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Protein transfer-mediated modification of virus-like particles and plasma membrane vesicles: A means to adjuvant and deliver tumor antigens

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Doctor of Philosophy

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ABSTRACT

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The optimal size and particulate nature of nano and microparticles make them ideal antigen delivery vehicles for immune activation. Encapsulation or surface attachment of antigen to these particles by chemical means has shown to enhance antigen-specific immune responses compared to administration of soluble antigen. However, chemical modifications can alter antigen immunogenicity and modify the particulate structure leading to decreased antigen-specific immunity. Herein, we propose a protein transfer method to modify lipid-based nano and microparticle surfaces with antigen and immunostimulatory molecules (ISMs). By protein transfer, glycosylphosphatidylinositol (GPI)-anchored forms of antigen and/or ISMs were incorporated stably onto influenza H5 virus-like particles (VLPs) or tumor-derived plasma membrane vesicles (PMVs) within 4 h. Multiple GPI-anchored proteins could be incorporated onto the particles simultaneously, and the incorporated proteins remained functional. Vaccination with GPI-GM-CSF-incorporated-VLPs led to enhanced VLP and virus-specific T helper 1 (Th1) and T helper 2 (Th2)-type IgG responses compared to unmodified VLPs as well as complete protection against a heterologous H5N1 viral challenge unlike vaccination with unmodified VLPs. Further, vaccination using influenza VLPs incorporated with a GPI-anchored form of tumor associated antigen, HER-2, led to enhanced protection against a HER-2-expressing murine breast cancer cell line compared to vaccination with corresponding levels of GPI-HER-2 alone. HER-2-specific antibody production characteristic of a Th1 and Th2-type immune response was also elevated in these mice. Similarly, vaccination with protein transferred-GPI-HER-2-PMVs also increased HER-2-specific Th1 and Th2-type immune responses and enhanced protection against live cell challenge of two murine breast cancers that express HER-2. Additionally, inclusion of GPI-IL-12 and GPI-B7-1 along with GPI-HER-2 on the PMVs by protein transfer led to complete protection against HER-2-expressing D2F2/E2 tumors in a prophylactic setting and delayed tumor growth with partial regression in a therapeutic setting.

These studies suggest that protein transfer can be used to modify the surfaces of VLPs and PMVs to enhance the inherent particle immunogenicity, and to enhance immunogenicity against exogenously incorporated tumor antigens. These results have implications in enhancing the potency of particle-based vaccines against influenza viruses and the efficacy of protein-based vaccines against cancer.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter I

Chapter II

Chapter III

Chapter IV

CHAPTER I:

Introduction

Excerpts taken from the following publication:

Lipid-mediated Cell Surface Engineering. Micro- and Nano Engineering of the Cell Surface, 2014. Chapter 6: Pages 121-141 (ISBN:978-1-4557-3146-6). (Edited by Weian Zhao and Jeffrey M. Karp, Elsevier Publications)

Cell membranes

The term "cell" was first coined in 1665, in Robert Hooke's *Micrographia* (1). Since then, breakthroughs in the field of cellular biology have given us not only a tremendous understanding of the cell and its components but also the ability to modify it through various means. In particular, our understanding of the cell membrane structure, its role in cellular function, molecules expressed on it and their function have enabled us to engineer the cell surface for desired purposes.

In 1972, S.J. Singer and Garth L. Nicholson proposed the fluid mosaic model for cell membranes (2).The fluid mosaic model represents the cellular membrane as a fluid phospholipid bilayer decorated with proteins and oligosaccharides. This model also places an emphasis on the vast variety of structures that the phospholipid bilayer consists of, breaking the components down into three major categories: proteins, lipids, and oligosaccharides. Additionally, the lipids of the cell membrane exist as separate domains called lipid rafts. Lipid rafts are highly enriched with sphingolipids, complex glycosphingolipids and cholesterol (3-6), and they play a vital role in cell membrane functions.

Cell membranes have been associated with a variety of cellular functions. Not only do they provide a barrier between interior cellular components and extracellular space, but cell membranes also are important for cell-to-cell communication and effector functions of the immune cell. Adhesion receptors found on the cell surface help to mediate these cellular communications as well as allow for targeted delivery of effector molecules. Additional cell surface receptors also bind to growth factors and cytokines allowing signals to be sent from outside the cell to inside further permitting

communication between different cell types. These receptors selectively mediate communication between the extracellular and intracellular environment since not all molecules can pass easily through the cell membrane. These selective signals delivered by cell surface receptors determine cell survival, function, and in general, cell fate. Other cell surface expressed proteins, such as major histocompatibility complex class I (MHC I), also display internal peptide antigens on the cell surface facilitating immune surveillance by immune cells. This immune surveillance component of the cell membrane becomes important during infections, such as viral infections, as it is involved in detection and termination of sick and infected cells. Therefore, the cell membrane plays a vital role in determining the fate of the cell during disease state.

Modification of cell membranes

Consequently, cell membrane surfaces can be engineered to manipulate cell functions. Cell surface modifications serve as useful tools for a variety of applications such as targeting cells for drug delivery, activation and amplification of immune cell responses, studying receptor functions, and development of cellular vaccines for tumor immunotherapy.

Genetic engineering techniques have been used to modify cell surfaces. These genetic engineering methods involve gene transfection in which the gene encoding the protein of interest is taken up within cells so that the cells translate and express the molecule of interest on the cell surface. Although this process is successful in leading to protein surface expression, many drawbacks exist: development of a culturable cell line or the availability of a large number of cells is needed, difficulty in transfecting many cell

lines, the requirement of viral vectors, the amount of protein expressed by the cells is not controllable, and it is a time-consuming process. To counteract these drawbacks, surface engineering methods to exogenously place proteins on cell membranes have been developed. This phenomenon that decorates cell surfaces is known as ‘protein transfer’ or ‘protein painting’ (7).

Protein transfer modification of cell membranes

One means of protein transfer involves attaching a lipid hydrophobic anchor to a protein, and then exogenously adding the modified protein to the cells of interest. Exchange between cholesterol derivatives found in lipid suspensions with those in erythrocyte ghost membranes have been detected upon incubation for 24 h (8, 9), therefore cholesterol-tethering has been used in protein transfer to incorporate exogenously added proteins onto lipid bilayers of cells (Figure 1A). Further, palmitoylation, a process that involves the lipid modification of proteins in which a hydrophobic fatty acid palmitate group is added covalently to the protein of choice (10), has been used to increase the hydrophobicity of proteins and allow for incorporation into lipid bilayers (Figure 1B). The addition of hydrophobic sequences from transmembrane anchored proteins to superantigens has also resulted in antitumor immune responses from the incorporation of the chimeric protein onto cell membranes (11). Other systems include a two-step method in which a scaffold is incorporated into cell membranes through lipid-interactions and this scaffold in turn binds to the protein-of-interest thus attaching the protein-of-interest indirectly to the cell surface (12, 13). Such lipid-mediated surface engineering techniques allow for the anchored protein to be

spontaneously incorporated into the cell membrane, and into lipid rafts in particular (14), thus permitting the cell surface to be decorated by the protein in a controllable and time efficient manner without any genetic modification. Lipid-mediated protein transfer methods are quick and efficient ways to engineer cell membrane surfaces resulting in controllable expression of functional proteins.

Although many advantageous methods exist to exogenously incorporate proteins onto cell surfaces by lipid-mediated protein transfer, limitations also occur. The production of cholesterol-tethered biomolecules is not an easy task; it involves many chemical additions and the linkage between the biomolecule and cholesterol derivative is vital for incorporation and ligand binding. Moreover, lipids present in circulation may bind to these administered conjugates and block incorporation onto the defined target cells. The degree of palmitoylation is also important for functional antigen binding as well as expression of functional protein. Increasing palmitoylation led to decreased ability of the incorporated antibody to bind antigen (15), and since palmitoylation occurs non-specifically throughout the protein, the protein functionality can also be affected. Therefore, we have proposed to use a different lipid-mediated protein transfer method to modify cell membrane surfaces that involve the use of glycosylphosphatidylinositol (GPI)-anchors attached to proteins (Figure 1C).

Surface Engineering with GPI-anchored Proteins

Proteins are expressed on the membrane of a cell through multiple mechanisms. Aside from commonly found transmembrane anchored proteins, lipid anchored proteins also exist. Lipid anchoring of proteins via glycophosphatidyl inositol (GPI) is predicted

to be found in 460 proteins (16). The GPI-anchor which anchors proteins to the outer leaflet of the cell membrane does not span the entire lipid bilayer as transmembrane proteins do (3). This anchor consists of two or three hydrophobic fatty acid chains that insert into the outer leaflet of the lipid bilayer exposing the anchored proteins to the outside environment. The core structure of the GPI-anchor is conserved in eukaryotes and consists of a phosphoethanolamine group, followed by a glycan region, followed by a phospholipid tail. The phosphoethanolamine group is covalently attached to the C-terminal end of the GPI-anchored protein (GPI-AP) by an amide bond. The conserved GPI backbone structure is "EtN-P-Man-Man-(EtN-P)Man-GlcN-PI, where EtN is ethanolamine, P is phosphate, Man is mannose, GlcN is glucosamine, and PI is phosphatidylinositol. The glycan region can vary between species and proteins via side chain modifications; however the core backbone is conserved (17, 18). Inositol attaches the glycan region to the phospholipid tail, which consists of two long fatty acid chains that are either saturated or unsaturated, and vary in length. This phospholipid tail is responsible for inserting into the lipid bilayer and anchoring the GPI-AP to the cell surface. In addition to serving as a membrane anchor, other functions of the GPI-anchor include signal transduction, membrane trafficking, and targeting proteins to lipid rafts (17).

The discovery and purification of the enzyme phosphatidylinositol phospholipase C (PI-PLC) from the bacteria, *Bacillus cereus*, in 1976 (19) further led to the discovery of the GPI-anchor as a means for membrane anchoring proteins. PI-PLC treatment led to the release of many proteins from tissues, thus suggesting that these proteins are anchored via a phosphatidylinositol group. Moreover, phase separation with Triton X-114 (TX-114)

can be used to characterize GPI-APs (20). At 0˚C, the nonionic detergent TX-114 forms a homogenous mixture with GPI-APs and an aqueous solution, however at 37˚C, TX-114 separates away from the aqueous phase. GPI-APs are found in the TX-114 phase whereas other transmembrane and soluble proteins are found in the aqueous phase; therefore TX-114-mediated phase separation also verifies the presence of a GPI-anchor (20). Upon further characterization, the structure of the GPI-anchor found in many GPI-APs became evident. However, some GPI-APs are resistant to PI-PLC and phospholipase D cleavage. These GPI-APs contain an extra fatty acid chain (palmitate) attached to the inositol ring, thus they have a total of three fatty acid chains anchoring the protein to the cell membrane (21).

The addition of the GPI-anchor is a post-translational modification. First, proteins destined to become GPI-anchored are translated in the endoplasmic reticulum (ER) with a hydrophobic amino acid GPI-anchor signal sequence. This GPI-anchor signal sequence, located at the C-terminal end of the protein inserts into the ER membrane and immediately gets cleaved. Upon cleavage, the GPI-anchor which is biosynthesized on the outer leaflet of the ER, flips to the luminal side and attaches to the C-terminal end of the newly cleaved protein via transamidination. Then the GPI-anchored protein is transported to the Golgi where further fatty acid remodeling occurs (18). Finally the GPI-AP is translocated to the outer leaflet of the cell membrane. In polarized epithelial cells, the GPI-anchor localizes the GPI-AP at the apical end of the cell. This was believed to be due to the association of the GPI-anchor with lipid rafts, however recently it has been shown that the presence of N-glycans and cholesterol plays a role in targeting the GPI-AP to the apical end (22).

Although GPI-APs are stably expressed on cell surfaces, transfer of GPI-APs has been detected between cell membranes *in vivo*. Kooyman et al. (23) demonstrated that the complement regulatory GPI-APs, human CD59 and decay-accelerating factor (DAF), that had restricted expression within erythrocytes in transgenic mice, were found to be transferred to the endothelium throughout the mice. Furthermore, these transferred GPI-APs remained functional in cardiac endothelial cells as well in preventing complement-mediated lysis.

Expression of proteins on the cell surface by protein transfer of GPI-APs

Purified GPI-APs contain the unique property of being able to re-incorporate into cell membranes and still retain their biological activity. In 1984, Medof et al. showed that the purified GPI-AP, DAF, was able to reincorporate into erythrocyte membranes by simply incubating purified DAF with erythrocytes (24). This reconstitution of DAF prevented complement protein C4b2a activity on the erythrocytes in a DAF-dose dependent manner. Furthermore, in the acquired disorder Paroxysmal Nocturnal Hemoglobinuria (PNH), the absence of DAF in erythrocytes from PNH patients leads the erythrocytes to be susceptible to complement induced lysis. However, this lysis is not detected-when exogenous DAF is reconstituted back into erythrocyte membranes (25). Additionally, in PNH patients, another complement regulatory protein, homologous restriction factor (HRF) later known as CD59, is also absent from erythrocyte membranes. Incubation of purified radiolabeled HRF with erythrocytes led to insertion of HRF into erythrocyte membranes and the inserted HRF remained biologically functional in preventing complement lysis by C5b-9 (26-30). Oligodendrocytes that also lacked

CD59 became resistant to complement induced lysis after exogenously incorporating purified CD59 into their membranes as well (31). Another complement regulatory protein which behaved similarly to DAF and CD59 is C8-binding protein (C8bp). In a comparable manner, C8bp, which is also not found in erythrocytes obtained from PNH patients, can be inserted into erythrocyte membranes exogenously after purification of the protein and again inhibit complement induced lysis of erythrocytes (32).

Additionally, adhesion molecules such as lymphocyte function-associated antigen 3 (LFA-3) are also found to be deficient on erythrocytes obtained from PNH patients (33). LFA-3 is expressed as a GPI-anchored form in erythrocytes whereas nucleated cells express both GPI-anchored forms and polypeptide anchored forms (34). Consequently, partial deficiency of LFA-3 was observed in nucleated cells from PNH patients. The interaction between LFA-3 on erythrocytes and its receptor CD2 on T-cells induces formation of T-cell rosettes (35), however, erythrocytes from PNH patients did not form rosettes upon incubation with CD2 expressing Jurkat cells. Interestingly, when exogenous purified GPI-anchored LFA-3 was added back to erythrocytes from PNH patients, rosettes were formed, suggesting that protein transfer of exogenous LFA-3 leads to stable as well as functional LFA-3 expression on the cell surface (33). Also, exogenous incorporation of purified CD16B (Fcγ receptor III), another naturally occurring GPI-AP, onto both a human T cell leukemia and B lymphoblastoid cell lines demonstrated that protein transferred CD16B on nucleated cells can still retain its function and mediate endocytosis of its ligand (36). Another GPI-AP, Thy-1, was able to exogenously incorporate onto murine Thy-1-negative cells. After this protein transfer, staining of the exogenously incorporated Thy-1 was similar to staining patterns of endogenously

expressed Thy-1 and the lateral mobility of both endogenously and exogenously inserted Thy-1 was similar as determined by fluorescence recovery after photobleaching (FRAP) (37). Furthermore, even non-mammalian GPI-APs, such as membrane form of variant surface glycoprotein (mfVSG) expressed on the parasite, *Trypanosoma brucei*, can also be purified and exogenously incorporated onto cell surfaces with similar lateral mobility as endogenously expressed mfVSG (38). However, PI-PLC treatment of purified GPI-APs decreases the insertion levels of these proteins onto cell membranes suggesting that protein transfer is due to the presence of the GPI-anchor (27, 33, 37). All of the above examples show that purified GPI-APs can be incorporated via the GPI-anchor into cell membranes by simply exogenously adding the purified GPI-APs and that after incorporation onto cell membranes, the biological function and lateral mobility of the protein remains similar to that of the endogenously expressed protein counterpart.

Characteristics of GPI-AP protein transfer

Protein transfer of GPI-APs onto cell membranes (Figure 1C) occurs in a concentration dependent manner. As higher concentrations of GPI-APs are added to the membranes, increased incorporation occurs (24, 36, 39). However, a saturation point is expected to occur in which all GPI-AP sites on the membrane will be occupied and no more GPI-APs can incorporate by protein transfer without disrupting the integrity of the membranes. Furthermore, GPI-anchor mediated protein transfer is time dependent. As the time for protein transfer is increased, increased expression of GPI-APs on membrane surfaces occurs. Incorporation was detected in as little as 20 minutes post incubation of GPI-APs with membranes and increases dependent on the incubation time upto at least 4

hours (24, 36, 39). Medof et al. noticed that incorporation of radiolabeled-DAF into erythrocyte membranes occurred best at 37˚C followed by 20˚C with no detectable DAF incorporation occurring at 0˚C (24). Poloso et al. detected a similar phenomenon in which protein transfer occurs best on cell membrane vesicles at 37˚C followed by 25˚C followed by 4˚C (39). These observations suggest the necessity of membrane mobility for the incorporation of GPI-APs. Plasma membranes are most fluid at 37˚C compared to lower temperatures, therefore, the dynamics between GPI-AP micelles and the lipid bilayer occurs more readily at 37˚C (40).

Optimum incorporation also occurs without the presence of certain serum proteins. Incorporation of GPI-APs is inhibited by the presence of human serum lipoproteins, such as HDL and LDL, and fatty-acid binding proteins, such as bovine serum albumin (BSA) and orosomucoid (24, 33, 36). High concentrations of ovalbumin on the other hand do not affect incorporation of GPI-APs onto cell membranes (24, 36). Once incorporated onto the cell surface by protein transfer, GPI-APs remain stably integrated into the cell membrane of erythrocytes and cannot be removed by washing even with high salt concentrations (24). After protein transfer, GPI-APs can however be removed by solubilization with detergents, such as NP-40. However, incorporation onto live or irradiated nucleated cells leads to loss of incorporated proteins under culture conditions (41).

Protein transfer leads to the incorporation of exogenously added GPI-APs into lipid rafts of cell membranes (14, 42). After Triton X-100 lysis of cells that have been protein transferred with GPI-APs, the GPI-APs are detected in detergent insoluble microdomains as are other lipid raft specific proteins (42). Furthermore, enhanced

incorporation of green fluorescent protein (GFP)-labelled-GPI-anchors into lipid rafts was also associated with the presence of lipid raft-associated molecules (cholesterol, sphingomyelin, and dipalmitoyl-phosphatidylethanolamine) further proving that protein transferred-GPI-APs associate with lipid raft structures (14). Upon initial incorporation of GPI-APs onto cell membranes by protein transfer, the GPI-APs are widely distributed throughout the cell membrane as observed by confocal microscopy (43). However, hours after incorporation, the GPI-APs localize into lipid raft microdomains as detected by confocal microscopy or by observing association of GPI-APs with lipid raft domains after lysis with Triton X-100 (42, 43).

Similar to the erythrocyte incorporated proteins described earlier, these protein transferred GPI-APs expressed on nucleated cells and cell membranes retain their functional biological activity in comparison to endogenously expressed forms of the proteins (36, 39, 41, 44). Not only do exogenously incorporated GPI-APs retain the functional extracellular activity of the GPI-AP, but they can also retain functional intracellular activity (42, 43). Exogenously incorporated GPI-APs onto cells led to association of the GPI-AP with active intracellular kinases (42, 43). Upon antibody crosslinking of exogenously incorporated GPI-B7-1, DAF, or CD59, phosphorylation induced by active kinase activity was detected (42, 43) as well as an induced transient Ca^{2+} flux signal (43).

It has also been observed that cells transfected to express the GPI-anchored form of the cytokine, GM-CSF, not only expressed functional surface-bound-GM-CSF that was susceptible to PI-PLC cleavage, but GM-CSF was also detected in the supernatant in a soluble form. This soluble GM-CSF was released by either shedding or proteolytic

cleavage and was not released by secretion (45). Similarly, IL-2 was detected in the supernatant of cultured cell lines transfected to express GPI-IL-2, however GPI-IL-12 was not secreted when expressed on CHO cells (46, 47). Therefore, this dual ability of cytokines to not only be expressed on cell surfaces by a GPI-anchor but also to be shed into the extracellular space could lead to a cytokine gradient allowing for the infiltration and activation of immune cells.

More importantly, after protein transfer of GPI-APs onto the lipid bilayers, the newly incorporated GPI-APs remain functionally active. Although it has been stated that naturally occurring GPI-APs can be reincorporated exogenously by protein transfer and retain their biological functional activity, even recombinant GPI-APs, such as GPI-B7-1 and GPI-IL-12, after protein transfer retain their biological functional activity (39, 41, 44, 48, 49). Protein transferred-GPI-B7-1 and GPI-IL-12 incorporated on tumor cells and tumor membrane vesicles retained their ability to induce T-cell proliferation.

Advantages and limitations of GPI-AP protein transfer

Modification of membranes and cell surfaces provides a wide range of application possibilities. Protein transfer allows for membrane modification in an easy, quick and efficient manner compared to the traditional genetic engineering approaches. (1) With protein transfer, purified GPI-APs incorporate onto cell membranes in a matter of minutes with maximal incorporation occurring around 2-4 hours (39), whereas genetic engineering approaches take much longer from the order of days to months to establish cultured transfected cell lines. (2) Genetic engineering makes modifying some cell lines extremely difficult especially when primary cell lines, such as bone marrow progenitors

and primary cultures, are hard to transfect. Also, protein transfer can be performed on small cell numbers and does not require cell division to occur. (3) Using protein transfer, the amount of incorporation can be finely controlled by adjusting the amount of exogenously added GPI-APs. (4) Finally, multiple GPI-APs can be incorporated onto the same cell membranes by protein transfer whereas double and triple transfections by genetic engineering are more difficult to attain (50).

However, membrane anchors are known to affect the functionality of external domains of some proteins (51). It has been noted that PI-PLC treatment of some GPI-APs, renders some antibodies non-responsive to the protein even if the antibody binds the GPI-AP in the presence of the GPI-anchor (52-54). Therefore, the addition of a GPI-anchor can affect the structural conformation and function of proteins. Also, if important antibody epitopes are found at the C-terminal end of a protein, the addition of the GPI-anchor at the C-terminus, may alter the antigenicity of the protein. Similarly, the GPI-anchor that attaches the protein close to the membrane with less than 10-14 Å space between the protein and the membrane surface as determined by fluorescence resonance energy transfer (FRET) (54, 55). This close proximity to the membrane surface may alter accessibility of important antibody binding sites. Therefore it is imperative that the functionality of each GPI-AP be tested before use. A bottleneck of the protein transfer method using GPI-APs, is the purification of GPI-APs. Purification using immunoaffinity chromatography is the best defined manner for obtaining pure fractions of GPI-APs, however optimization of the process for each individual GPI-AP is time-consuming. Despite these disadvantages, when optimal purification of GPI-APs that

retain their biological functionality is attainable, protein transferred GPI-APs provide an efficient method to controllably modify membrane surfaces.

Protein transfer of antigen-presenting cells with GPI-anchored MHC complexes

During viral infections, CTL responses are important to combat infected cells. For a CTL to become activated, the T-cell has to be engaged with MHC I that expresses the viral peptide, however, many viruses downmodulate the expression of MHC molecules on the cell surface to escape from the immune system. Therefore, Huang et al, constituted a method to control the amount of MHC:peptide complexes expressed on a cell by protein transfer (56). Their model system utilized a HLA-A2.1 MHC complexed with a hepatitis B virus (HBV) peptide that induces a dominant T-cell response. They constructed a GPI-anchored form of HLA-A2.1 by attaching a GPI-anchored signal sequence to the heavy chain and contransfected Schneider S2 *Drosophila melanogaster* cells with the beta 2 microglobulin gene. Upon purification of this HLA-A2:GPI, they were able to show that the MHC complex incorporated into HLA-A-negative and HLA-B-reduced cells in a time, temperature, and concentration dependent manner. They were able to detect incorporation by flow cytometry within 1 minute of incubation with cells, however maximal incorporation occurred after 1h incubation at 37˚C. Furthermore, incorporation occurred best at 37˚C compared to either 4˚C or 25˚C. Additionally, incorporation was inhibited by the presence of 10% fetal bovine serum (FBS) but if FBS was added after protein transfer, then no affect on cell surface expression of the MHC complex was noticed. Peptide was loaded onto the GPI-MHC molecules at 4˚C overnight before protein transfer leading to stable expression of the MHC-peptide complex and

MHC:peptide specific cell lysis by CTLs after protein transfer. These results showed that protein transfer with GPI-anchored MHC complexes could be used to express desired quantities of specific MHC complexes on cell surfaces. Thus, antigen presenting cells (APCs) can also be decorated with desired MHC:peptide complexes allowing further studies of APC and T-cell interactions to elucidate specific peptide mediated T-cell activation and tolerization.

Protein transfer of GPI-APs in tumor immunotherapy

Another application of protein transfer using GPI-APs is in tumor immunotherapy. Cancer cells have the ability to continuously evade the immune system therefore, finding a way to activate the immune system against these cells is key to combating tumors. One method in which tumor cells evade the immune system is through the downregulation of costimulatory molecules, such as B7-1, on tumor cell surfaces. This downregulation renders tumor-specific T-cells anergic and nonresponsive to the tumor. Therefore, tumor cells have been transfected to express B7-1 in order to improve the immunogenicity of cell-based therapeutic vaccines. However, in order to transfect tumor cells with immune stimulatory molecules, the establishment of primary tumor cells lines from patients' tumor tissue is required. However, tumor cells derived from patients are extremely difficult to culture; therefore, McHugh et al. proposed the use of protein transfer to express GPI-anchored B7-1 on tumor cells or on tumor membrane vesicles (TMVs) (44). The TMVs that are derived from the homogenization of tumor cells still contain tumor-associated antigens along with a lipid bilayer, thus they can be modified with GPI-APs by protein transfer. It was shown that TMVs derived from EG7 tumors, a

murine T-cell lymphoma line, could be protein transferred to express GPI-B7-1 on the surface. They found that these protein transfer-modified TMVs could protect against challenge with EG7 tumor cells. Furthermore, B7-1-modified TMVs led to T-cell proliferation and cytolytic T-cell activity in vaccinated mice *ex vivo* against the parental tumor (44).

Additionally, protein transfer was also shown to be conducted on TMVs derived from surgically removed human tumor tissue. These TMVs were stably incorporated with GPI-B7-1 by protein transfer in a concentration-, time-, and temperature-dependent manner. These incorporated TMVs were functionally active in stimulating T cell proliferation (39).

Many cytokine therapies, such as the administration of the cytokine IL-2, have been used as a tumor immunotherapy in order to activate immune cells against the tumor (57). Although cytokine administration has worked in activating the immune system, systemic toxicity also results which impedes the success of the treatment. Therefore, human TMVs modified by protein transfer to express GPI-cytokines were used as a method of delivery of not only the cytokine in a less toxic manner, but also tumor associated antigens (39, 48). Using protein transfer, purified GPI-anchored-IL-12 was incorporated onto human and murine TMVs from mammary carcinomas, renal cell carcinomas, melanoma, and B-cell lymphoma cultured cell lines, and the resulting TMVs expressed functionally active IL-12 that could induce T cell proliferation and IFN-γ secretion. IL-12 also worked well in combination with CD80 or CD40 to enhance T-cell proliferation and IFN-γ secretion (48).

Taken together, protein transfer of costimulatory molecules and cytokines that have been converted into GPI-anchored forms has led to a promising approach for inducing antitumor immunity. Because GPI-anchored cytokines are attached to the membrane surface, a slow-release depot of the cytokine is ensured circumventing the systemic toxicity associated with administration of soluble cytokines.

Nano and microparticles

Protein transfer of GPI-APs has previously been used to modify surfaces of whole cells and cell derived-membrane vesicles. We aimed to expand the versatility of this protein transfer approach by modifying either nano-sized or micro-sized particles to (1) enhance the immunogenicity of the nanoparticles and (2) deliver antigens to the immune system.

Nanoparticles range in size with a diameter of 1 nm to 100 nm (58), whereas microparticles range from 100 nm to 1000 nm (59). Particles have been widely used as vaccine candidates to elicit immunity against specific antigens encapsulated within or attached to the particles. Particles mimic the shape and structure of many pathogens therefore particulate structures are optimal in activating an immune response (58, 60). However, particle size may affect the location and type of immune response initiated. Smaller particles that range from 20 – 200 nm were shown to be able to traffic freely to draining lymph nodes, whereas larger particles (500 – 2000 nm) mostly associated with dendritic cells at the vaccination site which were then required for antigen migration to lymph nodes (61). Furthermore, particles around 30 – 40 nm localized more readily to LNs showing an optimal size for LN activation (61, 62). Moreover, DC uptake depends

on particle size as well in which particles smaller than 200 nm in diameter can undergo receptor-mediated endocytosis whereas larger particles that are greater than 500 nm undergo phagocytosis or micropinocytosis (63). Apart from antigen delivery, particle size has also been shown to have an effect on cellular processes. Nanoparticles of size 40 – 50 nm had the most enhanced effect on membrane receptor internalization out of particles ranging from 2 – 100 nm after binding (64). Further, expression of multiple ligands on particles within a specific density and surface curvature enhances receptor-ligand avidity (58). For example, Herceptin binding affinity to its receptor ErbB2 when solubilized is 10^{-10} M, whereas that of Herceptin expressed on a 70-nm particle is 1.5 x 10^{-13} M (64). Therefore, particles are able to mediate cellular responses as well as act as delivery vehicles.

More importantly, the immunogenicity of antigen associated to particles is enhanced compared to administration of soluble antigen. Many mechanisms as to why this occurs has been proposed. Incorporating antigen to particles can increase the antigen half-life compared to soluble administration (65). This is due to the particulate structure stabilizing the antigen from proteolytic degradation. Further, incorporation onto particles may allow for a slow release depot of the antigen to the immune system, allowing for a more prolonged immune response (65, 66). Particles smaller than 10 μm are also more readily taken up by APCs, therefore, incorporating antigen within particles can enhance uptake and presentation of antigen by APCs (66). Moreover, many particles, such as aluminum salts that are used as adjuvants, can directly stimulate the immune system to enhance cytokine production. Although adsorption of antigen onto aluminum salts was found to lead to degradation of antigen, this in turn may increase proteolytic cleavage of

antigen and thus lead to enhanced antigen presentation by the immune system (66). Therefore, antigens presented in particulate form to the immune system enhances the immunogenicity of the antigen and is beneficial as a vaccination strategy.

Many mechanisms exist to incorporate antigens onto particles. Current methods include encapsulation or adsorption. Entrapment of antigen within particles is performed so that controlled release of the antigen results, however a "burst release" is often seen instead where large amounts of antigen are quickly released (67). For encapsulation of antigen within particles, antigens are present during particle formation. This presence can lead to harmful exposure of antigen to organic solvents required to make the particles (66). Furthermore, encapsulating DNA or antigen within particles can alter the particle structure (68). For example, encapsulation of DNA and non-viral antigens within VLPs derived from murine polyomavirus proteins, increased the VLP diameter and inclusion of non-viral proteins altered the formation of VLPs as the VLPs did not localize to the nucleus (68). On the other hand, adsorption consists of covalently attaching the antigen to the surface of particles. Adsorption usually occurs through a chemical modification which can in turn alter the protein structure and thus the antigenicity of the protein as well.

To overcome the disadvantages of the current methods used to incorporate antigen into micro and nanoparticles, we propose to use the protein transfer technology to modify the surfaces of particles. Herein, we show that tumor associated antigens and immunostimulatory molecules (ISMs) can be converted into GPI-anchored forms and used to incorporate onto the surfaces of nano and microparticles that contain lipid bilayers, such as influenza virus-like particles (VLPs) and tumor derived plasma

membrane vesicles (PMVs), by protein transfer. Protein transfer occurred within a matter of hours and expression of the added GPI-AP could be controlled by adjusting the concentration of GPI-AP used during incubation with the particles. Protein transfer resulted in stable expression of the incorporated antigen or ISM, and the incorporated ISMs remained functionally active. We show that protein transfer can be used as a means to (1) enhance the immunogenicity of intrinsic viral antigens present on influenza VLPs, (2) incorporate a tumor antigen onto VLPs and PMVs to elicit an antitumor immune response, and (3) incorporate a tumor antigen along with ISMs simultaneously onto particles to enhance the antitumor immune response elicited. Therefore, the protein transfer technique is very versatile and can be used to not only introduce antigens onto particles that contain a lipid bilayer, but also enhance either intrinsic particle immunogenicity or the immunogenicity of exogenously incorporated antigens.

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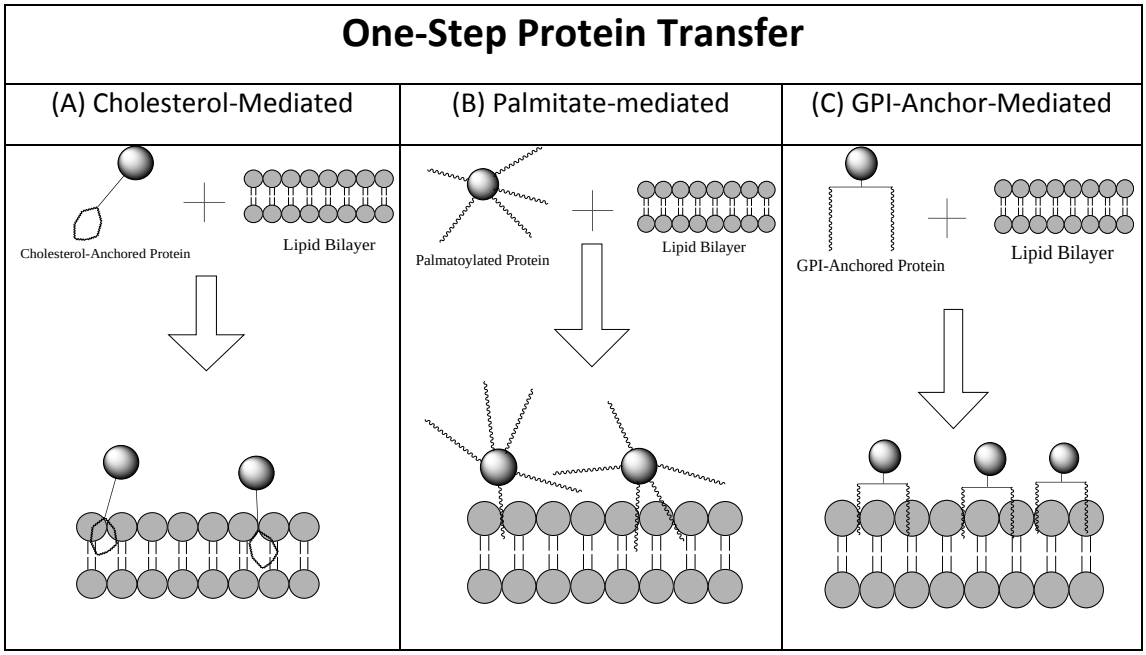
Figure Legends

Figure 1. One-Step protein transfer mediated by (A) cholesterol anchoring, (B) palmitoylation, or (C) GPI-anchoring of proteins.

Figure 1.


One-Step Protein Transfer
(A) Cholesterol-Mediated
(B) Palmitate-mediated
(C) GPI-Anchor-Mediated
Cholesterol-Anchored Protein
Lipid Bilayer
Palmatoylated Protein
Lipid Bilayer
GPI-Anchored Protein
Lipid Bilayer

CHAPTER II:

Protein transfer-mediated surface engineering to adjuvantate virus-like nanoparticles for enhanced anti-viral immune responses

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

Data presented in Figure 5 was generated with the cooperation of Min-Chul Kim, Yu-Na Lee and Sang-Moo Kang.

Data presented in Supplemental Figure 4A was generated with the cooperation of Emory University Robert P. Apkarian Integrated Electron Microscopy Core

Data presented in Supplemental Figure 4C was generated with the cooperation of Hyo-Jick Choi and Carlo Montemagno.

