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Brianne Caoyonan April 15, 2014

Intravenous vs. Subcutaneous prophylactic vaccination of protein transferred 4T07TMVs against 4T07-HER2 murine breast cancer

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Abstract

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There are many different avenues of dealing with breast cancer today, including: early detection, surgery, radiation, chemotherapy, and hormone therapy for ER, PR, and HER2 positive breast cancer. However, many of these hormone therapies lead to resistance and sometimes relapse or progression. Immunotherapy provides another level of defense by activating the immune system against tumor cells and, thereby, minimizing effects on healthy tissue. In this study we aimed to develop tumor membrane vesicles (TMVs) modified by protein transfer to express HER2, with or without additional glycosyl phosphatydlionsitol anchored immunostimulatory molecules (GPI-ISMs), in order to activate a HER2 specific response against 4TO7RGhHER2 murine breast cancer cells. These different 4TO7 TMVs, were made from 4TO7RG tumors, modified by protein transfer with GPI-anchored HER2, IL-12, and B7-1, and injected intravenously or subcutaneously for prophylactic vaccination against 4TO7RGhHER2 experimental metastasis. Mice vaccinated with 4TO7ts TMVs protein transferred with HER2 induced greater IgG responses against 4TO7RGhHER2 cells and greater protection against lung metastasis compared to mice vaccinated with unmodified 4TO7ts TMVs or soluble GPI-hHER2. Subcutaneous vaccination was also found to be more highly immunogenic overall compared to intravenous vaccination based on increase IgG responses against 4TO7HER2 cells and decreased lung metastasis. These results are promising for therapeutic studies aimed to activate HER2 specific immune responses in order to achieve tumor regression.

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I would like to thank Dr. Selvaraj for allowing me to join the lab as an honors student in spite of my inexperience. You were a constant guide in my development as a young scientist and have taught me more than just protocols, but how to ask the right questions. I came into your lab focused on my interest in cancer as a disease, and I came out having learned so much about the complexities of immune activation against this disease and the ongoing research regarding these complexities. I am truly thankful for the part I have been given to play in the fight against cancer.

I would like to acknowledge Ms. Jaina Patel, specifically, for your infallible patience and guidance. In the last year, you have given me, not only, a vast amount of knowledge about the role of immunotherapy in cancer research, but you have taught me more about the research process than I have learned in my four years of undergrad. Your persistence and enthusiasm have been an extraordinary motivation throughout this year and I could have not completed this project without your help.

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Table of Contents

Figures

Introduction

Breast Cancer

According to the American Cancer Society, approximately 232,670 will be diagnosed with invasive breast cancer in 2014 and 40,000 of these women will die from the disease [1]. There are many factors that contribute to the risk of breast cancer including: age, diet, hormone levels, breast density and genetics. The increasing knowledge of these factors also increases awareness for the need of early detection and breast self-awareness. The prevalence of the mammography has increased ~37% since 1987 and has remained stable since 2005. Additional methods of screening, such as, MRI, clinical breast exams (CBE), and breast ultrasound, have been suggested for women with either an increased risk of breast cancer, older women, or for women that have received a diagnostic mammogram [1]. Overall, early detection is a key component in decreasing breast cancer morbidity.

Many cancers, including, but not limited to, breast cancer, overexpress human epidermal growth factor receptor 2 (HER2), which often make these tumors faster growing and more likely to recur resulting in poor prognoses [2]. However, HER2 overexpression also provides a target for therapy which limits systemic toxicity by non-targeted chemotherapies. These therapies are mainly monoclonal antibodies, such as, trastuzumab and pertuzumab, against HER2 [1]. However, 70% of HER2 positive cancers treated with these monoclonal antibodies develop *de novo* resistance to the treatment [3].

Cancer and the Immune System

The goal of immunotherapy is to arm the immune system against tumor cells and, ultimately, to lead to an anti –tumor response [4]. Studies have shown that this response can be mediated by

both the innate and acquired arms of the immune system through their varying methods of pathogen recognition [5]. It may not be a classical pathogen, but cancer is a disease that can infiltrate and spread throughout the body. Moreover, in the 1890s, physician William B. Coley found that when cancer patients had concomitant bacterial infections, some of these patients' tumors shrank and many seem to be completely cured [6], suggesting similar immune pathways are employed against pathogens and aberrant cancer cells [7]. However, tumor antigens are not highly immunogenic on their own [8]. Not only are malignant cells veiled under the identity of self, but many mechanisms of immune evasion by these cells have been identified such as, inhibitory T-cell pathways and regulatory immune cell pathways [9]. Furthermore, many tumors modify their environment to be, not just tumor tolerant, but to be, tumor promoting, through a complex web of signals by cytokine expression, tumor cells [10], immune cells [11] and other non-malignant tumor associated cells [12]. Therefore, there are many barriers preventing anti-tumor responses, but the goal of immunotherapy is to overcome these barriers and tip the balance from tumor promotion to tumor elimination.

The main anti-tumor responses are mediated by T cells, which can specifically target tumor antigens and, furthermore, avoid non-specific damage to normal tissues. T-cells require two signals to become activated. The first is the binding of MHC-peptide on antigen presenting cells (APCs) to a T-cell receptor (TCR) and the second is induced by costimulatory molecules, such as, B7-1, expressed on APCs [13-15]. Many different strategies have been developed, and have even made it to clinical trials, to increase the immunogenicity of tumor cells including: systemic administration of cytokines [16], DNA vaccines, heat shock proteins, dendritic cells (DCs) and tumor cells, both autologous and allogeneic, modified by transfection to present tumor antigens [17]. Many of these immunotherapy approaches use cytokines to increase the

immunogenicity of cancer cells and have succeeded but not without unwanted hematologic toxicity and other toxic effects [18, 19]. Thus, there must be an equilibrium at which immunotherapy may overcome the immunosuppressive nature of a tumor yet induce minimal toxicity. Furthermore, there have been various studies on the specific cytokines and immunostimulatory molecules (ISMs) that would illicit the best innate and acquired immune response against tumor cells such as, IL-12 [20, 21] and B7-1 [22-24].

IL-12 is a cytokine that indirectly promotes the differentiation and maturation of DCs [25] and CD4+ T cells (into Th1 cells which secrete high levels of interferon-γ (IFN-γ)) [26] by the production of proinflammatory cytokines including IFN-γ, TNF-α, IL-6, and GM-CSF. It has also been reported to act as an anti-apoptotic factor for DCs, furthermore, the lack of IL-12 produced by DCs led to early cell death [27].

B7-1 was the first of the B7 family to be discovered on activated B cells [28]. It is a costimulatory molecule that is required for the activation and survival of T cells. Moreover, antitumor immunity was authenticated by *in vivo* studies demonstrating that CD8+ cytotoxic T lymphocytes (CTL) better reduced the tumorigenicity of murine malignancies that were transfected with B7-1 and B7-2 [29].

TMVs and Protein Transfer of GPI-anchored Immunostimulatory Molecules

IL-12 has potent anti-tumor activity but this is limited in clinical application due to severe systemic toxicity [30]. However, this toxicity has been shown to be alleviated by anchoring these cytokines to a membrane prior to administration [4, 31]. The size affecting diffusion of membrane vehicles (cells or tumor membrane vesicles) carrying these immunostimulatory molecules allows for immune activation to occur locally at the vaccination site, thereby, slowing

systemic circulation [30, 32]. Not only, does anchoring reduce toxicity, it also reduces fragility of cytokines, which, in some cases become ineffective after administration due to rapid degradation in vivo [30]. However not all cytokines and immunostimulatory molecules have endogenous anchors to a lipid membrane, therefore, our lab has established glycosyl phosphatydlionsitol (GPI)- anchored forms of a variety of immunostimulatory molecules, including B7-1 and IL-12. Studies in our laboratory have shown that GPI-anchored molecules spontaneously incorporate into bi-lipid membranes through lipid moiety [33-40]. The process of incorporation is much quicker than gene transfer, only taking a few hours, and multiple molecules are able to be transferred to one membrane simultaneously, which can also incorporate at different levels depending on the concentration of the molecules during the reaction. These constructed GPI-anchored proteins are not disrupted by the addition of this GPI-anchor, meaning they retain the function associated with their extracellular domain [4 (references therein)].

These GPI-proteins can be protein transferred to any bi-lipid membrane. Our lab has developed a system of transfer to tumor membrane vesicles (TMVs) which are derived from tumor cells. Therefore, they include all tumor associated antigens (TAA) within the membrane without any replicative capacity. By decorating these TMVs with GPI-immunostimulatory molecules, B7-1 and IL-12, the direct targeting of tumor associated antigens by APCs and T-cells is feasible after these cells engage with these decorated TMVs.

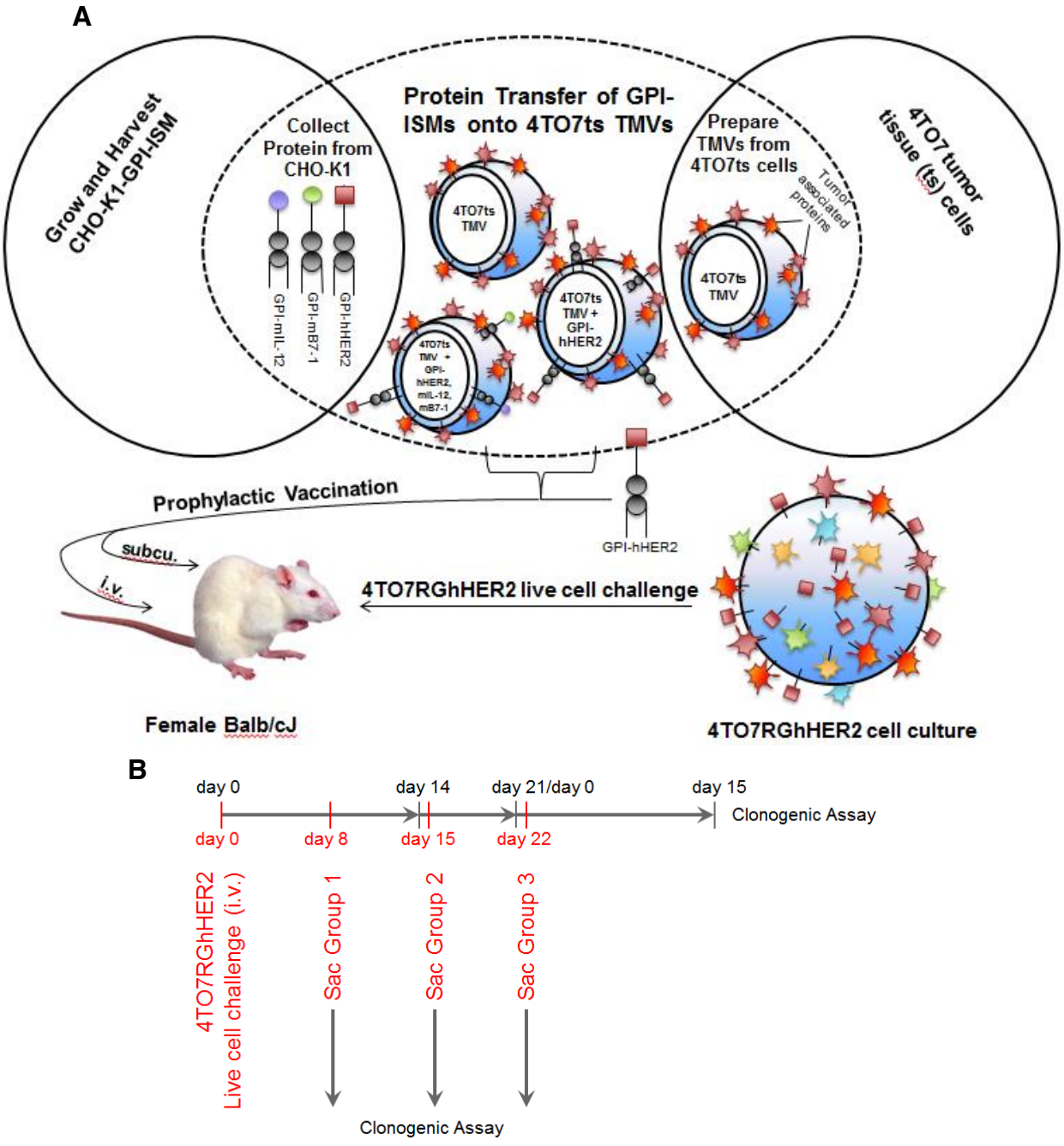
Experimental Design

4TO7 is a low level metastatic murine breast cancer, isogenic to 4T1– a highly metastatic breast cancer – both of which were isolated from a 410.4 mammary tumor that spontaneously arose in a Balb/cfC3H mouse [41]. There are two other sister lines from this tumor (67 and 168) that, all

together, reflect the spectrum metastasis and are often used to study the sequential stages of progression in breast carcinoma.

Neither 4TO7 cells, nor any of its sister lines, express HER2, endogenously. In order to simulate HER2 positive breast cancers in the 4TO7 model, 4TO7 cells were transfected with hHER2 (pcDNA3zeo). These hHER2 positive 4TO7 cells were tested previously in our lab for tumorigenicity using BALB/c mice. Initially, 4TO7RGhHER2 cells were injected subcutaneously to observe tumorogenesis; however, many of the tumors regressed and only 2/3 mice had continuously growing tumors when the number of cells used to challenge was increased. Mice were then injected intravenously with 4TO7RGhHER2 cells to test tumorigenicity in the lungs. Morbidity increased for mice that received 4TO7RGhHER2 cells intravenously in relation to mice that received 4TO7RGhHER2 cells subcutaneously. Intravenous challenge of 4TO7RGhHER2 cells was compared to intravenous challenge of 4TO7RG and 4TO7B7/IL12 cells. Mice challenged with 4TO7B7/IL-12 survived the longest in comparison to the other two lines which elicited comparable levels of morbidity with 4TO7RGhHER2 cells leading to an only slightly longer (3-5 days more) survival. Based on these previous observations, 4TO7RGhHER2 cells were injected intravenously and were tested as model of experimental metastasis in BALB/c mice in the following study. To test the efficacy of the aforementioned immunostimulatory molecules (B7-1 and IL-12) in a prophylactic setting, mice were vaccinated, prior to intravenous challenge with live 4TO7RGhHER2 cells (experimental metastasis), with soluble GPI-HER2 and three distinct groups of protein transferred 4TO7ts TMVs: unmodified 4TO7ts TMVs, 4TO7ts TMVs + GPI-HER2, 4TO7ts TMVs + GPI-HER2 + GPI-IL-12 + GPI-B7-1 (Fig. 1a).

As a preliminary experiment, kinetics of 4TO7RGhHER2 experimental metastasis were determined prior to these prophylactic vaccination studies (Fig. 1b). The results of the 4TO7RGhHER2 kinetics studies were then used to formulate the prophylactic vaccination strategy timeline (Fig. 1c).



