative of the primary tumor from which it was derived, comparative analysis revealed that along with secondary aberrations, there was a preservation of genomic alterations in breast and pancreatic cancer lines that were originally found in the primary tumor tissue [28-30]. Additionally, the use of allogeneic cellular vaccines, which are composed of two or three established human cell lines, may circumvent the limitations of establishing autologous vaccines from each patient [31].

Concluding Thoughts

In spite of it all, great promise remains in the area of breast cancer vaccine development particularly with the identification of key tumor-associated antigens namely HER-2, MUC-1, carcinoembryonic antigen (CEA) and alpha-lactalbumin [32]. The most relevant clinical application of cancer vaccines, including our proposed cellular vaccines expressing GPI-ISMs, would be an adjuvant setting to prevent tumor recurrence or in a minimum residual disease setting. In our constant quest to cure cancer, a number of hurdles must be conquered including the immune suppressive microenvironment, the thin line between anti-tumor immunity and autoimmunity and the development of therapeutic resistance, which are further confounded by the variability in hosts' ability to respond appropriately to an emerging tumor. While cancers are constantly becoming "smarter", we as cancer researchers are also becoming more aware and astute to the crafty strategies

exercised by tumors. In doing so, we are devising novel approaches, in the form of monotherapies and combinatorial therapies, to more effectively combat this deadly disease.

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