Abstract

Recombinant virus-like nanoparticles (VLPs) are a promising nanoparticle platform to develop safe vaccines for many viruses. Herein, we describe a novel and rapid protein transfer process to enhance the potency of enveloped VLPs by decorating influenza VLPs with exogenously added glycosylphosphatidylinositol-anchored immunostimulatory molecules (GPI-ISMs). With protein transfer, the level of GPI-ISM incorporation onto VLPs is controllable by varying incubation time and concentration of GPI-ISMs added. ISM incorporation was dependent upon the presence of a GPI-anchor and the incorporated proteins were stable and functional for at least 4 weeks when stored at 4°C. Vaccinating mice with GPI-GM-CSF-incorporated-VLPs induced stronger antibody responses and better protection against a heterologous influenza virus challenge than unmodified VLPs. Thus, VLPs can be enriched with ISMs by protein transfer to increase the potency and breadth of the immune response, which has implications in developing effective nanoparticle-based vaccines against a broad spectrum of enveloped viruses.

Key words: Virus-like particles; protein transfer; Influenza; GPI-anchored proteins; GM-CSF.

Background

Current influenza vaccine production using embryonic hen eggs takes months and is not able to scale-up efficiently to meet global vaccine requirements for a pandemic. Further, immunogenicity of the current vaccine is subtype specific and does not generate effective cross-protection against heterologous viral strains (1, 2). Therefore, there is an urgent need to develop a quick, safe, and broadly protective influenza vaccine. Recent animal studies and human clinical trials suggest that virus-like nanoparticles (VLPs) can be used as an alternative influenza vaccine (3-5). VLPs are derived from budding membranes of cells transfected to express viral matrix and envelope proteins and, therefore, mimic viral shape and structure through the expression of repetitive viral antigens without viral genomes. Influenza VLPs are typically 80-120 nm in diameter (6), allowing for trafficking to lymph nodes as well as optimal uptake by localized dendritic cells upon vaccination (7, 8). Therefore, VLP vaccination elicits both a strong humoral and cellular adaptive immune response against the virus (3, 9-11). Furthermore, VLP-based vaccines can quickly be produced in large quantities compared to conventional influenza vaccines. However, VLPs, similar to conventional influenza vaccines, induce mainly subtype specific protection with minimum cross protection against heterologous viral strains.

To enhance VLP immunogenicity for stronger cross-protection, VLPs have been genetically modified using the recombinant baculovirus (rBV) system to express immunostimulatory molecules (ISMs), such as membrane-bound cytokines, GM-CSF, CD40L, and flagellin, on the VLP surface (12-14). Another approach to introduce proteins onto VLPs is by pseudotyping, which involves fusing peptides or proteins-of-

interest and viral proteins together on the VLPs (15, 16). Genetic modification is time-consuming and other limitations include increased insect-cell cytopathology as a result of increased multiplicity of infection when using multiple rBVs (17). More importantly, the level of ISM and viral protein incorporation cannot be controlled by these genetic approaches and the creation of fusion proteins may affect protein functionality.

Herein we show for the first time that purified GPI-anchored-immunostimulatory molecules (GPI-ISMs) can be stably incorporated onto influenza VLPs in a concentration dependent manner within 2 hours (Figure 1) using a protein transfer technology. Vaccination of mice with VLPs modified by protein transfer with GPI-GM-CSF led to enhanced anti-heterologous virus antibody responses and complete protection against a heterologous viral challenge, which was not elicited by unmodified VLPs. The ease of protein transfer makes this method an attractive model system to adjuvantate enveloped VLP-based nanoparticle vaccines and enhance cross-protection against heterologous influenza viruses.

Methods

Animals:

Female BALB/c mice (6-8 weeks old) were purchased from Jackson Laboratory and experiments were conducted as per approved IACUC guidelines of Emory University and Georgia State University.

DNA Constructs:

To construct GPI-GM-CSF, nucleotides corresponding to the GPI-signal sequence from CD59 was attached to the C-terminus of mouse GM-CSF DNA as previously described(18-20). Similarly, GPI-ICAM-1 was constructed by attaching CD59 GPI-signal sequence to the extracellular domain of mouse ICAM-1 using an AflII restriction enzyme site(21). GPI-IL-12 was made by attaching CD59 GPI-signal sequence to mouse IL-12 p35 followed by an IRES sequence and mouse IL-12 p40-CD59 or soluble IL-12 p40. The GPI-IL-12 construct was placed in a pUB6^{blast} vector (Invitrogen), whereas GPI-GM-CSF and GPI-ICAM-1 were placed in a pcDNA3 (Neo) vector (Invitrogen).

Cell lines:

CHO-K1 cell transfectants were selected and maintained in RPMI 1640 and 10% Cosmic Calf Serum (Invitrogen) containing either 400 µg/ml Geneticin® selective antibiotic (G418 Sulfate) for cells transfected with GPI-ICAM-1 and GPI-GM-CSF or with 10 µg/ml Blasticidin (Invitrogen) for cells transfected with GPI-IL-12. Cell pellets of CHO-K1 cells transfected to express GPI-ISMs were lysed with 50 mM Tris-HCl pH 8, 2% n-octyl-β-D-glucopyranoside (A.G. Scientific), 1:100 dilution of Protease Inhibitor Cocktail (Sigma), 5 mM EDTA, 20 mM sodium iodoacetate, and 2 mM PMSF overnight

at 4˚C. The lysate was centrifuged at 14,000 rpm for 1 h at 4˚C and used for purification of GPI-APs.

Sheep red blood cells (RBCs) were obtained from HemoStat Laboratories. Bone marrow (BM) cells were obtained from tibias and femurs of BALB/c mice and cultured in RPMI, 10% CCS, 1% PenStrep, 50 μM β-mercaptoethanol at 37˚C, 5% CO_2, as previously described(22).

Antibodies:

Anti-mGM-CSF (A2F17107) secreting hybridoma was a kind gift from Dr. Michael B. Prystowsky, University of Pennsylvania(23) and anti-mICAM-1 (YN1/1.7.4) secreting hybridoma was a kind gift from Fumio Takai, University of British Columbia, Canada. Antibodies were purified from hybridoma supernatant by using a Gammabind plus Sepharose column (GE Healthcare). Purified anti-mIL-12 p40 mAb (C17.8) was obtained from Bio X Cell. Immunoaffinity columns were made by coupling mAbs to CNBr-activated-Sepharose beads (GE Healthcare).

PI-PLC treatment of transfected CHO-K1 cells:

Transfected CHO-K1 cells were washed and resuspended in PBS/EDTA/0.1% ovalbumin to a final concentration of 10 x 10^6 cells/ml. 1U PI-PLC from *Bacillus cereus* (Invitrogen) that specifically cleaves the GPI-anchor was added to 1ml of cell suspension and incubated at 37˚C for 45 min. Cells were washed and protein expression was analyzed by flow cytometry.

GPI-ISM purification:

GPI-ISMs were isolated using mAb-affinity chromatography from the detergent lysate of CHO cell transfectants as described previously (24, 25). Briefly, CHO-K1 cells transfected to express GPI-APs were grown in large quantities using roller bottles. Cell pellets were then lysed with 50 mM Tris-HCl pH 8, 2% n-octyl-β-D-glucopyranoside (A.G. Scientific), 1:100 dilution of Protease Inhibitor Cocktail (Sigma), 5 mM EDTA, 20 mM sodium iodoacetate, and 2 mM PMSF overnight at 4˚C. The lysate was centrifuged at 14,000 rpm for 1h at 4˚C and the supernatant was passed through a 70 µM cell strainer to remove cell debris. The lysate was first passed through a Sepharose 4B bead (Sigma-Aldrich) pre-column followed by the corresponding affinity chromatography column. The affinity column was then washed with 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 200 mM NaCl followed by a wash with 20 mM Tris-HCl, pH 7.5, 0.1% n-octyl-β-D-glucopyranoside. GPI-ICAM-1 and GPI-GM-CSF were then eluted with 100mM Triethylamine, 1% n-octyl-β-D-glucopyranoside, pH 11.6, whereas GPI-IL-12 was eluted with 100 mM Glycine-HCl pH 2.8 containing 1% n-octyl-β-D-glucopyranoside, and 10 mM sodium iodoacetate. The eluted fractions were subjected to SDS-PAGE and analyzed by western blot and silver staining. The protein containing fractions were combined in a 10-14 kDa MWCO dialysis bag (Fisherbrand) and concentrated using polyvinylpyrrolidone (Sigma-Aldrich). The concentrated proteins were dialyzed with 3 exchanges of 500 ml PBS containing 0.05% n-octyl-β-D-glucopyranoside and stored at 4˚C until use. Purified GPI-ICAM-1 concentration was quantified using a micro BCA kit (Thermo Scientific). GPI-GM-CSF and GPI-IL-12 quantifications were performed by

direct ELISA comparing to a standard curve obtained using recombinant soluble mGM-CSF (Peprotec) or mIL-12 p75 (eBioscience).

Incorporation of GPI-ISMs onto sheep RBCs by protein transfer:

To test if the purified GPI-ISMs retained the GPI-anchor and the ability to incorporate onto the lipid bilayer of cell membranes, protein transfer was initially performed on sheep RBCs. GPI-ISMs were incubated with 2 x 10^6 washed sheep RBCs in PBS/0.1% ovalbumin in an end-over-end rotation for 4 h at 37˚C. Unincorporated GPI-ISMs were washed out by centrifugation using FACS buffer (PBS, 5 mM EDTA, 1% CCS). Incorporation was analyzed by flow cytometry.

H5 and H1 VLP preparation:

H5 VLPs and H1 VLPs were made and purified using the recombinant baculovirus (rBV) expression system as previously described(10, 26). In brief, rBVs expressing influenza M1 protein from H1N1 influenza A/PR/8/1934 virus and rBVs expressing influenza HA proteins from H5N1 influenza A/Indonesia/05/05 virus or from H1N1 influenza A/PR/8/1934 virus, were generated, and then co-infected into Sf9 insect-cells to generate H5 or H1 VLPs, respectively. After 2-3 days, infected cell culture supernatants were clarified by centrifugation (6000 rpm, 30 min at 4˚C) and concentrated by the QuixStand hollow fiber based ultrafiltration system (GE Healthcare, Piscataway, NJ). VLPs were purified by sucrose gradient ultracentrifugation with 20% and 60% (wt/vol) layers at 30,000 rpm using a SW32Ti Rotor (Beckman) for 60 min at 4˚C and were collected from the 20%-60% interphase. VLPs were pelleted by ultra-centrifugation at

30,000 rpm using a SW32Ti rotor for 30 min at 4˚C. The VLP pellet was resuspended in PBS and stored at -80˚C until further studies. VLPs were quantified by the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) and HA content on the VLPs was approximately 11% of total VLP protein as detected by ELISA(11). Endotoxin levels were tested in the VLP preparations before and after protein transfer using the ToxinSensor™ Chromogenic Limulus Amebocyte Lysate Endotoxin Assay Kit (GenScript).

Incorporation of GPI-ISMs onto VLPs by protein transfer:

H5 VLPs and H1 VLPs were made using the recombinant baculovirus (rBV) expression system and purified using sucrose gradient centrifugation as previously described (10, 26). rBVs expressing influenza M1 protein from H1N1 influenza A/PR/8/1934 virus and rBVs expressing influenza HA proteins from H5N1 influenza A/Indonesia/05/05 virus or from H1N1 influenza A/PR/8/1934 virus, were used to generate H5 or H1 VLPs, respectively. Purified GPI-ISMs were centrifuged at 13,200 rpm for 1 h at 4˚C. Supernatant from the centrifuged GPI-ISM fractions were incubated with H5 VLPs in end-over-end rotation at 37˚C for 6 h in the presence of 0.22 μm filtered PBS or PBS/0.1% ovalbumin. GPI-ISM-VLPs were pelleted at 13,200 rpm at 4˚C for 30 min to remove unincorporated GPI-ISMs. VLP pellets were resuspended in PBS. Quantification of resulting protein transferred-VLPs was performed by a quantitative ELISA using known concentrations of VLPs as standards and quantification of incorporated GPI-ISMs was determined by quantitative ELISA using known concentrations of recombinant soluble GM-CSF or IL-12 as explained in supplementary materials.

Quantitative ELISA:

A direct ELISA was performed to quantify incorporated GPI-GM-CSF. Briefly, 100 µl of 1 µg/ml of GPI-GM-CSF-VLPs was coated onto a 96-well flat bottom ELISA plate in triplicate. As a standard, varying concentrations of recombinant soluble mouse GM-CSF (Peprotec) were coated on the plate. The plate was blocked with PBS/0.05% Tween/3%BSA, and stained with anti-GM-CSF antibody followed by the horseradish peroxidase (HRP)-conjugated-rabbit-anti-rat-IgG secondary Ab (Thermo Scientific). Tetramethylbenzidine (TMB) (BioLegend) was used to develop color that was then stopped with 2N H_2SO_4 and read at 450 nm.

Electron microscopy analysis of VLPs:

Either unmodified VLPs or VLPs modified by protein transfer with GPI-ICAM-1 were analyzed using transmission electron microscopy at the Emory University Robert P. Apkarian Integrated Electron Microscopy Core facility.

Physicochemical analysis of protein transfer-modified VLPs:

The size and zeta potential of VLP preparations were measured using the Malvern Zetasizer Nano ZS. Analysis was carried out using 1 ml of 0.2 mg/ml of VLPs at 4°C in either PBS (pH 7.4) for size analysis or 10 mM sodium phosphate buffer (pH 7.4) for zeta potential analysis.

Bone marrow-derived cell proliferation assay:

Bone marrow cells were obtained from tibias and femurs of BALB/c mice as previously described (22). Mouse bone marrow cells (1×10^5) were cultured for 4 days in 100 µl of medium containing 1 µg/ml of protein transferred-VLP per well in triplicate in a 96-well flat bottom plate. Four days after culture, 20 µl of CellTiter 96® Aqueous One Solution (Promega) was added. The plate was placed in a 37˚C, 5% CO_2 incubator for 1 h and read at 490 nm.

Stability analysis of GPI-GM-CSF incorporated onto VLPs by protein transfer:

GPI-GM-CSF was protein transferred onto H5 VLPs and the resulting GPI-GM-CSF-VLPs were stored at 4˚C. At day 0, 4, 7, 14, 21 and 28, aliquots of protein transferred-VLPs were washed by centrifugation, resuspended in Leammli sample buffer which was then boiled and frozen at -20°C. Western blot and Image J analysis for GM-CSF and VLP-specific proteins were performed to determine GM-CSF:VLP ratios.

Vaccination and challenge studies:

BALB/c mice were immunized on day 0 and boosted on day 36 with 100 µl of 0.5 µg protein transferred-H5 VLPs in PBS on the left hind flank subcutaneously. Control groups received 100 µl PBS or mock protein transferred-H5 VLPs without GPI-ISMs. For influenza challenge, mice were challenged intranasally with influenza A/Vietnam/1203/2004 (rgH5N1), 1LD in 50 µl (11).

To determine viral titer, mice were euthanized 4 days post infection and lungs were individually homogenized. Supernatants of lung homogenates were serially diluted

and inoculated into 9-11 day old fertilized eggs. The presence of virus was tested with the allantoic fluid 3 days later by hemagglutination assay. Fifty percent egg infectious dose (EID_{50}) titers were calculated by the Reed and Muench method (27).

To determine the presence of virus-specific antibody secreting cells within different mouse organs after challenge, mice were sacrificed 4 days post challenge with influenza A/Vietnam/1203/2004 (rgH5N1). One day before sacrificing mice, H5 VLP or inactivated influenza virus (2 µg/ml) was used as a coating antigen on ELISA plates with overnight incubation at 4°C. Wells were washed with PBS and blocked with RPMI media containing 10% FBS for 2 h at 37°C. Spleens, lungs, and bone marrows from mice (n = 3) were pooled and homogenized. The homogenate was passed through a cell strainer (Fisher Scientific) and the cells were treated with red blood cell (RBC) lysis buffer (Sigma, St. Louis, MO) to remove RBCs. The collected cells were then added into the plates (0.5×10^6 spleen or bone marrow cells/200 µl/well; 0.2×10^6 lung cells/200 µl/well) and incubated for 1 or 5 days at 37˚C, 5% CO_2. The supernatant was removed and virus-specific antibody bound to the wells was quantified by ELISA using secondary anti-mouse-IgG-HRP antibody and TMB substrate.

Detection of serum IgG responses:

ELISA was used to detect serum anti-viral IgG responses by coating 100 µl per well of 1 µg/ml VLP or inactivated virus to a 96-well flat bottom ELISA plate. Diluted serum was added as primary antibody followed by goat-anti-mouse IgG-HRP, rat-anti-mouse-IgG1-HRP, rat-anti-mouse-IgG2a-HRP, or goat-anti-mouse-IgG2b-HRP (Southern Biotech) as the secondary antibody.

Statistical Analysis:

All data are expressed as mean ± standard error of the mean (SEM). GraphPad Prism 6 was used to perform statistical analysis. Student's t-test, One-way ANOVA or Two-way ANOVA using Tukey's multiple comparisons post-test was used for analysis as described in the figure legends. A $p < 0.05$ value was considered significant.

Results

Protein transfer of GPI-ISMs onto influenza VLPs:

GPI-ISMs (GPI-ICAM-1, GPI-GM-CSF, GPI-IL-12) were purified from CHO-K1 cell transfectants by affinity chromatography (Supplementary Figs. 1 and 2) and incubated with VLPs. Incorporation levels were directly proportional to the concentrations of GPI-ISMs added to H5 VLPs during protein transfer (Figure 2A). By quantitative ELISA using known concentrations of recombinant soluble GM-CSF to generate a standard curve, protein transferred-GPI-GM-CSF constituted 8.9% to 38.5% of total VLP protein depending on the concentration GPI-GM-CSF added during protein transfer. The kinetic analysis of protein transfer revealed that the level of GPI-ICAM-1 incorporation onto VLPs was dependent on incubation time with saturation reaching within 2 h (Figure 2B). Protein transfer was not limited to H5 VLPs but could also be extended to H1 VLPs derived from the expression of H1N1 influenza A/PR/8/1934 hemagglutinin and matrix 1 proteins (Figure 2C). These results show that the level of incorporation of GPI-ISMs onto influenza VLPs during protein transfer can be controlled by varying the concentration of purified GPI-ISMs and/or time. To determine whether more than one GPI-ISM could be incorporated simultaneously, H5 VLPs were tested for protein transfer with either a fixed concentration of GPI-ICAM-1 and varying concentrations of GPI-IL-12 (Supplemental Figure 3A) or with a fixed concentration of GPI-IL-12 and varying concentrations of GPI-ICAM-1 (Supplemental Figure 3B). Both GPI-ISMs incorporated onto influenza VLPs in a concentration dependent manner after protein transfer, and incorporation of one GPI-ISM did not affect incorporation levels of the other at the tested concentrations.

Protein transfer of GPI-ISMs onto influenza VLPs depends on an intact GPI-anchor and does not affect the integrity of VLPs:

The GPI-anchor consists of two fatty acid chains that allow for insertion of the GPI-ISMs into the lipid bilayer by protein transfer (28-30). Phosphatidylinositol-specific phospholipase C (PI-PLC) cleaves the GPI-anchor from the ISM leaving the remaining protein domain intact (31). To determine if incorporation of GPI-ISMs onto influenza VLPs by protein transfer is GPI-anchor-dependent, GPI-ICAM-1 was subjected to PI-PLC digestion before or after protein transfer (Figure 2D). PI-PLC treatment decreased the level of VLP-incorporated-GPI-ICAM-1 and also abolished the ability of GPI-ICAM-1 to incorporate onto VLPs by protein transfer. Both of these actions of PI-PLC were blocked by $ZnCl_2$, a potent inhibitor of the PI-PLC enzyme. A similar effect on GPI-IL-12 was observed. Moreover, recombinant soluble IL-12 that lacks a GPI-anchor did not incorporate onto the VLPs upon incubation. When fatty acid binding proteins, BSA or orosomucoid, were added to compete during protein transfer, the level of GPI-ICAM-1-incorporation onto VLPs decreased compared to the control (Figure 2E). These results show that protein transfer mediated incorporation of GPI-ISMs onto VLPs is a specific process dependent on an intact GPI-anchor lipid moiety. Further, neither the structural integrity of the VLPs as assessed by electron microscopy (Supplemental Figure 4A) nor the concentration of influenza-specific proteins on the VLPs (Supplemental Figure 4B) were affected by the protein transfer process. Physicochemical characterization of the VLPs before and after protein transfer showed that the size and zeta potential of VLPs slightly lowered after protein transfer (Supplemental Figure 4C). By the Malvern Zetasizer Nano ZS analysis, the average size of the VLPs was 214.5 ± 38.7 nm before

and 191.0 ± 25.8 nm after protein transfer (Supplemental Figure 4C, left panel). Compared to electron microscopy in which VLPs appeared approximately 100 nm in diameter, the size is increased by Zetasizer analysis perhaps due to VLP aggregates. VLP aggregates were also seen by electron microscopy analysis supporting this idea. Further, the average polydispersity index of the VLPs was 0.226 ± 0.043 before and 0.454 ± 0.069 after protein transfer with GPI-ICAM-1 (data not shown). The increase in PDI after protein transfer suggests that there was a broader range of particle sizes after protein transfer with GPI-ICAM-1, however this may be due to the presence of GPI-ICAM-1 micelles in solution as these samples were not centrifuged to remove unincorporated GPI-ICAM-1. On the other hand, the zeta potential measures the net charge around the particles. In general, a larger net charge is associated with more stable particles. After protein transfer, the zeta potential decreased from -10.6 mV to -12.7 mV (Supplemental Figure 4C, right panel) suggesting that incorporation of GPI-ISMs by protein transfer does not destabilize the VLPs. Further, endotoxin levels were undetectable in protein transfer-modified-VLPs at the concentration that we used for administration (data not shown). This was not unexpected as VLPs are produced in insect cells whereas endotoxin concerns derived from vaccines are usually from production using bacterial cultures.

Protein transferred-influenza VLPs retain functional GPI-ISMs and are unaffected by storage:

GM-CSF stimulates bone marrow (BM) cell proliferation (12); therefore, we tested whether GPI-GM-CSF-protein transferred-VLPs (GPI-GM-CSF-VLPs) retained

ISM-functionality by inducing BM cell proliferation. After 4 days of culture, GPI-GM-CSF-VLPs induced 16-fold enhanced BM cell proliferation as compared to unmodified VLPs (Figure 3A). The co-incorporation of GPI-GM-CSF along with the cell adhesion molecule, GPI-ICAM-1, by protein transfer did not alter proliferation levels (data not shown) suggesting that ICAM-1 interaction with its receptor on BM cells does not further augment GPI-GM-CSF-VLP-induced proliferation. These results demonstrate that protein transferred-GPI-GM-CSF on VLPs retains its activity and is capable of stimulating cellular functions similar to soluble GM-CSF.

Vaccines often require storage, so we tested the ability of protein transferred-VLPs to retain functional GPI-ISMs after storage at 4˚C, at which VLP vaccines remain stable long-term (32). Storage of protein transferred-GPI-GM-CSF-VLPs for 4 weeks at 4˚C did not reduce the amount (Figure 3B) or the functionality (Figure 3C) of GM-CSF on the protein transferred-VLPs, as freshly prepared and stored GPI-GM-CSF-VLPs induced similar levels of BM cell proliferation.

Vaccination with protein transferred-influenza VLPs enhances anti-H5N1 viral serum antibody responses:

Influenza VLP vaccination induces strong antibody-responses against homologous viruses (10, 33, 34); however antibody responses against heterologous influenza viruses are weaker. We hypothesized that incorporating GPI-ISMs onto H5 VLPs by protein transfer would optimally activate immune cells leading to enhanced anti-heterologous viral immune responses. To test the *in vivo* efficacy of protein transferred influenza H5 VLPs, we analyzed serum antibody responses in vaccinated mice against either H5 VLPs,

homologous inactivated H5N1 influenza A/Indonesia/05/2005 virus, or heterologous inactivated H5N1 influenza A/Vietnam/1203/2004 virus by ELISA. Mice vaccinated with GPI-GM-CSF-VLPs demonstrated significantly enhanced (6-fold higher) anti-homologous and anti-heterologous serum IgG levels against inactivated H5N1 virus compared to mice vaccinated with unmodified VLPs (Figure 4A) on d17 after boost. Interestingly, although GPI-ICAM-1 (Supplementary Figure 5) and GPI-IL-12 (data not shown) remained functional *in vitro* after purification, GPI-ICAM-1-VLPs and GPI-IL-12-VLPs did not augment humoral immunity against virus (Figure 4A). Further, VLPs simultaneously protein transferred with both GPI-GM-CSF and GPI-IL-12 enhanced anti-homologous and anti-heterologous viral IgG similar to GPI-GM-CSF-VLPs (Figure 4A) suggesting that the enhanced antibody response is contributed mostly by GM-CSF. Also, vaccination with 0.5 μg of GPI-GM-CSF-VLPs that express 0.029 μg GM-CSF/μg VLP or 0.089 μg GM-CSF/μg VLP resulted in similar homologous inactivated H5N1 influenza A/Indonesia/05/2005 virus and heterologous inactivated H5N1 influenza A/Vietnam/1203/2004 virus specific IgG responses (Supplemental Figure 6).

Since humoral immune responses depend on T-cell help, IgG antibody subtype responses were examined to investigate the type of T-cell response elicited by VLP vaccination. IgG2a and IgG2b antibody subtypes correlate with a $CD4^+$ T helper 1 (Th1) response whereas IgG1 correlates with a Th2 response (35). A single vaccination of mice with GPI-GM-CSF-VLPs led to a 3-fold enhancement of anti-VLP-specific IgG compared to unmodified VLPs, however after boost, the fold enhancement narrowed to 1.4 (Figure 4B). GPI-GM-CSF-VLP vaccination enhanced 3-fold anti-VLP-specific IgG2a and anti-homologous Indonesia virus-specific IgG2a and IgG2b (Figure 4C)

antibody responses as well as increased 2 to 3-fold anti-heterologous Vietnam virus-IgG2a and IgG2b responses (Figure 4D). Interestingly, virus-specific IgG1 antibody responses were also elevated by 2-fold in mice vaccinated with GPI-GM-CSF-VLPs, suggesting an enhancement of both Th1 and Th2 responses in these mice.

VLPs protein transferred with GPI-GM-CSF provide complete protection against a heterologous virus challenge:

Vaccination with unmodified VLPs provides protection against challenge from homologous virus (10, 13); however, complete protection is not detected against heterologous viral strains (13). To determine if the enhanced antibody response detected in mice vaccinated with GPI-GM-CSF-VLPs corresponded to better heterologous protection, an intranasal challenge with the heterologous influenza A/Vietnam/1203/2004 (rgH5N1) virus (1LD) was conducted. Mice vaccinated with GPI-GM-CSF-VLPs exhibited significantly minimal changes in body weight (Figure 5A) and complete (100%) protection against a heterologous challenge (Figure 5B), whereas unvaccinated mice or mice vaccinated with unmodified VLPs had marked weight loss resulting in only 60% survival. Mice vaccinated with GPI-GM-CSF-VLPs also displayed a decreased viral titer whereas unmodified VLP vaccinated mice showed a similar lung viral titer as control mice suggesting effective control of viral replication by GPI-GM-CSF-VLPs (Figure 5C).

To determine if virus-specific long-lived memory B cells were present after vaccination with GPI-GM-CSF-VLPs, mice were challenged with rgH5N1 virus 21 weeks post boost. BM and lung cells derived from mice vaccinated with GPI-GM-CSF-

VLPs displayed enhanced antibody production against H5 VLPs, inactivated H5N1 Indonesia virus, and Vietnam virus compared to VLP vaccinated mice after both 1 and 5 days of culture. However, spleen cells demonstrated enhanced anti-viral IgG responses only after 5 days of culture with antigen (Figure 5D).

Discussion

Safety issues, concerns about production in chicken eggs, and difficulty in manufacturing conventional whole inactivated or attenuated influenza virus-based vaccines severely limit the current vaccine production system to meet pandemic threats. Therefore VLP-based vaccines, which are easier to produce in large-scale quantities and safer due to the lack of viral genomes, are considered to be a strong alternative to currently used virus-based vaccines. VLPs, due to their nanoparticulate and repetitive antigenic nature induce a strong immune response to viral proteins upon vaccination (33). However, although VLP-based influenza vaccines lead to complete protection against challenge with homologous virus, protection against heterologous viral strains is weak (13, 36). Herein, we demonstrate a protein transfer approach to adjuvantate enveloped VLPs with ISMs in a controlled manner and within a relatively short amount of time. In this method, a simple incubation of influenza VLPs with purified GPI-ISMs led to spontaneous incorporation of the GPI-ISMs onto the surface of VLPs in a time and concentration dependent manner. This protein transfer method relied on the presence of the GPI-anchor, occurred within two hours, and allowed for more than one GPI-ISM to be incorporated simultaneously onto the VLPs. The incorporated protein was expressed stably, as protein transfer-modified-VLPs can be stored for a month without losing the function of the incorporated molecules.

Our results demonstrated that mice vaccinated with protein transfer-modified-influenza H5 VLPs had enhanced anti-VLP and anti-viral (homologous and heterologous) serum IgG responses compared to unmodified VLP-vaccinated mice. Subtype analysis of anti-viral IgG antibodies showed that vaccination with GPI-GM-CSF-VLPs augmented anti-viral IgG2a and IgG2b responses, suggestive of a protective Th1 phenotype,

however anti-viral IgG1 subtype was also elevated demonstrating that the inclusion of GPI-GM-CSF onto these biodegradable nanoparticles enhances both Th1 and Th2-type immunity. Furthermore, this enhancement in anti-viral IgG responses correlated with protection against a heterologous viral challenge. Skountzou *et al*. demonstrated that vaccination with genetically engineered SIV VLPs expressing GPI-GM-CSF leads to enhanced SIV-specific IgG and neutralizing antibodies compared to VLPs administered with similar amounts of recombinant soluble GM-CSF (12). This suggests that augmentation of the immune response by protein transferred GPI-GM-CSF-VLPs could be due to administering cytokines physically attached to the nanoparticulate VLP surface, which allows for localized effects of the cytokine that can further aid in activating an immune response against VLP-associated antigens. Cytokine immobilization onto the VLPs not only reduces systemic toxicity associated with administered soluble cytokines, but also may allow for a slow release depot of the cytokine at the vaccination site thus increasing the cytokine half-life. Furthermore, because the receptor for GM-CSF is expressed on antigen presenting cells, such as DCs and B cells, engulfment of VLPs by phagocytosis or micropinocytosis (37) and activation of these cells could also be further enhanced by the presence of GM-CSF on the VLP surface.

The advantage of protein transfer to modify VLPs over gene transfer is the rapid process and that the level of incorporation can be easily controlled. Previous studies show that GPI-GM-CSF or CD40L incorporation onto SIV VLPs by gene transfer resulted in only 0.1% or 0.14% expression of total VLP proteins, respectively (12), however by protein transfer we are able to achieve GPI-GM-CSF expression up to 38.5% under the conditions that we tested. We also found that vaccination with protein transfer-

modified VLPs expressing 0.089 µg GM-CSF/µg VLP or 0.029 µg GM-CSF/µg VLP led to similar anti-viral IgG responses suggesting that low doses of GM-CSF incorporation can also induce anti-viral immune responses. Further, incorporating GPI-GM-CSF onto VLPs enhances immunity against viral-proteins, thereby decreasing the VLP dosage required for protection. This dose sparing outcome will increase vaccine availability and reduce production costs, which will be highly advantageous when quick large-scale vaccine production is required under the pressure of pandemic threats.

This method of incorporating novel antigens onto VLPs by protein transfer has the potential to be expanded to include additional viral proteins onto the VLPs. For example, cross protection against different viral influenza strains can be induced by protein transferring different viral strains of GPI-anchored-HA onto VLPs. Recent studies have shown that a higher density of antigen in VLPs increases antigen-specific immune responses (2, 38-41). Therefore, similarly the density of endogenously expressed HA antigen on the VLPs can be further increased by protein transfer of exogenously added GPI-HA for a better immune response.

It is well established that recombinant VLPs are easier to produce in large quantities and are as effective as currently used virus-based influenza vaccines produced in egg-based technologies. Our approach of adjuvanting VLPs with GPI-ISMs described in the current work further enhances the immunogenicity and breadth of the immune response and thus adds another layer of advantage to VLP-based influenza vaccines. Another advantage in vaccine production over the traditional egg-based technologies may be the stock preparation of GPI-ISM or GPI-HA, which can be coupled with influenza VLP vaccines by protein transfer especially with the possibility of a

potential pandemic at any time regardless of season. Also, production of influenza VLPs in insect cells does not have the safety concerns over handling highly pathogenic avian influenza viruses that are often lethal to embryos and can be a potential problem in the traditional egg-grown vaccine technologies.

In summary, we have demonstrated that protein transfer of GPI-ISMs onto nanoparticulate influenza VLPs leads to stable incorporation of the GPI-ISMs on the VLPs in a time and concentration dependent manner, and the incorporated GPI-ISM remains functional even after storage. Moreover, we have demonstrated the enhanced efficacy of protein transfer-modified-VLP vaccines in an influenza viral model. Therefore, protein transfer of GPI-ISMs onto VLPs provides an easy means to adjuvantate the immunogenicity of viral proteins expressed on the VLPs and thus allows for the development of effective enveloped VLP-based vaccines.

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Figure Legends:

Figure 1. Schematic of protein transfer of GPI-anchored proteins onto influenza VLPs. Transmembrane or secretory ISMs can be converted to GPI-anchored forms by attaching the DNA sequence correlating to the extracellular domain of transmembrane proteins or secretory ISMs to the DNA sequence corresponding to the GPI-anchor signal sequence from naturally occurring GPI-anchored CD59. The DNA constructs are then inserted into mammalian cell expression plasmid vectors and the resulting DNA plasmid constructs are used to transfect CHO-K1 cells. Transfected CHO-K1 cells are grown in large quantities using roller bottles, lysed, and then the GPI-ISMs are purified by mAb-affinity chromatography. Protein transfer is performed by incubating enveloped influenza VLPs with purified GPI-ISMs at 37°C for 2-6 h. Unincorporated GPI-ISMs are washed out by centrifugation at 13,200 rpm at 4˚C for 30 min. Resulting incorporation is detected by western blot analysis. CM – cell membrane; ECD – extracellular domain; HA - influenza hemagglutinin protein; M1 - influenza matrix protein 1.

Figure 2. Protein transfer of GPI-ISMs onto influenza VLPs depends on the GPI-anchor. (**A**) Concentration dependence of protein transfer. Varying concentrations of GPI-ICAM-1, GPI-IL-12, or GPI-GM-CSF were incubated with H5 VLPs at 37˚C for 2 h in PBS/0.01% ovalbumin. The VLPs were washed twice with PBS by centrifugation and the resulting VLPs were analyzed by western blot. (**B**) Kinetics of protein transfer. H5 VLPs were protein transferred with GPI-ICAM-1 for different time periods at 37˚C. ICAM-1 incorporation was analyzed by SDS PAGE and western blot analysis. (**C**) Protein transfer of GPI-ISMs onto H1 VLPs. GPI-ICAM-1 or GPI-GM-CSF was protein

transferred onto H1 VLPs. (**D**) Effects of PI-PLC treatment. VLPs protein transferred with GPI-ICAM-1 were treated with PI-PLC and analyzed by western blot (top panel). PI-PLC treatment was also performed on GPI-ICAM-1 or GPI-IL-12 before subjecting to protein transfer with H5 VLPs (bottom two panels). (**E**) Fatty acid binding proteins compete with and inhibit incorporation of GPI-ISMs onto H5 VLPs. During protein transfer, 1% BSA or 1% orosomucoid was incubated with H5 VLPs and GPI-ICAM-1. Due to availability and ease of purification, most of the protein transfer optimization studies were performed with GPI-ICAM-1.

