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Investigation of a Xenogeneic Vaccine in a Murine Breast Cancer Model

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An abstract of
A thesis submitted to the Faculty of the
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Abstract

Investigation of a Xenogenic Vaccine in a Murine Breast Cancer Model

By Sara He

Tumor cells escape immune system surveillance by inducing immune tolerance. Therefore, finding ways to break the tumor-induced immune tolerance is a fundamental aspect in developing effective cancer therapies. Tumor modification with immunostimulatory molecules (ISMs) such as co-stimulatory molecules and cytokines has successfully been shown to induce an antitumor response. Xenogeneic immunization using non-self heterogeneic proteins with the goal of inducing a cross-reactive response against a shared antigen through shared epitopes has been proven to be an effective strategy for breaking immune tolerance against tumor associated antigens. In our studies, CHO cells expressing human HER2 along with cytokines, IL-2, IL-12, GM-CSF, and transmembrane protein B7-1 are used as a xenogeneic vaccine against a murine mammary tumor cell line, TUBO. Since xenogeneic cells cannot stimulate T cells directly, the ultimate goal is to determine whether vaccination with a xenogeneic CHO cell line expressing xenogeneic human HER2 along with immunostimulatory molecules can induce a potent antitumor immune response through indirect-priming. The study investigates the effects of B7-1, and glycosyl-phosphatidylinositol (GPI)-anchored IL-2, IL-12, and GM-CSF on indirect priming using a breast cancer model. Because the delivery method of a vaccine has proven to be important and can affect the immune response, vaccines were delivered in a live cell, irradiated cell, or cell membrane form. Mice were vaccinated and challenged 30 days later with BALB/c mice derived mammary carcinoma, TUBO. Tumors grew in vaccinated mice at a reduced rate compared to unvaccinated mice. Tumors grew at the slowest rate in mice vaccinated with the live cell vaccine. However, no difference was seen in mice vaccinated with the irradiated cell vaccine as compared to unvaccinated mice. Tumor incidence was also reduced in several groups vaccinated with the live and irradiated cell vaccine. However, statistical tests showed there was no significant difference in average tumor size between vaccinated and unvaccinated groups of mice. The present study established a xenogeneic vaccine model system which can be used to study the effectiveness of various vaccination strategies in inducing indirect and cross priming.

Investigation of a Xenogeneic Vaccine in a Murine Breast Cancer Model

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INTRODUCTION

Cancer immunotherapies aim to stimulate and supplement the immune system's ability to recognize and eliminate cancer. Understanding the mechanism and requirements for stimulating an antigen specific immune response has led to promising approaches for cancer treatment such as peptide vaccines, DNA vaccines, hybrid tumor cells, dendritic cells modified to express tumor antigens, heat shock proteins, tumor cells transfected with immunostimulatory molecules, and novel protein transfer method has also been developed in our laboratory that utilizes glycosyl-phosphatidylinositol (GPI)-anchored immunostimulatory molecules on tumor cell membranes. A developing method for cancer treatment also includes the use of xenogeneic antigens that is proven to be effective in cancer treatment. [1-3].

This study evaluates the antitumor effects of vaccinating mice with xenogeneic CHO cell vaccines expressing xenogeneic human HER2 with GPI-anchored immunostimulatory molecules (ISMs) IL-2, IL-12, GM-CSF, and B7-1, alone or in combination as a model for a breast cancer vaccine. Breast cancer is one of the leading types of cancer among women in the United States and 230,480 new cases of breast cancer were diagnosed and 39,520 deaths occurred in 2011 alone [105]. New breast cancer treatment strategies are constantly being devised and remain an important precedence in research and medicine. This experiment employs co-stimulation molecules and cytokines as a breast cancer treatment approach. B7-1 is necessary for producing the co-stimulatory signal required for T cell activation[4], IL-2[5] and IL-12[6] are involved in immune activation and signaling , and GM-CSF is involved in stimulating macrophage and dendritic cell activation[7]. Because immunostimulatory molecules can interact synergistically, their effects alone or in combination are tested to determine if co-expression can enhance an antitumor response[8]. The introduction will provide an overview of cancer and the immune

system and cancer immunotherapy development in order to provide a framework for why we used our approach.

The Immune System and Escaping Immune Surveillance

The development of cancer vaccines has been fraught with numerous challenges and many cancers are able to escape immune surveillance and adapt immunosuppressive mechanisms that make them poor stimulators of the immune system. Tumor cells bear a striking resemblance to normal cells in humans which makes it difficult to distinguish non-cancerous and cancerous cells. Vaccination approaches that optimally stimulate the immune system against tumor specific antigens need to be developed in order to combat the poor antigenicity and immunosuppressive ability of cancer. In cancer immunotherapy the goal is to stimulate the immune system to first recognize and mount an immune response that ultimately leads to the elimination of cancer.

Tumors evade immune system recognition by down regulating molecules that activate and stimulate the immune system. Even though the immune system is continually surveying for foreign invaders such as viruses, bacteria, and mutated or damaged cells such as tumor cells, tumor cells still escape immune surveillance. The immunosurveillance hypothesis first proposed by Paul Ehrlich in 1909 and later by Sir Macfarlane Burnet and Lewis Thomas in the 1950s was initially dismissed due to lack of scientific evidence. The hypothesis proposed that immune cells survey the body and eliminate neoplastic cells during early stages of transformation into cancer cells [9]. The cancer immunosurveillance hypothesis was eventually broadened into one term: cancer immunoediting. The cancer immunoediting hypothesis proposes immune-tumor cell interactions facilitate tumor progression by sculpting the immunogenic phenotype of the

developing tumor. This process occurs in three stages: elimination, equilibrium, and escape. During the elimination phase, immune cells recognize and destroy transformed cells. For the equilibrium phase, the immune system and tumor cells enter a dynamic equilibrium stage. Lastly, the escape phase is when cancer cells confer resistance to immune detection and elimination[10]. The role of the immune system is critical for preventing cancer formation. Transplant patients on chronic immunosuppressive therapies have a 5-6% chance of developing cancer within the first few years after transplantation[11]. People with autoimmune disorders such as HIV have a higher risk of developing cancers such as Hodgkins lymphoma[12].

For a normal immune response to occur T cells receive an antigen specific signal from the interaction of the T cell receptor with a peptide-MHC complex and co-stimulation signaling from adhesion molecules such as B7-1 and CD28 [13]. Dendritic cells (DCs) are the main antigen presenting cells (APCs) in the immune system that can provide both these signals and elicit an immune response[14]. Cancer escapes immune surveillance in multiple ways. Altered antigen presentation is a way tumor cells escape immune recognition where an antigen is altered and not recognized by the immune system. This potentially allows tumor cells to proliferate [15, 16]. Tumor cells can express MHC molecules and therefore are capable of providing the primary signal for T cell activation; however, they can lack the costimulatory signal. Thus another way tumors evade immune surveillance is failure to express costimulatory molecule B7-1[17, 18]. Tumors can also escape the immune system by down regulation of MHC expression which ultimately reduces antigen presentation. Tumor antigens must be presented along the cell surface with MHC class I in order to activate cytotoxic T cells, the cells thought to be the main effectors in mediating an antitumor response [13, 17]. Along with MHC down regulation, tumor cells can express immunosuppressive molecules such as PD-L1 which can induce apoptosis or

inhibit activation of tumor antigen-specific T cells [19]. Tumors can also alter the function and activation of DCs and cause an expansion in CD4+CD25+ regulatory T cells that play a role in cancer expansion by suppressing cytotoxic T cell and DC function [20].

The fundamental goal in cancer vaccine development is to discover ways to counteract tumor evasion strategies. Thus vaccine development aims to increase tumor cell recognition and elimination by stimulating T cells and APCs to be more effective at antigen presentation. Different strategies have evolved in stimulating recognition and elimination of tumor cells using cytokines.

GPI-anchored Cytokines and Vaccine Strategies

Cytokines play important roles in the immune system for defending against bacteria, pathogens, and viruses. IL-2 is a potent mediator of the immune system and historically known as a T cell growth factor that is produced primarily by recently activated T cells to support further expansion[21]. IL-12 is produced by macrophages and B lymphocytes and stimulates IFN-γ production from NK cells and T cells [22-25]. IL-12 induces cytokine production proliferation, and enhances cytotoxic activity of T and NK cells[26-28]. B7-1 is found on antigen presenting cells and is important for the co-stimulatory signal that activates T cells through the CD28 receptor [29]. Granulocyte macrophage-colony stimulating factor (GM-CSF) is an important cytokine for the generation of dendritic cells, the most potent antigen presenting cell[7]. Because if their functional role in the immune system, DCs are attractive targets for vaccine development and have been extensively tested for their ability to induce an immune response against tumors[30]. Administration of cytokines IL-2 [31], IL-4[32], and IL-12[23, 33] in murine models has been shown to induce an antitumor immune response.

However, positive effects of immunostimulatory molecules in murine models do not always translate to humans. The administration of cytokines, such as IL-12, in humans has resulted in systemic toxicity and risky side effects [34]. Systemic IL-2 administration initially was thought to be a promising clinical therapy but further tests showed side effects such as impaired renal function, hypotension, vascular leaks, and life-threatening skin reactions [35]. Gene transfer of immunostimulatory molecules onto tumor cells has also been examined as a method for inducing an antitumor immune response. Vaccination with tumor cells transfected to express IL-2 in mice has shown to induce protective immunity against a parental tumor challenge [36] and cause tumor regression [37]. Gene transfer of other cytokines such as Il-12 and GM-CSF onto tumor cells also induces tumor immunity[38-40]. An alternative method is to administer cytokines that have been attached to the surface of the cell membrane by a GPI anchor.

Expression of GPI-IL-12 on tumor-cell membranes can induce T-cell proliferation, IFN-γ production and tumor immunity in highly tumorigenic murine mastocytoma model [41]. Another advantage of GPI-anchored immunostimulatory molecules is GPI-anchored cytokines may create a slow-release depository at the site of vaccination which can prevent toxicity associated with systemic cytokine administration [42]. Previous studies have also shown that GPI anchoring of GM-CSF was able to induce bone marrow cell and T-cell proliferation *in vivo*[43]. When GPI-anchored GM-CSF is transfected onto tumor cells it can induce an anti-tumor response and complete tumor rejection [39, 44]. The structure of GPI-anchored GM-CSF on the cell surface also has the benefit of being able to directly target dendritic cells through their GM-CSF receptor [43]. Lastly, GPI-anchored GM-CSF is also found to partially shed and dissociate from the cell membrane so irradiated tumor cells or membrane particles carrying GPI-

GM-CSF on the surface release the cytokine similarly to biodegradable micro particles[45] . This can facilitate a slow and longer lasting release of GM-CSF. In previous studies, GPI anchored mouse IL-12 expressed on the surface of tumor cells were able to stimulate T cell proliferation and induce antitumor responses in mice [41].

Previous studies have demonstrated that administering cytokines in murine tumor models have elicited antitumor responses and immune stimulation. Tumor cells genetically modified to express cytokines IL-2, IFN-γ, TNF, IL-7, and IL-4 have also been shown to be effective in inducing an antitumor response [46]. However, immunizations with cytokine modified tumor cells has not been shown to be superior to vaccinations with a mixture of tumor cells with adjuvant Cornebacterium parvum [46]. In order to improve vaccine immunogenicity, the addition of B7-1 transforms tumor cells into competent antigen presenting cells (APCs) resulting in T cell activation and antitumor responses[13]. Direct use of APCs is an important factor in cancer vaccine development and finding ways to stimulate APCs is important in order to have an effective cancer treatment. This study evaluates the ability of GPI-anchored IL-2, IL-12, GM-CSF, and B7-1 to stimulate indirect priming and activation of T cells through dendritic cells, the most powerful APC.

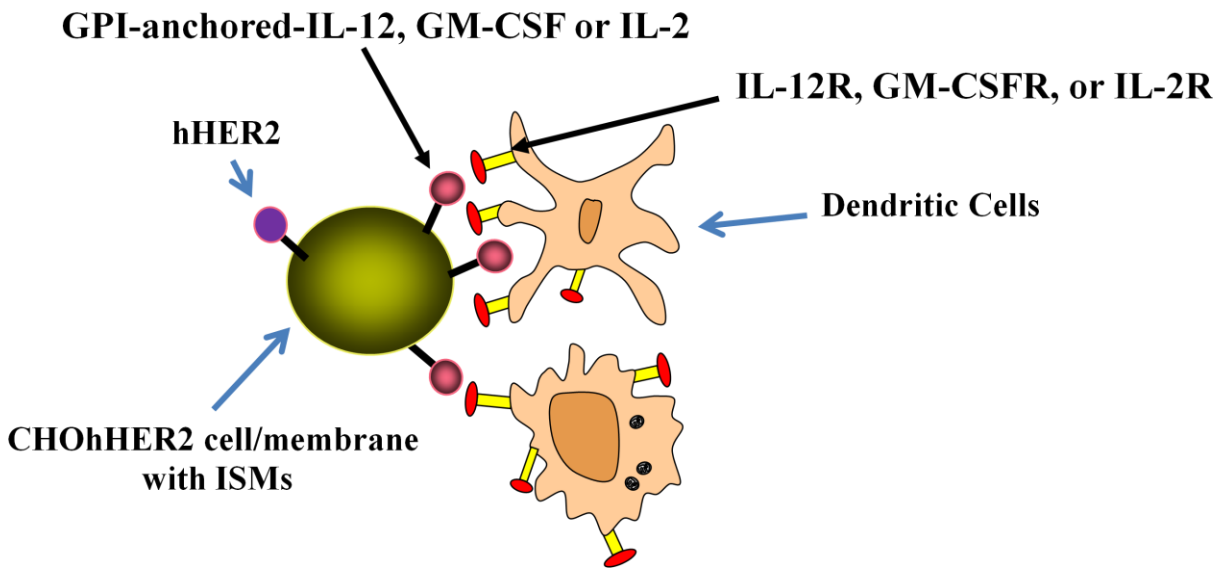


Figure 1. Stimulation of Dendritic cells by GPI-anchored ISMs. GPI-anchored IL-12, GM-CSF, and IL-2 can stimulate DCs through ISM specific receptors on DCs.

Developing Effective Cancer Immunotherapies

The understandings of antigen-specific immune responses have led to a number of promising immunotherapies and cancer vaccine strategies. One strategy employs APCs as vaccines. Vaccinations with dendritic cells pulsed with tumor antigens [47], tumor peptides [48] and tumor associated heat-shock proteins have all been strategies used previously in cancer immunotherapies. These strategies have been shown to result in antigen-specific immune responses. Sources of tumor associated antigens can come from apoptotic cells, tumor cells, cell lysates, and antigens encoded by DNA or RNA [49]. Heat shock proteins from tumor cells have also shown to induce DC maturation and antigen presentation which ultimately resulted in an antitumor response [50]. More recently, DCs pulsed with HER-2/neu peptides and administered to patients proved effective in inducing a strong, long-lasting immune response and reducing or eliminating HER-2/neu expression in patients with ductal carcinoma *in situ* [51].

Tumor and dendritic cell fusion vaccines have also been effective in reducing tumor incidence and prolonging survival in murine models [52].

How the antigen and vaccine is presented to the immune system affects its response and immunogenicity. Even though live cells and irradiated cells contain the same type of antigens and immunostimulatory component, the quality and quantity of the immune response can be affected by how the antigen and vaccine are presented. Irradiated cell vaccines vary in their ability to produce an immunogenic response. In a B16 melanoma model, irradiation of tumor cells alone did not produce an immune response but when the cells expressed GM-CSF, an effective and long lasting anti-tumor response was generated [39]. However, a tumor vaccine model using MethA cells demonstrated a single vaccination of irradiated cells can lead to immunity but the equivalent of whole cells lysates did not have any immunogenic effect [53]. In bacteria, irradiated vaccines have been shown to preserve adjuvant and antigenic properties and inhibit the role of regulatory T cells that can inhibit immune responses after vaccination [54]. Irradiation has also been shown to increase the expression of IL-2 on plasmocytoma cells and boost the antitumor response when compared to the effects of administering live cells expressing IL-2[55].

Another vaccination strategy involves vaccinating with cell membranes expressing immunostimulatory molecules. Gene transfer of immunostimulatory molecules on live cells as a vaccination strategy has been demonstrated to induce an antitumor response and result in tumor regression. However, gene transfer requires transfecting primary tumor cell lines which can be a difficult and time consuming process. In a clinical setting, gene transfer is impractical due to limiting factors such as isolating cells from primary tumors and low rate of gene uptake [56]. Cell membrane vaccines offer advantages over live or irradiated cell vaccines. Expression of

GPI-linked IL-12 on tumor cell membranes has been shown to induce IFN-γ, T cell proliferation, and tumor immunity in a murine mastocytoma model [41]. Vaccination with cell membranes also offers advantages such as the ability to be frozen and stored for at least 2 years with minimal consequence to the ISM expression [57]. Membranes do not actively divide so the GPI-anchored molecules cannot be lost through cell division. Because multiple vaccination strategies offer different advantages we have chosen to use live cell, irradiated cell, and cell membrane vaccines.

Xenogeneic Vaccine

Vaccines for infectious diseases elicit immune responses against foreign antigens. Cancer vaccines using tumor antigens most often target antigens perceived by the immune system as self. The immune system has evolved mechanisms to limit the response to antigens perceived as self and T cells with high affinity for self-antigens are typically deleted during development. Vaccine strategies using xenogeneic tumor specific antigens or molecules may be able to circumvent tumor-induced immune tolerance.

Immunotherapies using xenogeneic tumor antigens offer certain advantages. Structural differences in xenogeneic tumor associated antigens can induce specific antitumor responses during early and late stages of cancer development [58-60]. Vaccination of mice with xenogeneic rat thyroid medullary carcinoma cells engineered to secrete IL-2 prevented tumor formation when challenged with Lewis lung carcinoma cells [61]. Previously it has been shown that vaccines prepared from human and bovine endothelial cells was able to induce an antitumor response in mouse tumor models [3]. Vaccines using xenogeneic antigens have been shown to be more effective than vaccines using self-antigens. Rats vaccinated with xenogeneic human glioma membrane proteins were able to stimulate tumor-specific CTLs and CD8+ and CD4+ T

cell infiltration at the tumor site, however, self-rat glioma membrane proteins produced no effect [62]. Xenogeneic vaccines have also been tested clinically. In Phase I clinical trials, xenogeneic monkey fibroblast cells were genetically engineered to express human IL-2 and injected into patients with metastatic tumors. Results showed vaccine induced the production of T cell and NK cell derived cytokines IL-2 and IFN-γ [63]. The structural differences between xenogeneic and self-antigens have been demonstrated to be effective in inducing immune responses. However, antitumor immune responses from xenogeneic vaccines must be induced through indirect and cross priming because the vaccine cannot directly stimulate T cells.

