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Satoshi Yamanaka April 17, 2013

The Effect of Vaccination of TMVs Expressing GPI-mIL-12 and GPI-mGM-CSF on a Colon Cancer Model

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An abstract of a thesis submitted to the Faculty of Emory College of Arts and Sciences of Emory University in partial fulfillment of the requirements of the degree of Bachelor of Science with Honors

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ABSTRACT

Colorectal cancer is currently treated by surgical removal of the affected tissue. Surgery is often unsuccessful, and chemotherapy is administered to help prevent post-surgical relapse. Immunotherapy provides an alternative for the systemic administration of toxic chemicals; its effects are localized to the tumor and can be personalized for each patient. This study explored the development of active immunotherapy against the CT 26 murine colon cancer model using tumor membrane vesicles (TMVs) expressing IL-12 and GM-CSF cytokines. The naturally soluble cytokines were modified with glycosylphosphatidylinositol (GPI)-anchors to incorporate them onto membrane surfaces by a novel protein transfer method. Vaccination with CT26 TMVs expressing GPI-mIL-12 and GPI-mGM-CSF was unable to provide antitumor immunity to any significant extent than compared to unvaccinated mice. None of the treatments with TMVs expressing GPI-mIL-12 alone, GPI-mGM-CSF alone, or both GPI-mIL-12 and GPI-mGM-CSF were able to induce complete tumor rejection. Future studies will need to increase the dosage of TMVs or increase the amount of GPI-IL-12 and GPI-GM-CSF incorporation onto the TMVs in order to prepare a more immunogenic vaccine capable of inducing tumor rejection in a therapeutic setting.

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INTRODUCTION

Colorectal Cancer

Colorectal cancer is the third most common type of cancer in both women and men. The American Cancer Society estimates that 102,480 new cases of colon cancer will occur in 2013; it is also estimated that 50,830 Americans will die due to colorectal cancer this year [1]. Screening through flexible sigmoidoscopy or colonoscopy is recommended for adults older than fifty. Advocacy of early detection has helped reduce incidence in this group by 4.1% per year from 2005 to 2009 [2]. However, only 39% of colorectal cancers are diagnosed at the early localized stage. The 5-year survival rate for patients with colorectal cancer is 64%; at 10 years, the survival rate declines to 58%. For patients with advanced stages of colon cancer, the primary treatment is surgical resection. The surgery is often followed by adjuvant chemotherapy, commonly using 5-fluorouracil, oxaliplatin, and irinotecan [2].

Patients may also be treated with FDA approved antibody-based drugs such as bevacizumab, cetuximab, and panitumumab, which target various tumor-associated antigens expressed on colon cancer cells [2]. Bevacizumab suppresses tumor growth by binding to vascular endothelial growth factors (VEGF) expressed on colon cancer cells. VEGF normally stimulates endothelial cell growth and promotes angiogenesis. By binding to VEGF, bevacizumab inhibits angiogenesis and reduces the supply of nutrients available for tumor cell growth. Cetuximab and panitumumab inhibit the effect of epidermal growth factor receptors (EGFR), which stimulate tumor cell proliferation, survival, and differentiation. The blocking of EGFR by cetuximab and panitumumab prevents ligands such as EGF and tumor growth factor-alpha (TGF-α) from binding to

the receptor, resulting in the inhibition of intracellular signal cascade for tumor cell proliferation [3]. Unlike chemotherapy, antibody immunotherapy does not have the undesired side effect of systemic toxicity. However, passive immunotherapy with antibodies can become ineffective if the tumor cell mutates or alters the expression of the targeted surface antigen.

Tumor Membrane Vesicles

There are many approaches to stimulate the immune system. In contrast to passive immunotherapy, active immunotherapy stimulates the response of host lymphocytes specific to the presented antigen. One of the methods currently under investigation is to administer tumor membrane vesicles derived from tumor cells (TMVs). Membrane vesicles naturally form from various cells through the budding of the inner leaflet of the cell membrane into endosomes. Endocytosed surface proteins, cytosolic proteins, and RNA are loaded into the endosomes, which are then released from the cell as exosomes [4]. Surface proteins expressed on exosomes interact with receptors on neighboring cells, thereby activating downstream cellular machinery. Exosomes may also promote intercellular communication through internalization of the vesicles by surrounding cells, effectively delivering protein and RNA contents into the recipient cell [5]. As a result of secreting exosomes, cells create a microenvironment which promotes cell-cell communication and cell proliferation. Shedding of membrane vesicles is also increased in tumor cells, and exosomes have been found to promote immunosuppressive behavior in surrounding tumor cells. Increased amounts of sphingomyelin, a membrane phospholipid, are involved in exosome-mediated

angiogenesis [6]. Exosomes may also have high expression of MHC class I chain A, which binds to natural killer group 2, member D (NKG2D) receptors on natural killer (NK) cells to reduce their immune function [7]. In addition, increased expression of Fas ligand on exosomes may cause lymphocyte apoptosis by binding to Fas receptors on cytotoxic T cells [8].

In contrast to exosomes which display immunosuppressive characteristics, studies have found that *in vivo* vaccination of artificially produced exosomes, or tumor membrane vesicles (TMVs) can have immunogenic effects in the host. Vaccination with TMVs collected from cell cultures resulted in 90% tumor-free mice when challenged with a murine mammary adenocarcinoma cell line. The injected TMVs transfer their antigens to dendritic cells (DCs), which then process and present the tumor-associated antigens to CD4+ and CD8+ T cells [9]. Activated CD8+ T cells play a key role in antitumor immune responses through cell-mediated cytotoxicity. Cytotoxic T lymphocytes (CTLs) are activated by “Signal 1” received from MHC class I presentation of a foreign antigen and by “Signal 2” from co-stimulatory receptor CD28 binding to B7-1/2. In addition, recent studies have shown that antigen-specific CTL activation requires “Signal 3,” or IL-12 binding to its receptor; the transmission of “Signal 3” from DCs to CTLs requires direct antigen uptake by the DCs [10]. CTLs may also indirectly reject tumors by secreting IFN-γ, which activate macrophages by binding to receptors on the cell surface [11]. Antitumor immunity also depends on CD4+ T cell differentiation into Th1 and Th2 cells, which are involved in macrophage activation and antibody response [12].

Protein Transfer Method

The immunogenic effect of tumors can be further bolstered making tumors express cytokines. Currently, the more common method to induce expression of immunostimulatory molecules such as cytokines is gene transfer. However, gene transfer has several disadvantages, such as limited multiple immunizations, time consumption, and difficulty with expressing multiple proteins. An alternative to gene transfer is a novel protein transfer method. This protein transfer method allows for the incorporation of the protein in a few hours, expression of multiple proteins on the same membrane, and the use of non-viral membranes [13]. Protein transfer requires the use of a glycosylphosphatidylinositol (GPI)-anchored protein. The GPI-anchor is naturally expressed by cells on many proteins, including the complement regulatory protein CD59. It allows the protein to be anchored into the cell membrane by hydrophobic interactions with the lipid bilayer. The anchor is composed of carbohydrates and amino acids in the sequence Mana1–2Mana1–6Mana1–4GlcN followed by a phosphatidylinositol moiety [14]. The GPI-anchor attachment signal sequence from CD59 can be attached to the end of the gene of a naturally soluble cytokine. The altered gene is then translated into a product that has a GPI-anchor attached to the C-terminus of the extracellular domain, and the GPI-anchored protein can then be spontaneously incorporated onto cell membranes. Modification with the GPI-anchor does not affect the natural functions of most proteins. GPI-modified hIL-12p70 was folded correctly by cells transfected to express the protein, and the protein could successfully induce T cell proliferation [15]. Similarly, GPI-anchored forms of GM-CSF and B7-1 retain their immunostimulatory functionality [16], [17]. In other studies, however, GPI-anchor modification alters the

structure of the original protein. One study has found that the replacement of the transmembrane domain of CD16 with the GPI-anchor resulted in a change in the structural conformation of the extracellular domain [18].

Incorporation onto cell membranes is optimal at 37°C and is dependent on the concentration of protein used and the duration of incubation [19]. A study with GPI-B7-1 and GPI-ICAM-1 has shown that the two GPI-anchored proteins can be simultaneously incorporated onto microparticle surfaces via protein transfer [20]. This method of protein expression is applicable in clinical settings because the incorporated proteins can remain stably expressed on microparticle surfaces when stored at 4°C. After seven days at 4°C, incorporation level is decreased to 70% of original incorporation level [20]. Stored at 37°C, almost all of the proteins remain incorporated for up to four days [16]. TMVs also have a long shelf-life and can be stored at -80°C for a month without alterations in endogenous surface protein expression. Membranes also retain their ability to stimulate T cells for up to two years after storage [16].

Because of the significant advantages of protein transfer, this present study used this procedure to express GPI-mIL-12 and GPI-mGM-CSF onto TMVs. The modified TMVs were used to vaccinate BALB/c mice in order to study the effect of GPI-mIL-12 and GPI-mGM-CSF on CT26 colon cancer growth.

IL-12

IL-12 is a immunostimulatory cytokine secreted by macrophages and DCs. Among the many functions it performs, IL-12 was initially found to activate NK cells and enhance NK cell-mediated cytotoxicity [21]. IL-12 has also been found to promote the

differentiation of CD4+ T cells into Th1 helper cells that secrete high concentrations of IFN-γ [22]. In addition, IL-12 induces the production of GM-CSF, IL-1β, IL-6, TNF-α, and IFN-γ by DCs [23]. Recent studies have further demonstrated that IL-12 secreted by DCs binds to IL-12 receptors on naïve T cell surfaces, thereby signaling the T cells to differentiate into CTLs [24]. These effects lead to improved antigen presentation by DCs and to the proliferation of antigen-specific CTLs that play a key role in antitumor immunity. Clinical trials have demonstrated that administration of recombinant hIL-12 resulted in systemic toxicity, illuminating the risks of utilizing soluble IL-12 in cytokine therapy [25]. However, gene transfer of mIL-12 cDNA into CT26 tumor cells successfully resulted in the regression of subcutaneous and metastatic CT26 tumors [26]. IL-12 has also been shown to have a synergistic effect with IL-10 and B7-1 on CT26 tumor growth [27], [28]. Results from such studies show that IL-12 is a suitable candidate for the study of combined cytokine immunotherapy.