Figure 3. Functionality and storage of protein transferred VLPs. (**A**) VLPs protein transferred with GPI-GM-CSF induce proliferation of BM cells as described in Methods section. The protein transferred GPI-GM-CSF constituted approximately 38.5% of total VLP protein in GPI-GM-CSF-VLPs. (**B**) Protein transferred VLPs retain incorporated GPI-GM-CSF after storage. VLPs protein transferred with GPI-GM-CSF were stored at 4˚C for up to 4 weeks. Relative intensity was calculated by comparing the ratio of GM-CSF-to-VLP proteins at each storage time point to the ratio at day 0. (**C**) Protein transferred GPI-GM-CSF-VLPs stored for 4 weeks induce BM cell proliferation. BM cells were cultured in triplicate for 4 days with freshly prepared or stored GPI-GM-CSF-VLPs. Proliferation of BM cells was detected by CellTiter 96® Aqueous One Solution. (Statistical analysis: (**A**) One-way ANOVA – Tukey's multiple comparisons test, $p < 0.0001$, (**C**) multiple t-test; $p < 0.0001$).

Figure 4. Vaccination of mice with H5 VLPs protein transferred with GPI-GM-CSF leads to enhanced anti-homologous and anti-heterologous H5N1 inactivated virus and anti-VLP serum IgG. (**A**) Anti-homologous H5N1 influenza A/Indonesia/05/2005 and anti-heterologous H5N1 influenza A/Vietnam/1203/2004 specific total IgG in vaccinated mice. Serum was collected from groups of vaccinated mice (n = 5) 17 days after boost. Using 1:1000 diluted serum, an ELISA was performed to detect anti-homologous and anti-heterologous influenza virus specific total IgG. (**B**) H5 VLP-specific IgG subtype responses upon vaccination with protein transferred GPI-GM-CSF-VLPs. Serum was collected from groups of vaccinated mice (n = 5) 28 days after initial vaccination (left) and 7 days after boost (right). A 1:5000 diluted serum was used in an ELISA against coated H5 VLPs. (**C**) Inactivated homologous H5N1 influenza A/Indonesia/05/2005 virus-specific serum IgG subtypes. Serum was collected from groups of vaccinated mice (n = 5) 6 weeks after boost and 1:1000 dilution of serum was used in an ELISA against coated inactivated H5N1 influenza A/Indonesia/05/2005 virus. (**D**) Inactivated heterologous H5N1 influenza A/Vietnam/1203/2004 virus-specific serum IgG subtypes. Serum was collected from vaccinated mice (n = 5) 6 weeks after boost and 1:1000 diluted serum was used in an ELISA against coated inactivated H5N1 influenza A/Vietnam/1203/2004 virus. (Statistical analysis: (**A**) One-Way ANOVA – Tukey's multiple comparisons test, (**B-D**) T-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Figure 5. GPI-GM-CSF-VLP vaccination induces complete protection after a heterologous H5N1 influenza A/Vietnam/1203/2004 virus challenge. Groups of mice

(n = 5) were vaccinated and boosted 36 days later with 0.5 µg GPI-GM-CSF-VLPs protein transferred to incorporate 0.089 µg GM-CSF/µg VLP. Mice were then challenged intranasally with heterologous influenza A/Vietnam/1203/2004 (rgH5N1) virus (1LD) and (**A**) body weight and (**B**) survival was measured. Mice that displayed <75% of initial body weight were sacrificed according to IACUC regulations. (**C**) Decreased lung viral titers in mice (n = 3) vaccinated with GPI-GM-CSF-VLPs 4 days post challenge with heterologous rgH5N1. Lungs homogenates were used to determine viral titers using 9-11 day old embryonated eggs. (**D**) Enhanced generation of virus-specific-IgG antibody responses by cells from spleen, bone marrow, and lungs of GPI-GM-CSF-VLP vaccinated mice. Spleen, bone marrow and lung cells of mice vaccinated with PBS, unmodified VLPs, or GPI-GM-CSF-VLPs were cultured in plates coated with either PBS, H5 VLPs, homologous H5N1 influenza A/Indonesia/05/2005 or heterologous H5N1 influenza A/Vietnam/1203/2004 inactivated virus as antigen and the production of antigen-specific IgG was detected by ELISA. (Statistical analysis: (**A**) Repeated Measures Two-Way ANOVA, (**C**) One-Way ANOVA – Dunnett's multiple comparisons test; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ (**D**) Two-Way ANOVA – Tukey's multiple comparisons test; * $p < 0.0001$).

Supplementary Figure Legends:

Supplemental Figure 1. SDS PAGE of affinity purified GPI-ISMs. GPI-ICAM-1, GPI-GM-CSF, and GPI-IL-12 were purified from transfected CHO-K1 cell lysates by single step affinity chromatography. Purified GPI-ISMs were subjected to SDS PAGE, (**A**) western blot and (**B**) silver staining analysis.

Supplemental Figure 2. Purified GPI-ISMs incorporate onto the surface of sheep RBCs by protein transfer. Purified GPI-ISMs were incubated with sheep RBCs (2 x 10^6) in PBS with 0.1% ovalbumin for 4 h at 37°C. The RBCs were washed by centrifugation in PBS and then analyzed by flow cytometry.

Supplemental Figure 3. Two GPI-ISMs can incorporate simultaneously onto influenza VLPs by protein transfer. (**A**) H5 VLPs were protein transferred with a constant concentration of GPI-ICAM-1 and increasing concentrations of GPI-IL-12. (**B**) H5 VLPs were protein transferred with a constant concentration of GPI-IL-12 and increasing concentrations of GPI-ICAM-1. The results are a representative of at least three separate experiments.

Supplemental Figure 4. The structural integrity and physicochemical properties of GPI-ISM-protein transferred-VLPs. (**A**) Electron microscopy of H5 VLPs modified by protein transfer. Representative electron microscopy images are depicted of influenza H5 VLPs before (left panel) and after (right panel) protein transfer with GPI-ICAM-1. Scale bar, 100 nm. (**B**) Protein transfer does not affect VLP protein content. H5 VLPs were protein transferred with or without GPI-ICAM-1 at 37˚C and analyzed by SDS PAGE and western blot analysis against ICAM-1 (top) or against H5 VLP by using serum from mice challenged with H5N1 virus (bottom). (**C**) Physicochemical properties of protein transfer-modified-VLPs. H5 VLPs were protein transferred with or without GPI-ICAM-1 at 37˚C for 6 h. The size (left panel; n = 28) and zeta potential (right panel;

n = 15) of H5 VLPs before and after protein transfer were measured using the Malvern Zetasizer Nano ZS. (Statistical analysis: (**C**) T-test. * $p < 0.05$, ** $p < 0.01$).

Supplemental Figure 5. Purified GPI-ICAM-1 retains functionality. To test the ability of GPI-ICAM-1 to bind to LFA-1-expressing cells, a plate binding inversion assay was conducted. 100 µl of 100 µg/ml of GPI-ICAM-1 were coated in triplicate onto ELISA plate wells and 250,000 LFA-1-expressing SKW3 cells were allowed to adhere to GPI-ICAM-1. The plate was then inverted in a trough containing PBS/0.1% cosmic calf serum/1 mM $MgCl_2$ for 1 h and the number of bound SKW3 cells was counted using a hemocytometer. SKW3 cells bound to wells coated with GPI-ICAM-1, however binding was significantly inhibited by blocking with an anti-ICAM-1 mAb. The results are a representative of at least three separate experiments. (Statistical analysis: One-Way ANOVA – Tukey's multiple comparisons test, **** $p < 0.0001$, ns – not significantly different).

Supplemental Figure 6. Vaccination with protein transfer-modified GPI-GM-CSF-VLPs that express either 0.029 µg GM-CSF/µg VLPs or 0.089 µg GM-CSF/µg VLPs induces similar levels of homologous and heterologous virus-specific IgG responses. Mice were vaccinated and boosted with 0.5 µg of unmodified VLPs or 0.5 µg of protein transfer-modified-GPI-GM-CSF-VLPs that expressed either 0.029 µg GM-CSF/µg VLPs or 0.089 µg GM-CSF/µg VLPs. Serum was collected from groups of vaccinated mice (n = 5) 6 weeks after boost and 1:5000 dilution of serum was used in an ELISA against coated inactivated homologous H5N1 influenza A/Indonesia/05/2005 virus (left panel) or

heterologous H5N1 influenza A/Vietnam/1203/2004 virus (right panel). (Statistical analysis: One-Way ANOVA – Tukey's multiple comparisons test, **** $p < 0.0001$).

Figure 1.

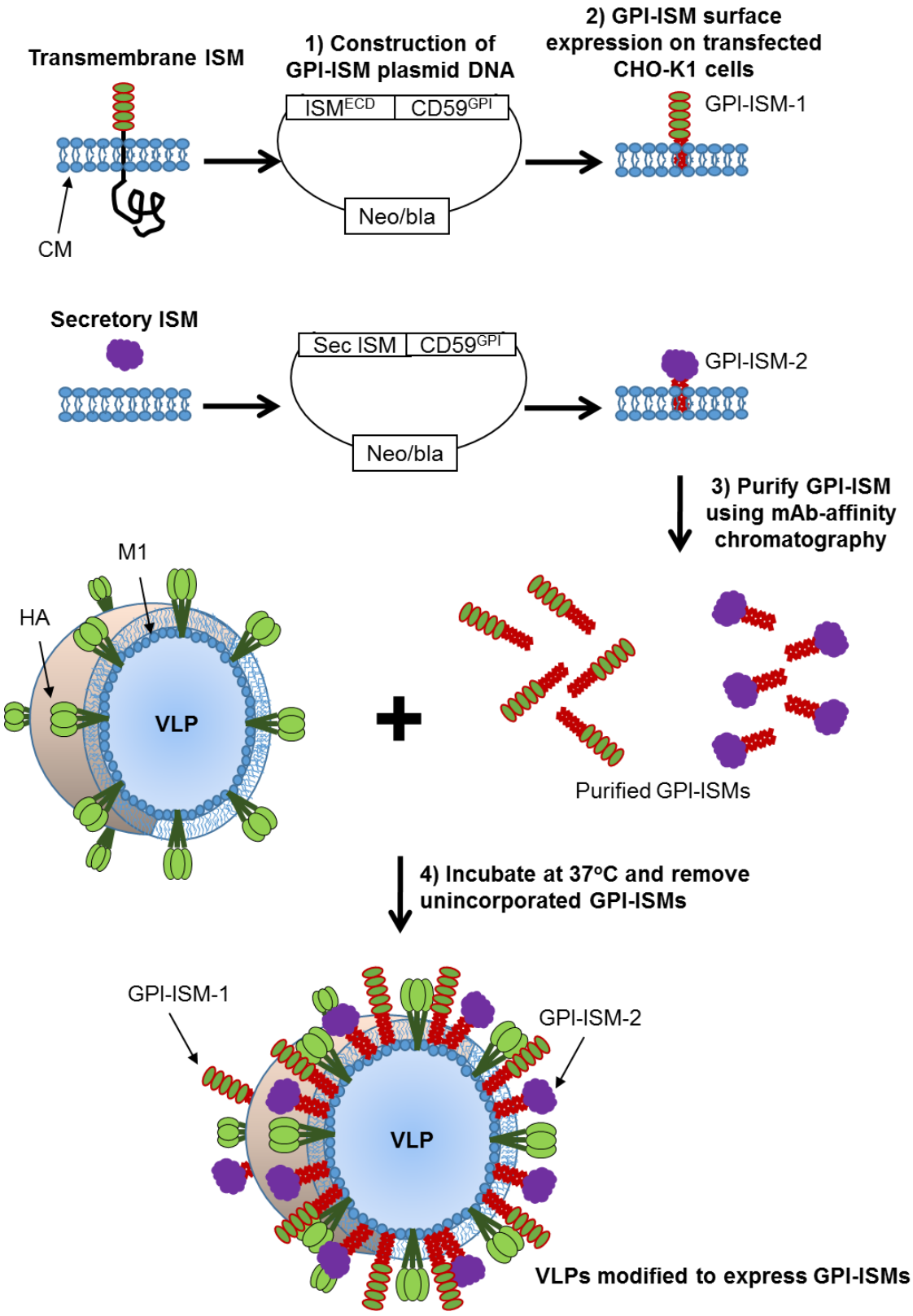

Transmembrane ISM
1) Construction of GPI-ISM plasmid DNA
2) GPI-ISM surface expression on transfected CHO-K1 cells
ISM^ECD
CD59^GPI
GPI-ISM-1
Neo/bla
CM
Secretory ISM
Sec ISM
CD59^GPI
GPI-ISM-2
Neo/bla
3) Purify GPI-ISM using mAb-affinity chromatography
M1
HA
VLP
Purified GPI-ISMs
4) Incubate at 37°C and remove unincorporated GPI-ISMs
GPI-ISM-1
GPI-ISM-2
VLP
VLPs modified to express GPI-ISMs

Figure 2.

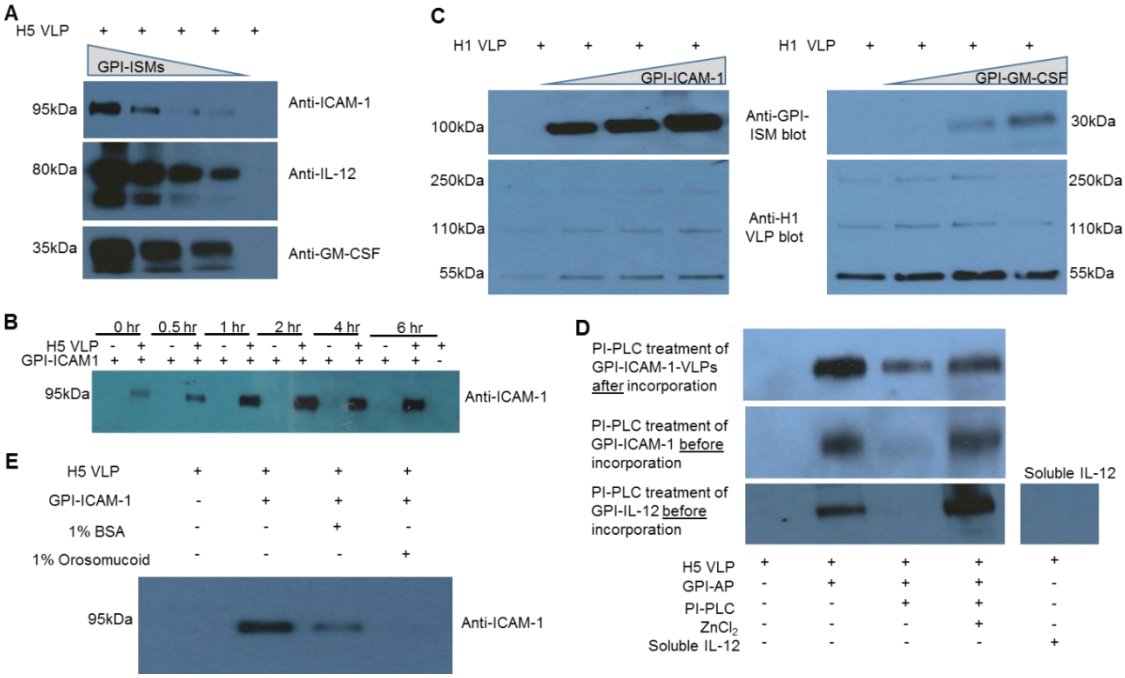

A
H5 VLP + + + + +
GPI-ISMs
95kDa
Anti-ICAM-1
80kDa
Anti-IL-12
35kDa
Anti-GM-CSF
C
H1 VLP + + + +
GPI-ICAM-1
100kDa
250kDa
110kDa
55kDa
Anti-GPI-ISM blot
Anti-H1 VLP blot
H1 VLP + + + +
GPI-GM-CSF
30kDa
250kDa
110kDa
55kDa
B
0 hr 0.5 hr 1 hr 2 hr 4 hr 6 hr
H5 VLP - + - + - + - + - + - + +
GPI-ICAM1 + + + + + + + + + + + + -
95kDa
Anti-ICAM-1
D
PI-PLC treatment of GPI-ICAM-1-VLPs after incorporation
PI-PLC treatment of GPI-ICAM-1 before incorporation
PI-PLC treatment of GPI-IL-12 before incorporation
Soluble IL-12
H5 VLP + + + + +
GPI-AP - + + + -
PI-PLC - - + + -
ZnCl2 - - - + -
Soluble IL-12 - - - - +
E
H5 VLP + + + +
GPI-ICAM-1 - + + +
1% BSA - - + -
1% Orosomucoid - - - +
95kDa
Anti-ICAM-1

Figure 3.

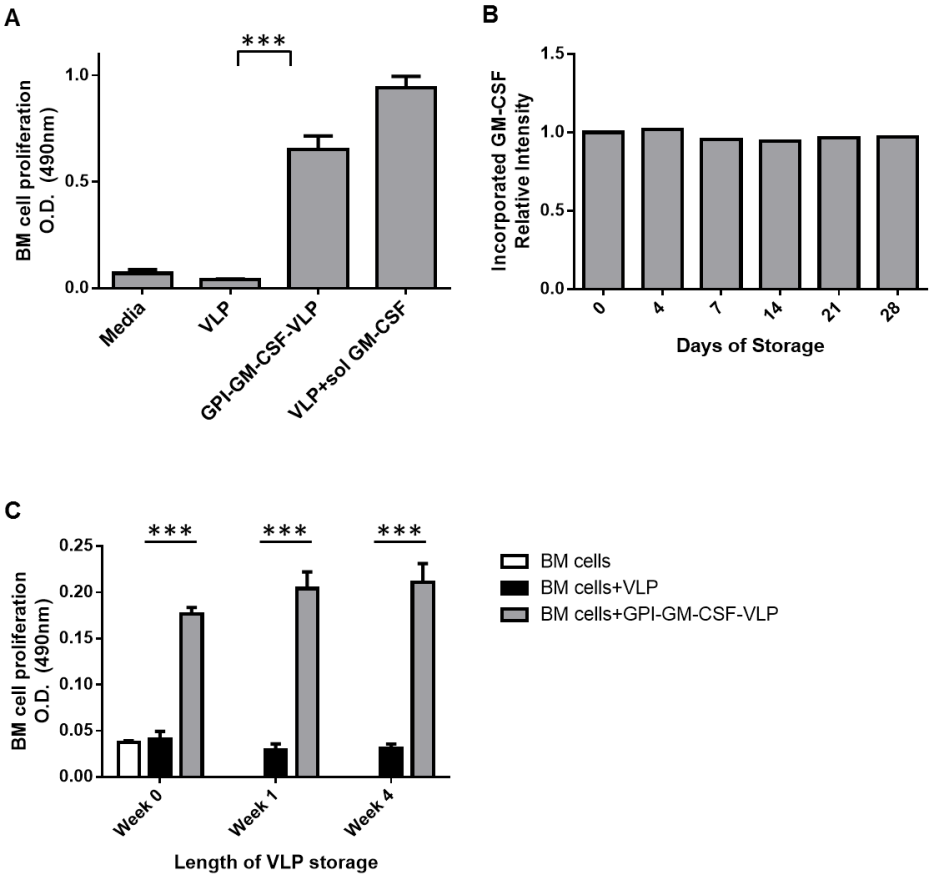
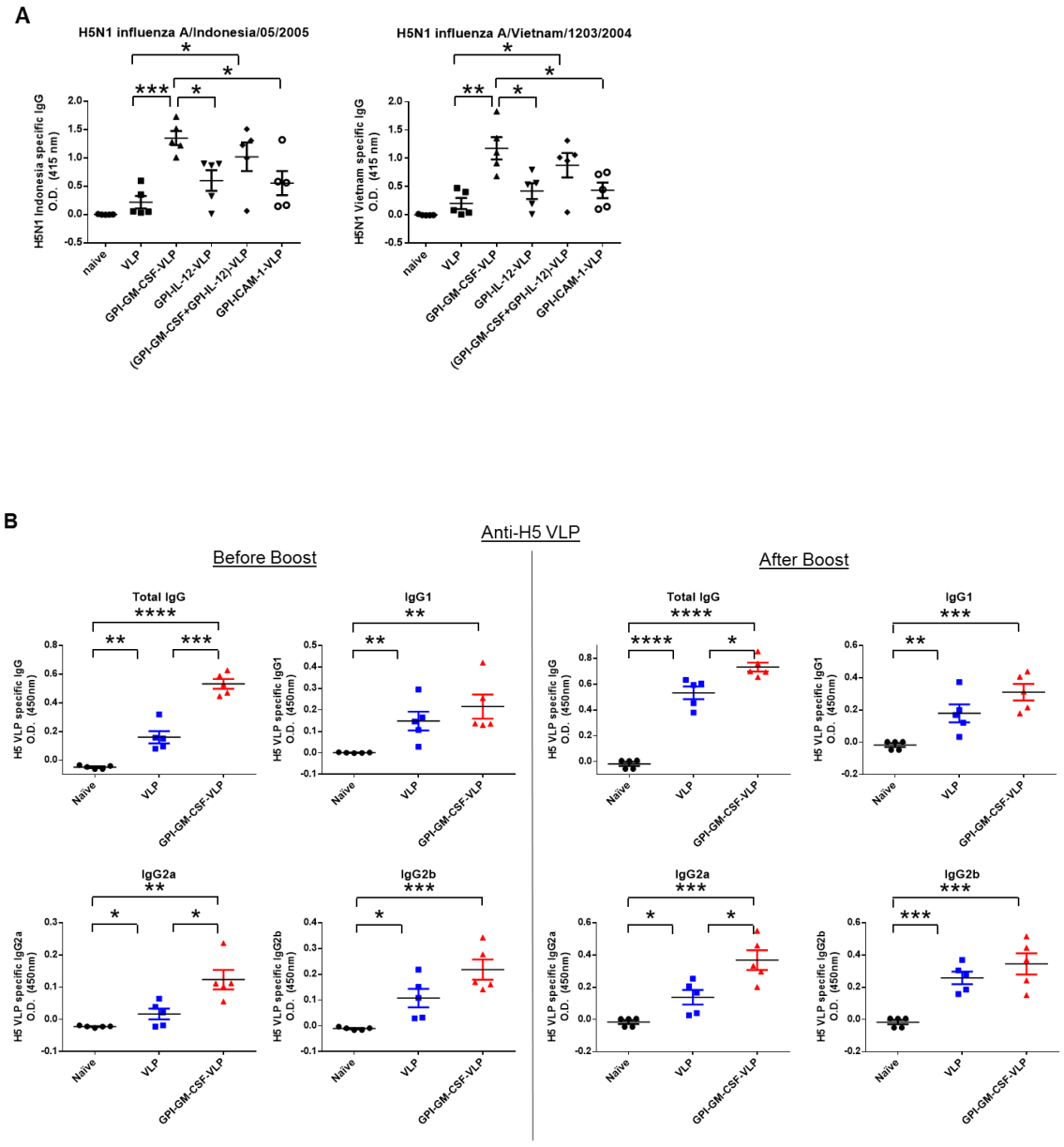

A
***
BM cell proliferation O.D. (490nm)
1.0
0.5
0.0
Media
VLP
GPI-GM-CSF-VLP
VLP+sol GM-CSF
B
Incorporated GM-CSF Relative Intensity
1.5
1.0
0.5
0.0
0
4
7
14
21
28
Days of Storage
C
***
***
***
BM cell proliferation O.D. (490nm)
0.25
0.20
0.15
0.10
0.05
0.00
Week 0
Week 1
Week 4
Length of VLP storage
BM cells
BM cells+VLP
BM cells+GPI-GM-CSF-VLP

Figure 4.


A
H5N1 influenza A/Indonesia/05/2005
H5N1 Indonesia specific IgG O.D. (415 nm)
naive
VLP
GPI-GM-CSF-VLP
GPI-IL-12-VLP
(GPI-GM-CSF+GPI-IL-12)-VLP
GPI-ICAM-1-VLP
H5N1 influenza A/Vietnam/1203/2004
H5N1 Vietnam specific IgG O.D. (415 nm)
B
Anti-H5 VLP
Before Boost
After Boost
Total IgG
IgG1
IgG2a
IgG2b
H5 VLP specific IgG O.D. (450nm)
H5 VLP specific IgG1 O.D. (450nm)
H5 VLP specific IgG2a O.D. (450nm)
H5 VLP specific IgG2b O.D. (450nm)
Naïve
VLP
GPI-GM-CSF-VLP

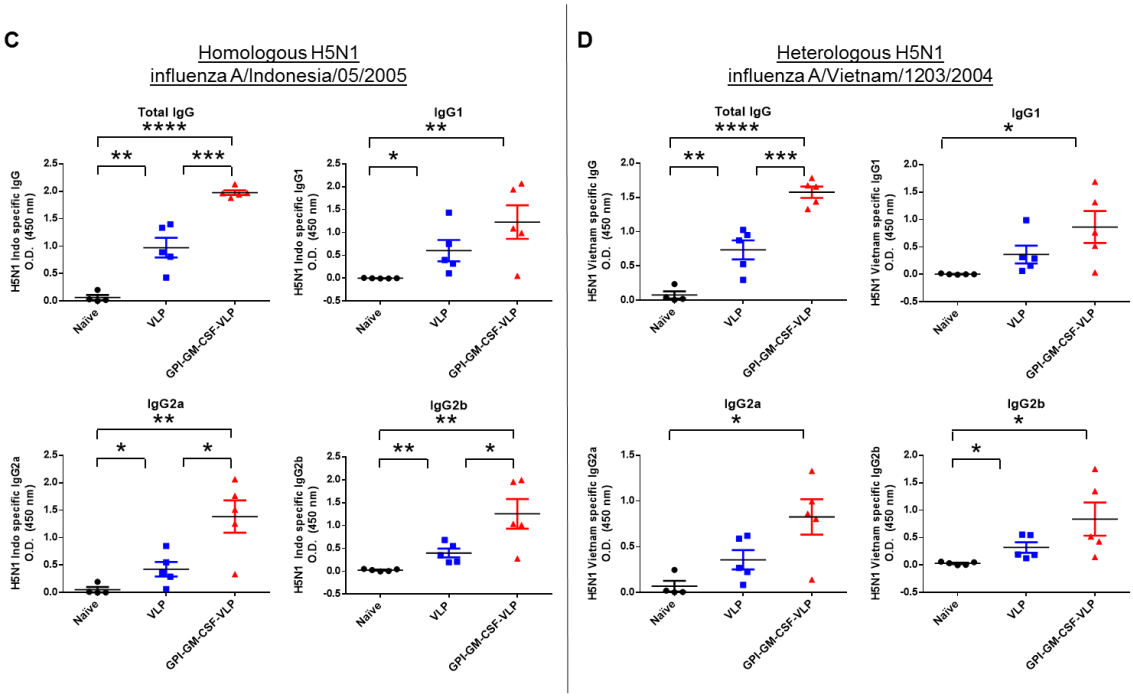
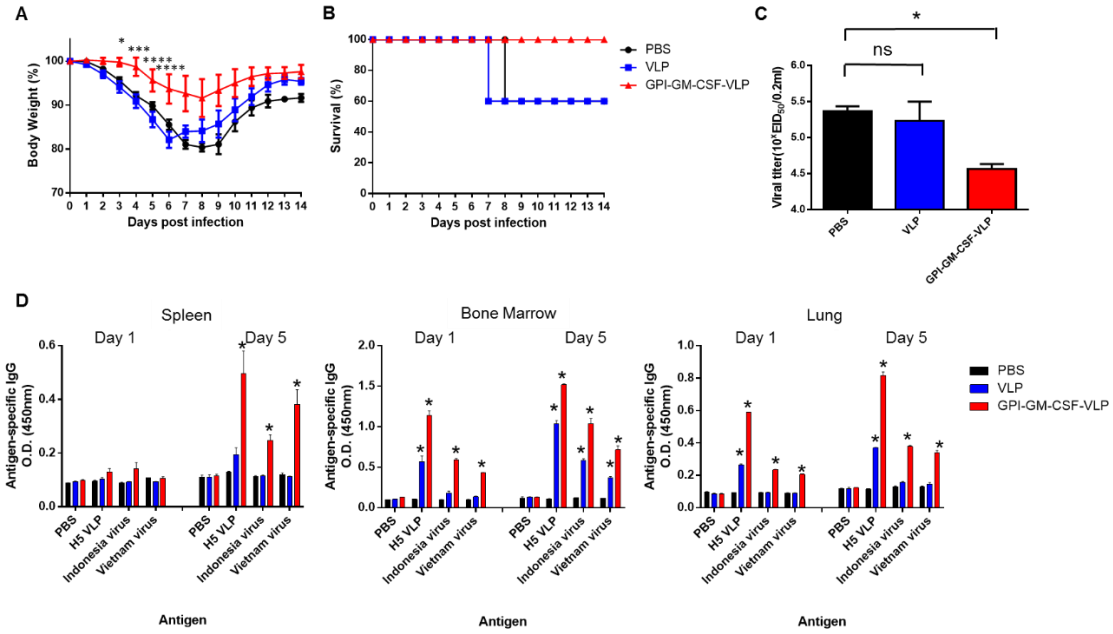
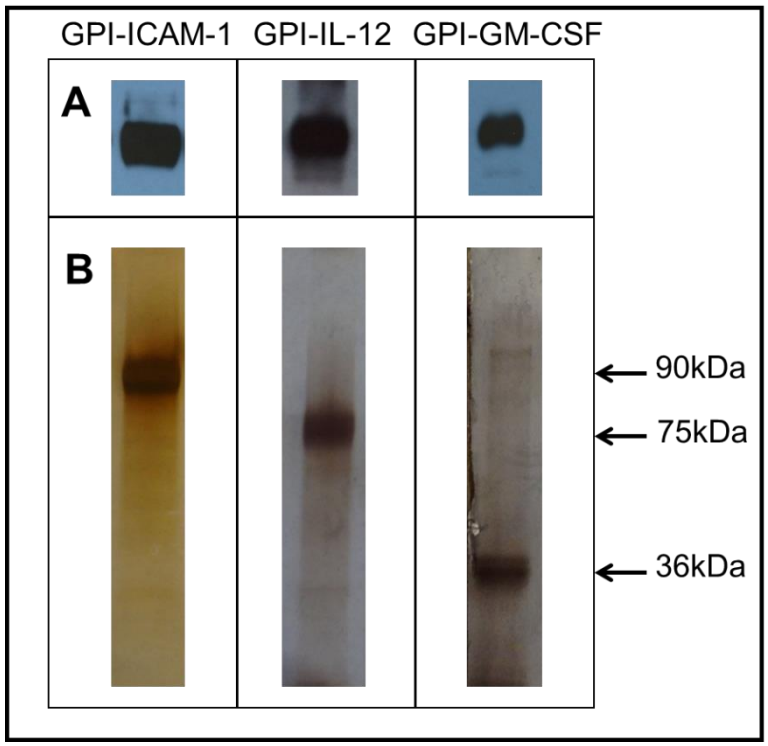

C
Homologous H5N1
influenza A/Indonesia/05/2005
Total IgG
IgG1
IgG2a
IgG2b
H5N1 Indo specific IgG O.D. (450 nm)
H5N1 Indo specific IgG1 O.D. (450 nm)
H5N1 Indo specific IgG2a O.D. (450 nm)
H5N1 Indo specific IgG2b O.D. (450 nm)
D
Heterologous H5N1
influenza A/Vietnam/1203/2004
H5N1 Vietnam specific IgG O.D. (450 nm)
H5N1 Vietnam specific IgG1 O.D. (450 nm)
H5N1 Vietnam specific IgG2a O.D. (450 nm)
H5N1 Vietnam specific IgG2b O.D. (450 nm)
Naive
VLP
GPI-GM-CSF-VLP

Figure 5.


A
Body Weight (%)
Days post infection
B
Survival (%)
Days post infection
PBS
VLP
GPI-GM-CSF-VLP
C
Viral titer(10^1EID50/0.2ml)
ns
*
PBS
VLP
GPI-GM-CSF-VLP
D
Spleen
Bone Marrow
Lung
Day 1
Day 5
Antigen-specific IgG O.D. (450nm)
PBS
H5 VLP
Indonesia virus
Vietnam virus
Antigen

Supplemental Figure 1.



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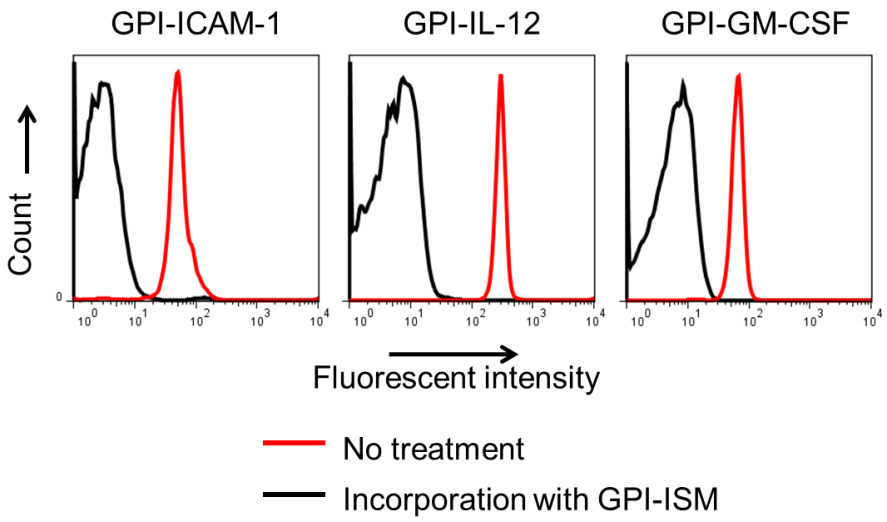

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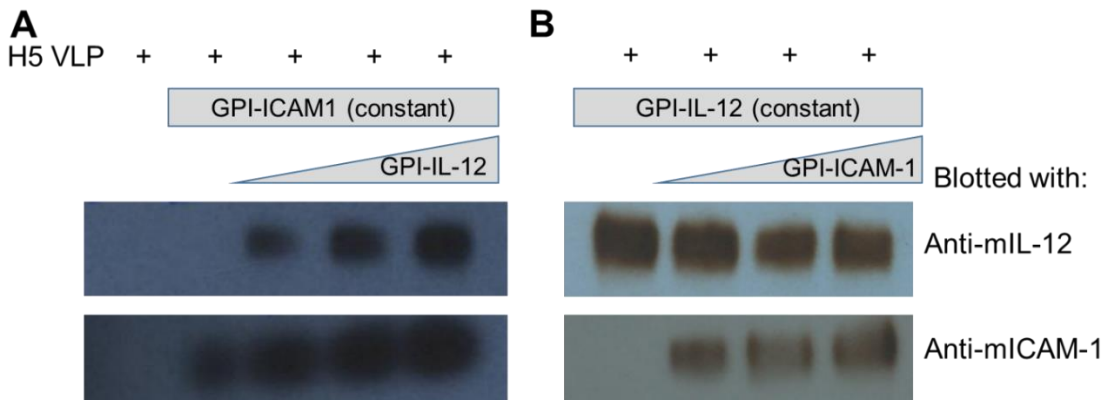
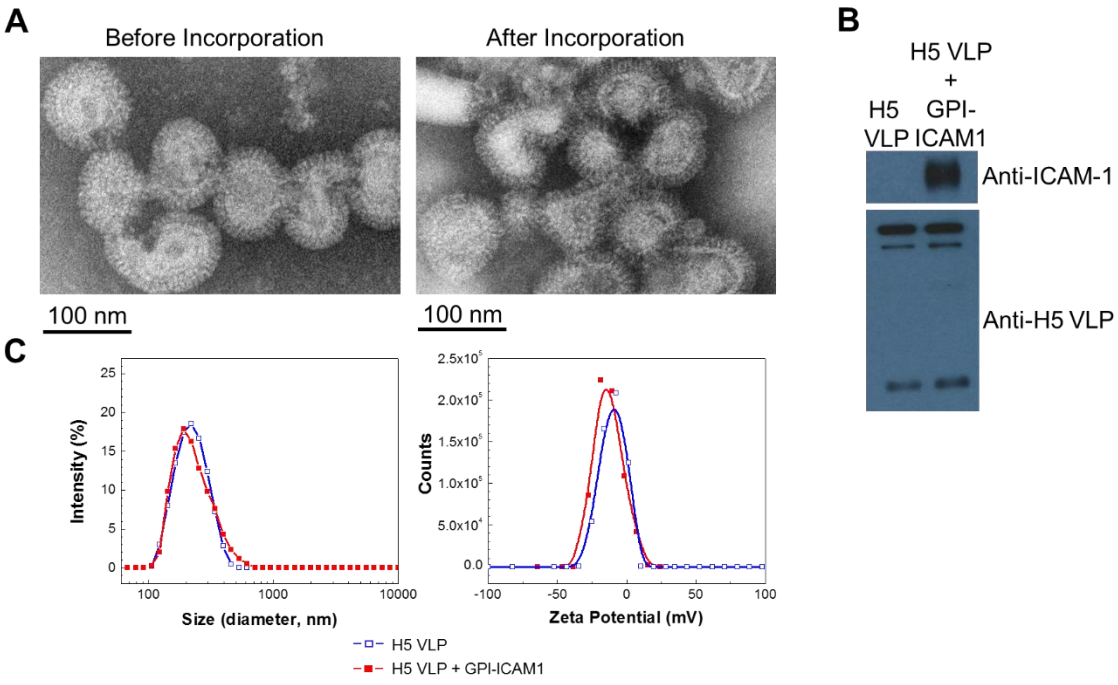


Supplemental Figure 4.



