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Nikhil Amaram April 17, 2013

Adjuvating Melanoma TMVs By Incorporating GPI-anchored Immunostimulatory Molecules To Induce B16 Melanoma Tumor Suppression

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Abstract

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By Nikhil Amaram

The body's immune system makes up the first line of defense against foreign pathogens and abnormal host cells as well. However, this system is not perfect and at times either of these two antagonists can evade elimination. In the case of a foreign pathogen, it may be called a disease or sickness, but for abnormal cells it is called a cancer. Many studies have tried utilizing the immune system to prime it against these cancers and help the body eliminate a tumor on its own. Our research lab has designed a new way of approaching tumor vaccines. If successful, a primary tumor could be removed from a patient and tumor membrane vesicles (TMVs) could be made from them. In addition, the lab has also created ISMs linked to a glycosylphophatidylinositol (GPI) anchor which allows these ISMs to incorporate even soluble cytokines into the membranes. This study aimed to test this combined vaccination model using both GPI-anchored ISMs and TMVs created from B16F10 cells, a popular model for melanoma. Successful incorporation of GPI-anchored IL-12 and GM-CSF was shown onto TMVs and were able to induce higher tumor protection than unmodified TMV in a prophylactic experiment. One mechanism for tumor-based immunity was found in TMVs incorporated with GPI-anchored GM-CSF through the use of antibody production in response to the vaccine. However, mice vaccinated with GPI-anchored IL-12 and GPI-anchored IL-12 and GM-CSF showed the greatest tumor prevention and are possible targets for a therapeutic vaccine using the same vaccination design.

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INTRODUCTION

Inherently the immune system of the body mediates the targeting and elimination of tumors within the body. At times abnormal cells may escape clearance and propagate to develop into a cancer. Immunotherapeutic strategies in oncology are designed to prime and supplement the immune system of the patient in an effort to clear the cancer through the protective measures induced by the immune system. Positive results have been found for some of these strategies which include DNA-vaccines (26), heat shock proteins (27), hybrid tumor cells (29), peptide vaccines, systemic administration of cytokines (10), dendritic cells (DCs) modified to present tumor antigens (30), and whole tumor cells trasfected to produce cytokines or costimulatory molecules (15). Our laboratory has proposed a tumor vaccine model using glycosyl-phosphatidylinositol (GPI)-anchored immunostimulatory molecules (ISMs) which can be transferred onto membranes isolated from the tumor (19, 31).

In this study we have evaluated the effects of expressing GPI-anchored interleukin 12 (IL-12) and granulocyte macrophage colony-stimulating factor (GM-CSF) either alone or in combination on the surface of tumor membrane vesicles (TMVs) made from murine melanoma tumor cells (B16F10) as a model for human melanoma vaccines. Melanoma is the source of most skin cancer deaths in the United States resulting in over 8000 deaths each year with 45,000 new cases of the cancer occurring each year from 2004-2006 (22). Both IL-12 and GM-CSF are cytokines known to be involved in immune cell signaling and activation but through different mechanisms(23). Since both of these cytokines have had successes in other

studies(23-26), we studied the effects of these in a membrane vaccine setting both individually and together to determine synergistic effects that may complement the antitumor effects of the TMV.

Ultimately, our goal is to create the most successful vaccine using membranes modified through protein transfer with these cytokines to target both innate and adaptive branches of the immune system and create an antitumor response. Successful results would show that the modified TMV vaccine may be used therapeutically in humans to induce tumor regression and protect against metastasis. The introduction that follows outlines an overview of the immune system in a cancer setting as well as recently studied tumor vaccine studies in comparison to the proposed method in this study.

Elements of the Immune System

The immune system provides defensive measures necessary to deal with foreign pathogens and abnormal body cells which may bring harm to the organism (32). Most cells of the immune system can be separated into two broad categories depending on the type of response. These would include the innate immune system and the adaptive immune system. The cells that comprise the innate immune system are nonspecific and attack based on pathogenic motifs. These cells do not change over the lifetime of the organism and are the first line of defense against harmful agents. Included in the innate system are barriers such as the skin and mucus as well as cellular barriers that block pathogen invasion.

Cells that respond to abnormal cells that may result in cancer include macrophages, natural killer (NK) cells, dendritic cells, and neutrophils. These cells

are not specific to a single antigen but recognize a broad group of antigens or cell signals. For example, macrophages recognize pathogen features such as lipopolysaccharides and double stranded RNA through the use of toll like receptors(29). Recognition through these receptors induces phagocytosis of the pathogen by the macrophage. NK cells recognize the presence of major histocompatibility complexes (MHC) molecules on cells. Normally present on cells, down regulation of MHC molecules from the cell membrane is one of the methods virus infected pathogens use to evade clearance (33). MHC, which normally inhibits NK cell activation, once gone causes the NK cell to release cytotoxic granules containing perforin and granzymes that kill the cell . Dendritic cells play an important role in activating the adaptive system towards pathogens and are the most prevalent form of antigen presenting cells (APCs). These cells can phagocytize pathogenic cells and present elements of these cells to T cells to activate them.

The adaptive immune system forms the second line of defense if the innate immune system is unable to clear the foreign pathogen or abnormal cell population on its own. Also known as the specific immune response, cells and proteins in this branch are highly specific for their targets unlike the innate immune system. Cells that are under this category include lymphocytes, which are T cells and B cells.

Stimulation of immature T cells through methods such as APC presentation causes these cells to differentiate into either cytotoxic T cells (CD8+ or CTLs) or helper T cells (CD4+). CD4+ T cells fall are divided into two main categories depending on targets of activation: T_H1 and T_H2. Cells under the T_H1 subset promote an adaptive response through the secretion of certain specific cytokines. This

includes the production of IL-2 which increases active CTL and NK cell levels in the body (32). Cytokines used by CD4+ cells to induce IL-2, IL-12, INF-γ, and TNF-α(28). These in turn target CTLs, which have direct lytic activity towards the cells they recognize. Like NK cells, CTLs release granules similar to NK cells which lyse the target. However CD4+ cells in the T_H2 subset are tailored towards activation of the humoral response through the use of a different cytokine repertoire. Cytokines used in the T_H2 subset are IL-2, IL-6, and IL-10. Studies have shown that the response produced by the T_H2 subset has lower antitumor response than that of T_H1 (34). B cells on the other hand induce humoral immunity through the use of antibodies. These cells can recognize antigen in the blood and if enough B cell receptors are activated, can produce antibodies against the target and through this promote phagocytosis of the target through macrophages. B cells can also be activated through an immunological synapse with CD4+ T cells in the T_H2 subset that are initially activated through APCs. Activated plasma B cells can also produce memory B cells through proliferation, which can be activated on subsequent exposures to the pathogen.

Immunosurveillance on Cancerous Phenotypes

The presence of tumor infiltrating lymphocytes into the patient's tumor is considered a positive sign than the lack of these cells. This was shown in a study when tumor infiltrating lymphocytes from patients with melanoma recognized and killed melanoma cells with the same MHC molecules (35). Supported by studies that show that immunocompromised patients have been found to have a higher risk of developing tumors than do healthy, immunocompetent individuals (36). Findings

from these studies were the first to suggest that there were antigens specific to a tumor. Tumor associated antigens are proteins expressed in tumors either as proteins present only in tumors, mutated proteins, and those expressed at abnormally high levels. As a result of their abnormal function or behavior, tumor antigens clearly mark cancer cells as different from the host's own cells. (37). Together these show that the immune system actively antagonizes cancerous cells.

Though the immune system has many tools through which it can eliminate a multitude of foreign pathogens and abnormal cells, some have been able to evade capture through certain mechanisms. Such is the case in cancer where an abnormal population of cells develops strategies by which it can evade immune responses. Studies have shown that tumors that develop in immunocompetent mice are less susceptible to immunological clearance than those in immunocompromised mice (38). This suggests that tumor cell populations that developed in competent mice have undergone measures to avoid detection and clearance by the immune system. Tumor cells that survive each round of immune system clearance proliferate and survive and produce populations better able to evade the immune system through multiple rounds. Thus multiple mutations in transformed cells avoid the pressures and become candidates for tumor formation.

Methods of Evasion By Tumor Cells

Tumor cells use a variety of methods to evade immune system regulation. One way in which this occurs is by down regulating antigen presentation by decreasing expression of membranous MHC molecules, adhesion molecules (such as the ICAM family), and costimulatory molecules (such as the B7 family)(38). Some

tumor cells increase production of inhibitory cytokines and other molecules. This can include increased levels of PD-L1 and CTLA-4 which counteract the effects of costimulatory molecules and decrease lymphocyte activation (39,40). Tumor cells may also hide in immunologically privileged areas in which it is too risky to perform routine surveillance. These sites include the eyes and testicles where tumor cells can reside to avoid directed killing by the immune system (41,42). T cells can also become the target of tumor cells directly. In some cancer patients, T cell precursors and memory cells are lower than normal or nonexistent and those that are still present exhibit signaling defects which prevent them from exerting their antitumor effects. Thus tumor directed T cells are unable to be stimulated by MHC interactions and become anergic, halting the advance of the best chance at tumor eradication by the body (43).

Several changes occur in the immune system of patients with cancer that can mediate lower antitumor protection. In a 2008 study, findings showed that the T_H2 response in melanoma patients was significantly higher than in healthy patients. Most notably was that there was an increase in IL-4, IL-10, and IL-13 that resulted in chronic systemic inflammation (1). At the same time melanoma patients have lower levels of T_H1 based cytokines which, if present, could cause the tumor to go into remission (2). By shifting the response away from a cytotoxic response, the tumor is able to avoid full clearance and is able to continue its growth. However, if the introduction of tumor infiltrating lymphocytes with IL-2 is done, even metastatic melanomas regressed supporting this description (3). Overall the main goal in tumor vaccine development is the creation of a vaccine that successfully combats

the tumors evasive properties. In this way, these vaccines target APCs and T cells to become more effective in presenting tumor antigens as well as recognizing and clearing the tumor. Many different strategies To achieve this effect have been tested to varying degrees of success. These strategies include directly modified APCs, modified whole tumor cells to express cytokines or costimulatory molecules, costimulation blockades as well as others. Several of these strategies based around the use of immunostimulatory molecules in tumor vaccine development are outlined below.

Systemic Cytokine Administration and Costimulation Blockades

Immunostimulatory molecules are one of the most important mediators of the anti-tumor response. Through the use of cytokines, one could stimulate specific targets of the immune system in an effort to combat the cancer itself. Thus adjuvanting vaccines with cytokines has become a popular candidate for an effective cancer vaccine. Studies with mice have shown that administration of cytokines such as GM-CSF (8) , IL-2 (10), IL-6 (11), or IL-12 (9). Uses of these cytokines have shown tumor protection through activation of the immune system. For example, IL-12 administration results in an increase of IFN-γ which increases levels of CD4+ and CD8+ cells.

However, despite promising results from studies with cytokine administration, some problems have arisen. In the case of IL-12, systemic toxicity can be a risk dependent on the amounts and frequency of injection (44). Data from administration with IL-2 show a larger risk in toxicity as some doses needed to be skipped for safety measures (10). Studies on GM-CSF however have shown little to

no toxic results when used as an adjuvant with other vaccines (8). Still, administering cytokines, including those such as IL-2 and IL-12, are popular methods of adjuvanting other forms of vaccines in many cancers such as melanoma, prostate, and ovarian cancer. The use of these adjuvants against toxic effects is still being examined.

Yet another administration treatment is the use of costimulation blockades. Often the mechanisms by which a tumor may be cleared are blocked through inhibitory molecules such as CTLA-4 (4-6) and PD-L1 (6,7). Activation of these molecules accounts for one way that cancers evade the immune system. Therefore, one possible target would be to block the inhibition of this signal and promote T cell activation. Administration of antibodies against the inhibitory molecule (such as anti-CTLA-4), has been shown to increase tumor protection and can be combined with other strategies for better anti-tumor activation (4-6).

Vaccines Using Antigen Presenting Cells

Instead of trying to induce activity of T cells indirectly through cytokines, one method being tested is the modification of APCs themselves. Early methods utilized peptide pulsed dendritic cells in which antigen specific immunity was established against tumors, in particular was the activation of CTLs (12). DCs have also been pulsed with the tumor lysate itself to similar degrees of effectiveness (13). Other targets of APC vaccines include loading of tumor cells, DNA and RNA encoded antigens (45), apoptotic cells, heat shock proteins, or fusion with cells. This vaccine model is attractive due to the direct priming of many APCs since, commonly, presentation of tumor specific antigens would be little. Additionally, DCs are able to

prime both CD4+ and CD8+ T cells without the need to assume that antigens are being adequately presented as in other models. Vaccination with pre-primed DCs have shown increased T cell activation and tumor regression and are currently in clinical trials for human use (46). Further research is necessary to correctly develop and distribute this type of vaccine.