GM-CSF

GM-CSF, or granulocyte-macrophage colony stimulating factor, is secreted by macrophages, T lymphocytes, fibroblasts, and endothelial cells. GM-CSF was originally discovered to stimulate the proliferation and differentiation of macrophages and granulocytes, such as neutrophils, eosinophils, and basophils [29]. The protein acts as a chemoattractant for monocytes and neutrophils, facilitating inflammatory immune response [30]. GM-CSF has also been found to enhance the cytotoxic activity of macrophages by increasing secretion of TNF-α [31]. In addition, GM-CSF binds to receptors on DC surfaces, and in response DCs induce the activity of CTLs [32].

Lymphocytes in mice carrying GM-CSF-expressing tumors have been found to lyse an increased number of tumor cells, demonstrating that GM-CSF can generate an immune-dependent antitumor response [33]. Furthermore, GM-CSF has been found to generate a dose-dependent antitumor response against various tumor cell lines. In mice challenged with TC-1, a murine lung cancer model, high doses of GM-CSF were found to promote tumor growth by inducing granulocytosis and lymphopenia [34]. In a murine colon cancer model, MC38, tumor cells expressing reduced amounts of GM-CSF exhibited increased cell viability, growth, and colony formation. The same study found that immune-independent effects of GM-CSF on the CT26 cell line were insignificant [33]. Antitumor effects of GM-CSF, especially its CTL-dependent effects, could possibly be enhanced when administered in combination with cytokines such as IL-12, which induce CTL activation.

CT26 Murine Colon Cancer Model

The antitumor effect of TMVs expressing IL-12 and GM-CSF was studied on the CT26 colon cancer model. This cell line is a colon adenocarcinoma originating from colon epithelial cells in BALB/c mice, induced by the carcinogen N-nitroso-N-methylurea. CT26 tumor cells primarily metastasize to the lungs, as well as to the ovaries, adrenals, kidneys, mesentery, heart, liver, and the diaphragm. The cell line is an experimental metastatic model; *in vivo* metastatic models are studied by injecting CT26 cells into the tail vein. CT26 cells have been categorized as Grade IV tumor cells, which are characterized by undifferentiation and rapid growth of cells [35]. CT26 tumors escape immunosurveillance through low expression of B7-1 on the cell surface [36]. The cell

line also has an over-expression of CD74, which binds to the macrophage migration inhibitory factor to signal an anti-apoptotic mechanism in the cells [37]. Because of characteristics similar to colon cancer in humans, CT26 has been extensively used to investigate the efficacy of potential immunotherapies.

METHODS

Cell cultures: CT26 cells were originally purchased from ATCC; the cells were thawed in the lab and cultured in RPMI 1640 media (with L-glutamine) with 10% Hyclone Cosmic Calf Serum (Thermo Scientific®), 50 U/mL penicillin, 50 µg/mL streptomycin, and 0.5 µg/mL Fungizone. Expression of MHC class I, CD47, IL-12, ICAM-1, PDL-1, GM-CSF, HSA, and B7-1 on the surface of CT26 cells was analyzed by flow cytometry. Freshly thawed cells were cultured a week before each *in vivo* cell injection.

CHOK1-mIL-12-a-CD59-b-sol and CHOK1-hHER2-mGM-CSF-CD59 were previously established by transfection and were cultured in RPMI 1640 media (with L-glutamine) with 10% cosmic calf serum, 50 U/mL penicillin, 50 µg/mL streptomycin, 0.5 µg/mL Fungizone, 10 µg/mL blasticidin. Each cell line was also cultured in 2 L roller bottles to harvest a mass quantity of cells for protein purification. In the roller bottles, each cell line was cultured in RPMI 1640 media (with L-glutamine) with 10% cosmic calf serum, 1 µg/mL blasticidin, 10 mM HEPES, and 2 mM L-glutamine. At 90% confluency, the cells were detached with PBS/5mM ethylenediamine tetracetic acid (EDTA) and centrifuged to collect the cell pellet.

Mouse model: Female BALB/c mice, six to eight weeks old, were purchased from Jackson Laboratory. Mice challenged with live CT26 cells were sacrificed if their tumors

grew to a diameter of more than 2.0 cm, or if their tumors became ulcerated. All mice were maintained in accordance with IACUC approved institutional guidelines and protocols at Emory University. Tumors were removed and collected after each *in vivo* experiment.

GPI-anchored protein purification: GPI-anchored mIL-12 and mGM-CSF were purified from CHOK1-mIL-12-a-CD59-b-sol Clone F8 Pan 1 and CHOK1-hHER2-mGM-CSF-CD59, respectively. The cells were collected in 8-12 gram pellets and lysed overnight at 4°C in a solution of 50 mM Tris pH 8.0, 2% octyl-β-D-glucopyranoside (OG), 1 mM $ZnCl_2$, 1:100 dilution of protease inhibitor cocktail (from Sigma®), 5 mM EDTA, 5 mM iodoacetic acid, and 2 mM phenylmethanesulfonylfluoride (PMSF). The cell lysate solution was centrifuged at 14,000 rpm for 1 hour at 8°C, and the resulting supernatant was passed through a cell strainer. To purify GPI-mIL-12, CHOK1-mIL-12-a-CD59-b-sol cell lysate was passed through an affinitiy chromatography column with sepharose beads coupled to R2-9A5, an anti-mIL-12p70 antibody. The column was eluted over eight fractions with a pH 2.80 solution of 100mM glycine, 1% OG, and 10 mM iodoacetic acid; the elutions were neutralized with 1 M Tris pH 11.0. To purify GPI-mGM-CSF, CHOK1-hHER2-mGM-CSF-CD59 cell lysate was passed through a column with beads coupled to A2/F17-107, an anti-mGM-CSF antibody, and the column was eluted with a pH 11.5 solution of 100 mM triethylamine and 1% OG. The eight elution fractions were neutralized with 1 M Tris-HCl pH 0.4.

The collected elution fractions were analyzed for the presence of GPI-mIL-12 and GPI-mGM-CSF through SDS-PAGE in 10% gel under non-reducing conditions, followed by Western blot and silver staining. The purified fractions of each GPI-anchored protein

were combined together in a dialysis bag and concentrated by polyvinylpyrrolidone treatment. Because the fractions contained 1% concentration of OG, they were diluted by washing the dialysis bags in PBS/0.1% OG solutions for 48 hours. Protein concentration in the dialyzed solutions was analyzed by ELISA.

Protein transfer: To test the ability of purified GPI-anchored proteins to incorporate onto membranes, GPI-mIL-12 and GPI-mGM-CSF were incorporated onto sheep red blood cells (RBCs) by protein transfer. Eppendorf tubes were coated overnight with PBS/0.1% ovalbumin to prevent the sticking of GPI-anchored proteins to the tube wall. RBCs were washed twice with plain PBS at 2000 rpm for 5 min. To optimize incorporation, sheep RBCs were diluted to 10×10^6 cells/mL in PBS/0.1% ovalbumin. RBCs were then rotated for 4 hours at 37ºC with GPI-anchored proteins to incorporate the proteins into the cell membrane. Protein transferred RBCs were then analyzed by flow cytometry to observe the level of protein incorporation.

Once the incorporation of GPI-mIL-12 and GPI-mGM-CSF on RBC surfaces was confirmed, the GPI-anchored proteins were transferred onto CT26 TMVs. Eppendorf tubes were briefly washed with PBS, and CT26 TMVs were incubated with the GPI-anchored proteins in PBS/0.1% ovalbumin solution for 4 hours at 37°C.

CT26 tumor membrane vesicles: CT26 cells grown in culture were harvested and centrifuged. The cell pellet was homogenized using a Polytron homogenizer in a buffer (20 mM Tris pH 8.0, 10 mM NaCl, 0.1 mM $MgCl_2$, 0.2% NaN_3, and 0.1 mM PMSF) four times for seven to eight seconds each, with one minute of cooling on ice between each homogenization. The homogenate was centrifuged for 1 hour at 4°C and 23,000 RPM over a 41% (w/v) sucrose gradient. The interphase was collected and washed with

homogenization buffer, and the pelleted TMVs were stored at -20°C in PBS/10 mM HEPES. The amount of TMVs in solution was determined by total protein expression using a bicinchoninic acid assay (Thermo Scientific®) with bovine serum albumin standards.

In vivo *experiments:* To assess the tumorigenicity of CT26, BALB/c mice were injected with 50,000 or 100,000 live CT26 cells suspended in 100 µL of PBS. The mice were injected subcutaneously in the left hind flank. The desired rate of tumor growth was indicated by the formation of a palpable tumor in 10-14 days post challenge.

A second experiment involved the prophylactic vaccination of BALB/c mice with 20 µg, 50 µg, or 100 µg of unmodified CT26 TMVs in 100 µL of PBS. The purpose of this experiment was to determine the maximum amount of TMVs that would provide the not inhibit tumor growth. This was to ensure that the immunogenic effect of GPI-mIL-12 and GPI-mGM-CSF expressed on CT26 TMVs could be observed in the subsequent vaccination study. The mice were vaccinated with the TMVs 21 days before challenge, and were given another vaccination 7 days before challenge. Both doses of vaccinations were given subcutaneously in the left hind flank. The mice were challenged with 50,000 live CT26 cells subcutaneously in the right hind flank, and tumor size (length and width) was measured every 3 to 4 days.

To determine the immunogenic effect of expression of GPI-mIL-12 and GPI-mGM-CSF on CT26 TMVs, BALB/c mice were vaccinated with 50µg of CT26 TMVs expressing GPI-mIL-12, GPI-mGM-CSF, or a combination of the two cytokines. Using the protein transfer method, CT26-GPI-mIL-12 TMVs were prepared by incubating 64.6 ng of GPI-mIL-12 per µg of CT26 TMVs in 200 µL of PBS/0.1% ovalbumin. CT26-GPI-

mGM-CSF TMVs were prepared by incubating 59.3 ng of GPI-mGM-CSF per μg of TMVs. CT26-GPI-mIL-12-mGM-CSF TMVs were prepared by incubating 64.6 ng of GPI-mIL-12 and 29.7 ng of GPI-mGM-CSF per μg of TMVs in 200 μL of PBS/0.1% ovalbumin. The initial vaccination was given 21 days before challenge, followed by a boost 7 days before challenge. Vaccinations were given subcutaneously in the left hind flank. The mice were challenged with 50,000 live CT26 cells in the right hind flank. Blood serum from each mouse was collected prior to the initial vaccination and the live cell challenge. Serum samples were tested for the presence of antibodies against CT26 cells by flow cytometry.

All of the *in vivo* experiments were performed with five mice in each control and experimental group.