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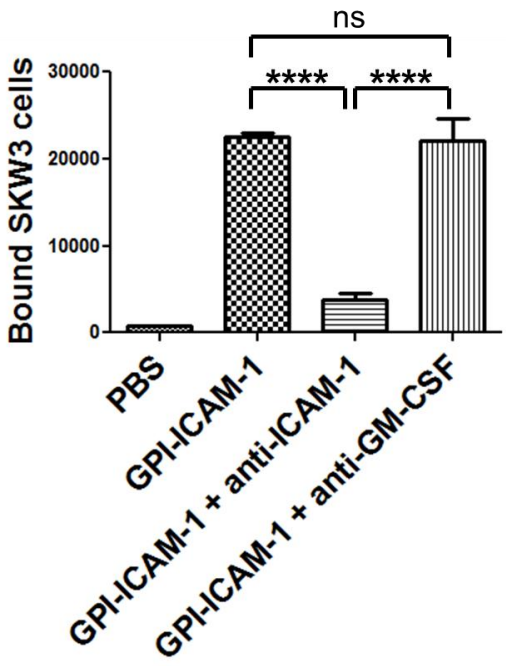
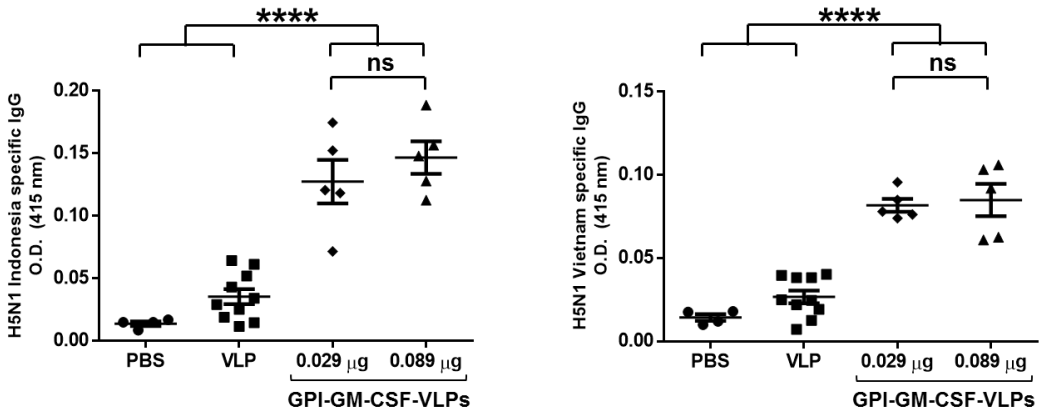


Supplemental Figure 6.



CHAPTER III:

Influenza virus-like particles engineered by protein transfer with tumor-associated antigens induces protective antitumor immunity

Figures 1-5, as presented in this Chapter, have been accepted for publication in

Biotechnology and Bioengineering, February 2015. PMID: 25689082.

Supplemental Figure 1 is unpublished.

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

Abstract

Delivery of antigen in particulate form using either synthetic or natural particles induces stronger immunity than soluble forms of the antigen. Among naturally occurring particles, virus-like particles (VLPs) have been genetically engineered to express tumor-associated antigens (TAAs) and have shown to induce strong TAA-specific immune responses due to their nano-particulate size and ability to bind and activate antigen-presenting cells. In this report, we demonstrate that influenza VLPs can be modified by a protein transfer technology to express TAAs for induction of effective antitumor immune responses. We converted the breast cancer HER-2 antigen to a glycosylphosphatidylinositol (GPI)-anchored form and incorporated GPI-HER-2 onto VLPs by a rapid protein transfer process. Expression levels on VLPs depended on the GPI-HER-2 concentration added during protein transfer. Vaccination of mice with protein transferred GPI-HER-2-VLPs induced a strong Th1 and Th2-type anti-HER-2 antibody response and protected mice against a HER-2-expressing tumor challenge. Soluble form of GPI-HER-2 induced only a weak Th2 response under similar conditions. These results suggest that influenza VLPs can be enriched with TAAs by protein transfer to develop effective VLP-based subunit vaccines against cancer without chemical or genetic modifications and thus preserve the immune stimulating properties of VLPs for easier production of antigen-specific therapeutic cancer vaccines.

Keywords: protein transfer, virus-like particles, breast cancer, HER-2, vaccine

Running title: Protein transfer of tumor antigens onto VLPs

Introduction

Overexpression of the tumor associated antigen (TAA) human epidermal growth factor receptor 2 (HER-2) found on a subset of breast cancers is correlated with poor prognosis and clinical outcome (1, 2). Therefore, HER-2 is an ideal candidate for targeted immunotherapies against cancer. Currently, multiple antibody-based treatment regimens exist to target HER-2 in HER-2-positive breast cancer (3). However, these passive antibody-mediated immunotherapies do not develop cellular immune responses and thus eventually fail. To fully combat cancer, active immunotherapies which develop humoral and cellular adaptive immune responses are needed.

Active immunotherapies include HER-2-expressing tumor cells that also secrete the cytokine GM-CSF (4, 5), patient derived peripheral blood mononuclear cells (PBMCs) activated with fusion proteins consisting of HER-2 and GM-CSF (6, 7), *ex vivo* loaded dendritic cells transfected with adenoviruses encoding HER-2 along with immunostimulatory molecules (8-10) or pulsed with HER-2 peptides (11, 12), as well as HER-2 encoding DNA vaccines (13-16), HER-2 peptide-based vaccines (17-19), and protein-based vaccines (20). Although these immunotherapies induce HER-2-specific immunity, complete remission is not seen. Further, obtaining and maintaining patient-derived DCs and PBMCs for *ex vivo* manipulation is expensive. Peptide-based and protein-based vaccination strategies are also poorly immunogenic due to stability issues and DNA-based vaccines do not allow for targeted expression. Therefore, there is a need for a more efficient approach to deliver the HER-2 antigen to the immune system.

Delivering antigens to the immune system as a particulate form has been shown to induce a stronger antigen-specific immune response than soluble antigens (21, 22). In

this regard, many biocompatible micro/nano-particles have been explored as delivery platforms. Among these, virus-like particles (VLPs) have shown to be effective. VLPs are nanoparticles that resemble viral counterparts in size and structure however they lack viral genome. They are derived from the expression of viral envelope and/or capsid proteins and express repetitive molecules in an ordered structure. The nanosize and particulate nature of VLPs makes them ideal for uptake by antigen presenting cells (APCs) (23, 24) and the presence of highly ordered repetitive structures on their surface provides a danger signal to initiate immune activation (25). These features make VLPs strong immunogens that induce robust cellular and humoral anti-viral immune responses upon vaccination (26).

The intrinsic immunogenicity of VLPs has been exploited to deliver tumor antigens to elicit antigen-specific immunity. Non-enveloped VLPs derived from murine polyomavirus VP1 and VP2 protein have been modified through genetic means to express HER-2 by fusing the extracellular domain of HER-2 to the VP2 protein (27). Vaccination with these chimeric HER-2-expressing VLPs led to protection against HER-2-expressing tumors. Further, vaccination with VLPs produced using rBVs expressing Gag and mTrop2, an antigen associated with pancreatic cancer, enhanced tumor infiltrating lymphocyte populations and generated mTrop2-specific antibodies and cytotoxic T lymphocytes that led to enhanced survival of pancreatic tumor-bearing mice (28). Both of these strategies involve genetic alterations to produce chimeric VLPs, however genetic engineering to produce chimeric VLPs may affect VLP proteins leading to lower VLP production and altered immunogenicity. Further, although genetic modification may produce a homogeneous product, the amount of expression of TAAs

per VLP cannot be easily manipulated due to the limitations of gene transfer technologies.

In this report we investigated whether enveloped VLPs, such as influenza VLPs, can be used as a tumor antigen delivery vehicle without the use of genetic manipulation but using a protein transfer approach. Previously, our lab has shown that cells or membrane vesicles that contain lipid bilayers can be modified by protein transfer to homogenously express glycophosphatidylinositol (GPI)-anchored immunostimulatory molecules, B7-1 or IL-12 (29-32). Protein transfer involves a simple incubation of purified GPI-proteins with cells or membrane vesicles for 2-4 hours and leads to stable expression of the incorporated protein which in turn remains functional (29-32). Influenza VLPs are enveloped VLPs that can be derived from the expression of hemagglutinin (HA) and matrix (M1) proteins and contain lipid bilayer envelopes derived from host cells. Vaccination with influenza VLPs has led to robust cellular and humoral immunity against HA expressed on the VLPs (33-36).

Based on these observations, we hypothesized that enveloped VLPs such as influenza VLPs can be modified to express TAAs by protein transfer similar to cellular membranes and used to induce tumor immunity. Herein, we show for the first time that influenza VLPs can be modified by protein transfer to express a TAA to induce TAA-specific immunity. We found that vaccination with GPI-HER-2 anchored to VLPs (GPI-HER-2-VLPs) by protein transfer led to enhanced protection against HER-2-expressing tumor growth in a murine breast cancer model. Further, GPI-HER-2-VLPs led to HER-2-specific IgG production and enhanced HER-2-specific Th1-type immunity compared to vaccination with GPI-HER-2 alone.

Methods

Animals, antibodies, and cell lines: 6-8 week old female BALB/c mice were purchased from Jackson Laboratory. All experiments were conducted in accordance with Emory University IACUC guidelines. Hybridoma secreting anti-HER-2 (TA1) monoclonal antibody (mAb) was obtained from ATCC. CHO-K1 cell transfectants expressing either full-length transmembrane HER-2 or GPI-HER-2 were maintained in RPMI 1640 and 10% Cosmic Calf Serum (Invitrogen) containing 10 μg/ml Blasticidin (Invitrogen). Panning was used to select for cells that expressed high levels of HER-2 and expression was analyzed by flow cytometry. Sheep red blood cells (RBCs) were purchased from HemoStat Laboratories. D2F2 and D2F2/E2 cells were a kind gift from Wei-Zen Wei from Wayne State University (37, 38). In brief, the D2F2 cell line is a murine breast cancer cell line that was transfected with full length transmembrane HER-2 DNA to create the HER-2-expressing cell line, D2F2/E2.

GPI-HER-2 DNA Construct: GPI-HER-2 was constructed by attaching the nucleotides corresponding to the GPI-signal sequence from CD59 to the C-terminal end of the extracellular domain sequence of HER-2 (GenBank X03363.1; amino acids 1-652) as previously described (31, 39, 40). An EcoRI restriction enzyme site was placed separating the two sequences. Nucleotide 1305 (1479 of Genbank sequence) was changed from a T to a C to remove an internal EcoRI restriction enzyme site in HER-2 without changing the amino acid sequence. HindIII and KpnI restriction enzyme sites were introduced into the N-terminal end before the HER-2 sequence. The resulting GPI-HER-2 construct was then placed in a $pUB6^{blast}$ vector (Invitrogen) and transfected into CHO-K1 cells to establish transfectants expressing GPI-HER-2.

Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment of transfected CHO-K1 cells: Transfected CHO-K1 cells were washed and resuspended in PBS/EDTA/0.1% ovalbumin to a final concentration of $10x10^6$ cells/ml. 1U PI-PLC from *Bacillus cereus* (Invitrogen) that specifically cleaves the GPI-anchor was added to 1ml of cell suspension and incubated at 37˚C for 45 min. Cells were washed and protein expression was analyzed by flow cytometry.

Purification of GPI-HER-2: Cell pellets of CHO-K1 cell transfectants expressing GPI-HER-2 were lysed with 50 mM Tris-HCl pH 8, 2% n-octyl-β-D-glucopyranoside (A.G. Scientific), 1:100 dilution of Protease Inhibitor Cocktail (Sigma), 5 mM EDTA, 20 mM sodium iodoacetate, and 2 mM PMSF overnight at 4˚C. The lysate was centrifuged at 14,000 rpm using a JA-20 rotor (Beckman) for 1 h at 4˚C. The supernatant was passed through a Sepharose 4B bead (Sigma-Aldrich) pre-column. The effluent was then passed through a TA1 mAb-affinity chromatography column followed by a wash with 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 200 mM NaCl and then with 20 mM Tris-HCl, pH 7.5, 0.1% n-octyl-β-D-glucopyranoside. GPI-HER-2 was eluted from the column with 100mM Triethylamine, 1% n-octyl-β-D-glucopyranoside, pH 11.6. Eluted fractions were analyzed by SDS PAGE followed by western blot using the anti-HER-2 mAb TA1 or silver stain. Fractions that contained purified GPI-HER-2 were concentrated in a 10-14 kDa MWCO dialysis bag (Fisherbrand) using polyvinylpyrrolidone (Sigma-Aldrich) and then dialyzed with PBS containing 0.05% n-octyl-β-D-glucopyranoside. Quantification of GPI-HER-2 was performed using a micro BCA kit (Thermo Scientific).

Protein transfer of GPI-HER-2 onto sheep RBCs: Sheep RBCs were washed with PBS two times by centrifugation followed by a wash with PBS containing 0.1% ovalbumin. 14 µg of GPI-HER-2 was then incubated with 2 x 10^6 sheep RBCs in 200 µL PBS containing 0.1% ovalbumin for 4 h at 37°C in an end-over-end rotation. Unincorporated GPI-HER-2 was washed out by centrifugation at 2000 rpm for 5 min using PBS, 5 mM EDTA, and 1% cosmic calf serum.

VLP preparation: VLPs were produced using the recombinant baculovirus (rBV) expression system as described (34, 41). Briefly, rBVs individually expressing influenza M1 protein and HA protein from H1N1 influenza A/PR/8/1934 virus were co-infected into Sf9 insect cells to produce VLPs. The cell culture supernatant containing the VLPs was collected after 2-3 days and centrifuged (6000 rpm, 30 min at 4˚C). VLPs were concentrated by the QuixStand hollow fiber ultrafiltration system (GE Healthcare, Piscataway, NJ). The VLPs were then purified by 20%-60% (wt/vol) sucrose gradient ultracentrifugation at 30,000 rpm using a SW32Ti Rotor (Beckman) for 60 min at 4˚C. The VLPs were collected at the interphase between 20% and 60% sucrose layers, diluted in PBS and pelleted at 30,000 rpm for 30 min at 4˚C. The VLP pellet was resuspended with PBS and stored at -80˚C until use. Quantification of VLPs was performed by the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA).

Protein transfer of GPI-HER-2 onto VLPs: Purified GPI-HER-2 was first centrifuged at 13,200 rpm for 1 h at 4˚C to exclude any precipitate formed during storage and then used for protein transfer. Approximately 250 µg of purified GPI-HER-2 was incubated with 1 mg of VLP in 4 ml PBS for 4 h at 37°C in an end-over-end rotation. To remove

any unbound GPI-HER-2, the VLPs were pelleted by centrifugation at 35,000 rpm for 1 h at 4˚C using a SW-41Ti rotor (Beckman) and the supernatant containing unincorporated GPI-HER-2 was removed. Then the protein transferred VLPs were resuspended in PBS and stored at 4˚C until use. GPI-HER-2 incorporation onto VLPs was analyzed by western blot using TA1 mAb and quantified by Image J. Known concentrations of purified GPI-HER-2 were used to make a standard curve.

Quantification of GPI-HER-2-VLPs by ELISA: GPI-HER-2-VLPs and known concentrations of unmodified VLPs were coated onto 96-well ELISA plates overnight at 4˚C. Polyclonal anti-VLP mouse antibodies were used as a primary antibody against the VLPs followed by secondary antibody detection using goat-anti-mouse-IgG-HRP (Southern Biotech). The TMB-1 substrate (BioLegend) was used to develop color followed by addition of 2N H_2SO_4 to stop the reaction. Absorbance was read at 450 nm. Values from known concentrations of unmodified VLPs were used as standards to quantify GPI-HER-2 VLPs.

Vaccination and tumor challenge studies: BALB/c mice were vaccinated at day 0 and boosted at day 14 with 25 µg of GPI-HER-2-VLPs each time in 100 µl PBS subcutaneously on the left hind flank. For tumor challenge, mice were challenged with 2 x 10^5 D2F2/E2 (HER-2 positive) cells or 2 x 10^5 wild-type D2F2 (HER-2 negative) cells, in 100 µl PBS on the right hind flank 7 days after boost. Tumor area was measured by multiplying the length and width of each tumor.

Detection of anti-HER-2 serum IgG responses: Flow cytometry (FACSCalibur, Becton Dickinson) analysis of serum anti-HER-2 IgG responses was carried out using a

1:200 diluted serum (obtained 7 days post boost) from vaccinated mice as primary antibody and 2.5 x 10^5 D2F2/E2 cells in 100 µl FACS Buffer (PBS, 5 mM EDTA, 1% cosmic calf serum). Goat-anti-mouse-FITC (Jackson Immunoresearch) was used as the secondary antibody. To determine HER-2 specific IgG subtypes, a cell ELISA (42, 43) using 5 x 10^4 D2F2/E2 cells per well with 50 µl of 1:100 diluted serum obtained from mice 7 days after boost was performed. The secondary antibody used was rat-anti-mouse-IgG1-HRP, rat-anti-mouse-IgG2a-HRP, or goat-anti-mouse-IgG2b-HRP (Southern Biotech) and the absorbance was measured at 450 nm. D2F2 cells were used as a negative control.

Detection of anti-VLP serum IgG responses: ELISA of serum from vaccinated mice collected 7 days after boost was performed by plating 100 µl of 1 µg/ml VLP in triplicate in 96-well flat bottom ELISA plates overnight at 4°C. The unbound VLPs were removed and then the wells were blocked with PBS containing 0.05% Tween-20 and 3% bovine serum albumin. A non-saturating (1:10,000) dilution of serum was added to wells as the primary antibody. Goat-anti-mouse IgG-HRP was used as the secondary antibody followed by detection with TMB-1 substrate (BioLegend). The color development was stopped with 2N H_2SO_4 and absorbance was read at 450 nm.

Statistical Analysis: All data are expressed as mean ± SEM. GraphPad Prism 6 was used to perform statistical analysis. Student's t-test, One-way ANOVA or Two-way ANOVA using Tukey's multiple comparisons post-test was used for analysis as described in the figure legends. A $p < 0.05$ value was considered significant.

Results

Construction and expression of GPI-anchored-HER-2:

HER-2 is a transmembrane protein that contains both an extracellular and intracellular domain. Since protein transfer requires a GPI-anchor, HER-2 was converted into a GPI-anchored form by attaching DNA encoding the GPI-signal sequence obtained from the naturally GPI-anchored protein, CD59, to the C-terminal end of DNA encoding the extracellular domain of HER-2. The resulting GPI-HER-2 DNA construct was placed in a pUB6^{blast} vector that was transfected into CHO-K1 cells. To determine the presence of the GPI-anchor, transfected CHO-K1 cells were subjected to PI-PLC treatment. PI-PLC is a bacterial enzyme that specifically cleaves the GPI-anchor and releases GPI-anchored proteins from the cell surface (44). CHO-K1 cells expressing full-length transmembrane HER-2 showed similar HER-2 surface expression whether subjected to PI-PLC treatment or not (Figure 1A; left panel). However, CHO-K1 cells transfected with the GPI-HER-2 DNA construct showed 97.3% decrease in HER-2 surface expression after PI-PLC treatment (Figure 1A; right panel) suggesting that HER-2 expressed on these cells contains a GPI-anchor.

Purification of GPI-HER-2:

GPI-HER-2 was purified from transfected CHO-K1 cell lysates using mAb-affinity chromatography. Analysis of purified GPI-HER-2 by SDS-PAGE followed by western blot (Figure 1B, top panel) and silver staining (Figure 1B, bottom panel) showed that the molecular weight of GPI-HER-2 is approximately 90 kDa.

To determine if purified GPI-HER-2 contained an intact GPI-anchor and was capable of incorporating onto cell membranes, protein transfer of GPI-HER-2 was

performed on sheep RBCs for 4 h at 37°C as an assay system. As shown in Figure 1C, purified GPI-HER-2 was incorporated onto sheep RBCs after protein transfer as detected by flow cytometry analysis suggesting that purified GPI-HER-2 contained an intact GPI-anchor.

Protein transfer-mediated incorporation of GPI-HER-2 onto VLPs:

Immunization of antigen in a particulate form enhances antigen-specific immune responses compared to immunization of antigen in a soluble form (21, 22). Therefore, we hypothesized that VLPs modified to express HER-2 by protein transfer would induce enhanced HER-2-specific immunity compared to administration of HER-2 alone. Since protein transfer led to incorporation of GPI-HER-2 onto sheep RBCs that contain lipid bilayers, we also hypothesized that purified GPI-HER-2 could incorporate onto lipid envelopes of influenza VLPs. As shown in Figure 2, GPI-HER-2 was able to incorporate onto influenza VLPs by protein transfer. As the concentration of purified GPI-HER-2 incubated with VLPs was increased, HER-2 incorporation on the VLPs increased as well showing that protein transfer of GPI-HER-2 onto VLPs is concentration dependent (Figure 2A, upper panel). Further, the overall structure (i.e. epitopes recognized by the anti-VLP antibodies) of the VLPs was not significantly modified after protein transfer as detected by western blot (Figure 2A, bottom panel). Incubation of 200 µg of GPI-HER-2 with 800 µg VLPs during protein transfer resulted in incorporation of 0.235 µg of GPI-HER-2 per µg of VLP as detected by ELISA and ImageJ analysis. This was further confirmed by western blot analysis of GPI-HER-2 present in the solution before and after protein transfer, which showed that 80.6% of the 200 µg of GPI-HER-2 was incorporated onto VLPs by protein transfer (Figure 2B).

Protein transfer results in stable expression of incorporated GPI-HER-2:

Next, we determined the stability of GPI-HER-2 incorporated onto VLPs by protein transfer. GPI-HER-2-modified VLPs were stored at 4°C for 7 days. As shown in Figure 2C, HER-2 expression on the VLPs remained stable during this time period. From day 7 to day 10, the GPI-HER-2-modified-VLPs were then transferred to storage at room temperature. Storage at room temperature for an additional 3 days also did not affect the expression levels of HER-2 on the protein transfer-modified-VLPs (Figure 2C).

Vaccination with GPI-HER-2-VLPs induces HER-2-specific antibodies:

To determine if vaccination with VLPs protein transferred with GPI-HER-2 (GPI-HER-2-VLPs) induced HER-2-specific immunity, antibody responses were analyzed after vaccination of mice with 25 µg GPI-HER-2-VLPs on day 0 and day 14. Serum was collected from vaccinated mice 7 days post boost and analyzed by flow cytometry using D2F2/E2 cells. A strong anti-D2F2/E2 serum IgG antibody response was detected in GPI-HER-2-VLP-vaccinated mice whereas unmodified VLP-vaccinated mice did not show any detectable levels of anti-D2F2/E2 IgG antibodies (Figure 3A). Interestingly, vaccination with purified GPI-HER-2 also induced anti-D2F2/E2 antibody levels similar to GPI-HER-2-VLPs. Further, minimum or no serum IgG antibody was detected against the parental HER-2-negative D2F2 (Figure 3B) cells showing that reactivity against D2F2/E2 was from HER-2 specific antibodies. Mice vaccinated with unmodified VLPs or GPI-HER-2-modified-VLPs displayed a similar serum antibody response against viral proteins present on VLPs (Figure 3C), suggesting that protein transfer does not affect the antigenicity of intrinsic VLP proteins.

Vaccination with GPI-HER-2-VLPs induces HER-2-specific Th1 and Th2-type immunity:

Th1-type immune responses play an important role in antitumor immunity (45) and many studies have shown that both Th1 and Th2-type cellular immunity play synergistic roles in eradicating tumors (46). To elucidate the type of immunity induced by vaccination with GPI-HER-2-VLPs, a cell ELISA was conducted on sera from vaccinated mice using D2F2/E2 cells and HER-2-specific IgG subtypes were analyzed. IgG2a and IgG2b subtypes are indicative of a Th1-type immune response whereas IgG1 production represents a Th2-type immune response. As shown in Figure 4, GPI-HER-2-VLP vaccination significantly raised levels of anti-HER-2 IgG2a (Figure 4B) and IgG2b (Figure 4C) antibody subtypes compared to those induced by soluble GPI-HER-2 vaccination. Whereas IgG1 levels, which is the predominant subtype in soluble GPI-HER-2 vaccinated mice, were similarly detected in both groups (Figure 4A). These results suggest that delivery of an antigen physically linked to nanoparticulate VLPs induces both Th1 and Th2-type IgG isotype responses as opposed to the soluble form of antigen which induces mostly Th2-type antibody responses.

Immunization with GPI-HER-2-VLPs protects mice against HER-2-expressing tumor challenge:

To test the protective efficacy of GPI-HER-2-VLPs, mice were vaccinated and boosted 14 days later with 25 μg of VLPs, GPI-HER-2-VLPs, or similar concentrations (0.757 μg) of soluble GPI-HER-2. At 7 days after boost, vaccinated mice were challenged with 2 x 10^5 D2F2/E2 cells, a HER-2 expressing murine breast cancer cell line. Mice vaccinated with GPI-HER-2-modified-VLPs had on average significantly smaller tumors compared to mice vaccinated with similar concentrations of GPI-HER-2

alone (Figure 5A, left panel). Further, a higher percentage of mice remained tumor free in the GPI-HER-2-VLP vaccinated group (66.67%) by the end of the study (day 58 post challenge) compared to either PBS (10%), unmodified VLPs (0%) or GPI-HER-2 alone (20%) (Figure 5A, right panel) vaccinated groups. The HER-2-specific antibody response in the vaccinated mice that succumbed to tumor growth was comparable to the antibody response in the vaccinated mice that cleared the tumor (Figure 3A). At present, it is not clear as to why some of these vaccinated mice succumbed to tumor growth despite the production of HER-2-specific antibodies. Intriguingly, the average tumor size in unmodified VLP vaccinated groups was significantly lower compared to control mice suggesting that VLP vaccination alone affected tumor growth, perhaps through activation of a more robust polyclonal immune response that could have primed mice against the HER-2 antigen expressed on the tumor cells used for challenge (Figure 5A, left panel). Mice vaccinated with GPI-HER-2 alone also portrayed some protection against tumor growth compared to control mice although not as enhanced as GPI-HER-2-VLP vaccinated mice (Figure 5A). On the other hand, when GPI-HER-2-VLP vaccinated mice were challenged with HER-2-negative D2F2 cells, tumors grew steadily similar to unvaccinated mice (Figure 5B, left panel) with 100% tumor incidence (Figure 5B, right panel). These results show that vaccination with VLPs protein transferred with GPI-HER-2 induced anti-tumor immune responses that are specific to the HER-2 antigen delivered by the VLPs.

Further, a dose titration study was conducted to determine the best vaccination strategy using protein transfer-modified GPI-HER-2-VLPs to induce protection against challenge with HER-2-expressing D2F2/E2 cells. Mice were vaccinated with a single

vaccination using either 10 µg, 25 µg, or 50 µg of protein transfer-modified-GPI-HER-2-VLPs on day 0 followed by challenge using 200,000 D2F2/E2 cells on day 22. Vaccination with increasing doses of GPI-HER-2-VLPs led to enhanced tumor protection by decreasing tumor growth (Supplemental Figure 1A) and incidence (Supplemental Figure 1B) in the challenged mice. Vaccination using 50 µg of protein transfer-modified-GPI-HER-2-VLPs led to the best protection with only 33% tumor incidence compared to the other vaccination doses tested which showed 66% tumor incidence or compared to unvaccinated mice (100% tumor incidence). These results suggest that even a single vaccination of GPI-HER-2-VLPs is sufficient for tumor protection.

Discussion

VLPs are nanoparticles that express antigens with a pattern of repetitive structures. The ordered and repetitive nature of antigen presentation on VLPs are suggested to enhance antibody production (47) by acting like pathogen associated molecular patterns (PAMPs), which produce danger signals activating both innate and adaptive immune responses (25). The particulate form and size of VLPs further allow for optimal uptake by APCs, in particular dendritic cells (48). In addition, the nanosize of VLPs is likely to permit for direct transport of VLPs to the lymph nodes for further activation of immune cells (23, 24). Notably, cross-presentation of VLP-expressing proteins is considered to activate both $CD4^+$ helper T-cells and cytotoxic T lymphocytes (CTLs), and incubation of VLPs with splenocytes was shown to induce IgG2a producing plasma cell differentiation (49). These characteristics might have contributed to making

VLPs strong immunogens. Thus, the potent inherent immunogenicity of VLPs make them ideal candidates for antigen delivery.

Our approach described here made use of this inherent immunogenicity of influenza VLPs to induce immunity against the new antigen HER-2 protein transferred onto the nanoparticles. Herein, we show as a proof of concept that protein transfer is a simple and rapid modification system to introduce TAAs onto lipid enveloped VLPs for induction of antitumor immunity. Protein transfer mediated modification of VLPs by GPI-HER-2 is concentration dependent and occurred within 4 h. The level of incorporation of GPI-HER-2 on VLPs can be varied by simply adjusting the concentration of purified GPI-HER-2 added during protein transfer. After incorporation, HER-2 was expressed on the VLPs stably for at least one week at 4°C and even for 3 days at room temperature. We demonstrated that mice vaccinated with GPI-HER-2-VLPs induced strong anti-HER-2 specific IgG responses and protected mice against challenge with HER-2-expressing D2F2/E2 cells. Interestingly, GPI-HER-2 attached to the VLPs by protein transfer induced both Th1 and Th2-type anti-HER-2 antibody responses whereas GPI-HER-2 alone induced mostly Th2-type antibody responses. The Th1-type antibody induction by GPI-HER-2-VLPs is likely due to the intrinsic nature of the nanoparticulate VLPs that have been shown to enhance Th1 and Th2-type immune responses against viral antigens (36, 49, 50). These results suggest that delivering antigen attached to these nanoparticles by protein transfer has the ability to modulate immune responses toward a more dual Th1/Th2 phenotype that appears correlative with enhanced therapeutic efficacy. A dual Th1/Th2 phenotype response is highly desirable in

a tumor immunotherapy setting, as a combination of Th1 and Th2-tupe responses has shown to be more effective in combating tumor growth (46, 51, 52).

The VLP-based delivery system described here has many advantages over other approaches to express TAAs on VLPs. When engineering chimeric VLPs to express tumor antigens by genetic means, the expression of tumor antigens on the VLPs cannot be easily controlled. Further, inclusion of tumor antigens may alter proteins intrinsic to the VLPs which may in turn influence the structural integrity of the VLPs. For generation of VLPs using the recombinant baculovirus (rBV) expression system, the multiplicity of infection (MOI) of rBVs for expression of viral capsid and envelop proteins have to be optimized to produce sufficient quantities of VLPs with high viral protein expression. Introducing foreign antigens such as TAAs onto VLPs using this rBV expression system, alters the optimized MOIs needed for viral protein expression and thus may lower VLP production. More importantly, post-translational modification of proteins expressed in insect cells is different from that of mammalian cells, which may limit proper conformation of mammalian cell-derived tumor antigens. Protein transfer would overcome these drawbacks and allow for easy modification of VLP surfaces after production. Further, protein transfer-mediated modification of enveloped VLPs does not involve any chemical changes (22) thus preserving the immune stimulating properties of VLPs as well as antigenicity of the incorporated TAA. For use in protein transfer, the protein antigens are precisely modified at the C-terminus, which does not affect the functionality or antigenicity of the proteins. We found that protein transfer of HER-2 did not alter the antigenicity of viral proteins expressed on the VLPs, as GPI-HER-2-VLPs still induced VLP-specific serum IgG responses similar to unmodified VLPs (Figure 3C).

For protein transfer to incorporate antigens onto the surface of lipid bilayers, only a GPI-anchor attached to the antigen is needed (5, 53-55). Since more than one GPI-anchored protein can be incorporated onto VLPs by protein transfer simultaneously, this approach can be used to expand the repertoire of immunity induced to target multiple TAAs. Thus, protein transfer-mediated incorporation of TAAs onto influenza VLPs provides a quick and simple method for developing antigen-specific therapeutic vaccines to induce antitumor immune responses to treat cancer.

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Dr. Selvaraj is a co-founder & equity holder of Metaclipse Therapeutics Corporation - a startup company formed to develop therapeutic cancer vaccines for humans using the protein transfer technology described here for which he is a co-inventor.

Figure Legends

Figure 1. Construction and purification of GPI-HER-2. (**A**) HER-2 expressed on the surface of GPI-HER-2-CHO-K1 cell transfectants is GPI-anchored. PI-PLC treatment (red) was performed on CHO-K1 cells transfected with full-length transmembrane (TM) HER-2 (left panel) and CHO-K1 cells transfected with the GPI-HER-2 DNA construct. Surface expression was analyzed by flow cytometry. (**B**) Purification of GPI-HER-2. GPI-HER-2 was purified from the lysate of transfected CHO-K1 cells by mAb-affinity chromatography. HER-2 containing fractions were pooled, concentrated and dialyzed with PBS containing 0.05% octyl-β-glucopyranoside. The resulting concentrated fraction was subjected to SDS-PAGE under non-reducing conditions in a 10% gel followed by western blot (top panel) or silver stain (bottom panel) analysis. (**C**) Purified GPI-HER-2 retains the GPI-anchor domain and the ability to incorporate onto cell surfaces. Protein transfer of purified GPI-HER-2 was performed on sheep RBCs (2×10^6) in PBS containing 0.1% ovalbumin for 4 h at 37°C. The sheep RBCs were then washed with PBS by centrifugation and incorporation was analyzed by flow cytometry.