Cross Priming

The mechanism behind xenogeneic vaccines in this study involves cross priming. Because CHO cells are foreign and do not express MHC class I or II, they cannot directly stimulate T cell activation. Therefore, an antitumor immune response would have to be induced through indirect and cross priming. Tumor specific T cell responses from cross-priming result from the cross-presentation of tumor antigens by professional APCs, such as dendritic cells. Cross priming takes place when an exogenous antigen, not expected to gain access to the cytoplasm of an APC, are presented on MHC class I molecule [64-67]. MHC class I along with the antigen induces a CD8+ cytotoxic T cell (CTL) response which can stimulate an antitumor response. CTL responses play a critical role in the elimination of tumor cells and cancer vaccines seek to generate tumor specific CTLs that will ultimately initiate an antitumor response. Cancer immunotherapies can stimulate the production of tumor specific CTLs through utilizing tumor associated antigens (TAAs) [68]. In order for cross priming to occur, the antigen must be presented through DCs. Dendritic cells are capable of ingesting tumor cells and presenting tumor associated antigens to the immune system in order to generate a CTL response. Cytotoxic

T cells are critical for the induction of a strong antitumor response and generation of CTLs [69, 70] is a goal in many cancer vaccine strategies. In order to generate CD8+ T-cells, priming by potent antigen presenting cells must occur.

Evidence exists in cross priming where antigens not expected to gain access to the cytoplasm of an APC are somehow presented on an MHC class I molecule [71-73]. In the 1970s, Bevan and colleagues first proposed cross priming by demonstrating that immunization with lymphoid cells congenic for minor histocompatibility antigens resulted in CD8+ T cell generation that were restricted by host MHC class I molecules [64]. The explanation was that the minor histocompatability antigens were transferred from donor cells to APCs for CD8+ T cell priming [64]. Dendritic cells are believed to play an essential role in cross presentation and subsequent stimulation and initiation of tumor specific responses [74-76]. A previous study shows that DC based vaccines can indirectly prime CD8+ T cells *in vivo* by transferring antigens to endogenous cells that present them to CD8+ T cells [77]. Bringing exogenous antigens to the MHC class I-restricted processing pathways through professional APCs is a considered a critical component for inducing an antitumor response. Tumor peptides and antigens are taken by dendritic cells and cross presented on MHC class I molecules to induce antigen-specific CTL responses upon successful T-cell receptor-mediated recognition [44, 67, 78-80]. Studies have also shown that viral antigens from apoptotic cells can be acquired by DCs and mediate an antiviral CTL response through MHC class I restricted CD8+ T cells. DCs fed with apoptotic tumor cells can lead to effective priming of tumor specific CTLs in *in vivo* animal studies [81, 82] and in *in vitro* models [72]. Differences in the processing and presentation of TAAs can be affected by the source of the maturation of DCs, tumor-derived materials, and the T-cell population available for stimulation [71, 82-84]. Even though DCs are shown to be capable of

cross presentation, tumors can also secret immunosuppressive factors such as IL-10, TGF-β, VEGF, and prostaglandin E-2 that prevent DC function and differentiation [74, 85].

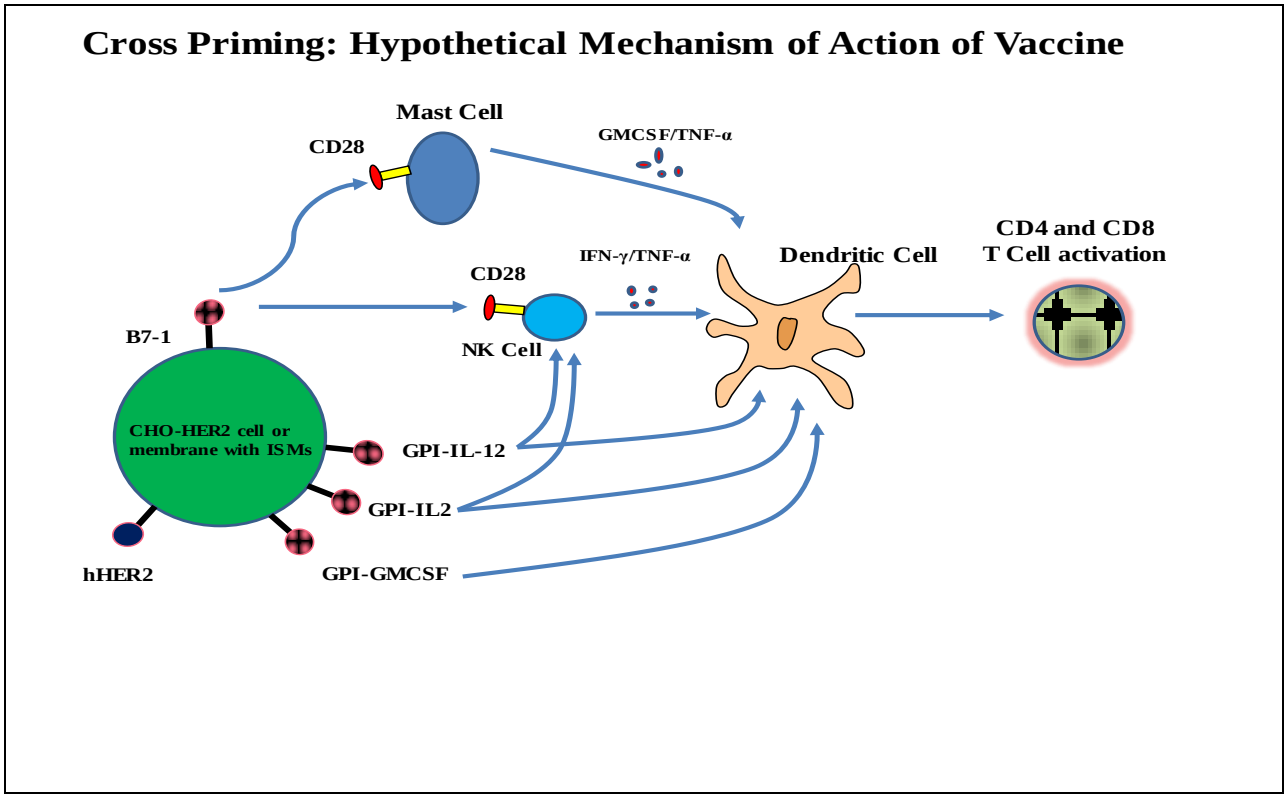


Figure 2. A Hypothetical Mechanism of Vaccine. CHOhHER2 cells or membranes with ISMs can indirectly stimulate T-cell proliferation. Cytokines can induce T cell proliferation through DC stimulation. B7-1 can also bind to CD28 expressing NK cells and mast cells. After binding, the NK and mast cells release IFN-γ and TNF-α which stimulate DCs, which can result in T cell proliferation.

CHO cell Vaccine Model

In this study Chinese Hamster Ovarian (CHO) cells will be utilized as a vaccine to induce HER2/neu immunity in BALB/c mice. CHO cells are fast becoming prominent in the biotechnology field due to its resilience, ability to grow in different culture conditions, and adaptability when it comes to genetic alterations. Recombinant and glycoproteins used in protein

therapeutics have provided innovative and effective therapies for a number of diseases including cancer and infertility. The proteins are generally synthesized on a large scale in mammalian cells because bacteria, such as *E.coli,* lack the appropriate machinery to synthesize complex proteins. Today CHO cells are responsible for producing around 70% of all recombinant protein therapeutics [86]. CHO cells are known for their adaptability in different culturing conditions and can be genetically manipulated without any consequences to cell viability or growth. They also have an ability to uptake DNA at high levels [86]. CHO cells have been previously used to study cancer in mice. CHO cells transfected to express human TNF-α were injected into mice to determine if it would affect metastasis in nude mice [87].

HER2/neu

HER2 is an epidermal growth factor receptor 2 responsible for controlling normal cell growth and differentiation. Ligand binding to HER2 causes receptor dimerization that generates intracellular signals for growth and differentiation. Few HER2 molecules exist on the cell surface in normal cells making growth signals controllable but over expression of HER2 causes enhanced responsiveness to growth factors that can result in malignant growth and tumors. HER2 over expression is also linked to other types of cancers including ovarian and gastrointestinal related cancers [88] and is associated with aggressive tumor growth and metastasis. Information from clinical trials also suggests HER2/neu overexpression is associated with resistance to chemotherapy[89]. This explains the poor prognosis in HER2 positive breast cancer but because HER2 is highly specific it presents a promising target in breast cancer therapies [90]. Herceptin is a monoclonal antibody directed against HER2 and can reduce tumor size [91] and angiogenesis [92]. Even though Herceptin is a promising treatment, the majority of

patients who respond initially develop Herceptin resistance within 1 year [93, 94]. Thus, more effective breast cancer immunotherapies need to be developed.

In this study, TUBO, a cloned cell line that expresses high levels of HER2/neu generated from a spontaneous mammary gland tumor from BALB-neuT mice will be used to study cross priming and if there is an antibody response generated against TUBO cells.

Rationale and Hypothesis

In this experiment the CHO cell line will be transfected with the human HER2 antigen in addition to immunostimulatory molecules B7-1, IL-2, IL-12, and GM-CSF in order to induce an antitumor effect. Because the model uses xenogeneic CHO cells along with heterologous hHER2, the antitumor effect is predicted to be due to cross priming. Since CHO cells are foreign to the mouse's immune system, this may induce a stronger and more potent response against the CHO cell and hHER2 than if hHER2 was simply presented as DNA or on a self-cell. A decrease in overall tumor size and incidence from vaccinated mice was expected. CHO cells are xenogeneic therefore they cannot directly stimulate mouse T cells, thus, these studies are also a way to study the effects of cross priming in the immune system.

EXPERIMENTAL DESIGN

The first phase of the experiment involved the single or double transfection of previously established CHO cells expressing human HER2 with immunostimulatory cytokine molecules IL-2, IL-12, B7-1, and GM-CSF. Once stable expression of the ISMs was established, the anti-

tumor effects of ISM expressing CHOhHER2 cells were investigated. The following phases were addressed in this study:

I. *ISM expressing CHOhHER2 cell lines establishment*: Cells were transfected via lipofection of a pUB6 plasmid containing ISM cDNA and selection of cells expressing ISMs was performed by magnetic bead isolation and cell panning. Protein expression was confirmed by performing fluorescent activated cell sorting (FACS) analysis. Phosphatidylinsotisol phospholipase-C (PIPLC) treatment was used to verify the cytokines are GPI-anchored.

II. *Vaccination of mice with ISM expressing CHOhHER2 cells*: In three separate experiments mice were vaccinated with 3 x 10^5 live or irradiated cells, or 50μg of cell membranes made from ISM expressing CHOhHER2 cells. The purpose was to see if the cells had an anti-tumor effect and which delivery method would be the most effective. Mice were directly challenged with wild-type tumor cells to investigate possible antitumor effects of the vaccine.

Materials and Methods

Cell Culture

CHOhHER2 cells were maintained in RPMI (Cellgro, Mediatech) supplemented with 10% cosmic calf serum (Hyclone), 1% Penicillin/Streptomycin (Cellgro), and zeocin (200μg/mL). Transfected cells were maintained in the same media but with blasticidin added (20μg/mL). TUBO cells were maintained in DMEM- F12 (50/50) with 10% CCS and 1% Penicillin/Streptomycin. All cells were cultured at 37°C at 5% CO2.

DNA constructs

cDNA of murine IL-12, GM-CSF, and IL-2 were previously constructed in our laboratory and inserted into pUB6 vectors. cDNA of hHER2 was also constructed in our laboratory and inserted into a pCDNA3 vector. IL-12, GM-CSF, and IL-2 were linked with a GPI-anchor signal sequence as described by Poloso [43], McHugh [95], and Nagarajan [41].

Establishment of CHO Cell Lines Expressing ISMs

CHO cells were plated in six well plates at 300,000 cells/well with viability greater than 90% in RPMI with 10% CCS and incubated for 24 hours. Cells were transfected using Lipofectamine 2000 (Invitrogen) and optimem (Invitrogen). Reagents were allowed to warm to room temperature then 520ul of optimem and 20ul of Lipofectamine were incubated for 5 minutes in an eppendorf tube. 40µg of DNA for single transfections (B7-1, IL-2, IL-12, GM-CSF) or 20ug each for double transfections (B7-1/IL-2, IL-2/IL-12, etc) were added and incubated for an additional 20 minutes. Medium from the wells were removed and saved in a centrifuge tube (conditioned media) and replaced with plain RPMI. The content of one eppendorf tube was added evenly between two wells. The transfecting cells were incubated at 37°C at 5% CO_2 for 6-7 hours. Transfection media was removed and replaced with 2.5mL of conditioned media and 2.5mL of RPMI with 10% CCS. 24 hours after transfection, cells were transferred to a T75 flask and grown in RPMI with 10%CCS and 1% P/S. Magnetic bead isolation was performed 48 hours post transfection.

Magnetic Bead Isolation

Magnetic bead isolation utilizes antibody conjugated magnetic beads for physically isolating cells. Cells are incubated with a primary antibody and the secondary antibody is

conjugated to the magnetic bead. A magnet isolator is then used to select for cells expressing the transfected protein.

Magnetic bead isolation was performed using Sheep anti-rat magnetic beads (Dynal Biotech Dynabeads). Recently transfected cells were detached from culture flasks using PBS/EDTA (5mM EDTA) and centrifuged at 1200rpm for 5 minutes and transferred into eppendorf tubes with 200µl of Phosphate Buffered Saline (PBS). Cells were counted using a hemocytometer and incubated with the primary antibody to detect the expression of the transfected molecule (1G10, rat anti-mB7-1: S4B6, rat anti-mIL-2: A2F17, rat anti-mGM-CSF: 9A5, rat anti-IL-12p75) at 50µl per 500,000 cells for and placed in a rotator (Cole-Parmer Instrument Company) at 4°C for 30 minutes. Cells were centrifuged at 1200rpm for 5 minutes and resuspended in200µl magnetic bead buffer. Magnetic beads were calculated at 4 beads/cell then washed and re-suspended in 1mL magnetic bead buffer (PBS with 0.1% BSA, 2mM EDTA, pH 7.4) and added to cells. Cell and magnetic bead suspension were placed in a rotator at 4°C for 30 minutes. Tubes were placed against a magnetic bead separation magnet (Miltenyi Biotech) for 2 minutes and the supernatant was removed using a pipette. The tubes were removed from the magnet and re-suspended in magnetic bead buffer. This was performed three times and after the final wash, cells that remained bound to the magnetic beads were re-suspended in 1mL culture media and placed in 1 well of a six well plate with 5mL of RPMI with 10%CCS, 1% P/S, 400ug/mL zeocin, and 20ug/mL blasticidin. Cells were cultured at 37 °C and 5% CO_2. FACS analysis was performed to determine the expression of the ISMs and subsequent rounds of cellular panning were carried out to obtain a homogenous, high expressing population of cells expressing each ISM.

Cell Panning

Cell panning selects for high protein expressing cells using antibodies bound to a bacterial petri dish. Primary antibodies for specific ISMs are recognized and bind to secondary antibodies attached to a petri dish and cells expressing high levels of the protein are added to the dish. Cells expressing high levels of the ISM attach to the dish and low ISM expressing cells are washed away. Cell panning was performed against one ISM at a time. For double transfectants, panning had to be performed twice sequentially, once for each immunostimulatory molecule. Reagents were all handled inside a tissue culture hood and sterile filtered beforehand using a 0.22μm filter. Cell panning was performed using whole molecule Goat anti-Rat IgG (Sigma-Aldrich) secondary antibody diluted at 10μg/mL in PBS. 5mL of the secondary antibody solution was added to a bacterial petri dish (BD Falcon) and left at room temperature for 2 hours. A spot marked with an X was used to designate a spot on the dish where all reagents are added. Secondary antibody was removed and washed with 10mL of cold PBS for 2-3minutes to remove any unbound IgG. The primary antibody (1G10, rat anti-mB7-1: S4B6, rat anti-mIL-2: A2F17, rat anti-mGM-CSF: 9A5, rat anti-IL-12p75) was added and allowed to incubate at room temperature for 30-40 minutes. During the incubation period, cells were removed with PBS/EDTA and spun for 5min at 1200rpm and re-suspended in 5mL ice cold PBS/EDTA. Primary antibody was removed and the plate was washed 2x with 10mL PBS for 2-3minutes. The cells suspended in PBS/EDTA were added to the plate and placed at 4^{o}C for 30-40minutes. The plate was placed under a microscope to check for adherent cell. The plate was washed 5-10X with cold PBS/EDTA to remove non-adherent cells. Cells were placed in RPMI with selection and placed in the incubator. Cells were transferred to T75 flasks (Corning) when cell density increased and expression of cells were then checked with FACS analysis once confluent.

Flow Cytometry Analysis

Fluorescent activated cell sorting (FACS) staining uses fluorescently labeled antibodies to detect protein expression on the cell surface. Cells are incubated with a primary antibody specific to the protein and then incubated with a secondary antibody conjugated to a fluorescent protein. In this case, the fluorescent protein is fluorescein isothiocyanate (FITC) which fluoresces when excited with a beam of light with its specific wavelength or frequency. The entire experiment was performed on ice.

A 96-well v-bottom plate was presoaked in 200µl FACS buffer (PBS, 1%EDTA, 1% CCS) for 10 minutes at room temperature. Cells were removed with PBS/EDTA and centrifuged at 1200rpm for 5 minutes and viability was checked to make sure it was 90% with a hemocytometer (10ul cell suspension with 10ul trypan blue). Supernatant was removed and cells were re-suspended in cold PBS/EDTA at 5 million cells/mL. FACS buffer was removed from the well and 50µl of cell solution was placed into each well with 50µl of primary antibody, re-suspended, and placed at 4°C on a shaker for 30 minutes. The plate was centrifuged for 2 minutes at 1300rpm. Supernatant was removed and cells were washed twice in 200µl of FACS buffer. FITC conjugated Goat anti-rat IgG was diluted 1:50 in FACS buffer and added to the wells and placed back on the shaker for 20-30 minutes. After incubation, the plate was centrifuged at 1300rpm for 2 minutes and the supernatant was removed. Cells were washed 2X with FACS buffer and re-suspended in 150µl of FACS buffer followed by 150µL of PBS with 2% formalin. Samples were put into labeled microtubes in a microtube box and covered with aluminum foil. The samples were analyzed via FACS analysis and FlowJo analysis software.