RESULTS

Surface protein characterization of the CT26 cell line

Analysis by flow cytometry showed that the CT26 cell line expresses moderate levels of MHC class I and B7-1 molecules on the cell surface (Fig. 1, Table 1). The level of B7-1 expression on the CT26 cell line used in this study was higher than the level of expression reported by other studies [36]. Higher level of B7-1 expression may have may have allowed CD28, a co-stimulatory molecule on T cell surfaces, to bind to B7-1, leading to an increased recognition of tumor cells by immune cells and thus a reduction in the tumorigenicity of CT26 cells. CT26 cells also express very limited levels of CD47, ICAM-1, PD-L1, and HSA (Fig. 1). Analysis showed that CT26 cells do not express IL-12 or GM-CSF, since both cytokines are naturally secreted in soluble forms. Therefore,

artificial expression of IL-12 and GM-CSF on CT26 membranes by protein transfer could be expected to have an effect on CT26 tumor growth.

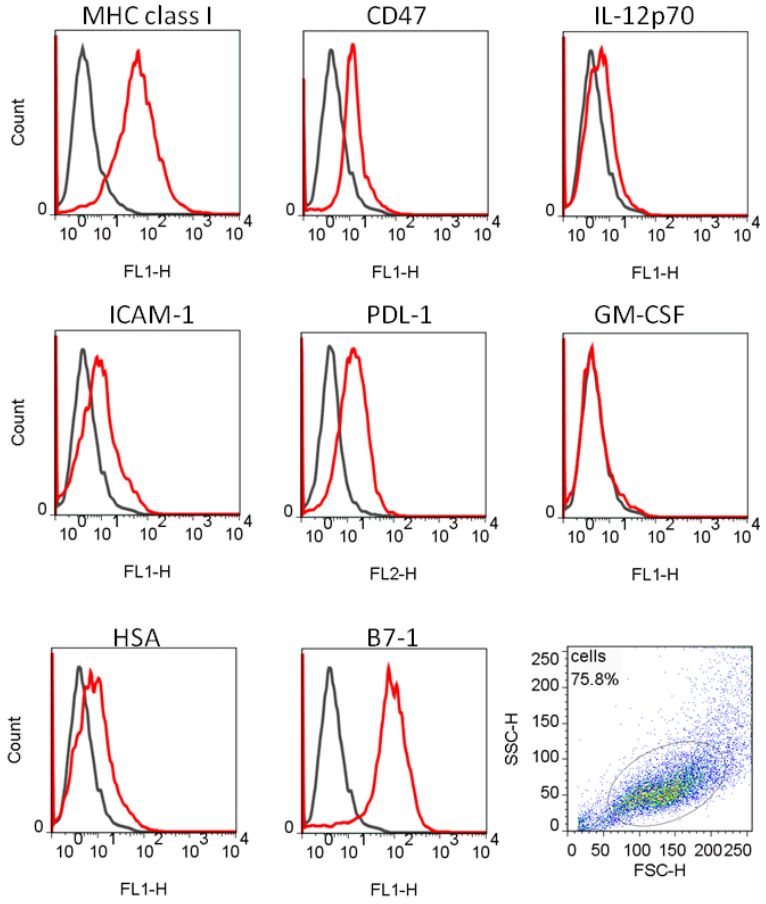

Figure 1: Surface protein characterization of CT26 cell line by flow cytometry. Black peaks represent isotype control; red peaks represent proteins of interest.

Table 1: Mean fluorescent intensities (MFI) of peaks for the surface protein characterization of the CT26 cell line by flow cytometry, as shown in Figure 1. MFI was calculated by subtracting the MFI of the isotype control from that of the protein of interest.

Surface molecule	MFI
MHC class I	66.47
CD47	13.49
IL-12	1.14
ICAM-1	3.24
PD-L1	13.13
GM-CSF	-0.80
HSA	3.35
B7-1	75.79

Purification of GPI-mIL-12 and GPI-mGM-CSF

Analysis of purification fractions of GPI-mIL-12 and GPI-mGM-CSF showed that both proteins were successfully eluted from their affinity chromatography columns. GPI-mIL-12 was more clearly detected in both the silver stain and western blot analyses, and bands at 70 kDa indicated the presence of GPI-mIL-12 in seven of the eight fractions (Fig. 2A, B). GPI-mGM-CSF could not be detected as well as GPI-mIL-12 with either analysis (Figure 2C, D). However, analysis by Western blot showed bands at 30 kDa in Fractions 2 and 3, indicating the presence of purified GPI-mGM-CSF in the solutions.

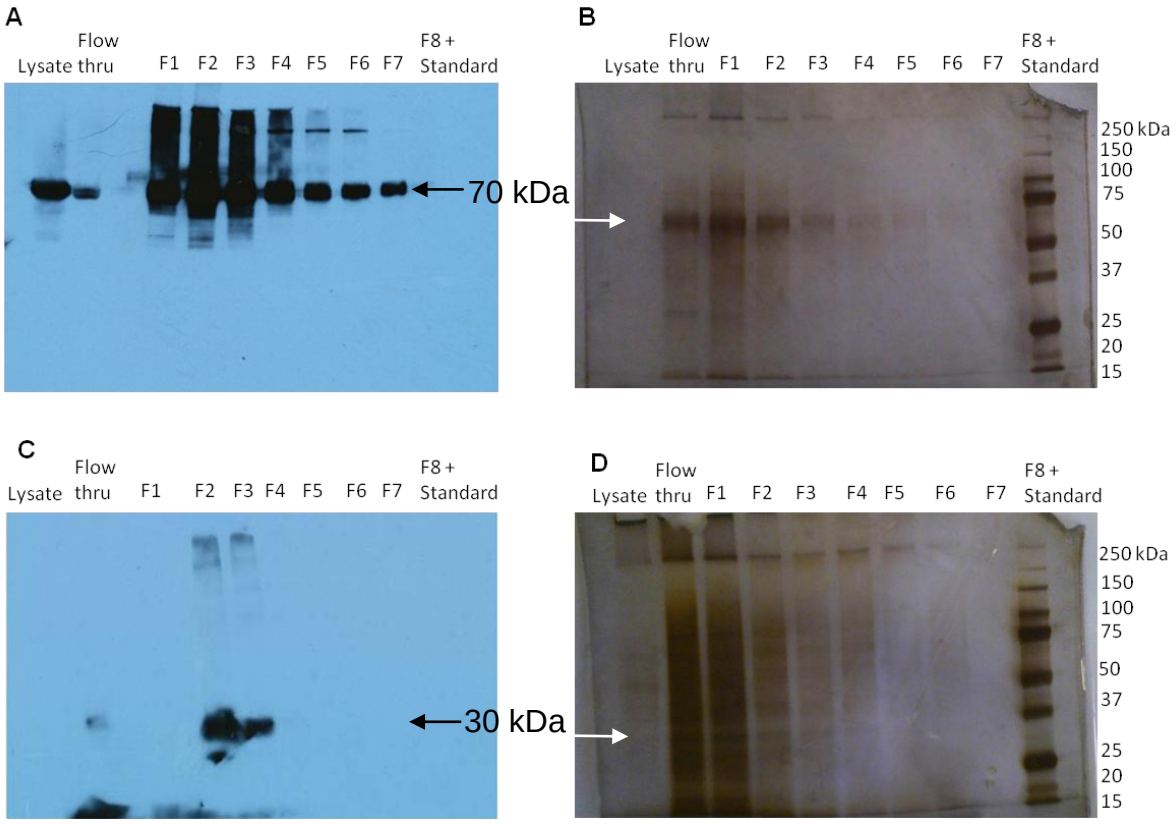

Figure 2: Analysis of purified elution fractions of GPI-mIL-12 and GPI-mGM-CSF. (A) and (B) show analysis of GPI-mIL-12 by western blot and silver stain, respectively. The Western blot gel was blotted with anti-mIL-12 antibody C17.8 and detected with HRP-conjugated goat anti-rat antibody. (C) And (D) show analysis of GPI-mGM-CSF by western blot and silver stain, respectively. The Western blot gel was blotted with anti-mGM-CSF antibody A2/F17-107 and detected with HRP-conjugated goat anti-rat antibody. Western blots for both proteins were exposed for 15 seconds.

GPI-mIL-12 and GPI-mGM-CSF incorporation onto sheep red blood cells (RBCs)

Purified GPI-mIL-12 and GPI-mGM-CSF were incorporated by protein transfer onto sheep RBCs to test the GPI-modified proteins' ability to stably attach to cell membranes. Protein transfer was performed by incubating 1.29 µg of GPI-mIL-12 and 1.19 µg of GPI-mGM-CSF with 2×10^6 red blood cells for four hours at 37°C. The protein transfer procedure resulted in the expression of both proteins on RBC surfaces (Fig. 3, Table 2).

The shifts in mean fluorescent intensity (MFI) demonstrated that the protein transfer method was capable of inducing expression of desired proteins on membrane surfaces.

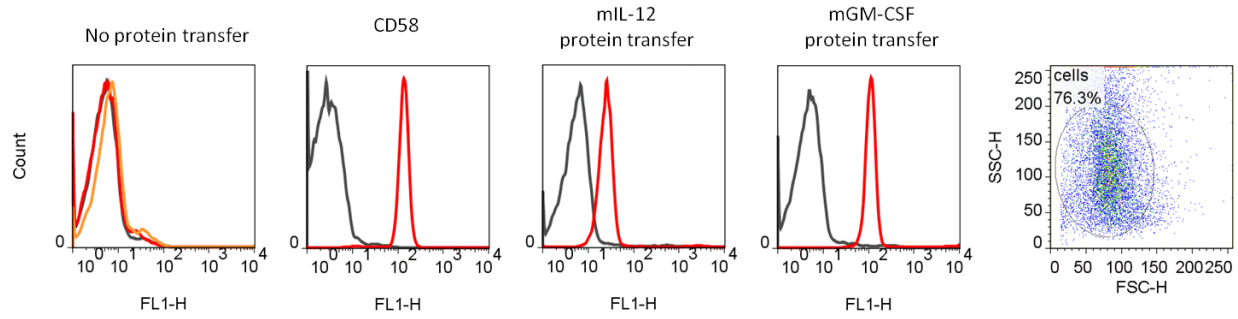

Figure 3: Incorporation of GPI-mIL-12 and GPI-mGM-CSF onto sheep RBCs. Levels of incorporation were detected by flow cytometry using C17.8 (anti-mIL-12) and A2/F17-107 (anti-mGM-CSF) as primary antibodies and FITC-conjugated goat anti-rat antibody as the secondary antibody. Peaks showing level of protein incorporation were compared against RBCs stained with the secondary antibody alone.