Figure 2. Protein transfer of GPI-HER-2 onto influenza VLPs. (**A**) Concentration dependent-incorporation of GPI-HER-2 onto influenza VLPs by protein transfer. Purified GPI-HER-2 was incubated with 10 µg VLPs at different concentrations for 4 h at 37°C. Incorporation of HER-2 was detected by subjecting VLPs to SDS-PAGE and western blot analysis (top panel). The presence of VLP proteins was analyzed (bottom panel) by staining with a 1:1000 dilution of VLP-specific serum from vaccinated mice. (**B**) High level of added GPI-HER-2 incorporates into VLPs by protein transfer. Supernatant before (lane 1) and after (lane 2) protein transfer of 200 µg GPI-HER-2 with

800 µg VLPs was analyzed by SDS-PAGE and western blot by blotting against HER-2. 80.6% GPI-HER-2 incorporated onto VLPs as analyzed by Image J. (**C**) GPI-HER-2 incorporated into influenza VLPs remains stably associated with VLPs after storage. From day 0 to day 7, GPI-HER-2-VLPs were stored at 4°C. The GPI-HER-2-VLPs were then transferred to room temperature for another 3 days (from day 7 to day 10*). On each day, an aliquot of GPI-HER-2-VLPs was washed with PBS by centrifugation at 13,200 rpm for 30 min. The VLP pellet was then resuspended with Laemmli sample buffer and then subjected to SDS PAGE and western blot analysis to detect VLP and GPI-HER-2 concentrations using anti-VLP serum and TA1 mAb, respectively.

Figure 3. Vaccination with GPI-HER-2-protein transferred-VLPs induces HER-2-specific antibody responses. (**A**) Protein transferred GPI-HER-2-VLP vaccination induces HER-2-specific serum IgG. Mice were vaccinated with GPI-HER-2-VLPs (25 µg) on day 0 and day 14, and serum was collected from vaccinated mice on day 21. Flow cytometry analysis was performed to detect anti-D2F2/E2 (HER-2-positive cell line) specific (n = 5) or anti-D2F2 (HER-2-negative cell line) specific (n = 15) IgG antibodies by using 1:200 diluted serum and 250,000 cells per well. (**B**) Minimum or no serum IgG was detected against D2F2 cells showing HER-2-specific antibody reactivity against D2F2/E2. (**C**) Influenza VLPs protein transferred with or without GPI-HER-2 induce similar levels of VLP-specific serum IgG antibodies. ELISA was performed using 1:10,000 diluted serum collected 1 week post boost from groups of vaccinated mice (n = 15).

Figure 4. Vaccination with GPI-HER-2-VLPs enhances Th1-type antibody responses. Mice were vaccinated with GPI-HER-2-VLPs (25 µg) on day 0 and day 14, and serum was collected on day 21. Anti-D2F2/E2-specific serum (**A**) IgG1, (**B**) IgG2a, and (**C**) IgG2b subtype antibodies were analyzed by cell ELISA using 1:200 diluted serum from vaccinated mice (n = 5) with 50,000 D2F2/E2 cells per well.

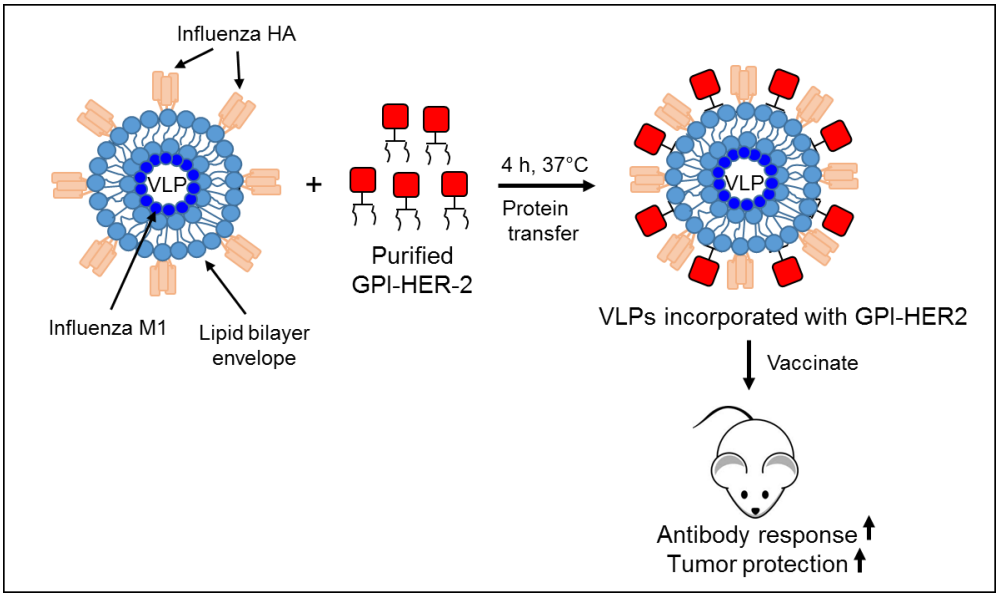
Figure 5. Vaccination with GPI-HER-2-VLPs induces protection against challenge with a HER-2-expressing breast cancer cell line. (**A**) Tumor growth (left panel; n = 5) and incidence (right panel; n = 9 or 10) after vaccination with protein transferred GPI-HER-2-VLPs followed by D2F2/E2 cell challenge. Mice were vaccinated with GPI-HER-2-VLPs (25 µg) on day 0 and day 14, and then challenged with 200,000 D2F2/E2 cells on day 21. (**B**) GPI-HER-2-VLPs did not protect against challenge with HER-2-negative D2F2 breast cancer cells. Vaccinated mice were challenged with 200,000 D2F2 (HER-2 negative) cells 7 days post boost. Tumor growth (left panel) and incidence (right panel) was measured.

Supplemental Figure Legends

Supplemental Figure 1. Tumor growth and incidence of a HER-2-expressing breast cancer cell challenge after vaccination with different doses of GPI-HER-2-VLPs. (**A**) Tumor growth (n = 3) after vaccination with protein transferred GPI-HER-2-VLPs followed by D2F2/E2 cell challenge. (**B**) Percent tumor free survival (n = 3) after vaccination with protein transferred GPI-HER-2-VLPs followed by D2F2/E2 cell challenge. Mice were vaccinated with either 10 µg, 25 µg, or 50 µg of GPI-HER-2-VLPs

on day 0 and then challenged with 200,000 D2F2/E2 cells on day 22. Tumor growth (left panel) and incidence (right panel) was measured.

Graphical Table of Contents.



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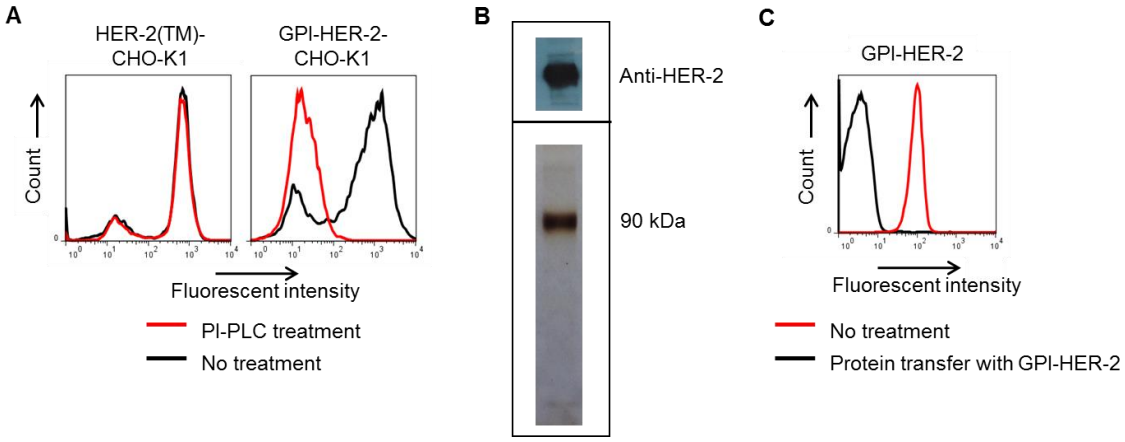
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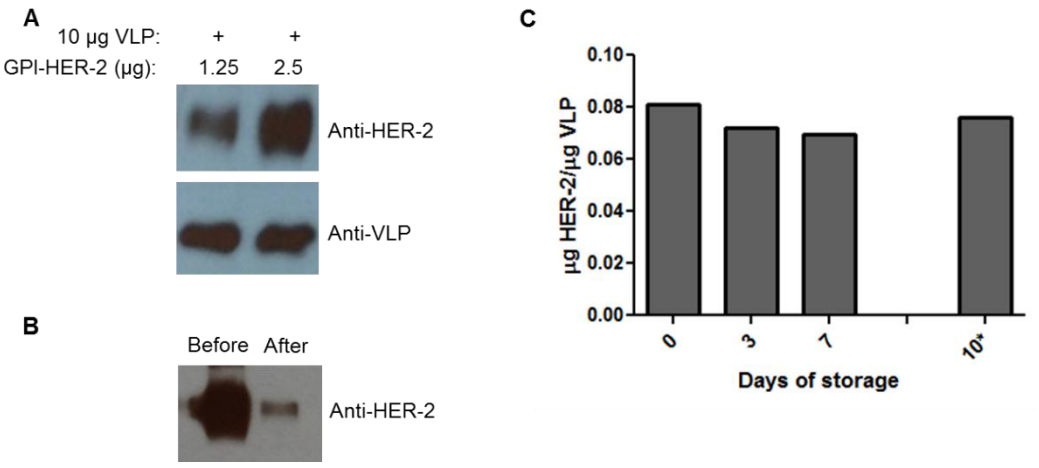
HER-2(TM)-CHO-K1
GPI-HER-2-CHO-K1
Count
Fluorescent intensity
PI-PLC treatment
No treatment
B
Anti-HER-2
90 kDa
C
GPI-HER-2
Count
Fluorescent intensity
No treatment
Protein transfer with GPI-HER-2

Figure 2.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10 µg VLP: + +
GPI-HER-2 (µg): 1.25 2.5
Anti-HER-2
Anti-VLP
B
Before After
Anti-HER-2
C
µg HER-2/µg VLP
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0.08
0.06
0.04
0.02
0.00
0
3
7
10+
Days of storage

Figure 3.

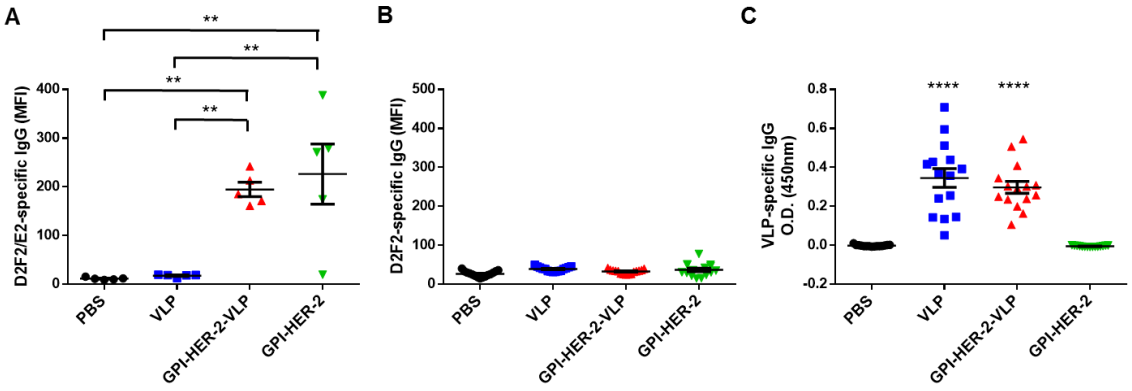
A
B
C
D2F2/E2-specific IgG (MFI)
D2F2-specific IgG (MFI)
VLP-specific IgG O.D. (450nm)
PBS
VLP
GPI-HER-2-VLP
GPI-HER-2
**
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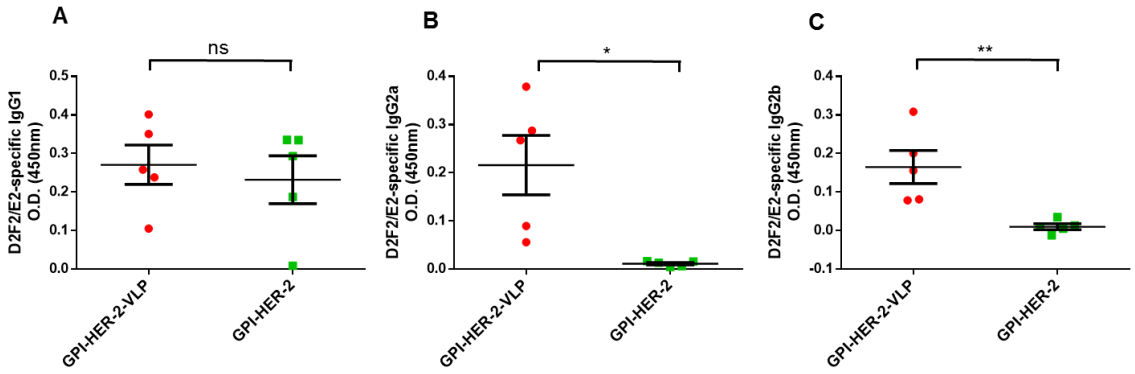
Figure 4.


A
ns
D2F2/E2-specific IgG1 O.D. (450nm)
0.5
0.4
0.3
0.2
0.1
0.0
GPI-HER-2-VLP
GPI-HER-2
B
*
D2F2/E2-specific IgG2a O.D. (450nm)
0.4
0.3
0.2
0.1
0.0
GPI-HER-2-VLP
GPI-HER-2
C
**
D2F2/E2-specific IgG2b O.D. (450nm)
0.4
0.3
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0.1
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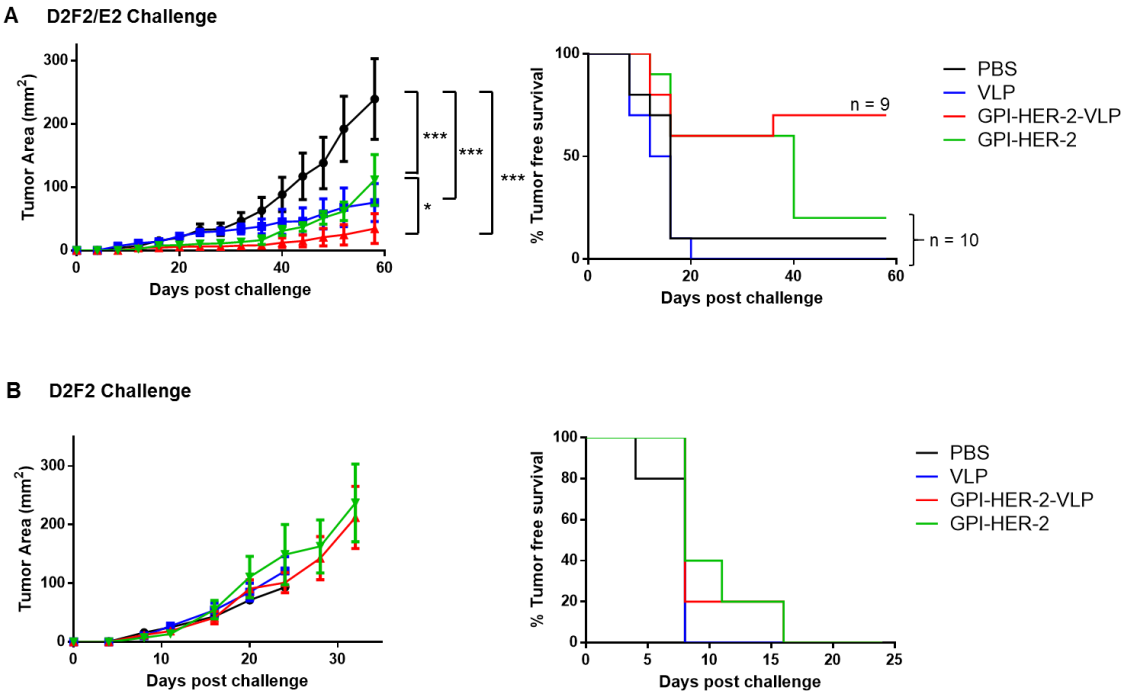
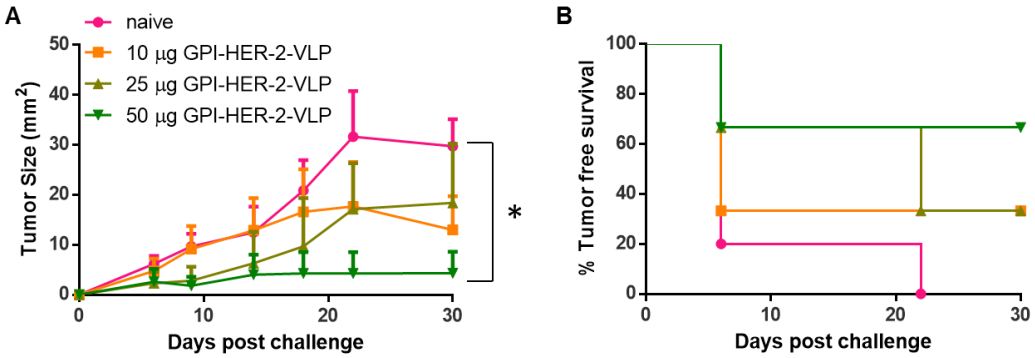
-0.1
GPI-HER-2-VLP
GPI-HER-2

Figure 5.


A
D2F2/E2 Challenge
Tumor Area (mm2)
Days post challenge
***
***
***
*
% Tumor free survival
n = 9
n = 10
PBS
VLP
GPI-HER-2-VLP
GPI-HER-2
B
D2F2 Challenge
Tumor Area (mm2)
Days post challenge
% Tumor free survival
PBS
VLP
GPI-HER-2-VLP
GPI-HER-2

Supplemental Figure 1.



CHAPTER IV:

Plasma membrane vesicles engineered by protein transfer as an adjuvanted antigen delivery platform

The data as presented in this chapter in unpublished.

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

Data presented in Figure 4 was generated with the cooperation of Brianne Caoyonan.

Data presented in Figure 12 was generated with cooperation of Erica N. Bozeman and Lily Yang.

Abstract:

Delivery of antigens encapsulated in particulate materials induces stronger immune responses than soluble antigens. Particulate materials such as liposomes and synthetic particles made from many biodegradable polymers have been used to deliver tumor antigens to enhance antitumor immunity. However, these approaches are limited by the complexity of particle production that affects stability and immunogenicity of the antigens. To overcome these limitations, herein we describe a tumor antigen delivery system that makes use of plasma membrane vesicles (PMVs) derived from biological materials such as cultured tumor cells or tissues for elicitation of effective antitumor immune responses. We show that these nanoparticulate PMVs can be easily modified within 4 h by a protein transfer method to express a glycosylphosphatidylinositol (GPI)-anchored form of the breast cancer antigen, HER-2. Protein transfer resulted in stable dose-dependent incorporation of HER-2 on the PMV surface and immunization of mice with GPI-HER-2-modified PMVs induced strong anti-HER-2 antibody responses and protection from tumor challenge using two different murine breast cancer models. Further, incorporation of the immunostimulatory molecules IL-12 and B7-1 could occur simultaneously with HER-2 on the PMVs by protein transfer, and the resulting modified-PMVs led to enhanced tumor protection and induction of beneficial Th1 and Th2-type HER-2-specific immune responses. Since protein antigens can be easily converted to GPI-anchored forms, these results demonstrate that isolated plasma membrane vesicles can be modified with desired antigens along with immunostimulatory molecules by protein transfer and used as a vaccine delivery vehicle to elicit potent antigen-specific immunity.

Key words: Plasma membrane vesicle, nanoparticle, protein transfer, breast cancer, tumor antigens

Introduction:

The identification of tumor-associated antigens (TAAs) has led to major advancements in the field of cancer immunotherapy (1). To elicit an immune response against TAA-bearing tumor cells, TAA-derived peptides and proteins have been delivered to the immune system by a variety of ways. These include direct administration of TAAs as vaccines in the presence of adjuvants, dendritic cells (DCs) loaded *ex vivo* with TAA proteins or peptides, and DNA encoding TAAs delivered directly or by viral vectors (2-4). However, poor immunogenicity and short half-life of soluble proteins and peptides, the need for standardization and extensive *ex vivo* preparation required for loading DCs (2, 3), the poor delivery of DNA vaccines, and safety issues with the use of viral vectors (4) have necessitated the development of new approaches to deliver TAAs to boost anti-tumor immune responses.

Successful delivery of antigens to develop effective antigen-specific immunity, requires for the antigens to be delivered without degradation to antigen presenting cells (APCs) (5). Numerous studies have demonstrated the use of particle-based delivery systems for protein and peptide-based vaccines as a highly promising approach. Nanoparticles and microparticles that are particulate in nature and optimal in size for phagocytosis and uptake by APCs (6), also have the ability to increase antigen half-life and deliver antigens in a sustainable manner by encapsulating or attaching antigens onto the surface (7). These properties make particles an efficient vaccine delivery system (7-9). Many tumor, viral and parasitic antigens have been delivered using particles (10), which has resulted in augmented immunity against the antigen compared to soluble antigen administration (7, 9).

A variety of particle-based vaccines, such as lipid-based particles as well as natural and synthetic polymer-based biodegradable particles (11), have been used as antigen delivery vehicles to elicit an antigen-specific adaptive immune response. Nonetheless, these approaches require complex particle production. Encapsulating antigens during particle formation also often leads to exposure of antigens to toxic organic solvents (7). On the other hand, attachment of antigens to particle surfaces may involve chemical modification that can further affect antigen stability and immunogenicity, as well as alter particle formulation. Further, lack of complete biodegradability and biocomptability of particles leads to further toxicity concerns (8), and production of reproducible large quantities of uniform particles may also raise cost issues (8). These caveats emphasize the need to develop biocompatible antigen delivery systems as vaccines.

In the present study, we describe the use of plasma membrane vesicles (PMVs) as a promising biological particle-based tumor antigen delivery system. These vesicles are distinct from exosomes that are secreted by cells and are approximately 110 nm in diameter (12, 13). PMVs are particles prepared from homogenization of cells or tissues, followed by purification using sucrose gradient centrifugation (14-19). Homogenization of cells results in the formation of vesicles from plasma membranes; thus PMVs contain lipid bilayers, making them amenable to modification by protein transfer. Protein transfer makes use of glycosylphosphatidylinositol-anchored immunostimulatory molecules (GPI-ISMs) to modify cell or membrane surfaces in a simple, rapid process whereby cells or membranes are incubated with purified GPI-ISMs for 2-4 hours (16, 17, 19-21). Incubation results in the spontaneous incorporation of the GPI-ISMs on to cell

membranes via the GPI-anchor in a concentration, time and temperature-dependent manner(16, 17, 19) and expression of incorporated GPI-APs on PMVs is not affected even after storage of the protein transferred vesicles at -80°C for at least 2 weeks (19)

Previously, ISMs such as the transmembrane co-stimulatory molecule B7-1 and the soluble cytokine IL-12 were converted to GPI-anchored forms and incorporated successfully onto PMVs to enhance immunity against antigens native to the PMVs (16, 17, 19). Protein transferred PMVs with incorporated GPI-B7-1 or GPI-IL-12 induced enhanced T-cell proliferation (16, 17, 19), and PMVs displaying GPI-B7-1 also induced tumor-specific T-cell mediated cytotoxicity, and protection from parental tumor challenge in mice (16). Although ISMs have been incorporated onto PMVs by protein transfer for elicitation of immunity against intrinsic antigens expressed on PMVs, the protein transfer of GPI-anchored antigens and the elicitation of immunity against incorporated foreign antigens has yet to be investigated. Such information will have implications in the delivery of a wide array of antigens to induce immunity against tumors and pathogens.

The ability to convert both transmembrane and soluble proteins into GPI-anchored forms suggests that TAAs can be converted into a GPI-anchored form, incorporated onto PMVs by protein transfer, and delivered to the immune system. Herein, we present a PMV-based adjuvanted co-delivery system of a breast cancer antigen, human epidermal growth factor receptor-2 (HER-2). HER-2 along with membrane-bound ISMs, B7-1 and IL-12, were incorporated onto breast cancer derived-PMVs by protein transfer. Vaccination with modified tissue-derived induced complete protection against a HER-2-expressing tumor cell challenge along with delayed tumor

growth and partial regression of established tumors. The protection induced was found to be both cellular and humoral mediated, in which the delivery of GPI-HER-2 on PMVs enhanced HER-2-specific serum IgG compared to soluble GPI-HER-2 administration and elicited both a T helper 1 (Th1) and Th2-type immune response.

Materials and Methods:

Animals:

6-8 week old BALB/c mice or C57BL/6 mice were purchased from Jackson Laboratories. All experiments were conducted per Emory University approved IACUC guidelines.

DNA Constructs:

To construct GPI-HER-2, the nucleotide sequence corresponding to the GPI-signal sequence from human CD59 was attached to the nucleotide sequence corresponding to the extracellular domain of HER-2 (GenBank X03363.1; amino acids 1-652) as previously described (22, 23) with an EcoRI restriction enzyme site. To remove an internal EcoRI site, the T nucleotide at position 1305 (GenBank sequence position 1479) was replaced with a C. Mouse GPI-B7-1 was constructed by attaching the CD59 GPI-signal sequence nucleotides to the extracellular domain of mouse B7-1 (GenBank BC131959.1, nucleotides 45-800) via an EcoRI restriction enzyme site. Further, amino acid 253 was changed from a lysine to alanine by changing nucleotides AAG to GCG. To construct GPI-IL-12, the p35 subunit of mouse IL-12 was attached to the CD59 GPI-signal sequence. This sequence was then followed by an IRES along with the soluble

p40 subunit of mouse IL-12. All three constructs were inserted in a pUB6^{blast} vector (Invitrogen).

Cell lines:

CHO-K1 cells, maintained in RPMI with 5% bovine calf serum (BCS), were transfected with HER-2-CD59, B7-1-CD59, or IL-12-a-CD59-b-sol DNA constructs using Lipofectamine 2000 (Invitrogen). The transfected CHO-K1 cells were selected with 20 µg/ml Blasticidin and maintained in RPMI with 5% BCS with 10 µg/ml Blasticidin. High expressing transfected CHO-K1 cells were selected for by panning and expression was analyzed by flow cytometry. 4TO7 cells are an aggressive, non-metastatic murine breast cancer cell line derived from a mammary tumor spontaneously grown in a Balb/cfC3H mouse (24). D2F2 and D2F2/E2 cells are murine breast cancer cell lines that were a kind gift from Wei-Zen Wei (Wayne State University) (25, 26). D2F2/E2 cells were created by transfecting D2F2 cells with full-length transmembrane HER-2 DNA. E0771 cells were generated by Francis M. Sirotnak (Sloan-Kettering Institute) (27) and were a kind gift from Subra Malarkannan (The Med. College of Wisconsin). The cell line is derived from the mammary gland of C57BL/6 (28). E0771 cells are highly aggressive and metastatic (29).

Antibodies:

Anti-HER-2 monoclonal antibody (mAb)-secreting hybridoma, TA1, was obtained from ATCC. Anti-HER-2 mAbs were purified from the supernatant by a Gammabind-plus Sepharose column (GE Healthcare). Purified anti-mIL-12 p40 mAb,

(C17.8) and anti-mB7-1 mAb (1G10) was obtained from Bio X Cell. Purified mAbs were coupled to CNBr-activated Sepharose beads (GE Healthcare) to create mAb-affinity columns for purification. Anti-CD4 hybriboma (GK1.5) and anti-CD8 hybridoma (H35) were a kind gift from Aaron Lukacher, Emory University. The anti-CD4 and anti-CD8 mAbs were purified from the hybridoma culture supernatant by a Gammabind-plus sepharose column. Goat-anti-mouse IgG-FITC and Goat-anti-rat IgG-FITC (Jackson Immunoresearch) was used for flow cytometry analysis.

PI-PLC treatment:

The enzyme phosphatidylinositol-specific phospholipase C (PI-PLC) specifically cleaves the GPI-anchor. Therefore, to determine if transfected CHO-K1 cells expressed the GPI-anchored form of each protein, transfected CHO-K1 cells were subjected to PI-PLC treatment. 1 ml of a 10 x 10^6 cell/ml cell suspension of transfected CHO-K1 cells in PBS/EDTA/0.1% ovalbumin was incubated with 1U of PI-PLC from *Bacillus cereus* (Invitrogen) at 37°C for 45 min. The cells were then washed three times and surface protein expression was analyzed by flow cytometry using a FACSCaliber.

Purification of GPI-anchored proteins:

10-20 g of transfected CHO-K1 cells were lysed with 50 mM Tris-HCl pH 8, 2% n-octyl-β-D-glucopyranoside (A.G. Scientific), 5 mM EDTA, 20 mM sodium iodoacetate, and 2 mM PMSF for at least 6 h at 4˚C. The lysate was centrifuged at 14,000 rpm for 1 h at 4˚C using a JA-20 rotor. The supernatant containing GPI-APs were first passed through a Sepharose 4B pre-column (Sigma-Aldrich), followed by the respective

mAb-affinity chromatography column. The columns were then washed with 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 200 mM NaCl followed by 20 mM Tris-HCl, pH 7.5, 0.1% n-octyl-β-D-glucopyranoside, 10 mM sodium iodoacetate. GPI-HER-2 and GPI-B7-1 were eluted from the column using 100mM Triethylamine, 1% n-octyl-β-D-glucopyranoside, pH 11.6, and GPI-IL-12 was eluted using 100 mM Glycine-HCl pH 2.8 containing 1% n-octyl-β-D-glucopyranoside, and 10 mM sodium iodoacetate. The eluted fractions were analyzed by SDS PAGE followed by western blot and silver stain. Fractions containing the GPI-anchored protein (GPI-AP) were combined and concentrated using polyvinylpyrrolidone (Sigma-Aldrich) in a 10-14 kDa MWCO dialysis bag (Fisherbrand), followed by dialysis with three exchanges of PBS containing 0.01% n-octyl-β-D-glucopyranoside. A micro BCA kit (Thermo Scientific) was used to quantify concentrated purified GPI-HER-2 and GPI-B7-1. A direct ELISA with known concentrations of recombinant soluble mIL-12 p75 (eBioscience) as standards was used to quantify purified GPI-IL-12 concentrations.

Protein transfer of purified GPI-APs onto sheep RBCs:

To test if purified GPI-APs can incorporate onto cell membrane lipid bilayers by protein transfer, sheep red blood cells (RBCs) were used as an assay system. Sheep RBCs were washed twice with PBS. RBCs were then resuspended in PBS with 0.1% ovalbumin and 200 µl of 10 x 10^6 RBCs/ml were incubated with purified GPI-AP for 4 h at 37°C under solution end-over-end rotation. Unincorporated protein was washed off by centrifugation with PBS and RBCs were then stained and analyzed for the incorporated protein by flow cytometry.

PMV preparation and characterization:

BALB/c mice were inoculated with 400,000 4TO7 regrowth (RG) or D2F2 cells and C57BL/6 mice were inoculated with 400,000 E0771 cells. After tumor growth, mice were sacrificed and tumor tissue was isolated and stored at -80˚C until homogenization. For homogenizing tumor tissue into membrane vesicles, tumor tissue was thawed on ice and minced with scissors. Ice cold 1 mL of homogenization buffer (20 mM Tris pH 8.0, 10 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM PMSF) was added to 0.5 g of tumor tissue and the tissue was homogenized four times for 7-8 seconds with 1 min intervals between each homogenization. The homogenized solution was then centrifuged at 1200 rpm for 5 min and the supernatant was collected. Homogenization of the remaining tumor tissue was repeated and the resulting supernatants was placed on 41% sucrose gradient and ultracentrifuged at 23,000 rpm, 1 h, at 4˚C using a SW-41 rotor. Purified PMVs were collected at the interphase and washed with PBS by centrifugation at 13,200 rpm, 1 h, 4˚C. PMVs were similarly prepared from tumor cells. PMVs were stored in PBS at 4°C short-term (3 months) and at -20°C long-term. A micro BCA kit (Thermo Scientific) was used to quantify PMV concentrations. Flow cytometry analysis was used to characterize protein expression on the TMVs and size distribution was assessed by zetasizer analysis (Malvern Zetasizer Nano ZS Serial # MAL1047760) and electron microscopy.

Protein transfer of GPI-APs onto PMVs:

In order to modify PMVs with GPI-APs by protein transfer, purified GPI-APs were centrifuged at 13,200 rpm for 1 h, at 4°C. GPI-APs from the supernatant were then

added to PMVs in PBS. 250 µg GPI-HER-2, 50 µg GPI-IL-12 and 50 µg GPI-B7-1 were added to 1 mg 4TO7 PMVs in 8 mL total PBS for PMVs prepped for *in vivo* vaccination. The solution was rotated end-over-end at 37°C for 4 h. The PMVs were then centrifuged at 13,200 rpm, 1 h, at 4°C. The pelleted PMVs were resuspended in PBS and centrifuged again to wash off unincorporated proteins. Finally, the PMVs were resuspended in PBS and stored at 4°C until use. Western blot and flow cytometry analysis was used to determine incorporation. The amount of HER-2 incorporation on PMVs after protein transfer was calculated by western blot and Image J analysis using known concentrations of GPI-HER-2 as standards.

Experimental metastasis model:

BALB/c mice were vaccinated and boosted with 25 µg of PMVs in 200 µl PBS intravenously (i.v.) 14 days apart. Vaccinated mice were then challenged with 100,000 4TO7-HER-2 cells i.v. 7 days post boost. 15 days post challenge, mice were sacrificed and lungs were isolated and homogenized using 1 mg/ml collagenase Type IV (Sigma-Aldrich) in HBSS (without calcium, magnesium and phenol red) for 2 h at 37°C. The homogenate was then passed through a cell strainer to create a single cell suspension. Lung cells were plated in tissue culture plates with 10 ml of DMEM containing 10% bovine calf serum, 1% penicillin/streptomycin, 0.8% nystatin, 5mM L-glutamine, 5mM HEPES, and 5 µg/ml 6-thioguanine. The cells were cultured at 37°C 5% CO_2 for 8 days. Media was removed and each plate was washed with PBS. Cells were detached with PBS containing 5 mM EDTA and centrifuged. Cell pellets were resuspended in equal

volumes and cell counts for each sample were obtained by collecting the number of cellular events collected within 30 seconds using a BD FACSCalibur flow cytometer.

Tumor challenge studies:

For prophylactic vaccination studies, female BALB/c mice were immunized subcutaneously (s.c.) with 25 µg of PMVs in 100 µl PBS on the left hind flank followed by a booster injection (s.c) with 25 µg of PMVs in 100 µl PBS on the left hind flank 14 days later. Mice were then challenged with 200,000 D2F2/E2 cells in 100 µl PBS s.c. on the contralateral flank 7 days after boost. Tumor area was measured by multiplying tumor length by width. For therapeutic vaccination studies, BALB/c mice were challenged s.c. with 200,000 D2F2/E2 cells on the right hind flank on day 0. Day 3 and day 6 post challenge, mice were vaccinated s.c. with 100 µg of protein transferred-PMVs in 100 µl PBS on the left hind flank.

For cell depletion studies, mice were vaccinated and boosted s.c. as described above with 25 µg of PMVs in 100 µl PBS. Mice were given intraperitoneal i.p. administration of 200 µg anti-CD4 Ab (GK1.5), 200 µg anti-CD8 Ab (H35), or 200 µg of anti-CD4 with 200 µg anti-CD8 Ab in 200 µl PBS 3 days before challenge with D2F2/E2 cells, and days 2, 7, 12, and 18 post challenge.

Serum antibody analysis studies

Flow cytometry was used to determine the presence of breast cancer-specific antibodies in serum collected 7 days post boost from mice vaccinated with protein transferred-PMVs. 1:200 diluted serum in 100 µl FACS Buffer (PBS, 5mM EDTA, 1%

bovine calf serum) was added to 250,000 D2F2/E2, D2F2, or 4TO7 cells and incubated for 30 min at 4°C. Cells were then washed, and stained with FITC-goat-anti-mouse antibody (Jackson Immunoresearch). The presence of D2F2/E2-specific antibodies was analyzed by flow cytometry.