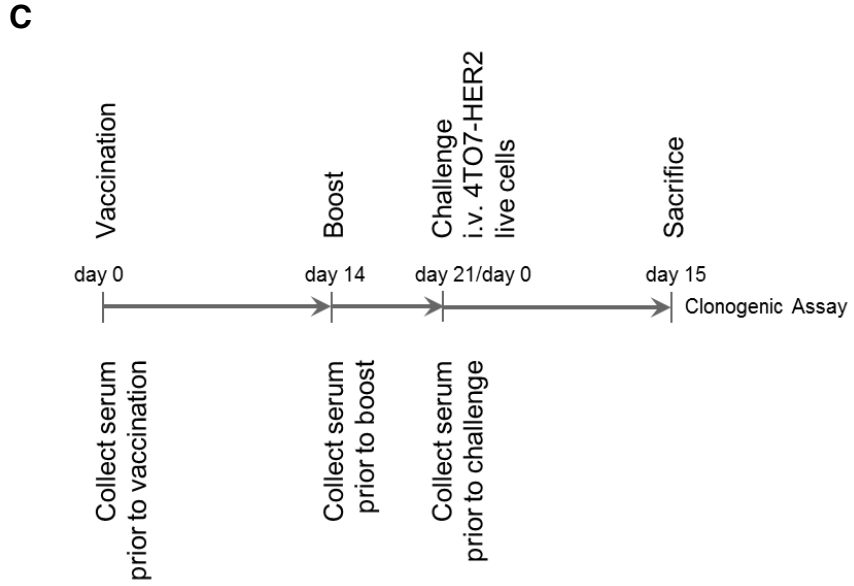


Figure 1: Overall experimental design. (A) Schematic of overall experimental design. CHO-K1 cells expressing -hHER2-CD59, -mIL-12-a-CD59-b-sol, GPI-mB7-1-short (left circle) were cultured and harvested. The GPI-ISMs were collected and purified, and protein transferred onto 4TO7ts TMVs which were made from 4TO7 tumor tissue (right circle). Three separate types of TMVs were made using 4TO7ts TMVs and different GPI-ISMS (middle oval). These TMVs were used for prophylactic vaccination in female BALB/cJ mice, 6-8 weeks in age, through either subcutaneous or intravenous injection. Two weeks later, these mice were challenged with live 4TO7RGhHER2 (sort, pan 6) cells. (B) Challenge and euthanizing ("sac") strategy (red) to determine the kinetics of 4TO7RGhHER2 metastasis. Three groups (n=3) were challenged on day 0 then euthanized at 3 different time points: day 8, day 15, and day 22. (C) Timeline of vaccination strategy. Mice were inoculated with 4TO7ts TMVs (depicted in A) on day 0. They were given a boost with the same type of TMV on day 14, and challenged on day 21/ day 0. The mice were bled before, but on the same day as, inoculation, boost, and challenge.

Materials and Methods

Cell culture: 4TO7RGhHER2 (sorted, pan 6) cells were thawed from -80°C and were grown at 37°C and 5% CO_2 in DMEM with 4.6g/L glucose, L-glutamine, sodium pyruvate, 10% BCS, and 200µg/mL Zeocin.

CHO-K1-hHER2-CD59, CHO-K1-mB7-1-CD59, and CHO-K1-mIL-12-a-CD59-b-sol cells were grown at 37°C and 5% CO_2 in 1640 RPMI with 5% BCS and 10µg/mL Blasticidin in T150 flasks until 90% confluent. These cells were then detached using 0.25% trypsin and seeded into either glass or plastic ribbed roller bottles along with 500mL RPMI 1640, 5% BCS, and 5mM HEPES and grown at 37°C.

Harvesting roller bottle cells: Once confluent, CHO cells were collected from roller bottles. Suspended cells were centrifuged in Beckerman JA-14 rotor jars at 1500rpm, 4°C, for 10 minutes. Adherent cells were detached using 1X PBS 1mM EDTA and centrifuged at 1500rpm, 4°C, for 10 minutes. All cells were washed with 1X PBS 1mM EDTA and suspended in 50 mL. These cells were counted and checked for viability by diluting at 1:1 with trypan blue. This final suspension was then centrifuged at 1500rpm, 4°C, for 10 minutes into a single pellet, weighed, and frozen at -80°C.

Fluorescence-activated cell sorting (FACS): In a 96-well, v-bottom, plate 200µL of FACS buffer (PBS/ 1%BCS/ 1%EDTA) was added and allowed to incubate for 10 minutes at room temperature. Cells needed for staining were detached, counted using 1:2 dilution trypan blue, and resuspended at $5x10^6$-$10x10^6$ cells/mL (only cells that had >80% viability were used for staining). After 10 minutes of incubation the FACS buffer was removed by swiftly inverting the plate. The plate was then placed on ice and 50µL of cells were added to each coated well. To the cells, 50µL of the necessary primary antibody was added. One well, for each cell type, was left without primary antibody, and was used for either secondary antibody only or isotype control in the case of a directly conjugated antibody. The plate was then covered and placed on a shaker for 30 minutes at 4°C. The plate was then centrifuged at 1300rpm, 4°C, for 2 minutes and the supernatant removed by gently but swiftly inverting the plate. The pellet was then resuspended with 150µL FACS buffer and pelleted two times for washing. This final pellet was then resuspended using 50µL of a 1:100 dilution of appropriate FITC conjugated antibody. The plate was once again shaken at 4°C for 30 minutes. After the secondary incubation, the plate was centrifuged at 1300rpm, 4°C, for 2 minutes and the supernatant removed by gently but swiftly inverting the plate and washed with FACS buffer two times as described before. The final pellet

was resuspended in in 150µL FACS buffer and fixed by immediately mixing with 150µL (an equal amount) of 2% formalin (PBS/ 2% formaldehyde) in FACS tubes. Fixed suspension could then be stored in the dark at 4°C for 2-3 days until they could be analyzed by flow cytometry.

If TMVs were stained, all samples were prepared in Eppendorf tubes, and instead of 50µL of cells, 20µg of TMVs were used per tube (20µg TMVs/50µL FACS buffer). All incubations took place on ice, without shaking, and each sample was washed with only 100µL of FACS buffer and centrifuged at 13,200 rpm for 10 minutes at 4°C. They were resuspended in a 100µL suspension which was fixed in 100µL of formalin.

Primary antibodies used include: TA1 (mouse mAb) anti-hHER2, M1/42 (mouse mAb) anti-mMHC ClassI, M1/69 (rat mAb) anti-HSA, YN (rat mAb) anti-ICAM1, C17.8 (rat mAb) anti-mIL-12, 1G10 (rat mAb) anti-mB7-1. For directly conjugated antibodies 50µL of 1:100 dilution was used along with a separate 50µL 1:100 diluted isotype (negative) control. Directly conjugated antibodies used in these experiments include: PE-anti-PDL1, PE-anti-IL-12, and FITC-anti-B7-1. If the plate used both directly conjugated and supernatant antibody, the directly conjugated antibody and isotype control was added at the same time as the secondary.

GPI-protein purification using affinity chromatography: Frozen CHO-K1 cell pellets were removed from -80°C and allowed to thaw and then lysed with 5-10 mL of ice cold lysis buffer per 1g of cells. In cold dH_2O, a solution of 50 mM Tris.HCL buffer pH 8, 2% octyl-beta-D-glucopyranoside (1 g to 100 mL), 20 mM Iodoacetic acid, 5mM EDTA, and 1 mM Zinc Chloride was made. Just before the cells were added, 2mM PMSF from a 200 mM in 100% ethanol stock was added, dropwise, to the solution. Cells were stirred with lysis buffer at 4°C for 4 hours. The lysate was then centrifuged in ultracentrifuge tubes using Beckman centrifuge rotor type 21 at

14000 rpm, 4°C for 60 min. The supernatant was passed filtered through a 70µm cell strainer, and 50µL if this lysate supernatant was saved in an eppendorf tube for later analysis. Before loading the lysate into the affinity columns, both pre-column (containing 10 mL plain Sepharose-4B beads) and main column (containing sepharose beads coupled to antibody against the desired protein) were pre-equilibrated with wash buffer-3 containing 20 mM Tris-HCL pH 7.5, 0.1% 1-octyl-beta-D-glucopyranoside, and 10 mM Iodoacetic acid sodium salt.

The pre-column was connected to the main column through a plastic tube and the lysate loaded onto the pre-column, so that it passed through the main column and into a beaker at approximately one drop per 6 -10 seconds. After 90% of the lysate passed through the column, the flow through could be saved to be passed through the column again after washing.

After the supernatant passed through the column completely, the pre-column and main column were washed with 50 mL of wash buffer-1. The wash buffers were passed through the column at a faster flow rate than the lysate. The upper column was then disconnected from the pre column. The main column washed two times with approximately 10 mL of wash buffer-1 to remove any contaminating proteins, allowing the liquid to pass through the column completely between each wash. The main column was then washed again 3 to 4 times with wash buffer-3, once again, allowing the liquid to pass through the column completely between each wash. Once the column was washed of any contaminating proteins, the specifically bound GPI proteins were eluted very slowly from the column. The elution buffer was prepared depending on the desired protein and column. For GPI-IL-12 it was prepared with 100 mM glycine – HCL pH 2.8, 200 mM NaCl, 1% octyl-beta-D-glucopyranoside, and 10 mM iodoacetic acid sodium salt. While fore GPI-B7-1 and GPI-HER2 were eluted with 100mM Triethylamine, and 1%O.G., pH 11.6. Before adding the

elution buffer to the column, 7-8, 2 mL, low retention Eppendorf tubes were prepared for collecting 1.5 mL eluted fractions. In each tube the appropriate neutralization buffer was added for neutralization of the elution buffer. The elution buffer used for GPI-IL-12 was neutralized using 100µL 1M Tris-HCl, pH 11.0 while 200µL of low pH neutralization buffer was used for GPI-HER2/B7-1 elution buffers. When the elution was complete, the column was washed with 1M Tris – HCL pH 7.5 until the column was completely neutralized and then washed a final time with wash buffer-3.

All fractions were analyzed for protein content by Western blot and Silver Stain.

Western Blot (WB) analysis: All Western blot analysis were electrophoresed in 10% Mini-PROTEAN® TGX™ precast polyacrylamide gels from Bio-Rad© Laboratories, Inc. with varying capacities (i.e. number of wells, or maximum well volume).

Part I: All protein samples were kept at 4°C or on ice before preparation. For a 30µL gel/20µL max volume, 10µL (or ½ the maximum volume) of each protein sample was placed into separate Eppendorf tubes and resuspended with an equal volume 2x Laemmli buffer. For an internal control, 1µg of purified TA1 (mouse anti-hHER2) was used if the samples required an anti-mouse primary antibody, and 2.45µg of purified C17.8 (rat anti-mIL-12) was used if the samples required an anti-rat primary antibody. All sample volumes were made equal by adding PBS. After the samples were mixed with the 2x Laemmli buffer, pre-boiled water was cooled with ice to ~80°C. The tubes were heated in the water for 5 minutes and then centrifuged at room temperature 14,000 rpm for 1 minute. While the samples were heated, the gel was removed from all packaging and placed in a Western blot electrophoresis case along with enough 1x SDS-running buffer to cover the wells. This buffer can be saved and re-used up to three times for

Western blot. The SDS-running buffer was made at 10x with 1090 mL dH_2O, 30 g Tris Base, 144g glycine, 10 g sodium dodecyl sulfate (SDS) and stored at room temperature. Since the WB case held two gels, if only one gel was needed, an empty gel case was used in place of a second gel. Samples were removed from the centrifuge and the maximum volume of each sample was loaded into separate wells. The samples ran for 50 minutes at 150V constant volts

Part II: While the samples were running, 4 filter papers and 1 nitrocellulose membrane were cut to the size of the gel and soaked in transfer buffer at room temperature for at least 20 minutes. The transfer buffer was previously made with 9.0 g Tris, 43.2 g glycine, 600 mL methanol, and 2.4 L dH_2O for a total volume of 3 L and stored at room temperature. When the samples were finished running, the gel was removed from the electrophoresis case, removed from its casting case, and rinsed in transfer buffer for 2-5 minutes. While the gel was rinsing, two of the filter papers and nitrocellulose membrane were removed from the transfer buffer and placed on the transfer plate. When the gel was rinsed, it was placed on top of the membrane, and the excess membrane around the gel was cut away with a razor. The last two filter papers were placed on top of the gel and a test tube was rolled over the top to remove any bubbles. Finally, the cover transfer plate was placed, carefully, on top as to ensure the bottom and top plates were not touching. The transfer ran for 60 minutes at 100mA constant amps.

While the samples were transferring, 5% milk was prepared for blocking. For one gel, 2.5 g of blotting grade milk (instant non-fat dry milk) was stirred into 50 mL of TTBS which was previously made with 7.26 g Tris Base (20 mM), 24 g NaCl (137 mM), 3 mL Tween-20 (0.01%), into 3 L dH_2O and the pH adjusted with HCl to pH 7.6. After the transfer was complete, the nitrocellulose membrane was placed in a tray with 50 mL milk, on a horizontal shaker for 1 hour

at room temperature, or covered overnight at 4°C. After blocking, the milk was poured off the membrane into a beaker for later use and, the membrane was then immersed in the appropriate primary antibody and shaken for 40 minutes (see FACS method for specific antibody). After 40 minutes, the primary antibody was removed and stored at 4°C for later use (used for up to 5 different membranes). The membrane was rinsed with TTBS, first quickly (5s) then again for 10 minutes on the shaker.

Before staining the protein with the secondary antibody, the desired method of development was determined. For analysis of protein fractions eluted from an affinity column, alkaline phosphatase (AP) conjugated secondary antibody was used, and results were visualized directly on the membrane. For protein quantification, horseradish peroxidase (HRP) conjugated secondary antibody was used, and results were visualized by film.

After the second rinse, the TTBS was removed and the membrane covered with 10 mL of milk. The appropriate secondary antibody was then added and mixed into the milk. For all secondary antibody 2 µL of antibody were added to 10 mL of milk except for HRP conjugated anti-rat, in which case only 0.5 µL of antibody were required for 10 mL of milk. The membrane, milk, and antibody were shaken together for 40 minutes. The milk/antibody was discarded and the membrane rinsed quickly with TTBS and then three more times on the shaker for 5 minutes each. While the membrane was rinsing, the appropriate developing substrate was prepared. For AP conjugated antibodies, 66 µL of nitroblue tetrazolium (NBT) and 33 µL of 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) were mixed into 10 mL WB substrate buffer and poured onto the membrane. To stop the development, the substrate was removed and replaced with dH_2O.