Immunostimulatory Molecule Gene Transfected Vaccines

While recognition of peptides through major histocompatability complexes plays a major role in lymphocyte activation, other costimulatory signals are necessary for these cells to act. These often consist not only of membrane proteins such as the B7 family (47) but also cytokines such as IL-2 and IL-12 (48). Even if tumor cells express MHC on their surface, the lack of a costimulatory stimulus through one of these sources prevents activation of the immune response. In addition, if the tumor specific lymphocyte recognizes the tumor antigen multiple times without costimulation, the immune cell itself may undergo clonal anergy and become ineffective in inducing an immune response (49). One method to combat this form of evasion is to create a tumor cell that expresses this secondary signal through gene transfection. In this method, tumor cells would then express membrane bound costimulatory molecules (50) or begin secretion of activating cytokines upon transfection (9,24).

Some of these vaccines are currently in clinical trials and have shown promise in a human model system. One example of a transfected gene vaccine is the GM-CSF secreting melanoma model (14,15). In a cutaneous melanoma setting, patients showed partial regression and a strong humoral response (14). Noted

heavy infiltration of CD4+ and CD8+ T cells into the site of the tumor even in patients in the advanced, terminally ill stages of the cancer was seen(18). Other targets of gene transfer include IL-2 and INF-γ which have both shown to protect against tumor growth and promote antitumor activity (16,17). Though results in clinical trials show decreased levels of regression than in murine models, continuing research on this vaccination method may produce a successful vaccine.

One problem associated with this form of vaccination is that they often require the use of retroviruses to introduce the gene . Unfortunately, this can prevent multiple immunizations using the same vector with the risk of replacing the gene with another one (51). Using viral vectors also means that the vector must integrate into the genome without disrupting or activating genes essential for survival. Use of this method requires tumor cell lines to be established which may be doable for some patient tumors. Even if possible, cell lines would need to be processed and selected for the best expression of the immunostimulatory gene. This is therefore a lengthy process requiring constant testing of tumor lines before an actual vaccine is produced. Even if shown to work in a clinical setting, production of the vaccine may not be time or cost effective for a large population.

Protein Transfer of Immunostimulatory Molecules

Research from our laboratory has shown that it is possible to associate an immunostimulatory molecule to a tumor cell without the use of gene transfection (19, 20, 31). By attaching immunostimulatory molecules to a glycosylphosphatidylinositol (GPI) anchor, we have shown that these molecules will spontaneously incorporate into hydrophobic structures such as a lipid membrane

within a matter of hours. The gene itself creating this modified protein consists of the ISM gene ligated to the GPI coding segment such as on the CD59 gene, a naturally occurring GPI-anchored complement regulating protein. Despite being attached to the GPI-anchor, the modified protein does not lose its functional capabilities (20). Incorporation of these proteins into membranes simply takes 2-4 hours at 37˚C. This allows association of ISMs onto tumor cells in which cell lines cannot be maintained and avoid the need for subsequent pannings to induce increased expression, thus saving time. Use of GPI-anchored proteins also allows vaccine developers to control the amount of ISM expression on the target surface. Control of expression is often not available using other methods especially in the case of soluble cytokines where the vaccine can continually produce the ISM. Furthermore, multiple GPI-anchored ISMs can be incorporated simultaneously onto lipid bilayers (20).

As noted above, cytokines have been shown to induce strong immune responses toward specific targets in the body such as tumor cells. With the use of a GPI-anchor, we can also attach soluble molecules such as cytokines to the surface of membrane vesicles. The use of tumor membrane vesicles (TMVs) would constitute an ideal method of vaccination using GPI-anchored ISMs. These vesicles are prepared from homogenized tumor cells and consist of their lipid bilayers and the proteins it contains reflect the proteins present when it was part of the whole cell (66). TMVs do not maintain cellular processes as whole cells and therefore expression of incorporated proteins is maintained. Our laboratory has shown advances using this method with GPI-anchored IL-12. Using murine mastocytoma

cells, GPI-anchored IL-12 was transferred onto cell membrane vesicles and vaccinated. Vaccinated mice were tumor free up to 55 days and the anchored cytokine induced INF-γ production, T cell proliferation, and as a result protection against tumor growth (19).

Studies in our laboratory have also shown successful usage of GM-CSF modified to express the GPI anchor and attach onto membranes (20). Other studies have shown that GM-CSF stimulates dendritic cell proliferation, which is important in inducing lymphocyte activation and can induce antitumor activity (15). GPI-anchored GM-CSF also retains the ability to promote bone marrow cell proliferation which creates an important immune cell subpopulation such as DCs (21). Anchored GM-CSF also sheds itself from the membrane at about 10-15% after 72 hours at 37˚C but did not shed at 4˚C (20). This shedding may occur through the activation of proteolytic cleavage and may create a local cytokine release system in addition to the membrane associated version. Release may correlate with the attraction of APCs to the site of vaccination and facilitate antigen uptake and presentation.

EXPERIMENTAL DESIGN

The study was split into three segments. The first segment involved the purification of GPI-anchored ISMs and the creation of TMVs from B16F10 cells. At this point, two preliminary mice were performed to obtain parameters for the final experiment. Once GPI-anchored ISMs and B16F10 TMV were obtain, protein transfer was done of the ISMs onto the TMVs. Mice were then vaccinated with TMVs modified with GPI-anchored ISMs and challenged with live B16F10 cells . These segments are shown below (Fig. 1).

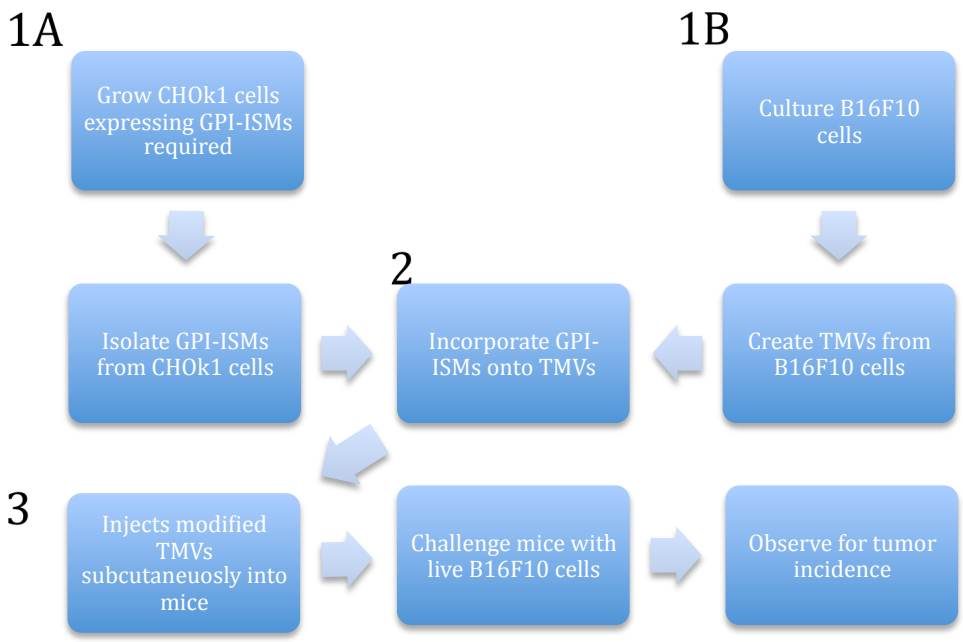


Figure 1. Experimental design. In phase 1A, CHO cells expressing GPI-anchored ISM were grown and the ISMs isolated from their membranes (upper left). In 1B, B16f10 cells were grown and TMVs prepared from them (upper right). In 2, ISMs were incorporated into TMVs (middle). In 3, these modified TMVs were injected into mice and challenged (bottom row).

Purification of GPI-anchored ISMs from transfected CHO cells. CHO-K1-mGM-CSF-hHER2-CD59 and CHO-K1-mIL-12-CD59 cells expressing GPI-anchored murine forms of GM-CSF and IL-12 respectively were cultured. These were grown in

roller bottles and after confluent were harvested. Cells were lysed and the lysate was analyzed using a dot blot analysis. GPI-anchored protein was extracted into fractions using affinity chromatography and fractions analyzed through Western blot. Fractions containing the eluted protein were concentrated using polyvinylpyrrolidone, dialyzed, and stored till further use.

Homogenization of B16F10 TMVs. B16F10 cells were cultured and after confluent, were harvested. Expression of certain surface proteins was analyzed using FACS analysis. B16F10 cells were also homogenized to create TMVs. TMVs were tested using FACS analysis for certain proteins.

B16F10 live cell murine challenge. Mice were challenged with two different amounts of B16F10 cells and were observed for tumor growth (Fig. 2A). This was to determine a cell load injection needed for tumor development in mice.

Unmodified B16F10 TMV vaccination. Groups of mice were vaccinated with different TMV amounts and given a booster injection a week later. Mice were then challenged with B16F10 live cells and observed for tumor growth. Experiment was used to determine amount of TMV to be used in the modified TMV experiment.

GPI-anchored ISM incorporation onto TMVs. TMVs previously prepared were incubated with purified GPI-anchored ISMs. Modified TMVs were analyzed using FACS analysis for successful incorporation (Fig 2B).

Modified B16F10 TMV vaccination. This test studied effectiveness of the assembled vaccine by examining tumor development in mice after vaccination and challenge. Modified TMVs were injected into mice follow by two booster injections of similarly modified TMVs every seven days. Seven days after the final booster

injection, mice were challenged with live B16F10 cells and observed for tumor growth (Fig 2C). Mice were also bled before each vaccination and antibody response examined through FACS analysis.

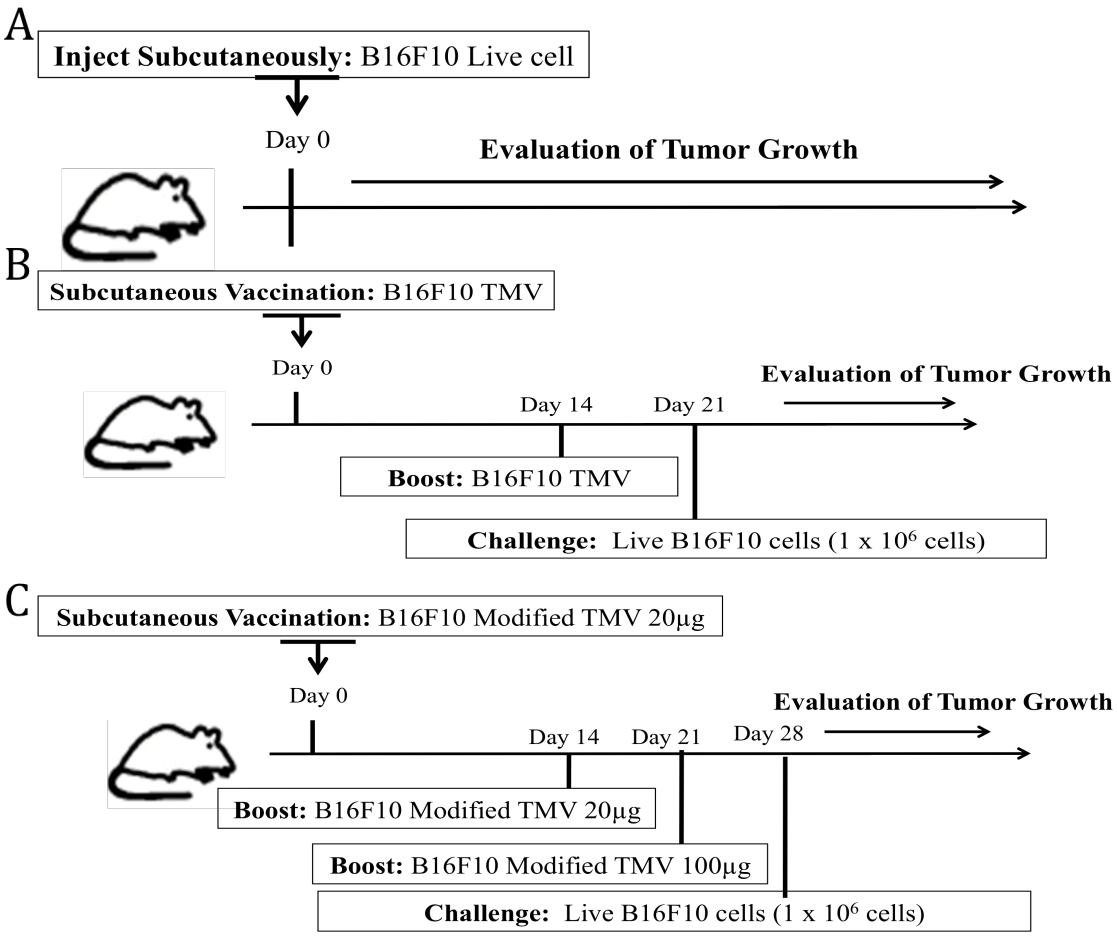


Figure 2. Overview of mice experiments. Two preliminary experiments with mice (A and B) were done for the final mice experiment. Experiment A tested the presence of tumors in mice injected with one of two different cell amounts. Experiment B tested the tumor protection of unmodified TMVs using different injection amounts. Experiment C tested tumor protection when mice were vaccinated with GPI-anchored ISM modified TMV.