Cell ELISA was used to determine the presence of HER-2 specific IgG subtypes, in which 50,000 D2F2/E2 cells were incubated with 100 µl of 1:100 diluted serum (collected day 7 post boost) in FACS buffer per well in triplicate for 30 min at 4°C. Cells were washed and then 50 µl of 1:2000 dilution of secondary antibody, rat-anti-mouse-IgG1-HRP, rat-anti-mouse-IgG2a-HRP, or goat-anti-mouse-IgG2b-HRP (Southern Biotech) was added. 100 µl of TMB-1 substrate (BioLegend) was added to the cells to develop color. Color was stopped with the addition of 2N H_2SO_4. Absorbance was read at 450 nm.

Results:

Production and characterization of PMVs

To generate sufficient quantities of PMVs for our studies we used the aggressive murine breast cancer cell line, 4TO7, as a source. Frozen pellets of *in vitro* cultured cells or tissues from tumors grown in mice were homogenized and PMVs were isolated by centrifugation over a 41% sucrose layer. The interphase containing PMVs (15) was collected and used for further studies. Our established protocol resulted in 1 mg of PMVs from homogenization of 2×10^8 cultured 4TO7 cells and 5 mg PMVs from 1 gram of 4TO7 tumor tissue. Zetasizer analysis showed that the PMVs derived from cultured cells were 410.2 ± 6.8 nm in diameter with a polydispersity index of 0.595 ± 0.02 and a zeta

potential of -12.0 ± 0.6 mV (Figure 1A). Electron microscopy analysis showed particles of round vesicular shape with diameters ranging from approximately 100 nm to 200 nm (Figure 1B – left panels). 4TO7 tumor tissue derived PMVs were approximately 347 ± 3.6 nm in diameter with a polydispersity index of 0.560 ± 0.1, and a zeta potential of -17.1 ± 1.5 (Figure 1A). Electron microscopy results of tissue derived PMVs showed a wide distribution on vesicle sizes ranging from approximately 100 nm to 400 nm (Figure 1B – right panels). Flow cytometry analysis of cell surface markers expressed on 4TO7 cells showed that PMVs obtained from cultured cells and tissues (Figure 1C and 1D) expressed adhesion molecules, such as HSA, as well as major histocompatibility complex class I (MHC I) molecules. Also CD107a, a marker for degranulation on $CD8^+$ T-cells and natural killer (NK) cells, was found to be expressed on both cell-derived and tissue-derived PMVs, although expression levels of all of these surface markers were higher in cell derived PMVs compared to tissue-derived PMVs (Figure 1C and 1D). Interestingly, PMVs obtained from tumor tissue expressed additional surface markers such as MHC Class II found on activated antigen presenting cells and GR1, usually expressed on neutrophils and myeloid derived suppressor cells (Figure 1C and 1D). The enhanced expression of these surface molecules on PMVs from tumor tissue may result from infiltration of immune cells into the tumor microenvironment. Overall, the size and particulate nature of these biologic PMVs are ideal for uptake by APCs (6, 7) and surface expression of adhesion and immune molecules on the PMVs may further enhance binding with cognate receptors found on immune cells thus promoting the ability of PMVs to act as a delivery vehicle.

Construction and purification of GPI-HER-2

PMVs are derived from cell membranes and thus contain a lipid bilayer making them amenable to protein transfer. In order to test whether the PMVs can be used as a scaffold to deliver TAAs by protein transfer we aimed to deliver the breast cancer antigen, HER-2. However, protein transfer-mediated modification of PMVs requires proteins to have a covalently attached lipid tail which spontaneously anchors the protein into the lipid bilayer through a lipid-lipid interaction (14, 18, 21). Therefore, we converted the transmembrane HER-2 antigen into GPI-anchored-HER-2 by attaching the DNA sequence encoding the GPI-anchor signal sequence from naturally GPI-anchored CD59 to the DNA sequence encoding the extracellular domain of HER-2 in a pUB6^{blast} vector (Figure 2A). CHO-K1 cells transfected with HER-2-CD59 plasmid were subjected to PI-PLC treatment that specifically cleaves the GPI-anchor. The HER-2 expression level on CHO-K1 cells transfected with full-length transmembrane HER-2 was not affected by PI-PLC treatment (Figure 2A, left panel) whereas HER-2 expression was reduced by 99.99% on CHO-K1 cells transfected with HER-2-CD59 after PI-PLC treatment (Figure 2B, right panel). Therefore, this demonstrates that HER-2-CD59 (GPI-HER-2) was anchored onto the cell membrane via the GPI-anchor.

GPI-HER-2 was purified from transfected CHO-K1 cells by affinity chromatography (Figure 2C), in which 13 g of GPI-HER-2-transfected-CHO-K1 cell pellet yielded approximately 2 mg of purified GPI-HER-2. Purified fractions were concentrated and dialyzed with PBS containing 0.01% octyl-β-glucopyranoside. Protein transfer was then performed on sheep red blood cells (RBCs) as an assay system to evaluate if GPI-HER-2 could incorporate onto cell surfaces. Purified GPI-HER-2

incorporated onto sheep RBCs upon protein transfer suggesting that purified GPI-HER-2 retains an intact GPI-anchor (Figure 2D).

Incorporation of GPI-HER-2 with GPI-ISMs onto 4TO7 PMVs by protein transfer

Vaccination with soluble protein is poorly immunogenic due to the short *in vivo* half-life and susceptibility to enzyme degradation. Therefore nanoparticles and microparticles have been used as delivery vehicles to increase the immunogenicity and protect proteins against degradation (7). We hypothesized that delivering HER-2 via membrane vesicles would induce strong HER-2-specific immunity. Since the presence of a lipid bilayer on PMVs makes them amenable to protein transfer of GPI-APs, we performed protein transfer of purified GPI-HER-2 onto PMVs derived from 4TO7 tumor tissue. Protein transfer resulted in GPI-HER-2 incorporation onto PMVs in a concentration dependent manner (Figure 3A). Similar results held true for protein transfer of GPI-HER-2 onto 4TO7 cultured cell derived PMVs (data not shown). Further, GPI-ISMs, GPI-IL-12 and GPI-B7-1, could be incorporated simultaneously along with GPI-HER-2 by protein transfer resulting in surface expression of HER-2 along with IL-12 and B7-1 on 4TO7 PMV surfaces (Figure 3B).

To determine if protein transfer affected PMV size, zetasizer analysis of 4TO7 PMVs before and after protein transfer was performed. 4TO7 PMVs simultaneously protein transferred with GPI-HER-2, GPI-IL-12, and GPI-B7-1 had a similar diameter (331.8 ± 12.2 nm) and polydispersity index (0.469 ± 0.1) (Figure 3C) compared to unmodified 4TO7 PMVs (347.1 ± 3.6 nm and 0.560 ± 0.1, respectively) (Figure 1A) suggesting that protein transfer mediated incorporation of new proteins onto PMVs does

not alter the size distribution of PMVs (Figure 3C). The zeta potential was increased to -8.7 ± 0.7 after protein transfer however it still reflected particle stability.

Vaccines often require controlled storage conditions. However, cold storage is not always feasible. Therefore, we tested whether incorporated HER-2 by protein transfer remained bound to PMVs after storage at room temperature. As shown in Figure 3D, HER-2 remained stably displayed on protein transfer modified 4TO7 PMVs after storage for at least 7 days at room temperature. Therefore, PMVs can be simultaneously modified to stably express GPI-HER-2 along with GPI-ISMs.

Vaccination with protein transferred (GPI-HER-2)-4TO7 PMVs induces protection against HER-2-expressing tumor challenge in an experimental metastasis model

To determine whether delivering TAAs using PMVs to the immune system results in antitumor immunity, the *in vivo* efficacy of GPI-HER-2-protein transferred PMVs was assessed. We established 4TO7 cells expressing HER-2 by transfecting the cells with a gene encoding full-length HER-2 to test the protective antitumor immunity. However, subcutaneous (s.c.) challenge of BALB/c mice with 4TO7-HER-2 cells resulted in spontaneous tumor regression, whereas intravenous (i.v.) administration led to metastatic growth of the tumor cells in the lungs (data not shown). Therefore, we used the 4TO7-HER-2 experimental metastasis model to determine if vaccination with 4TO7 PMVs protein transferred with GPI-HER-2 conferred protection against metastatic growth in lungs. Mice were given two immunizations of either unmodified 4TO7 PMVs, GPI-HER-2-protein-transferred-4TO7 PMVs (H-PMVs), 4TO7 PMVs protein transferred simultaneously with GPI-HER-2, GPI-IL-12 and GPI-B7-1 (HIB-PMVs) or with similar

concentrations of soluble GPI-HER-2 alone. The mice then received a challenge with live 4TO7-HER-2 cells i.v. 7 days post boost. Mice immunized with 4TO7 PMVs protein transferred with GPI-HER-2 had significantly decreased numbers of 4TO7-HER-2 cells in the lungs compared to control mice or mice vaccinated with unmodified 4TO7 PMVs (Figure 4) although mice in all vaccinated groups did not show any differences in body weight (data not shown). Further, vaccination with 4TO7 PMVs protein transferred with GPI-HER-2 induced significantly less metastasis of 4TO7-HER-2 cells in the lungs compared to mice vaccinated with similar concentrations of soluble GPI-HER-2. However, addition of GPI-ISMs did not alter the protection induced by GPI-HER-2 PMVs. These results demonstrate that delivery of GPI-HER-2 using 4TO7 PMVs induces antitumor immunity sufficient to confer protection against metastatic tumor growth without further addition of ISMs.

Vaccination with protein transferred (GPI-HER-2)-4TO7 PMVs induces protection against subcutaneously administered syngeneic breast cancer cells

Due to the difficulty in longitudinal follow up of tumor growth in metastatic mouse models, we chose to use another syngeneic breast cancer model, the D2F2/E2 cell line that expresses HER-2. D2F2/E2 cells are derived from transfection of full-length HER-2 in the parental D2F2 breast cancer cell line (25, 26). Subcutaneous challenge with D2F2/E2 cells leads to steady tumor growth that can be measured longitudinally to follow the effect of immune responses on tumor growth without euthanizing mice. BALB/c mice immunized with protein transferred-4TO7 PMVs were challenged with D2F2/E2 tumor cells 7 days post boost. As shown in Figure 5A and 5B, mice vaccinated

with GPI-HER-2-incorporated 4TO7 PMVs (H-PMVs) showed delayed D2F2/E2 tumor growth compared to control mice or mice vaccinated with unmodified 4TO7 PMVs. The tumors in H-PMV vaccinated mice developed 32 days post challenge compared to 4 days post challenge in control mice. The late appearance of tumors in vaccinated mice may be from clones that lost or down regulated HER-2 expression due to *in vivo* immune selection pressures causing immunoediting in tumor cells to escape the immune system (30). Also, D2F2/E2 tumors grew in mice without antibiotic selection, which may further lead to decreased HER-2 expression on the tumor cells. Therefore, to determine if the tumors that developed in vaccinated mice still expressed HER-2, we excised the tumor and allowed for expansion *in vitro* for a week. Flow cytometry analysis revealed that cells derived from progressed tumors in H-PMV vaccinated mice were HER-2-negative or had low levels of HER-2 expression, and by day 52, tumors in control mice also had decreased HER-2-surface expression (Supplemental Figure 1). HER-2 expression on D2F2/E2 cells grown without selection for 8 days did not show a significant decrease in HER-2 expression by flow cytometry analysis (data not shown) suggesting that *in vivo* immune selection pressure results in the decreased HER-2 expression in these isolated tumors.

Previous studies have shown that inoculation of 4TO7 cells that expressed both GPI-IL-12 and B7-1 led to complete protection against a 4TO7 wild-type concomitant challenge compared to mice inoculated with 4TO7 cells expressing either GPI-IL-12 or B7-1 (31). Therefore, to further enhance the immunity induced by GPI-HER-2 PMVs, mice were vaccinated with 4TO7 PMVs that were modified by protein transfer to express GPI-IL-12 and GPI-B7-1 on the surface along with GPI-HER-2 (HIB-PMVs). Mice

vaccinated with HIB-PMVs were completely protected against challenge with D2F2/E2 cells (Figure 5A and 5B), suggesting that the inclusion of the ISMs, IL-12 and B7-1, on 4TO7 PMVs with GPI-HER-2 augmented the antitumor immune response. Interestingly, mice vaccinated with unmodified PMVs had reduced average tumor sizes compared to control mice suggesting induction of a robust polyclonal immune response against D2F2/E2 tumor cells by unmodified PMV vaccination.

To further elucidate the specificity of immunity induced upon mice vaccinated with HIB-PMVs, we examined whether vaccinated mice that remained tumor free after D2F2/E2 challenge were protected against the parental HER-2-negative tumor cell line D2F2. Upon challenge of tumor free mice with D2F2 cells 65 days post D2F2/E2 challenge, control and vaccinated mice observed similar D2F2 tumor growth (Figure 5C), suggesting that immunity induced with HIB- PMV vaccination was HER-2-specific. To determine if immunity was induced against antigens expressed on the PMVs used for vaccination other than HER-2, HIB-PMV vaccinated mice that remained tumor free after D2F2/E2 challenge were challenged with 4TO7 tumor cells. 4TO7 tumors grew steadily in control and vaccinated mice (Figure 5D), suggesting that the immunity induced is dominantly against the incorporated TAA, HER-2.

Since administration of soluble cytokines, such as IL-2 and IL-12, lead to systemic toxicity (32, 33), we determined whether membrane-bound IL-12 and B7-1 caused systemic toxicity when delivered attached to PMVs. One of the major indications of IL-12 induced toxicity is the release of inflammatory cytokines such as IFN-γ (34). Serum concentration levels of IFN-γ in mice vaccinated with unmodified PMVs, H-PMVs or HIB-PMVs were all similar to IFN-γ levels in control mice (Supplemental

Figure 2), suggesting that vaccination with PMVs modified by protein transfer to express HER-2 and membrane-bound ISMs does not lead to systemic toxicity. These results show that vaccination with PMVs modified to express HER-2 along with immunostimulatory adjuvants further enhance HER-2-specific immunity without any overt toxicity concerns.

HIB-PMVs protect therapeutically against D2F2/E2 tumor growth

Although prophylactic vaccination with HIB-PMVs conferred protection against HER-2-expressing tumor challenge, therapeutic protection against an existing tumor is more clinically relevant. Therefore, we wanted to determine the therapeutic efficacy of vaccination with HIB- PMVs after challenge with D2F2/E2 tumor cells. Mice were implanted with tumor cells and then vaccinated on days 3 and 6. As shown in Figure 6, vaccination with HIB-PMVs showed a significant delay in tumor growth compared to control mice by day 45 post implant and tumors in 20% of mice fully regressed suggesting that HIB-PMV vaccination controls HER-2-expressing tumor growth therapeutically.

Role of $CD4^+$ and $CD8^+$ T lymphocytes induced by PMVs modified with GPI-HER-2 and GPI-ISMs in antitumor immunity

T-cells play a role in combating tumors. $CD4^+$ T-cells help to setup an anti-tumor cytokine milieu to direct the type of immunity induced whereas $CD8^+$ T-cells play a prominent role in killing tumor cells. Both are important in combating tumor growth (35-37). To elucidate the nature of HIB-PMV vaccine induced T-cell immunity responsible for protection against HER-2-expressing tumor growth, $CD4^+$ and/or $CD8^+$ cells were

depleted in the effector phase after PMV vaccination as well as before and after D2F2/E2 challenge (Figure 7A). HIB-PMV vaccinated mice showed 0% tumor incidence upon D2F2/E2 challenge whereas control-PBS mice showed 100% tumor incidence. Mice depleted of $CD4^+$ cells showed 40% tumor incidence and mice depleted of $CD8^+$ cells showed 20% tumor incidence. Mice depleted of both $CD4^+$ and $CD8^+$ cells showed 40% tumor incidence during the depletion treatment, however after depletion was discontinued, tumor incidence reduced to 20% as tumors in 20% of the mice regressed (Figure 7B). These results suggest that $CD4^+$ and $CD8^+$ cells play a role in the effector phase of an anti-HER-2-specific immune response after vaccination with protein transferred HIB-PMVs, however the role is partial in terms of protection.

The partial protection seen in the T-cell-depleted mice may be due to the presence of HER-2-specific antibodies against D2F2/E2 tumors. We observed that vaccinated mice depleted of $CD4^+$ and/or $CD8^+$ cells had similar amounts of anti-D2F2/E2 specific serum antibodies (Figure 7C), suggesting that the presence of anti-HER-2 antibodies could be involved in inhibiting D2F2/E2 tumor growth when tumors are administered in a prophylactic setting.

4TO7 PMVs protein transferred with GPI-HER-2 induce HER-2-specific IgG

Since we noticed that HIB-PMV vaccination induced a D2F2/E2-specific serum antibody response, we wanted to test its efficacy in preventing D2F2/E2 tumor growth. We transferred serum collected from mice that were vaccinated with H-PMVs, HIB-PMVs, or similar concentrations of GPI-HER-2 into naïve mice one day before and three days after challenge with D2F2/E2 cells. Mice that received serum from HIB-PMV

vaccinated mice had significantly smaller average tumor sizes compared to mice that received serum from H-PMV or GPI-HER-2 vaccinated mice (Figure 8). These results suggest that vaccination with protein transfer-modified PMVs that express HER-2 along with ISMs, IL-12 and B7-1, induced a protective humoral immune response.

To further elucidate the mechanism of antitumor immunity induced with PMV vaccination, we determined the specificity and type of the antibody response after vaccination with PMVs. Serum was collected 7 days post boost from mice vaccinated with 4TO7 PMVs protein transferred with GPI-HER-2 with or without GPI-ISMs. We included a group where mice were vaccinated with GPI-HER-2 alone. In this group we vaccinated mice with 0.85 μg GPI-HER-2 since the PMV vaccination dose of 25 μg expressed approximately 0.85 μg of GPI-HER-2. Flow cytometry analysis showed that serum from mice vaccinated with GPI-HER-2-protein transferred-4TO7 PMVs induced strong anti-D2F2/E2-specific antibodies (Figure 9A; left panel). Interestingly, mice vaccinated with soluble GPI-HER-2 produced significantly lower levels of anti-D2F2/E2 specific IgG in comparison. Serum IgG antibody binding to D2F2/E2 cells was HER-2-specific as no detectable response was seen against D2F2 or 4TO7 cells (Figure 9A; middle and right panel, respectively). Further, probing of antibodies produced upon vaccination with GPI-HER-2-PMVs via western blot analysis showed that antibodies were HER-2 specific and not against other antigens present on the PMVs (Supplemental Figure 3).

T-helper 1 (Th1)-type immunity has been shown to be protective in combating tumors (38), however induction of both Th1 and Th2-type immunity are beneficial in tumor protection (39-41). To further elucidate the type of HER-2-specific immunity

induced upon PMV vaccination, D2F2/E2-specific IgG subtypes were analyzed by cell ELISA. IgG1 subtypes correlate with a Th2-type immune response, whereas IgG2a and IgG2b correlate with a Th1-type immune response (42). Mice vaccinated with 4TO7 PMVs protein transferred with GPI-HER-2 alone (H-PMVs) or with GPI-IL-12 and GPI-B7-1 (HIB-PMVs) induced strong anti-D2F2/E2 IgG1, IgG2a, and IgG2b responses compared to mice vaccinated with GPI-HER-2 (Figure 9B). Interestingly, mice vaccinated with GPI-HER-2 induced only detectable anti-D2F2/E2-specific IgG1 responses albeit at lower levels and no detectable IgG2a and IgG2b responses (Figure 9B). These results suggest that vaccination with soluble GPI-HER-2 induces only weak Th2-type immunity, whereas vaccination with GPI-HER-2 incorporated onto 4TO7 PMVs by protein transfer induces stronger Th1 and Th2-type immunity.

Since protein transfer-modified-PMVs that express HER-2 induced a Th1-type antibody response whereas GPI-HER-2 administration alone did not, we next determined if protein transfer-modified-PMVs also induced Th1-type cytokines. Splenocytes from mice vaccinated with protein transfer-modified-PMVs were stimulated with 4TO7 cells transfected to express full-length HER-2 for 2 days. Analysis of the supernatant obtained after 2 days of stimulation showed that splenocytes from mice vaccinated with 4TO7 PMVs protein transferred to express GPI-HER-2 with or without GPI-ISMs led to enhanced IFN-γ production compared to splenocytes from mice vaccinated with unmodified PMVs (Figure 10). Further, inclusion of ISMs on PMVs led to enhanced IFN-γ production compared to H-PMVs suggesting that HER-2-expression on PMVs leads to a Th1-type immune response whereas inclusion of GPI-ISMs on PMVs further enhances Th1-type cytokine production.

Delivery of GPI-HER-2 using PMVs derived from syngeneic and allogeneic breast cancer tissues confers similar protection against D2F2/E2 tumor growth

We have shown that protein transfer of GPI-HER-2 onto PMVs derived from 4TO7 tumor tissue confers protection against HER-2-expressing tumor challenge, therefore we wanted to determine if similar protection was observed when protein transfer of GPI-HER-2 was performed on PMVs derived from syngeneic and allogeneic breast tumor tissues. PMVs were prepared from tumor tissue obtained from BALB/c mice challenged with D2F2 cells or from C57BL/6 mice challenged with E0771 cells. BALB/c mice were given two immunizations with 25 μg of GPI-HER-2-protein transferred-4TO7, D2F2, or E0771 PMVs, followed by challenge with D2F2/E2 cells 7 days post boost. Mice vaccinated with PMVs protein transferred with GPI-HER-2 had similar decreased levels of average tumor growth compared to control mice and mice vaccinated with unmodified PMVs (Table 1). Therefore, protein transfer of GPI-HER-2 onto PMVs regardless of whether they are derived from syngeneic or allogeneic breast tumor tissues induces similar level of protection against HER-2 expressing tumor cells.

Delivery of GPI-HER-2 using PMVs derived from cultured 4TO7 cells confers protection against D2F2/E2 tumor growth

Tumor tissues contain tumor infiltrating immune cells that include tumor-specific effector T-cells as well as many immunosuppressive cells such as regulatory T-cells and myeloid derived suppressor cells (MDSCs) (43, 44). Consequently, PMVs derived from tumor tissue may contain membrane vesicles from these immunosuppressive cells as well as express immunosuppressive molecules. Therefore, we determined if PMVs derived

from cultured tumor cells that are void of immunosuppressive cells leads to similar protection compared to PMVs derived from tumor tissue. Mice were immunized with 4TO7 cultured cell-derived PMVs that were protein transferred with GPI-HER-2, followed by challenge with D2F2/E2 cells 7 days post boost. Vaccination with GPI-HER-2-modified cell culture-derived 4TO7 PMVs also led to delayed tumor growth compared to control mice with 40% of mice remaining tumor-free by day 32 post challenge (Figure 11A). Protection induced was HER-2-specific as challenge of immunized mice with HER-2-negative D2F2 tumor cells led to steady tumor growth (Figure 11B).

Vaccination using 4TO7 TMVs protein transferred to incorporate GPI-HER-2 results in prolonged antigen presence at vaccination site.

Vaccination with antigen encapsulated onto particles has shown to enhance antigen presence at the vaccination site for at least 7 days and thus preventing quick antigen clearance (within 6 h) that is seen with administration of soluble antigen (9). To test if the enhanced HER-2-specific tumor protection seen with vaccination using HER-2-incorporated-PMVs was due to enhanced antigen persistence at the vaccination site, we performed *in vivo* imaging studies using fluorescently-labeled GPI-HER-2 that was incorporated onto 4TO7 PMVs by protein transfer. As shown in Figure 12A and 12B, incorporation of GPI-HER-2 onto PMVs led to antigen persistence at the vaccination site for at least 6 days, suggesting that enhanced HER-2-specific immunity induced by H-PMV vaccination could be due to the enhanced antigen exposure at the vaccination site.

Discussion

Incorporation of antigen onto micro and nanoparticles enhances antigen stability and increases antigen presence at the vaccination site (9). The unique size and particulate structure of nano and microparticles additionally makes them ideal for optimal engulfment and uptake by APCs, thus directly targeting incorporated antigens to the adaptive immune system. Further, the size of these particles may allow for direct transport of the particles and incorporated antigen to draining lymph nodes for additional immune cell activation. Antigen-encapsulated particles have been shown to induce both an enhanced antigen-specific antibody response as well as Th1 and Th2-type cytokine production compared to administration of soluble antigen (9). In addition, inclusion of antigen within particles enhances antigen specific central memory T cell responses compared to soluble antigen injected alongside particles (9). These immunogenic characteristics of particles make them ideal antigen delivery candidates.

Herein, we use these intrinsic immune activation properties of particles to deliver the tumor associated antigen HER-2. In our approach, we developed biological plasma membrane vesicles prepared from tumor cells or tumor tissue as a model system to test the use of PMVs as a particle-based delivery vehicle. These PMVs are biologically compatible making them less toxic to APCs (8, 45). Homogenization of tumor cells or tissues resulted in vesicles of an average size range from 300 – 420 nm in diameter and thus the PMVs produced are of an optimal size to be taken up by antigen presenting cells, such as dendritic cells found in the vaccination site (6). More importantly, these PMVs were derived from cell membranes and thus contained a lipid bilayer making them susceptible to modification by protein transfer using GPI-APs.

We show herein that these biological PMVs could be modified by protein transfer to express GPI-HER-2. The level of incorporation by protein transfer occurred in a concentration dependent manner. Therefore, antigen levels can be easily adjusted by varying the concentration of purified antigen incubated with the PMVs during protein transfer. Moreover, incorporation of HER-2 occurred simultaneously along with the incorporation of ISMs, GPI-IL-12 and GPI-B7-1. Thus, this approach allows for a controlled level of antigen delivery along with targeting or immune activation molecules on the same vesicles. In addition, incorporation took place within a matter of hours and the incorporated antigen remained stably expressed on the PMVs for at least a week after protein transfer even after storage of the PMVs at room temperature.

Currently, the methods available to incorporate new antigens onto surfaces of synthetic and biological particles involve chemical modification of both antigen and particle. Often times such chemical modifications may alter antigenicity of the protein or nature of the particles. However, protein transfer-mediated-incorporation of GPI-HER-2 onto PMVs occurs by the interaction of the lipid moiety of the GPI-anchor with the lipid bilayer of the cell surface plasma membrane, avoiding such deleterious effects. GPI-anchor attachment to the protein is only added to the C-terminal end. Attachment does not alter the functionality of proteins as soluble and transmembrane proteins can be converted to GPI-anchored forms and remain functionally active (Nagarajan Cancer Res 2002, Poloso Molec Immunol 2001, McHugh PNAS 1995). Our purification protocol described here allows for purification of these proteins with an intact GPI-anchor, which can be used in protein transfer to decorate PMVs with desired protein molecules for targeted delivery.

Vaccination of mice with protein transfer-modified-PMVs led to complete protection against challenge with HER-2-expressing tumors prophylactically as well as delayed tumor growth therapeutically when GPI-HER-2 was incorporated alongside GPI-IL-12 and GPI-B7-1. Vaccination also protected mice against metastatic tumor growth in an experimental metastasis model. However, the ability to deliver antigen for an effective antitumor immune response was not restricted to PMVs from a single tumor. PMVs from many different tumor tissues, whether obtained from syngeneic or allogeneic mice, or from cultured 4TO7 cells, also were capable of being modified by protein transfer to express GPI-HER-2 and induce similar protection against a HER-2-expressing tumor challenge.

Administration of soluble proteins is known to induce a poor immune response probably due to soluble proteins being more prone to degradation *in vivo* or lack of activation of APCs during uptake of soluble proteins (7). However, a robust adaptive immune response is induced when the antigens are delivered as a particulate form via micro or nanoparticles where they are more persistent *in vivo* due to slow release (9). Also, particulate delivery can activate APCs since phagocytosis of particulate materials can accompany cell activation (7, 8). Our results showed that vaccination with GPI-HER-2-protein transferred-PMVs increased HER-2 antigen persistence at the vaccination site and enhanced anti-HER-2 IgG antibody responses compared to administration of soluble GPI-HER-2. Both Th1-type and Th2-type antibody immune responses were generated by vaccination with GPI-HER-2-protein transferred-PMVs whereas only Th2-type immune responses were detected upon soluble GPI-HER-2 vaccination. The induction of Th1-type immunity may be due to the particulate nature of the PMVs since nanoparticles and

microparticles have been shown to induce more of a Th1-type immune response (46, 47). The induction of tumor specific B-cell, Th1, and Th2-type immune responses has shown to play important roles in the eradication of tumors (35) making protein transferred-PMVs ideal candidates for an effective vaccine against cancer.

Moreover, protein transfer allows for simultaneous incorporation of more than one GPI-AP onto the PMVs, thus allowing for different ISMs to incorporate onto the PMVs along with the targeted TAA. We observed that the inclusion of IL-12 with B7-1 on the PMVs alongside HER-2 led to long-lasting protection (up to at least 52 days) against D2F2/E2 challenge, suggesting that vaccination induced sufficient immune stimulation to eradicate HER-2-expressing tumor cells upon challenge (Figure 5). The combination of membrane-bound IL-12 and B7-1 on 4TO7 cells has previously been shown to enhance anti-tumor immunity *in vivo* compared to individual ISM expression through the reduction of immunosuppressive regulatory T-cells and myeloid derived suppressor cells at the tumor site (31). In addition, CD28, the receptor for B7-1, is expressed on T-cells, NK cells, and mast cells, and the IL-12 receptor is expressed on NK cells, T-cells, and APCs (14). Therefore expression of B7-1 and IL-12 on PMVs along with HER-2 could be leading to more efficient delivery of HER-2 to immune cells, such as DCs, along with widespread immune activation.

This versatile biological membrane vesicle protein delivery approach may be further expanded to elicit an immune response against numerous TAAs including soluble and transmembrane antigens. Individual as well as multiple TAAs can be expressed on the PMVs simultaneously for delivery to broaden the immune response generated. Also, the PMVs, depending upon the cell source, may contain naturally occurring cell adhesion

molecules, which will further enhance cell-to-cell interaction for efficient antigen delivery. Moreover, through the inclusion of different combinations of membrane-bound-ISMs along with TAAs, immunity can be skewed towards a desirable result allowing for a more controlled immune response depending on the disease. Therefore, the rapid simultaneous protein transfer of GPI-TAAs along with GPI-ISMs onto PMVs can be used as an augmented vaccine in a tumor setting.

In summary, the ability of PMVs derived from biological materials to be rapidly modified by protein transfer to express antigens along with ISMs, allows for an attractive single antigen delivery system that not only enhances antigen immunogenicity but also broadens the scope of antigen-specific immunity induced thus providing broader implications in subunit vaccine production against various diseases.

Acknowledgements:

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Financial & competing interest disclosure

Dr. Selvaraj is a co-founder & equity holder of Metaclipse Therapeutics Corporation - a startup company formed to develop therapeutic cancer vaccines for humans using the protein transfer technology described here for which he is a co-inventor.

Writing assistance was not utilized in the production of the manuscript.

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Figure Legends

Figure 1. Characterization of PMVs. PMVs were produced from 4TO7 cultured cells or from 4TO7 tumor tissue obtained from subcutaneous inoculation in a BALB/c mice with 4TO7 cells. (**A**) Cultured cell derived and tissue derived 4TO7 PMVs portray similar size, polydispersity index and zetapotential. (**B**) Electron microscopy of PMVs derived from 4TO7 cultured cells versus 4TO7 tumor tissue. (**C**) Flow cytometry analysis of 4TO7 cell cultured derived PMVs. (**D**) Flow cytometry analysis of 4TO7 tumor tissue derived PMVs.

Figure 2. **Construction, expression and purification of GPI-HER-2.** (**A**) Construction of GPI-HER-2. The extracellular domain of HER-2 is attached to GPI-anchor signal sequence from CD59 and cloned into pUB6 vector (**B**) PI-PLC treatment of CHO-K1 cells transfected with HER-2. PI-PLC treatment was performed on CHO-K1 cells transfected with full-length transmembrane (TM) HER-2 (left panel) or on CHO-K1 cells transfected with HER-2-CD59 (right panel) and analyzed by flow cytometry. Grey – not treated with PI-PLC. Red – after PI-PLC treatment. (**C**) Purification of GPI-HER-2. GPI-HER-2 was purified from transfected CHO-K1 cells by mAb affinity chromatography. SDS PAGE and western blot (top panel) or silver staining (bottom panel) was performed on the purified fractions. (**D**) Protein transfer of purified GPI-HER-2 onto sheep red blood cells (RBCs). Sheep RBCs were incubated with purified GPI-HER-2 for 4 h at 37°C. Flow cytometry analysis was performed to determine incorporation levels. Grey – no protein transfer. Red – GPI-HER-2 added during protein transfer.

Figure 3. Protein transfer of GPI-HER-2 with GPI-ISMs onto 4TO7 TMVs. (A) Protein transfer mediated incorporation of GPI-HER-2 onto 4TO7 PMVs is concentration dependent. 20 μg of 4TO7 PMVs were protein transferred with increasing concentrations of GPI-HER-2. Incorporation was analyzed by SDS PAGE and western blot analysis against HER-2. (**B**) Flow cytometry analysis of 4TO7 PMVs protein transferred with GPI-HER-2 +/- GPI-ISMs. 4TO7 PMVs were protein transferred with GPI-HER-2 along with GPI-IL-12 and/or GPI-B7-1. Then PMVs were stained with fluorescein conjugated mAbs and analyzed by flow cytometry. (**C**) Protein transfer of GPI-APs onto 4TO7 PMVs does not affect TMV size. The diameter, PDI and zeta potential of unmodified or protein transferred 4TO7 TMVs were analyzed using a Zetasizer. (**D**) HER-2 incorporated onto 4TO7 PMVs by protein transfer remains stably expressed at room temperature. 4TO7 PMVs modified by protein transfer with GPI-HER-2 were stored at room temperature. Each time point, PMVs were washed by centrifugation with PBS and HER-2 and PMV analysis was determined by SDS PAGE and western blot analysis by staining against HER-2.

Figure 4: Immunization of mice with 4TO7 PMVs protein transferred with GPI-HER-2 confers protection against experimental metastasis of HER-2-expressing tumor cells. Groups of mice (n = 5) were vaccinated and boosted on day 14 with 25 μg protein transferred PMVs i.v. Mice were then challenged 7 days post boost with transfected 4TO7-HER-2 tumor cells i.v. for experimental metastasis of the tumor cells to the lungs. Mice were sacrificed day 15 post challenge and experimental metastasis of

4TO7-HER-2 cells to the lungs were analyzed by counting the number of 6-thioguanine resistant cells after 8 days of selection.

Figure 5. Vaccination with 4TO7 PMVs protein transferred simultaneously with GPI-HER-2 along with GPI-IL-12 and GPI-B7-1 protects mice against challenge with HER-2 expressing tumor cells. Groups of BALB/c mice (n = 5) were vaccinated and boosted with 25 μg of protein transferred 4TO7 PMVs on day 14 and then challenged with 200,000 D2F2/E2 (HER-2 positive) tumor cells. Protein transferred PMVs expressed similar levels of HER-2 at 0.034 μg HER-2/μg PMV (**A**) Tumor incidence and (**B**) tumor area were measured. Tumor free mice from (GPI-HER-2+GPI-IL-12+GPI-B7-1)-4TO7 group were then rechallenged 65 days post boost with either (**C**) 200,000 D2F2 (HER-2 negative) cells or (**D**) 200,000 4TO7 (HER-2 negative) cells. Tumor area and incidence in each mouse was measured.

Figure 6. Vaccination with (GPI-HER-2+GPI-IL-12+GPI-B7-1)-4TO7 controls the growth of established tumors. Groups of mice (n = 5) were injected s.c. with 200,000 D2F2/E2 cells on the right hind flank on day 0. On days 3 and 6 (indicated by black arrows), the challenged mice were vaccinated with 100 μg of 4TO7 PMVs protein transferred with GPI-HER-2, GPI-IL-12, and GPI-B7-1 s.c. on the left hind flank. Tumor area was measured by multiplying the length and width of the tumor. (Statistical analysis: Repeated measures Two-Way ANOVA – Bonferroni's multiple comparisons test).