Blots stained with HRP conjugated antibodies were developed with 750 µL Luminol/Enhancer and 750 µL Stable Peroxide Buffer from Pierce ECL Western Blotting Substrate Kit. In a dark room, the TTBS was removed from the membrane and all of the ECL substrate (1500 µL) was slowly drizzled on top of the membrane so it was completely covered. The membrane was allowed to develop for 40 seconds and then the substrate poured off. The membrane was then place between two transparencies and the air bubbles were removed by sliding them towards the edge of the membrane. The membrane and transparencies were then placed in a film case with a piece of film placed on top. Separate sections of film were exposed to the HRP reaction for increments anywhere between 10 seconds and 20 minutes depending on the level of detection. After the film was exposed, it was placed in a developing machine.

Silver Stain (SS) analysis: All Silver Stain analysis were electrophoresed in 10% Mini-PROTEAN® TGX™ precast polyacrylamide gels from Bio-Rad© Laboratories, Inc. with varying capacities (i.e. number of wells, or maximum well volume).

*Part I:*Part I of silver stain is the same as Part I of Western blot, however, unlike WB, the SDS-running buffer must be freshly made for silver stain.

Part II: While the samples were running, a 100 mL solution of 50% methanol in dH_2O was prepared and poured into a clean glass tray. When the samples were finished running, the gel was removed from the electrophoresis case, removed from its casting case, and placed in the 50% methanol solution on the shaker for 15 minutes. Meanwhile, another 100 mL solution of 5% methanol in dH_2O was prepared. After 15 minutes, the 50% solution was poured of the gel and replaced with the 5% solution and left on the shaker for another 15 minutes. Afterward, the 5% methanol solution was poured off the gel and 200 mL of dH_2O was poured into the tray. To the

dH_2O, 200 µL of dithiothreitol (DTT) was added and allowed to shake for 15 minutes. During this incubation, the silver stain solution was made by stirring 0.1 g $AgNO_3$ into 100 mL dH_2O until dissolved. The DTT solution was poured off and the gel rinsed with dH_2O for no more than 5 seconds. The silver stain solution was then added to the gel and placed on the shaker for at least 15 minutes. Meanwhile, a developing solution was made by stirring 6.0 g of sodium carbonate anhydrous, and 200 µL of formaldehyde into 200 mL dH_2O. The silver stain was poured off and the gel rinsed quickly, three times for 10-15 seconds each, with dH_2O. To develop the gel, 1/3 of the developing solution was used to quickly rinse the gel and then poured off. Then, the remaining developing solution was added to the gel. The reaction was stopped by adding acetic acid until the fizzing stopped (~3 mL) and rinsed with dH_2O.

Polyvinylpyrrolidone mediated protein concentration: A ~6 in piece of MWCO 10-14 kDa dialysis membrane was cut from a roll kept at 4°C. The membrane was then place in a tray with dH_2O and opened gently as not to tear it. The inside of the membrane was flushed out with water and then left in the dH_2O and allowed to soak for 20 minutes. One end of the membrane was closed with a knot so no liquid could leak out. The membrane was emptied of any water and all eluted fraction (see *GPI-protein collection through affinity chromatography)* that were determined to contain the desired protein were transferred to inside the dialysis bag which was then closed at the other end with a knot. In a tray, the dialysis bag with samples was placed on top of 2 tbls of polyvinylpyrrolidone (PVP) and more PVP was sprinkled on top to cover the bag. The bag was checked every half hour for 2-4 hours until the final volume inside the bag reached ~1 mL. To speed up the process, the wet PVP was removed from around the bag and replaced with fresh PVP. Once ~1 mL was left, the bag was rinsed completely clean of PVP with dH_2O and then purified, immediately, by dialysis.

Dialysis mediated protein purification: While concentrating the protein samples with PVP, three 500 mL aliquots of dialysis buffer was prepared by adding 0.01% (wt/vol) octyl glucoside (o.g) to 500 mL 1x PBS. One end of the rinsed dialysis membrane was then placed in a beaker with one 500 mL aliquot of dialysis buffer and stirred at 4°C overnight. The buffer was then exchanged the next morning and once that same night.

The next morning, the bag was removed from the buffer and cut at one end, near the knot, and the sample was collected from inside and transferred to a low retention Eppendorf tube.

Protein transfer of GPI-protein onto sheep erythrocytes: A ~1mL aliquot of sheep red blood cells, that were kept at 4°C, was transferred to a 15 mL conical tube and rinsed twice with PBS by centrifugation at 2000 rpm for 5 minutes, and the supernatant was removed by aspiration. The final pellet was then resuspended in ~mL PBS/0.1% (wt/vol) ovalbumin. The RBCs from this suspension were counted using a hemocytometer (without trypan blue) at a 1:10 or a 1:100 dilution. Based on the count, the cells were diluted to 10 x 10^6 cells/mL in PBS/0.1% ovalbumin. In a mini Eppendorf tube, 200 µL of the RBC suspension was mixed with 40 µL, (1:5) of the purified GPI-protein to be tested for GPI anchor. For each sample, one mini Eppendorf tube was prepared without GPI-protein for a negative control. All the tubes were then rotated at 37°C for 4 hrs. Meanwhile, separate 1.5 mL Eppendorf tubes were prepared for rinsing the samples by adding 1 mL of FACS buffer. After the incubation, the RBCs were transferred to the new Eppendorf tubes, resuspended in the FACS buffer, and centrifuged at 2000 rpm for 5 minutes at 4°C. The supernatant was aspirated and the pellet resuspended in 100 µL of FACS buffer. The samples were then analyzed by FACS (see *FACS).*

Bicinchoninc assay (BCA) for estimation of protein concentration: A 1:10 (or 1:50, or 1:100) dilution of the sample being tested was prepared for 100 µL/well for three wells of a flat bottom 96-well plate. Nine bovine serum albumin standards of known concentrations (200 µg/mL, 40 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1 µg/mL, 0.5 µg/mL and 0 µg/mL) were prepared for 100 µL/well for three wells. Enough working reagent for 100µL/well was made from and according to the Micro BCA Protein Assay Kit by Thermo Scientific. The samples and standards were added to the plate and the working reagent added to each well using a multichannel pipet. The plate was incubated at 37°C for 2 hours. Afterwards, the reaction was quantified by 590 nm light absorbance. The absorbance levels of the standards were plotted as a linear function of protein concentration, and the protein concentration of the sample was extrapolated from this function.

Protein transfer of GPI-ISMs onto 4TO7ts Tumor Membrane Vesicles (TMVs): For one group of mice (n=5) and expecting 50% recovery, 500 µg of TMVs are used for protein transfer, however, in these experiments, two groups of mice used the same type of TMVs, therefore, all quantities were doubled for protein transfer. For each separate TMV group 1000 µg of 4TO7ts TMVs were used for protein transfer. For 4TO7ts TMVs + HER2 and 4TO7ts TMVs + HER2 + mIL-12 + mB7-1, 250 µg of HER2 were used for protein transfer. Finally, for 4TO7ts TMVs + HER2 + mIL-12 + mB7-1, 50 µg of each ISM were used for protein transfer. The volumes corresponding to the specified amount of TMVs and protein were calculated along with the amount of PBS to bring the final volume up to 8 mL. Before using the GPI-ISMs, the stock tubes were centrifuged at 13,200 rpm for 1h at 4°C. During this time, PBS needed for a final volume of 8 mL, filtered through 0.2 µm filter, was added to a separate 15 mL conical for each type of TMV. After the 1 hour centrifugation, the protein was added carefully, without disturbing the centrifuged

suspension, and the TMVs were added to the PBS in the conical. All tubes were sealed with parafilm and secured to an end over end rotator at 37°C and allowed to rotate, at ~ 20 sec/rotation, for 4 hours. After the incubation, the tubes were centrifuged, briefly, at 4000 rpm, for 2 minutes. Each tube was split into 6 Eppendorf tubes (~1.3 mL per tube) and centrifuged at 13,200 rpm for 1 hour at 4°C. Carefully, as to not disturb the pellet, the supernatant was removed and saved in a separate conical for later analysis. The first pellet was resuspended with 200 µL of PBS and then transferred to the second tube and used to resusupend the second pellet. Then, 200 µL of PBS were placed in each of the remaining four tubes and used to resuspend and transfer the other 4 pellets to that second tube for a total volume of 1mL.

Quantification of GPI-protein incorporated onto TMVs:

The concentration of TMVs in solution after protein transfer was quantified using BCA estimation. The amount of GPI-ISM per µg of TMV was quantified using either Western blotting or ELISA.

Western Blot: Performed as previously described using HRP conjugated secondary antibody. Various dilutions of the sample TMVs were run by side various dilutions of known concentrations of GPI-ISM. The each band area was then quantified using ImageJ software and the areas of the known concentrations were used to plot area as a linear function of amount of protein. The sample concentration was then extrapolated from this function.

ELISA (enzyme-linked immunosorbent assay): ELISA was used after Western blotting was insufficient for quantification of mIL-12 and mB7-1 per µg of TMVs. All protein and antibodies were kept on ice at all times. Samples were diluted to ensure that they fall within the range of the dilutions of purified GPI-ISMs. All dilutions were prepared with ELISA coating

buffer and enough for 6 wells at 100 µL per well, and 3 wells were used for primary and secondary antibody staining while the other three were used for secondary antibody only. The TMVs and purified proteins were added to a 96-well flat bottom ELISA plate, and the plate was incubated with the TMVs and proteins overnight at 4°C. The next day, the samples removed from the wells and washed, three times, with 200 µL per well of PBS/0.05%Tween. The wells were blocked using 200 µL per well of PBS/0.05%Tween/3% BSA and incubated at 37°C for 90 minutes. After blocking, 100 µL of primary antibody (see *FACS* for specific antibody) were added to the wells designated for primary and secondary staining while 100 µL of blocking buffer were added to the secondary only wells, and the plate was incubated at 37°C for 1 hour. The liquid was then removed from the wells and the wells washed three times with 200 µL of PBS/Tween per well. A 1:5000 dilution (in blocking buffer) of HRP-rabbit anti-rat (for C17.8 and 1G10) secondary antibody was prepared and 100 µL added to each well. The plate was then incubated at 37°C for 40 minutes. During this incubation, the Thermo Scientific Pierce TMB Substrate Kit was brought to room temperature. The antibody was removed from the plate and washed five times with 200 µL per well of PBS/Tween as previously described. Equal parts of the two room temperature TMB substrates, TMB solution and peroxide solution, were mixed in equal parts and 100 µL were added to each well. The reaction was carried out at room temperature, and the color development was stopped by adding 100 µL 2M sulfuric acid.

In vivo studies using BALB/cJ mice

Model: Female BALB/cJ mice, six to eight weeks old, were purchased from Jackson Laboratory, housed and maintained according to IACUC approved institutional guidelines and protocols at Emory University.

Prophylactic vaccination using 4TO7ts TMVs: TMV vaccinations were prepared by resuspending 25 μg of TMVs in 200 μL per mouse. Enough vaccine was prepared for two groups of mice of n =5 and each mouse received an inoculation and a boost, a total of 20 injections/4000 μL of vaccine. All inoculations were performed on day 0 and the boosts performed on day 14. For subcutaneous vaccination, the left hind flank of the mouse was shaved and disinfected with an alcohol swab. The vaccine tube was vortexed and ~600 mL were sucked up into a 1 mL insulin syringe to be injected into 3 mice. Before the last 2 mice, more vaccine had to be loaded into the syringe. The mice were placed under a heat lamp to increase vasodilation of the tail vein, and the mice were restrained using tube restraint while 200 μL were injected into the tail vein similarly to subcutaneous injection.

Intravenous live 4TO7RGhHER2 cell challenge: 4TO7RGhHER2 cells were previously grown as described in *Cell Culture* and checked for hHER2 expression through *FACS*. They were detached using PBS/EDTA and were counted and checked for viability. A 100 μL aliquot was saved for FACS analysis, and the rest was resuspended in PBS at 10^5 cells/200 μL.

Intravenous injections were once again performed by Chris Pack as previously described. All mice were weighed every 3-4 days until euthanized.

Serum antibody assay: Five mice were bleed before any injections, and all mice were bled from the cheek at day 14 (prior to the boost) and at day 21 (prior to the challenge). Approximately 100μL of blood was collected into microtainer serum separator tubes which were then centrifuged at 13,200 rpm for 10 minutes at 4°C. The top layer/serum was transferred into an eppendorf tube and stored at -20°C until needed.

Serum was used to stain 4TO7RG and 4TO7RGhHER2 cells. All steps were performed as in *FACS* except the serum was used as the primary antibody at a 1:100 dilution and FITC goat anti-mouse was used as the secondary antibody.

Clonogenic assay: Prior to assay, DMEM media with 10% serum, 1% penstrep, 0.8% nystatin, 5mM addition L-Glutamine, and 5mM HEPES along with separate aliquot of DMEM that contained 2.5 mg of 6-thioguanine in 500 mL in addition to the aforementioned components. For tissue dissociation, 2mL of 2 mg of Sigma collagenase type IV /mL 1x HBSS (without calcium, magnesium, and phenol red) was filtered through a syringe filter into separate 15 mL conical tubes for each mouse. These tubes were kept at 4°C until ready for use. Six well plates were then prepared for the lung samples (3 wells per mouse) by adding 2 mL 1x HBSS to the first two wells and the third was left dry.

Mice were euthanized and sprayed down with 70% ethanol and placed in the hood. The mouse was pinned down by the hands and feet of the mouse and the skin above the abdomen cut open to access the rib cage. To remove the lungs, the rib cage was cut down the diaphragm up to the throat, and, with a tweezers, the lungs were pulled out with the heart by cutting the inferior vena cava. The lungs were then placed in the first well with HBSS and left until lungs were removed. The lungs were then gently washed by moving them around with a tweezers. Before transferring to the second wash well, the heart was separated from the lungs. After the second wash, the lungs were transferred to the dry well and minced into small pieces with scissors. These small pieces were transferred into the 15 mL conical tubes containing 2 mL of 2 mg/mL collagenase using 2 mL HBSS. The final volume was 4 mL with a final concentration of 1 mg/mL collagenase. All the samples were rotated end over end at 37°C for 2 hours at ~10 seconds per rotation. After the

incubation, each sample was transferred into a 70 μm cell strainer in a 50 mL conical tube, and, using the rubber end of a 5 mL syringe plunger, the tissue was broken up and the cells pushed through the cell strainer. A separate strainer was used for each mouse and a separate plunger was used for each group which was rinse with PBS between each mouse. The strainer was rinsed every now and then with DMEM complete media (without 6-thioguanine) to bring the total volume to 10 mL. Each sample was centrifuged at 1200 rpm for 5 minutes at 4°C and rinse two more times with DMEM complete. The final pellet was resuspended in 11 mL of DMEM complete with 6-thioguanine. Before plating the cells, 1 mL of DMEM complete with 6-thioguanine was added to each well of 6-well plates. One plate was to be used for three dilutions (1:2, 1:4, and 1:8) for two different mice. First, 10 mL of the 11 mL cell suspension was added to a 110x20 mm tissue culture dish, and the remaining 1 mL was added to the left most well of three on a 6 well plate, which already contained 1 mL media. The cells were resuspended with the media and, then, 1 mL of the suspension was transferred to the next well, resuspended and, then 1 mL of that suspension was transferred to the last well and resuspended. After resusupending the last well, 1 mL of that suspension was discarded. The tissue culture plates and 6-well plates were then placed at 37°C, 5% CO_2. After 24 hours, 3 mL of media were added to each of the wells in the 6-well plates. The cells were allowed to grow at 37°C, 5% CO_2 until the control showed a sufficient amount of colonies (5-7 days). The 6-well plates were then removed from the incubator to be stained. The media was removed, and each well was rinsed with 3 mL PBS and immediately stained with 1 mL of a crystal violet stain, made with 6% (vol/vol) glutaraldehyde and 0.5% crystal violet (wt/vol) in dH_2O, for 30-40 minutes. The crystal violet was dumped out of the wells into the sink and the sink filled with several inches of water. Each

plate was submerged gently into the water to rinse off excess crystal violet and then inverted onto paper towels to dry for 24 hours.