We hypothesize that when challenged, mice vaccinated with TMVs modified with GPI-anchored IL-12 and GM-CSF will experience greater tumor protection

than those vaccinated with only one of the GPI-anchored TMVs, unmodified TMVs, and unvaccinated mice.

MATERIALS AND METHODS

Cell culture. B16F10 cells were grown in 1640 RPMI with 10% CCS, 0.02% amphotercerin. CHO-K1-mGM-CSF-CD59 and CHO-K1-mIL-12-CD59 cells were grown in RPMI 1640 with 10% CCS, 1% Penicillin/Streptomycin, 0.02% amphotericin, and 10ug/mL blasticidin. Cells were kept at 37˚C and 5% CO_2 until 90% confluent. These CHO-K1 cells were also grown in 1L grooved roller bottles using 500mL of RPMI 1640, 10% CCS, 1% Penicillin/Streptomycin, 0.02% amphotericin, and 1ug/mL blasticidin and grown under the same conditions. Cells were detached using PBS with 5% EDTA. CHO-K1 cell pellets were frozen at -80˚C after collected.

Fluorescence-activated cell sorting (FACS) analysis. FACS buffer was prepared before staining containing 1% CCS and 1% EDTA in PBS. In a v-bottom 96 well plate, 350µL of FACS buffer to each well to be used and was incubated for 10 minutes at room temperature. Cells to be stained were harvested from flasks or in the case of TMV staining, 20µg of TMV per well was collected. In the case of cells, cells were counted and viability checked to be at least above 90%. Cells and TMVs were centrifuged and resuspended in FACS buffer to give $5x10^6$ cells/mL or 20µg of TMV per 50µL FACS buffer. FACS buffer was removed from wells on plate using an aspirator. To each coated well, 50µL of the cell or TMV suspension was added as well as 50µL of a primary antibody. Primary antibodies consist of A2F27-107 for mGM-CSF, C17.8 for mIL-12, m1/142 for mMHC, Y1/1.7.4, m1/169 for HSA, and 1G10 for mB7-1. In the case of a negative control or directly conjugated antibody, 50µL FACS buffer was added instead. Plate was sealed with a cover and shaken at

4˚C for 30 minutes. Plate was then centrifuged for 3 minutes at 1500 rpm and the supernatant was removed using an aspirator. Cells and TMVs in each well were resuspended in 200µL FACS buffer and the plate was centrifuged again for 3 minutes at 1500 rpm. This washing step was repeated one more time and the supernatant was removed using an aspirator. FITC conjugated secondary antibody was diluted 1:50 and 50µL of the dilution was added to each well depending on the type of secondary. Goat anti-mouse antibody used for MHC-1 sample and for others goat anti-rat antibody was used as a secondary antibody. In the case of directly conjugated antibodies, these and their isotype controls were diluted 1:100 and 50µL of the dilution were added to wells. CD47 was FITC conjugated and PD-L1 was PE conjugated. Plate was then covered and shaken for 30 min at 4˚C. Plate was then centrifuged for 3 minutes at 1500 rpm and the supernatant was removed using an aspirator. Cells and TMVs in each well were resuspended in 200µL FACS buffer and the plate was centrifuged again for 3 minutes at 1500 rpm. This washing step was repeated one more time and the supernatant was removed using an aspirator. Microtubes were counted and labeled with one per well. To each tube 150µL of 2% Formalin in PBS was added. Each well was then resuspended in 150µL FACS buffer and the suspension was added to the labeled microtubes and mixed thoroughly. These were covered to keep light out and kept at 4˚C till flow cytometry was used.

Affinity chromatography of GPI-ISMs: Lysing solution containing 50mM Tris-HCl pH 8.0, 2% octyl glucoside, 1mM $ZnCl_2$, 1:100 dilution of protease inhibitor (Sigma P8340-5ML), 5mM EDTA, 20mM Iodoacetic acid, 2mM PMSF, in dH_2O was made with 5mL of the solution per gram of transfected CHO-K1 cells expressing a

GPI-ISM. Frozen CHO-K1 pellets were removed from -80˚C and resuspended in the lysing solution. The were stirred at 4˚C overnight with a stir bar. The following day old Sepharose beads from large pre-column were removed and the column itself was washed with dH_20. Pre-column was rinsed with PBS and 20mL of Sepharose beads were added. Sepharose beads were coupled to A2F17-107 antibodies for mGM-CSF column or coupled to m142 antibody for a mIL-12 column. Coupled beads were placed in separate small columns and one used depended on protein being purified. This was then washed three times using PBS. Pre-column was then attached to a small column containing antibodies against the desired GPI-ISM using a plastic tube. Cell lysate was removed from 4˚C was then poured into the pre-column and the rate of flow from the small column was slowed to 1 drop per 6 seconds. The lysate was collected at the bottom and once finished, the entire set up was washed once by passing through wash buffer 1. Elution buffer at a pH depending on the GPI-ISM being eluted was then made. To elution tubes, HCL or NaOH was added in an amount such that 1.5mL of the elution buffer would neutralize it. The small column was washed three times using wash buffer 3 and the resulting flow through was once again removed. Tube on small column was tightened to restrict flow and 1.5mL of the elution buffer was added to the top of the small column. Flow was restored at 1 drop every10 seconds into one of the elution tubes with a neutralizing reagent. After eluting a fraction, the tube was inverted at least 2 times, kept at 4˚C, and another 1.5mL of elution buffer was added. This continued for at least five fractions. After the last fraction, Tris-HCl with pH

dependant on the elution buffer was added to neutralize the column detected using pH strips. Flow was restricted and extra Tris-HCl added once pH was close to 7.0.

Dot blot analysis. A 5x2 cm section of nitrocellulose was cut with scissors. Nitrocellulose was labeled and a drop of the sample was placed onto it. In addition, a drop of water was used for a negative control and a drop known to contain the tested protein was used as a positive control. Nirtocellulose was left to completely dry. Cellulose membrane was then removed from the apparatus and blocked using TTBS with 5% milk on a horizontal shaker for one hour at room temperature. Nitrocellulose was then quickly rinsed with TTBS. Primary antibody was for sample was removed from 4˚C and 10 mL of this was shaken with the membrane for one hour at room temperature. Primary antibody was removed and saved. Membrane was rinsed with TTBS and shaken with TTBS for 5 minutes. Milk, 10mL of which, and 0.5µL of HRP-goat-anti-rate secondary antibody were shaken together with the membrane. Membrane was rinsed with TTBS and shaken with TTBS three times for five minutes each. Using the GE Western blot developer kit, the developing reagent was mixed for at least 2mL and the membrane, developing solution, developing case, developing film, and two clear, projector sheets were taken to a darkened room. Membrane was covered in the developing solution and was allowed to sit for 30 seconds. Developing solution was removed. The membrance was then placed in between two projector sheets and excess liquid was squeezed out. Lights were turned off. Membrane and projector sheets were then placed inside the developing case and held together for at least 30 seconds with a sheet of developing film. Film was then removed from case and processed for results.

Western blot analysis. Hot water was boiled in a 1L beaker. Into Eppendorf tubes, 10µL of sample and 10µL of 2x Laemmli buffer was placed and sealed. Water was cooled to around 90˚C using ice. These were placed in nearly boiling water and allowed to sit for 5 minutes. Samples were centrifuged for 1 minute at 14,000 rpm at room temperature. Meanwhile tape was removed from a 10% gel non-reducing and placed in a Western blot case. The case was filled with 900mL SDS- running buffer. Samples were removed from centrifuge and 10µL of each sample were loaded into separate wells. The case was then closed and cords plugged into the back. The gel was run for 50 minutes at 150 volts. Nitrocellulose was cut to the size of the gel as well as 4 similarly sized filter papers. These were placed into quick transfer buffer solution and shaken for 20 minutes at room temperature. Gel was removed and placed into quick transfer buffer and shaken for 5 minutes. It was then placed into dH_2O and shaken for 5 minutes. On quick transfer machine, two of the filters were placed down first, then the cellulose membrane, followed by the gel, and lastly the other two papers. Excess liquid was squeezed out and transfer buffer was poured on top of the filters. The machine was close and run at 25 volts for 20 minutes. Cellulose membrane was then removed from the apparatus and blocked using TTBS with 5% milk on a horizontal shaker for one hour at room temperature. Nitrocellulose was then quickly rinsed with TTBS. Primary antibody was for sample was removed from 4˚C and 10 mL of this was shaken with the membrane for one hour at room temperature. Primary antibody was removed and saved. Membrane was rinsed with TTBS and shaken with TTBS for 5 minutes. Milk, 10mL of which, and 0.5µL of HRP-goat-anti-rate secondary antibody were shaken together with the

membrane. Membrane was rinsed with TTBS and shaken with TTBS three times for five minutes each. Using the GE Western blot developer kit, the developing reagent was mixed for at least 2mL and the membrane, developing solution, developing case, developing film, and two clear, projector sheets were taken to a darkened room. Membrane was covered in the developing solution and was allowed to sit for 30 seconds. Developing solution was removed. The membrance was then placed in between two projector sheets and excess liquid was squeezed out. Lights were turned off. Membrane and projector sheets were then placed inside the developing case and held together for at least 30 seconds with a sheet of developing film. Film was then removed from case and processed for results.

Polyvinylpyrrolidone mediated concentration. A 12,000 kDa dialysis bag was tied at one end and fractions obtained from affinity chromatography were placed inside. The other end was tied and the bag was placed into an open top plastic container. The bag was covered with a layer of dry polyvinylpyrrolidone (PVP) and let sit at 4˚C until the PVP became a gel like consistency. The layer of PVP was then pushed off the dialysis bag to the sides of the container and replaced with another layer of PVP on top of the bag. Replacements continued till the desired volume was achieved. When this occurred, the final layer of PVP was removed from the bag. The bag was then taped on one end to the inner rim of a 1L beaker. The beaker was filled with 500mL of PBS with 0.005% octyl glucoside so that the bag is mostly covered and a stir bar placed inside the beaker. The PBS solution plus the bag was then stirred for 6-8 hours. After every 6-8 hours, the solution in the beaker was replaced with 500mL of fresh PBS with 0.005% octyl glucoside. This continued

for a total of five times. Once completed, the bag was removed from solution, one of the tied ends was cut, and the solution inside the bag was placed in a low binding Eppendorff tube. The tube was then stored at 4˚C for future use.

B16F10 TMV preparation. Solubilization buffer was prepared with 20mM Tris pH8.0, 10mM NaCl, 0.1mM $MgCl_2$, .02% NaN_3, and 0.1mM PMSF. B16F10 cells were harvested from flasks and dissociated using PBS with 5% EDTA. Cells were then lysed in a hypotonic solubilization buffer and a Polytron homogenizer was rinsed with 70% ethanol. The resulting lysate was then homogenized using a Polytron homogenizer using 4 8-second pulses on power level 5. Between pulses, the lysate was cooled for 1 min on ice. Homogenate volume was then brought up to 8mL with solubilization buffer. Homogentate was centrifuged at 1200rpm for 5 minutes and supernatant was transferred to a Beckman tube. Using a Pasteur pipette, 4mL of 41% (w/v) sucrose in solubilization buffer was poured through the pipette to the bottom of the tube. This was centrifuged at 23,000 rpm for 1 hour at 4˚C. The cloudly interface in the tube was transferred to a new Beckman tube and diluted using twice the amount of solubilization buffer as the interface collected. This was centrifuged at 14,000 rpm for 1 hour at 4˚C. Supernatant was removed and the pellet was resuspended in PBS with 10mM HEPES buffer and stored at -20˚C.

BCA assay for protein concentration. Sample that was being tested was diluted 1:10 and 1:100 for at least 150 µL per three wells in a flat bottom 96-well plate. In addition, standards using known concentrations of bovine serum albumin were used 150µL per well for three wells. Working reagent as mixed using reagents in the micro BCA Protein Assay Kit made by Thermo Scientific. To each sample

150µL of the working reagent was added and the plate was incubated at 37˚C for two hours. Every 30 minutes the plate was checked to avoid oversaturation. After this time had passed, absorbance of the plate was read in a plate reader at 562nm.

B16F10 live cell mouse challenge. C57BL6 female mice were chosen for the experiment and all other following mice experiments. This was chosen since the mouse line was the original host from which the B16F10 cell line was harvested. Two sets of five mice were shaved on the lower half of the body and ears punched for identification. One day later, they were wiped on the right hind flanks using alcohol swabs. Each mouse in the first set was then injected with $2x10^5$ cells in 100µL PBS. Each mouse in the second set was then injected with $1x10^6$ cells in 100µL PBS. Both groups were then observed for tumor growth in the following weeks. After tumor on a mouse reached $200mm^2$ in size, the mouse was sacrificed. The epidermis was then peeled off and the tumor scraped off into an Eppendorf tube. These tubes were then stored at -80˚C.