Table 2: Mean fluorescent intensities (MFI) of peaks for the incorporation of GPI-mIL-12 and GPI-mGM-CSF onto RBCs, as shown in Figure 3. Detection of CD58, a cell adhesion molecule inherently expressed on red blood cells, was used as a positive control. The MFI was calculated by subtracting the MFI of the isotype control from that of the protein of interest.

Protein	MFI
GPI-mIL-12	60.2
GPI-mGM-CSF	240.9
CD58	134.36
No protein transfer RBC GPI-mIL-12	3.98
No protein transfer RBC GPI-mGM-CSF	15.68

GPI-mIL-12 and GPI-mGM-CSF incorporation onto CT26 TMVs

To test the incorporation capability of GPI-mIL-12 and GPI-mGM-CSF onto TMVs, increasing amounts of each GPI-anchored protein were incubated for four hours at 37°C with 50 μg of TMVs. Analysis by flow cytometry showed that GPI-mIL-12 and GPI-mGM-CSF are individually able to incorporate in a dose-dependent manner onto CT26 TMVs (Figs. 4 and 5). Compared to GPI-mIL-12, GPI-mGM-CSF showed greater levels of incorporation, possibly as a result of reduced steric hindrance among individual proteins due to the small size of GM-CSF. GPI-mIL-12 and GPI-mGM-CSF were also simultaneously transferred onto the same TMVs at low, middle, and high doses of each protein. In this dual incorporation setting, the levels of incorporation of were significantly greater when compared to the levels of incorporation of GPI-mIL-12 (Fig. 5). For the same amounts of GPI-mIL-12 incubated, the levels of expression indicated by MFI were reduced by more than half in the dual-incorporation setting compared to the levels of expression in the single-incorporation setting (Fig. 5). This again suggests that GPI-mGM-CSF is able to incorporate in more hydrophobic regions of membranes than GPI-mIL-12 due to its smaller molecular size. However, even with GPI-mGM-CSF out-competing GPI-mIL-12, both proteins were able to show incorporation in a dosage-dependent manner, indicating that the two proteins could be simultaneously expressed on TMVs.

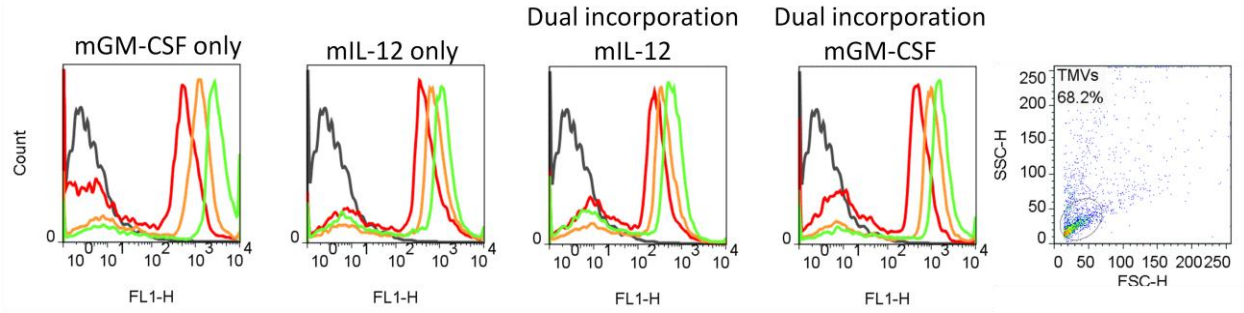

Figure 4: Incorporation of GPI-mIL-12 and GPI-mGM-CSF on CT26 TMVs. Levels of incorporation were detected by flow cytometry using C17.8 and A2/F17-107 as primary antibodies and FITC-conjugated goat anti-rat antibody as the secondary antibody. Peaks corresponding to levels of incorporation were compared against CT26 cells. CT26 TMVs were either incubated with GPI-mIL-12 alone, GPI-mGM-CSF alone, or with both proteins. Red, orange, and green peaks (in order) indicate the level of protein expression after incubation with the lowest, middle, or highest dose of protein.

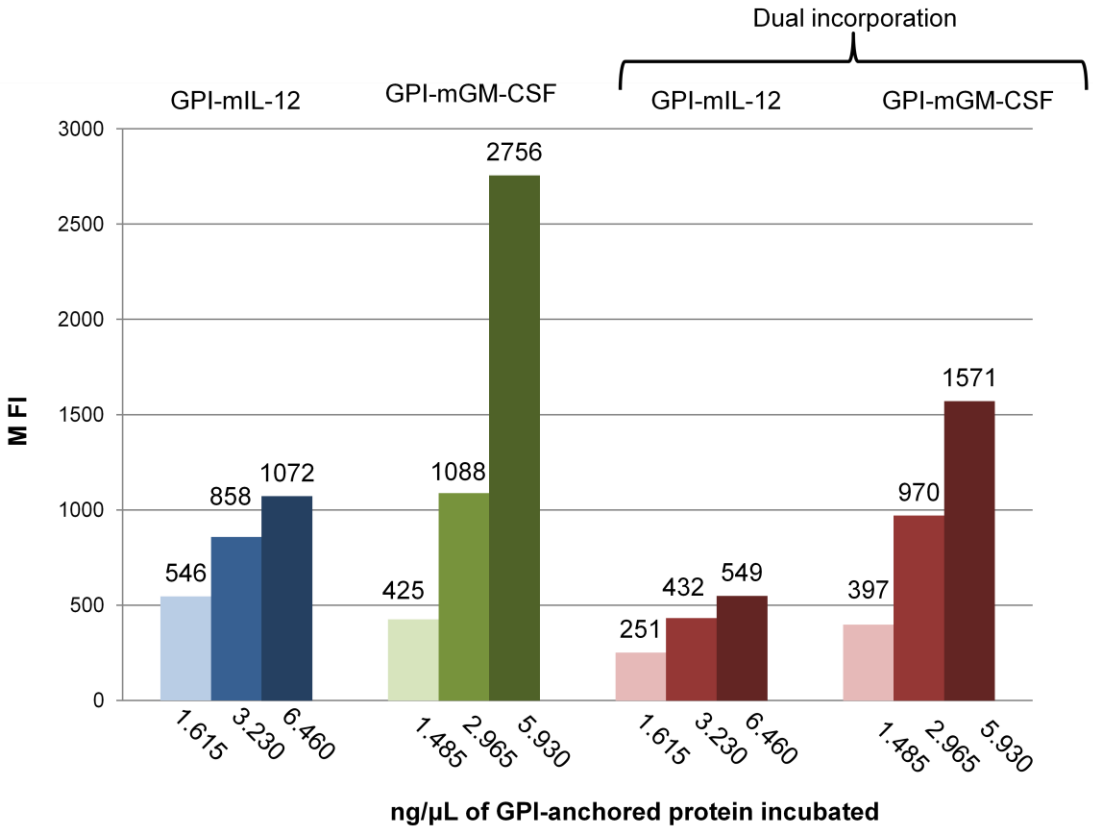

Figure 5: Mean fluorescent intensities (MFI) of peaks for the incorporation of GPI-mIL-12 and GPI-mGM-CSF on CT26 TMVs, as shown in Figure 4. MFIs indicating levels of incorporation of GPI-mIL-12 and GPI-mGM-CSF at different incubation doses are displayed above each bar. MFI of the control (CT26 cells only) was constant for all samples at 12.2. Each MFI was calculated by subtracting the MFI of the isotype control from that of the protein of interest. To test the GPI-anchored proteins' ability to incorporate simultaneously onto the same membrane surface, GPI-mIL-12 and GPI-mGM-CSF were incubated together at different doses (dual incorporation). GPI-mIL-12 at 1.615 ng/µL was incubated with 1.485 ng/µL of GPI-mGM-CSF; GPI-mIL-12 at 3.230 ng/µL was incubated with 2.965 ng/µL of GPI-mGM-CSF; GPI-mIL-12 at 6.460 ng/µL was incubated with 5.930 ng/µL of GPI-mGM-CSF.

Live CT26 cell challenge

To determine the tumorigenicity of CT26 cells, BALB/c mice were challenged with 50,000 or 100,000 live CT26 cells from culture. To ensure that the cells had not lost their tumorigenicity from being passaged too many times, a fresh batch of CT26 cells

was cultured a week before challenge. Subcutaneous injection of both doses of CT26 cells resulted in the formation of observable tumors ten days after challenge (Figs. 6A and B). Statistical analysis by Student's *t*-test comparing average tumor sizes showed no significant difference between the two challenged groups (Fig. 6C). However, challenge with 50,000 CT26 cells showed a longer delay in the development of tumors in all the mice in the group (Fig. 6D). Therefore, subsequent *in vivo* experiments were performed by challenging vaccinated mice with 50,000 CT26 cells. Mouse #2 in the high-dose challenge group failed to show any growth in its tumor size after the development of a palpable tumor on Day 10 (Fig. 6B). This may have been the result of a procedural error during the injection of the CT26 cells.