Live cell count: After 8 days of growing, the tissue culture dishes (from the clonogenic assay) were removed from the incubator and placed at room temperature overnight. The next day, the plates were rinsed twice with non-sterile PBS and one more time with PBS/EDTA which was dumped into the sink. The plates were placed at 37°C and allowed to detach with the residual PBS/EDTA for 20 minutes. After 20 minutes, 5 mL FACS buffer was added to each plate which was used to rinse the plate with a transfer pipet. The cells were then transferred to a 15 mL conical tube, and the remaining cells were rinsed from the plate by adding 3 mL FACS buffer and then 2 mL more FACS buffer for a total volume of 10 mL. The samples were centrifuged at 1200 rpm for 5 minutes at 4°C. The supernatant was removed by dumping it in the sink, and 450 µL of FACS buffer was added to resuspend the pellet. A separate FACS tube was prepared for each sample with 300 µL of formalin which was used to fix 300 µL of each sample. The cells were counted by running each fixed sample through the flow cytometer for 30 seconds.

The remaining sample was used to test hHER2 expression. Each sample was added to two wells of a 96-well v-bottom plate, one for primary (TA1) and secondary (anti mouse) staining and one for secondary only staining. The staining was performed as in *FACS.*

Kinetics of 4TO7RGhHER2 metastasis: A separate experiment to determine kinetics of 4TO7RGhHER2 experimental metastasis was performed by challenging three groups (n=3) of female BALB/cJ mice with live 4TO7RGhHER2 cells. Each group was euthanized at different time points (day 8, day 15, and day 21) for a clonogenic assay on the lungs. Cells from the clonogenic assay that were grown in the tissue culture dishes were not used for crystal violet

staining. After growing in 6-thioguanine for 8 days, adherent cells were harvested and stained for hHER2, MHC class I, HSA, ICAM-1, and PDL1 (see *FACS*) to determine changes in expression due to the in vivo environment.

Results

Surface protein expression profile of 4TO7RGhHER2 culture cells

Analysis of protein expression of 4TO7RGhHER2 cells by flow cytometry indicated relatively high leves of hHER2, very high levels of HSA, moderate levels of mMHC Class I, and very low levels of ICAM-1 as indicated by mean MFI (Fig. 2). PDL1 expression was tested later (Fig. 4) which was found to be very low (data not shown).

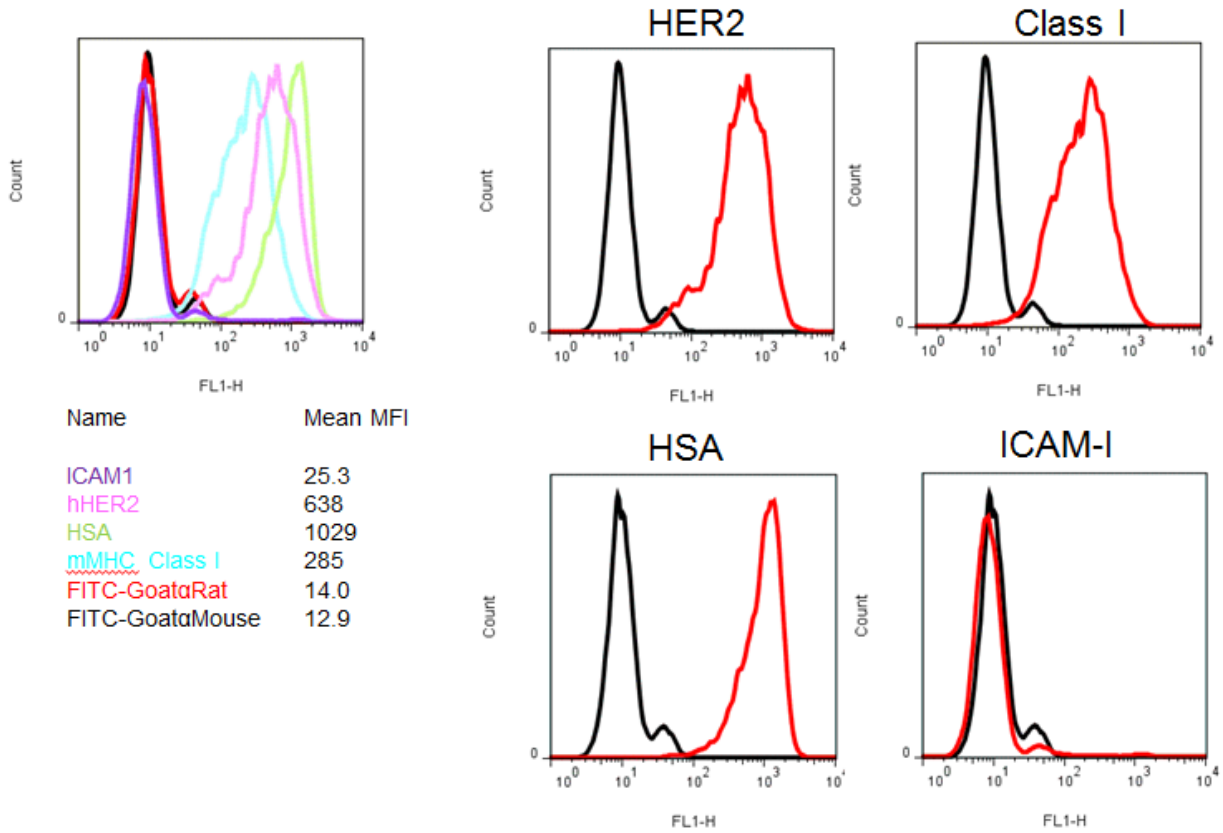


Figure 2: Surface protein expression profile of 4TO7hHER2 culture cells. 4TO7hHER2 cells were stained for levels of hHER2, MCH ClassI, heat shock antigen (HSA), and ICAM-1. Red peaks denote expression of the protein

of interest as compared to secondary antibody control (black). Superimposed histograms (left) show protein levels relative to eachother.

Kinetics of 4TO7RGhHER2 metastasis

In order to observe the kinetics of metastasis of 4TO7RGhHER2 live cells, three groups of female BALB/cJ mice (n=3) were intravenously challenged with 100,000 live 4TO7RGhHER2 cells. Each group was euthanized, along with two unchallenged (null) mice, at a different time point after the challenge to observe the metastatic cell burden in the lungs. The first was euthanized and analyzed by clonogenic assay on day 8 after the challenge. Mouse 3 of the 3 was the only mouse that showed 4TO7 colonies in the clonogenic assay after six days of growth in 6-thioguanine media (Fig. 3). The second group was euthanized on day 15 and all mice showed cell growth after 6 days. However, mouse 1 of 3 showed more growth than both of the others. The third group was euthanized on day 22, and though all three mice yielded cell growth, mouse 3 of 3 overgrew by day 6 after euthanization while the other 2 mice grew moderately.

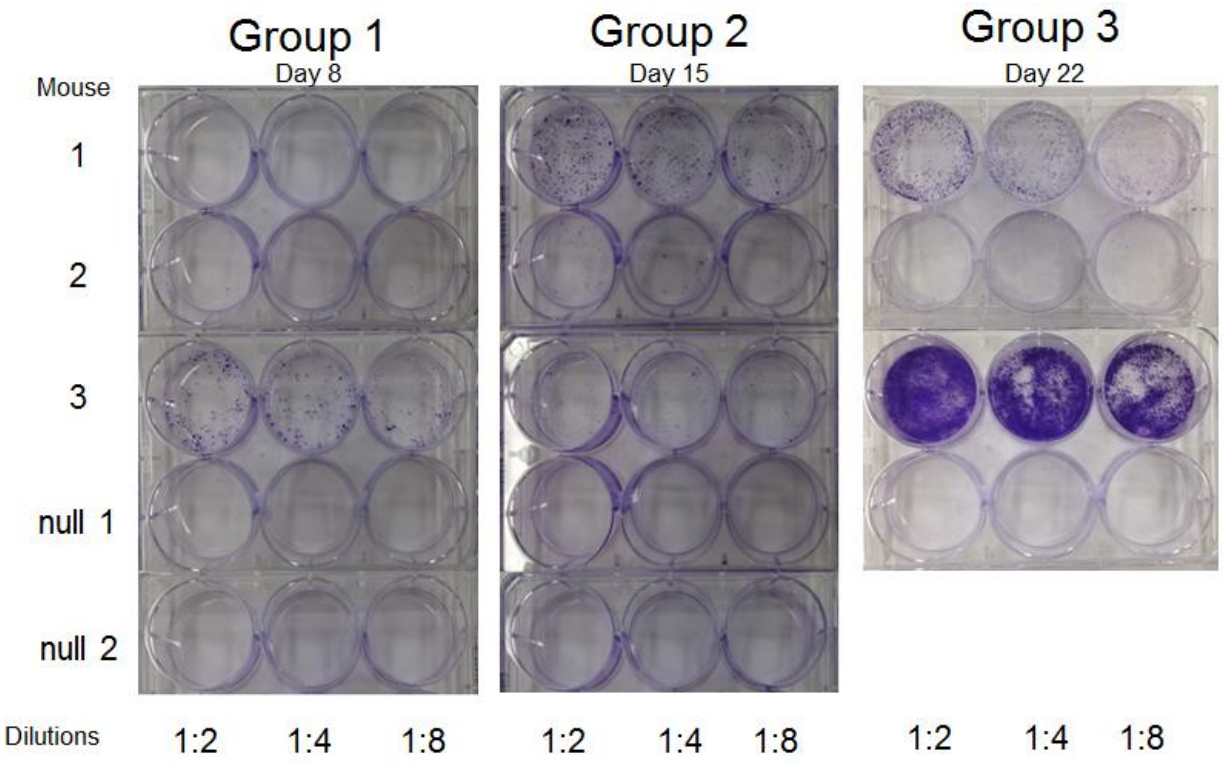


Figure 3: Kinetics of 4TO7RGhHER2 metastasis clonogenic assay. Three groups of female BALB/cJ mice (n=3) were challenged (i.v.) with 100,000 live 4TO7hHER2 cells. Each group was then euthanized at a different time point, day 8, day 15, and day 22, and their lungs were harvested for analysis of live metastatic cells by clonogenic assay. Challenged mice are denoted by numbers 1-3, while null 1 and 2 are unchallenged mice.

The same cells that were harvested to observe the kinetics 4TO7RGhHER2 lung metastasis by clonogenic assay, on day 8, 15, 22, were stained for hHER2, MHC class I, HSA, ICAM-1 and PDL1 alongside 4TO7RGhHER2 culture cells (Fig. 4).

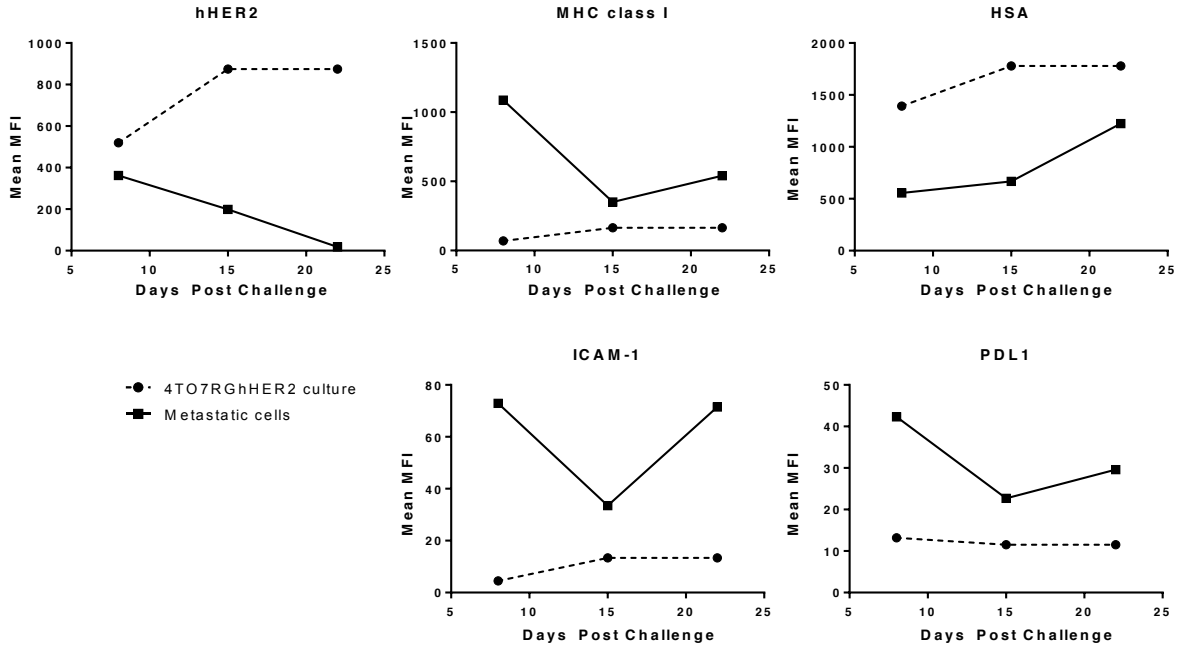


Figure 4: Kinetics of 4TO7RGhHER2 metastasis protein expression changes. Cells harvested from lungs groups euthanized on day 8, 15, and 22 were grown in 6-thioguanine for 8 days. Adherent cells (solid lines) were harvested and stained alongside 4TO7RGhHER2 culture cells (dotted lines) for hHER2, MHC class I, HSA, ICAM-1, and PDL1 see to determine changes in expression due to the in vivo environment.

Overall, hHER2 and HSA expression levels were lower in 4TO7RGhHER2 cells harvested from the lungs compared to 4TO7RGhHER2 cell culture. Although HSA expression is lower in cells grown in vivo than in cells grown in vitro, the expression seemed to increase over time when grown in vivo in contrast to hHER2 expression which seemed to disappear in a linear fashion and almost completely disappeared after 22 days of growing in vivo even though the metastatic burden increased. Expression of MHC class I, ICAM-1 and PDL1 all increased in vivo compared to 4TO7RGhHER2 cell culture, however, there does not seem to be any specific trend in the changes of expression.