B16F10 TMV Immunization experiment to determine concentration of TMV to inject. Four sets of five mice were shaved on the lower half of the body and ears punched for identification. The following day B16F10 TMV dilutions were made to 1000µg/mL, 500µg/mL, and 200µg/mL in PBS. Each mouse was wiped using an alcohol swab on the left hind flank and each group was injected with 100µL of one of the TMV dilution. The fourth group was vaccinated with 100µL PBS. One week later, mice were wiped on the right hind flank using an alcohol swab and injected with $1x10^6$ cells per 100µL in the disinfected area. Mice were then observed for tumor growth in the following weeks.

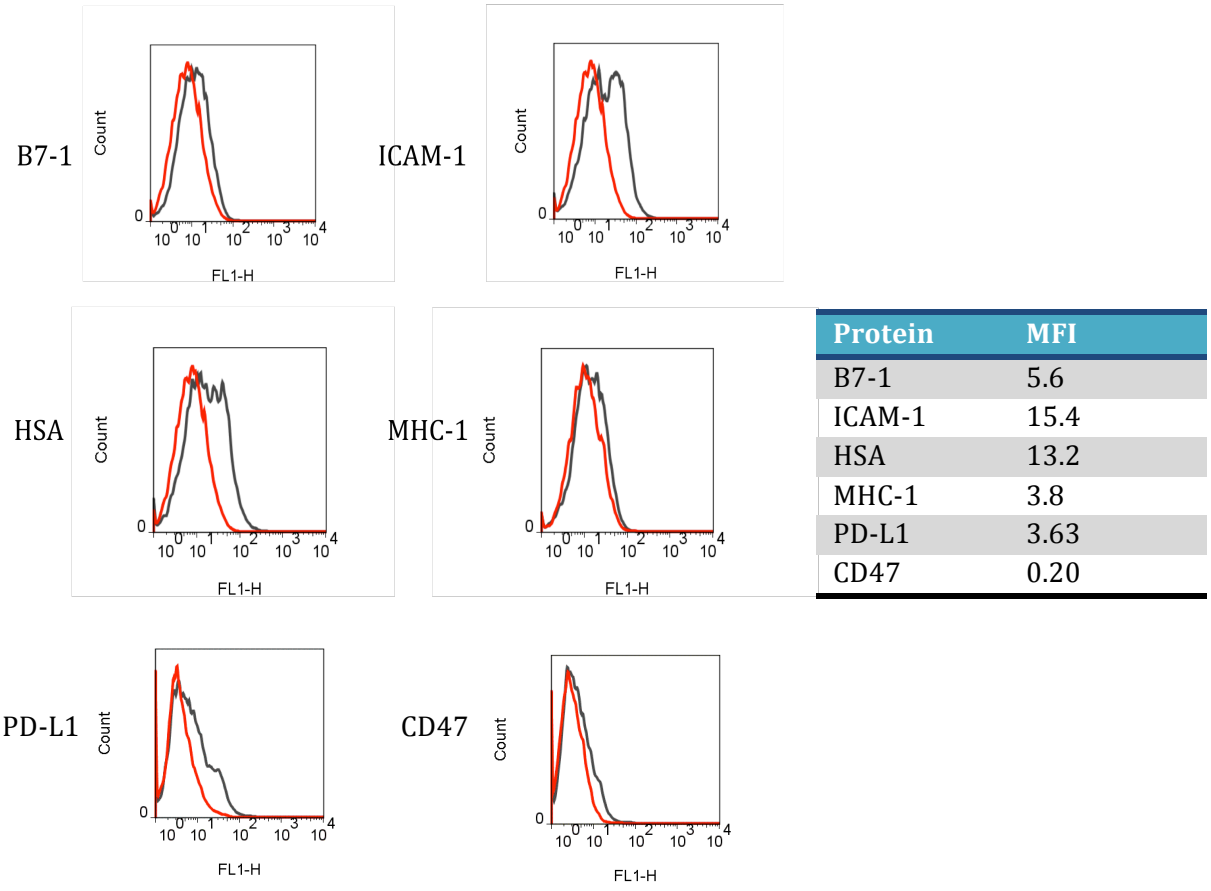
GPI-ISM incorporation onto B16F10 TMVs. TMV and GPI-ISMs were centrifuged at 13,200 rpm for 1 hour. Mini Eppendorf tubes were rinsed with PBS and excess PBS was removed with an aspirator. PBS/0.1% ovalbumin was filtered 3 times using a 0.22 micron filter with a new syringe and filter after each pass through. PBS/0.1% ovalbumin, TMV, and GPI-ISMs were added to coated tube so that the final volume was 500μL. Tubes were then rotated at 37˚C for 4 hours. After incubation, the suspension transferred to a fresh tube and centrifuged at 13,200 rpm for 1 hour at 4˚C. Supernatant was saved and the pellet was resuspended in 100μL PBS and transferred to a new Eppendorf tube with 500μL PBS. This was centrifuged for 1 hour and the supernatant was saved. The resulting pellet was resuspended to the desired volume.

Modified B16F10 TMV prophylactic vaccination and mouse challenge. B16F10 TMVs incorporated with either mIL-12, mGM-CSF, both, or neither were diluted in PBS to make a 200μg/mL concentration. On day 0, four groups of five mice were disinfected using an alcohol swab on the left hind flank and each group was injected with 100μL of one of the different TMV groups. Two groups were injected in the same area using 100μL PBS. On day 7, the procedure was repeated in the same area. On day 14, the same procedure was done except with TMV concentrations of 1000μg/mL instead. On day 21, all groups injected with TMVs and one group that was not, were disinfected on the right hind flank and injected with $1x10^6$ B16F10 cells per 100μL in the disinfected area. All mice were then observed for tumor growth in the following weeks.

Serum antibody assay. Mice were scruffed such that they were unable to move their head. Using a lancet, a hole was punched through their cheek pouch and the resulting blood was collected in a serum separator tube. Once at least 50 cc of blood was collected, the tube was sealed using a cap. Sealed tubes were then centrifuged at 13,200 rpm for 10 minutes. Tubes were removed from centrifuge and serum was collected from the top layer formed. Collected serum was then stored for future use at 4˚C. Serum was removed and FACS analysis was done suing the serum as a second antibody a live cell B16F10. A 1:50 dilution of the serum was used instead of a primary antibody at the same volume for FACS analysis. Secondary antibodies used were FITC conjugated goat anti-mouse antibodies. Results analyzed using a one-way ANOVA with a Bonferroni post test to determine significant results (Fig. 17).

RESULTS

Characterization of the B16F10 cell line. B16F10 cells were first characterized before prior to TMV creation. Figure 3 shows that this population expresses low levels of B7-1, MHC-I, HSA, and ICAM-1. Tests examining PD-L1 and CD47 presence on the cell were conducted at a later time and were found at low levels on the cell membrane as well.



Protein	MFI
B7-1	5.6
ICAM-1	15.4
HSA	13.2
MHC-1	3.8
PD-L1	3.63
CD47	0.20

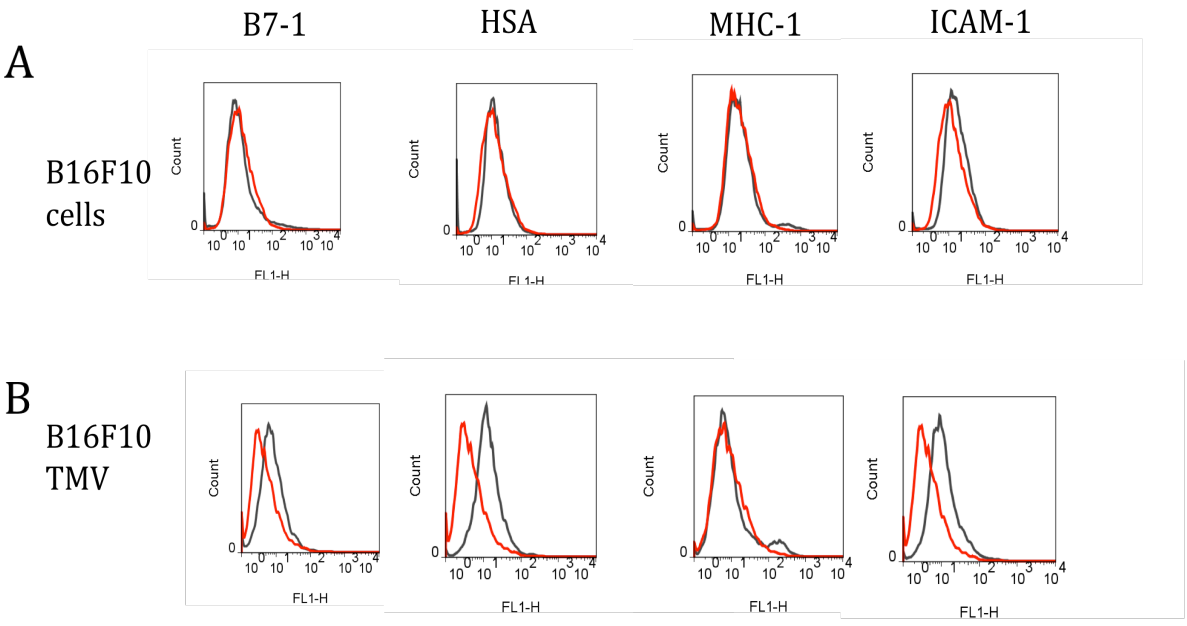
Figure 3. FACS analysis of B16F10 live cells for membrane proteins. In 3A, FACS analysis on different proteins was done to check their expression on the cell *in vitro*. The red line shows fluorescence intensity without the presence of a primary antibody (a negative control). The black line represents fluorescence intensity in the presence of primary and secondary antibodies (shows expression). Mean fluorescence intensity for each test with background noise taken into account located in 3B.

B16F10 TMV preparation and characterization.

TMVs were prepared from various pellets of B16f10 cells and quantified using a BCA assay. Concentration of TMV varied with cell count of pellets and seemed to be based on the process of homogenization itself (Table 1). Amounts produced were still adequate for future experiments in the study and the five different aliquots were stored at -20˚C for future use. Characterization was also done on TMVs and compared with B16F10 cells (Fig. 4). PD-L1 and CD47 expression were not tested on TMVs.

Cell Count	Protein (µG/mL)
$5.66x10^7$	78.0
$6.42x10^7$	96.8
$8.32x10^7$	67.2
$1.15x10^8$	2319
$2.22x10^8$	1609

Table 1. TMV concentrations from B16F10 whole cells. B16F10 cells were harvested from flasks, counted, and homogenized. Concentrations of TMV for each cell count is given above.



B16F10 Live Cell

Protein	MFI
B7-1	0
HSA	6.3
MHC-1	7.2
ICAM-1	6.7

B16F10 TMV

Protein	MFI
B7-1	25.3
HSA	0.9
MHC-1	17.6
ICAM-1	7.0

Figure 4. Characterization of B16F10 TMVs against B16F10 live cell. Both B16F10 TMVs and live cells were characterized using FACS analysis. The red line shows fluorescence intensity without the presence of a primary antibody (a negative control). The black line represents fluorescence intensity in the presence of primary and secondary antibodies (shows expression). Much like the previous characterization, live cells (A) showed little expression as did TMVs (B). MFIs for both live cell and TMV are on the magnitude of 10^1 from the negative control.

Expression of membranes proteins tested was once again low and almost nonexistent. Similar results were found when testing TMVs. Mean fluorescence intensity (MFI) shows the magnitude of this expression on the order of 10^1. We can therefore conclude that these TMVs do not express these proteins either.

Purification of GPI-anchored ISMs from transfected CHO cells. After CHO cells were collected and lysed, dot blot analysis was used to determine the presence of GPI-anchored GM-CSF (Fig. 5). Darkened spot where lysate was tested was similar to the positive control confirming that GM-CSF was within the lysate. Following this affinity chromatography was done and a Western blot was used to determine successful elution of protein into fractions (Fig. 6). Most of the protein had eluted into fractions 1-4 which were then concentrated to 3mL using polyvinylpyrrolidone. Weight of GM-CSF was found near 25kDa and IL-12 near 75-80 kDa. Similar process was done to GPI-anchored IL-12 (not shown). BCA assay

was used to determine concentrations of 59.316 µg/mL of GM-CSF and 64.607 µg/mL of IL-12 for the concentrated protein solution.