PIPLC Treatment

PIPLC (phosphatidylinositol phospholipase-C) treatment is used to cleave the lipid portion of a GPI-anchor and is used to confirm proteins are indeed GPI-linked. Each PIPLC treatment was done with a control (tube without PIPLC). CHOhHER2 transfectants were disassociated with PBS/EDTA and centrifuged at 1200rpm for 5 minutes. Cell viability (>90%) and cell count was performed using a hemocytometer. Cells were washed once in PIPLC buffer (PBS/EDTA with 5mg/mL ovalbumin) and re-suspended to 5 x 10^6 cells/mL in PIPLC buffer. 1mL of cell suspension was placed into 10mL round bottom tubes and PIPLC at a 1:1000 dilution (1µl in 1 mL) was added. Tubes were incubated in a 37°C water bath for 45 minutes with occasional shaking every 15 minutes to ensure even mixing of cells with the enzyme. After incubation, cells were centrifuged at 1200rpm for 5 minutes and washed twice with 10mL of FACS buffer. The cells were then stained for FACS analysis as described previously and analyzed.

Membrane Preparation

Cell membranes were prepared as described [96]. Cells were grown in large quantities in roller bottles with RPMI, 10% CCS, 0.5% Penicillin/Streptomycin, 0.2% Fungizone, and 1% HEPES buffer. After cells reached 95% confluency, they were removed using PBS/EDTA and centrifuged for 10minutes at 1200rpm. Cells were re-suspended in 1mL cold homogenization buffer (20mM Tris pH 8.0, 10mM NaCl, 0.1mM MgCl2, 0.02% NaN_3, and 0.1mM PMSF added immediately before use). A 41% sucrose gradient was prepared using the homogenization buffer. Cells were homogenized with a Polytron homogenizer for four 7-8 second pulses on power level 5 with 1 minute cooling on ice between pulses. The cell suspension was brought up

to 8mL with homogenization buffer and added to a new Beckman centrifuge tube with 4mL of 41% sucrose gradient for a total volume of 12mL. Membranes were centrifuged at 23Krpm for 1 hour at 4°C with a SW41 router ultracentrifuge. After centrifugation, the cell membrane interphase was collected and placed into eppendorf tubes and centrifuged at 14K rpm for 1 hour at 4°C. The supernatant was removed and the membrane pellet was re-suspended in 1ml of PBS with 10mM HEPES buffer. Cell membranes were stored at -20°C until performing protein estimation.

Protein Estimation

Protein estimation was performed to determine the amount of protein from the cell membrane preparation. Protein concentrations were determined by the Bio-Rad dye binding method using bovine serum albumin (BSA) as a standard. The experiment was done using a 96-well flat bottom plate in triplicates. 150 µl of the standard and unknown were plated in triplicates along with 150 µl of the working reagent and the plate was incubated for 2 hours at 37°C. After incubation, the plate was allowed to cool down to room temperature. The plate was read using a Softmax plate reader and a standard curve was prepared by plotting the average BSA standard versus its concentration in µg/mL in a linear form. A linear regression was used in order to determine the concentration of protein from the samples from the membrane preparation.

Animals

Female BALB/c mice were provided from Jackson Laboratories at 6-8 weeks of age. All mice were maintained in Emory University animal facilities according to the Institutional Animal Use Committee regulations.

Mouse Vaccinations and Challenge

All vaccinations were done subcutaneously in the right flank using a $27^{1/2}$ gauge 1mL needle (BD Precision Glide Needle). Each experimental group had 5mice/group. For the live cell vaccine, ISM expressing CHOhHER2 cells were removed with PBS/EDTA and centrifuged at 1200rpm for 5minutes. Cell count and viability was determined by the trypan blue exclusion method to ensure viability was >90%. Cells were then resuspended at a concentration of 3×10^6 cells/mL in PBS. Mice were shaved to expose the skin on the hind flanks of the mice. 100µl of the cell suspension were injected subcutaneously into the right flank of the mouse giving a total of 300,000 cells injected per mouse. Mice were visually monitored for any adverse side effects a day after vaccination.

For irradiated cells, vaccinations cells were grown in culture without selection overnight, removed 24 hours later with PBS/EDTA and centrifuged for 1200rpm at 5 minutes. Cells were re-suspended in plain RPMI and irradiated at 80Gy in Emory Whitehead Research Building facilities. Cells were centrifuged and re-suspended in PBS at $3x10^6$cells/ml. Injections were also performed in the right flank with a $27^{1/2}$ gauge needle injecting a total of 300,000 cells per mouse.

Membrane vaccinations were also performed the same as the live cell vaccine and each mouse was vaccinated with 50µg of cell membranes. The amount of membrane required to vaccinate 5 mice at 50µg/mouse was taken from the membrane preparation stock and placed in an eppendorf tube. Membranes were centrifuged at 14Krpm for 1 hour and re-suspended in 500µl PBS. Cell injections were also performed using a $27^{1/2}$ gauge needle and 100µl of re-suspended membrane was injected per mouse.

Mice were challenged 4 weeks post vaccination. All mice were challenged with 200,000 TUBO cells subcutaneously in the left flank. TUBO cells were removed using PBS/EDTA and centrifuged for 5 minutes at 1200rpm. The supernatant was removed and cell count and viability were performed. Cells were re-suspended at $2x10^6$ cells/mL and 100µl of the suspension was injected into the left flank of the mouse. Mice were monitored for the next 2-3 days for side effects. When tumors started appearing they were measured with vernier calipers by 2x2 perpendicular measures and the tumor size (mm^2) was determined by multiplying the two diameters. Mice were euthanized when the tumor size reached $>2cm^2$.

Mouse Serum Antibody Analysis

Serum was collected from the vaccinated mice prior to tumor challenge in order to analyze if any antibodies against TUBO, 4T07hHER2, CHOhHER2, and CHO-K1 cells were produced. Mice were bled prior to challenge with TUBO cells by puncturing the cheek pouch saphenous vein using a lancet. Blood was collected in blood collection tubes. After blood was collected, it was centrifuged at 13.2Krpm for 10minutes and the clear liquid serum was collected and stored at -20^oC until analyzed by flow cytometry. Serum from each vaccinated mice group was stained against TUBO, 4T07hHER2, CHOhHER2, and CHO-K1 cells at 1:10 and 1:100 dilution and results were analyzed through FACS analysis.

Results

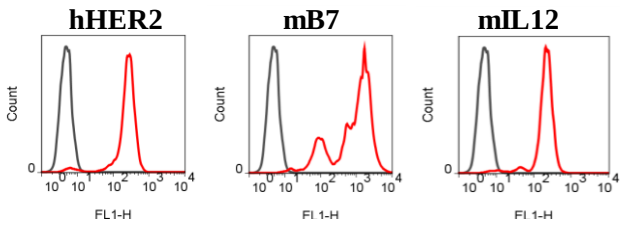
CHOhHER2 Transfectants

The initial part of the experiment involved establishing CHOhHER2 cells that express the murine form of GPI-anchored, IL-2, IL-12, GM-CSF, and transmembrane B7-1. The immunostimulatory molecules were transfected to be expressed alone or in double combination (Table 1). After transfecting the cells, magnetic bead isolation was performed in order to isolate cells expressing the protein and multiple rounds of panning were performed to increase the expression of the molecule (Figure 3). The expression of the protein throughout the establishment process was profiled through FACS analysis using a FITC conjugated secondary antibody. The MFI level is listed (Table 2). The ratio of ISMs in double transfectants is also given. The expression of the ISMs were all high, however, CHOhHER2 IL-2/IL-12 transfectants had a small negative population for IL-2. For the single transfectant groups, IL-12 had the highest expression (MFI=1529) and B7-1 had the lowest expression (MFI=654). Group CHOhHER2 B7/IL-12 also had a small low expressing population.

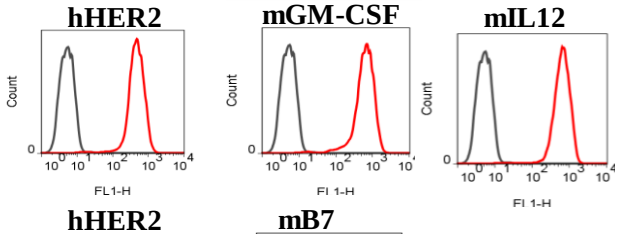
Table 1. List of EstablishedCHOhHER2 Transfectants

CHOhHER2 mB7-1	CHOhHER2 mIL-2
CHOhHER2 mIL-12	CHOhHER2 mGM-CSF
CHOhHER2 mIL-2/mIL-2	CHOhHER2 mB7-1/mIL-12
CHOhHER2 mGM-CSF/IL-12	CHOhHER2 mGM-CSF/IL-2
CHOhHER2 mGM-CSF/mB7-1	CHOhHER2 mB7-1/IL-2
CHOhHER2	

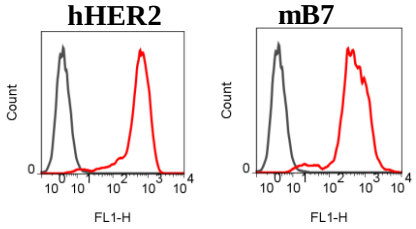
ChohHER2 mB7/mIL12



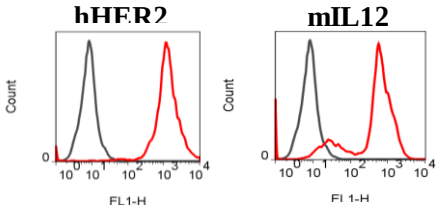
ChohHER2 mGMCSF/mIL12



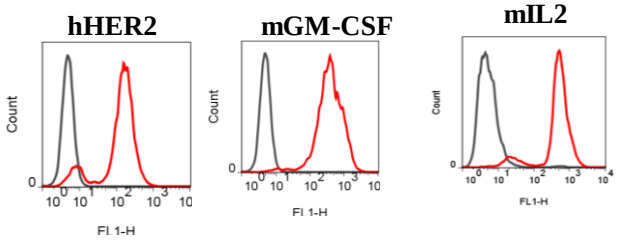
ChohHER2 mB7



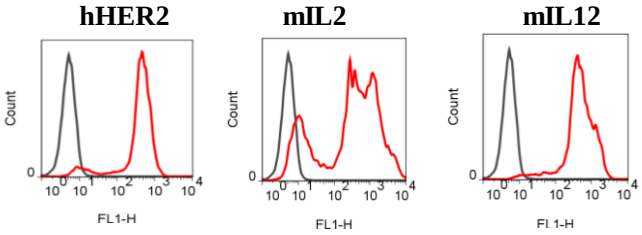
ChohHER2 mIL12



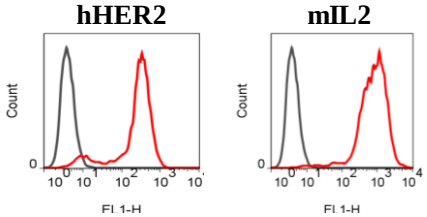
ChohHER2 mGMCSF/mIL2



ChohHER2 mIL2/mIL12



ChohHER2 mIL2



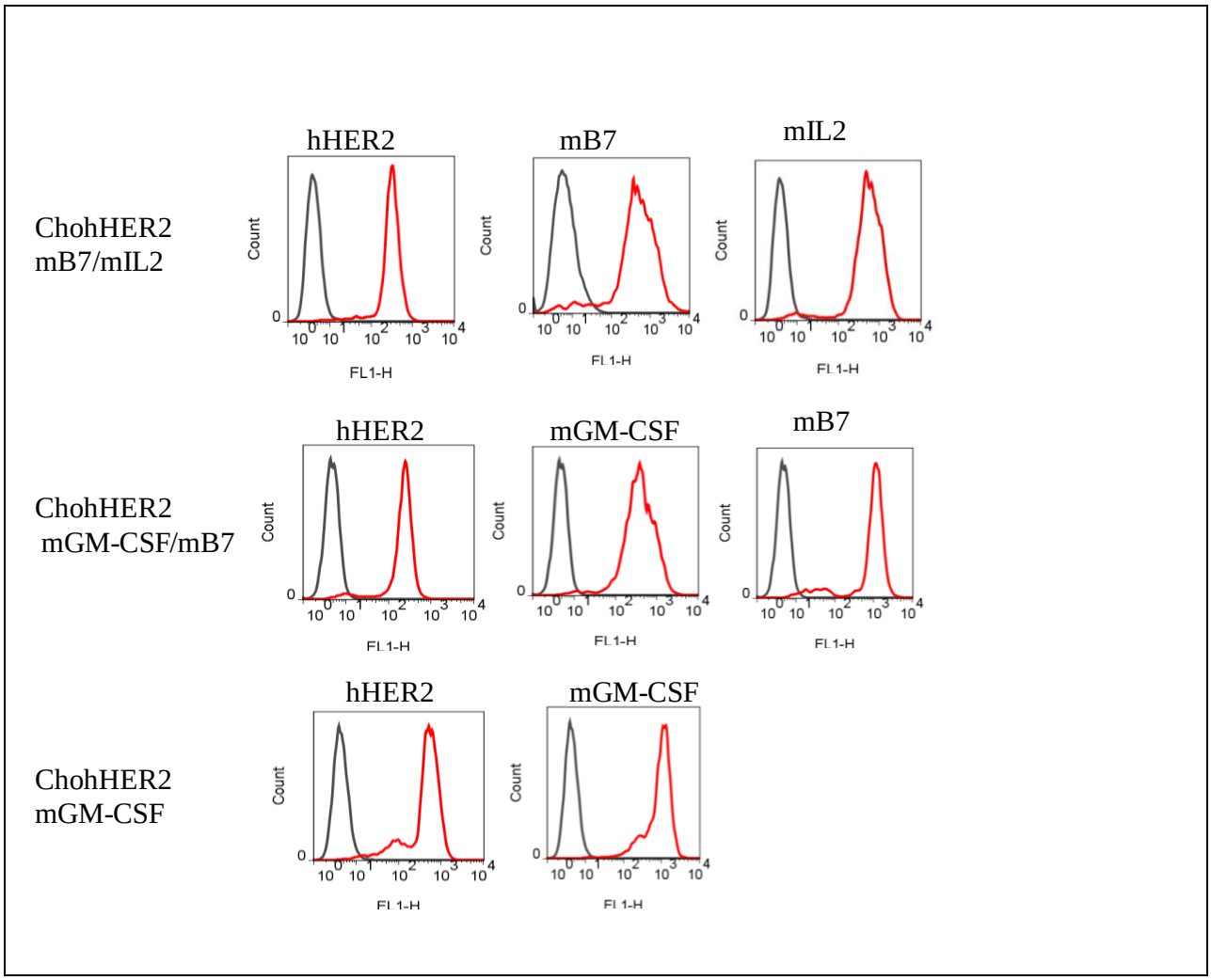


Figure 3. FACS analysis of CHOhHER2 Transfected with ImmunostimulatoryMolecules. CHOhHER2 cells were analyzed for the expression of the GPI-anchored immunostimulatory molecules and cytokines B7-1, IL-12, IL-2, and GM-CSF. FACS analysis results revealed cells were all positive for the ISMs at high levels.

Table 2. MFI values for CHOhHER2 transfectants. MFI values were determined through FACS analysis. (A) MFI values for single transfectants. (B) MFI values for double transfectants. MFI ratio for double transfected cells is also provided.

A.

Cell Line/Single Transfectant	hHER2 (MFI)	ISM (MFI)
CHOhHER2 mIL-2	309	981
CHOhHER2 mGM-CSF	509	1009
CHOhHER2 mIL-12	565	1529
CHOhHER2 mB7-1	622	654

B.

Cell Line	hHER2(MFI)	MFI	MFI	MFI ratio
CHOhHER2 mB7-1/mIL-2	406	mB7-1=1236	mIL-2-=657	1.88 :1
CHOhHER2 mGM-CSF/mB7-1	565	mGM-CSF=500	mB7-1=764	1: 1.52
CHOhHER2 mGM-CSF/mIL-2	577	mGM-CSF=678	mIL-2=1297	1: 1.92
CHOhHER2 mGM-CSF/mIL-12	502	mGM-CSF=678	mGM-CSF=672	1:1
CHOhHER2 mIL-2/mIL-12	428	mIL-2=731	mIL-12=636	1: 1.08
CHOhHER2 mB7-1/mIL-12	260	mB7-1=1236	mIL-12=216	5.72 : 1

PIPLC treatment

After establishing the CHOhHER2 cell lines with the ISMs, enzymatic treatment with PIPLC was performed to confirm the cell surface expressed molecules are indeed GPI-linked. The level of expression for the molecules was measured by flow cytometry and the mean fluorescent intensity (MFI) was used as an indicator of the ISMs expression. PIPLC treated cells

were compared to non-PIPLC treated (control) cells in order to determine the decrease in MFI. For single transfectants (Figure 4) both IL-12 and IL-2 showed a large decrease in protein expression upon enzyme treatment. IL-12 expression decreased 92%, IL-2 expression decreased 52.1%, and GM-CSF expression decreased 69.5% confirming GPI-linkage. Double transfectants were also treated with PIPLC in order to determine if the ISMs were GPI-anchored (Figure 5). For group (3A) IL-2/IL-12 there was a 47.2% decrease in expression for IL-2 and a 50.3% decrease in expression for IL-12 after PIPLC treatment. This verifies the molecules are indeed GPI-anchored. Group (3B) GM-CSF/B7-1 had an MFI decrease of 43.1% for GM-CSF and 12.36% decrease for B7-1. Even though B7-1 is transmembrane rather than GPI-anchored the procedure alone such as incubating the cells at 37^{o}C in a water bath for 45 minutes may have affected the expression of B7-1 slightly. The secondary antibody control may not have been added causing the peak to not shift to the right. This could have affected the MFI. Group (3C) GM-CSF/IL-2 also showed a large decrease in expression for both molecules GM-CSF (72.6%) and IL-2 (87.7%) which confirms they are GPI-anchored. For group (3D) GM-CSF/IL-12 there was also a significant decrease in GM-CSF (73.1%) and IL-12 (81.3%) expression also showing they are GPI-anchored. Group (3E) IL-2/IL-12 also showed a decrease in MFI value for IL-2 (47.2%) and IL-12 (50.3%). For group B7-1/IL-2 there was a 3.49% decrease in B7-1 expression which confirms that it is not GPI-linked. The results from the PIPLC treatment showed large shifts in MFI levels after treatment which confirms the transfectants are GPI-anchored. The lower decrease in MFI for transfectants with B7-1 also verifies it is a transmembrane molecule.