GPI-hHER2 protein analysis

CHO-K1-hHER2-CD59 showed moderate hHER2 expression after harvest from roller bottles (Fig. 5a) and the GPI-hHER2 protein from these cells was collected through affinity chromatography and successfully eluted from the column (Fig. 5b).

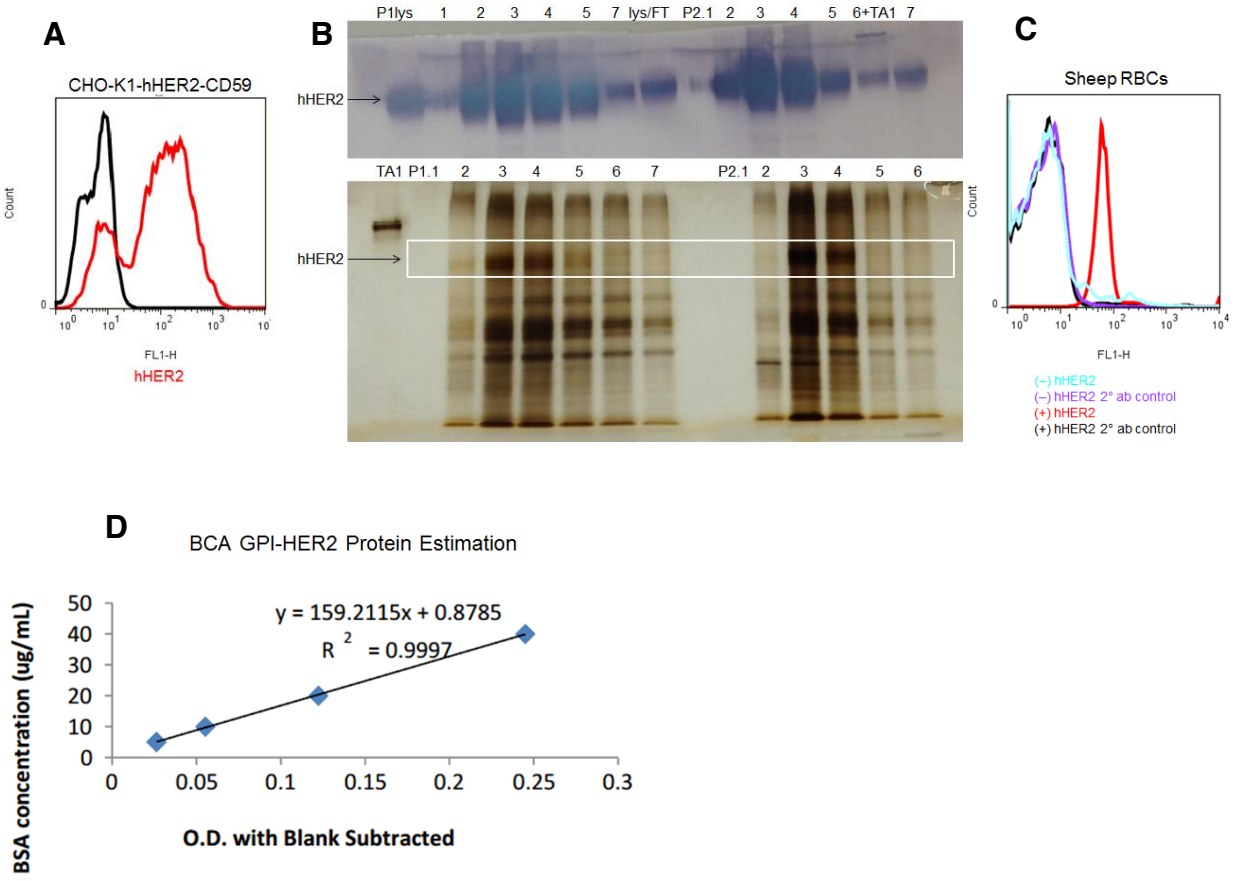


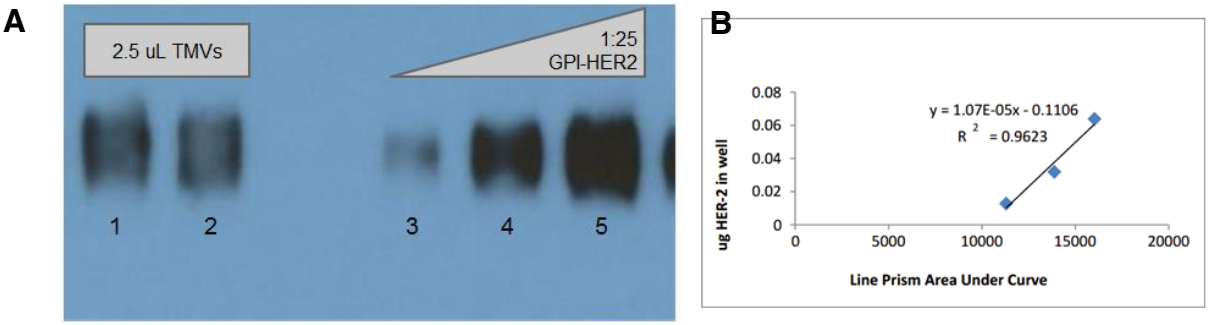
Figure 5: GPI-hHER2 protein analysis. (A) CHO-K1-hHER2-CD59 cells harvested from roller bottles were stained for hHER2 expression. From these CHO cells, GPI-hHER2 was collected in fractions, through affinity chromatography, (B) which were analyzed for protein content through Western blotting and purity through Silver Staining. (C) These protein fractions were purified and consolidated into one sample which was then tested for GPI-anchor functionality by incorporation onto sheep erythrocytes. Red peak shows hHER2 incorporation, as compared to secondary antibody control (black). The blue peak represents the negative control of erythrocytes without hHER2 as compared to a parallel secondary antibody control (purple). (D) Concentration of GPI-hHER2 was determined to be 259.8061µg/mL through BCA analysis as a function of optical density (O.D.) at 590 nm.

Western blot analysis shows the presence of hHER2 in all fractions from pass 1 and pass 2 with the highest amounts in fractions P1.2, 3, 4, 5 and P2.2, 3, 4, 5. The Silver Stain shows a moderate

amount of contaminating protein which suggests the column was not washed effectively before eluting the desired protein. All fractions containing hHER2 were combined, concentrated and dialyzed. This final sample was then tested for GPI-anchor functionality. GPI-hHER2 was successfully incorporated into sheep erythrocyte membranes when analyzed by FACS (Fig. 5c). This final sample was also analyzed by BCA for estimation of hHER2 concentration which was found to be 259.8061 µg/mL. Analysis on GPI-mIL-12 and GPI-mB7-1 not shown since the samples used were previously tested for GPI-anchor functionality and protein concentration prior to these experiments.

GPI-Protein incorporation onto 4TO7ts TMVs

Once the functionality of the GPI-anchor and the concentration of each desired GPI-protein sample were established they could be used for protein transfer onto TMVs. GPI-hHER2 (Fig. 5) was used with previously tested samples of GPI-mIL-12 and GPI-mB7-1 for protein transfer onto 4TO7ts TMVs. Based on Western blotting, there were 13.368 ng GPI-hHER2 per µg 4TO7ts TMV + GPI-hHER2 and 11.024 ng GPI-hHER2 per µg 4TO7ts TMV + GPI-hHER2 + GPI-mIL-12 + GPI-B7-1 after protein transfer (Fig. 6a,b) which is consistent with previous data which shows decreased incorporation of one type of GPI-protein when transferred simultaneously with other GPI-proteins.



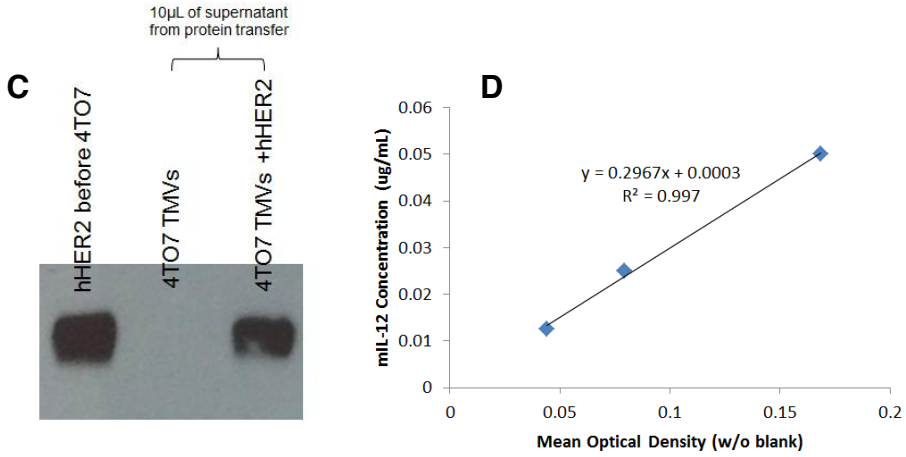


Figure 6: GPI-Protein incorporation onto 4TO7ts TMVs. GPI-hHER2, GPI-mIL-12, and GPI-mB7-1 were protein transferred onto 4TO7ts TMVs. (A) Western blotting was performed on 4TO7ts TMVs + GPI- hHER2 (lane 1) and 4TO7ts TMVs + GPI-hHER2 + GPI-mIL-12 + GPI-mB7-1 (lane 2) by side known concentrations of GPI-hHER2 (12.7728 ng in lane 3, 31.932 ng in lane 4, 63.864 ng in lane 5) to determine the concentration of hHER2 per µg of TMVs. (B) The blot was analyzed through IMAGEJ in which each band produced a curve with a certain area. The amount of hHER2 in each well was plotted as a linear function of this area which was used to determine the concentration of GPI-hHER2 on the 4TO7ts TMVs. Based on these analyses, there are 13.368 ng GPI-hHER2 per µg 4TO7ts TMV + GPI-hHER2 and 11.024 ng GPI-hHER2 per µg 4TO7ts TMV + GPI-hHER2 + GPI-mIL-12 + GPI-B7-1. (C) The amounts of hHER2 in the supernatant before and after protein transfer onto 4TO7ts TMVs were compared using Western analysis. Based on IMAGEJ analysis, ~34.4% of the GPI-hHER2 in solution incorporated into TMVs. (D) ELISA was used to determine concentration of GPI-mIL-12 on 4TO7ts TMVs by comparing the optical density (415 nm) of known concentrations of IL-12 to the optical density produced from the 4TO7ts TMVs + GPI-hHER2 + GPI-mIL-12 + GPI-mB7-1. Based on the linear function produced, there were 12.728 ng GPI-mIL12/µg 4TO7ts TMVs.

Western analysis was performed on supernatants of samples before and after protein transfer showed 34.4% incorporation of GPI-hHER2 from solution (Fig. 6c). Western blot analysis was also performed to determine the level of incorporation of GPI-mIL-12 and GPI-mB7-1 but was found to be insufficient since IL-12 stained too heavily and mB7-1 was undetectable (blots not shown). The concentration of incorporated GPI-mIL-12 per µg of TMVs was determined through ELISA and found to be 12.728 ng GPI-mIL12/µg 4TO7ts TMVs. ELISA was also performed to determine concentration of incorporated GPI-mB7-1, but the purified mB7-1 samples used for standards were contaminated with antibody making the control and the stained samples indistinguishable.

FACS analysis of GPI-ISM incorporation onto 4TO7ts TMVs

The three different types of modified 4TO7ts TMVs were analyzed by FACS analysis to observe relative levels of incorporation (Fig. 7).

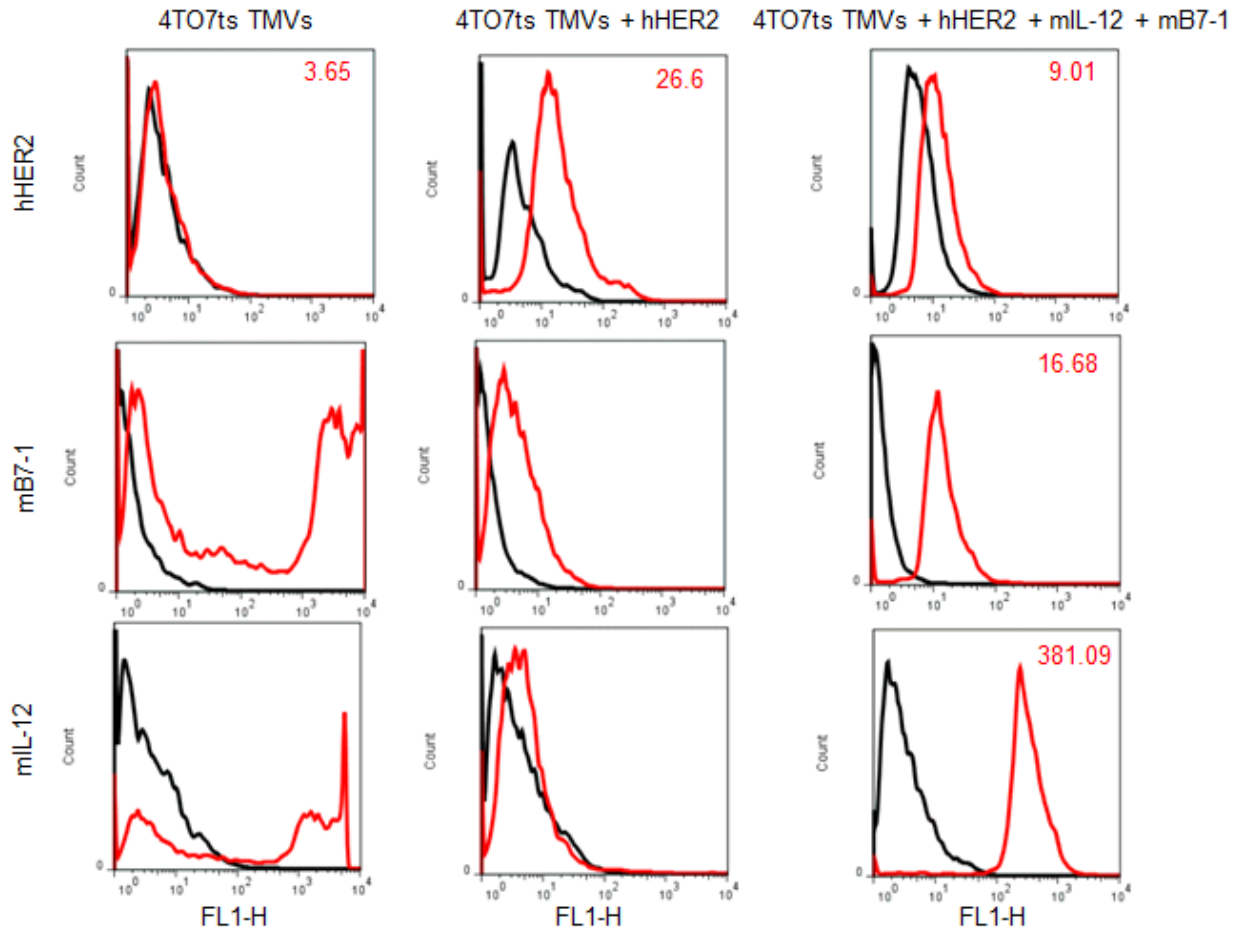


Figure 7: FACS analysis of GPI-ISM incorporation onto 4TO7ts TMVs. hHER2 was stained with mAb TA1. B7-1 and IL-12 were double stained with B7-1-FITC conjugated antibody and IL-12-PE conjugated antibody. 4TO7ts TMVs (left) shows no incorporation hHER2 and according to the dot plot (data not shown) apparent staining of mB7-1 and mIL-12 is inaccurate due to double staining. 4TO7ts TMVs + hHER2 (middle) show incorporation of hHER2. 4TO7ts TMVs + hHER2 + mIL-12 + mB7-1 show incorporation of all three. The red numbers in the corners are the mean MFIs of the protein of interest (red peaks) with the mean control MFI (black peaks) subtracted.