A B C D

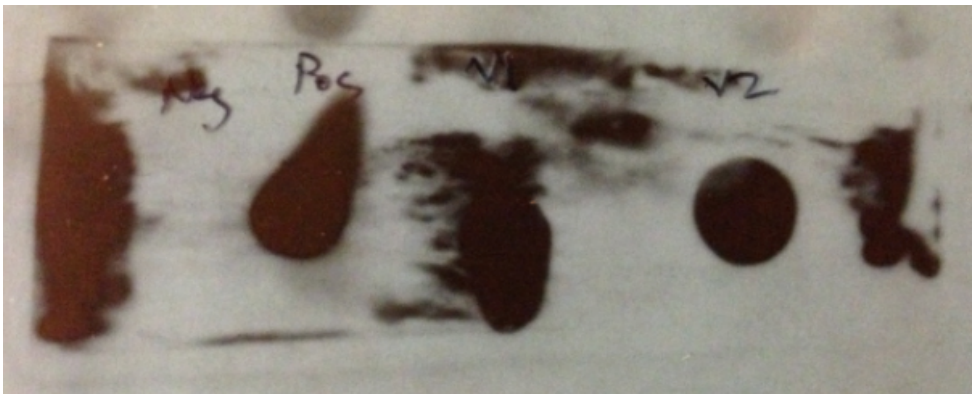


Figure 5. Dot blot analysis of CHO-mGM-CSF-CD59 lysate. Prior to affinity chromatography, lysate was checked for GM-CSF proteins to determine if protein may be purified in later stages. Spot below 6A is the negative control with dH2O and 6B is previously purified GM-CSF. Dark spots below 6C and 6D indicate that GM-CSF was present in the sample.

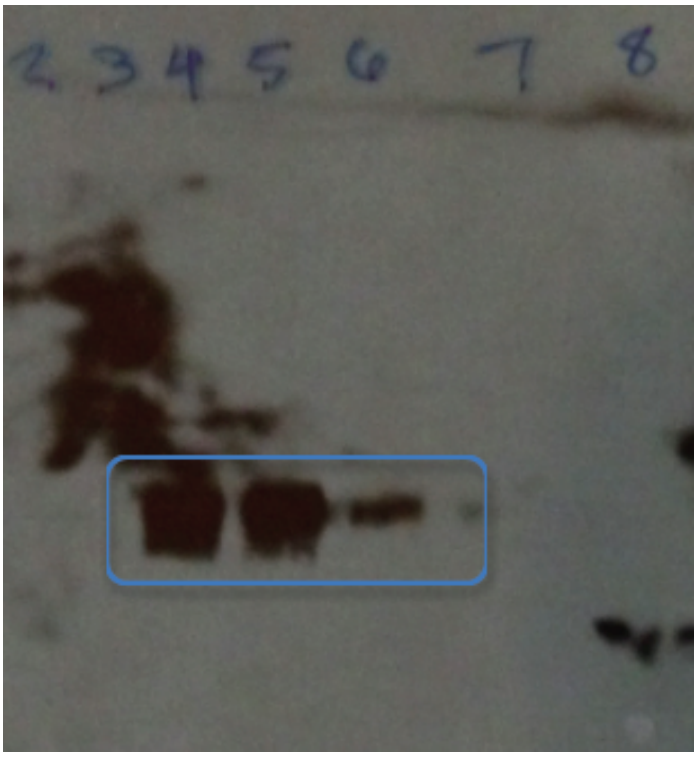
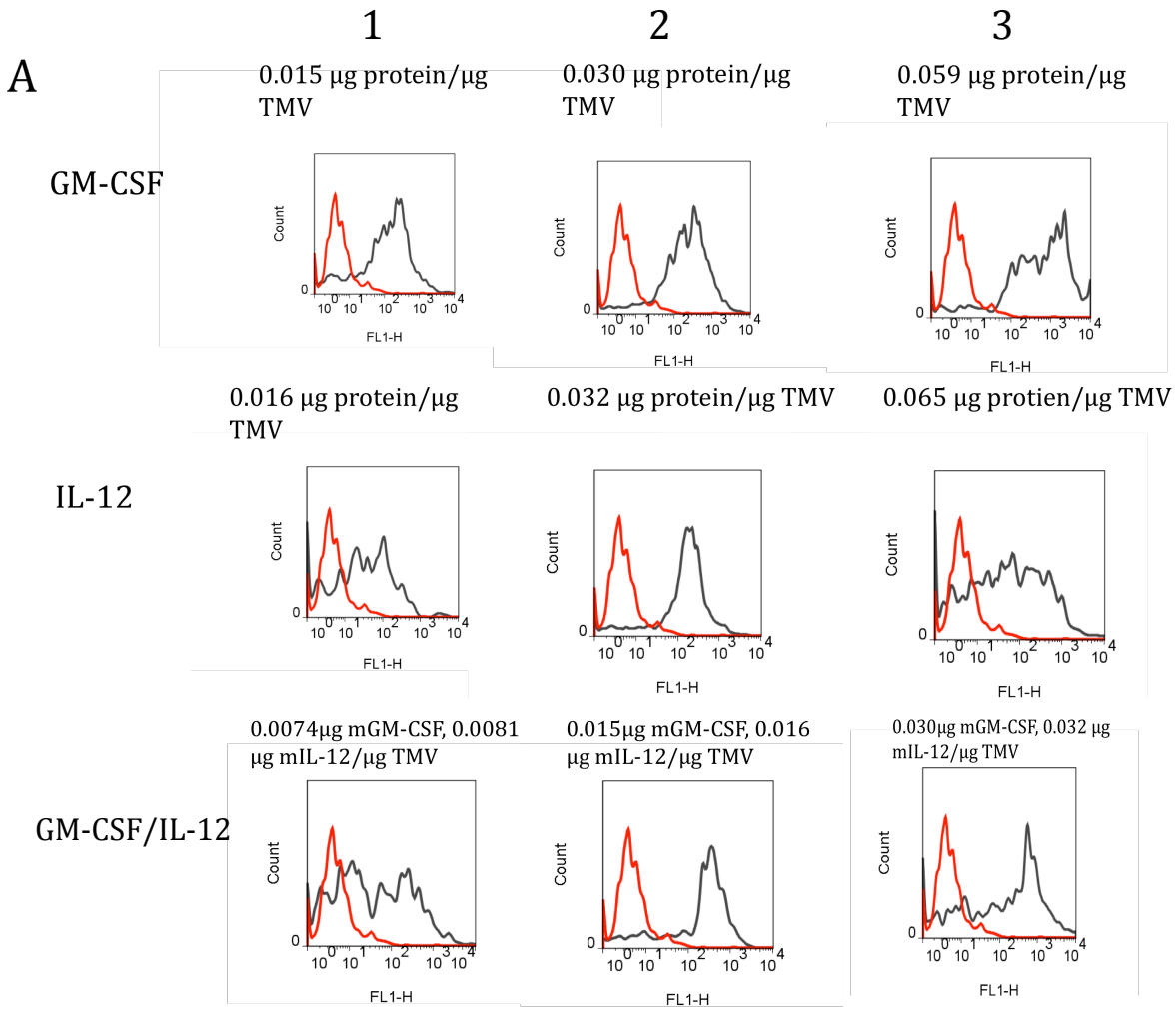


Figure 6. Western blot of purified GPI-GM-CSF from affinity chromatography. Fractions obtained through chromatography were tested for successful elution of the

product. Numbers represent columns. Column 3 shows the lysate passed through affinity chromatography. Columns 4-7 show fractions collected from affinity chromatography. Square contains bands containing the protein found in fractions 1-3.

Incorporation of GPI-anchored ISMs onto B16F10 TMVs. Experiment was done to test what protein amount was successful in incorporating onto TMVs. Below were the different concentrations tested for either GPI-anchored GM-CSF, GPI-anchored IL-12, or a combination of both (Fig. 7). Results showed that the most stable incorporation of GPI-anchored GM-CSF occurred using 0.030µg protein/µg TMV and that 0.032µg protein/µg TMV of GPI-anchored IL-12 was most stable.

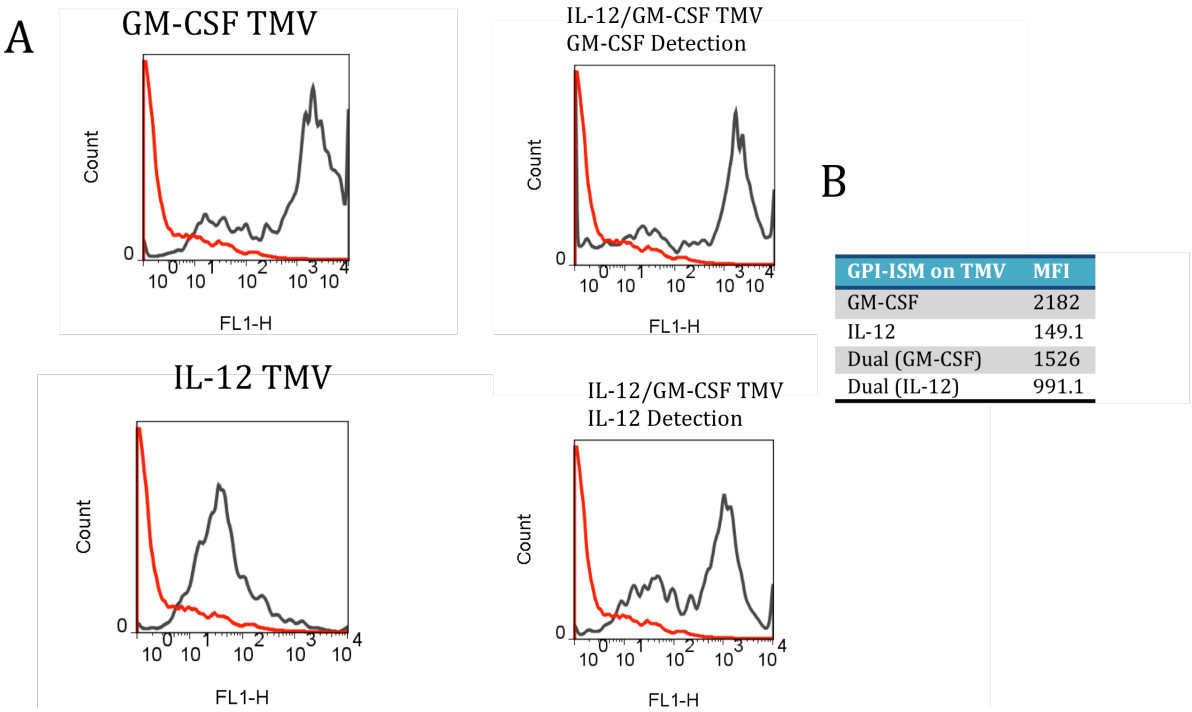


B

Modified TMV	MFI Column 1	MFI Column 2	MFI Column 3
GM-CSF	270.2	399.2	1321
IL-12	103.2	245.2	256.2
GM-CSF/IL-12	199.2	349.2	601.2

Figure 7. FACS analysis of TMVs incorporated with GPI-anchored molecules. FACS analysis on modified membranes was done to check successful incorporation of GM-CSF, IL-12, and both together (A). The red line shows fluorescence intensity without the presence of a primary antibody (a negative control). The black line represents fluorescence intensity in the presence of primary and secondary antibodies (shows expression). MFIs for these with MFIs from the negative control subtracted can be found in B.

However separate tests detecting GM-CSF or IL-12 were not done for the dual incorporated vaccine. From this data it was decided that 0.030μg protein/μg TMV would be used for GPI-anchored GM-CSF, 0.032μg/μg TMV would be used for GPI-anchored IL-12, and 0.015μg protein/μg TMV of GPI-anchored GM-CSF and 0.032μg/μg TMV of GPI-IL12 would be used for the dual vaccine. Further incorporation showed of TMVs with these amounts showed different amounts of incorporation.

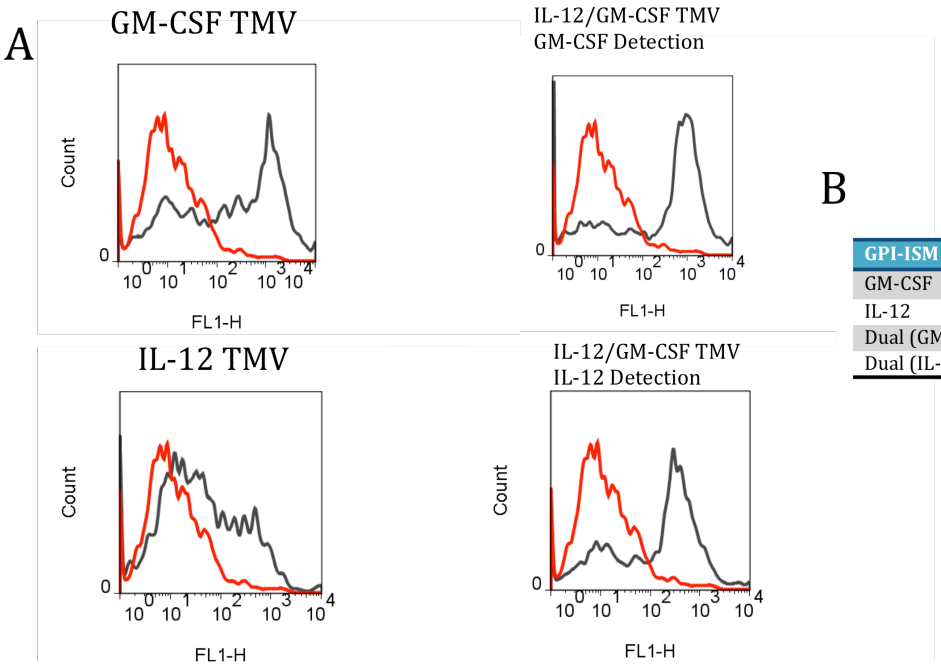


GPI-ISM on TMV	MFI
GM-CSF	2182
IL-12	149.1
Dual (GM-CSF)	1526
Dual (IL-12)	991.1

Figure 8. FACS analysis of TMVs incorporated with GPI-anchored ISMs for 20µg vaccinations. FACS analysis was done on modified TMVs to be used for 20µg injections (A) and MFIs (B) showed that there was increased incorporation of GPI-anchored GM-CSF. The red line shows fluorescence intensity without the presence of a primary antibody (a negative control). The black line represents fluorescence intensity in the presence of primary and secondary antibodies (shows expression).