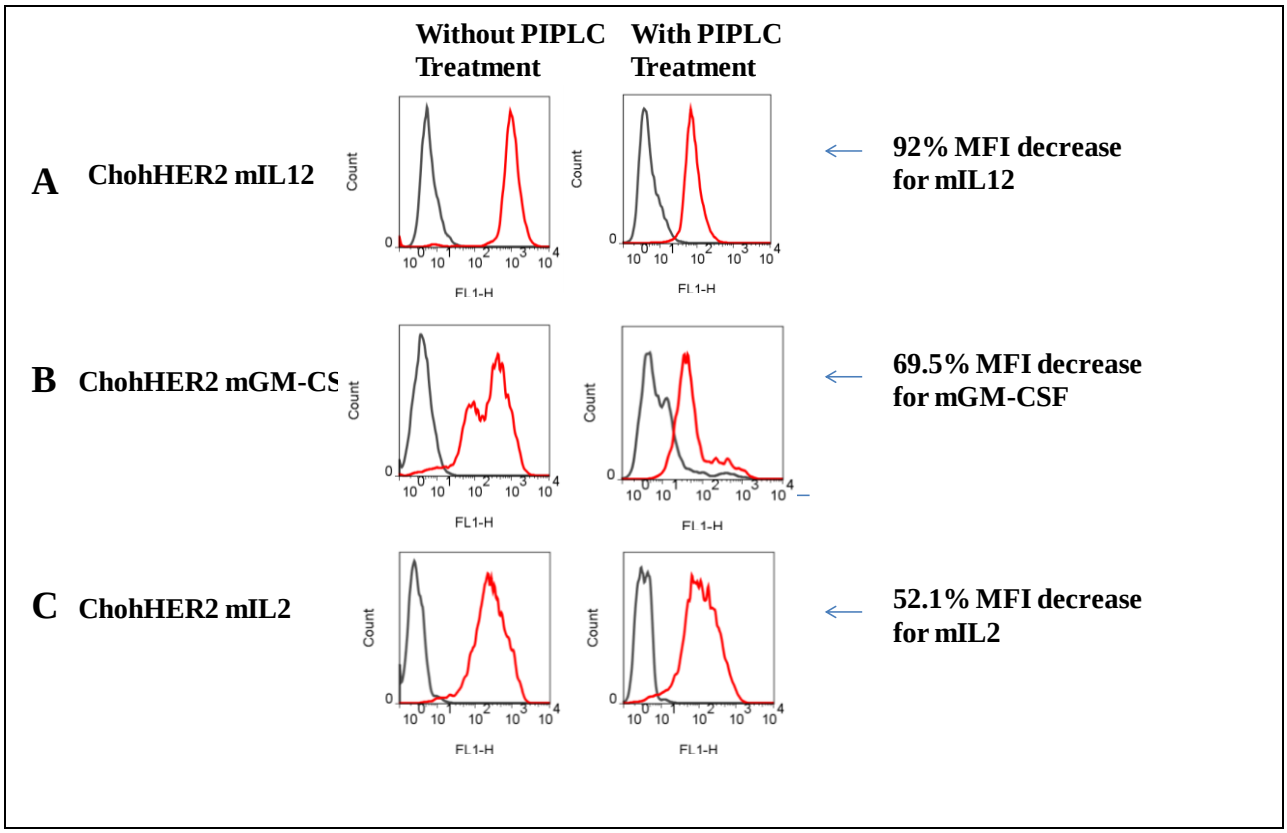
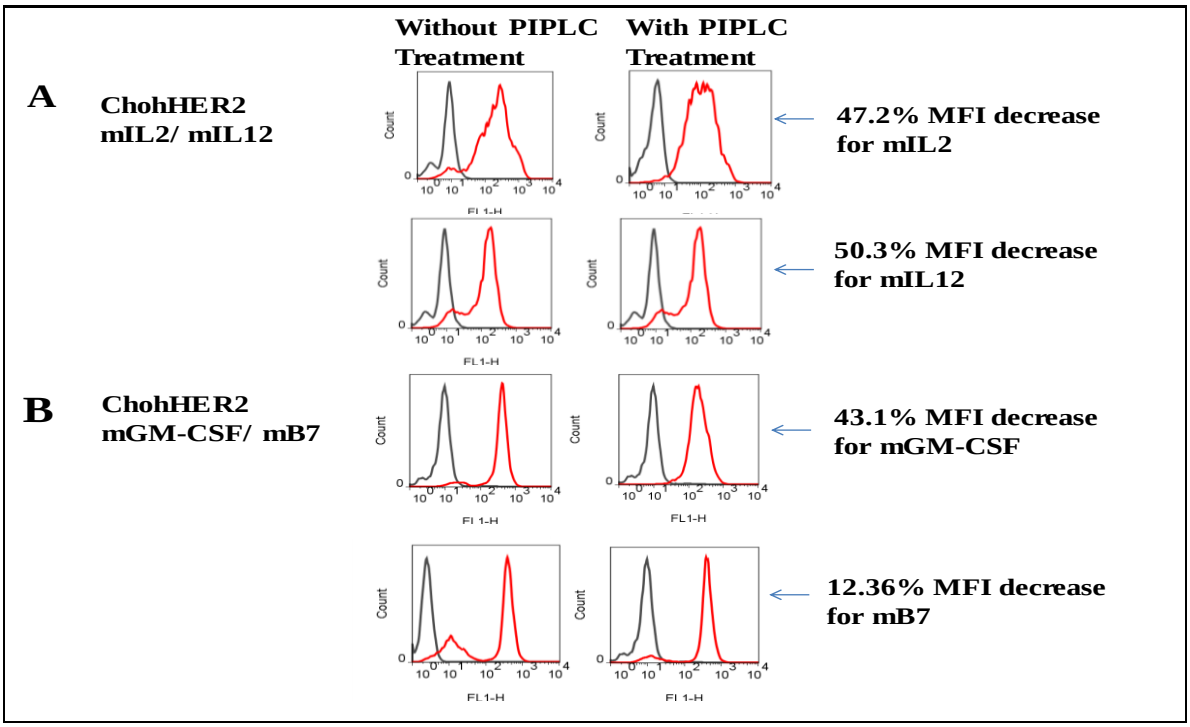


Figure 4. PIPLC treatment for single transfectants. PIPLC (phosphatidylinositol phospholipase-C) enzymatic treatment was used to confirm GPI-linkage of (A) IL-12, (B) GM-CSF, and (C) IL-2. GPI-anchored IL-12 showed a 92% decrease in expression and IL-2 also showed a 52.1% decrease in expression verifying the GPI-linkage. GM-CSF also showed a 69.5% decrease in expression upon treatment indicating it is GPI-anchored as well. All MFI's were determined by FACs analysis.



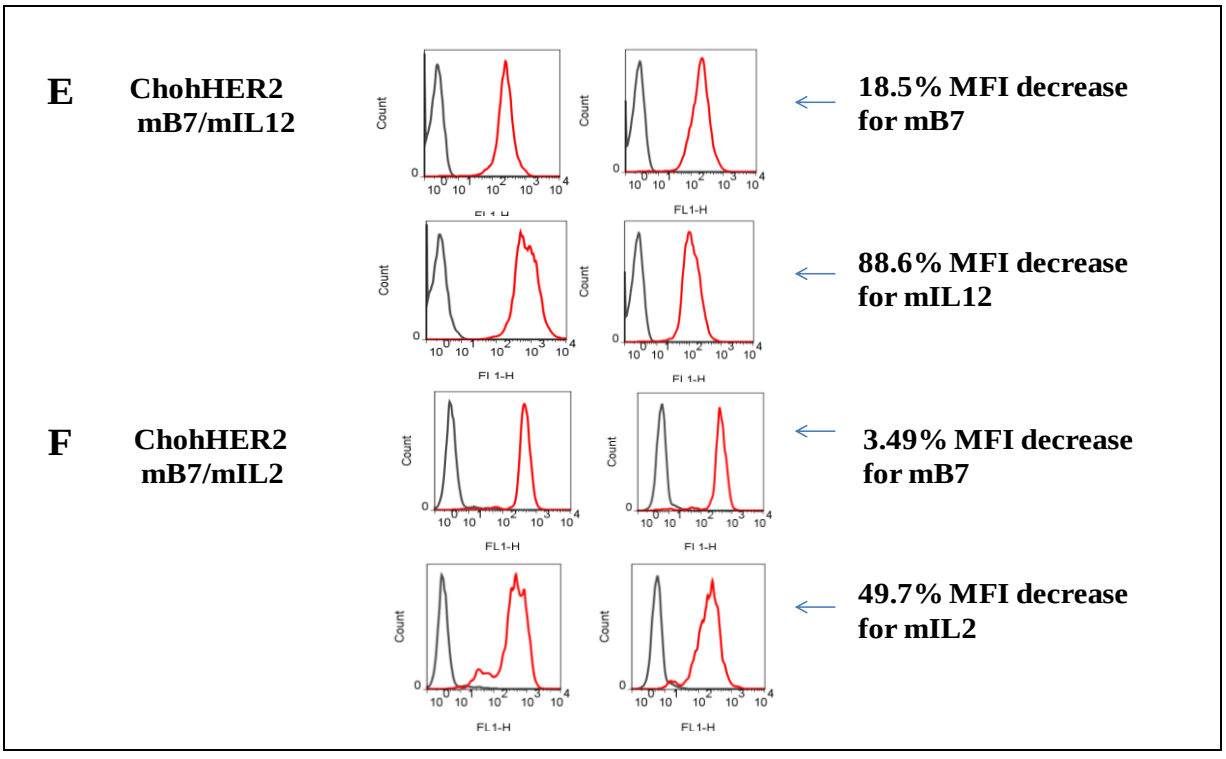


Figure 5. PIPLC Treatment for Double Transfectants. The enzyme PIPLC was also used to cleave the GPI-anchor on the ISM to confirm its GPI-linkage. (A) IL-2/IL-12 had a 47.2% decrease in expression for IL-2 and a 50.3% decrease in expression for IL-12 is shown after PIPLC treatment

showing GPI-linkage being cleaved. (B) GM-CSF/B7-1 had a MFI decrease of 43.1% for GM-CSF And 12.36% for B7-1. Even though B7-1 is transmembrane rather than GPI-anchored the procedure alone may have affected the expression. (C) GM-CSF/IL-2 showed a large decrease in expression for both molecules GM-CSF (72.6%) and IL-2(87.7%) confirming GPI-linkage. (D)GM-CSF/IL-12 also showed a significant decrease in GM-CSF(73.1%) and IL-12(81.3) expression also showing it is GPI-anchored. (E) B7-1/IL-12 and (F)B7-1/IL-2 also showed decreases in MFI value for IL-2(49.7%) and IL-12(88.6%). For B7-1 in group B7-1/IL-12 there was an 18.5% decrease which may be attributed to the treatment but for B7-1 in group B7-1/IL-2 there was only a 3.49% decrease which confirms that it is not GPI-linked.

Table 3. MFI values of cells before and after PIPLC treatment. Cells were treated with enzyme PIPLC and the MFI before and after treatment was calculated to determine the decrease in MFI which translates to a decrease in molecule expression. The decrease in MFI confirms that the ISMs are GPI-anchored.

Cell Lines	MFI before PIPLC Treatment	MFI after PIPLC Treatment	% Decrease in MFI
CHOhHER2 mIL-2	378	181	52.11%
CHOhHER2 mIL-12	1067	90.6	92%
CHOhHER2 mGM-CSF	401	122	69.5%
CHOhHER2 mIL-2/mIL-12	mIL-2- 288 mIL-12- 144	mIL-2-152 mIL-12-71.5	mIL-2- 47.2% mIL-12-50.3%
CHOhHER2 mGM-CSF/mB7-1	mGM-CSF- 390 mB7-1-283	mGM-CSF- 222 mB7-1- 248	mGM-CSF- 43.1% mB7-1- 12.6%
CHOhHER2 mGM-CSF/mIL-12	mGM-CSF- 424 mIL-12- 566	mGM-CSF- 114 mIL-12- 102	mGM-CSF- 73.1% mIL-12- 81.3%
CHOhHER2 mB7-1/mIL-12	mB7-1- 281 mIL-12-936	mB7-1-229 mIL-12- 106	mB7-1- 18.15% mIL-12-88.6%
CHOhHER2 mB7-1/mIL-2	mB7-1- 458 mIL-2- 509	mB7-1- 442 mIL-2-256	mB7-1- 3.49 mIL-2- 49.7
CHOhHER2 mGM-CSF/mIL-2	mGM-CSF-723 mIL-2-878	mGM-CSF-525 mIL-2-562	mGM-CSF- 72.6% mIL-2- 87.7%

Results from cell membrane preparation

Cells were grown in large quantities and homogenized in a homogenization buffer. Cell membranes were extracted using a 41% sucrose gradient. Protein estimation was performed to quantify the membrane yield. The results are shown in Table 4.

Table 4 . Cell Membrane Preparation. Cell count was determined via the trypan blue method. Final cell count, weight of the cell pellet, and final protein estimation are listed.

Cell Line	Total Cell Count at Harvest	Weight of Cell Pellet (g)	Final Protein Estimation(µg)
ChohHER2 mB7-1	$977x10^6$	1.92	3674
ChohHER2 mIL-2	$897\ x10^6$	1.60	4324
ChohHER2 mIL-12	$784\ x10^6$	1.73	3210
ChohHER2 mGM-CSF	$935\ x10^6$	1.79	3814
ChohHER2 mIL-2/mIL-12	$914\ x10^6$	1.98	5630
ChohHER2 mB7-1/mIL-2	$935\ x10^6$	1.65	3270
ChohHER2 mGM-CSF/mB-7	$1210\ x10^6$	2.16	4912
ChohHER2 mGM-CSF/mIL-12	$621\ x10^6$	1.42	3674
ChohHER2 mB7-1/mIL-12	$731\ x10^6$	1.56	4079

***In vivo* mouse experiments and challenge**

BALB/c mice were vaccinated subcutaneously(n=5/group) in the right flank with $3x10^5$ live or irradiated cells or 50µg of membranes made from the CHOhHER2-ISM expressing cells. 30 days after vaccinations, mice were challenged with $2x10^5$ live TUBO cells and tumor growth was monitored there on (Figure 6). Prior to challenge TUBO cells were analyzed by FACS analysis to confirm the expression of the rat HER2/neu protein (Figure 7).

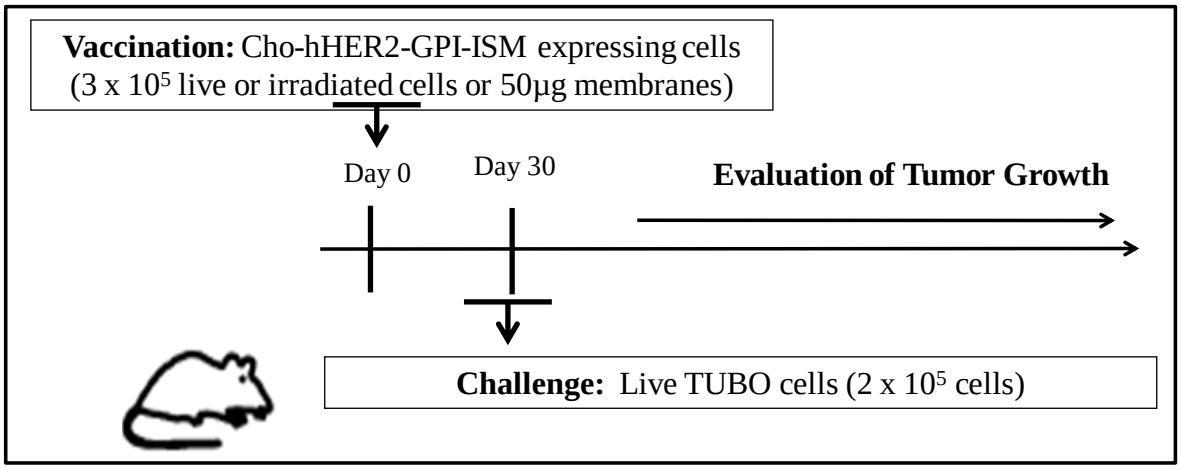


Figure 6. Mouse vaccine studies timeline. Mice were shaved and vaccinated with $3x10^5$ live, irradiated, or membrane vaccines. 30 days post vaccination mice were challenged with $2x10^5$ live TUBO cells.

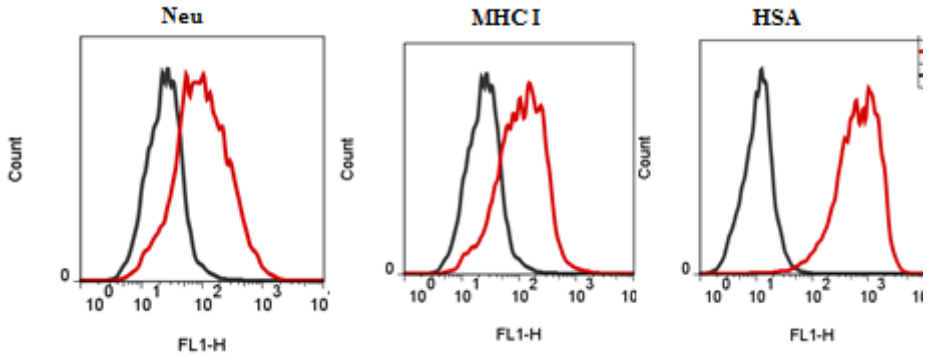


Figure 7. Characterization of TUBO cells: TUBO cells were characterized by FACS analysis. TUBO cells express the rat HER2/neu protein (MFI=159), HSA protein (MFI=848), and MHC class I (MFI=159).

Results

Live Vaccine Results

Single Transfectant Vaccine Studies: BALB/c mice (n=5/group) were vaccinated with $3x10^5$ live cells expressing a single immunostimulatory molecule and challenged 30 days later with wild-type TUBO cells. The tumor growth rate for vaccinated mice was slower than the tumor growth rate for unvaccinated control mice. The individual tumor size for each mouse is given in Figure 8. Group B7-1, IL-2, and IL-12 all had tumors that started to appear on day 13. All mice in group B7-1 developed tumors which grew the fastest out of all the other groups. Mouse 5 in Group IL-12 remained tumor free during the whole study and the remaining mice formed tumors on day 13 which grew the slowest and remained the smallest compared to the other groups. Group IL-2 had a steady increase in tumor size; however, 2 mice (mouse 1 and mouse 5) remained tumor free. The tumors in the GM-CSF group formed the latest at day 15 and had the slowest growth. 3 mice (mouse 1, 2 and 5) formed tumors that remained below $50mm^2$ with the

exception of mouse 3 and mouse 4 which never formed a tumor. The vaccinated mice groups grew tumors at a reduced rate than the unvaccinated control group implying there is some antitumor effect from the vaccine. The control group mice all developed tumors that progressed until they were sacrificed on day 38.