The 4TO7ts TMVs without any protein modifications showed no expression of hHER2 and, according to the dot plot (not shown), peaks indicating mIL-12 and mB7-1 incorporation were erratic due to the sample running low and the machine sucking up air. The 4TO7ts TMVs +

hHER2 showed incorporation of hHER2 and no incorporation of GPI-mIL-12 or GPI-B7-1 indicated by MFI. The triple protein transfer group showed different levels of incorporation, the highest level being mIL-12 and lower levels of hHER2 and mB7-1.

After prophylactic vaccination of with these three different types of protein modified 4TO7ts TMVs, all mice were challenged, intravenously, with 10^5 4TO7RGhHER2 live cells with a relatively high expression level of hHER2 as indicated by MFI (Fig. 8).

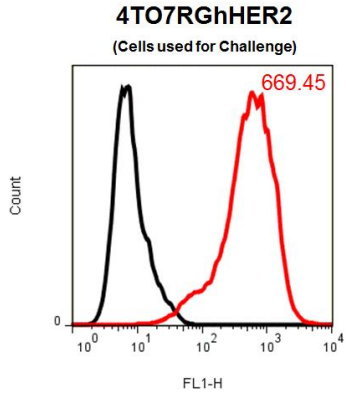


Figure 8: HER2 expression on 4TO7RGhHER2 cells used for intravenous challenge after prophylactic vaccination. These 4TO7RGhHER2 cells were stained in at the time of challenge to observe actual HER2 expression injected. Red number in the corner is the mean MFI of the protein of interest (red peaks) with the mean control MFI (black peaks) subtracted.

Weight changes post 4TO7RGhHER2 challenge

After each mouse was challenged with 4TO7RGhHER2 live cells, each mouse was weighed to monitor weight changes (Fig. 9).

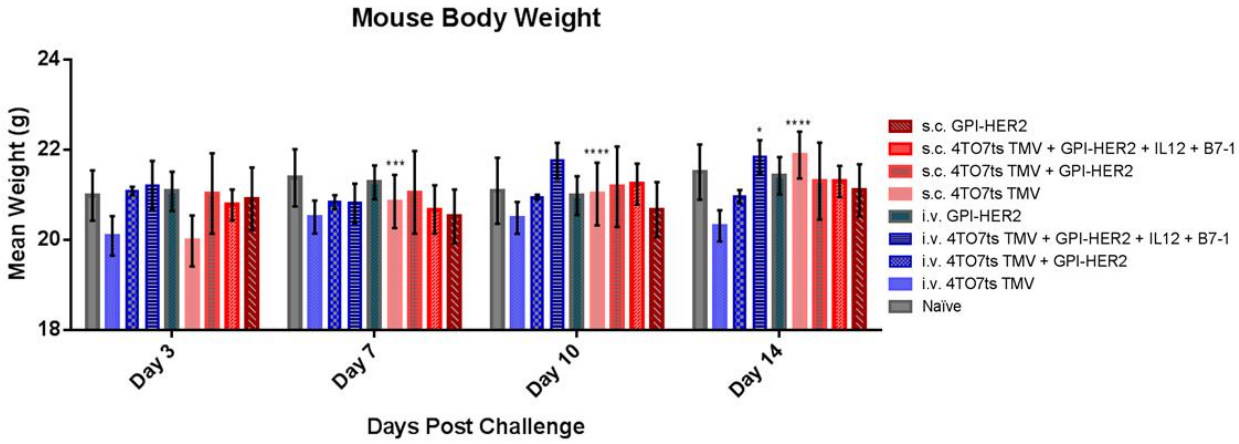
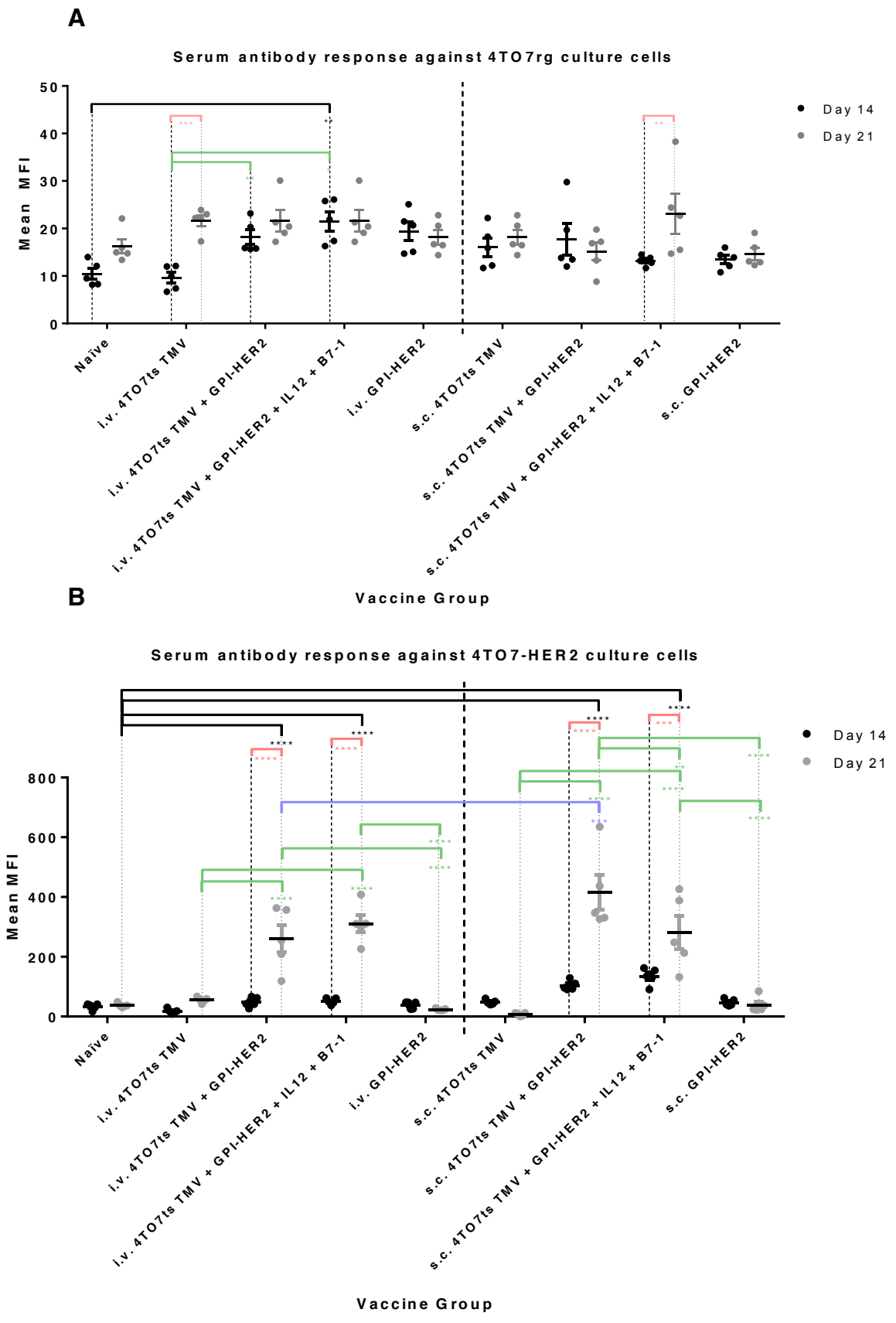


Figure 9: Weight changes post 4TO7RGhHER2 challenge. Each mouse was weighed every 3-4 days for two weeks post challenge. The mean weight of each mouse group on a particular day was compared to the mean weight of that same group on day 3 (using Two-Way ANOVA followed by Bonferroni multiple comparisons test) to observe any statistical differences from near the start of challenge. Mean ± SEM depicted, and all p-values are adjusted by Bonferroni correction for multiple comparisons (* $p < 0.05$, **** $p < 0.0001$).

None of the groups showed any significant weight loss, however, the group vaccinated, subcutaneously, with 4TO7ts TMVs gained a significant amount of weight from day 3 after the challenge. This is probably due to the fact that they were severely dehydrated on the day they were challenged as shown by their severe weight loss and scruffed appearances. This was further confirmed when they regained weight and healthy demeanors when treated with purified gel water packs for dehydration. The i.v. 4TO7ts TMV + HER2 + IL2 + B7-1 group also gained a significant amount of weight by day 14 after the challenge.

Serum antibody response is specific to hHER2

Both, 4TO7RG and 4TO7RGhHER2 cells were stained with serum collected from mice on day 14 (pre-boost) and day 21 (post-boost) (Fig. 10).


A
Serum antibody response against 4TO7rg culture cells
Mean MFI
50
40
30
20
10
0
Day 14
Day 21
Naïve
i.v. 4TO7ts TMV
i.v. 4TO7ts TMV + GPI-HER2
i.v. 4TO7ts TMV + GPI-HER2 + IL12 + B7-1
i.v. GPI-HER2
s.c. 4TO7ts TMV
s.c. 4TO7ts TMV + GPI-HER2
s.c. 4TO7ts TMV + GPI-HER2 + IL12 + B7-1
s.c. GPI-HER2
Vaccine Group
B
Serum antibody response against 4TO7-HER2 culture cells
Mean MFI
800
600
400
200
0
Day 14
Day 21
Naïve
i.v. 4TO7ts TMV
i.v. 4TO7ts TMV + GPI-HER2
i.v. 4TO7ts TMV + GPI-HER2 + IL12 + B7-1
i.v. GPI-HER2
s.c. 4TO7ts TMV
s.c. 4TO7ts TMV + GPI-HER2
s.c. 4TO7ts TMV + GPI-HER2 + IL12 + B7-1
s.c. GPI-HER2
Vaccine Group

C

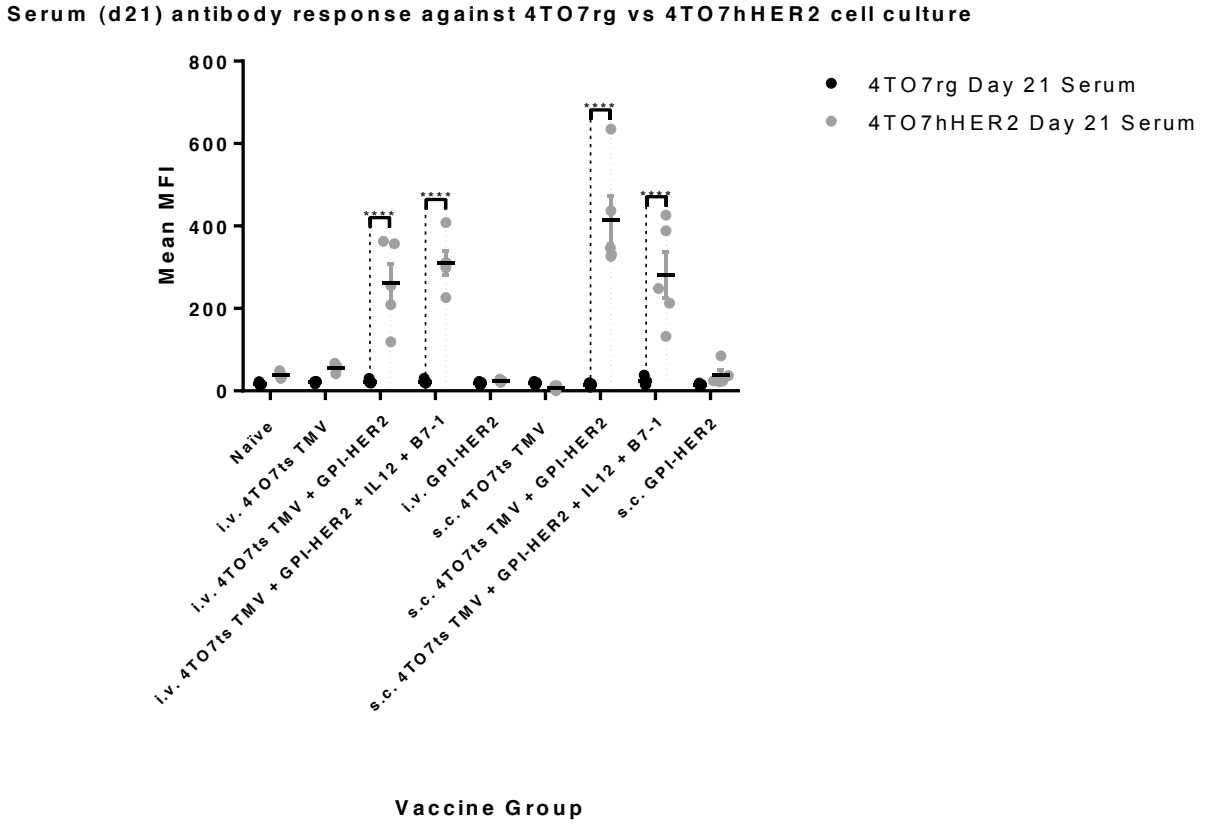


Figure 10: Serum antibody responses to 4TO7RG vs 4TO7RGhHER2. (A) 4TO7RG cells were stained with blood serum collected from mice on day 14 (pre-boost) and day 21 (7 days post-boost). Using Two-way ANOVA followed by Bonferroni multiple comparisons test, the mean of each group, on day 14 (heavy dotted lines) and day 21 (light dotted lines) separately, was compared to the mean of every other group mean on day 14 and day 21 respectively. Black brackets denote significant differences between naïve and other groups. Green brackets denote significant differences between different vaccine within either i.v. or s.c. Pink brackets denote significant differences between day 14 and day 21 of each vaccine. (B) 4TO7RGhHER2 were stained with the same serum and analyzed by the same statistical tests as in (A). Blue brackets indicate significant differences between parallel i.v. and s.c. vaccine groups. (C) Day 21 serum responses to 4TO7rg cells were compared to day 21 serum responses to 4TO7hHER2 cells using Two-Way ANOVA followed by Bonferroni multiple comparisons test. Mean ± SEM depicted, and all p-values are adjusted by Bonferroni correction for multiple comparisons (* $p < 0.05$, **** $p < 0.0001$).