GPI-anchored GM-CSF incorporation increased from the last experiment while incorporation of GPI-anchored IL-12 stayed relatively the same when incorporated alone but increased in the dual incorporation (Fig. 8). Both TMV samples were kept in incubation for equal times and TMVs originated from the same cell pellet in spite of these results. Similar results were shown when modified TMVs were prepared for 100µg vaccinations (Fig. 9). IL-12 incorporation alone seemed to remain stable across all three incorporations but did see a decrease when TMVs were made for vaccination. GPI-anchored GM-CSF in particular was shown to show dramatic shifts in incorporation with MFIs of 399.2, 2182, and 782.4 in the three incorporations

done.



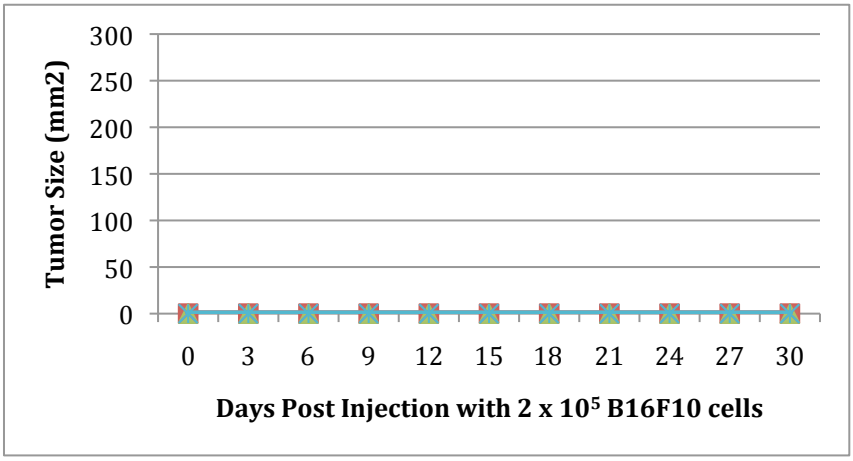
GPI-ISM on TMV	MFI
GM-CSF	782.4
IL-12	168.4
Dual (GM-CSF)	733.4
Dual (IL-12)	408.4

Figure 9. FACS analysis of TMVs incorporated with GPI-anchored ISMs for 100µg vaccinations. FACS analysis was done on modified TMVs to be used for 100µg injections (A) and MFIs (B) showed that there was increased incorporation of GPI-anchored GM-CSF. Not as much protein incorporated in each sample as in the 20µg injections but there was a slight increase in IL-12 incorporation when incorporated alone. The red line shows fluorescence intensity without the presence of a primary antibody (a negative control). The black line represents fluorescence intensity in the presence of primary and secondary antibodies (shows expression).

B16F10 live cell challenge of mice. Mice injected with $1x10^6$ B16F10 cells all obtained tumors by the end of the study (Fig. 11). Tumors first began forming at day 12 while the last tumor to begin development was detected by day 18. Mice were observed for a total of 27 days at which time tumors had reached over $200mm^2$ in size. However none of the mice injected with $2x10^5$ cells developed any tumors throughout the study. Mice in this group were kept for an additional seven days and still no tumors developed. Figure 10 shows that tumors took about 14 days to grow to $200mm^2$. Tumors were black and were not solid as characteristic of melanoma

tumors. From this study it was determined that mice in later trials should be injected with $1x10^6$ cells for adequate tumor incidence.

A



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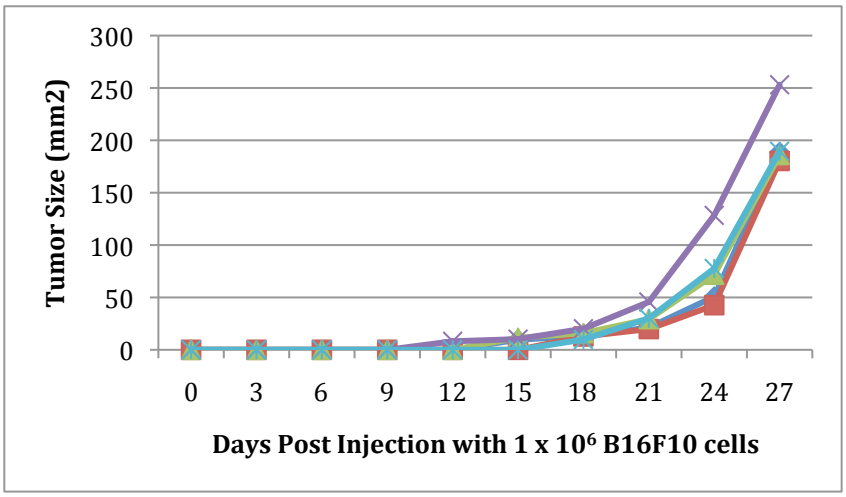

Figure 10. Tumor growth post challenge in groups challenged with $2x10^5$ or $1x10^6$ B16F10 cells. Mice challenged with $2x10^5$ cells (A) did not exhibit any tumor growth during the length of the experiment. Mice challenged with $1x10^6$ cells (B) all developed tumors and reached almost $200mm^2$ in size by day 27. Each colored line represents one mouse in each group

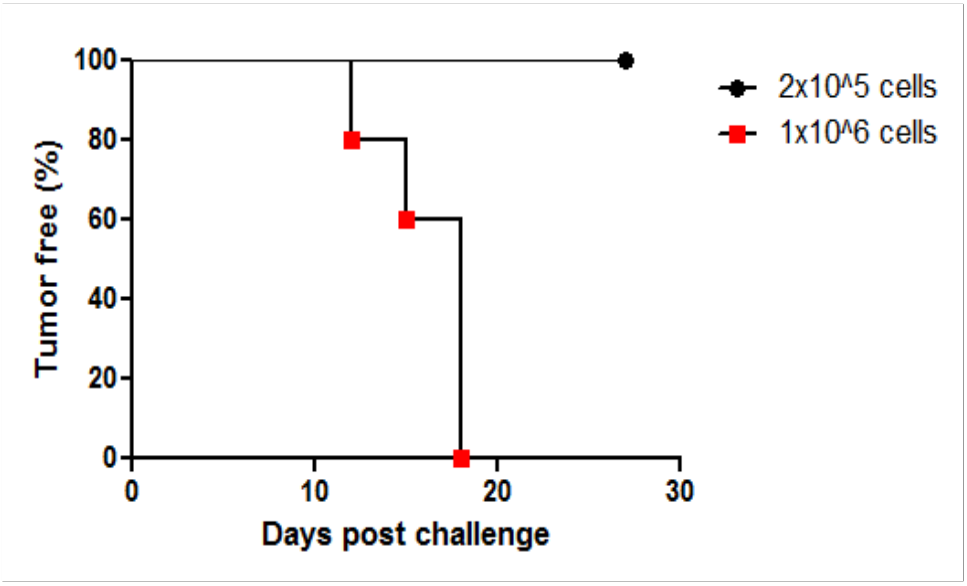

Figure 11. Kaplan-Meier curve of percent tumor free mice only challenged with live cells. Mice injected with $2x10^5$ exhibited no tumor development during the course of the experiment. Mice injected with $1x10^6$ cells first developed tumors 9 days ost challenge. All mice injected with this dose developed tumors.

Unmodified B16F10 TMV challenge of mice. TMVs were shown to induce immunity wihtout the need of GPI-anchored ISMs (Fig 13). Group injeced with 20µg TMV exhibit tumor protection in one mouse. All four of the other mice developed tumors, however two of these mice experienced necrosis (Fig 12). This is in comparison to an unvaccinated group in which all five mice developed tumors. Mice vaccinated with 50µg failed to induce protection in only two mice. Those vaccinate with 100µg however failed to induce protection in four of the five mice. Only one of the tumors in this group experienced necrosis. No abnormal discoveries were found in the naïve group. From this study a vaccination schedule was designed in which mice would be vaccinated twice with 20µg of TMV one week apart from each other with a 100µg vaccination a week later. Mice would were challenged seven days after vaccination with 100µg TMV.

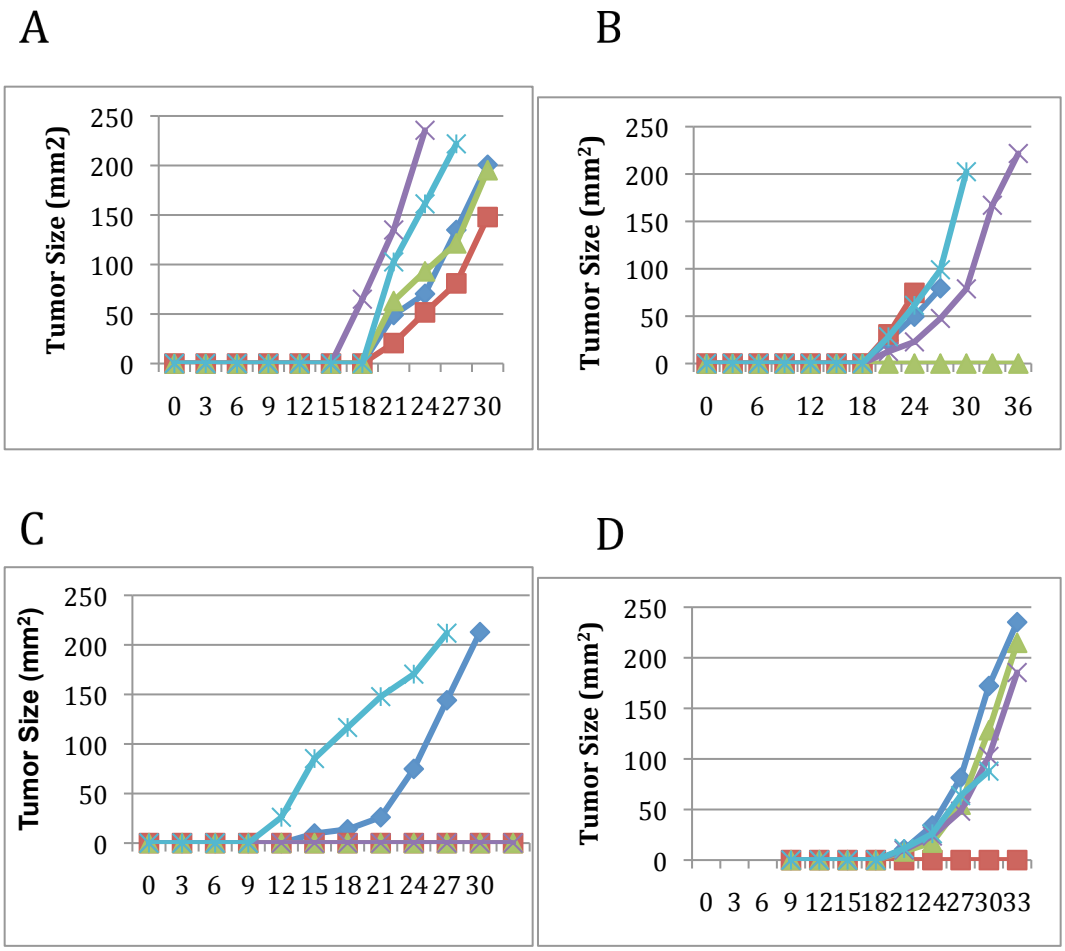

Figure 12. Tumor growth in mice vaccinated with unmodified B16F10 TMV and challenged with B16F10 live cells. The x-axis refers to the days post challenge with B16F10 live cells. Each colored line represent one mouse per group. Compared to mice in the the unvaccinated group (A), mice in the 20μg TMV vaccination (B) received induced tumor protection in one mouse. Better protection was achieved in the 50μg vaccination group (C) yet protection did not increase once dose reach 100μg (D).