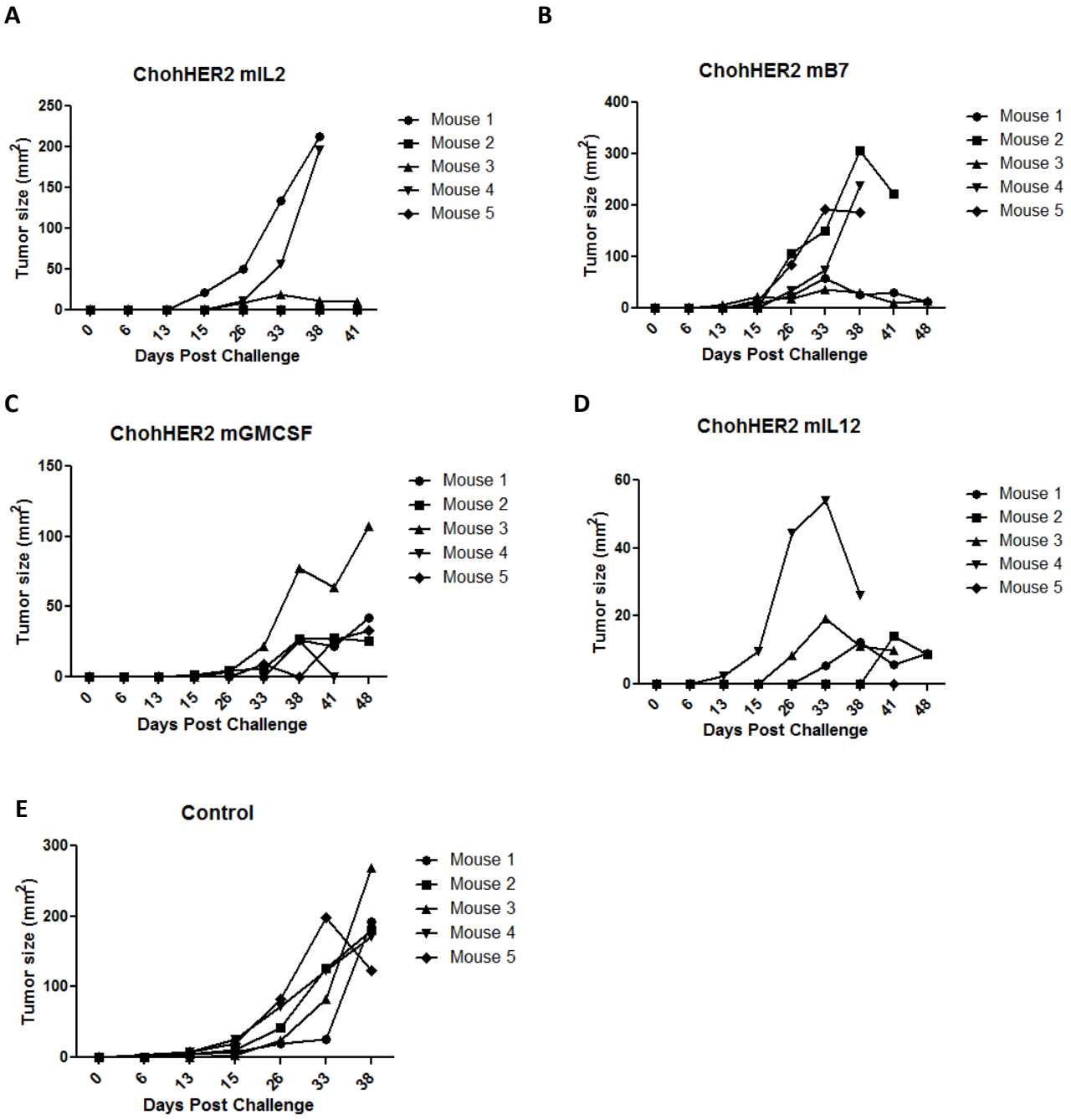


Figure 8. Tumor size for individual mice vaccinated with single transfectants. BALB/c mice (n=5/group) were vaccinated with the single ISM CHOhHER2 expressing cells and challenged 30 days later with $2x10^5$ live TUBO cells in 100µl of PBS. Mice were monitored every 2-3 days and tumor size was calculated by taking 2x2 perpendicular measurements (mm) and multiplying together to give mm^2. Graphs show reduced tumor growth compared to the control group.

Double Transfectant Vaccine: Mice (n=5/group) were vaccinated with CHOhHER2 cells that expressed combinations of two ISMs. Group B7-1/IL-12, GM-CSF/IL-2, IL-2/IL-12, and the control groups all had tumors form on day 13 post challenge. Tumor growth for B7-1/IL-2 was comparable to the unvaccinated control group and grew at almost the same rate. However B7-1/IL-12 group had one mouse (mouse 4) that remained tumor free and the remaining four tumor positive mice were sacrificed on days 38 and 41. The control mice tumors grew fast and all mice were sacrificed on day 38 post challenges. Group GM-CSF/IL-12 grew barely palpable tumors on day 13 that were not measurable until day 26. One mouse (mouse 4) remained tumor free and the tumor positive mice grew tumors steadily. Mice in group B7-1/IL-2 started forming barely palpable tumors on day 13 and the tumors were finally large enough to measure on day 26. One mouse from B7-1/IL-2 never formed a tumor (mouse 1) and one mouse grew a barely palpable tumor that completely regressed. Tumors for group IL-2/IL-12 grew at the slowest rate but all the mice were tumor positive. For group GM-CSF/B7-1, tumors started appearing on day 15; however, there was an accident in the animal facility with the watering system that caused 4 of the mice to drown on day 26 due to cage flooding. One mouse (mouse 4) survived and that mouse had very slow tumor formation. In the future, the GM-CSF/B7-1 vaccinations will be performed again to assess the antitumor effects of that ISM combination. Group GM-CSF/IL-2 had tumor growth on day 13 and all mice were tumor positive by day 26. However, mouse 5 remained tumor free. The results show there is reduced growth rate in tumor formation for mice vaccinated with live ISM expressing CHOhHER2 cells when compared to unvaccinated control mice.

Tumor Incidence Curve for Live Cell Vaccine: Group IL-2 had the most tumor free mice. The vaccinated group developed tumors at a slower rate and group IL-2 had the highest percentage of

tumor free mice at the end of the experiment. The control mice were all tumor positive by day 20 (Figure.10).

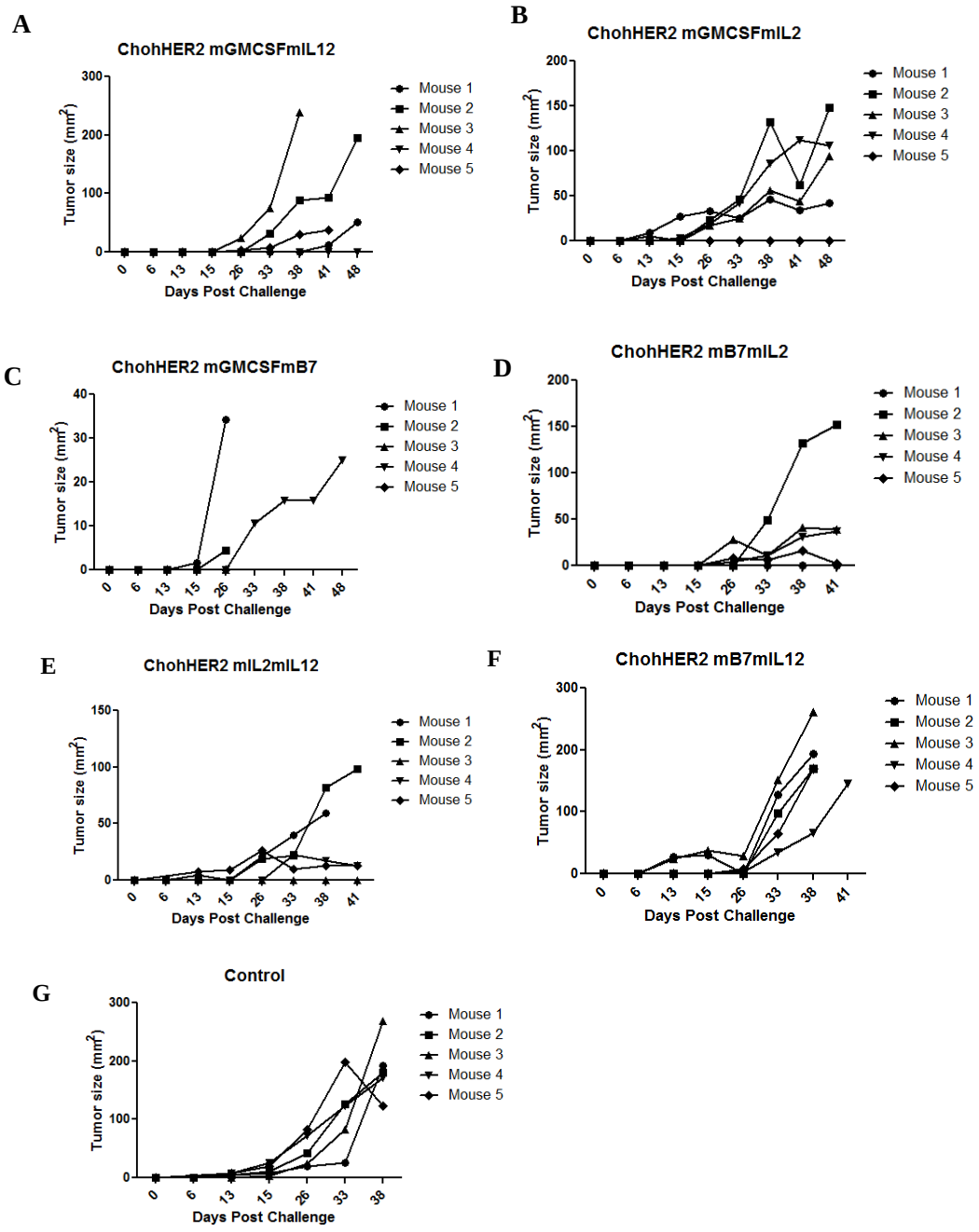


Figure 9. Tumor size for mice vaccinated with double transfectants and challenged with TUBO cells. BALB/c mice (n=5/group) were vaccinated with the single ISM CHOhHER2 expressing cells and challenged 30 days later with $2x10^5$ live TUBO cells in 100µl of PBS. Mice were monitored every 2-3 days and tumor size was calculated by taking 2x2 perpendicular measurements (mm) and multiplying together to give mm^2.

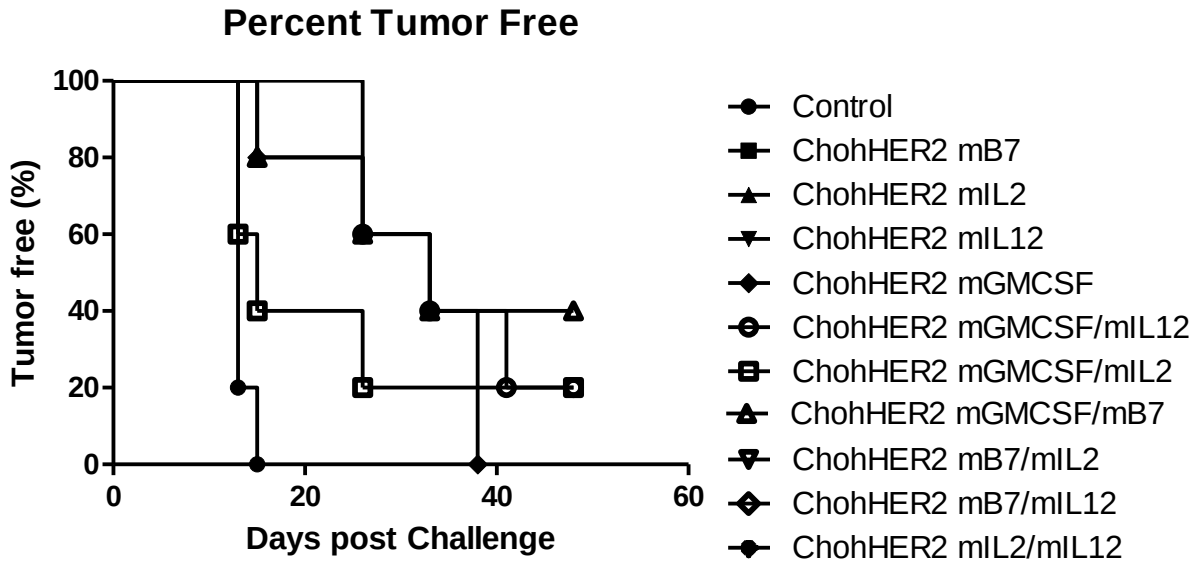


Figure 10. Tumor incidence for mice vaccinated with live cell vaccine. Tumor incidence curve shows tumor incidence in vaccinated groups. Control mice all were tumor positive before day 20 and the remaining groups still had tumor free mice after day 20.

Average tumor sizes: Tumor sizes of all the groups were averaged and compared. A One-way ANOVA test was used to analyze the tumor size averages of vaccinated mice to the tumor sizes of the control group. Results from the test indicate there is no significant difference between the tumor sizes of each vaccinated group against the control even though vaccinated groups did have smaller tumors and grew at a slower rate. Even though vaccinated mice did have decreased tumor sizes the difference was not enough to be considered significant.

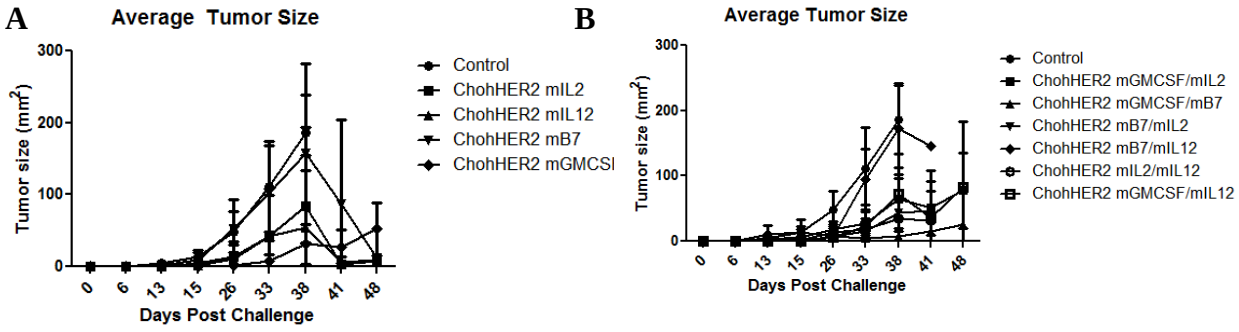


Figure 11. Average tumor sizes for live cell vaccines. (A) Average tumor size for single transfectants. (B)Average tumor size for double transfectants.

Irradiated Cell Vaccine Results

Irradiated Cell Vaccine for Single Transfectants: CHOhHER2-ISM expressing cells were irradiated at 80Gy then immediately injected subcutaneously into BALB/c mice at $3x10^5$ cells resuspended in 100ul PBS. 30 days later mice were challenged with $2x10^5$ live TUBO cells in 100uL of PBS. Tumors were measured in mm^2 by multiplying 2 perpendicular measurements (Figure 10). Barely palpable tumors began on day 12 and were able to be measured by day 17. Tumors grew at faster rates than mice vaccinated with live cell vaccine. No difference in tumor sizes was seen between the vaccinated and control group. However, for group GM-CSF the tumor size and rate of growth was reduced compared to the control and two mice remained tumor negative (mouse 3 and 5). Mouse 4 in group IL-12 remained tumor negative however the remaining tumor positive mice grew tumors at rates comparable to the control group. In summary, the effects of the irradiated vaccine were not as pronounced as the effects of the live cell vaccine.

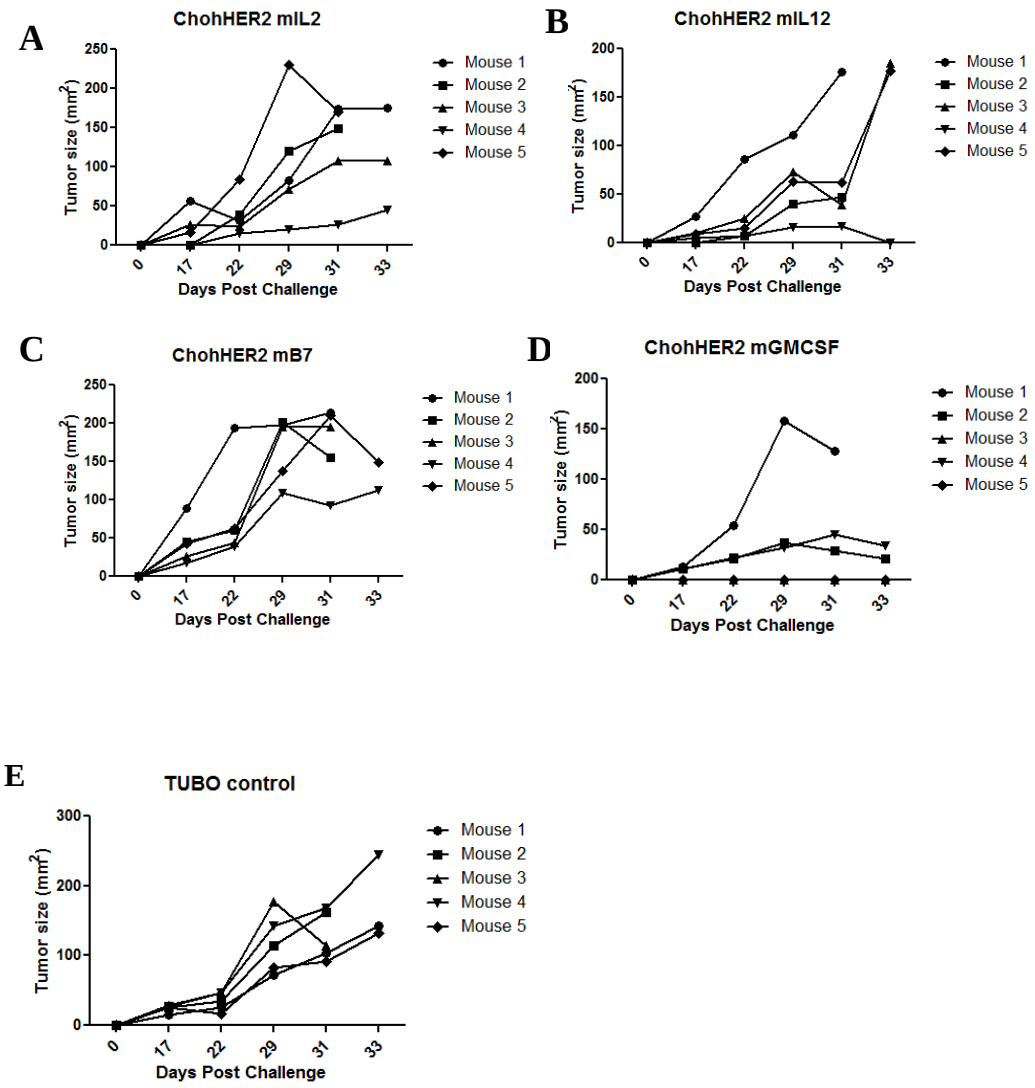


Figure 12. Growth rate of tumors for mice vaccinated with single transfectant irradiated vaccines: (A) IL-2 group mice grew tumors by day 17 that steadily increased similar to the control. (B) IL-12 also grew tumors at the same rate as the control group with one mouse remaining tumor free. (C) B7-1 group mice had fast tumor rate formations with all tumor positive mice. (D) GM-CSF vaccinated mice grew tumors at the slowest rate with 2 mice remaining tumor free during the experiment. (D) Unvaccinated control group mice were all tumors positive at steady rates.