Antibody responses, indicated by MFI, against 4TO7RG cells from mice vaccinated with i.v. 4TO7ts TMVs + hHER2 + mIL-12 + mB7-1 were significantly higher than unvaccinated mice (naïve) and mice vaccinated with i.v. 4TO7ts TMVs pre-boost (Fig. 10a). Antibody responses produced by mice vaccinated with i.v. 4TO7ts TMV + hHER2 were also significantly higher than mice vaccinated with i.v. 4TO7ts TMVs. There were no significant differences between i.v. and parallel s.c. groups. The only significant increases between day 14 and day 21 were within

the groups vaccinated with i.v. 4TO7ts TMV and s.c. 4TO7ts TMVs + hHER2 + mIL-12 + mB7-1 meaning all the rest of the groups antibody production against 4TO7RG cells did not increase with a boost unlike antibody production against 4TO7RGhHER2 which increased, significantly, in groups vaccinated with 4TO7ts TMV + hHER2 and 4TO7ts TMVs + hHER2 + mIL-12 + mB7-1 in both i.v. and s.c. (Fig. 10b). These same groups were also significantly higher, after the boost, than the naïve group, the parallel 4TO7ts TMVs, and the parallel GPI-hHER2. The antibody responses s.c. 4TO7ts TMV + hHER2 was also higher than s.c. 4TO7ts TMVs + hHER2 + mIL-12 + mB7-1, as well as, the parallel i.v. 4TO7ts TMVs + hHER2 suggesting, in this case, the s.c. method of vaccination is better than the i.v. method. When post-boost serum responses to 4TO7RG cells were compared to post-boost serum responses to 4TO7RGhHER2, groups vaccinated with 4TO7ts TMV + hHER2 and 4TO7ts TMVs + hHER2 + mIL-12 + mB7-1, in both i.v. and s.c., produced higher antibody responses 4TO7RGhHER2 than to 4TO7RG cells suggesting this response is specific to hHER2 (Fig. 10c).

Quantification of 4TO7RGhHER2 metastasis

After harvesting the lungs of all mice on day 15 post 4TO7RGhHER2 challenge, the level of metastasis was quantified in two ways: clonogenic assay and raw count of metastatic cells. The clonogenic assay allows visualization of single cells from the lungs when each of those cells grow into a colony, therefore, it is a representation of the number of cells that actual grew in the lungs. However, some of the plates stained in the clonogenic assay showed no visible colonies; while the undiluted suspension, grown in the tissue culture plates, showed some growth in respective plates. Therefore, cells from these plates were harvested and used for a secondary quantification. For both the cell count and the colony count, there was a significantly higher

metastatic burden in mice vaccinated with i.v. GPI-hHER2 when compared to unchallenged (null) mice (Fig. 11a) and a significantly lower metastatic burden in mice vaccinated with s.c. 4TO7ts TMV + hHER2 when compared to unvaccinated mice (Fig. 11b). This is consistent with the antibody response study which showed that the s.c. 4TO7ts TMV + HER2 vaccine led to the highest antibody response against 4TO7RGhHER2 cells of all vaccines. The naïve and the i.v. 4TO7ts TMV groups were found to be significantly higher than the null group only when comparing the cell counts but not the colony counts (Fig. 11b).

In order to see if the methods of counting were significantly different, the difference between the cell count and colony count for each group was tested using Two-Way ANOVA followed by Bonferonni multiple comparisons test (data not shown). No significant differences were found meaning the each method of counting is comparable. Therefore, in order to find overlapping differences, each count was normalized separately and plotted together (Fig. 11c). Each whole mean, encompassing both types of normalized counts, was compared to every other whole mean. The group vaccinated with i.v. GPI-hHER2 was found, still, significantly higher than the null group, however, no groups were found significantly different from the naïve group. The i.v. GPI-hHER2 was also found significantly higher than the groups vaccinated with i.v. 4TO7ts TMV + hHER2 and i.v. 4TO7ts TMV + hHER2 + mIL-12 + mB7-1 which were, in turn, found much lower than i.v. 4TO7ts TMV. No significant differences were found between s.c. vaccines, still, they were also not found different to their i.v. counterpart suggesting that they are comparable. Therefore, overall the 4TO7ts TMV and GPI-hHER2 vaccines led to higher metastatic burdens in comparison to 4TO7ts TMVs that were modified with GPI-hHER2, IL-12, and mB7-1.

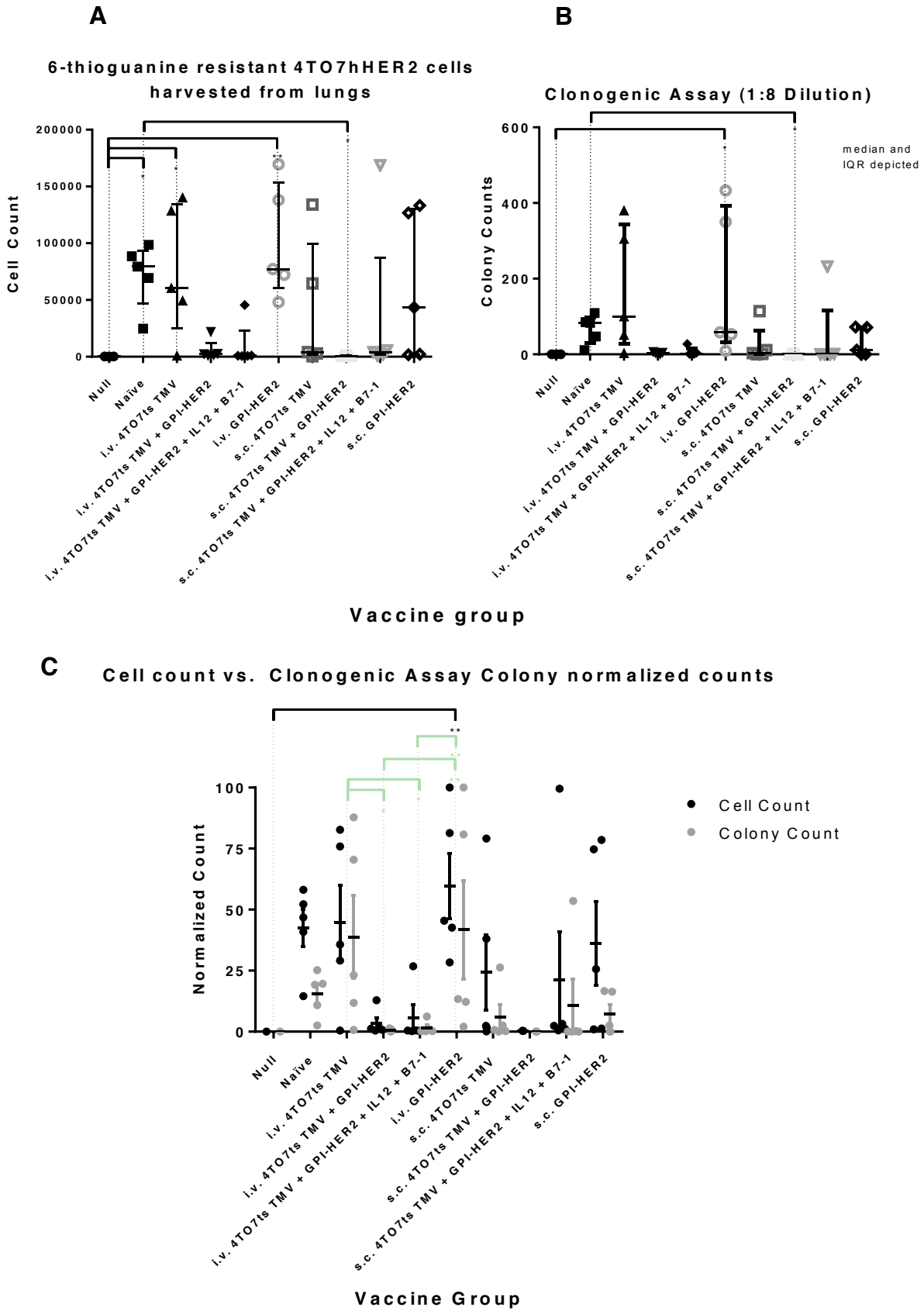

A
6-thioguanine resistant 4TO7hHER2 cells harvested from lungs
Cell Count
200000
150000
100000
50000
0
B
Clonogenic Assay (1:8 Dilution)
Colony Counts
600
400
200
0
median and IQR depicted
Null
Naïve
i.v. 4TO7ts TMV
i.v. 4TO7ts TMV + GPI-HER2
i.v. 4TO7ts TMV + GPI-HER2 + IL12 + B7-1
i.v. GPI-HER2
s.c. 4TO7ts TMV
s.c. 4TO7ts TMV + GPI-HER2
s.c. 4TO7ts TMV + GPI-HER2 + IL12 + B7-1
s.c. GPI-HER2
Vaccine group
C
Cell count vs. Clonogenic Assay Colony normalized counts
Normalized Count
100
75
50
25
0
Cell Count
Colony Count
Vaccine Group

Figure 11: Quantification of 4TO7hHER2 metastasis. Lungs were harvested from all mice 15 days after intravenous challenge with 4TO7hHER2 live cells and plated in 6-thioguanine media (A) Undiluted cells, growing in tissue culture dishes, were allowed to grow for 8 days, harvested, fixed in formalin and counted using flow cytometry. (B) Diluted cells, grown in 6-well plates, were stained with crystal violet. The 1:8 dilution well was used for counting stained colonies. Due to non-Gaussian distributions (i.e. there is no obvious visible mean) both (A) and (B) counts were analyzed using Kruskal-Wallis test followed by Dunn's multiple comparisons test which adjusts all p-values for multiplicity. Median ± IQR depicted. (C) In order to normalize the distributions, raw cell counts (black dots) and stained colony counts (gray) were normalized separately (the lowest of each count made equal to 1 and the highest made equal to 100). The whole mean (both types of counts) of each group were compared using Two-Way ANOVA followed by Bonferroni multiple comparisons test. Mean ± SEM depicted, and p-values are adjusted by Bonferroni correction for multiple comparisons (for all: * $p < 0.05$, **** $p < 0.0001$).

HER2 expression on lung metastatic cells

The same cell samples that were used for raw cell counts were stained for HER2 expression to observe if the vaccine produced a difference in expression. The mean MFI of the cells that were collected, was compared to mean MFI of the 4TO7RGhHER2 cells that were injected (Fig. 12a), mean MFI of cells from unvaccinated mice, and to mean MFI of cells from mice vaccinated mice.

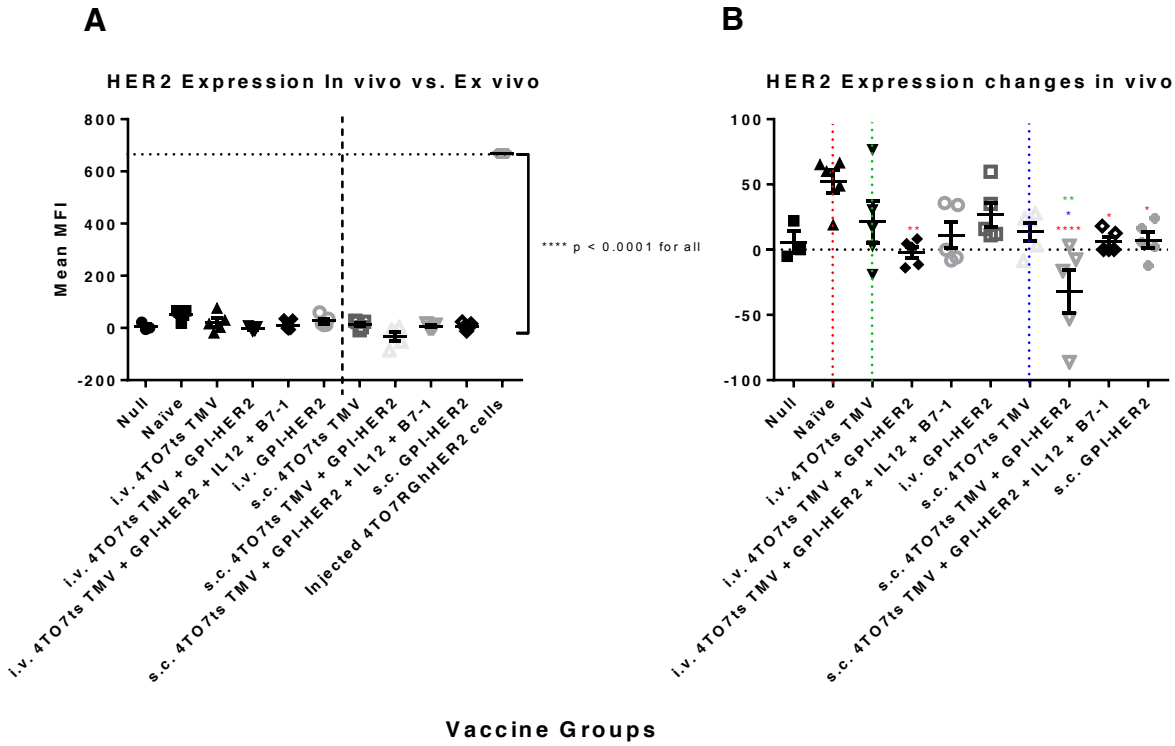


Figure 12: HER2 expression on lung metastatic cells. Undiluted cells, in tissue culture dishes, were stained for HER2 expression. (A) The raw mean MFI (hHER2 expression) of each vaccine group was compared to naive (red dotted line) mean and the raw mean MFI for HER2 expression of each group was compared to hHER2 expression of injected 4TO7RGhHER2cells (horizontal black dotted line). Statistical differences were tested through One-Way ANOVA analysis followed by Bonferroni multiple comparisons test. Mean ± SEM depicted, and all p-values are adjusted by Bonferroni correction for multiple comparisons (* $p < 0.05$, **** $p < 0.0001$).

All of the groups had significantly lower hHER2 expression compared to the injected cells after growing 15 days in vivo. However, mice vaccinated with 4TO7ts TMV + hHER2 (both i.v. and s.c.) had significantly decreased HER2 expression on the harvested metastatic cells than the naïve (Fig. 12b). Furthermore, even though both were significantly lower, s.c. 4TO7ts TMV + HER2 was still more significant which is consistent with the fact that it had the highest antibody response to 4TO7RGhHER2 cells. When compared to groups vaccinated with unmodified TMVs, it was also significantly lower. Groups vaccinated with s.c. 4TO7ts TMV + hHER2 + mIL-12 + mB7-1 and s.c. GPI-hHER2 were also significantly lower than the naive group.