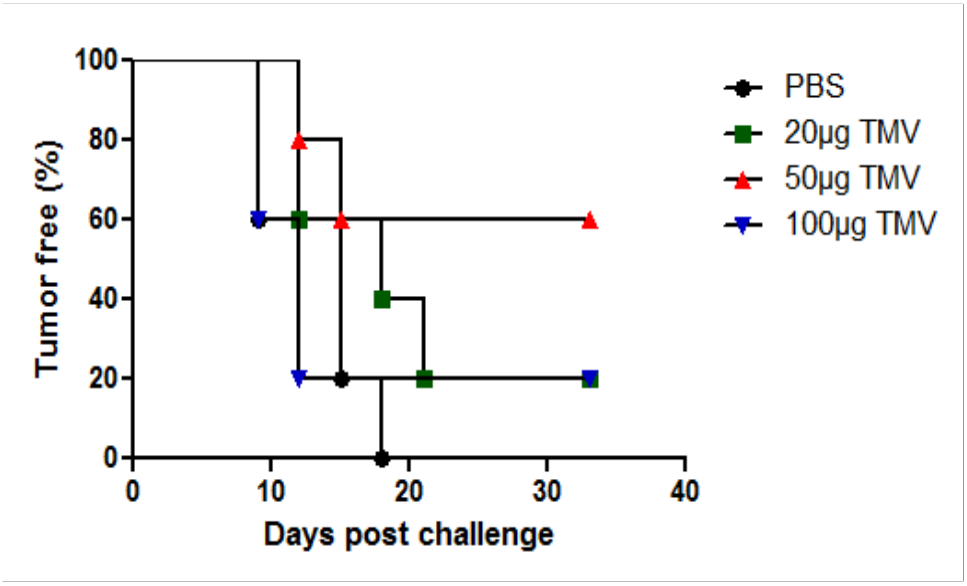

Figure 13. Kaplan-Meier curve of percent tumor free mice vaccinated with unmodified TMVs and challenged. Mice were monitered over the 30 days for the presence of palpable tumors. All vice unvaccinated mice obtained tumors. Four mice in both the 20µg and 100µg TMV vaccination groups developed a turmor. Two mice vaccinated with 50µg TMV obtained a tumor.

GPI-anchored ISM modified TMV challenge of mice. Various levels of tumor protection were induced dependant on the presence of GPI-anchored ISMs (Fig 15). Only four of the five unvaccinated mice developed tumors in this experiment. The group of mice vaccinated with unmodified TMV only only developed tumors in two mice and growth was delayed until day 22. Only one mouse vaccinated with GPI-anchored GM-CSF developed a tumor, yet unlike those in the unmodified TMV group, the tumor grew at the same time and rate us those in the unvaccinated group and necrosed at day 22(Fig. 14). No mice in either GPI-anchored IL-12 or dual GPI-anchored GM-CSF and IL-12 vaccination groups developed any tumors.

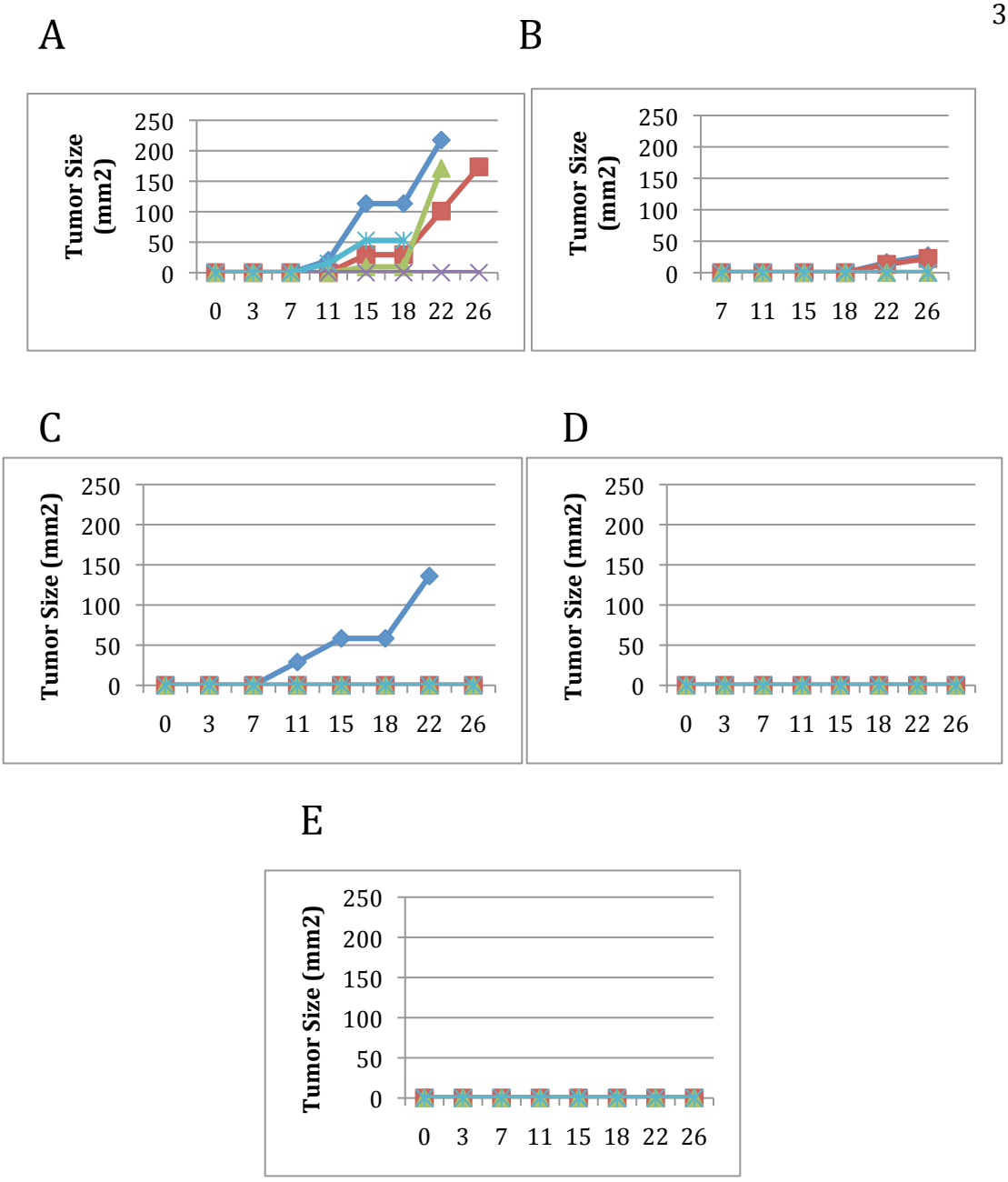

Figure 14. Tumor growth in mice vaccinated with modified B16F10 TMVs and challenged with B16F10 live cells. Mice were vaccinated subcutaneously on the left flank with either unmodified TMVs (B) or TMVs modified with GPI-anchored GM-CSF (C), IL-12 (D), or both GM-CSF and IL-12 (E). Unvaccinated mice where challenged as well for a positive control (A). Each colored line represents one mouse in each group.

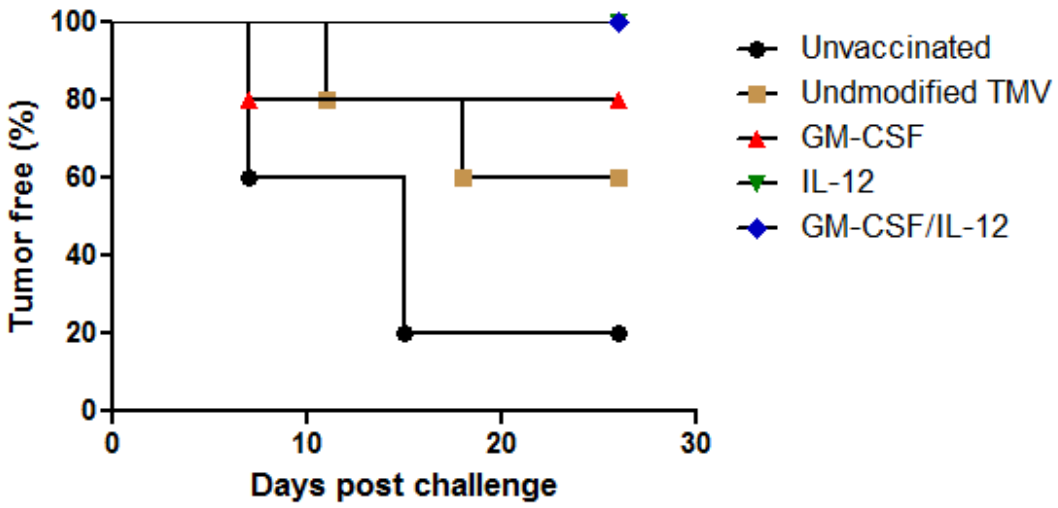

Figure 15. Kaplan-Meier curve of percent tumor free mice vaccinated with modified TMVs and challenged. Mice were monitered over the 26 days for the presence of palpable tumors. No tumor growth was exhibited in mice vaccinated with GPI-anchored IL-12 or GPI-anchored GM-CSF and IL-12 on TMVs. Only one mouse developed a turmor in the GPI-GM-CSF group.

Antibody assay of mice vaccinated with modified TMVs. Mice were bled prior to each vaccination and serum collected was tested for antibody production using FACS analysis (Fig. 16). Results showed a significant shift in intensity mainly in samples from mice vaccinated with GPI-achored GM-CSF whether it was incorporated alone ($p<0.01$) or with GPI-anchored IL-12 ($p<0.001$) (Fig.17) between mice vaccinated with unmodified TMV. Three mice vaccinated with GPI-anchored IL-12 seemed to exhibit a shift in intensity suggesting an increase in antibody production. However the shift detected was not statistically significant, yet the shift in IL-12 containing vaccines was significant. Mice vaccinated with unmodified TMV showed a slight shift on the magnitude of 10^1.

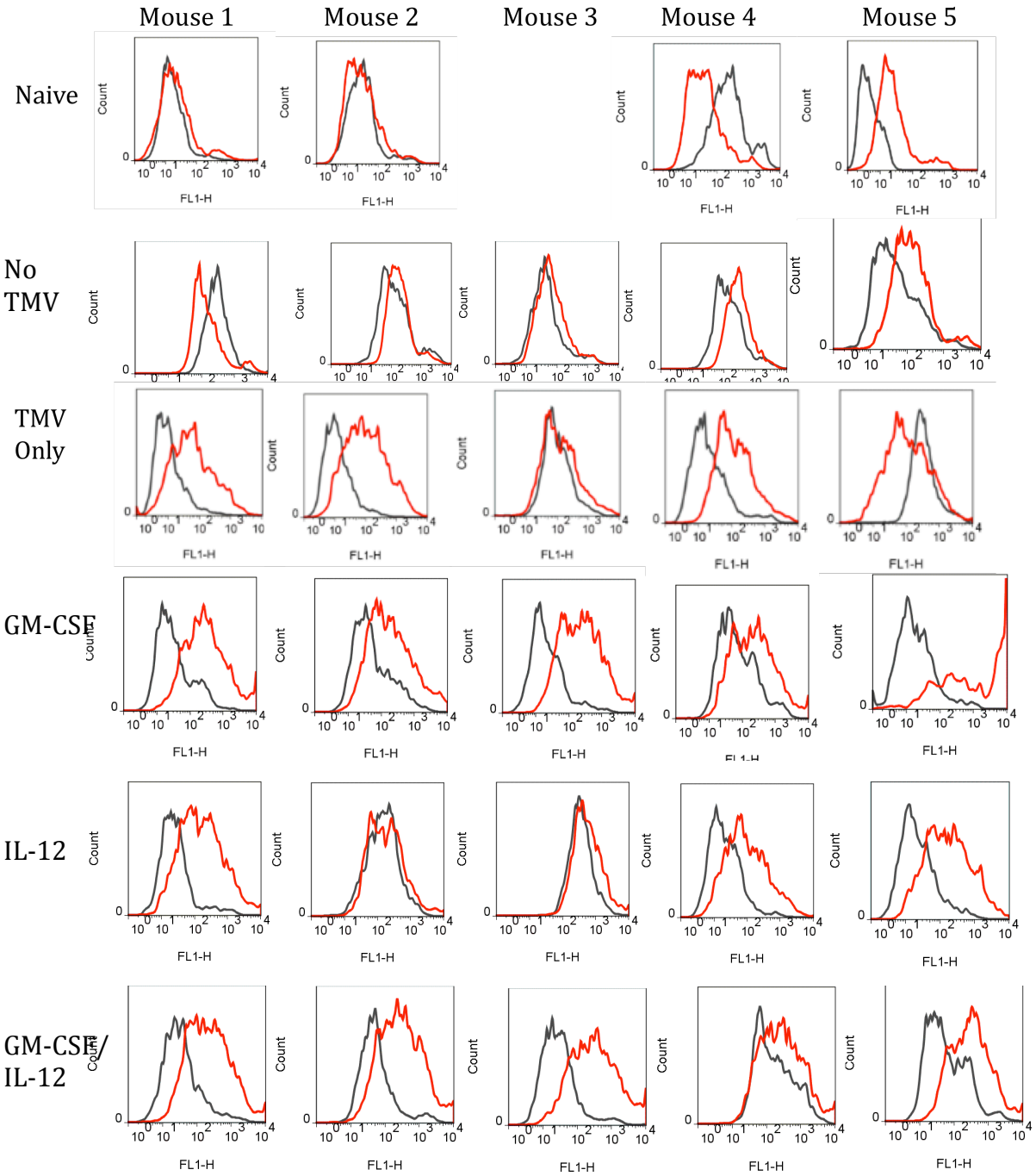

Figure 16. FACS analysis of antibody production in mice vaccinated with modified TMVs. Mice we bled and blood was centrifuged to separate serum. Serum was collected and studied using FACS for antibody production before vaccination and after the third vaccination. No mouse 3 in naïve group. Results from mouse 5 removed due to low FACS analysis count. Black line represents serum antibody before any vaccination and the red line represents serum antibody after the 100µg modified TMV vaccination.