Irradiated Double Transfectants: Mice vaccinated with CHOhHER expressing two ISMs were challenge with TUBO cells. No difference was seen between the vaccinated and control mice (Figure 12). Tumors formed in all the mice in groups GM-CSF/IL-12, B7-1/IL-12, and IL-2/IL-12. The tumors formed at rates comparable to the control mice. Mouse 2 in group B7-1/IL-2 remained tumor free and tumors formed in the remaining mice steadily at the same rate as the control group. Mouse 4 in group in GM-CSF/B7-1 remained tumor free and tumors formed in the remaining mice.

Analysis of average tumor size in irradiated cell vaccinated groups: Average tumor sizes for all the groups were compared against the control (Figure 12). Not much difference was seen in the control versus the vaccinated groups in terms of average tumor size except for group GM-CSF which had the smallest average tumor size at approximately three times lower than the control average tumor sizes. A One-way ANOVA was performed to see if significant differences did existed between the vaccinated and control groups. Results revealed there was no difference between the control and vaccinated groups. The statistical test also shows no significant difference in tumor sizes between control and vaccinated groups.

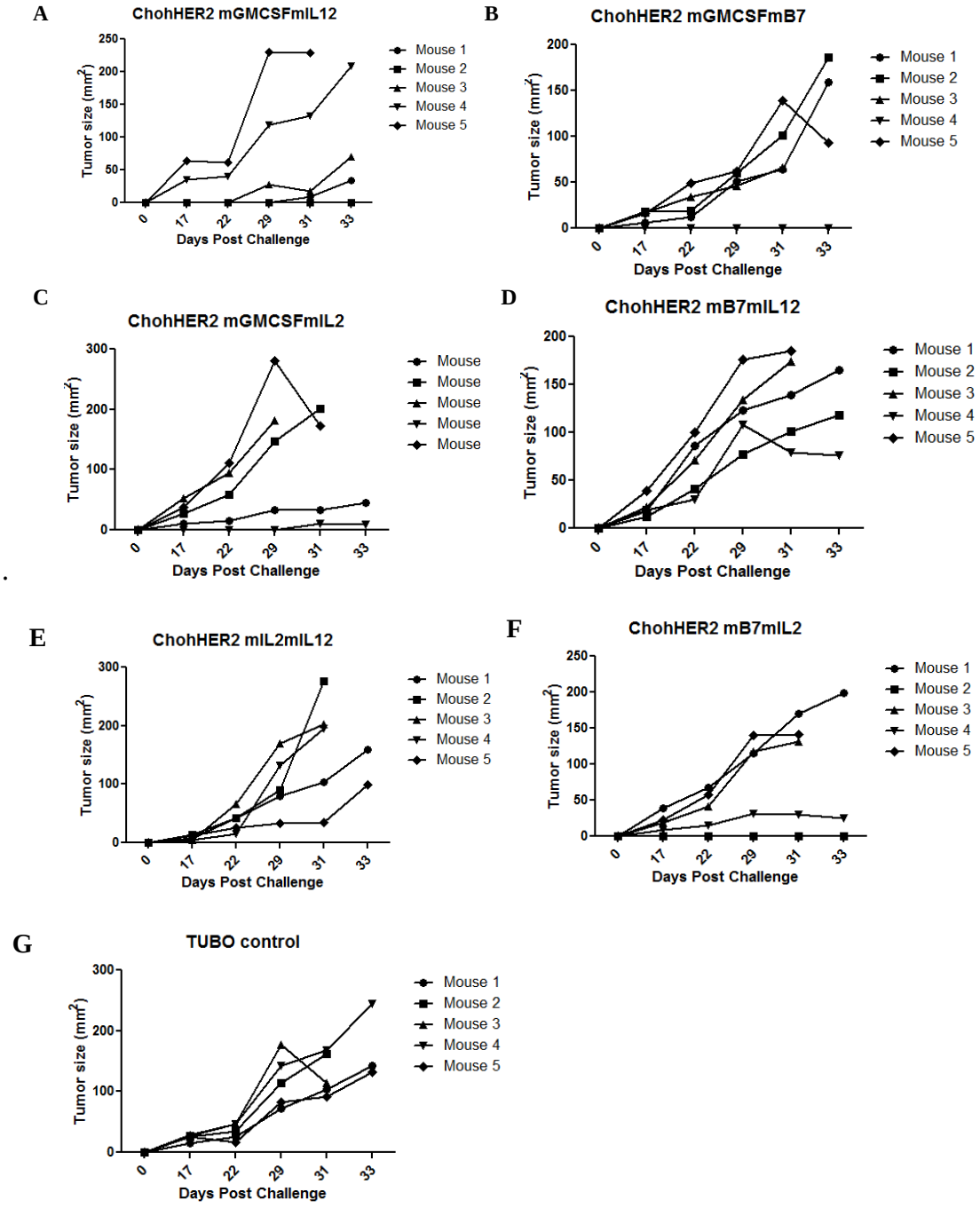


Figure 13. Tumor size for each individual mouse vaccinated with double transfectant irradiated vaccine. (A) GM-CSF/IL-12 had a fast increase in tumor volume. (B) GM-CSF/B7-1 group were all tumor positive and had tumors that grew at slower rates compared to the control. (C) GM-CSF/IL-2 had two mice with tumor formations comparable to the control group and 2 mice with slow tumor formation. (D) B7-1/IL-12 group had rapidly growing tumor sizes for all mice. (E) IL-2/IL-12 had all mice grow tumors at steady rates. (F)B7-1/IL-2 had a tumor free mouse and the remaining mice grew tumors at steady rates. (G) Unvaccinated control mice group with all tumor positive mice that increased inside.

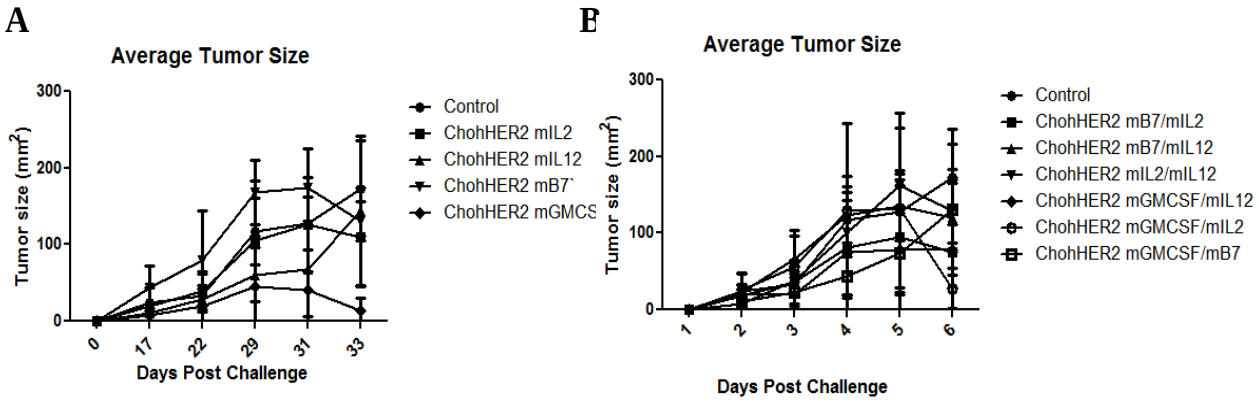


Figure 14. Average tumors sizes for irradiated cell vaccine. No significant difference in tumor size was found between the averages from vaccinated groups compared to the control groups. (A) Average tumor sizes for mice vaccinated with single transfectants. (B) Average tumor sizes for mice vaccinated with double transfectants.

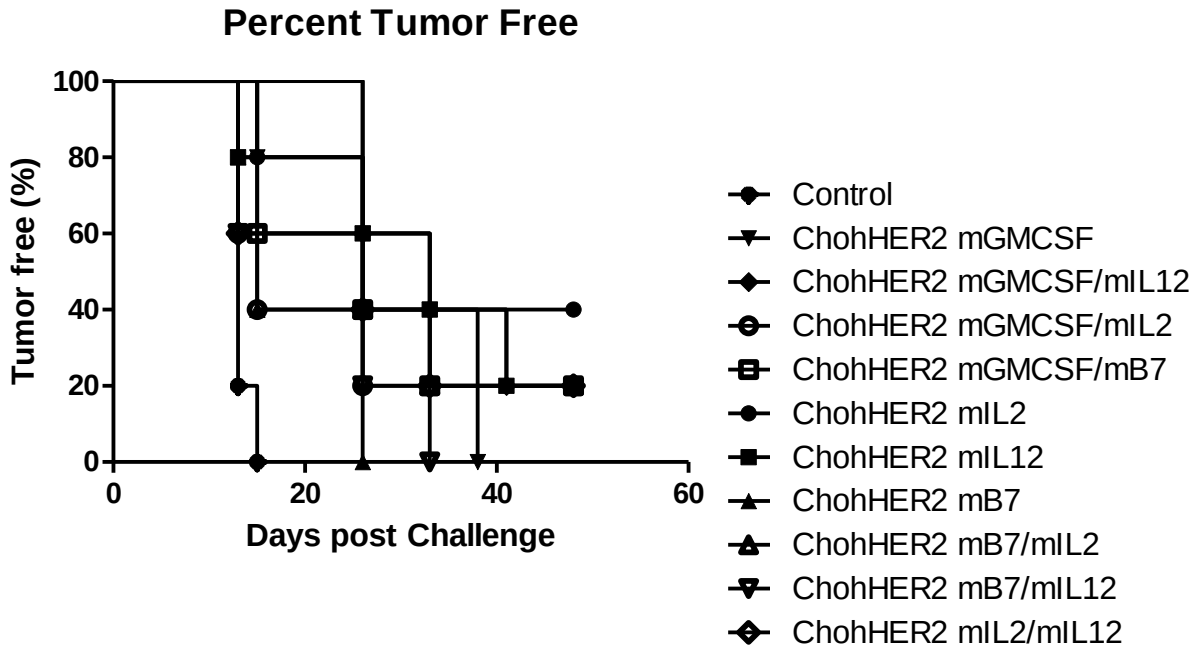


Figure 15. Tumor Incidence for Mice Vaccinated with Irradiated Cell Vaccines. Curve shows the different time points at which tumors developed for each group. All control mice developed tumors by day 20 and group IL-2 had two mice remain tumor free.

Tumor Incidence Curve for Irradiated Cell Vaccinated Mice: Groups IL-12, GM-CSF, GM-CSF/IL-12, B7/IL-2, and GM-CSF/B7 each had one mouse remain tumor free. The tumor incidence curve indicates which time points a mouse develops a tumor (Figure 15).

Conclusions for Irradiated Vaccine Study: No apparent differences between control and vaccinated mice were detected. A One-way ANOVA statistical test did not reveal any significant differences between control and vaccinated groups. The group vaccinated with GM-CSF did have a measurable decrease in tumor size but statistical tests did not indicate it was significant. Vaccinations with irradiated CHOhHER-ISM expressing cells were not effective in reducing tumor size.

***Membrane Vaccine Studies Results*:** Mice vaccinated with CHO cell membranes expressing ISMs started developing tumors however they are not large enough to measure at this stage of the experiment. A list of tumor positive mice for each group is given for day 16 post vaccination (Table 4). Mice vaccinated with IL-12, B7/IL-12, and GM-CSF/IL-2 membranes remained tumor free up to this point. The control mice started forming tumors except for 1 mouse. Group B7/IL-2 has 3 tumor positive mice and group GM-CSF/B7 has one tumor positive mouse. Tumors formed in 4 mice for groups GM-CSF/IL-12, IL-2/IL-12, GM-CSF, and IL-2. At this current stage tumor formation is too early to assess the effects of the vaccine however delayed tumor formation for some of the groups may indicate an effect from the vaccine.

Table 4. Tumor incidence for mice vaccinated with cell membrane 16 days post challenge. The number of tumor positive mice in each group and percent tumor free (n=5/group).

Cell Membrane Line	Tumor (+) mice	Percent Tumor Free (%)
CHOhHER2 mIL-12	0	100
CHOhHER2 mB7/mIL-2	3	40
CHOhHER2 mGM-CSF/mIL-12	4	20
CHOhHER2 mGM-CSF/mB7	1	80
CHOhHER2 mIL-2/mIL-12	4	20
CHOhHER2 mGM-CSF	4	20
CHOhHER2 mGM-CSF/mIL-2	0	100
CHOhHER2 mB7/mIL-12	0	100
CHOhHER2 mB7	2	60
CHOhHER2 mIL-2	4	20
Control	4	20

Analysis of Serum for anti-hHER2 antibody

Serum was collected from the blood of vaccinated mice prior to challenge. Serum was analyzed through FACS analysis to determine if any antibodies against TUBO, 4T07hHER2, CHOhHER2, and CHO-K1 cells were produced. TUBO cells were stained against to determine if any cross-reactive antibodies were generated against rat HER2/neu. Serum was stained against 4T07hHER2 in order to examine to analyzed antibody production against hHER2. The MFI values between the groups were compared to assess antibody production differences.

Serum Analysis of mice vaccinated with whole live cells

Antibodies from the serum of vaccinated mice were detected using FACS analysis (Table 5). Mice vaccinated with CHOhHER2 IL-12 produced the highest levels of antibodies for CHO-K1 (MFI=865) cells and CHOhHER2 (MFI=600) cells. Serum from mice vaccinated with CHOhHER2 GM-CSF/B7-1 and GM-CSF also had high MFI levels against CHO-K1 cells and CHOhHER2. However, high antibody levels against CHO-K1 and CHOhHER2 did not coincide with high antibody levels against 4T07hHER2 or TUBO cells. Antibodies against 4T07hHER2 were present in vaccinated mice serum however their levels were not as high when compared to CHO-K1 and CHOhHER2 antibodies. Low antibodies were generated against TUBO cells and groups that generated the highest antibody levels against TUBO were GM-CSF/IL-2 (MFI=156) and IL-12 (MFI=104.7). Mice vaccinated with CHOhHER2 IL-12 did have lower tumor sizes than the control as well as mice vaccinated with CHOhHER2 GM-CSF/IL-2. The highest antibody levels against TUBO cells were from mice vaccinated with CHOhHER2 GM-CSF (MFI=204) and those mice did have the slowest tumor growth rate and overall tumor size. The GM-CSF group also did have high antibody generation in the CHO-K1 (MFI=539), and CHOhHER2 (MFI=494) groups when compared to the other groups. Group B7-1/IL-12 serum had a low MFI value when stained against TUBO. Unvaccinated mice did have the lowest MFI when stained against CHO-K1, CHOhHER2, 4T07hHER2, and TUBO. This suggests antibody generation was a result of the vaccinations.

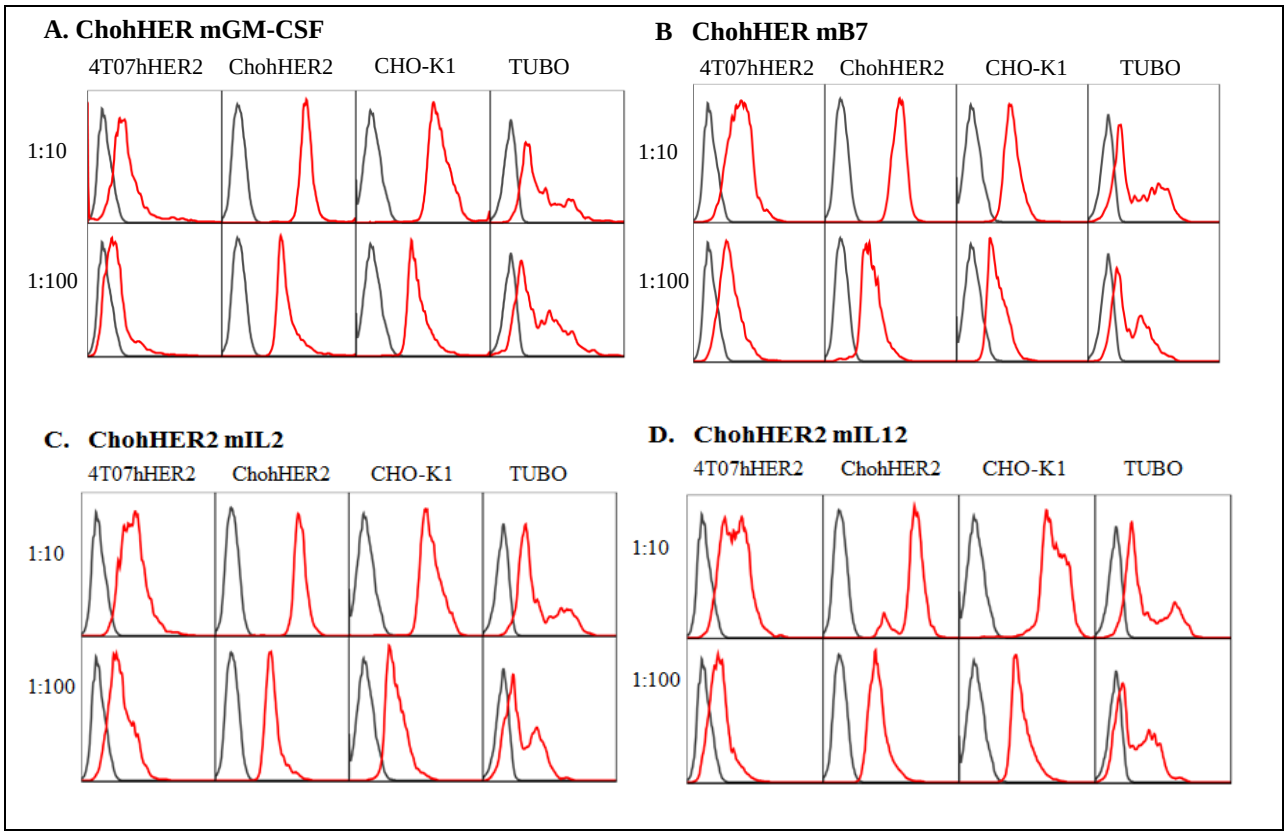


Figure 16. FACS analysis of serum from mice vaccinated with live cells for single transfectants when stained against 4T07hHER2, CHOhHER2, CHO-K1, and TUBO. Serum was analyzed at 1:10 and 1:100 dilutions in FACS buffer. Black: Isotype control and Red:ISM expression. Shifts if MFI levels were observed between the 1:10 and 1:100 dilutions. (A) CHOhHER2 mGM-CSF. (B) CHOhHER2 mB7-1. (C) CHOhHER2 mIL-2. (D) CHOhHer2 mIL-12.