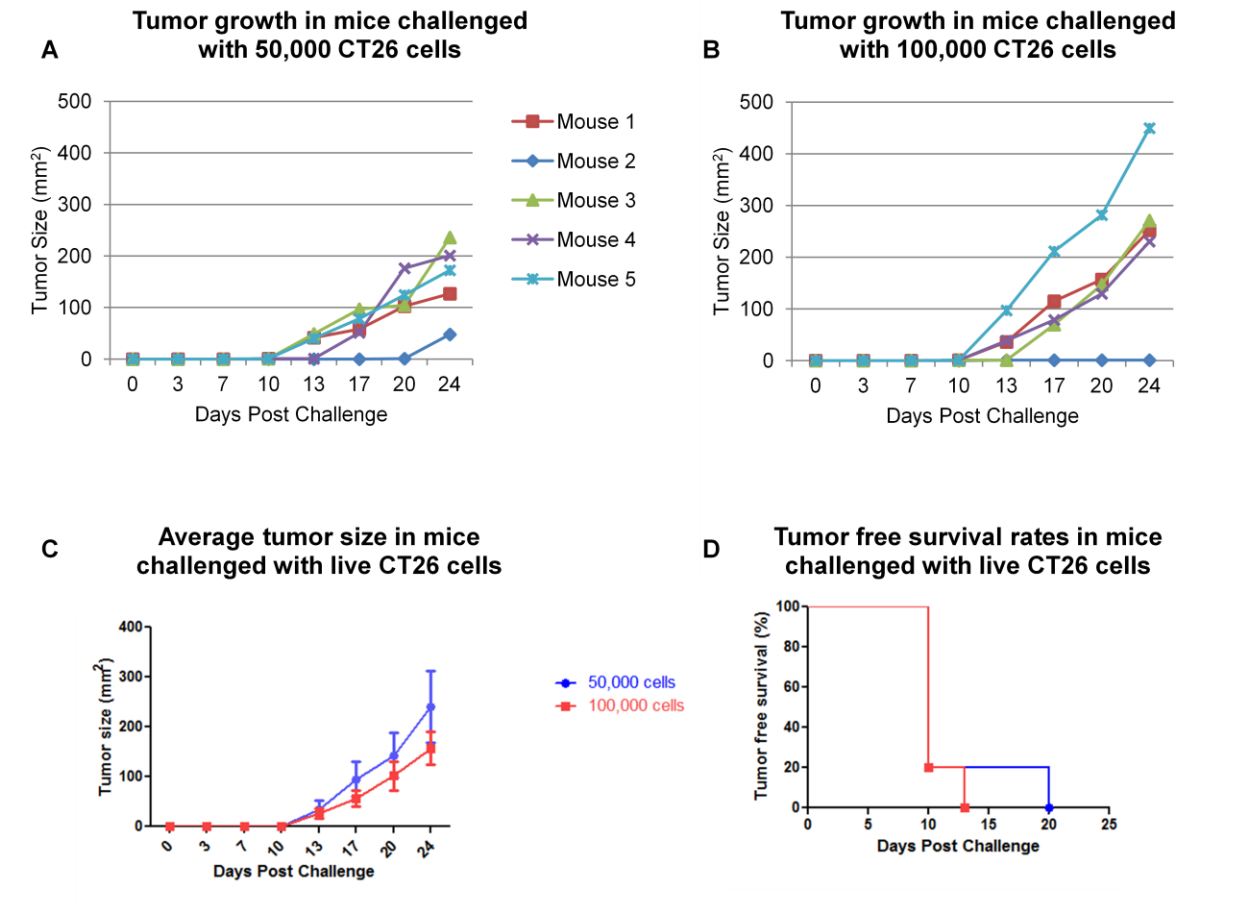

Figure 6: Tumor growth in mice challenged with live CT26 cells. BALB/c mice were challenged subcutaneously with 50,000 (A) or 100,000 (B) CT26 cells. (C) Average tumor size of mice challenged with 50,000 cells compared to average tumor size of mice challenged with 100,000 cells. Analysis by Student's *t*-test showed no statistical significance in the difference between average tumor sizes resulting from challenge with 50,000 or 100,000 CT26 cells ($p > 0.05$). (D) Tumor free survival rate in mice challenged with live CT26 cells.

Vaccination with CT26 TMVs

BALB/c mice were vaccinated with various doses of CT26 TMVs to test the immunogenicity of the TMVs. Experimental groups were given vaccinations of 10 µg, 50 µg, or 100 µg of CT26 TMVs suspended in 100 µL of PBS. The control group was vaccinated with 100 µL of PBS. The initial vaccination was given subcutaneously 21

days before challenge, followed by a booster vaccination 7 days before challenge. Mice were challenged s.c. with 50,000 live CT26 cells.

Vaccination with 100 μg of CT26 TMVs provided the greatest amount of antitumor protection, as indicated by the slowest rate of tumor growth (Fig. 7D). The group of mice vaccinated with 100 μg also had the longest delay in tumor development, though four of five mice in the group ultimately developed tumors (Fig. 7F). However, analysis by Dunnett's post-test revealed no significant differences between the mean tumor sizes of the treatment groups and that of the control group (Fig. 7E). This may have been the result of high variance of tumor sizes in unvaccinated mice, whose tumor sizes ranged between 0 and 287 mm^2 on Day 24 (Fig. 7A). If tumor sizes of mice in the control group had been more uniform, statistical test would have found a significant difference between the mean tumor size in mice vaccinated with 100 μg and the mean tumor size in unvaccinated mice.

Compared to the amount of protection provided by vaccination with 100 μg of CT26 TMVs, vaccination with 50 μg resulted in more moderate rates of tumor growth (Fig. 7C). Because the immunogenic effect of IL-12 and GM-CSF expression on TMVs could be observed and compared more easily if tumors are capable of developing to moderate sizes, the subsequent experiment was performed with vaccinations with 50 μg of CT26 TMVs.

Mouse #2 in the 10 μg vaccination group, Mouse #4 in the 100 μg group, and Mouse #4 in the control group failed to develop tumors throughout the experiment (Figs.7A, B, and D). This may have been due to injection errors during vaccination or challenge.

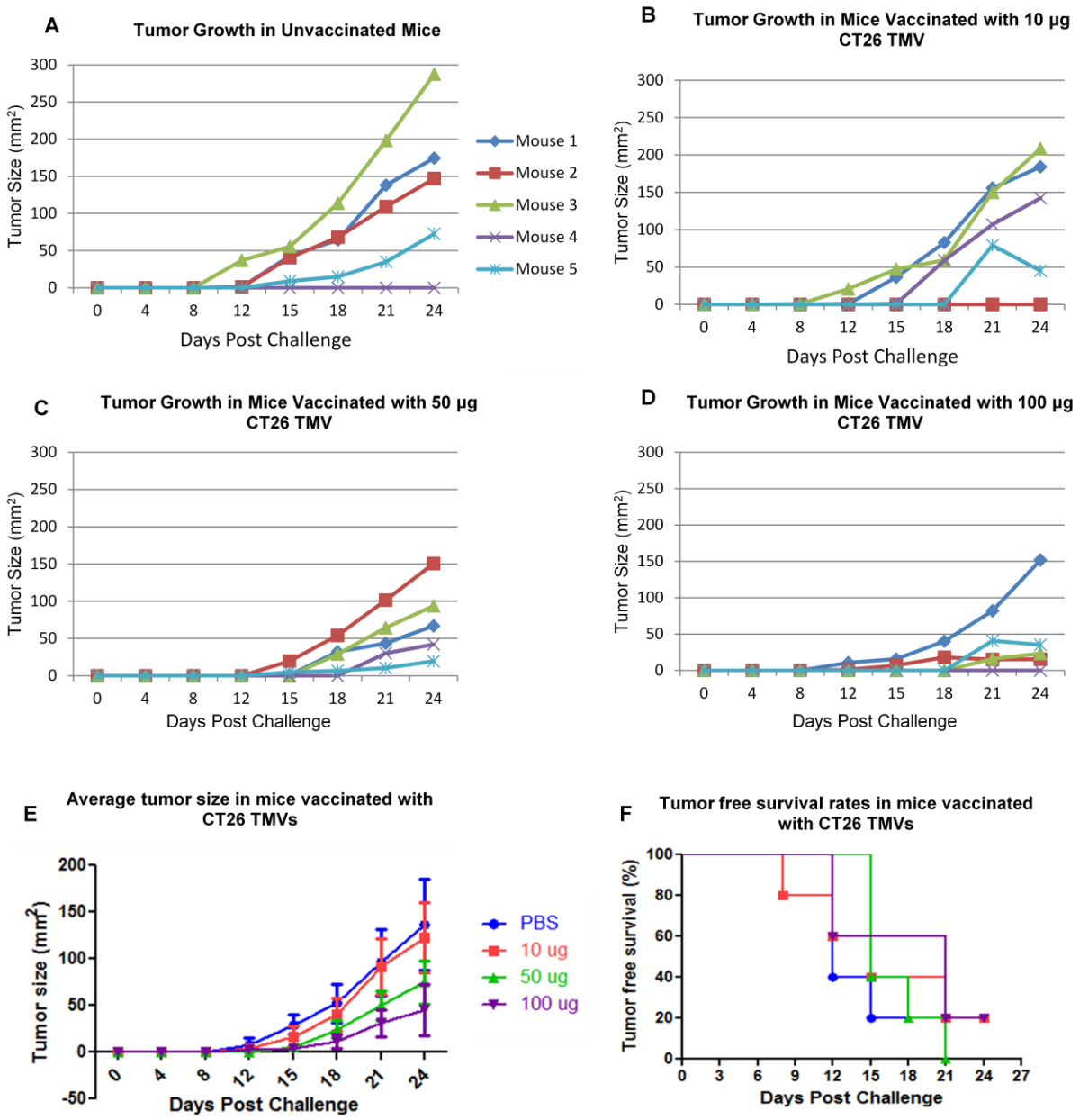

Figure 7: Tumor growth in BALB/c mice vaccinated with (A) PBS only, (B) 10 µg CT26 TMVs, (C) 50µg CT26 TMVs, or (D) 100 µg CT26 TMVs. Mice in all groups were vaccinated 3 weeks and 1 week prior to challenge; mice were challenged with 50,000 CT26 cells. (E) Comparison of average tumor sizes of mice in all vaccination groups by one-way analysis of variance. Comparison of each vaccination group (10 µg, 50 µg, and 100 µg TMVs) to the control group (PBS only) by Dunnett's post-test showed no statistical significance in the difference between the average tumor sizes of the groups ($p > 0.05$). (D) Tumor free survival rate in mice vaccinated with CT26 TMVs.

Vaccination with CT26 TMVs expressing GPI-mIL-12 and GPI-mGM-CSF

The effect of IL-12 and GM-CSF expression on CT26 tumor growth was tested by vaccinating BALB/c mice with 50 µg of CT26 TMVs expressing GPI-mIL-12, GPI-mGM-CSF, or both. The same vaccination schedule was followed as in the previous experiment.

Comparing the tumor growth in individual mice of the vaccinated groups to the tumor growth in mice of the unvaccinated group, the expression of GPI-mIL-12 and GPI-mGM-CSF on CT26 TMV surfaces had a very limited effect on CT26 tumor growth (Fig. 8). Mean tumor sizes in each vaccination group was compared with the mean tumor size of the unvaccinated control group by Dunnett's test. The statistical test found no significant difference between the mean tumor size in any of the treatment groups and the mean tumor size in the control group (Fig. 9A). The mean tumor growth curves of all the control and treatment groups overlapped to a great extent, indicating that the vaccination did not have significant immunogenic effects in mice. All groups had high incidence rate, and none of the vaccinations were able to provide complete protection from tumor growth (Fig. 9B).

Some mice in the control group, CT26-GPI-mIL-12 TMV vaccinated group, and CT26-GPI-mGM-CSF TMV vaccinated group had to be sacrificed before the trial end date due to the development of ulcerated tumors (Figs. 8A, C, and D). Mouse #3 in the control group failed to develop any tumors, most likely due to injection errors (Fig. 8A).