Discussion

Overall the inclusion of HER2 in 4TO7ts TMVs did increase IgG responses to 4TO7RGhHER2 cells over 4TO7RG cells, and in turn increased protection against lung colonization by 4TO7RGhHER2 cells. Previous studies have shown that B7-transduced tumors increased protective immunity, particularly through the activation of cytotoxic T lymphocytes (CTL), against wild-type tumors when compared to nontransduced tumors [42]. Therefore, this protection could be due to the direct activation of T-cells by costimlation with B7-1 and IL-12 complexing to CD28 and IL-12R respectively, or it could be an indirect activation of T-cells through APCs or other immune cells [24].

Since the results of these experiments showed growth in all groups it would be pertinent to establish differential survival probabilities based on vaccine groups. Furthermore, it also may be beneficial to perform T-cell response assays for mechanistic evidence of activation, such as,

increased production of IFN-ɣ or Treg and MDSC assays to observe if these vaccines decrease immunosuppressive pathways incurred by the tumor microenvironment [31].

Mice vaccinated subcutanesouly with 4TO7ts TMVs + GPI-HER2 had the highest IgG response and protection against 4TO7RGhHER2 lung colonization. Furthermore, unlike the i.v. GPI-HER2 and i.v. 4TO7ts TMVs vaccinated groups, which had significantly higher lung metastasis than unchallenged (null) mice (Fig. 11), none of the s.c. vaccine groups had a significantly higher metastatic burden compared to the unchallenged group. This suggests that subcutaneous vaccination, over all, leads to lower lung metastasis in relation to groups vaccinated intravenously, thus, the method of subcutaneous vaccination serves as a better method of protection against 4TO7hHER2 experimental metastasis. This may be due to the size effect diffusion of these molecules. They may diffuse more slowly subcutaneously, allowing enough time for surrounding APCs and T-cells to come into contact with and be activated by these TMVs.

Since HER2/neu is a self-antigen, it is tolerogneic. Based on mouse models transgenic for HER2, it is apparent that HER2 self-tolerance is caused by the deletion or inactivation T cells with high activity against HER2 [43]. HER2 also downregulates antigen-presentation machinery which, further, leads to an immune escape phenotype [44]. Mice vaccinated intravenously with GPI-HER2 had significantly higher metastatic incidence than unchallenged mice, and mice vaccinated intravenously with 4TO7ts TMVs protein transferred with HER2. It is possible that in the absence of a membrane anchor, soluble GPI-HER2 further suppresses antigen-presentation machinery and further increases inactivation of T-cells ultimately leading to tumor promotion.

Immune activation has been shown to down regulate HER2 expression both in vivo and in vitro [45]. The pathway in which HER2 down regulates immune responses and the pathway in which immune activation down regulates HER2 expression seem to work in opposing fashion. While the APC machinery is suppressed by HER2 over-expression, upon immune activation with the appropriate costimulation, HER2 expression goes down. The problem with this is decreased HER2 expression also allows tumor cells to circumvent HER2 specific immunity especially if the vaccines inducing this response contain only low levels of HER2 (Fig. 7) [46, 47]. This may be why the vaccines used in these experiments did not induce complete protection against lung metastasis.

In the kinetics studies, HSA levels increased the longer 4TO7RGhHER2 cells were grown in vivo (Fig. 4). Upregulation of HSA in tumor cells have been shown to be important to the progression of tumorogenisis, furthermore, its deletion has been shown to completely inhibit the formation of solid tumors [48]. In that same experiment, PD-L1 was shown to increase, subtly, above 4TO7RGhHER2 culture levels. Tumor cells often upregulate PD-L1 which upon contact with its receptor PD-1 present on T-cells inhibits their effector function [49]. In concert with decreased HER2 expression, the upregulation of HAS and PD-L1, could attribute to the incomplete protection against 4TO7RGhHER2 lung metastasis among vaccinated groups. It may be possible to include a PD-L1 and HSA inhibitors along with the vaccines to compare lung colonization or overall survival.

Conclusion

Overall, the inclusion of HER2 in 4TO7ts TMVs did increase IgG response to 4TO7RGhHER2 cells in comparison to 4TO7RG cells, and, in turn, increased protection against lung colonization by 4TO7RGhHER2 cells. However, the addition of B7-1 and IL-12 did not lead to a significant increase in protection compared to 4TO7ts TMVs protein transferred with GPI -HER2 alone. Mice vaccinated subcutaneously with 4TO7ts TMVs + GPI-HER2 had the highest IgG responses and protection against 4TO7RGhHER2 lung colonization, furthermore, none of the s.c. vaccines had a significantly higher metastatic burden compared to the unchallenged group, unlike several of the i.v. vaccines, suggesting that the method of subcutaneous vaccination serves as a better method of protection against 4TO7hHER2 experimental metastasis.

References

1. American Cancer Society. Cancer Facts & Figures 2013. Atlanta: American Cancer Society; 2013.
2. Lianos GD, Vlachos K, Zoras O, Katsios C, Cho WC, Roukos DH. Potential of antibody-drug conjugates and novel therapeutics in breast cancer management. Onco Targets Ther. 2014;7:491-500.
3. Pohlmann P. R., Mayer I. A. & Mernaugh R. Resistance to Trastuzumab in Breast Cancer. Clin. Cancer Res. 2009;15:7479–7491
4. Shashidharamurthy R, Bozeman EN, Patel J, Kaur R, Meganathan J, Selvaraj P. Immunotherapeutic strategies for cancer treatment: a novel protein transfer approach for cancer vaccine development. Med Res Rev. 2012;32(6):1197-219.
5. Ja¨ger E, Chen YT, Drijfhout JW, Karbach J, Ringhoffer M, Ja¨ger D, Arand M, Wada H, Noguchi Y, Stockert E, Old LJ, Knuth A. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: Definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. J Exp Med 1998;187:265–270.
6. Coley, W. B. 1893a Treatment of malignant tumors by repeated inoculations of erysipelas: with a report of ten original cases. Am. J. med. Sci. 1893;105:487–511.
7. Burkholder B, Huang RY, Burgess R, et al. Tumor-induced perturbations of cytokines and immune cell networks. Biochim Biophys Acta. 2014;1845(2):182-201.
8. Soong RS, Song L, Trieu J, et al. Direct T Cell Activation via CD40 Ligand Generates High Avidity CD8+ T Cells Capable of Breaking Immunological Tolerance for the Control of Tumors. PLoS ONE. 2014;9(3):e93162.

9. Andersen MH. The targeting of immunosuppressive mechanisms in hematological malignancies. Leukemia. 2014; doi: 10.1038/leu.2014.108
10. Kim, R., M. Emi, K. Tanabe, K. Arihiro. Tumor-driven evolution of immunosuppressive networks during malignant progression. Cancer Res. 2006;66:5527-5536.
11. DeNardo, D.G., L.M. Coussens. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. Breast Cancer Res. 2007;9:212.
12. Marsh, T., K. Pietras, S.S. McAllister. Fibroblasts as architects of cancer pathogenesis. Biochim. Biophys. Acta. 2013;1832:1070-1078
13. Johnson JG, Jenkins MK. Accessory cell-derived signals required for T cell activation. Immunol Res. 1993;12:48–64.
14. Chen L, Linsley PS, Hellstrom KE. Costimulation of T cells for tumor immunity. Immunol Today. 1993;14:483–486.
15. Lanier LL. Distribution and function of lymphocyte surface antigens. Molecules costimulating T lymphocyte activation and function. Ann NY Acad Sci. 1993;677:86–93.
16. Atkins MB, Lotze MT, Dutcher JP, et al. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. J Clin Oncol. 1999;17(7):2105-2116.
17. Guo C, Manjili MH, Subjeck JR, Sarkar D, Fisher PB, Wang XY. Therapeutic cancer vaccines: past, present, and future. Adv Cancer Res. 2013;119:421-475.
18. Alatrash G, Hutson TE, Molto L, et al. Clinical and immunologic effects of subcutaneously administered interleukin-12 and interferon alfa-2b: phase I trial of patients with metastatic renal cell carcinoma or malignant melanoma. J Clin Oncol. 2004;22(14):2891-900.

19. Siegel JP, Puri RK. Interleukin-2 toxicity. J Clin Oncol. 1991;9(4):694-704.

20. Ansell SM, Witzig TE, Kurtin PJ, Sloan JA, Jelinek DF, Howell KG, Markovic SN, Habermann TM, Klee GG, Atherton PJ, Erlichman C. Phase 1 study of interleukin-12 in combination with rituximab in patients with B-cell non-Hodgkin lymphoma. Blood. 2002;99:67–74.

21. Gokhale MS, Vainstein V, Tom J, et al. Single low-dose rHuIL-12 safely triggers multilineage hematopoietic and immune-mediated effects. Exp Hematol Oncol. 2014;3(1):11.

22. Chen L, Ashe S, Brady WA, Hellstrom I, Hellstrom KE, Ledbetter JA, McGowan P, Linsley PS. Costimulation of antitumor immunity by the B7 counter receptor for the T lymphocyte molecules CD28 and CTLA-4. Cell. 1992;71:1093–1102.

23. Gimmi CD, Freeman GJ, Gribben JG, Gray G, Nadler LM. Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. Proc Natl Acad Sci USA. 1993;90:6586–6590.

24. Cimino AM, Palaniswami P, Kim AC, Selvaraj P. Cancer vaccine development: protein transfer of membrane-anchored cytokines and immunostimulatory molecules. Immunol Res. 2004;29(1-3):231-40.

25. Nagayama H1, Sato K, Kawasaki H, Enomoto M, Morimoto C, Tadokoro K, Juji T, Asano S, Takahashi TA. IL-12 Responsiveness and Expression of IL-12 Receptor in Human Peripheral Blood Monocyte-Derived Dendritic Cells. The Journal of Immunology. 2000;165(1):59.

26. Manetti R, Parronchi P, Giudizi MG, et al. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. J Exp Med. 1993;177(4):1199-204.

27. Lenahan C, Avigan D. Dendritic cell defects in patients with cancer: mechanisms and significance. Breast Cancer Res. 2006;8(1):101.

28. Freeman GJ, Freedman AS, Segil JM, Lee G, Whitman JF, Nadler LM. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. J Immunol. 1989;143(8):2714-22.

29. Greaves P, Gribben JG. The role of B7 family molecules in hematologic malignancy. Blood. 2013;121(5):734-44.

30. Lotze MT, Matory YL, Ettinghausen SE, et al. In vivo administration of purified human interleukin 2. II. Half life, immunologic effects, and expansion of peripheral lymphoid cells in vivo with recombinant IL 2. J Immunol. 1985;135(4):2865-75.

31. Bozeman EN, Cimino-mathews A, Machiah DK, et al. Expression of membrane anchored cytokines and B7-1 alters tumor microenvironment and induces protective antitumor immunity in a murine breast cancer model. Vaccine. 2013;31(20):2449-56.

32. Leonard JP, Sherman ML, Fisher GL, et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. Blood. 1997;90(7):2541-8.

33. McHugh RS, Ahmed SN, Wang Y-C, Sell KW, Selvaraj P. Construction, purification and functional reconstitution on tumor cells of glycolipid-anchored human B7-1 (CD80). Proc Natl Acad Sci USA. 1995;92:8059–8063.

34. Nagarajan S, Anderson M, Ahmed SN, Sell KW, Selvaraj P. Purification and optimization of functional reconstitution on the surface of leukemic cell lines of GPI-anchored Fcγreceptor III. J Immunol Methods. 1995;184:241–251.

35. McHugh RS, Ahmed SN, Wang Y-C, Sell KW, Selvaraj P. Construction, purification, and functional reconstitution on tumor cells of a glycolipid anchored B7-1 molecule: A novel alternative to gene transfection in human immunotherapy. Proc Am Assoc Cancer Res.1995;36:494–

36. McHugh RS, Nagarajan S, Wang YC, Sell KW, Selvaraj P. Protein transfer of glycosylphosphatidylinositol-B7-1 into tumor cell membranes: A novel approach to tumor immunotherapy. Cancer Res. 1999;59:2433–2437.

37. Selvaraj P, Dustin ML, Silber R, Low MG, Springer TA. Deficiency of lymphocyte function associated antigen-3 (LFA-3) in paroxysmal nocturnal hemoglobinuria: Functional correlates and evidence for a phosphatidylinositol membrane anchor. J Exp Med. 1987;166:1011–1025.

38. Medof ME, Kinoshita T, Nussenzweig V. Inhibition of complement activation on the surface of cells after incorporation of decay-accelerating factor (DAF) into their membranes. J Exp Med. 1984;160:1558–1563.

39. Nagarajan S, Selvaraj P. Reconstitution of CD16 expression on nucleated cells using purified CD16. FASEB J. 1991;5:A1718–

40. Brunschwig EB, Levine E, Trefzer U, Tykocinski ML. Glycosylphosphatidylinositol-modified murine B7-1 and B7-2 retain costimulator function. J Immunol. 1995;155:5498–5505.

41. Heppner GH, Miller FR, Shekhar PM. Nontransgenic models of breast cancer. Breast Cancer Res. 2000;2(5):331-4.

42. Chen L, Mcgowan P, Ashe S, et al. Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. J Exp Med. 1994;179(2):523-32.

43. Occhipinti S, Sponton L, Rolla S, et al. Chimeric Rat/Human HER2 Efficiently Circumvents HER2 Tolerance In Cancer Patients. Clin Cancer Res. 2014;

44. Seliger B, Kiessling R. The two sides of HER2/neu: immune escape versus surveillance. Trends Mol Med. 2013;19(11):677-84.

45. Shi Y, Fan X, Meng W, Deng H, Zhang N, An Z. Engagement of immune effector cells by trastuzumab induces HER2/ERBB2 downregulation in cancer cells through STAT1 activation. Breast Cancer Res. 2014;16(2):R33.

46. Sas S, Chan T, Sami A, El-gayed A, Xiang J. Vaccination of fiber-modified adenovirus-transfected dendritic cells to express HER-2/neu stimulates efficient HER-2/neu-specific humoral and CTL responses and reduces breast carcinogenesis in transgenic mice. Cancer Gene Ther. 2008;15(10):655-66.

47. Ladjemi MZ, Jacot W, Chardès T, Pèlegrin A, Navarro-teulon I. Anti-HER2 vaccines: new prospects for breast cancer therapy. Cancer Immunol Immunother. 2010;59(9):1295-312.

48. Naumov I, Zilberberg A, Shapira1 S, et al. CD24 knockout prevents colorectal cancer in chemically induced colon carcinogenesis and in APC(Min) /CD24 double knockout transgenic mice. Int J Cancer. 2014;

49. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L. Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. Nat Med. 2002;8:793–800.