FACS analysis profile of serum antibody analysis of mice vaccinated with a live cell vaccine:

The FACS analysis charts show the shift in the peak when cells were stained with serum at 1:10 dilution versus a 1:100 (Figure 16 and 17). The peak shifted to the left (decreased MFI) when serum was diluted to 1:100. The peaks in expression shifted more to the right (higher MFI) for all the serum stained against CHOhHER2 and CHO-K1 cells. Serum stained against 4T07hHER2 and TUBO cells did have a slight MFI shift in expression, however, it was not as pronounced as the expression shift for serum stained against 4T07hHER2 and TUBO cells.

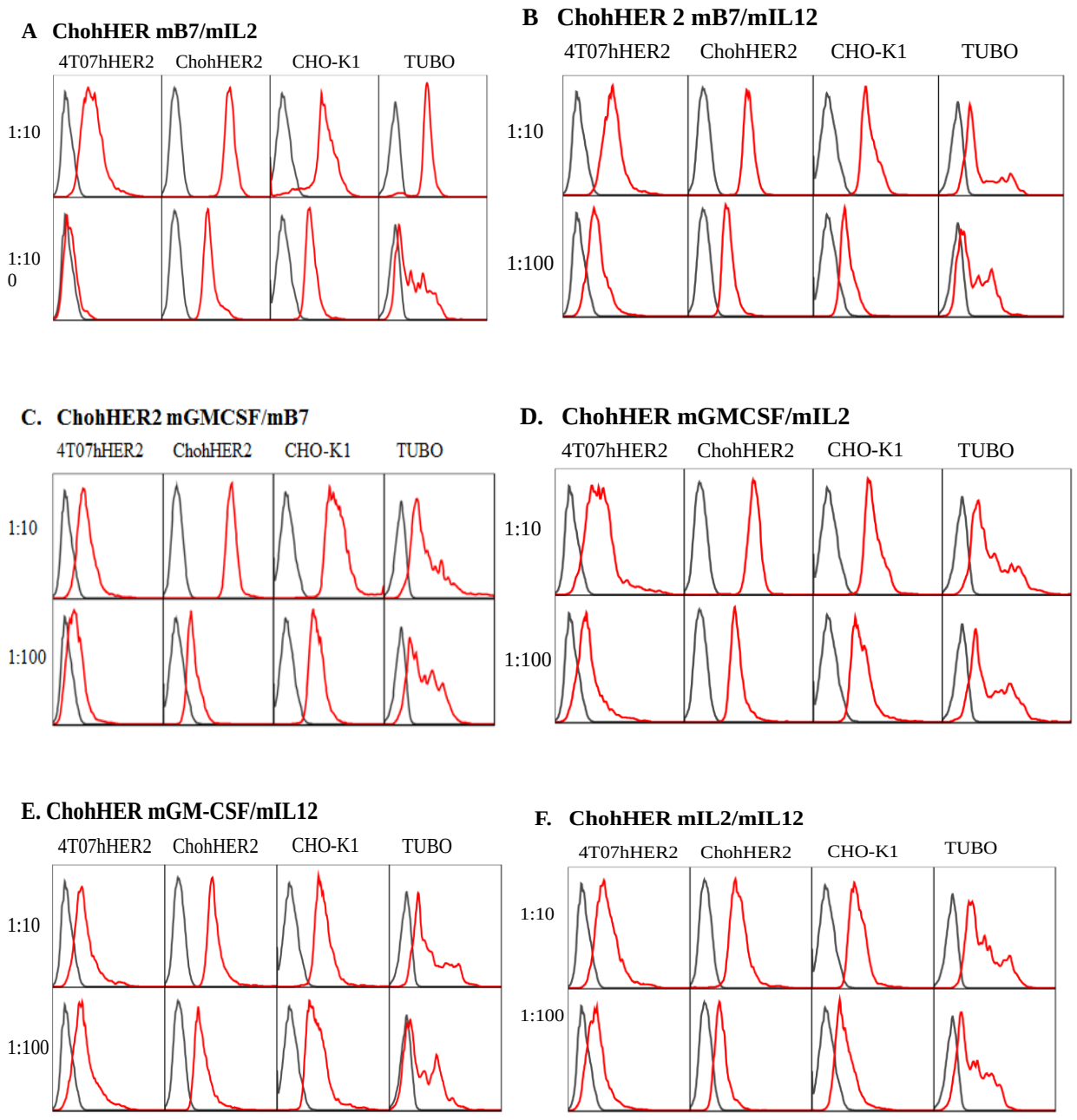


Figure 17. FACS analysis of serum from mice vaccinated with live cells for double transfectants when stained against 4T07hHER2, CHOhHER2, CHO-K1, and TUBO. Serum was analyzed at 1:10 and 1:100 dilutions in FACS buffer. Black: Isotype control and Red:ISM expression. (A) CHOhHER2 mB7-1/mIL-2. (B) CHOhHER2 mB7-1/mIL-12. (C) CHOhHER2 mGM-CSF/mB7-1. (D) CHOhHER2 mGM-CSF/mIL-2. (E) CHOhHER2 mGM-CSF/mIL-12. (F) CHOhHER2 mIL-2/mIL-12.

Table 5. Mean fluorescent ntensity (MFI) of serum from live cell vaccinated mice. Serum was collected from mice and stained at 1:10 and 1:100 dilutions against TUBO, CHO-K1, CHOhHER2, and 4T07hHER2.

Live Cell Vaccinations	CHO-K1 (MFI)		CHOhHER2 (MFI)		4T07hHER2 (MFI)		TUBO (MFI)	
	1:10	1:100	1:10	1:100	1:10	1:100	1:10	1:100
Naive	16.13	6.63	15.14	5.14	30.85	7.55	33.7	11.2
mIL-2/mIL-12	46.73	3.01	73.02	21.3	38.05	13.85	132	39.8
mIL-12	865.83	97.83	600.42	60.62	4.38	3.75	104.7	30.3
mIL-2	382.83	33.63	398.42	67.52	63.15	23.65	158	28.8
mGM-CSF/mIL-12	104.83	72.55	161.42	41.32	28.35	2.22	91.3	15.7
mGM-CSF/mIL-2	98.83	9.131	166.42	63.22	78.65	1.1	156.4	109.3
mGM-CSF	539.83	22.65	494.42	199.42	84.75	19.55	204	22.6
mGM-CSFmB7-1	440.83	48.13	380.58	79.42	24.65	7.15	184	66.7
mB7-1/mIL-12	79.63	4.17	95.42	1.78	61.35	15.05	62.6	26.7
mB7-1	66.43	20.73	206.42	5.9	35.75	16.35	95.1	50.8
mB7-1mIL-2	157.82	41.83	74.2	20.3	50.55	3.13	105.6	40.6

Serum analysis of mice vaccinated with irradiated cells

Analysis of serum from mice vaccinated irradiated cells stained against TUBO, 4T07hHER2, CHOhHER2, and CHO-K1 cells showed antibody formation for all the cells (Table 6). Cross reactive antibodies for 4T07hHER2 and TUBO were also produced at low levels. The antibody production was lower for irradiated cell vaccinated mice than mice vaccinated with live cells. Serum analysis results did not have any correlation to tumor size and a higher MFI was not associated with smaller tumor sizes. B7-1/IL-12 had the highest MFI (101.5) when stained against TUBO however the tumor size and growth were comparable to that of control unvaccinated mice. Group GM-CSF produced low levels of antibodies against TUBO cells

(MFI=50.2) which is similar to the level of antibodies produced for all other groups. However, GM-CSF did have the slowest tumor formation in the irradiated vaccine study. Serum stained against CHOhHER2 and CHO-K1 cells had the highest level of antibody production.

FACS analysis charts of serum from mice vaccinated with irradiated cell vaccine: FACS analysis charts show a decrease in the MFI peak when stained in a 1:100 dilution (Figure 18 and 19). The peak was farther to the right for CHOhHER2 and CHO-K1cells indicating more antibodies were produced against those cells. There is a slight shift in the peak when serum was stained against TUBO and 4T07hHER2, however, it was not as pronounced as it was for CHOhHER2 band CHO-K1.

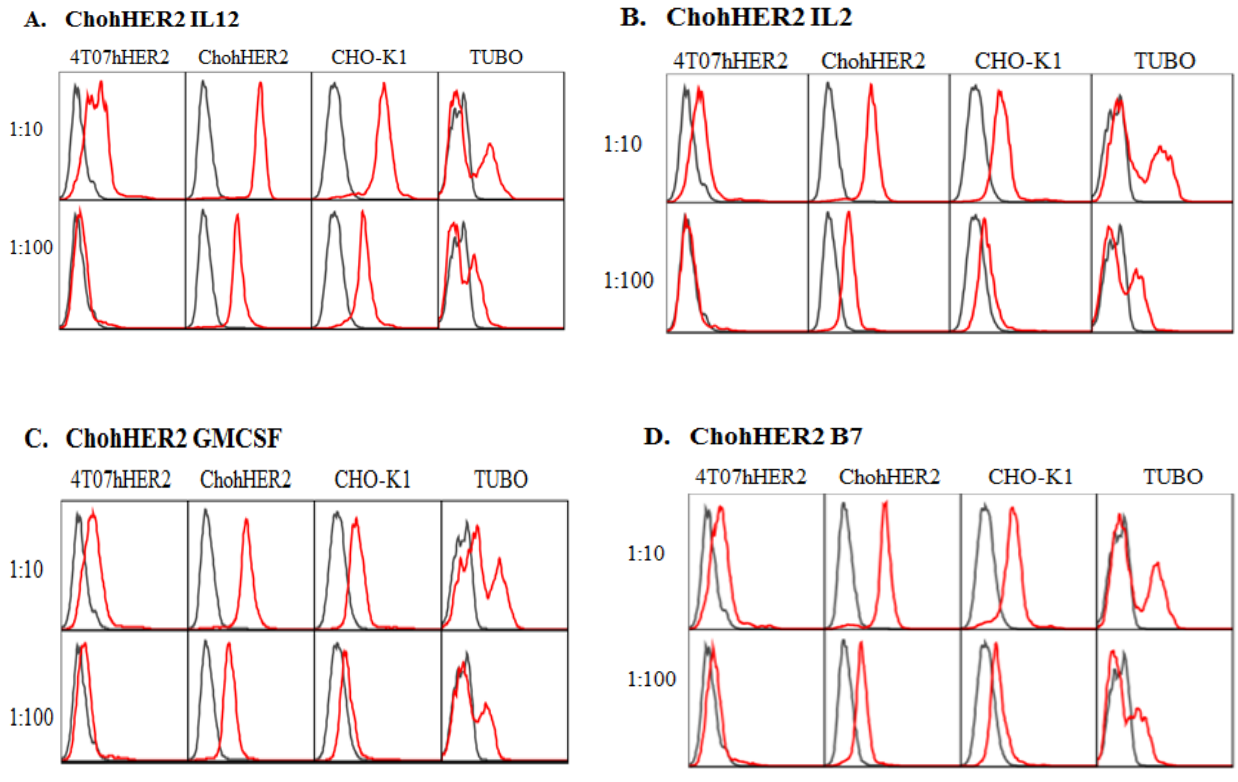


Figure 16. FACS analysis of Serum from Mice Vaccinated with Irradiated Cells for Single Transfectants when stained against 4T07hHER2, CHOhHER2, CHO-K1, and TUBO. Serum was analyzed at 1:10 and 1:100 dilutions in FACS buffer. Black: Isotype control and Red:ISM

expression. (A) CHOhHER2 mIL-2. (B) CHOhHER2 mIL-2. (C) CHOhHER2 mGM-CSF. (D) CHOhHER2 mB7-1

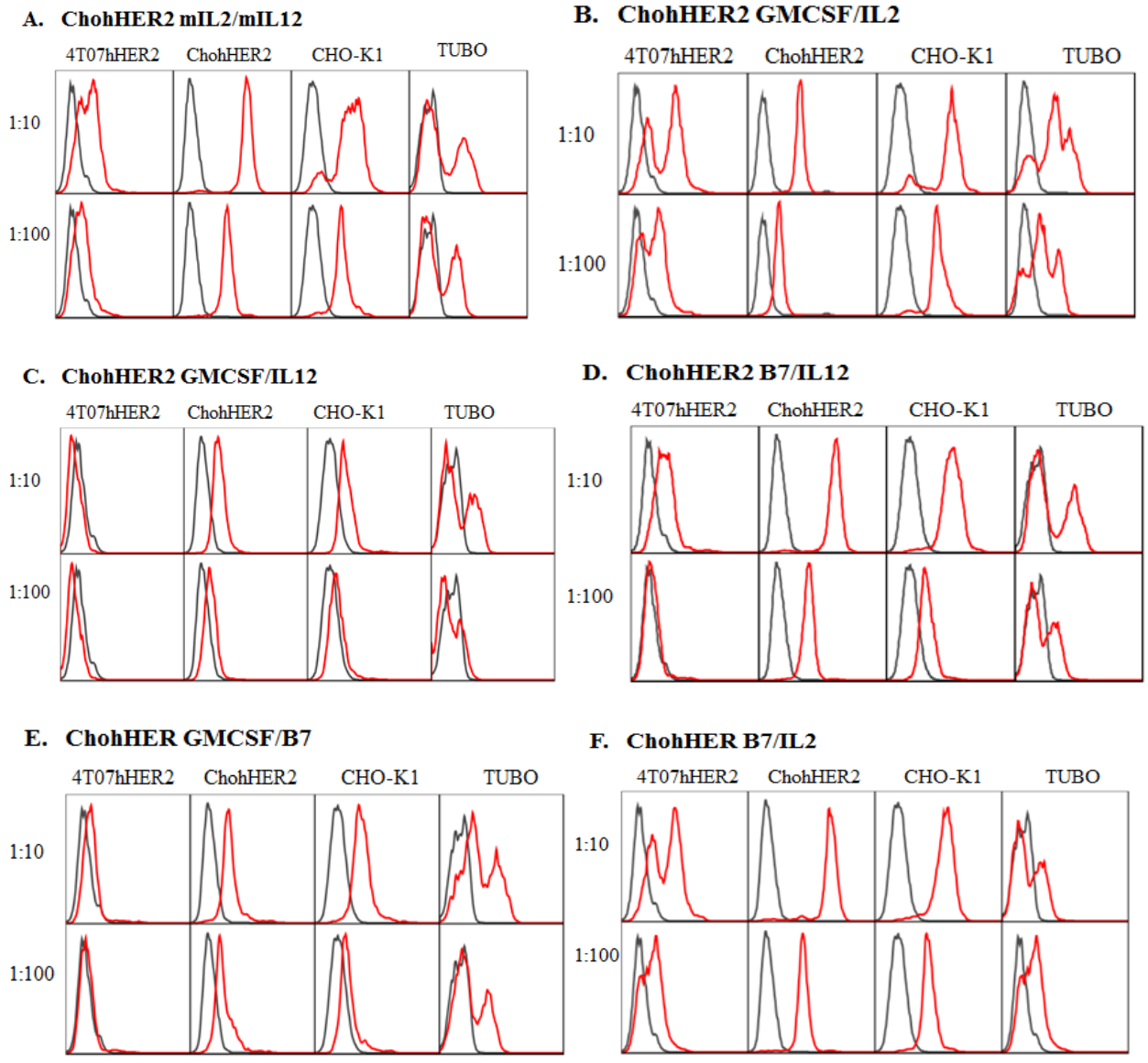


Figure 17. FACS analysis of serum from mice vaccinated with irradiated cells for double transfectants when stained against 4T07hHER2, CHOhHER2, CHO-K1, and TUBO. Serum was analyzed at 1:10 and 1:100 dilutions in FACS buffer. Black:Isotype control and Red: ISM expression. (A) mIL-2/mIL-12. (B) CHOhHER2 mGM-CSF/mIL-2. (C) CHOhHER2 mGM-CSF/mIL-12. (D) CHOhHER2 mB7-1/mIL-12. (E) CHOhHER2 mGM-CSF/mB7-1. (F) CHOhHER2 mB7-1/mIL-2.

Table 6. Mean fluorescent intensity (MFI) of serum from irradiated cell vaccinated mice. Serum was collected from mice and stained at 1:10 and 1:100 dilutions against TUBO, CHO-K1, CHOhHER2, and TUBO.

Irradiated Cell Vaccinations	CHO-K1 (MFI)		CHOhHER2 (MFI)		4T07hHER2 (MFI)		TUBO (MFI)	
	1:10	1:100	1:10	1:100	1:10	1:100	1:10	1:100
Naive	26.2	.01	12.0	.10	.43	.40	49.9	11.5
mIL-2/mIL-12	95	16	147	19.2	24.6	2.92	64.5	14.5
mIL-12	66.9	20.2	127	27.6	24.9	7.9	94.5	30.2
mIL-2	164	22.2	472	79.4	69.4	7.2	68.5	10
mGM-CSF/mIL-12	29.4	7.9	29.6	7.6	16.4	1.72	35.2	15.2
mGM-CSF/mIL-2	52.9	9.4	45.4	8.0	35.9	5.6	81.9	32.0
mGM-CSF	129	35.7	327	61.1	29.7	5.9	50.2	17.5
mGM-CSFmB7-1	67.7	7.8	99.5	9.5	18.4	2.26	75.0	17.1
mB7-1/mIL-12	443	26.3	101.5	15.3	73.9	4.10	101.5	15.2
mB7-1	91.6	22.1	71.2	16.2	49	6.9	70.7	17.1
mB7-1mIL-2	100	12.9	152.5	31.4	20.5	5.5	60.4	13.3

Serum Analysis of mice vaccinated with membranes: Analysis of serum from mice vaccinated with membranes stained against revealed low antibody levels for CHO-K1, CHOhHER2, 4T07hHER2, and TUBO cells. Analysis shows a higher fluorescence for 1:10 dilution than 1:100 dilutions (Figure. 17 and 18). A larger shift in MFI was seen when serum was stained with CHOhHER2 and CHO-K1 cells. Group IL-12 had high antibody levels for CHO-K1 and CHOhHER2. The GM-CSF/IL-2 group had high antibodies levels produced against TUBO, CHO-K1, and CHOhHER2. Serum from GM-CSF/IL-12 produced low level of antibodies against CHO-K1, CHOhHER2, TUBO, and 4T07hHER2.