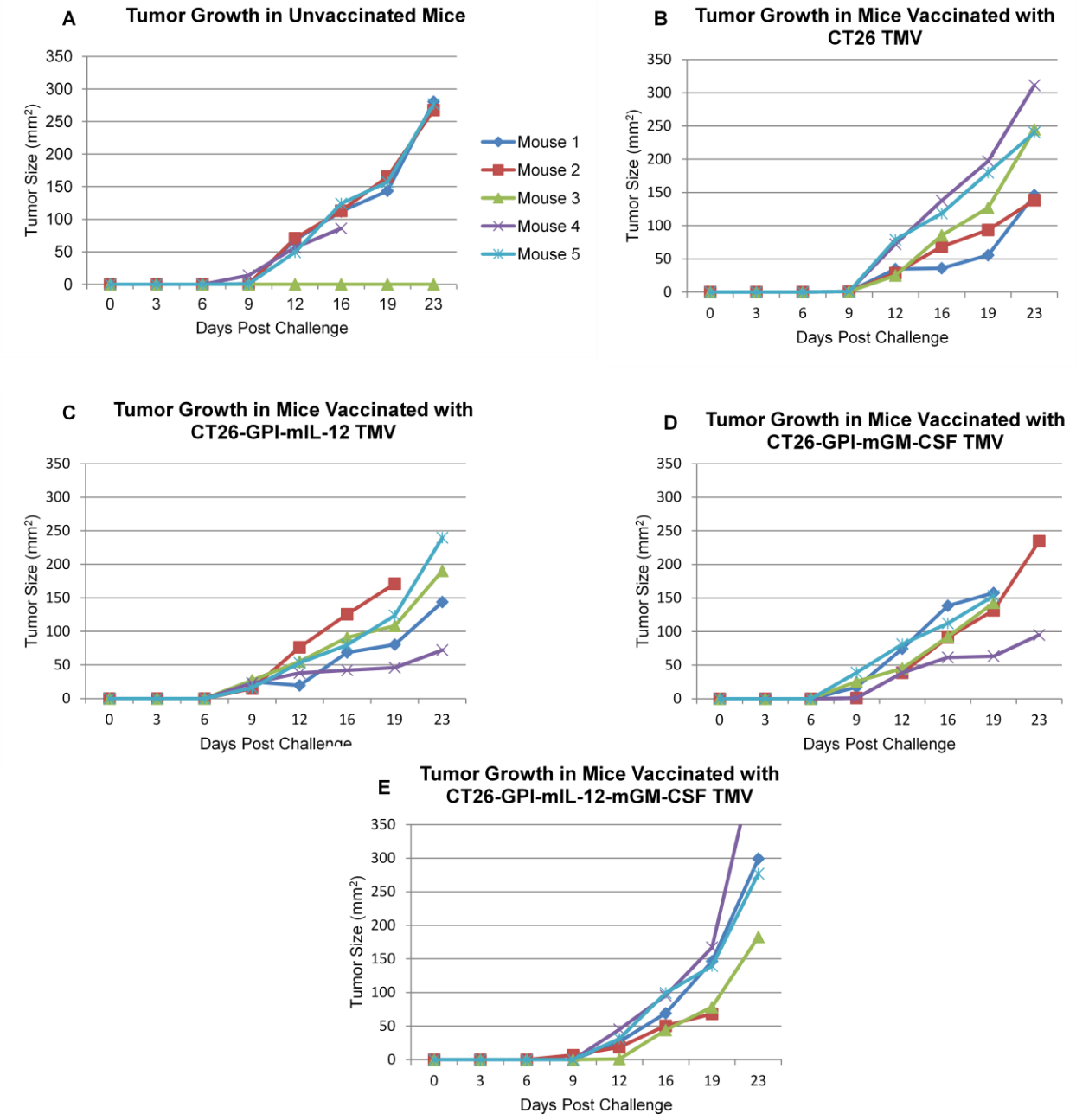

Figure 8: Tumor growth in BALB/c mice vaccinated with (A) PBS only, (B) 50 µg CT26 TMVs, (C) 50µg CT26-GPI-mIL-12 TMVs, (D) 50 µg CT26-GPI-mGM-CSF TMVs, or (E) 50 µg CT26-GPI-mIL-12-mGM-CSF TMVs. Mice in all groups were vaccinated 3 weeks and 1 week prior to challenge; mice were challenged with 50,000 CT26 cells.

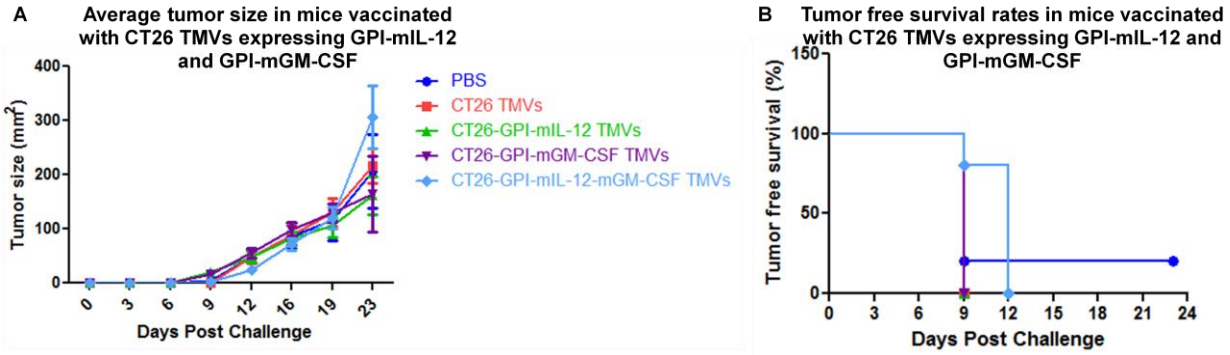

Figure 9: (A) Comparison of average tumor sizes of mice vaccinated with CT26 TMVs expressing GPI-mIL-12 and GPI-mGM-CSF by one-way analysis of variance. Comparison of each vaccination group (Fig. 8B-E) to the control group (Fig. 8A) by Dunnett's post-test showed no statistical significance in the difference between the average tumor sizes of the groups ($p > 0.05$). (B) Tumor free survival rate in mice vaccinated with CT26 TMVs expressing GPI-mIL-12 and GPI-mGM-CSF.

Production of anti-CT26 antibodies by vaccinated mice

The production of antibodies specific for CT26 tumor antigens in mice vaccinated with TMVs expressing GPI-mIL-12 and GPI-mGM-CSF was tested by flow cytometry analyis. Antibody production was tested by staining blood serum collected prior to the initial vaccination and serum collected 21 days after the vaccination (prior to challenge) on CT26 cells (Fig 10). For each group of mice, mean fluorescent intensities of blood serum collected prior to challenge was plotted against tumor sizes measured 19 days post challenge to find correlations between antibody production and tumor size (Fig. 12). Tumor sizes from Day 19 were plotted because a large number of mice had to be sacrificed on that day due to the development of ulcerated and over-grown tumors.

Mice vaccinated with CT26-GPI-mGM-CSF TMVs showed the highest increase in the level of antibody production against CT26 tumor antigens in blood serum

collected prior to the initial vaccination and serum collected prior to challenge (Fig. 10 and 11). Mice vaccinated with CT26-GPI-mIL-12-mGM-CSF TMVs did not show a similar amount of increase in antibody production, suggesting that the expression of GPI-mIL-12 on the TMV vaccine may have inhibited antibody production. No correlation was found between the levels of antibody production and tumor sizes in unvaccinated mice or in mice vaccinated with CT26-GPI-mIL-12, CT26-GPI-mGM-CSF, or CT26-GPI-mIL-12-mGM-CSF (Figs. 12A, C-E). Interestingly, levels of antibody production positively correlated with tumor sizes in mice vaccinated with unmodified CT26 TMVs (Fig. 12B). However, further investigation was not performed to explore a causality relationship between antibody production and tumor size.