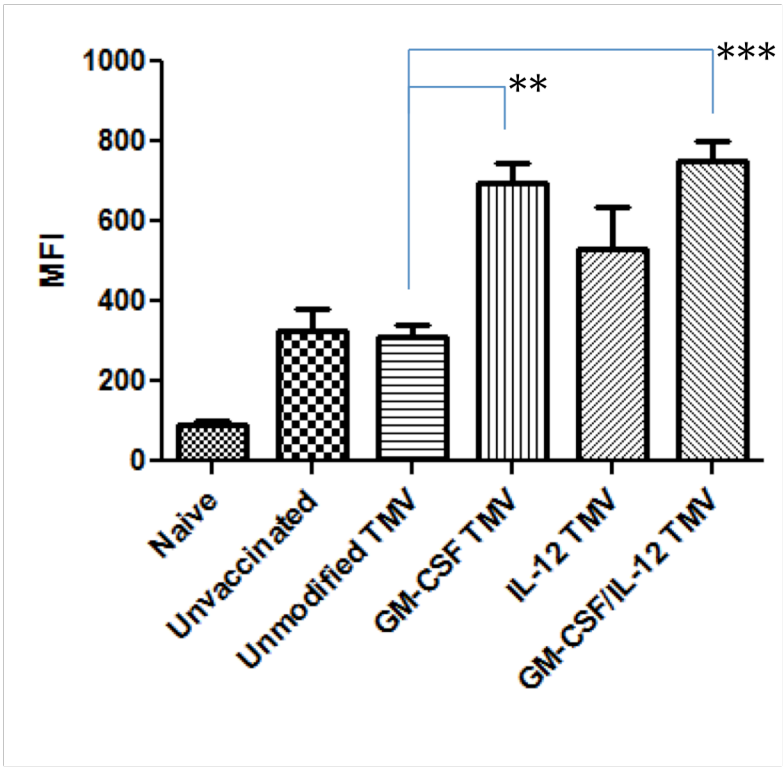

Figure 17. Comparison of antibody production between groups vaccinated with modified TMVs. MFIs from FACS analysis testing antibody production after 100μg modifeied TMV vaccination were collected and compared between groups . **indicates significant difference with $p<0.01$ and *** indicates significant difference with $p<0.001$.

DISCUSSION

The B16F10 cell line used in this experiment exhibit low levels of MHC-I, B7-1, ICAM-1, CD47, and PD-L1. Both B7-1 and ICAM-1 have been shown to be important in mediating TCR-antigen interactions in CD4+ T cells and are not commonly found on this cell line (57). Decreased levels of these molecules can therefore be considered ways in which the melanoma tumor may evade this branch of the immune system. This becomes more important when considering that the cell line does not express MHC-I, a primary recognition tool for CTLs which has also been confirmed (58). Without MHC-I, activated CTLs can depend on costimulatory proteins and adhesion proteins; two of which as described above are missing as well (55,56). However, cells expressed low levels of CD47 which is normally present on B16F10 cells (60). This lower expression of CD47 suggests that either the variant cell type used does not express CD47 or that a problem occurred during FACS analysis which would need to be reexamined. CD47 is a key mediator for innate immune functions on other cells yet paradoxically facilitates tumor cell survival and proliferation (59). PD-L1 levels were low in this cell line but other studies show that expression increases *in vivo* (62) especially in the presence of IFN-γ (61). TMVs formed from these cells exhibited similar characteristics even after being frozen and thawed suggesting that they remained stable and could be used for vaccination.

Dot blot analysis detected the successful production of GPI-GM-CSF from the lysate. Chinese hamster ovarian cells therefore can be used as a medium to produce GPI-GM-CSF. In addition, Western blot analysis shows the presence of IL-12 and GM-CSF in lysates of CHO cells expressing these GPI-anchored ISMs respectively. The

Western blot also showed that these proteins could be isolated using affinity chromatography. However this data alone does not imply that the GPI-anchor that was encoded onto the previously transfected gene remained attached to it. It has been shown through PIPLC treatment however that GPI-anchored GM-CSF and GPI-anchored IL-12 grown from CHO cells contain the GPI anchor, as treatment with PIPLC, cleaved the GPI-anchor and reduced ISM cell surface expression.

Here we show in this study that after incorporation of these anchored ISMs, they retained the GPI anchor even after storage at 4˚C for over a month. Proteins could therefore in a clinical setting be produced, collected in stock solution, and set for later use. This provides further evidence that a vaccine can be quickly designed as long as the GPI-anchored protein was collected beforehand. Incorporation however was not uniform as suggested by FACS analysis. Though the amount of protein used in each incorporation was similar, GPI-anchored GM-CSF exhibited better incorporation than GPI-anchored IL-12 so higher amounts of the IL-12 modified protein is necessary to achieve the same levels in future experiments. This may be due the smaller size GM-CSF in relation to IL-12 which would result in less steric hindrance. Subsequent protein transfers of these proteins onto TMVs exhibited the lack of uniformity in the amount of protein incorporated and incorporation of GPI-anchored GM-CSF varied the most suggesting that uniform incorporation of smaller ISMs may be harder to control.

Prior to the actual murine vaccination study it was necessary to determine what cellular load could induce tumor formation and growth. Many studies using B16F10 as a model have used different loads of cells to induce tumors. The $1x10^6$

cellular dose was able to produce tumors and thus used for further experiments while the lower dose of $2x10^5$ was not. Several other studies have used lower doses in metastatic studies where mice are injected into the tail vein (52-54) but others have used $1x10^6$ cells in metastasis. Metastasis to the lungs was not observed after 27 days at which time the mice were sacrificed. Despite the ability of the tumor cell line to induce metastasis, studies focusing on metastasis used tail vein injection to induce metastatic nodule growth rather than a subcutaneous injection (52-55).

The following murine study was used to determine the most useful TMV concentration to use to induce immunity. In comparison to the unvaccinated group in which all mice experienced tumor growth, only four of the five mice vaccinated with 20μg obtain tumors showing some protection. Even further, tumors from two of these mice underwent necrosis and tumor growth halted suggesting that even low amounts of TMV have limited protection. Mice in the 50μg group fared better and may be a vaccination strategy to consider for future experiments. The main anomaly with this experiment occurred in the 100μg group. Four of the mice vaccinated with 100μg experienced tumor growth with only one mouse experiencing tumor necrosis. Results may have occurred due to injection error of the vaccine. This was proposed since tumor protection has been shown in mice vaccinated with higher doses than their challenge dose.

Across all groups, tumor protection occurred to varying degrees in each vaccinated group in the modified TMV study. Again the TMV itself was shown to induce some protection alone as tumors only developed in two mice in comparison to the four in the unvaccinated group. However, tumors in these mice developed

much slower that in the unvaccinated group which may be due to the anti-tumor effects induced by the TMV itself. This may represent only limited time dependent immunity in some cases and continuous vaccination of TMVs and administration at a higher concentration may be needed to upkeep protection if used without GPI-anchored ISMs.

Groups of mice vaccinated with TMVs incorporated with GPI-anchored ISMs each exhibit stronger protection against B16F10 challenge than TMV alone. This is shown in the group of mice vaccinated with GPI-anchored GM-CSF. A tumor developed in only one mouse out of the five within this group. Unlike the tumors that formed in the unmodified TMV group, the tumor that developed in this mouse grew at the same time and rate as the tumors that developed in the unvaccinated group. This suggests failure of protection and not the time dependent protection as in the unmodified TMV group. This may indicate that in a larger sample size, those mice vaccinated with unmodified TMVs may also fail to achieve any protection. Still the other four mice did not develop tumors through out the rest of the study suggesting either complete protection or longer protection than the unmodified TMV. One reason that may be the source of tumor protection in this murine model is the recruitment and infiltration of DCs and increased levels of B7-1 found these APCs facilitated by GM-CSF interactions (24). In the presence of GM-CSF, APCs more readily pick up and present antigen which can therefore activate lymphocyte responses toward tumor cells.

However, the groups vaccinated with GPI-anchored IL-12 did not develop any tumors at all during the course of the study. Anti tumor protection in this case

may be due to IL-12 ability to induce INF-γ, the main mediator of immunity with IL-12 activity (60). INF-γ production in return induces CD8+ differentiation (63), activate macrophages (64), and up regulate MHC-1 and MHC-2 on cells (64). Since most mice vaccinated with any form of the TMV did not develop tumors, one potential future experiment could include rechallenging mice that did not develop tumors with B16F10 live cells. If the mice are protected from tumor growth, then the mice have developed full anti-tumor immunity against the tumor. This could be helpful in determining the use of this vaccine against remission, as is sometimes the case in a clinical setting.

Data suggests that B cells were activated to produce antibodies as shown through the antibody assay. The assay shows significantly higher levels of antibodies against B16F10 or in other words the tumor itself in mice vaccinated with GPI-anchored GM-CSF than in mice vaccinated with unmodified TMVs or those unvaccinated at all. This shows evidence towards another form of protection against the cancer. While literature suggests that there may be a similar antibody response in mice vaccinated with GPI-anchored IL-12, results showed that these were not significant. The result may stem from the large standard deviation due to two mice. A repeat of the mice study and antibody assay may provide more conclusive results. For mice vaccinated with GPI-anchored GM-CSF and GPI-anchored IL-12, a significantly higher antibody response was also detected similar to mice vaccinated with TMVs containing GPI-anchored GM-CSF alone. This response is at least partly due to the associated GM-CSF but it may be that IL-12 is working in conjunction with GM-CSF to produce antibodies. Other studies following this would need to

study CD8+ T cell infiltration to the vaccination site in these mice as have been shown in numerous studies and are important in antitumor response. Infiltration of macrophages and dendritic cells can also be studied in relation to both cytokines.

Ultimately, the goal of this study is to use the provided evidence towards successful use of GPI-anchored ISMs transferred onto TMVs in a therapeutic setting. Further studies will be necessary to elucidate the differences of TMV based vaccination versus whole cell vaccination in the presence of GPI-anchored ISMs. By performing protein transfer onto tumor cell membranes as opposed to live tumor cells, several problems are avoided. Tumor membrane vesicles developed from different tumors have been shown to keep expression of both MHC-I and MHC-II molecules. If used as a vaccine, these molecules could help facilitate both CD4+ and CD8+ T cell proliferation and activation which would aid in antitumor responses. Membranes can be prepared directly from a patient's tumor and can be frozen for later use for at least 2 years. Even after storage, GPI-anchored ISMs can be transferred onto these TMVs to create the vaccine. Membranes already modified with GPI-anchored ISMs can also be frozen and stored without losing expression of the transferred proteins (20).

Since it is not necessarily possible or feasible to prevaccinate a population against a certain tumor type, additional tests would have to be done in a therapeutic setting. The aim in this case is for a patient that has been diagnosed with melanoma prior to metastasis to have the tumor removed. The tumor membrane vesicles could then be made from the tumor cells and GPI-anchored ISMs can be transferred onto it. The patient could then be vaccinated to protect against remission and metastasis

and the vaccine could be stored for later use. The study conducted here only describes a prophylactic setting, but this data supports evidence that the vaccine could be used therapeutically. In particular that a combination of GPI-anchored ISMs could improve these TMV based vaccines. However, future study is required for conclusive evidence.

CONCLUSION

In this study we tested the use of unmodified TMVs and TMVs modified with either GPI-anchored GM-CSF, GPI-anchored IL-12, or a combination of both as a vaccine in a prophylactic setting. Firstly we showed that we were able to incorporate different GPI-anchored ISMs onto B16F10 TMVs multiple times for vaccination. Murine experiments further showed that the TMV itself had protective properties against tumors and that TMVs modified with GPI-anchored GM-CSF exhibited even more of an increase in protection. Overall, vaccinations utilizing GPI-anchored GM-CSF and IL-12 or simply IL-12 alone showed the greatest protection among all. Future studies will examine these vaccines in a therapeutic setting to determine their use in a clinical setting

Protein transfer of these ISMs onto TMVs may one day be used as an effective strategy to combat tumors in a therapeutic setting either alone or with other vaccination modalities. If allowed in a clinical setting, tumors from patients can be isolated and TMVs created from them in a short amount of time. Combine with the brief process of incorporating GPI-anchored ISMs, this strategy provides a time and cost effective way of creating a vaccine. Even if vaccines utilizing TMVs and GPI-anchored ISMs do not prove useful in a clinical setting, these two parts of the vaccine can be complemented with other strategies to create entirely different ones. These two modalities together may become a powerful alternative to initiating an immune response to create antitumor responses as a new form of tumor vaccines.

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