Figure 7. The role $CD4^+$ and $CD8^+$ T-cells play in (GPI-HER-2+GPI-IL-12-GPI-B7-1)-4TO7 PMV mediated protection. Groups of mice (n = 5) were vaccinated with protein transferred 4TO7 PMVs derived from tumor tissue. (A) Schematic of vaccination and depletion. Before and after challenge with 200,000 D2F2/E2 cells, mice were depleted of $CD4^+$, $CD8^+$ or both $CD4^+$ and $CD8^+$ cells. (B) Tumor growth and incidence of D2F2/E2 after $CD4^+$ and $CD8^+$ cell depletion. (C) Anti-D2F2/E2 specific serum IgG responses in vaccinated mice before depletion.

Figure 8. The role of antibodies in (GPI-HER-2+GPI-IL-12-GPI-B7-1)-4TO7 PMV mediated protection. Serum was collected from mice vaccinated with protein transferred 4TO7 PMVs derived from tumor tissue. Collected serum was adoptively transferred i.p. to naïve mice (n = 3) 1 day before and 3 days after challenge with 200,000 D2F2/E2 cells s.c. Tumor area was measured by multiplying the length and width of the tumor. (Statistical analysis: Repeated measures Two-Way ANOVA – Bonferroni's multiple comparisons test).

Figure 9. Vaccination of mice with 4TO7 PMVs protein transferred with GPI-HER-2 induces both Th1 and Th2-type HER-2-specific antibody production. (**A**) Total anti-HER-2 specific serum IgG antibody induced upon vaccination with 4TO7 PMVs protein transferred with GPI-HER-2. HER-2 expression was similar in all protein transferred samples at 0.034 µg HER-2/µg PMV. Serum was collected from groups of vaccinated mice (n = 5) day 7 post boost. Flow cytometry analysis using 1:200 diluted serum incubated with D2F2/E2, D2F2, or 4T07 cells was performed. (**B**) IgG antibody

subtype analysis of anti-HER-2 antibodies in sera from vaccinated mice. 1:100 diluted serum collected on day 7 post boost from vaccinated mice (n = 5) was incubated with 50, 000 D2F2/E2 cells per well in triplicate in a cell ELISA. The subtype of bound antibody was detected by adding peroxidase conjugated subtype specific antibodies.

Figure 10. Enhanced IFN-γ secretion by stimulated splenocytes from mice vaccinated with (GPI-HER-2-IL-12-B7-1)-4TO7 PMVs. Splenocytes from mice (n = 5) vaccinated and boosted with 25 µg protein transferred PMVs were stimulated for 2 days with mitomycin C-treated 4TO7-HER-2 cells. Supernatant was collected and IFN-γ concentrations were analyzed by sandwich ELISA in triplicate.

Figure 11. Vaccination with cell culture derived 4TO7 PMVs protein transferred with GPI-HER-2 protects mice against challenge with HER-2 expressing tumor cells. Groups of BALB/c mice (n = 5) were vaccinated and boosted with 25 µg of protein transferred 4TO7 PMVs on day 14. Protein transfer resulted in 0.01 µg HER-2/µg PMV. Vaccinated mice (n = 5) were challenged 7 days post boost with (**A**) 200,000 D2F2/E2 (HER-2 positive) cells or (**B**) D2F2 (HER-2 negative) cells. Tumor size (left panel) and tumor incidence (right panel) were measured. (Statistical analysis: Repeated measures Two-Way ANOVA - Tukey's multiple comparisons test was used to compare between PBS and (GPI-HER-2)-4TO7 PMV vaccinated groups.

Figure 12. Vaccination with H-PMVs leads to prolonged HER-2 persistence at the vaccination site. Purified GPI-HER-2 was fluorescently labeled with IRDye 800CW

(Licor) and then protein transferred onto 4TO7 PMVs. BALB/c mice (n=4) were injected s.c. on the hind flank with 25 µg of the resulting tagged H-PMVs. **(A)** Optical imaging was conducted using the Kodak *In vivo* FX imaging system (Carestream Health Inc.) at the indicated time points (ex: 720 nm; em: 790 nm). **(B)** Kodak MI imaging software was used to quantify the average fluorescence intensity at the injection sites. Regions of Interests (ROIs) were selected for measuring the mean fluorescence intensity (MFI) of the protein and corresponding body background. Signal to body background (S/B) ratio was calculated from the MFI of the injection area divided by of the body background area. Data shown in Results were the mean S/B ratio ± standard error from three to four mice.

Table Legends

Table 1. Vaccination with breast cancer derived PMVs protein transferred GPI-HER-2 induce similar protection against HER-2 expressing tumor challenge. Groups of BALB/c mice (n = 5) were vaccinated and boosted with 25 µg of PMVs derived from 4TO7, D2F2, or E0771 tumor tissue and protein transferred with GPI-HER-2. Mice were then challenged 7 days post boost with 200,000 D2F2/E2 cells. Tumor area was measured. Statistical Analysis: Two-Way ANOVA, multiple comparisons test; * $p < 0.05$.

Supplemental Figure Legends

Supplemental Figure 1. Expression of HER-2 on harvested D2F2/E2 tumor cells in mice prophylactically vaccinated with protein transferred (GPI-HER-2)-4TO7 PMVs. Tumors were harvested from mice, minced and grown in vitro in DMEM with 10% bovine calf serum for 8 days. Tumor cells were harvested with PBS/EDTA and HER-2 expression was analyzed by flow cytometry.

Supplemental Figure 2. Vaccination with protein transfer-modified PMVs did not induce systemic toxicity in mice. The concentration of serum IFN-γ was analyzed in vaccinated mice (n = 5) 3 days after boost by a sandwich ELISA.

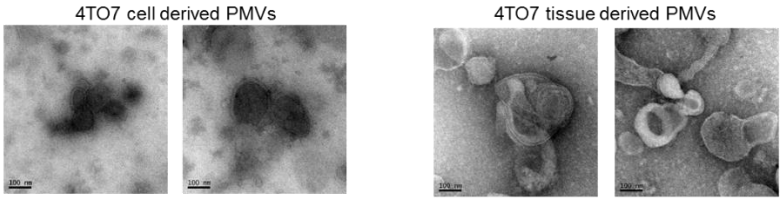
Supplemental Figure 3. Mice vaccinated with 4TO7 PMVs protein transferred with GPI-HER-2 induced HER-2 specific antibodies. Western blot analysis was performed after SDS PAGE of 4TO7 PMVs, D2F2 PMVs or purified GPI-HER-2 and blotting with 1:500 diluted pooled serum from mice vaccinated (n = 5) with protein transferred 4TO7 PMVs.

Figure 1.

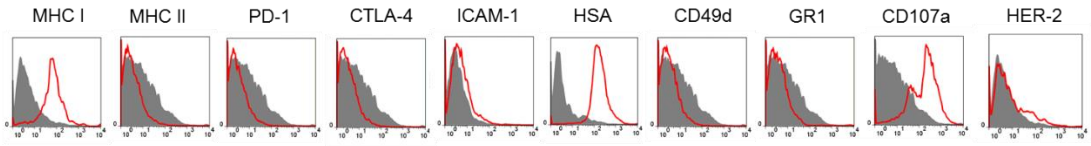
A

	Diameter (nm)	Polydispersity index (P.I.)	Zeta Potential (mV)
4TO7 cell derived PMVs	410.2±6.8	0.595±0.02	-12.0±0.6
4TO7 tissue derived PMVs	347.1±3.6	0.560±0.1	-17.1±1.5

B



C



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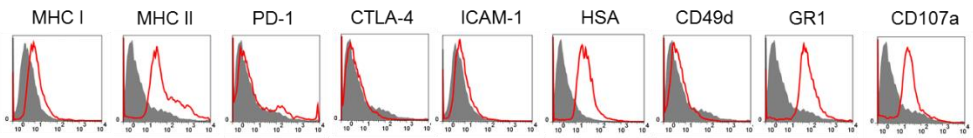


Figure 2.

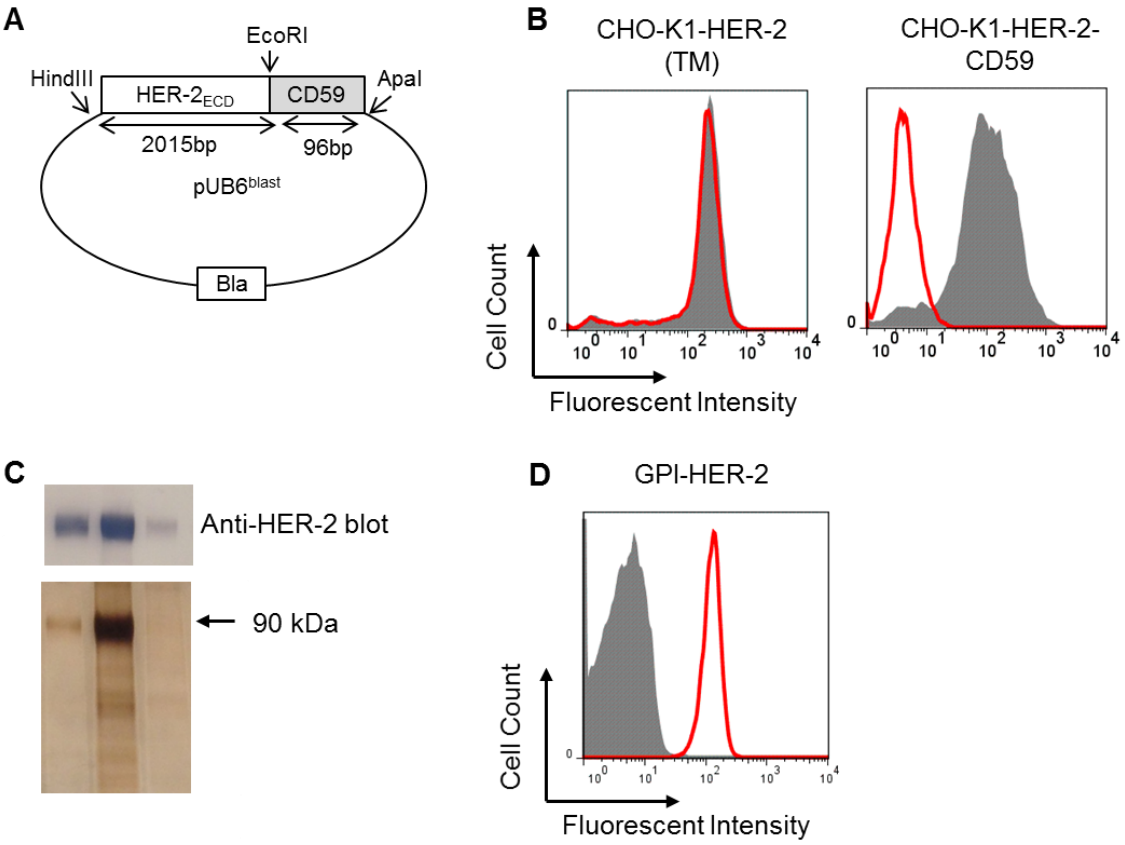
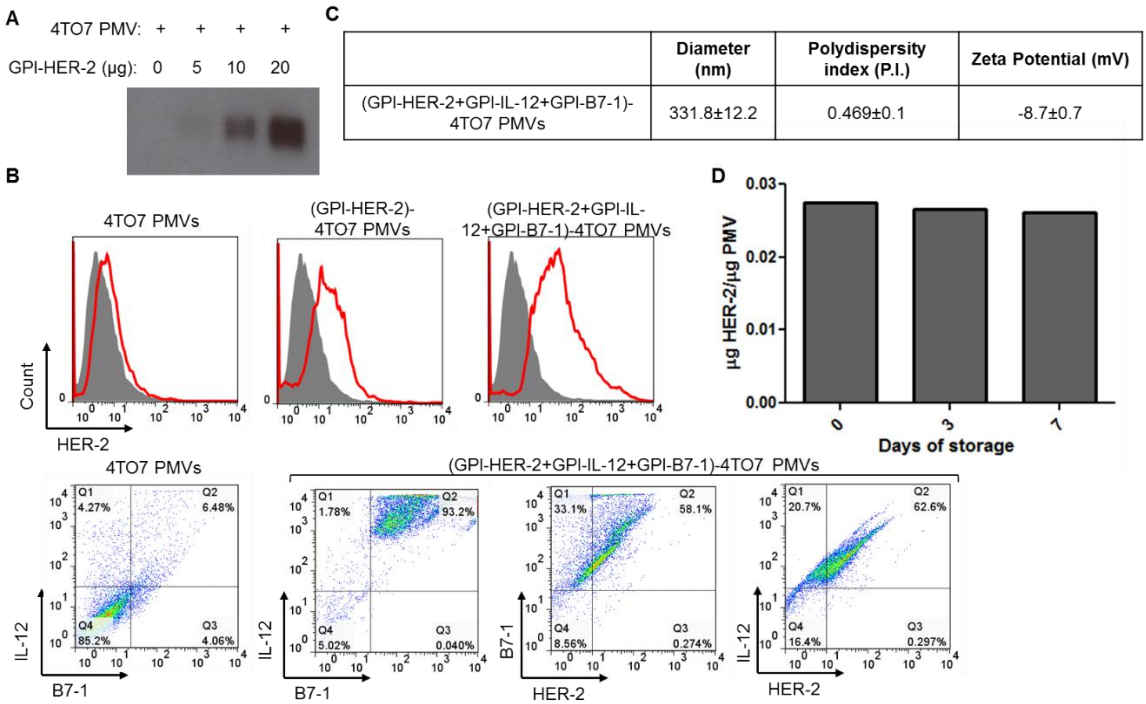

A
EcoRI
HindIII
HER-2ECD
CD59
ApaI
2015bp
96bp
pUB6blast
Bla
B
CHO-K1-HER-2 (TM)
CHO-K1-HER-2-CD59
Cell Count
Fluorescent Intensity
C
Anti-HER-2 blot
90 kDa
D
GPI-HER-2
Cell Count
Fluorescent Intensity

Figure 3.



C

	Diameter (nm)	Polydispersity index (P.I.)	Zeta Potential (mV)
(GPI-HER-2+GPI-IL-12+GPI-B7-1)-4TO7 PMVs	331.8±12.2	0.469±0.1	-8.7±0.7

Figure 4.

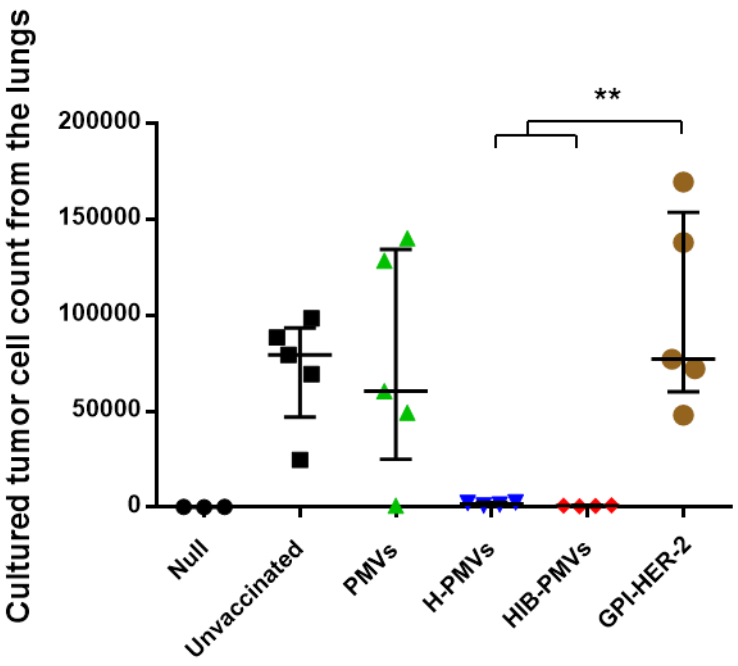

Cultured tumor cell count from the lungs
200000
150000
100000
50000
0
**
Null
Unvaccinated
PMVs
H-PMVs
HIB-PMVs
GPI-HER-2

Figure 5.

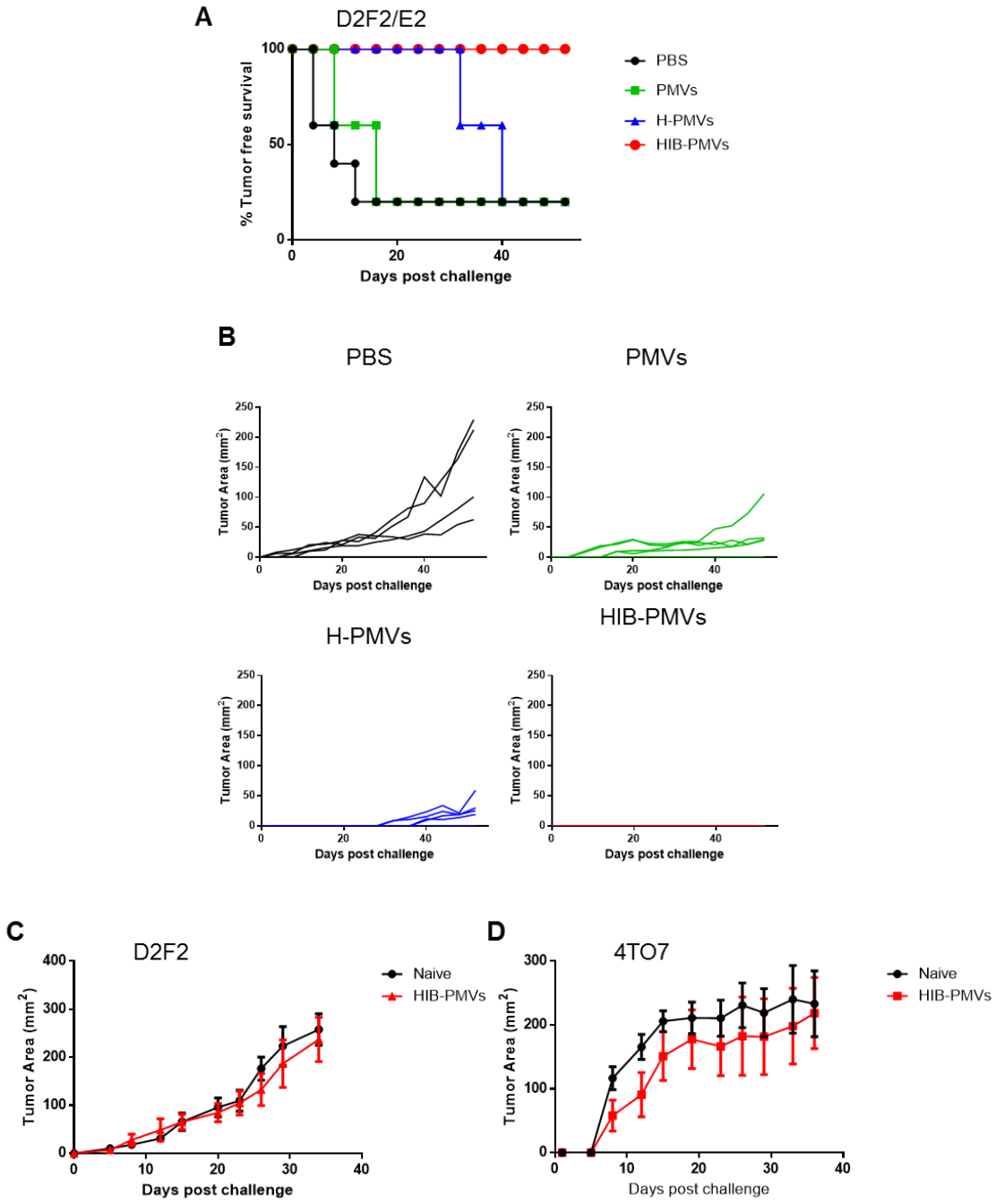

A
D2F2/E2
% Tumor free survival
Days post challenge
PBS
PMVs
H-PMVs
HIB-PMVs
B
PBS
PMVs
H-PMVs
HIB-PMVs
Tumor Area (mm2)
Days post challenge
C
D2F2
Naive
HIB-PMVs
Tumor Area (mm2)
Days post challenge
D
4TO7
Naive
HIB-PMVs
Tumor Area (mm2)
Days post challenge

Figure 6.

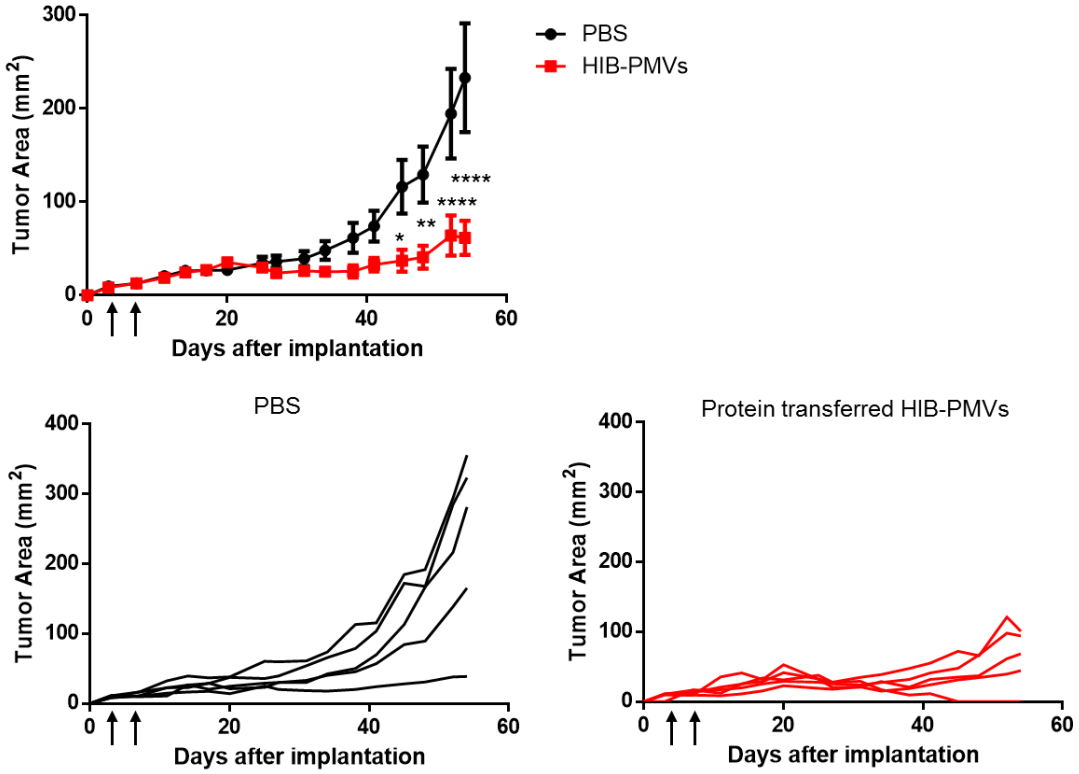

PBS
HIB-PMVs
Tumor Area (mm2)
Days after implantation
****
****
**
*
PBS
Protein transferred HIB-PMVs

Figure 7.

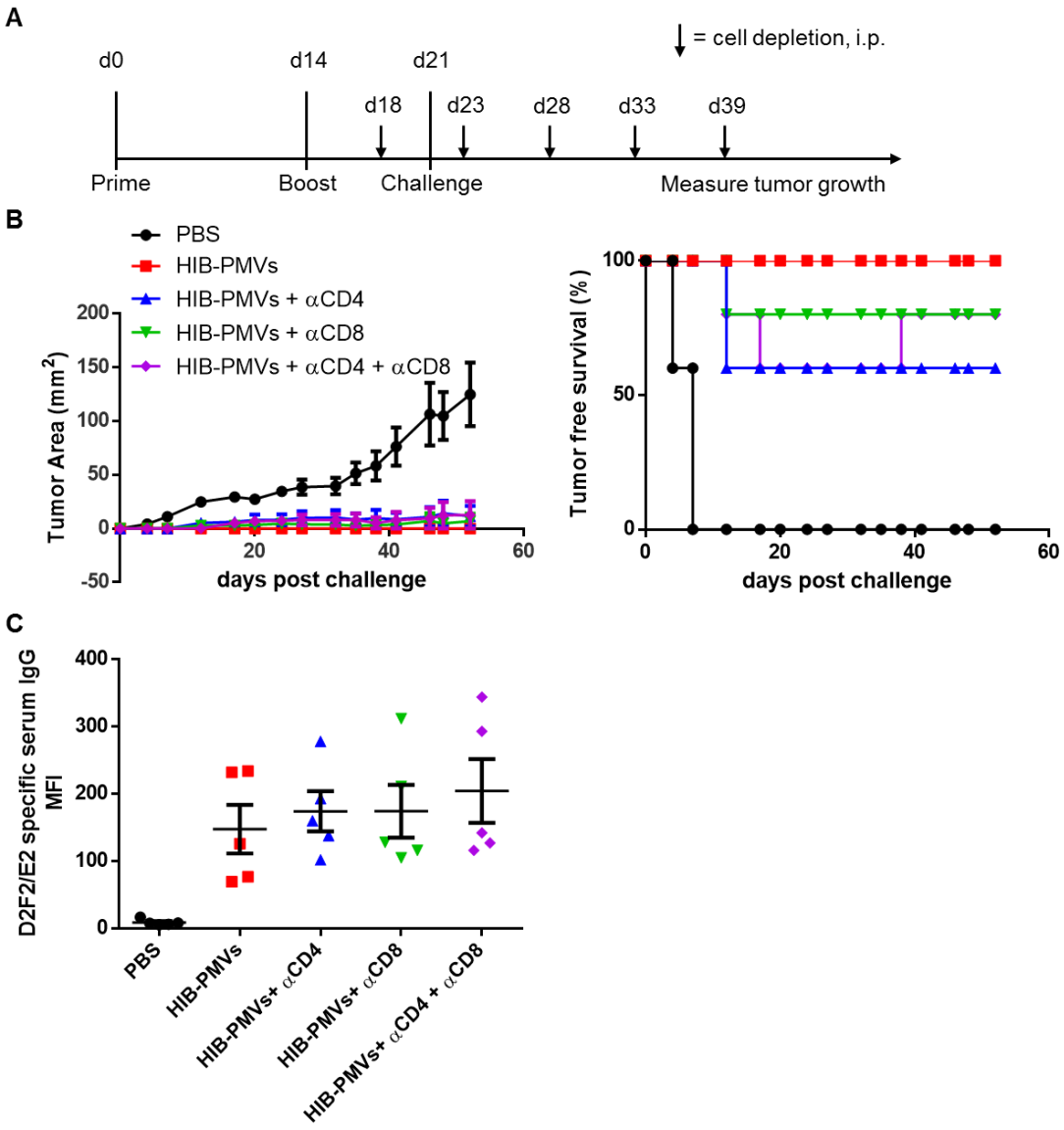

A
= cell depletion, i.p.
d0
d14
d21
d18
d23
d28
d33
d39
Prime
Boost
Challenge
Measure tumor growth
B
PBS
HIB-PMVs
HIB-PMVs + αCD4
HIB-PMVs + αCD8
HIB-PMVs + αCD4 + αCD8
Tumor Area (mm2)
days post challenge
Tumor free survival (%)
days post challenge
C
D2F2/E2 specific serum IgG MFI
PBS
HIB-PMVs
HIB-PMVs+ αCD4
HIB-PMVs+ αCD8
HIB-PMVs+ αCD4 + αCD8

Figure 8.

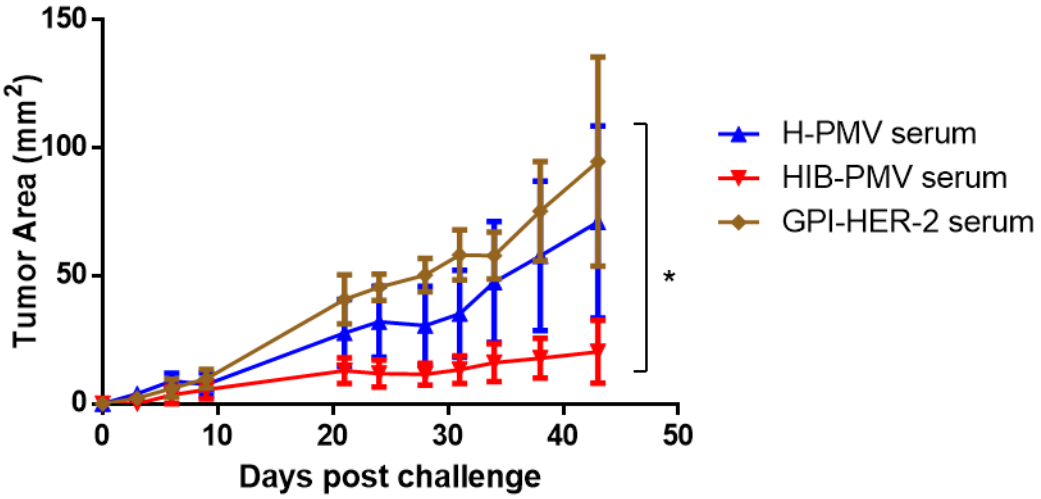

150
100
50
0
Tumor Area (mm2)
0
10
20
30
40
50
Days post challenge
*
H-PMV serum
HIB-PMV serum
GPI-HER-2 serum

Figure 9.

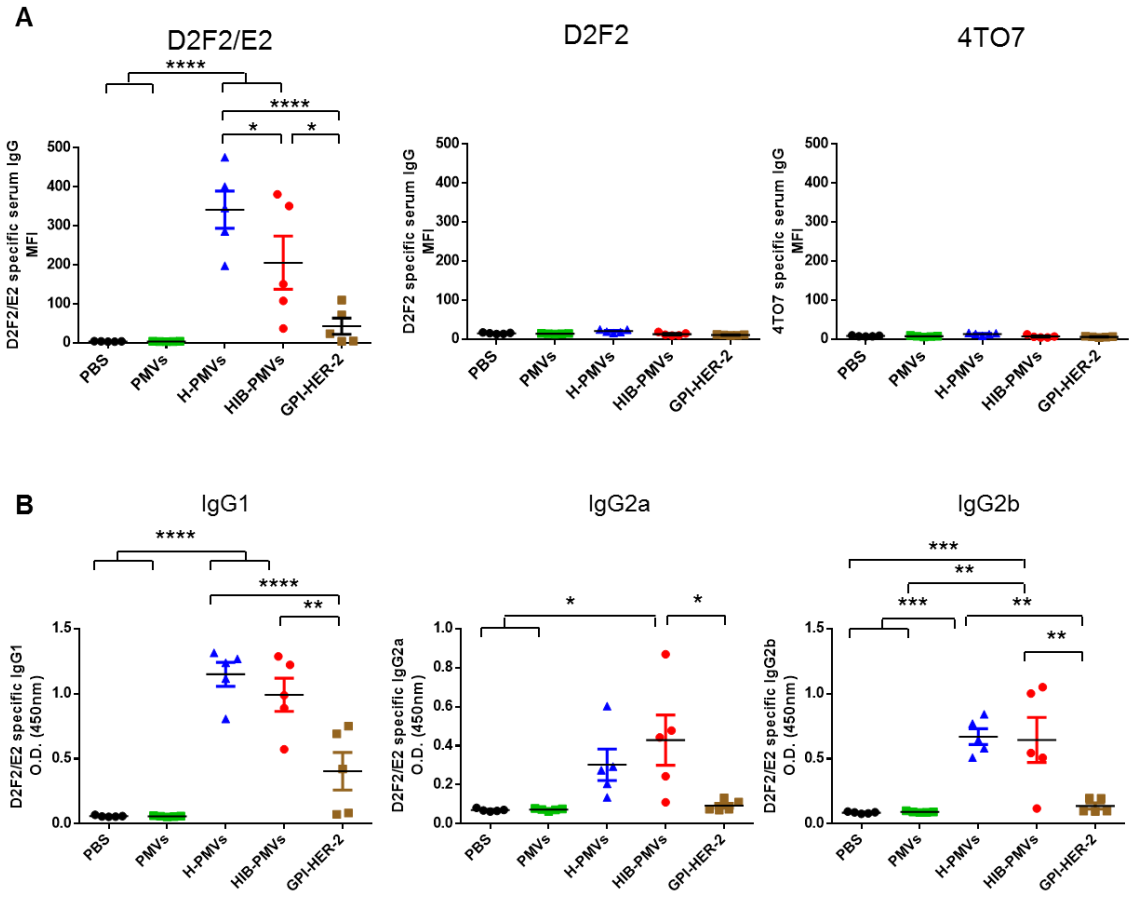

A
D2F2/E2
D2F2
4TO7
D2F2/E2 specific serum IgG MFI
D2F2 specific serum IgG MFI
4TO7 specific serum IgG MFI
PBS
PMVs
H-PMVs
HIB-PMVs
GPI-HER-2
B
IgG1
IgG2a
IgG2b
D2F2/E2 specific IgG1 O.D. (450nm)
D2F2/E2 specific IgG2a O.D. (450nm)
D2F2/E2 specific IgG2b O.D. (450nm)

Figure 10.

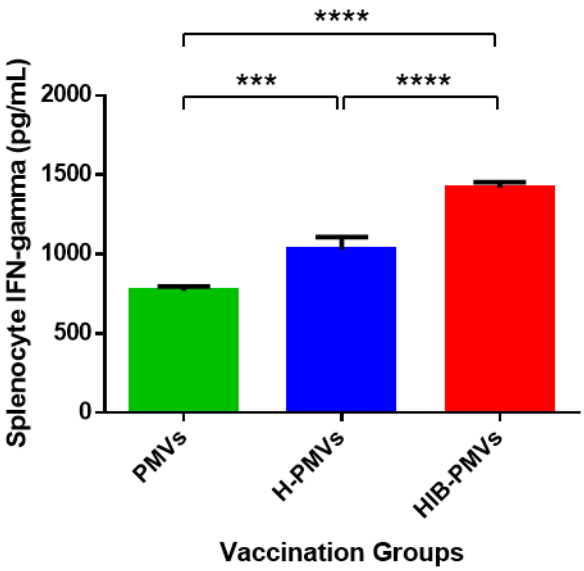

****
***
****
2000
1500
1000
500
0
Splenocyte IFN-gamma (pg/mL)
PMVs
H-PMVs
HIB-PMVs
Vaccination Groups

Figure 11.

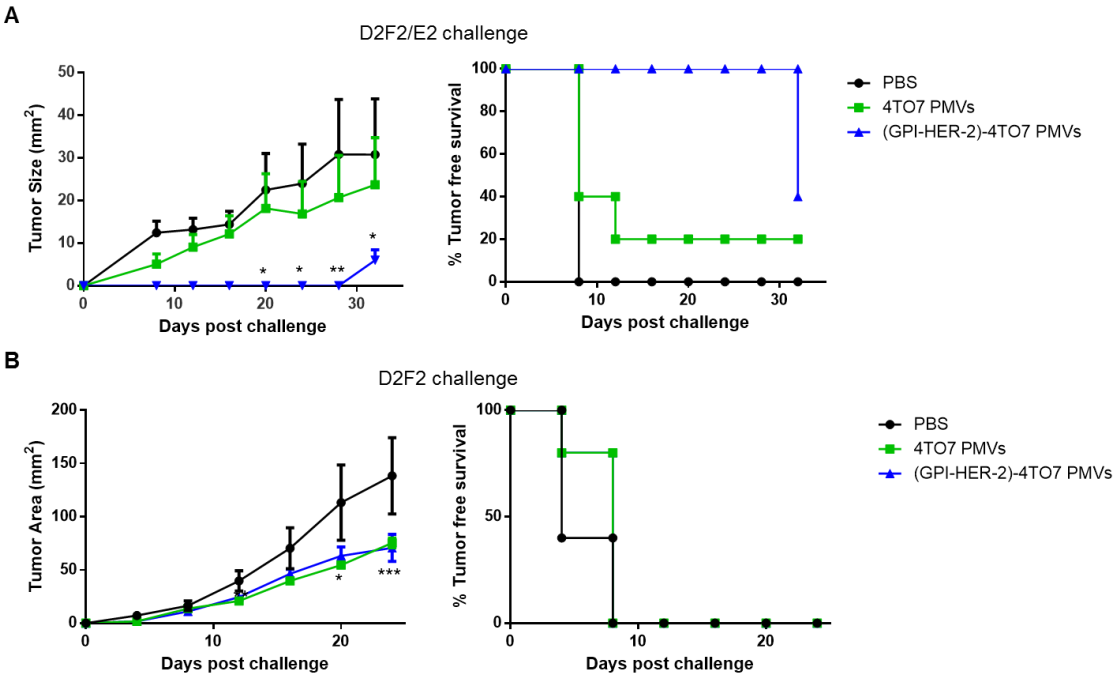

A
D2F2/E2 challenge
Tumor Size (mm2)
Days post challenge
% Tumor free survival
Days post challenge
PBS
4TO7 PMVs
(GPI-HER-2)-4TO7 PMVs
B
D2F2 challenge
Tumor Area (mm2)
Days post challenge
% Tumor free survival
Days post challenge
PBS
4TO7 PMVs
(GPI-HER-2)-4TO7 PMVs

Figure 12.