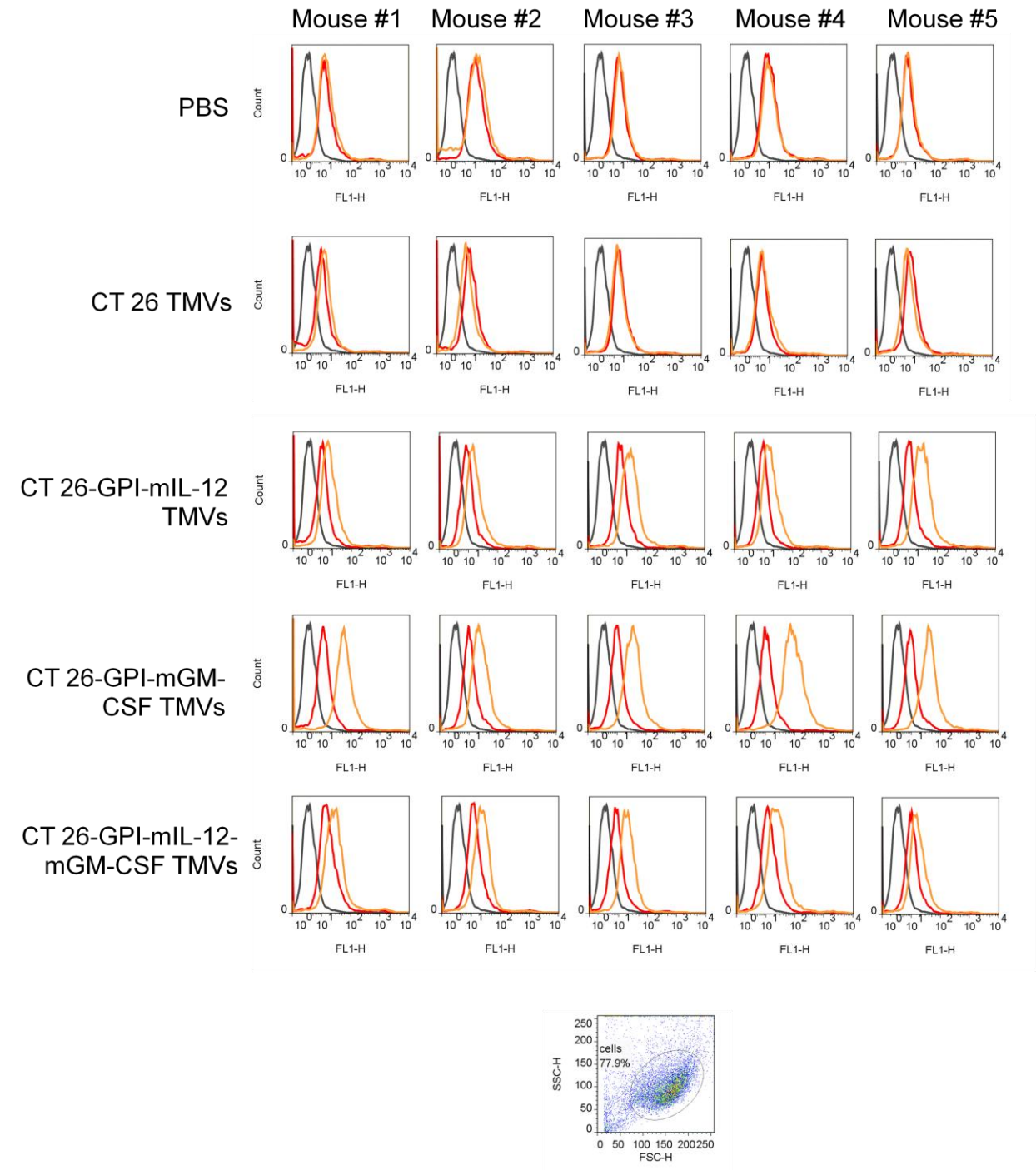

Figure 10: Flow cytometry analysis for production of antibodies against CT26 cells in the blood serum of BALB/c mice vaccinated with CT26 TMVs expressing IL-12 and GM-CSF. Each row shows antibody production in five mice from each vaccination group of the experiment. Blood serum was collected before the initial vaccination (red peaks) and before challenge with 50,000 live CT26 cells (orange peaks). Each serum was stained on CT26 cells at 1:10 dilution; antibody binding was detected using FITC-conjugated goat antimouse. Black peaks are isotype control, obtained from staining CT26 cells with FITC-conjugated goat antimouse only, with no serum.

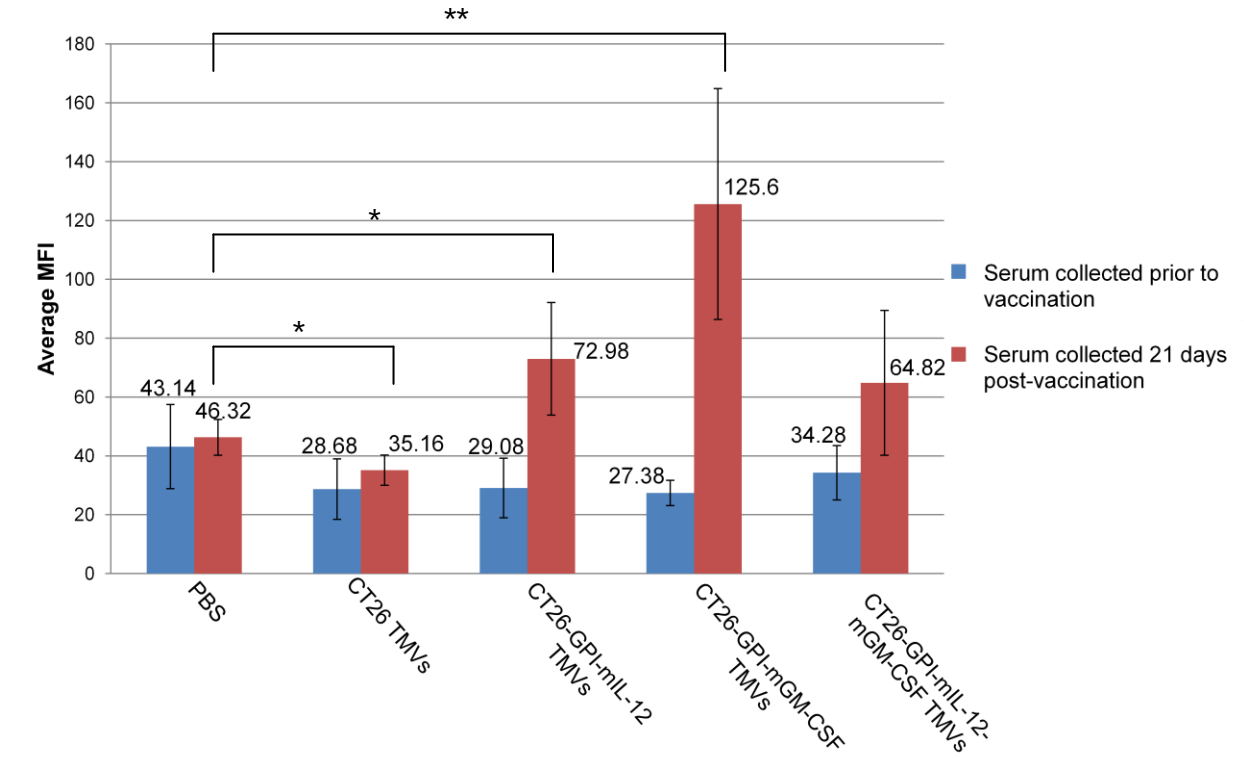

Figure 11: Average mean fluorescent intensities (MFI) of peaks for production of antibodies specific against CT26 tumor antigens in vaccinated mice, as shown in Figure 20. MFIs indicating levels of antibody production in each control and experimental group are displayed above each bar. Antibody production 21 days post-vaccination in each treatment group was compared against antibody production 21 days post-vaccination in the unvaccinated control group (PBS) and analyzed for statistical significance using unpaired Student's *t*-test with unequal variance. * indicates p-value < 0.05, ** indicates p-value <0.01.

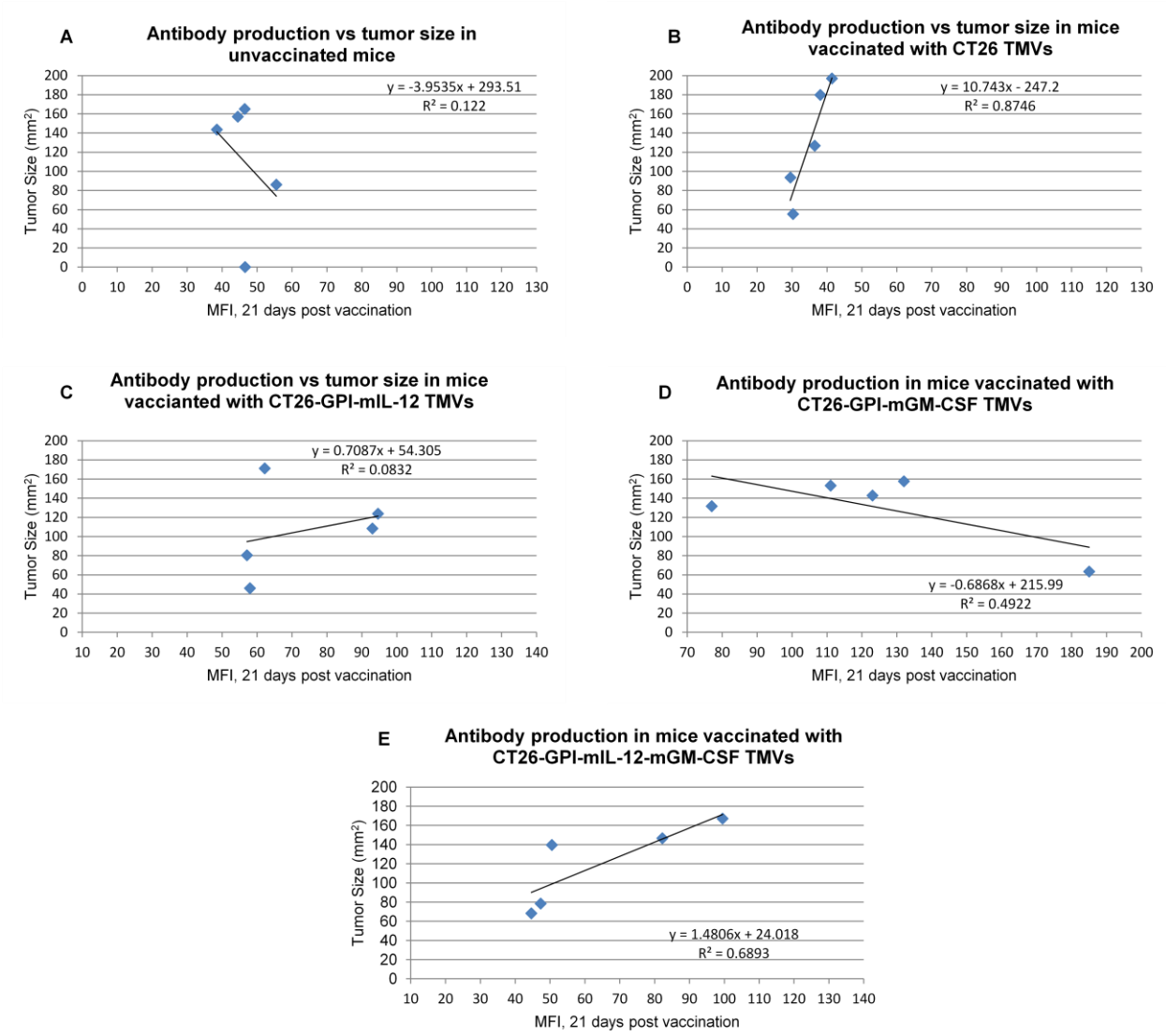

Figure 12: Level of production of antibody against CT26 cells in each mouse plotted against the tumor size developed in that mouse. Level of antibody production was plotted as MFI of blood serum collected 21 days post vaccination against live CT26 cells, as shown in Fig. 10. Tumor size was plotted as area measured in mice 19 days after challenge. MFI and tumor size were plotted for each group – (A) unvaccinated mice, (B) mice vaccinated with CT26 TMVs, (C) mice vaccinated with CT26-GPI-mIL-12 TMVs, (D) mice vaccinated with CT26-GPI-mGM-CSF TMVs, (E) mice vaccinated with CT26-GPI-mIL-12-mGM-CSF TMVs. Each plot shows a line of best fit and the coefficient of determination (R^2) for the equation of the line.

DISCUSSION

CT26 immunoescape through expression of surface molecules

Flow cytometry analysis of surface molecules expressed on the CT26 cell line showed low levels of expression of PD-L1, CD47, ICAM-1, and HSA (Fig. 1). Analysis of such markers expressed on CT26 cells provides insight into the various mechanisms by which CT26 colon cancer cells escape immunosurveillance. PD-L1, or programmed cell death ligand 1, is a transmembrane protein which binds to the PD-1 receptor expressed on T cells to inhibit T cell activation. Expression of PD-L1 on tumor cells can enhance their tumorigenicity by escaping tumor-specific cell-mediated cytotoxicity by CTLs [38]. In this way, low level of PD-L1 expression on CT26 cells may make the tumor cells less susceptible to cell-mediated cytotoxicity.