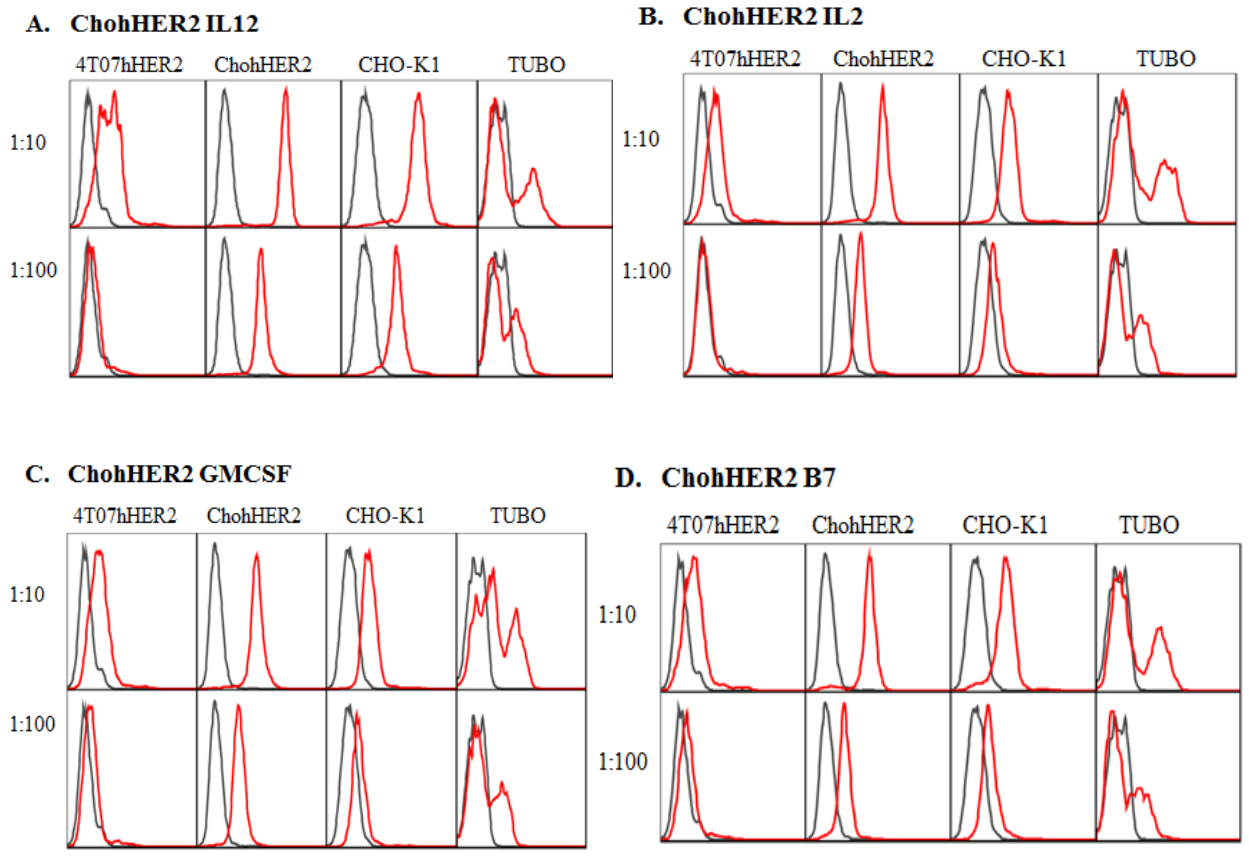


Figure 18. FACS analysis of serum from mice vaccinated with membranes for single transfectants when stained against 4T07hHER2, CHOhHER2, CHO-K1, and TUBO. Serum was analyzed at 1:10 and 1:100 dilutions in FACS buffer. Black:isotype control and Red:ISM expression. (A) CHOhHER2 mIL-12. (B) CHOhHER2 mIL-2. (C) CHOhHER2 mGM-CSF. (D) CHOhHER2 mB7-1

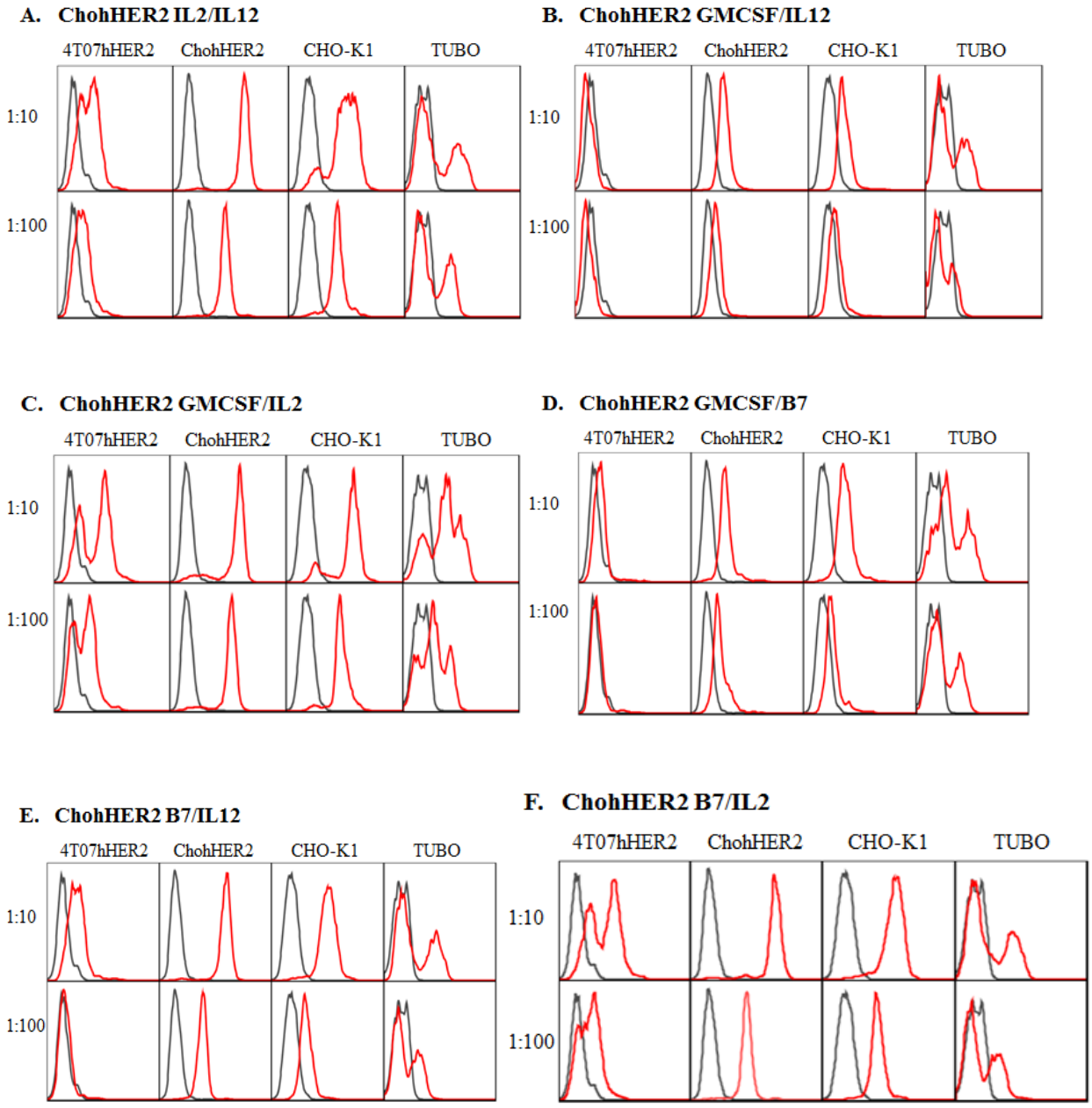


Figure 18. FACS analysis of serum from mice vaccinated with membranes for double transfectants when stained against 4T07hHER2, CHOhHER2, CHO-K1, and TUBO. Serum was analyzed at 1:10 and 1:100 dilutions in FACS buffer. Black:Isotype control and Red:ISM expression. (A) CHOhHER2 mIL-2/mIL-12. (B) CHOhHER2 mGM-CSF/mIL-12. (C) CHOhHER2 mGM-CSF/mIL-2. (D) CHOhHER2 mGM-CSF/mB7-1. (E) CHOhHER2 mB7-1/mIL-12. (F) CHOhHER2 mB7-1/mIL-2

Table 7. Summary mean fluorescent intensity (MFI) of serum from membrane vaccinated mice. Serum was collected from mice and stained at 1:10 and 1:100 dilutions against TUBO, CHO-K1, CHOhHER2, and TUBO.

Membrane Vaccinations	CHO-K1 (MFI)		CHOhHER2 (MFI)		4T07hHER2 (MFI)		TUBO (MFI)	
	1:10	1:100	1:10	1:100	1:10	1:100	1:10	1:100
Naive	19.5	8.5	13.2	5.3	7.5	1.15	37.2	5.5
mIL-2/mIL-12	150	31	192	71	27.1	7.3	34.1	12.9
mIL-12	206	49.3	226	46.6	21.1	1.67	15.7	3.9
mIL-2	40.1	10.3	62.5	11	11.6	1.63	41.3	6.1
mGM-CSF/mIL-12	19.9	7.8	12.6	.70	1.35	1.32	7.6	.26
mGM-CSF/mIL-2	197	92.2	224	129	43.9	12.2	43.9	12.2
mGM-CSF	27.2	10.2	49.3	18.4	1.4	1.67	27.2	7.00
mGM-CSFmB7-1	41.9	12.9	22.4	12.5	5.5	2.55	32.1	12.1
mB7-1/mIL-12	125	16.8	249	39.5	15	1.05	31.2	5.4
mB7-1	38.7	12	58.4	9.2	10.2	1.56	23	5.4
mB7-1mIL-2	174	52.7	400	56.5	35.9	7.5	21.6	7.2

Conclusion from Serum Analysis: The live cell vaccine produced the highest antibody levels when compared to the irradiated and cell membrane vaccine. Antibody levels were highest for CHO-K1 and CHOhHER2 and lowest for 4T07hHER2 and TUBO for the live cell, irradiated cell, and membrane vaccines. Cross reactive antibodies for 4T07hHER2 and TUBO were generated, however, at very low level.

Discussion

Successful transfectants of CHOhHER2 cells with immunostimulatory molecules B7-1, IL-2, IL-12, and GM-CSF alone or in combination were established. Further magnetic bead isolation and panning were successful in isolating cells with high expressing ISM proteins. Analysis of ISM expression was done via FACS analysis and enzymatic treatment with PIPLC also confirmed the GM-CSF, IL-2, and IL-12 were GPI-anchored. B7-1 is a transmembrane

protein, thus, not susceptible to PIPLC cleavage. However, B7-1 expression did decrease slightly upon treatment. B7-1 in GM-CSF/B7-1 decreased 12.36%. This may have been caused by the different background for the untreated cells. The peak for the background (cells with only FITC secondary antibody) in untreated cells was lower than the background for the treated cells. This may have been caused by not adding the secondary antibody. The procedure such as placing the cells in a water bath for 45 minutes may have affected the B7-1 expression. IL-12 was most susceptible to GPI-cleavage by PIPLC than IL-2 and GM-CSF. The question of why IL-12 is more susceptible to GPI-cleavage is not clear because the GPI constructs in the IL-2, IL-12, and GM-CSF cDNA vectors are identical. A possible explanation is that GPI sensitivity to cleavage can depend on the cell type it is expressed on and the molecule that it anchors[97].

The mice were vaccinated in three different ways to determine which delivery method is more effective in inducing cross-priming and stimulate immunity against tumors without directly interacting with T cells. Upon challenging with TUBO cells for the live cell vaccine most of the groups grew tumors at reduced rates than the control group. Vaccination with B7-1, IL-2, and IL-12 all had tumors appear on the same day as the control, day 13. The tumors from group B7-1 grew faster and larger than the other vaccinated groups. It is possible that B7-1 costimulation is less effective than the other immunostimulatory molecules, or is less effective alone than in combination. When B7-1 is in combination with IL-2, the tumors grew at slower rates than when B7-1 is expressed alone except for mouse 2 which did not develop a tumor. Tumor formation in the GM-CSF was delayed until day 15 with 1 mouse remaining tumor free. The remaining tumor free mice had reduced tumor growth with the sizes remaining below $50mm^2$ except for 1 mouse. The live vaccine studies showed a decrease in tumor growth rate compared to unvaccinated control. However, even though tumor growth grew at a slower rate, statistical

analysis through One-way ANOVA which compared the control group against vaccinated groups indicated the average tumor size differences were not statistically significant. Statistical tests were only performed for average tumor sizes and not for tumor incidence or mortality. In the future, statistical tests would be performed to analyze if tumor incidence and mortality between vaccinated and unvaccinated groups are significant. The statistics in these studies were performed by comparing the average tumor sizes of vaccinated mice with the unvaccinated mice control. Time points were not taken into account. However, in order to measure effectiveness of therapy, tumor volume at selected measurement times should have been used.

The irradiated cell vaccine did not have any significant effect on tumor growth and size. All of the B7-1, IL-2, IL-12, and GM-CSF groups except for GM-CSF alone did not appear to decrease tumor size or growth. GM-CSF was the only group with a decrease in tumor growth and two mice never developed tumors. Group IL-12 also had one mouse that never developed a tumor but the remaining mice grew tumors at a rate similar to the unvaccinated control. ISM expression also could have been lost through irradiation. Live cell vaccines can persist inside the host longer than irradiated cells can. Thus, the differential persistence of live versus irradiated cell vaccines can correlate to the potential immunity [98]. Since the irradiated vaccine was administered only once this may not have been enough to induce a potent immune response and a booster may have been needed in order to enhance the vaccine's efficacy. Vaccination with irradiated melanoma cells genetically modified to secrete GM-CSF enhanced antitumor immunity in a Phase 1 clinical trial of patients with non–small-cell lung cancer (NSCLC). The patients were given weekly doses of the vaccine for 3 weeks and every other week until the vaccine ran out [99]. The multiple doses of irradiated vaccines may have been beneficial in

boosting the immune response and future studies in this breast cancer model would utilize booster vaccinations as a way to increase vaccine immunogenicity.

The cell membrane vaccine was administered at 50µg per mouse in 100µl of PBS. The cell membrane vaccine is at a stage where it is too early to fully assess the impact of the vaccine on tumor growth. However, tumor formation has begun in the control groups, but GM-CSF/IL-2, B7/IL-12, and IL-12 have remained tumor free 16 days after tumor challenge thus far. The control group is tumor positive for 4/5 mice and so are groups GM-CSF/IL-12, IL-2/IL-12, GM-CSF, and IL-2. Even though tumor formation is at an early stage the delayed tumor appearance in some of the groups may indicate the vaccine having some effect. In future studies with membrane vaccines, a booster would be given to further stimulate the immune system.

Both live and irradiated vaccine studies yielded widely scattered results which might have affected the statistical analysis. This is unusual as the mice are genetically inbred mice and results within a vaccinated group should have been similar. The scatter in the results may have been contributed to human error in resuspending the cells in the syringe and some mice may have received more or less of the vaccine/tumor cells. The cells in the vaccine are also comprised of a heterogeneous population which may have contributed to the scatter. Some mice may have received a vaccine dose with cells expressing higher or lower levels of ISMs and this could have affected the immune response. In future studies more caution would be taken with resuspending the cell vaccines. Alternatively, the differences in tumor growth may be individual variation in eliciting antitumor immunity although mice are genetically matched.

Antibodies collected from serum of vaccinated mice pre-challenged were analyzed to see if antibodies were produced against TUBO, 4T07hHER2, CHOhHER2, and CHO-K1 cells.

Antibody production is considered an important mechanism of treating HER2/neu positive tumors such as TUBO cells. Herceptin, a monoclonal antibody that reduces the expression of HER2/neu, has shown to have antitumor effects [91]. Therefore, antibody production can be an important antitumor mechanism. Mice vaccinated with live cells produced higher antibody levels than serum from irradiated and membrane vaccinations. FACS analysis revealed high production of antibodies against CHOhHER2 and CHO-K1 cells. This was expected because the hamster derived CHO cells are more foreign than mouse derived 4T07hHER2 and TUBO cells. Cross reactive antibodies were also generated against 4T07hHER2 and TUBO cells. TUBO cells express the rat version of the HER2/neu protein that is responsible for carcinogenesis [99] and tumor progression [100-102]. The rat HER2/neu in TUBO cells are 92% homologous to hHER2 [102]. Production of cross reactive antibodies to TUBO may be attributed to the common epitopes such as the CD8 epitope (Her2p780-PYVSRLLGI) between rat, human, and mouse HER2 [103].

The induction of antibodies suggests cross priming of the immune system through dendritic cells. Because CHO cells cannot directly activate T cells, they must activate T cells through cross priming and indirect priming. Cross-priming is important for the induction of CD8+ T cells which are critical for antitumor responses. Previously it has been shown that vaccine-induced CD8+ T cells by DCs are enhanced by vaccine induced antibody production [104]. However further tests are needed to examine CD8+ T cell responses in these studies.

Much of the mechanism behind the xenogeneic vaccine is unknown and future experiments need to be performed in order to fully assess the effector mechanism of the vaccine. Immune memory studies would be included in future studies such as rechallenging tumor free mice in order to see if antitumor effects are long lasting. CTL studies would also need to be

performed to look at cross priming and tumor specific CD8+ T cells such as a chromium release CTL assay. Since CD8+ cytotoxic T cells are extremely important for inducing an antitumor response, stimulating tumor specific CTLs is crucial. Another study that could be done is to co culture DCs with CHOhHER2 membranes and then mix the DCs with HER2 specific T cells to see if they proliferate in response to HER2. The purpose of this would be to see if DC cells are capable of cross presenting the HER2 antigen and activating T cells. Realistically, CHO cells will probably never be used as a vaccine for humans. However, the study of xenogeneic vaccines can provide valuable information on the mechanism of cross priming and the induction of antitumor responses. Information of these experiments can be useful in the future for developing potential vaccines for humans.

Conclusion

Advances in science and medicine have exposed mechanisms behind initiating immune responses against types many types of cancers. These studies examined the expression of immunostimulatory molecules as a xenogeneic vaccine using xenogeneic hHER2 and xenogeneic CHO cells. Decreased tumor growth was exhibited in mice vaccinated with live cells however it was not enough to completely prevent tumor formation or regression. Mice vaccinated with the irradiated cell vaccine demonstrated no measurable antitumor effects which may have been contributed to the lack of a booster vaccine. Even though tumors are in an early stage of growth, mice vaccinated with CHOhHER2-ISM expressing cells show delayed tumor formation in some of the vaccinated groups. All three vaccines were able to stimulate antibody production at both low and high levels. Future studies with this xenogeneic vaccine model would hopefully incorporate cellular based assays to determine the mechanism behind the reaction. Further development and study of xenogeneic vaccines through cross priming may

reveal new ways to break immune tolerance, and provide potential immunotherapies for different types of cancers.

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