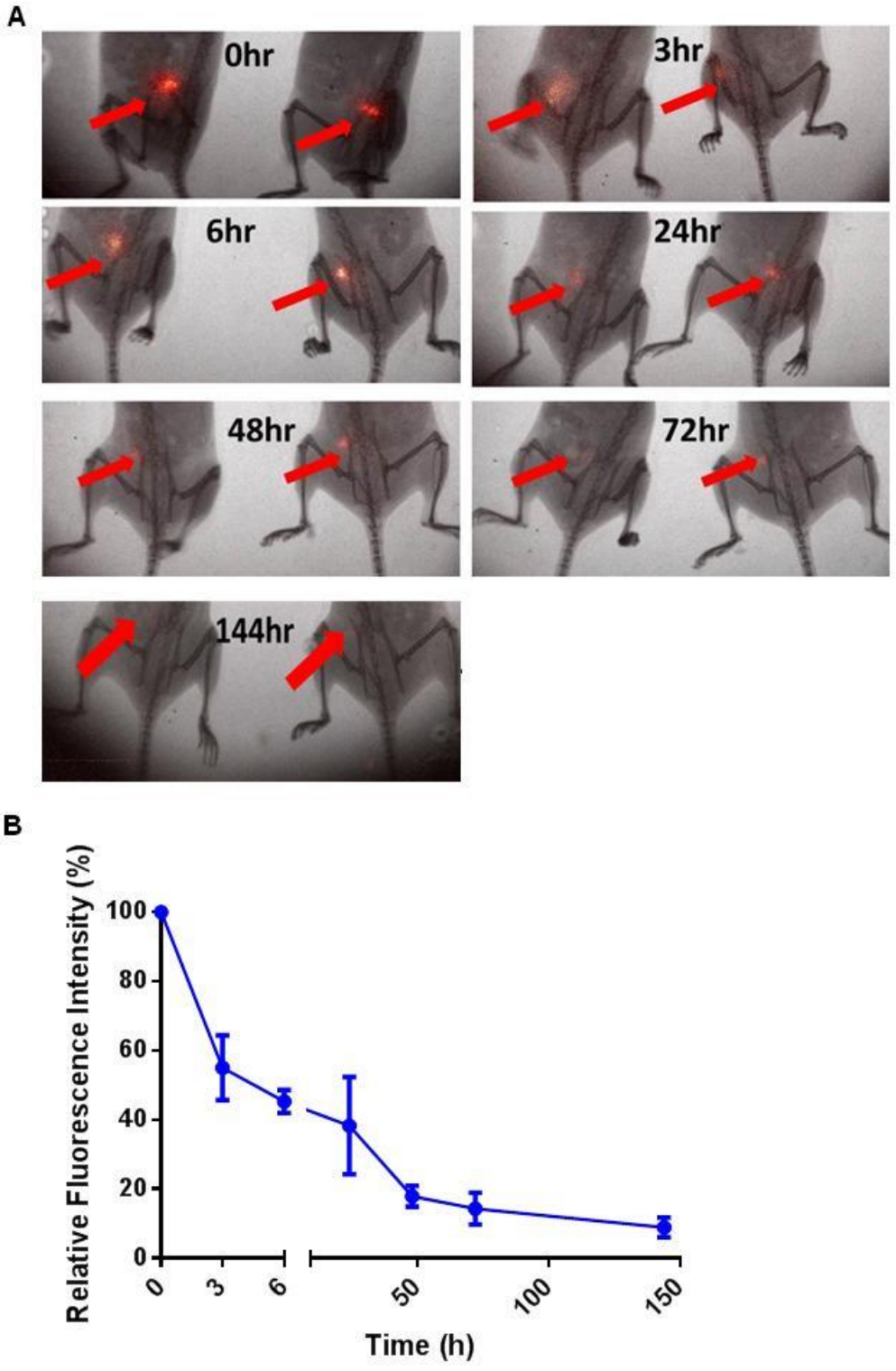
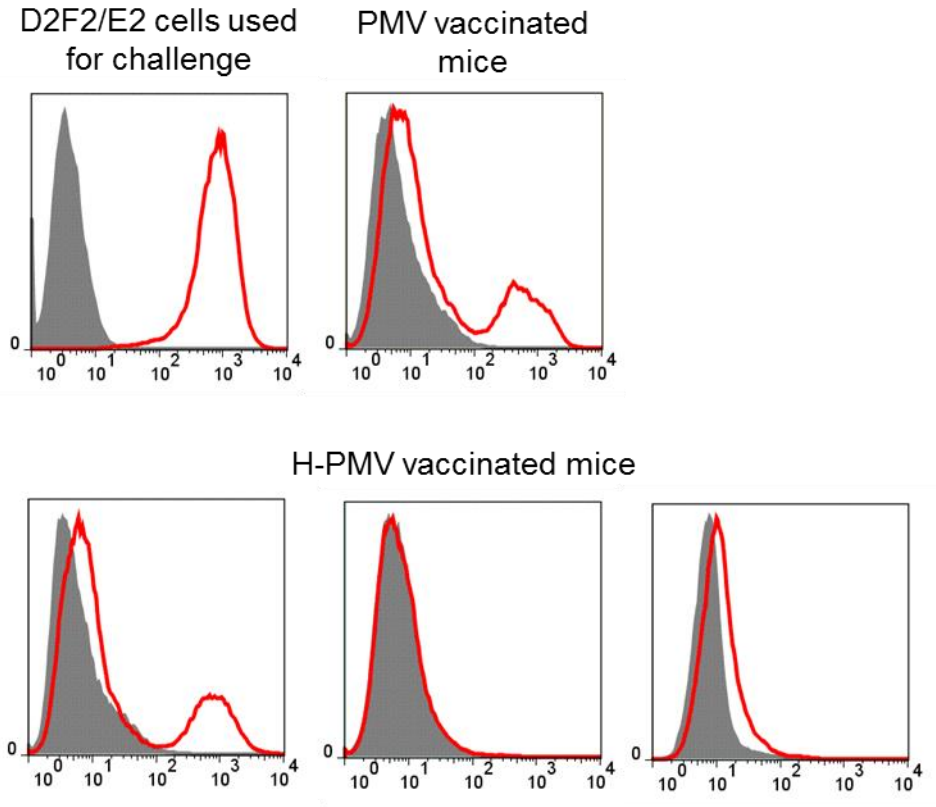

A
0hr
3hr
6hr
24hr
48hr
72hr
144hr
B
Relative Fluorescence Intensity (%)
100
80
60
40
20
0
0
3
6
50
100
150
Time (h)

Table 1. Vaccination with breast cancer derived PMVs protein transferred GPI-HER-2 induce similar protection against HER-2 expressing tumor challenge.

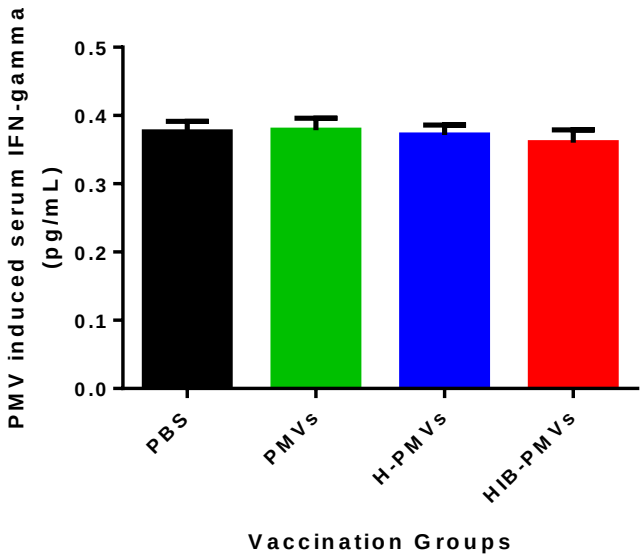
	Tumor Area (mm^2)						
Day post challenge	PBS	4TO7 PMVs	(GPI-HER-2)-4TO7 PMVs	D2F2 PMVs	(GPI-HER-2)-D2F2 PMVs	E0771 PMVs	(GPI-HER-2)-E0771 PMVs
0	0	0	0	0	0	0	0
17	26.0±19.7	32.6±14.1	11.5±10.5	26.3±25.6	11.5±10.8	41.7±13.9	10.3±15.5
38	56.8±40.0	40.7±31.9	33.0±13.0	74.7±64.6	18.9±15.3	70.6±41.4	23.4±23.5
57	133.4±91.5	101.7±83.5	63.4±40.6 *	175.2±164.1	51.1±54.3 *	170.9±136.9	62.0±45.1 *

Average ± SD

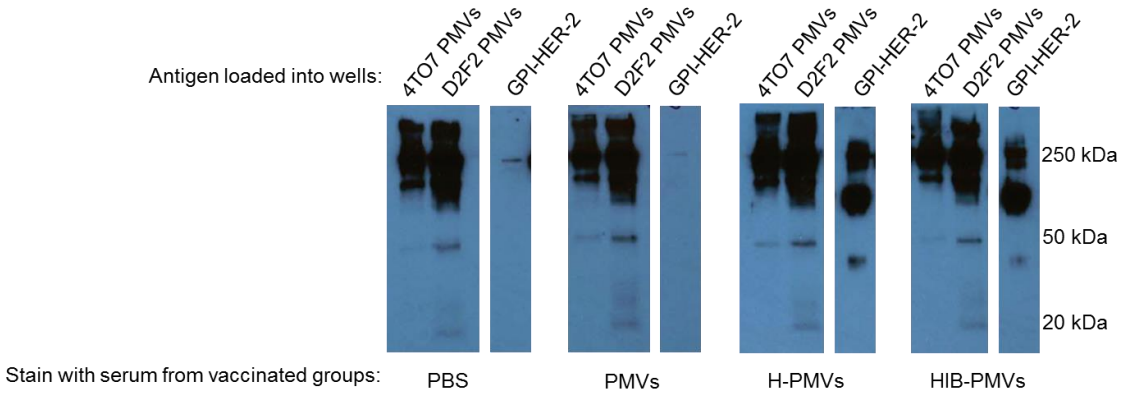
Supplemental Figure 1.



Supplemental Figure 2.



Supplemental Figure 3.



CHAPTER V:

Discussion

Antigens presented as a particulate form have enhanced immunogenicity compared to antigens presented as a soluble form, therefore the use of nano- and microparticles as an antigen delivery system has been widely studied. We demonstrated that lipid-based particles protein transferred to express GPI-APs on the surface can be used to deliver antigen and further enhance antigen-specific immune responses. For successful particulate-based antigen delivery, four criteria need to be met: 1) the antigen must be stabilized in order to prolong delivery to the immune system, 2) the particles must be able to deliver the antigen to immune cells, APCs in particular, that uptake and process the antigen, 3) an antigen-specific adaptive immune response must be generated and 4) the type of immunity induced should be controlled for specific situations.

Within this dissertation, we demonstrate that the protein transfer technology can be used to modify the surfaces of influenza virus-like particles to express ISMs which enhance the immunogenicity of the viral proteins expressed on the VLPs. We then showed that the intrinsic immunogenicity of the VLPs can be used to induce tumor protection by modifying VLPs to express GPI-HER-2, a tumor antigen, by protein transfer. Finally, protein transfer was used to modify tumor-derived PMVs to express exogenously added tumor antigens and that this delivery of PMV-incorporated-tumor antigen enhances the immunogenicity of the antigen compared to the soluble form. Previous studies have shown that protein transfer can be used to modify tumor-derived PMVs with ISMs to enhance the immunogenicity of intrinsically expressed PMV antigens, however herein, we show that protein transfer of GPI-ISMs with GPI-HER-2 onto PMVs can be used to enhance the immunogenicity of exogenously added tumor antigens as well.

Use of enveloped VLPs to enhance immunogenicity of tumor antigens

Tumor microenvironments are highly immunosuppressive with an accumulation of immunosuppressing cells, such as regulatory T-cells and myeloid derived suppressor cells, and factors, such as cytokines TGF-β and IL-10 (1, 2). The high amount of immunosuppression, makes it difficult for soluble antigen-based vaccines to elicit a protective immune response. Therefore a highly immunogenic carrier is needed. Influenza VLPs that are particulate, present antigen in a repetitive structure, and are thus able to induce both a T-cell and B-cell-mediated immune response, are promising candidates to be used as a highly immunogenic carrier for use in a tumor setting.

Our data shows that vaccination with GPI-HER-2-VLPs leads to enhanced HER-2-specific tumor protection compared to vaccination with similar levels of GPI-HER-2 alone (Chapter III). This heightened HER-2-specific immunity was attributed to the incorporation of HER-2 onto influenza VLPs as administration of unincorporated soluble GPI-HER-2 did not elicit the same immune response. VLPs have been shown to physically bind to APCs (DCs, macrophages, and B-cells) in particular (3, 4). VLPs are taken up by micropinocytosis and endocytosis which lead to activation of DCs (5). Aside from DCs, VLP uptake has also been shown to induce maturation of macrophages and monocytes (6). Further, direct VLP administration or DCs pulsed with VLPs can further lead to activation of spleen T-cells and B-cells as shown by increased surface expression of the activation marker CD69 and decreased expression of CD62L, a lymph node homing receptor (6). VLPs taken up by DCs have shown to be cross-presented by an endosome-to-cytosol pathway resulting in VLP-expressing-antigens presented onto MHC

I molecules to activate a $CD8^+$ T-cell response (7, 8). Therefore, VLP administration leads to broad immune cell activation.

The size and structure of influenza VLPs, which are approximately 100 nm in diameter, was not altered by protein transfer (Chapter II), therefore, GPI-HER-2-VLPs are likely to freely migrate into draining lymph nodes as well as be taken up by resident APCs at the vaccination site. Vaccination using GPI-HER-2-VLPs, in which HER-2 is in a particulate form, may stabilize HER-2 as well as induce uptake by APCs more readily while inducing maturation and activation of APCs simultaneously. After uptake, HER-2 presented onto VLPs can be processed onto MHC I (by cross-presentation) and MHC II molecules along with VLP antigens and can thus be presented to both $CD8^+$ and $CD4^+$ T-cells as well as B-cells.

Further, both $CD4^+$ and $CD8^+$ T-cells are important for tumor protection (9, 10). $CD4^+$ T-cells release Th1 or Th2-type cytokines to help control antitumor immune responses. Th1-type cytokines primarily activate $CD8^+$ cytotoxic T-cells and promote direct tumor killing by these cells, whereas Th2-type cytokines promote B-cell immune responses (11). A combination of both Th1 and Th2-type immunity has been shown to induce maximal tumor protection however (10, 12, 13). Therefore, the induction of both Th1 and Th2-type anti-tumor immune responses are important in combating tumors.

In Chapter III, we noted that administration of GPI-HER-2-VLPs led to enhanced Th1 along with Th2-type immune responses whereas administration of GPI-HER-2 alone led to only detectable levels of Th2-type immunity. This may be contributed to the ability of VLPs to induce both Th1 and Th2-type immune responses (14-16). Therefore,

including HER-2 on VLP surfaces can elicit broad HER-2-specific immunity which is needed for tumor protection.

We also noted in Chapter III, Figure 5A, that vaccination with unmodified VLPs delayed HER-2-expressing tumor growth. Previously, vaccination with inactivated influenza virus has shown to promote antitumor immune responses. Surgical removal of tumors induces NK cell dysfunction that then allows metastasis to occur, however perioperative vaccination using inactivated influenza virus before surgery reduces metastasis by reversing the dysfunction of NK cells (17, 18). IFN-α production in particular led to the activation of NK cells to kill tumor cells after surgery. Further, vaccination with influenza viral infection has also led to controlled tumor growth through tumor antigen-specific antibody and T-cell production (19). Inflammation elicited through influenza viral infection was thought to transiently express abnormal self-antigens that are also expressed on tumor cells, therefore an antibody and T-cell response against the common self-antigens aided to control tumor growth. In a similar manner, inflammation induced by vaccination with unmodified influenza VLPs could lead to transient expression of tumor associated antigens also expressed on the murine breast cancer cell line D2F2/E2, thus allowing for nonspecific activation against tumor antigens and/or activation of NK cells that could then aid to kill tumor cells. However, our results showed better protection when vaccination occurred with VLPs modified to express a strong tumor associated antigen (Chapter III, Figure 5A).

Use of plasma membrane vesicles to enhance immunogenicity

Protein transfer could also be used on microparticles that are derived from homogenization of tumor cells or tumor tissue. The resulting PMVs contain a lipid bilayer and are thus amenable to protein transfer using GPI-APs. We showed that protein transfer of GPI-HER-2 onto breast cancer cell and tumor tissue derived PMVs led to enhanced HER-2-specific immunity whereas co-incorporation with the ISMs, GPI-IL-12 and GPI-B7-1, led to complete protection against HER-2-expressing tumor challenge (Chapter IV).

PMVs are larger in size (approximately 350 - 410 nm diameter) compared to VLPs (approximately 100 nm diameter), therefore unlike VLPs, PMVs most likely localize at the vaccination site and do not freely migrate to lymph nodes. However, PMV size is optimal for DC uptake and presentation, resulting in PMV antigens being transported to the draining lymph nodes for further activation of T-cells and B-cells. Similar to VLPs, PMVs provide a particulate scaffold for HER-2 delivery that may enhance HER-2 uptake and presentation by DCs and stabilize HER-2 from proteolytic degradation preventing clearance from circulation. Our studies showed that PMVs derived from multiple breast cancer tumors led to similar HER-2-specific protection whether PMVs were from a syngeneic or allogeneic source, therefore, the PMV structure in particular is most likely necessary for eliciting HER-2-specific immunity in this case. In addition, vaccination using PMVs modified to express HER-2 led to enhanced Th1 and Th2-type HER-2-specific immunity as compared to vaccination using similar quantities of GPI-HER-2 alone (Chapter IV). This observation may be attributed to the particulate nature of PMVs, similar to what was seen with VLPs. Another important factor of PMVs

is that PMVs are biological materials derived from cells or tissue. Therefore toxicology issues that result from many synthetically made nano and microparticles may not arise here suggesting the use of PMVs to be a safer alternative.

Enhanced immunogenicity by GM-CSF

Vaccination with influenza H5 VLPs protein transferred to express GPI-GM-CSF led to enhanced immunogenicity against a heterologous H5N1 viral challenge compared to vaccination with unmodified VLPs (Chapter II). Although the mechanism is currently unknown, GPI-GM-CSF-VLP vaccination led to the formation of long-lived viral-specific antibody secreting cells in the spleen, bone marrow, and lungs (Chapter II, Figure 5D). VLPs in general have been shown to induce strong virus-specific antibody responses (20-22), therefore the use of VLPs with GM-CSF incorporated on its surface may further heighten this phenomenon.

DCs, key antigen presenting cells, are critical for activation of an adaptive immune response. Upon uptake of antigen from the environment with a danger signal, DCs start to mature and present antigen onto MHC I (by cross-priming) and MHC II molecules while increasing expression of costimulatory molecules. They then migrate to draining lymph nodes to activate T-cells and the adaptive immune system. Moreover, the immune stimulation activity of DCs can be enhanced by GM-CSF (23). GM-CSF has previously been shown to recruit immature DCs to the vaccination site (24) and studies which involved GM-CSF-secreting tumor cells suggests that GM-CSF presence leads to enhanced T-cell mediated tumor protection either through enhanced presentation of tumor antigen by DCs or by increased expression levels of co-stimulation molecules,

such as B7-1 (25, 26). Therefore, vaccination with GPI-GM-CSF-VLPs can release a slow cytokine gradient attracting DCs to the vaccination site, and interaction between GM-CSF expressed on the VLP surface and GM-CSFR expressed on the surface of DCs can facilitate binding to DCs as well as simultaneously activate DCs. GM-CSF expression on VLPs can thus lead to enhanced presentation of associated viral proteins displayed on the VLPs by DCs along with increased expression of costimulatory molecules.

Further, influenza VLPs are small enough to freely migrate to draining lymph nodes after vaccination. In the LNs, GPI-GM-CSF-VLPs may interact more readily with B-cells that also express the GM-CSF receptor (27, 28). GM-CSF has shown to effect both immature and mature B-cells leading to enhanced antibody production (27, 29). Also, Liu *et al*. showed that single vaccination with GM-CSF-expressing adenovirus along with L1 HPV VLPs led to similar levels of prolonged neutralizing antibodies compared to three vaccinations with L1 HPV VLPs alone (30), therefore GM-CSF expression reduces the antigen dose required to elicit a strong anti-viral antibody response.

In addition, GM-CSF incorporation can also lead to enhanced T-cell responses. Skountzou *et al.* demonstrated that vaccination using SIV VLPs expressing a GPI-anchored form of GM-CSF led to enhanced virus-specific Th1 and Th2-type cytokine production by $CD4^+$ and $CD8^+$ T-cells from the spleen (31). This demonstrates that incorporating GM-CSF onto VLP surfaces can stimulate a stronger antigen-specific T-cell response by increasing polyfunctionality of T-cells and enhancing the production of many different cytokines that are beneficial when inducing anti-viral immunity.

Taking all this into account, incorporating GPI-GM-CSF onto VLPs by protein transfer may reduce the vaccine dose required for optimal protection. To support this hypothesis, our results showed that a single vaccination with 0.5 μg of GPI-GM-CSF-VLPs led to enhanced VLP-specific IgG responses compared to a single vaccination with 0.5 μg of unmodified VLPs. This may be due to the interaction between GM-CSF expressed on the VLPs with APCs at the vaccination site leading to enhanced uptake of VLP proteins as well as activation of APCs or through GPI-GM-CSF-VLP interaction with B-cells in the draining LNs.

Enhanced tumor antigen-specific immune stimulation by IL-12 and B7-1

Both the cytokine IL-12 and the costimulatory molecule B7-1 have been widely studied for use in tumor immunotherapy (32-37). The receptors for IL-12 and B7-1 are expressed on a wide array of immune cells. The IL-12 receptor is expressed on T-cells, NK cells, and APCs whereas the B7-1 receptor, CD28, is expressed on T-cells, NK cells, and mast cells (38).

IL-12 stimulates both NK cells and CTLs (39), both of which are important in combating tumors. Therefore, IL-12 administration has been studied in tumor settings. Administration of recombinant soluble IL-12 or IL-12 plasmids in tumor bearing mice leads to tumor regression and prolonged survival in mice (39-42). The rejection seen was found to be mediated primarily through IFN-γ production and subsequent production of chemokines and cytokines that attract more $CD8^+$ T-cells into the tumor microenvironment (39, 41, 42). More recent analysis has also showed that IL-12 administration can affect tumor-associated macrophages to decrease production of tumor

promoting factors and increase production of proinflammatory factors (43). Although IL-12 has proved to be beneficial in combating tumor growth, administration does not come without side effects, such as systemic toxicity associated with IL-12 treatment (44, 45). However, Nagarajan *et al.* saw that inoculation of murine mastocytoma cells transfected to secrete soluble IL-12 showed more systemic IL-12 and IFN-γ production compared to cells transfected to express GPI-anchored-IL-12 (34). Therefore, attaching IL-12 via a GPI-anchor to the surface of PMVs by protein transfer may help to localize the effects of IL-12 to the vaccination site thus reducing systemic toxicity.

On the other hand, B7-1 is a costimulatory molecule required as 'signal 2' for the generation of an antigen-specific T-cell response. In immunocompetent mice, inoculation with murine melanoma cells that express the E7 gene from human papillomavirus 16 forms tumors however when transfected to express B7, local and distant tumors regressed (46). In addition, when mice were inoculated with melanoma cells transfected to express B7, tumor rejection occurred (47). In these cases, regression was mediated by $CD8^+$ T-cells (46, 47). In another study using B16 melanoma cells, inoculation with B16 cells that expressed high levels of B7-1 did not lead to tumor growth and B7-1-mediated regression was found to be NK cell and $CD8^+$ T-cell mediated (48). However, when B16 cells that expressed high levels of B7-1 were mixed with wild type B16 cells and then inoculated into mice, tumors began to grow (48). These results suggest that although B7-1 expression is important for tumor rejection, B7-1 expression alone is not always sufficient. Basker *et al*. aimed to enhance B7-1-mediated tumor protection by transfecting MHC II molecules onto sarcoma cells along with B7-1 which could be used to treat established tumors more efficiently than cells expressed with MHC

II or B7-1 alone (49). Rejection in this case was mediated by $CD4^+$ and $CD8^+$ T-cells (49).

However, the combination of both has presented more promising results. Putzer *et al*. showed that adenovirus vectors that express soluble forms of IL-12 along with the transmembrane form of B7-1 when injected intratumorally into mice with adenocarcinoma resulted in 70% tumor regression, whereas injection of adenoviruses that expressed only IL-12 or only B7-1 led to delayed tumor growth and no tumor regression. However, interestingly, when IL-12 and B7-1 were expressed in separate vectors and injected together, only 30% tumor regression resulted as compared to 70% tumor regression seen when both IL-12 and B7-1 were expressed in the same vector (36). Further inoculation of tumor cells that express both soluble forms of IL-12 and transmembrane forms of B7-1 lead to no tumor growth (37, 50). However, B-cell lymphoma A20 cells that secreted IL-12 without B7-1 expression also did not lead to tumor growth whereas B7-1 expression alone led to delayed tumor growth (50). In addition, vaccination with T-cell lymphoma-derived membranes that were protein transferred to express a GPI-anchored form of B7-1 induced minimal parental tumor-specific CTL lysis, however inclusion of soluble IL-12 along with B7-1-expressing membranes led to further enhanced specific lysis. Nonetheless, both vaccines protected against parental tumor growth (51). Due to systemic toxicity effects by soluble administration of IL-12 (44, 45), Pan *et al.* transfected colon adenocarcinoma cells with a membrane-bound form of IL-12 along with full-length B7-1 (52). This double surface expression prevented tumor growth and decreased lung metastasis, and intratumoral injection of an adenovirus vector that expressed both membrane-bound forms of IL-12

and B7-1 led to complete regression of local tumors and decreased growth of distal tumors (52). Furthermore, Bozeman-Cimino *et al*. showed that therapeutic vaccination with irradiated breast cancer cells transfected to express a GPI-anchored form of IL-12 with transmembrane-bound B7-1 led to decreased tumor growth in mice bearing the parental tumor (32). All of these studies showed the beneficial use of the IL-12 and B7-1 combination in a tumor setting. In addition, inclusion of ISMs, GPI-IL-12 and GPI-B7-1, on the PMVs along with HER-2 led to enhanced HER-2-expressing tumor protection compared to HER-2 expression alone (Chapter IV).

The antitumor effects of IL-12 are due to an increase in CTL and NK cell cytotoxicity, T-cell, NK cell, and NK T-cell activation, as well as production of IFN-γ by these cells. IFN-γ can then promote anti-angiogenic properties to prevent tumor spread (33, 53, 54) and induce production of chemokines IP-10 and Mig that attract T-cells into the tumor microenvironment (41, 42). The antitumor effects of B7-1 on the other hand mostly include stimulation of NK cells and $CD8^+$ T-cells (36, 46-48) and aid in effective antigen presentation (47). Therefore, the combination of both IL-12 and B7-1 in a tumor setting has thus been shown to generate stronger CTL responses (37, 50) and maximal proliferation of lymphocytes (50) and both $CD4^+$ and $CD8^+$ cells appear to aid in mediating the antitumor effects (50, 52). Enhanced production of IFN-γ also resulted after expression of both IL-12 and B7-1 on tumor cells (37). Further, co-expression of GPI-IL-12 and transmembrane B7-1 on breast cancer cells led to decreased numbers of MDSCs and Tregs at the tumor site and MDSCs in the spleen as well as reduced angiogenesis (32). Therefore, inclusion of both IL-12 and B7-1 on the PMV surface can

activate a variety of immune cells that are important for tumor killing and thus may enhance the HER-2-specific immunity seen in Chapter IV.

Advantages/disadvantages of protein transfer onto particles

Protein transfer allows for rapid and simple surface modifications of lipid-based particles within a matter of hours. Expression levels of incorporated GPI-APs can be controlled precisely by protein transfer without altering the functionality of the incorporated protein, and multiple GPI-APs can be incorporated simultaneously onto PMVs. Further, protein transfer results in stable expression of the incorporated GPI-AP even when stored at room temperature, therefore protein transfer-modified-particles can be easily stored for vaccine purposes. Moreover, the physical linkage of ISMs to particles prevents systemic toxicity effects and enhances immunity against other incorporated antigens and particle-intrinsic antigens.

Particulate delivery of antigens protects antigen from degradation and also leads to optimal uptake by APCs. Co-expression of antigen with incorporated ISMs leads to enhanced activation of APCs and immune cells. Further, the ability to incorporate multiple ISMs along with antigens can help to control the immune response generated. For example, the presence of the cytokine IL-12 leads to the generation of a Th1-type immune response (55) whereas the presence of IL-4 leads to more of a Th2-type immune response (56). Th1-type immunity has shown to be protective in tumor settings and viral infections, whereas Th2-type immunity plays important roles against extracellular pathogens (57). By protein transfer, a specific type of immune response against antigen-expressing-particles can be generated which suits the setting at hand.

For protein transfer to occur, GPI-APs are needed. Although both secretory and transmembrane proteins can be converted into GPI-anchored forms, the GPI-anchor can only be attached to the C-terminal end of a protein. Therefore, key functional and antigenic sites located at the C-terminal end of the protein may be affected. Hence, each protein converted into a GPI-anchored form needs to be tested. Furthermore, GPI-anchors are only found in eukaryotic systems and not prokaryotic systems (58), therefore, only eukaryotic systems can be used for large production of GPI-APs.

Future studies using protein transfer technology onto lipid-based particles

The protein transfer technology can be expanded in many aspects to generate immunity against a vast range of known antigens, whether they be viral or tumor antigens. Due to the ability of multiple GPI-APs being able to simultaneously incorporate onto lipid-based particles, the repertoire of immunity generated can be greatly extended. On the other hand, protein transfer can also be used to increase the expression levels of incorporated proteins by simply increasing the concentration of GPI-AP added during incubation. The level of antigen present in vaccines affects the immune response generated with higher antigen levels leading to enhanced antigen-specific immunity (59-61). Therefore, protein transfer can be used to increase antigen levels already found on particles, such as HA content found on influenza VLPs, or to increase exogenously added antigen levels. Modification of nanoparticles by genetic engineering on the other hand creates difficulties in generating particles with increased antigen expression as well as optimal particle formation.

We found that vaccination with two immunizations of 0.5 μg of protein transfer-modified GPI-GM-CSF-VLPs led to complete protection against challenge with a heterologous H5N1 virus (Chapter II, Figure 5A and 5B). We also showed that vaccination with two immunizations of 0.5 μg of GPI-GM-CSF-VLPs that express either 0.029 μg GM-CSF/μg VLP or 0.089 μg GM-CSF/μg VLP led to similar levels of virus-specific IgG serum responses (Chapter II, Supplemental Figure 6). However further studies need to be conducted that measures (1) the expression level of GPI-GM-CSF on the VLP surface that is required for protection, (2) the minimal dose of GPI-GM-CSF-VLPs required for protection, and (3) the number of vaccinations of GPI-GM-CSF-VLPs required for protection against heterologous viral challenge.

In addition, the mechanism of action that leads to protection by GPI-GM-CSF-VLPs needs to be addressed. The requirement for DCs and/or B-cells to lead to GPI-GM-CSF-VLP-mediated enhanced anti-viral antibody responses needs to be assessed to further delve into the mechanism of action leading to protection. Virus-specific T-cell responses will also need to be analyzed as VLP vaccination induces T-cell immunity alongside B-cell immunity (31, 62), which may be important for protection.

Vaccination using breast cancer 4TO7 tissue derived PMVs that were protein transferred to express GPI-HER-2, GPI-IL-12 and GPI-B7-1 led to complete protection against challenge with HER-2-expressing D2F2/E2 breast cancer cells (Chapter IV, Figure 5). Repeated vaccination with a strong immunogen has shown to induce immunity against the strong antigen as well as induce epitope spreading to initiate immunity against minor weaker antigens (63-66). Therefore, vaccination with the strong antigen HER-2, expressed on 4TO7 PMVs was thought to induce immunity against HER-

2 alongside minor antigens expressed on the 4TO7 PMV surface or against minor antigens expressed on D2F2 tumor cells. By our vaccination and boost strategy using 25 μg of HIB-PMVs, we did not see these results as antibody responses and tumor protection was found to only be specific for HER-2-expressing D2F2/E2 cells and not for the parental HER-2-negative D2F2 cells or 4TO7 cells (Chapter IV, Figure 5 and 9). Therefore, to induce immunity against other minor antigens that may be co-expressed on 4TO7 cells and D2F2 cells, the number of vaccinations and doses for HIB-PMVs will need to be further analyzed. Using the vaccination strategy presented here, we did not detect HER-2-specific CTL lysis by either *in vitro* or *in vivo* CTL assays, however if the dosage and number of vaccinations were altered, detectable CTL responses may be seen. Therefore, when using protein transfer to modify PMVs to express HER-2, the expression levels of HER-2 along with ISMs IL-12 and B7-1, the amount of protein transferred PMVs, and the number of vaccinations all need to be further optimized in order to obtain maximal tumor protection.

Protein transfer for personalized cancer immunotherapy

Not all tumors express known tumor-specific antigens and many tumors are heterogenous so that expression levels of different antigens vary amongst the cancerous cells that makeup a tumor. In these cases, PMVs can be derived from patient tumors which in turn can be modified with ISMs to generate an immune response against the associated tumor antigens already expressed on the PMVs. In this manner, all antigens within the patient tumor are still presented although specific antigens may be unknown and heterogeneity may occur. Generation of PMVs from tumor tissue not only expresses

cell-surface antigens, but MHC I molecules expressed on the PMVs may also express intracellular antigens. In this manner, patient-specific PMVs will have the capability to induce immunity against both surface and intracellular antigens. The inclusion of ISMs or adjuvants that promote tumor protection can also be included by protein transfer to further enhance immunity against the tumor antigens presented on the PMVS.

Implications of protein transfer-modified VLPs and PMVs

Protein transfer-mediated modification of lipid-based particles can be used in a wide range of situations from viral to tumor immunotherapy. The type of immunity induced can be tightly controlled by controlling the type of ISM and concentration incorporated thus providing specificity to different situations and diseases easily. The versatility, ease and speed of protein transfer allows for this approach to be easily scaled up for large production in a relatively short amount of time. This can prove beneficial when confronted with pandemics in which large quantities of vaccines may be needed quickly. All of these qualities of protein transfer make this technique highly cost effective. Therefore, this protein transfer technology can be used in a wide variety of applications to deliver antigen along with immune stimulation, thus enhancing the antigen-specific immune response generated.

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