While PD-L1 inhibits antitumor immune response, antigens such as HSA, MHC class I, and B7-1 expressed on CT26 cells have an immunostimulatory effect that may suppress tumorigenicity. HSA, or heat stable antigen, is a cell adhesion molecule which stimulates T cell proliferation and tumor-specific cell-mediated cytotoxicity [39]. Expression of HSA may be downregulated on CT26 cells to allow the tumor cells to escape recognition and killing by CTLs.

Moderate levels of MHC class I and B7-1 expression were not expected since both molecules are immunostimulatory. MHC class I presents peptides derived from tumor-associated antigens on the surface of tumor cells, which may be recognized by T cell receptors and activate antitumor immune response [40]. Increased levels of MHC class I expression may leave tumor cells more susceptible to recognition by immune cells and lead to cytotoxic killing of CT26 cells. Similarly, normal B7-1 expression

provides co-stimulatory signals for the activation of T cells when bound to CD28. Low levels of B7-1 favor the binding of immunoinhibitory molecule CTLA-4 to B7-1, resulting in the blocking of T cell activation. Studies have reported that CT26 cells escape immunosurveillance through low levels of B7-1 expression [36]. The CT26 cell line had an altered cell surface profile, suggesting that the cells may have been less tumorigenic than those used in other studies.

CD47 has both stimulatory and inhibitory effects on immune cells. CD47 is an integrin-associated protein expressed on macrophages, T cells, and B cells, and it regulates the proliferation, differentiation, and death of these cells. The binding of thrombospondin ligands to CD47 expressed on T cells results in T cell death due to the inhibition of protein kinase A [41]. CD47 have also been found to mediate the death of breast cancer cells in a similar manner through the binding of ligands to CD47 receptors expressed on tumor cell surfaces [42]. Such studies show that the expression of CD47 on CT26 cell surfaces may limit the tumorigenicity of CT26 cells if increased levels of the thrombospondin ligands are present in the tumor environment. On the other hand, CD47 acts as a “don’t eat me” signal against phagocytosis by macrophages. In this context, the expression of CD47 on CT26 tumors may aid with tumor growth by allowing tumor cells to escape elimination by phagocytosis [43].

Membrane incorporation of GPI-anchored protein via protein transfer

Purified GPI-mIL-12 and GPI-mGM-CSF incorporated onto sheep red blood cell and CT26 TMV surfaces, supporting findings by many other studies on membrane incorporation via the GPI-anchor [15], [16], [17]. GPI-anchor modification and the

protein transfer method helped expedite the preparation of TMV vaccines expressing GPI-mIL-12 and GPI-mGM-CSF on the membrane surface, requiring a simple 4-hour incubation process. In addition, the level of expression of GPI-mIL-12 and GPI-mGM-CSF could be easily controlled through the concentration of the GPI-anchored proteins incubated with TMVs (Figs. 3 and 5). The short incubation time required for expression, the ability to incorporate multiple proteins, and the dose-dependent incorporation of GPI-anchored proteins make the protein transfer method a clinically applicable procedure for delivering cytokines to the target site. In further experiments, the expression of IL-12 and GM-CSF on TMV surfaces could be easily increased by incubating higher concentrations of GPI-mIL-12 and GPI-mGM-CSF in the protein transfer process to prepare the vaccines.

Limited efficacy of TMV vaccines in prophylactic setting

Statistical analysis of data on tumor size in this experiment failed to show significant decrease in tumor size as the concentration of TMVs vaccinated increased (Fig. 7E). Comparing tumor sizes in each treatment group to the unvaccinated control group, analysis by Dunnett's test returned p-values greater than 0.05 for all treatment groups. Lack of statistical significance, however, most likely resulted from the high variance in tumor sizes in the unvaccinated control group (Fig. 7A). Comparison of tumor sizes in individual mice between each group, especially between the unvaccinated group and the group vaccinated with 100 μg of CT26 TMVs, clearly shows a reduction in the tumor sizes that developed in mice vaccinated with TMVs (Fig. 7A, 7D). This suggests that

TMVs are immunogenic and supports the futures study of TMVs as potential antitumor vaccines.

The expression of GPI-mIL-12 and GPI-mGM-CSF were not able to further enhance the immunogenicity of CT26 TMVs in mice. The high tumor incidence in all groups vaccinated with CT26 TMVs expressing GPI-mIL-12 and GPI-mGM-CSF suggest a limitation in the immunogenicity of the vaccines (Fig. 9B). Statistical analysis using Dunnett's test also showed no significant difference between the tumor sizes in vaccinated mice and tumor sizes in unvaccinated mice (Fig. 9A). None of the mice showed complete rejection of tumor growth, indicating that prophylactic vaccination with 50 μg of CT26 TMVs expressing GPI-mIL-12 and GPI-mGM-CSF does not confer complete immunity against tumor growth (Fig. 8). Follow-up experiments should vaccinate with higher doses of TMVs or higher levels of GPI-mIL-12 and GPI-mGM-CSF incorporation to test the immunogenicity of the vaccine at higher concentrations.

Although the abilities of GPI-mIL-12 and GPI-mGM-CSF to stably incorporate onto CT26 TMVs were tested at various concentrations of the proteins (Fig. 5), analysis by both Western blot and ELISA was unable to quantify the expression of GPI-mIL-12 and GPI-mGM-CSF on CT26 TMVs used in the actual vaccinations. Without any data to support the expression of GPI-mIL-12 and GPI-mGM-CSF on CT26 TMV vaccines, it may be possible that the expected immunogenic effect of GPI-mIL-12 and GPI-mGM-CSF expression was not observed due to the lack of stably incorporated GPI-anchored proteins on the TMVs.

The expression of IL-12 and GM-CSF were expected to enhance antitumor immune response because of their roles in increasing DC and CTL activity. IL-12 is

involved in the activation of CD4+ T cells into Th1 helper cells, which induces increased secretion of IL-12 and IFN-γ by macrophages [22]. In addition, IL-12 secreted by DCs activates CTLs, which are heavily involved in antitumor immune response [11]. IL-12 binding to receptors on DC surfaces allows DCs to transmit “Signal 3” to CD8+ T cells, inducing their activation into CTLs. The transmission of this signal by DCs further requires direct antigen uptake by the DCs, which may be facilitated by vaccinations using TMVs that present tumor-associated antigens on its surface [9], [10].

GM-CSF, on the other hand, has been known to induce the production of granulocytes and monocytes, which later differentiate into macrophages and DCs [29]. Clinical trials have found that administration of GM-CSF resulted in a threefold increase in the number of circulating DCs [44]. DCs play a key role in cell-mediated cytotoxicity by priming CTLs to recognize tumor-associated antigens. CTL activity may be further enhanced by GM-CSF-binding on CTL receptors, contributing to cell-mediated cytotoxicity in antitumor immune response [33].

Lack of correlation between antibody response and tumor size

Analysis of the level of antibody production against CT26 cells in mice blood serum did not show any correlation between the level of antibody production and tumor growth (Fig 12). Some mice vaccinated with CT26-GPI-mGM-CSF TMVs or CT26-GPI-mIL-12-mGM-CSF TMVs developed the smallest tumors in their groups and had the highest levels of anti-CT26 antibody production (Figs.8 and 10). However, it is difficult to evaluate the significance of antibody production in antitumor response. Studies have reported that antibody-secreting B cells inhibit antitumor immune response through IL-

10 secretions by downregulating DC activity and MHC class I expression on tumor cells [45]. In addition, antibody-dependent cell cytotoxic responses become ineffective once a tumor cell alters its surface marker profile.

Comparison of mean levels of antibody production among different vaccination groups showed that mice vaccinated with CT26-GPI-mGM-CSF TMVs produced more antibodies against CT26 cells than mice vaccinated with CT26-GPI-mIL-12 or CT26-GPI-mIL-12-mGM-CSF TMVs (Fig. 11). Lower levels of antibody secretion in mice vaccinated with TMVs expressing GPI-mIL-12 could be contributed to the role of IL-12 in blocking Th2 activation, which is involved in B lymphocyte activation [22]. The inhibitory effect of IL-12 on B cell activation may have resulted in decreased levels of antibody production in these mice compared to mice vaccinated with TMVs only expressing GPI-mGM-CSF.

No significant correlation between antibody production and tumor sizes were found overall in different vaccination groups (Fig. 12). This suggests that antitumor immune response is enhanced by increasing cell-mediated cytotoxicity against tumor cells rather than antibody-dependent cell cytotoxicity, which may become ineffective when tumor cells mutate and change surface antigen profiles. In contrast, cell-mediated cytotoxicity relies on the activation of CTLs specific for the tumor cells developed in the host.

CONCLUSION

The results from these experiments provide initial insight into the immunogenic effect of combined expression of GPI-mIL-12 and GPI-mGM-CSF in a murine cancer model. Although reduction in tumor sizes in mice vaccinated with TMVs expressing GPI-mIL-12 and GPI-mGM-CSF was observed, difference in mean tumor size from that observed in unvaccinated mice was not statistically significant (Fig. 9A). To more clearly determine the effect of expression of GPI-mIL-12 and GPI-mGM-CSF on colon cancer, the dose of vaccinated TMVs and the expression of the GPI-anchored proteins on TMV surfaces must be increased in future experiments. Moreover, further investigation is required to determine which immune cells are affected by the expression of GPI-mIL-12 and GPI-mGM-CSF. T cell proliferation assays may illuminate the role of CD4+ T cells in eliciting antitumor responses; cytotoxic T lymphocyte assays should also be performed to determine the involvement of CD8+ T cells.

This study was also limited to the effect of GPI-mIL-12 and GPI-mGM-CSF expression in a prophylactic setting, in which mice were vaccinated 21 and 7 days before challenge. The prophylactic experiment is necessary to establish the ability of a vaccine to confer complete immunity against colon cancer. However, the clinical applicability of cytokine therapy combined with TMV vaccinations cannot be determined from the present study alone. More clinically relevant system should be studied with therapeutic vaccinations, in which mice are vaccinated after challenge with CT26 cells. Tumor rejection in vaccinated mice in a therapeutic setting would suggest the vaccine's efficacy in a more realistic clinical setting, in which patients are treated with the vaccine after the detection of a developed tumor. Future experiments with the above

procedures and considerations are necessary to illuminate the extent and mechanisms of the vaccine’s immunogenicity.

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